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**Original papers** should normally be divided into abstract, introduction, materials and methods, results, discussion and references.

The section Decision Making and Problem Solving presents papers on health decision science specifically regarding hematological problems. Suitable papers will include those dealing with public health, computer science and cognitive science. This section may also include guidelines for diagnosis and treatment of hematological disorders and position papers by scientific societies.

Reviews provide a comprehensive overview of issues of current interest. No particular format is required but the text should be preceded by an abstract which should be structured as follows: background and objective, evidence and information sources, state of art, perspectives. Within review articles, *Haematologica* gives top priority to: a) papers on molecular hematology to be published in the section Molecular basis of disease; b) papers on clinical problems analyzed according to the methodology typical of Evidence-Based Medicine.

The section The Irreplaceable Image deals with short papers containing pictures of particular interest: bone marrow or peripheral blood smears, biopsies, physical and radiographic findings, etc. No more than three authors should sign these papers.

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Meeting Supplement

XIII Congress
of the Italian Association
of Immunopharmacology
September 9-11, 1998
Pavia, Italy

Chairmen
Prof. Antonio Crema
Prof. Antonio Dal Canton

PAPERS
On the cover. This figure shows a possible explanation for the inflammatory/immune response in the acute phase of atherosclerotic ischemic heart disease (see the complete legend at pag. 29).

**Haematologica**

is a Latin adjective, neuter and plural, used in this context as a substantive: it means “hematological subjects”.
The appropriate English translation is therefore **Journal of Hematology**.
Introduction

This supplement to Haematologica reports the proceedings of the XIII Congress of the Italian Association of Immunopharmacology, to be held in Pavia, September 9-11, 1998.

The Congress is an inter-disciplinary meeting aimed to discuss pathophysiological and clinical topics that are of common interest to the pharmacologist, the immunologist and the specialist in several branches of internal medicine.

The scientific program includes original papers and lectures tailored to present the state of the art in the field. Education and up-dating is granted by wide space for open discussion and interactive participation.

In brief, these are the main contents:
1. Effects of cytokines and other autacoids released in immunological reactions.
2. Inflammation and atherosclerosis in chronic neurodegenerative diseases.
3. Immunotherapy: including bacterial and chemical agents and cytokines in transplation, neoplastic disease, immunodeficiency, chronic infections, allergic, inflammatory or autoimmune disorders.
4. Immunotoxicology: adverse effects of drugs and xenobiotics on the immune system.

The congress venue, Pavia, is a quiet, nice town placed on the left bank of Ticinum. Pavia was the capital of the Lombard kingdom and site where the emperors of the Holy Roman Empire received their crown, in the Basilica of San Michele. Its middle-aged intact enviroment, its churches and palaces, the castle and the university (one of the most ancient in the world) are well worth of a visit.

Welcoming you to the Congress,

Antonino Mazzone M.D.,
Scientific Secretary
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Kaposi’s sarcoma (KS) is an angioproliferative disease occurring in four clinic-epidemiologic forms (Ensoli et al., 1991; Ensoli and Stürzl, 1998). Although the AIDS-associated KS (AIDS-KS) is the most aggressive, all forms of KS share the same immunological and histopathological features suggesting common etiological and pathogenic factors. Recent data indicate that at least in early stage KS is not a real sarcoma but an angio-hyperplastic-inflammatory lesion mediated by inflammatory cytokines and angiogenic factors, that is triggered or amplified by infection with human herpesvirus-8 (HHV-8). In addition, the HIV-1 Tat protein appears to be responsible for the higher grade of aggressiveness of AIDS-KS as compared to the other forms of KS. However, on time, reactive KS may progress to a sarcoma as suggested by evidence of monoclonality in late-nodular lesions.

Pathology of KS lesions: nature of the inflammatory cell infiltrate and origin of the spindle cells. Histologically, early lesions are characterized by an inflammatory-granulation type reaction with activated proliferating endothelial cells which form new blood vessels often abnormal. This can precede the appearance of the typical “spindle cells” [KS cells (KSC)] that are considered to be the tumor cells of KS. On time, the spindle cells become the predominant cell type and the lesions acquire a more monomorphic aspect (Ensoli et al., 1991; Ensoli and Stürzl, 1998).

The inflammatory cell infiltrate of KS is the first to appear and precedes the spindle cell formation (Fiorelli et al., 1998; Sirianni et al., 1998). Immunohistochemical studies indicate a prevalent infiltration of T cells dominated by CD8+ T cells but also containing CD4+ T cells, numerous monocyte-macrophages often with a spindle-like morphology, dendritic cells (FXIIIa+) and few B cells (Fiorelli et al., 1998; Sirianni et al., 1998; reviewed in Ensoli and Stürzl, 1998). The same features are also observed by analyzing tumor infiltrating lymphocytes (TIL) and macrophagic spindle cell cultures derived from the lesions (Sirianni et al., 1998).

Spindle cells are an heterogeneous cell population dominated by activated vascular endothelial cells mixed with macrophagic spindle-shaped cells (Uccini et al., 1994; Fiorelli et al., 1995; Fiorelli et al., 1998; Sirianni et al., 1998). Both endothelial spindle cells (E-KSC) and macrophagic spindle cells (M-KSC) have been established from the lesions and long-term cultured by utilizing the same IC expressed in the lesion but with modifications of IFN and interleukin-2 (IL-2) content (Nakamura et al., 1988; Salahuddin et al., 1988; Ensoli et al., 1989; Fiorelli et al., 1995; Sirianni et al., 1998). These cells possess the same phenotype as in situ KS spindle cells of both endothelial and macrophagic phenotype, respectively (Fiorelli et al., 1995; Sirianni et al., 1998).

The reactive or hyperplastic E-KSC are not transformed nor they induce tumors in nude or SCID mice, however, they promote highly angiogenic lesions of mouse cell origin that closely resemble early KS lesions (Salahuddin et al., 1988; Ensoli et al., 1994b; Fiorelli et al., 1995). These lesions regress as early KS can regress in humans and, as discussed below, are mediated by the angiogenic cytokines and growth factors produced by KS cells.

Immunopathology of KS patients and in individuals at high-risk of KS: CD8 T cell activation and Th-1 cytokine profile. All patients with KS or at high risk of KS have signs of immunodeficiency (reviewed in Ensoli and Stürzl, 1998). For example, homosexual men have increased blood levels of ICAM-1, soluble CD8, neo-protein levels and other signs of activation prior to HIV-1 infection or KS development (reviewed in Ensoli and Stürzl, 1998). African individuals are also immunologically probably due to frequent infections of different. Elderly men can present an oligoclonal CD8 expansion with increased production of IL-1 and tumor necrosis factor α (TNFα) and have no signs of immunosuppression at KS onset (Fagiolo et al., 1993). Post-transplanted individuals receive large quantities of alloantigens which lead to local foci of immunostimulated cells even during clinically induced immunosuppression. These and other observations suggest a role for a CD8 T cell activation and production of IC of the Th-1 type (γIFN and IL-2) in KS development (reviewed in Ensoli et al., 1991; Ensoli and Stürzl, 1998).

Activated peripheral blood mononuclear cells (PBMC) from both AIDS-KS and CKS patients produce high levels of γIFN and no or little IL-4 as compared to patients without KS but with other dermatological disorders (Sirianni et al., 1998). CD8 T cell activation and infiltration and production of IC by CD8 T cells and monocytes-macrophages is also found in KS lesions (Fiorelli et al., 1998). Thus, immunopathology is a trait of individuals developing KS and production of IC including γIFN. IL-1, TNFα appears to be key to KS development (Sirianni et al., 1998; Fiorelli et al., 1998). In fact, the administration of γIFN, IL-2 or TNFα to KS patients leads to disease.
progression or to KS development. A systemic increase of IC may be responsible of several features of KS patients such as i) the presence of circulating spindle cell precursors (see below); ii) activation of vessels and increased circulating levels of FVIII-RA, and iii) increased vascular adhesiveness with extravasation and tissue recruitment of lymphocytes and monocytes (Zietz et al., 1996).

Circulating spindle cell progenitors: a trait of KS patients. Circulating spindle cell precursors have been found in patients with all forms of KS and in individuals at high risk to develop KS (Browning et al., 1994; Sirianni et al., 1997; Colombini et al., submitted). In KS patients these cells arise spontaneously as spindle cells from the adherent cell fraction of cultured PBMC and more markers of tissue macrophages (Colombini et al., submitted). In addition, a proportion of these cells acquires expression of VE-cadherin, a marker of vascular endothelial cells, although they remain negative for FVIII-RA and CD34 (Colombini et al., submitted). This phenotype resembles an unusual cell type found in lymph nodes, the so called endothelial macrophages. The presence of these cells in the blood may suggest an explanation for the multifocal lesions developing in KS patients. In fact, they have the same phenotype of the M-KSC, and, as discussed below, in KS patients they are infected by HHV-8 (Sirianni et al., 1997) suggesting that they can carry the virus to tissues and differentiate in vivo in macrophages and endothelial macrophages as occurs in vitro.

Inflammatory cytokines are expressed in KS lesions and trigger lesion formation. A variety of IC are expressed in lesions from all forms of KS. These include γIFN, TNF, IL-1, IL-6, granulocyte/macrophages colony stimulating factor (GMCSF), and others (reviewed in Ensol et al., 1991; Starzl et al., 1995; Fiorelli et al., 1998; Sirianni et al., 1998, reviewed in Ensol and Starzl, 1998). They are produced by infiltrating lymphocytes and monocytes-macrophages. This IC production is associated with vessel activation (ICAM-1+, ELAM-1+, V-CAM-1+, DR4+, CD40+, up-regulation of αSβ1 and αVβ3 integrins) and increased vascular adhesion of inflammatory cells. The same IC are produced by activated PBMC and this mixture [T cell activated conditioned media (TCM)] or recombinant cytokines added together at the same concentration as found in TCM, mediate, in a synergistic fashion, phenomena that appear to be key to KS lesion formation and progression.

TCM or combined IC induce the long-term growth of cultivated E-KSC (Nakamura et al., 1988; Salahueddin et al., 1988; Ensol et al., 1989). Several of the IC present in TCM [IL-1α and β, TNFα and β, γIFN, Oncostatin M (OSM), and others] induce this growth that is mediated by a synergistic stimulatory effect on basic fibroblast growth factor (bFGF) production and release (Ensol et al., 1989; Ensol et al., 1994b; Fiorelli et al., 1995, 1996; Samaniego et al., 1995; Faris et al., 1996; Samaniego et al., 1997; Faris et al., 1998). bFGF, in turn, functions as an autocrine KS cell growth factor. IC also increase the in vivo angiogenic and KS-forming activity of KS spindle cells, suggesting that they can also maintain and enhance KS growth and progression (Barillari et al., 1992; Samaniego et al., 1995; Barillari et al., 1997, Samaniego et al., 1998).

IC support the establishment of M-KSC from the lesions and are capable of maintaining in culture KS-derived TIL with the same phenotype as those found in situ in KS lesions, whereas, in the absence of TCM, these cells undergo apoptosis and disappear rapidly (Sirianni et al., 1998).

The same IC activate endothelial cells to acquire the phenotypic and functional features of E-KSC. These include the spindle morphology, the expression of the same markers (downregulation of FVIII-RA, activation of ELAM-1, ICAM-1, V-CAM-1, DR, αSβ1, αVβ3 integrin expression) and production of angiogenic factors such as bFGF, vascular endothelial cell growth factor (VEGF) and other cytokines and chemokines expressed in primary lesions with effects on cell recruitment, growth, angiogenesis and lesion formation (discussed below) (reviewed in Ensol et al., 1991; Barillari et al., 1992; Barillari et al., 1993; Fiorelli et al., 1995; Samaniego et al., 1997; Samaniego et al., 1998; Faris et al., 1998; Fiorelli et al., 1998, reviewed in Ensol and Starzl, 1998). In addition, upon exposure to IC endothelial cells become angiogenic in nude mice and induce formation of KS-like lesions as E-KSC do (Ensol et al., 1994b; Fiorelli et al., 1995; Samaniego et al., 1997; Fiorelli et al., 1998, 1999). Similarly, inoculation of IC induces KS-like angiogenic lesions in mice, indicating that they can trigger a cascade of events leading to lesion formation (Samaniego et al., 1995; Samaniego et al., 1998, Fiorelli et al., 1998). IC also induce normal endothelial cells to become responsive to the effects of extracellular HIV-1 Tat protein, discussed below (Barillari et al., 1993; Barillari et al., 1997).

γIFN appears to be the major mediator of these changes although the other IC, particularly IL-1 and TNF, contribute to these effects in a synergistic manner (Fiorelli et al., 1995; Fiorelli et al., 1998).

Finally, IC activate HIV-1 transcription, replication and production of Tat in infected cells and they activate HHV-8 replication and increase viral load (Barillari et al., 1992, Ensol et al., 1993). Thus IC produced in KS lesions are capable of triggering a cascade of events leading to lesion formation and to maintenance and progression of KS.

Angiogenic molecules, growth factors and chemokines mediate KS lesion formation. E-KSC induce angiogenesis in the chorioallantoic membrane assay and highly angiogenic KS-like lesions after inoculation in nude mice (Salahueddin et al., 1988; Ensol et al., 1994b, Samaniego et al., 1995; Fiorelli et al., 1995; Samaniego et al., 1998). Inoculated KS-like lesions are of mouse cell origin, regress in time and are mediated by specific angiogenic factors produced by the cells. In particular, bFGF is a key mediator of lesion formation (Ensol et al., 1994a; Ensol et al., 1994b). Inoculation of bFGF in nude mice results in the formation
of KS-like lesions. bFGF is expressed at very high levels by E-KSC in vitro and in vivo and it is released by these cells in the absence of cell death or cell permeability changes. Finally, inhibition studies with specific neutralizing antibodies or antisense oligodeoxynucleotides directed against bFGF mRNA have shown that bFGF is required for the formation of KS-like lesions induced by inoculation of E-KSC in nude mice (Ensoli et al., 1994b). In addition to its paracrine activity, bFGF has autocrine activity in KS development because it stimulates proliferation of E-KSC and IC-activated endothelial cells. Both bFGF mRNA and protein are highly increased in tissue sections of KS primary lesions and in KS-like mice lesions (Ensoli et al., 1994a; Samaniego et al., 1998), which indicates that bFGF regulates angiogenesis and E-KSC growth in both humans and mice.

VEGF is another angiogenic factor expressed in both KS lesions and in cultured E-KSC. As for bFGF, VEGF expression in E-KSC is also induced by IC and by other cytokines found in KS lesions. VEGF synergizes with bFGF in inducing endothelial cell growth and angiogenesis as demonstrated by in vitro and mice studies. In addition, bFGF and VEGF synergize to induce edema as shown by injecting both cytokines alone and combined in guinea pigs. VEGF, however, does not induce the growth of E-KSC (Corneli et al., 1996; Samaniego et al., 1998).

HHV-8, a new herpesvirus associated with KS: a triggering event or a consequence of lesion formation? HHV-8 has been shown to be present in all forms of KS (Chang et al., 1994; de Lellis et al., 1995). Recent studies by PCR on PBMC and by serological assays indicate that HHV-8 is particularly prevalent in those geographical areas, including certain areas of Africa, Greece and Italy, with a high incidence of KS (Monini et al., 1996; Lennette et al., 1996). In these areas and, less frequently, in other areas of the world at a lower HHV-8 prevalence, the virus is also present in normal blood donors or in patients without KS (Monini et al., 1996). However, in these individuals, viral DNA and tissues appear to be much lower than in patients with KS. HHV-8 load in PBMC is also higher in HIV-infected individuals and in Africans as compared to other groups at risk of KS. Similarly, viral DNA and a positive serology is found more often in homosexual men, in Africans and in elderly men of high risk geographical areas (Lennette et al., 1996; Rezza et al., 1998). Since HHV-8 seroprevalence is low in areas at low incidence of KS and its detection can precede the onset of, these results suggest that HHV-8 is key to KS development but it requires additional factors to exert its effects in KS pathogenesis.

In PBMC, the virus is detected in B cells, but our recent data indicate that it is also present in monocytes-macrophages, dendritic cells, and, more rarely (in advanced KS), in T cells (Goletti et al., submitted; Colombini et al., submitted). Interestingly, HHV-8 is detected in the circulating monocytes and spindle cell progenitors of KS patients, suggesting that these cells may play a role in virus recruitment into tissues (Blasig et al., 1997; Siriani et al., 1997; Siriani et al., 1998; Colombini et al., submitted; Goletti et al., submitted).

At the lesion level, HHV-8 is present in endothelial and spindle cells mostly in a latent form, whereas mononuclear cells including monocytes-macrophages are lytically infected and may support virus production and spread to other cell types, as suggested by in situ hybridization results showing the recruitment of HHV-8 infected monocytes into KS tissues (Blasig et al., 1997; Stürzl et al., 1997; Stürzl et al., 1998). In fact, although circulating B cells are infected and may represent one of the major reservoir of the virus, they are few or absent in KS lesions, whereas monocytes and T cells are much more abundant. In addition, the virus is lost after culture of E-KSC from the lesions, but it is maintained in the M-KSC cultures derived from the lesions (Siriani et al., 1998).

The question whether extravasation of HHV-8 infected mononuclear cells into the tissue may be the initiating event of KS development or whether these cells are recruited secondarily into an early reactive focus of KS has not yet been solved. However, the higher viral load found in KS patients and in late-nodular lesions (Stürzl et al., 1996) suggest that individuals at risk of KS offer better conditions to virus growth and spread in the body. In fact, our yet unpublished data indicate that the same IC found increased in KS lesions can maintain and rescue virus growth, activate viral lytic replication and increase viral load in B cells and monocytes-macrophages, likely promoting viral transmission to other cell types (Colombini et al., submitted). In addition, increased IC such as γIFN and DR activation can be found in early lesions prior to HHV-8 PCR detection, suggesting that IC are, at least partially, responsible of virus growth and behavior in KS patients and in individuals at high risk to develop KS (Fiorelli et al., 1998).

HIV-1 Tat protein: a progression factor in AIDS-KS. All factors described above including HHV-8 are present in all forms of KS. However, AIDS-KS is more frequent and has a more aggressive course than the other KS forms, including AKS that acquires the most aggressive course after HIV-1 infection. Again, AIDS patients have at least 300-fold higher probability to get KS than individuals with primary immunodeficiency. This suggests that HIV-1 itself may play a role in KS development. Recent studies indicate that the Tat protein of HIV may be responsible for the aggressive nature of AIDS-KS.

Tat is a transcriptional activator of viral gene expression produced early after infection and essential for viral replication. During acute infection of T cells by HIV-1, Tat is released from the cells in an active form and via a leaderless secretory pathway (Ensoli et al., 1993; Chang et al., 1997). Extracellular Tat is capable of inducing the growth, migration and invasion of E-KSC and of IC-activated endothelial cells (Ensoli et al., 1990; Barillari et al., 1993). Tat also induces endothelial cells to express collagenase IV of the 72KD-type that is
known to be associated with angiogenesis and tumor growth. Finally, Tat induces E-KSC and endothelial cell adhesion and stimulates endothelial cells to undergo in vitro morphogenesis (Barillari et al., 1995; Albini et al., 1995). Thus, Tat mimics the effect of ECM proteins such as fibronectin and vitronectin that are known to play a key role in endothelial cell survival, adhesion, growth, invasion, and angiogenesis. However, all the effects of Tat on normal endothelial cells require a previous exposure of the cells to the same IC increased in KS patients and, again, γIFN appears to play a major role in inducing responsiveness to Tat (Fiorelli et al., 1995; Fiorelli et al., 1998; Fiorelli et al., submitted). This is due to both IC induction of the expression of α5β1 and αvβ3 integrins that function as the receptors for Tat and to the induction of bFGF expression that, in turn, induces the same integrins and it is required for Tat-angiogenic effect (Barillari et al., 1993; Fiorelli et al., 1995; Samaniego et al., 1997). In contrast, both α5β1 and αvβ3 integrins and bFGF are constitutively expressed by E-KSC that respond to Tat in the absence of other stimuli (Ensoli et al., 1994a; Samaniego et al., 1995). Consistent with these data, inoculation of Tat alone in nude mice does not lead to angiogenesis, however, when Tat is inoculated in the presence of suboptimal (non lesion forming) amounts of bFGF, it greatly enhances angiogenesis and KS-like lesion formation (Ensoli et al., 1994a). Similar synergistic effects are observed by inoculating mice with combined IC and Tat since IC induce both integrins and bFGF expression. Interestingly, Tat exerts this synergistic effect with bFGF but not with VEGF and recent studies suggest that this is due to the binding of Tat to bFGF-induced integrins (α5β1 and αvβ3) and not to αvβ5 that is induced by VEGF and is involved in its angiogenic pathway.

Tat effects are mediated by two domains the RGD and the basic region. The RGD region of Tat binds the α5β1 and αvβ3 integrins, induces the phosphorylation of the focal adhesion kinase p125 FAK (BE unpublished data) and promotes cell adhesion (when Tat is coated onto plates) or growth, migration, and invasion (when Tat is added to the cells in a soluble form). At the same time, Tat basic sequence binds to heparan sulfate proteoglycans of the cell surface and ECM and releases ECM-bound bFGF. This bFGF is the final mediator of Tat-induced cell growth.

Extracellular Tat is detectable in AIDS-KS lesions (Ensoli et al., 1994a). In addition, endothelial and spindle cells of KS lesions express both bFGF and α5β1 and αvβ3-Tat receptors and extracellular Tat co-stains with these receptors on spindle cells and activated vessels, suggesting that the mechanisms described here are operative in vivo and that Tat may play the higher frequency and aggressiveness of KS in the setting of HIV-1 infection (Ensoli et al., 1994a).

Oncogene expression in KS: BCL-2, a prognostic marker of progression. Recent data indicate that bcl-2 is expressed in endothelial and spindle cells of the lesions from all forms of KS and that its expression increases with lesion stage reaching the maximal levels in nodular lesions. Bcl-2 is also induced during angiogenesis suggesting that its expression may also be related to the angiogenic growth present in KS. The reasons for the induction of bcl-2 are under study, however, preliminary results suggest that the same IC and angiogenic factors present in KS lesions upregulate bcl-2 expression in endothelial and spindle cells. The role of bcl-2 in KS is proven by the results of clinical trials of KS patients with Taxol that have shown disease regression. Taxol, in fact, inhibits bcl-2 function and our unpublished work indicates that it blocks E-KSC growth and KS-like lesion formation in nude mice. Thus, bcl-2 expression coupled with cell growth stimuli may divert cells from apoptosis toward continued cell proliferation and this may represent a step toward the lesion transformation and monoclonality that has been observed in some nodular KS lesions.

References


Barillari G, Gendelman R, Gallo RC, Ensoli B. The Tat protein of human immunodeficiency virus type 1, a growth factor for AIDS Kaposi sarcoma and cytokine-activated vascular cells, induces adhesion of the same cell types by using integrin receptors recognizing the RGD amino acid sequence. Proc Natl Acad Sci USA 1993, 90, 7941-7945.

Barillari G, Fiorelli V, Gendelman R, et al. HIV-1 Tat protein enhances angiogenesis and Kaposi’s sarcoma (KS) development triggered by inflammatory cytokines (IC) or bFGF by engaging the αvβ3 integrin. J Acq Immum Def Synd Hum Retrov 1997, 14, A33.


Ensoli B, Nakamura S, Salahuddin SZ, et al. AIDS-Kaposi’s


Faris M, Ensoli B, Kokot N, Nel AE. Inflammatory cytokines induce the expression of bFGF isoforms required for growth of Kaposi's sarcoma and endothelial cells through the activation of AP-1 response elements of the bFGF promoter. AIDS, in press.


Sirianni MC, Vincenzi L, Fiorelli V, et al. g-interferon production in peripheral blood mononuclear cells (PBMC) and tumour infiltrating long lymphocytes from Kaposi's sarcoma patients: correlation with the presence of human herpesvirus-8 in PBMC and lesional macrophages. Blood 1998, 91, 968-976.


Chemokines are a superfamily of small proteins which play a crucial role in immune and inflammatory reactions and in viral infection. Most chemokines cause chemotactic migration of leukocytes, but these molecules also affect angiogenesis, collagen production and the proliferation of hematopoietic precursors. Based on a cysteine motif, a CXC, CC, C and CX3C family have been identified. The chemokine scaffold consists of an N-terminal loop connected via Cys bonds to the more structured core of the molecule (3 β sheets) with a C terminal α helix. About 50 human chemokines have been identified.

Chemokines interact with seven transmembrane domain, G protein-coupled receptors. Eight CC (CCR1 through 8), 5 CXC (CXCR1 through 5) and 1 CX3C (CX3CR1) receptors have been identified. Receptor expression is a crucial determinant of the spectrum of action of chemokines. For instance, recent results indicate that polarized Th1 and Th2 populations show differential receptor expression and responsiveness to chemokines. Emerging evidence shows that regulation of receptor expression during activation or deactivation of monocytes is as important as regulation of chemokine production for tuning the chemokine system.

In the context of our interest in chemokines and DC, we recently transfected tumor cells with MCP-3. After MCP-3 gene transfer, P815 mastocytoma cells grew, but underwent rejection. MCP-3-elicited rejection was associated with resistance to subsequent challenge with parental cells. MCP-3-elicited rejection was associated with profound alterations of leukocyte infiltration. TAM were already present in copious number, but T cells, eosinophils and neutrophils increased in tumor tissues after MCP-3 gene transfer. DC (e.g. Dec205+, high MHC class II+ cells) did not increase substantially in the tumor mass. However, in peritumoral tissues, DC accumulated in perivascular areas. In contrast with their behavior, in immunocompetent mice, MCP-3-transfected tumor cells grew normally in nude mice. Increased accumulation of macrophages and PMN was evident also in nude mice. Antibodies against CD4, CD8 and IFNγ, but not against IL-4, inhibited rejection of MCP-3 transfected P815 cells. An anti-PMN mAb caused only a retardation of MCP-3 elicited tumor rejection. Thus, MCP-3 gene transfer elicits tumor rejection by activating type I T cell-dependent immunity. It is tempting to speculate that altered trafficking of antigen presenting cells, which express receptors and respond to MCP-3, together with recruitment of activated T cells, underlies activation of specific immunity by MCP-3-transfected cells.

We have recently investigated how signals which induce maturation of dendritic cells affect their migration. These studies were based on parallel efforts in monocytes. Maturation of DC by CD40 ligation, or culture in the presence of inflammatory agonists, such as bacterial lipopolysaccharide, IL-1 and TNF, induces downregulation of the two main CC chemokine receptors expressed by these cells, CCR1 and CCR5, and abrogates the chemotactic response to their ligands, MIP-1α, MIP-1β, RANTES and MCP-3. Inhibition of chemotaxis was rapid (<1h), and included the unrelated agent fMLP. Concomitantly, in the same experimental conditions, the expression of CCR7 and the migration to its ligand ELC/MIP-3β, a chemokine expressed in lymphoid organs, were strongly upregulated, though with slower kinetics (24-48h). Rapid inhibition of responsiveness to chemoattractants present at sites of inflammation and immune reaction may be permissive for leaving peripheral tissues. Conversely, the slower acquisition of responsiveness to ELC/MIP-3β may guide subsequent localization of DC in lymphoid organs.

References

03 Cytokine involvement in pulmonary granulomatous disorders

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The definition of the importance of cytokine patterns in inflammation had a powerful impact on the field of granulomatous disorders affecting the pulmonary tract. In the last three years an enormous amount of detailed molecular and cellular information has been obtained on the cell-to-cell communications which lead to the accumulation of inflammatory cells during granulomas initiated by infectious agents, such as Mycobacterium tuberculosis, or in hypersensitivity lesions which are the result of an exaggerated immunological response against an undefined antigen which has persisted at the sites of disease involvement, such as in sarcoidosis and other multisystemic granulomatous disorders.

The first event in the development of the granulomatous lesions is the accumulation in involved organs of mononuclear inflammatory cells, mostly T cells and monocyte-macrophages which attempt to contain the stimulatory molecule. It is now known that chemotactic molecules molecules cooperate to immobilize leukocytes in perivascular foci of inflammation, thus contributing to the development of the central core of the granuloma. In particular, a positive signal for RANTES has been detected in sarcoid and tubercular lesions, whereas very few positive cells could be detected in the normal residual lymphoid tissue surrounding them or in reactive lymph nodes. Other molecules with lymphocyte-specific chemotactic activity (i.e., IP-10, Mig, I-Tac) have been detected in sarcoid lesions and, more recently, in tubercular granulomas (our preliminary data). Immunohistochemistry performed with an anti-IP-10 antibody in lymph nodes displaying abundant granulomas has shown that cells bearing IP-10 are mainly located inside granulomas. In particular, epithelioid cells and CD68 macrophages are stained, as these cells produce IP-10; in contrast, scattered IP-10 positive cells are present in the peri-granulomatous inflammatory reaction areas. However, IL-16 is expressed in areas where a perivascular accumulation of lymphocytes takes place. Collectively, these data emphasize the role of chemokines in the formation of the central core of the granuloma. It is likely that cell-to-cell and cell-to-matrix interactions accounting for the development of the granuloma modulate the local chemokine expression, contributing to the pathological outcome of the granulomatous lesion.

The different pattern of cytokine production by infiltrating T cells and, in particular, alteration of the Th1/Th2 balance may also influence the evolution of granulomatous inflammation. Lymphokine-producing CD4+ T cells can be subdivided into two broad categories of cells, called Th1 and Th2, based on their lymphokine production. There are data in experimental and human granulomatous disorders suggesting that during the formation of the typical hypersensitivity granuloma a Th1 CD4+ T-cell profile predominates, while the Th2 type is associated with...
impaled granuloma formation and reduced resistance to intracellular pathogens. In particular, sarcoid granulomas express a Th1 pattern of cytokine mRNAs, while tuberculous granulomas express either a Th1 or a Th0 profile. GM-CSF and LT-β mRNAs are more abundant in sarcoid than in tuberculous granulomas, whereas IL-8 mRNA is strongly expressed only in tuberculous lymph nodes. Increased expression of GM-CSF, TNF-α, TGF-β and IL-8 by granulomas correlates with the presence of florid granulomatous lesions, the absence of central necrosis, and the presence of neutrophil infiltration.

However, it is assumed that some granulomas are also characterized by a Th2 pattern. For instance, T cells surrounding granulomas that arise in response to parasite ova chronically release Th2 cytokines while producing small amounts of Th1 cytokines. Differences in the Th pattern may have an impact on the type of granuloma formed, its intensity and the extent of central necrosis. In general terms, we can assume that a Th1 pattern provides the maximum protective immunity to invading intracellular pathogens, including Mycobacterium tuberculosis, while Th2 cytokines are required for the infiltration and activation of eosinophils which contribute to the destruction of soluble, toxic antigens, as in the case of schistosomiasis. The Th1/Th2 pattern is essential not only in determining the type of granulomatous response but also in the regulation of local fibrogenetic processes. A hyperplasia of fibroblasts may be observed in granulomatous lesions, and it is thought that a switch to a Th2 T-cell pattern with concomitant release of IL-4 (a cytokine which is a chemotactic factor for fibroblasts and is able to stimulate the production of extracellular matrix proteins) may help the expansion of the mesenchymal cell population with increased deposition of extracellular matrix components in the environment surrounding granulomatous reactions.

The factors that regulate the type-1 versus type-2 patterns of cytokine production at sites of granulomatous inflammation are poorly characterized. Chemokines foster the formation of type-1 or type-2 granulomatous inflammation. In fact, MCP-1 not only favors the formation of the eosinophil-rich type-2 granulomas but also appears to have a broader role in the regulation of Th differentiation and expression at sites of granuloma formation. Two possible candidates for the regulation of Th patterns are IL-10, which promotes Th2-type immune response via the inhibition of Th1-type reactions, and IL-12 which has pro-inflammatory properties, induces the Th0 vs Th1 shift and stimulates the proliferation and the lytic activity of activated T cells. IL-12, in synergy with IL-15, also favors the contact between activated T cells and antigen presenting cells. Thus, the equilibrium between IL-10, IL-12 and IL-15 may in theory dictate the outcome of hypersensitivity-type granulomatous responses. As a matter of fact, expression of mRNA of the IL-12 p40 subunit and IL-15 can be demonstrated in the lung of patients with active sarcoidosis and tuberculosis.

There is clearly much more to be learned in order to define the regulation of the Th1 and Th2 cytokine patterns at sites of granuloma formation. The development of potential immunosuppressive strategies to inhibit cytokine activity will be a major challenge to researchers involved in the clinical management of patients with granulomatous disorders of unknown etiology.

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References
Accumulation of extracellular matrix proteins is a distinctive feature of progressive renal diseases, however all the events that follow the initial insult leading to glomerulosclerosis and interstitial fibrosis are not fully elucidated. Recently, our understanding of these mechanisms have been improving because of the flourishing of the cellular and molecular biology techniques. Some of these techniques have also been employed to the study of renal biopsies slowly leading toward the elucidation of processes occurring at the cellular and molecular levels. There is increasing evidence that cytokines are important mediators of inflammation and play a central role in the progression of renal disease. Inflammation with activation of resident cells and infiltration of mononuclear cells is a common feature of most nephropathies and both local and infiltrating cells are able to produce cytokines.

Cytokines. Cytokines are a family of molecules with different properties which regulate several aspects of the inflammatory process such as inflammation, cell proliferation and fibrosis. They form a complicate network and their interactions make the understanding of their biological role very difficult. A large body of literature has indicated that cytokines participate to the recruitment of infiltrating cells, some of them play a central role in the local inflammatory response and finally some are important as fibrogenic or healing factors.

Chemokines. A common feature of the majority of renal diseases is cellular proliferation and mononuclear cell infiltrate. Chemokines, such as MCP-1 and RANTES have an important role in the recruitment of blood derived cells. MCP-1 or monocyte chemotactic peptide-1 both attracts and activates monocytes. The expression of MCP-1 has been demonstrated in renal biopsies of patients with membranoproliferative glomerulonephritis secondary to cryoglobulinemia using a combination of immunohistochemistry and in situ hybridization. MCP-1 was expressed by resident cells as well as by infiltrating cells suggesting a role for monocytes and MCP-1 in the pathogenesis of cryoglobulinemia associated nephritis. An upregulation of MCP-1 protein was also observed in the area of focal glomerulosclerosis in a remnant kidney model in rats suggesting that MCP-1 contributes to macrophage infiltration which is a determinant of mesangial matrix expansion in this model. RANTES is a chemoattractant for lymphocytes, macrophages and eosinophils. It has been detected in endothelial and tubular epithelial cells during acute cellular rejection. The upregulation of RANTES may be important in the recruitment of blood cells at site of tissue injury during acute rejection.

IL-1, TNF and IL-6. There are many in vitro and in vivo data which favor a role for IL-1 and TNF in the pathogenesis of renal diseases. High levels of IL-1 and TNF have been demonstrated in isolated glomeruli from experimental nephritis. The expression of these cytokines is also upregulated in ANCA positive systemic vasculitis and in biopsies from renal transplant patients with acute rejection. IL-1 and TNF are not only produced by infiltrating cells but also by mesangial, endothelial and proliferating podocytes suggesting that they may be involved in the development of crescents. IL-6 is a multifunctional cytokine which regulates the immune response, acute phase reaction and hematopoiesis. The finding of severe mesangial proliferation and matrix expansion in IL-6 transgenic mice has prompted several studies on the role of this cytokine in renal disease. IL-6 was shown to be upregulated in IgA nephropathy patients and especially in patients with relevant signs of tubulointerstitial damage. Furthermore lectins present in the serum of IgA patients stimulates the release of IL-6 by mesangial cell cultures suggesting that they may be responsible for the glomerular deposition of IgA complexes.

TGF-β. Transforming growth factor is a pleiotropic cytokine which is important in the healing process. However overexpression of TGF-β induces the accumulation of extracellular matrix components and the development of fibrosis. Glomerulosclerosis is the result of an unbalance between synthesis and degradation of extracellular matrix proteins. In addition to increasing the synthesis of extracellular matrix components TGF-β also reduces its degradation in fact this cytokine upregulates production of plasminogen activator inhibitor downregulating the activity of plasminogen activator thus reducing the activity of metalloproteinases. Furthermore the inhibition of local TGF-β expression via introduction of antisense oligodeoxynucleotides suppressed the accumulation of ECM components in a model of anti-Thy 1 glomerulonephritis.

Conclusions. During the last decade great progresses have been made in our understanding the pathogenesis of renal diseases and on the role played by cytokine as mediators of renal damage. Even if more data are needed, these preliminary experimental studies may lead to new therapeutic approaches in clinical nephrology.

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References


05 Cytokines are "injurins" and cause hepatocyte growth factor release during dialysis

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We have previously shown that dialysis causes a striking rise in HGF serum levels and that serum factors ("injurins") released during dialysis stimulate peripheral blood mononuclear cells (PBMC) to produce HGF. Here we show for the first time in vivo that injurins include TNF-α and IL-6. IL-5, though able to stimulate HGF production in vitro, is not active as injurin during dialysis, suggesting that different injurins may regulate HGF release in different condition.

Hepatocyte growth factor (HGF) was originally identified in serum of partially hepatectomized rats and characterized as a potent stimulator of DNA synthesis in cultured hepatocytes. Subsequent studies have shown that HGF is a pleiotropic factor that induces scattering of cells colonies, angiogenesis, formation of epithelial tubules in vitro and regulates the synthesis of acute-phase proteins. HGF is produced in various organs by cells of mesenchymal origin following tissue injury. Injury to a particular organ induces a marked increase in HGF expression not only in the damaged tissues, but also in distant organs. The humoral factors signalling tissue injury to HGF producing cells are ill-defined substances named injurins. Recent studies suggest that injurins may be cytokines, because IL-1α, IL-1-β and TNF have been shown to stimulate in vitro HGF production. We have previously shown that dialysis causes a striking rise in HGF serum concentration and that serum factors released during dialysis stimulate peripheral blood mononuclear cells (PBMC) and human fibroblasts (MRC-5) to produce HGF. Since during dialysis peak serum levels of HGF and serum injurins activity parallel leukocyte activation and this, in turn, is associated with release of cytokines, we have studied whether cytokines, i.e. IL-1β, TNF and IL-6, act as injurins in serum of dialysis patients.

Bleed was collected from patients on regular dialysis treatment (RDT) and centrifuged. Part of serum was processed for IL-1 beta (Space, R&D System), IL-6 (Space, R&D System) and TNF-α (Bender) measurement by ELISA, and part was preincubated for 2 h with neutralizing anti-IL-1β (Boheringer), anti-IL-6 (Sigma) and anti-TNF-α (Space, R&D System) monoclonal antibodies.
PBMC were isolated from healthy subjects and conditioned with recombinant cytokines: IL-1β (Sigma), IL-6 (Space, R&D System), TNF-α (Sigma) (10 ng/mL). In addition, PBMC were conditioned with serum (10%) of healthy subjects (N) and of patients on RDT, with and without anti-cytokine antibodies. The release of HGF was measured in the supernatant after 48 h of incubation.

PBMC preparation and culture: twenty mL of whole blood were carefully layered over lymphocyte-separation medium (Flow Laboratories, Irvine, Scotland, UK) allowing gradient density centrifugation. Cell pellets were washed twice with RPMI 1640 (Flow Laboratories), counted using a coulter counter and then resuspended at 2×10⁶/mL, in Iscove’s (Flow Laboratories) containing 1% de-complemented fetal calf serum (Gibco), 100 IU/mL penicillin and 100 mcg/mL streptomycin (Gibco). PBMC were placed in polypropylene round bottom tubes at 37°C in 5% CO₂ saturated humidity incubator for 48 hours.

HGF was measured in cell culture supernatant by a commercial EIA (R&D Systems, Minneapolis, MN, USA).

Results show that IL-1β, TNF, IL-6 stimulate significantly PBMC to produce HGF (Figure 1). HGF release from PBMC conditioned with serum of dialysis patients is significantly suppressed by addition of anti TNF (53.54%) and anti IL-6 (42.66%) antibodies. Anti-IL-1β antibody does not contrast the effect of serum of dialysis patients on PBMC (14.69%) (Figure 2).

These results confirm that recombinant cytokines increase HGF release in vitro, and show for the first time that TNF is an effective stimulator of HGF production. In addition, our study gives the first evidence that cytokines act as injurins in vivo, i.e. in a clinical condition characterized by high HGF production. A humoral factor mediating a signal of injury to distal organs was purified from sera of rats with various hepatic and renal injury and proved to be an acid and heat stable protein with an apparent molecular mass of 10-20 kDa. Another factor stimulating HGF synthesis distinct from the previous one was isolated from porcine liver. Previously, we reported that humoral factors in dialysis patients induce HGF production by PBMC. Here we show that these factors include TNF and IL-6, i.e. that these cytokines behave as injurins. It is of interest that IL-1, though able to stimulate HGF production in vitro, is not active as injurin during dialysis, suggesting that different injurins may regulate HGF release in different conditions in vivo.

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References
In this study, we compared the convective and diffusive removal of IL-6 in three different dialytic treatments. Our result demostrate that in hemodiafiltration with polysulfone membrane the cytokine balance through the filter is negative, this effect resulting from the convective removal of IL-6. In conclusion, hemodiafiltration presents a double advantage: the use of syntetic and biocompatible membranes (polysulfone) with consequent low stimulation of PBMC activation and convective removal of circulating cytokines.

Bioincompatibility is a relevant characteristic of hemodialysis treatment; in fact, blood interaction with biomaterials may result in activation of leucocytes and production of cytokines.\(^1,2\)

We have demonstrated an enhanced production of interleukin 6 (IL-6) by peripheral blood mononuclear cells of uremic patients treated with cuprophan membrane,\(^3\) and high levels of plasma IL-6 were found by Herbelin et al. in these patients.\(^4\) IL-6, a pleiotropic cytokine, plays a relevant role in the regulation of the hepatic acute phase protein response to inflammation.\(^5\)

IL-6 removal during hemodialysis (HD) treatment has not been defined. Since IL-6 is peptide with molecular weight of 24 kDa, it may be removed both by membrane absorption and by convective mass transfer across membranes with high permeability.\(^6\)

In this work, we compared the convective and diffusive removal of IL-6 in three different dialytic treatments. The study was carried out in 15 patients (11 male and 4 female) on regular dialysis treatment (three times weekly), each session lasting 240 min. The operative conditions were as follows: 5 patients (dialytic age was 32±15 months) in bicarbonate dialysis with cuprophan membranes (HD-CU), 5 patients (dialytic age was 28±11 months) in bicarbonate dialysis with polysulfone high flux (HDF-PS), 5 patients (dialytic age was 34±13 months) in postdilutional hemodiafiltration with polysulfone high flux (HDF-PS), ultrafiltrate flow rate was 50 ml/min). In each patient two blood samples were simultaneously drawn at the hemofilter inlet and outlet, 60 min after start of dialytic session. Concentrations of IL-6 in plasma were determined by commercially available ELISA kits (RD System, Minneapolis, MN, USA) according to the manufacturers instructions.

Calculations: IL-6 mass transfer through the filter was calculated as the difference between the amount of the cytokine at the filter outlet minus the amount of IL-6 at the filter inlet, using the following formula: IL-6 (pg/min) = \((Q_{po} \times C_{po}) - (Q_{pi} \times C_{pi})\), where Qpi is inflowing plasma flow rate, and Qpo outflowing plasma flow rate, Cpi solute concentration in inflowing plasma and Cpo solute concentration in outflowing plasma.\(^7\)

IL-6 was detected in plasma of all patients and a significant difference was observed between HD-CU (43±14 pg/dL, p<0.01) and HD-PMMA (13±5 pg/dL), and HDF-PS (19.8±6.6 pg/dL).

Figure 1 shows that both in HD-CU and HD-PMMA IL-6 mass transfer is null. In contrast, in HDF-PS cytokine balance through the filter is negative, this effect resulting from the convective removal of IL-6 through the filter membrane from blood to dialysate. In hemodiafiltration, mass transfer of solutes through a membrane depends both on convection and diffusion, and diffusion play a major role in removing low-molecular weight solutes, whereas convection is essential for mass transfer of middle molecular weight solutes, as cytokines.\(^8\) Our results show that only HDF with high flux membrane removes significative amount of IL-6 through convective clear-
Inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor α (TNF-α), have been suggested to play an important pathogenic role in chronic arthritides. Among the various onset types of juvenile chronic arthritis (JCA) several data show that systemic JCA is characterized by prominent production of IL-6 and that overproduction of IL-6 explains the great majority of the clinical and laboratory features of this disease. This suggest that anti-IL-6 therapies may be effective in the treatment of systemic JCA.

A vast amount of evidence has pointed to proinflammatory cytokines, such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor α (TNF-α), as important mediators of inflammation and tissue damage in chronic arthritides. Anti-TNF-α therapies, that showed substantial clinical benefit in adult rheumatoid factor positive rheumatoid arthritis (RA), further supported the pathogenic role of proinflammatory cytokines and demonstrated the feasibility and clinical usefulness of anti-cytokine treatment in chronic arthritides.

The term juvenile chronic arthritis (JCA) encompasses different conditions characterized by chronic arthritis which are distinguished in onset types based on clinical symptoms at onset. In addition to clinical features, immunogenetic data support the concept that the clinical classification identifies different disease entities. While polyarticular and pauciarticular JCA are characterized essentially by joint involvement, systemic JCA (s-JCA) is characterized by the association of chronic arthritis with high spiking fever and with other systemic features, such as evanescent rash, hepatosplenomegaly, lymphoadenopathy, and serositis.

In this paper we review the evidence regarding the role of IL-6 in JCA, with particular emphasis on the role in s-JCA. The previously mentioned clinical features of s-JCA and the prominent laboratory evidence of inflammation characteristic of the disease suggest that increased production of inflammatory cytokines may play a relevant role. Studies on circulating levels of inflammatory cytokines showed that s-JCA is characterized by markedly elevated serum IL-6 levels, much higher than those found in patients with polyarticular or pauciarticular JCA, or with RF+ RA. On the contrary plasma IL-1b levels were found to be lower in s-JCA than in polyarticular JCA, while studies on circulating TNF-α levels have provided contradictory results. The levels of circulating IL-6 in s-JCA were found to be even higher when the amount of cytokine which circulates bound to its soluble receptor (sIL-6R) was determined. Indeed in patients with s-JCA IL-6 circulates in ng/mL amounts as IL-6/sIL-6R complex and IL-6/sIL-6R complex levels are strictly correlated with C-reactive protein (CRP) concentration.

The large amount of circulating IL-6 appears to be the main factor responsible for the extraarticular manifestation of s-JCA. The pivotal role of IL-6 in fever induction has been demonstrated in IL-6-/-/ mice and in patients with s-JCA an increase in circulating levels of IL-6 (2 to 8 fold), but not of IL-1b or TNF-α, precedes the fever peak. Serum IL-6 levels are correlated with CRP concentrations, while IL-1b and TNF-α levels are not, and IL-6 is a major inducer of liver acute phase protein production. Serum IL-6 levels also significantly correlated with platelet counts and IL-6 has been demonstrated to promote
megakariocyte growth and thrombocytopoiesis in vivo. Since IL-6 has a profound effect on bone metabolism (it induces and stimulates osteoclasts and IL-6/- mice do not develop osteoporosis following ovariectomy), elevated production of IL-6 appears also to explain the marked generalized osteoporosis which is a relevant complication of s-JCA.

Patients with s-JCA frequently develop a severe anemia which belongs to the anemia of chronic disease (ACD). In the ACD of RA, the most studied model of ACD, inhibition of erythroid progenitors growth and blunted erythropoietin production appear to be the most important contributors to anemia and are considered secondary to the inhibitory effect of IL-1 and TNF-α. On the contrary, we observed that iron deficient erythropoiesis is the most important determinant of anemia in patients with s-JCA and therefore that the ACD of s-JCA is different from that of adult RA. Prominent IL-6 production may well be responsible for the features of the ACD associated with s-JCA since IL-6 does not inhibit, but rather stimulates hypoxia-induced erythropoietin production and erythroid progenitor proliferation. IL-6 increases ferritin expression and hepatic uptake of serum iron resulting in reticuloendothelial iron block, mice with dysregulated IL-6 expression develop a microcytic anemia, and short-term administration of IL-6 to humans causes a marked decrease in serum iron and an increase in ferritin levels.

Recent data suggest also that prominent production of IL-6 is responsible for the growth impairment which is a frequent complication of severe and long-lasting s-JCA. We have recently found that the IL-6 transgenic murine lines with high levels of circulating IL-6 since early phases of life (NSE/hIL-6 mice) show a growth defective phenotype. This growth defect, that can be reversed by administration of a mAb to the murine IL-6R, is associated with normal growth hormone (GH) pituitary production, but markedly decreased insulin-like growth factor-I (IGF-I) levels, and administration of IL-6 to normal mice causes a decrease in IGF-I levels. IGF-I mediates GH effects in several peripheral organs including muscles and bones, and has a pivotal role in postnatal growth. Similarly to NSE/hIL-6 mice, patients with s-JCA have normal GH production, while levels of IGF-I are decreased. We also found that in patients with s-JCA plasma levels of IGF-I were inversely correlated to serum levels of IL-6.

In addition to explain the extraarticular features of s-JCA, IL-6 appears to be involved in the induction of joint inflammation and damage to articular and periarticular tissues. Through studies in IL-6/- mice, IL-6 was found to be necessary for the development of collagen induced arthritis, and to play a major role in leukocyte recruitment at inflammatory sites. Moreover, IL-6 has been shown to be involved in neoangiogenesis, synovial fibroblast proliferation and, as mentioned in bone resorption. Interestingly, in patients with s-JCA, serum IL-6 levels are directly correlated with the extent and severity of joint involvement. Moreover, although ng/ml amounts of IL-6 are present in synovial fluid from patients with all three JCA onset types, synovial fluid levels of IL-6 are significantly higher in s-JCA than in the JCA onset types or in adult RA.

In summary, IL-6 is measurable in serum and synovial fluid from all three JCA onset types. However, both serum and synovial fluid levels of IL-6 are much higher in patients with s-JCA suggesting that this disease is characterized by prominent IL-6 production. Overproduction of IL-6, but not of IL-1 or TNF-α, appears to explain most of the features of this disease, suggesting that anti-IL-6 therapies may provide a more satisfactory treatment of s-JCA.

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References
In the present study, we used IL-6 knockout mice (IL-6KO, kindly supplied by IRBM, Pomezia, Italy) to evaluate a possible role of IL-6 in the severe inflammatory response characterized by peritoneal exudation. High peritoneal levels of nitrate/nitrite and leukocyte infiltration into peritoneal exudate was induced by zymosan administration in WT mice. Peritoneal administration of zymosan (500 mg/kg, suspended in saline solution, i.p.) in the WT mice induced also a significant increase in the plasma levels of nitrite/nitrate and in the levels of peroxynitrite at 18 hours after zymosan challenge. IL-6KO mice, after zymosan administration, showed a significantly inhibition of the development of peritonitis. Taken together, the present date clearly demonstrate that IL-6KO are resistant to this systemic inflammation model.

Multiple organ failure (MOF) remains a principal cause of death after severe shock or trauma, with or without evidence of sepsis. Administration of zymosan, a nonbacterial, non endotoxic agent, produces acute peritonitis and multiple organ failure in experimental animals characterized by an increase of interleukin 6 (IL-6) associated with functional and structural changes in liver, intestine, lung and kidneys. IL-6 is a multifunctional cytokine that is produced by many different cell types including fibroblasts, macrophages/monocytes, T and B lymphocytes, endothelial cells, synovial cells, keratinocytes, and by a number of tumor-derived cell lines. In the present study we investigated the role of IL-6 on multiple organ failure. To this aim a genetically engineered mice, with lack functional IL-6 were utilized to provide evidence for the involvement of IL-6 in the inflammatory process.

All zymosan-injected mice developed an acute peritonitis, as indicated by the production of turbid exudate (Figure 1A). Trypan blue stain revealed a significant increase in the polymorphonuclear leukocytes in comparison to sham mice (Figure 1B). Nitrite/nitrate concentrations were also significantly increased in the exudate after zymosan challenge (Figure 1C). Sham animals demonstrated no abnormalities in the pleural cavity or fluid. The absence of IL-6 in mice resulted in a pronounced reduction of exudate volumes, leukocyte counts in the peritoneum, and significantly attenuated the zymosan-induced increase nitrite/nitrate exudate levels. The biochemical and inflammatory changes observed in the peritoneal cavity of zymosan-treated mice were associated with a significant elevation of plasma NOx. Nitrate/nitrite levels were significantly elevated in zymosan treated WT mice (110±9 µM) in comparison to control mice (18±5 µM, p<0.01). Injection of zymosan caused a significant increase in the rhodamine fluorescence of plasma, indicative of peroxynitrite-induced oxidation of dihydrorhodamine at eighteen hour after zymosan administration. At 18 hours after zymosan administration, lung and small intestine were investigated for tissue damage by histological examination and MPO activity, indicative of neutrophil infiltration was evaluated in the liver, lung and small intestine. MPO activity and MDA levels were significantly increase in all organs (p<0.01) at 18 hours after zymosan injection. At histological examinations lung, small intestine and liver reveal pathologic changes that correlate closely with the increase in MPO activity and MDA levels. Lung biopsies examination revealed inflammatory infiltration by neutrophil, macrophage and plasma cells. Histological examination of ileum section showed inflammatory infiltration by neutrophil, lymphocytes and plasma cells extending through the wall and concentrated below the epithelial layer. Occasionally focal ulceration, sometimes extending through the muscularis...
dysmetabolic changes paralleled the organ morphological alteration. For example, blood levels of alanine aminotransferase increased from basal levels of 44±6.2 U/L to 221±15.8 U/L at 18 h after zymosan administration (p<0.01). Zymosan-treated IL-6WT mice exhibited also a marked hyperbilirubinemia from basal levels of 0.11±0.04 mg/dL to 1.2±0.14 mg/dL. A significant increase of LDH and phosphatase alcaline was also found at 18 h after zymosan injection. Zymosan-treated IL-6KO mice show a significant reduction of alanine aminotransferase, bilirubine, LDH and phospholipase alkaline blood levels in the after zymosan injection.

Human and experimental MOF is invariably characterized by a well-defined sequence of organ failures, starting with the lungs, progressing to the clotting system, subsequently involving the bowel, the kidney, and ultimately resulting in liver failure. The mortality risk increases with the progression of organ failure. The main finding of the current study is that inhibition of IL-6 (by the use of genetically engineered animals) reduces PMN recruitment and accumulation into inflammatory tissue sites. Activation and accumulation of polymorphonuclear cells (PMNs) is one of the initial events of tissue injury due to release of oxygen free radicals, arachidonic acid metabolites and lysosomal proteases. Macrophage are multifunctional cell that play a central role in host defense. Not only do they process and exert antimicrobial activities they also act as secretory cells during an inflammatory tissue sites. Activation and accumula-

References


09 Effects of macrophage stimulating protein (MSP) on human monocyte/macrophages

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Macrophage stimulating protein (MSP), an 80-kDa serum protein originally identified for its ability to stimulate chemotaxis of murine resident peritoneal macrophages to CSa, is structurally related to the pleiotropic hepatocyte growth factor (HGF) and is the ligand for the heterodimeric tyrosine kinase receptor Ron. Besides the increase of motile and phagocytic activity in mouse resident peritoneal macrophages, MSP inhibits nitric oxide production in mouse macrophages. It also stimulates human and murine bone marrow megakaryocytopoiesis and induces proliferation and migration of murine keratinocytes as well as of epithelial cells. Recently, an altered inflammatory response was observed in knock-out mice lacking the STK/RON gene. The possible effects of MSP on human macrophages and its role in human pathophysiology is still elusive. Therefore, we evaluated the effects of MSP on different human macrophage populations: alveolar macrophages (AMs), peritoneal macrophages (PMs) from ascitic fluid of three cirrhotic patients; human monocyte-derived macrophages (MDMs). AMs were isolated from broncho-alveolar lavage and purified by adherence; PMs were obtained from ascitic fluid of three cirrhotic patients; human monocytes (HMs) were isolated from heparinized venous blood by standard procedure and purified by adherence; MDMs were prepared from monocytes cultured for 7-8 days (37°C, 5% CO2, RPMI 1640 containing 10% heat-inactivated calf serum and antibiotics) as described. Superoxide anion (O2−) production was selected as a parameter of cell activation and measured spectrophotometrically by evaluating SOD-inhibitable cytochrome C reduction. Recombinant MSP was obtained by the baculovirus expression system in conditioned medium of cultured insect cells. Although adherent HMs showed detectable RON mRNA expression, no significant O2− production was observed upon challenge with increasing concentrations of MSP. On the contrary, AMs, PMs and MDMs exhibited a significant respiratory burst in response to MSP along with RON mRNA expression. MSP was active in a dose-dependent manner on the three human macrophage populations evaluated, being the maximal effect observed at 1:3 dilution of the conditioned medium and still active at 1:500 dilution. O2− production was the highest in PMs and above the level obtained with standard stimuli, (e.g., PMA, FMLP). In the same cells, HGF was inactive. These results indicate that human macrophages of different origin express the RON gene and are activated by its ligand MSP. Interestingly, MSP does not evoke O2− production by HMs, but is active on the different human macrophage populations evaluated; therefore, it can be regarded as an activator of mature macrophages.

References


We have identified a new gene, glucocorticoid-induced leucine zipper (GILZ), encoding a member of the leucine zipper family. GILZ overexpression is induced by dexamethasone (DEX)-treatment in thymocytes and peripheral T cells. Furthermore GILZ expression selectively protects T cells from apoptosis induced by treatment with anti-CD3 monoclonal antibody. This antiapoptotic effect correlates with inhibition of Fas and Fas ligand expression and with the inhibition of IL-2/IL-2R system.

Apoptosis (programmed cell death, PCD) is involved in cell and tissues development as well as in the control of neoplastic growth. A number of stimuli can either induce or inhibit lymphocyte PCD through activation of molecules acting at different levels including cell membrane, cytoplasm and nucleus. The definition of the signalling pathways involved in the control of apoptosis has important implications to understand normal tissue development and drug resistance. In the T-cell lineage, several pathways have been identified which regulate apoptosis negatively or positively. In particular, apoptosis activated through the antigen (Ag) interaction with the T-cell-receptor (TCR)/CD3 complex is responsible for negative selection. Engagement of the TCR/CD3 complex, (either by APCs presenting antigenic peptide or by anti-CD3 monoclonal antibody, mAb), cytokines, coaccessory molecules and tissue microenvironment trigger a series of activation events which can contribute to regulate cell survival. Elevation of intracellular Ca2+, protein phosphorylation/dephosphorylation, upregulation of the antioxidant glutathione, expression of bcl-2/bcl-x and Fas/Fas ligand (Fas/FasL) systems, and activation of transcription factors, such as for instance NF-kB, myc, fos and jun, profoundly influence T-cell apoptosis. Some of these signals can induce apoptosis in thymocytes, mature T cells and T-cell hybridomas. In particular, activation of T-cell hybridomas leads to cell cycle arrest, followed by apoptosis. This activation-induced cell death (AICD) requires the interaction of Fas with FasL. Among different stimuli, glucocorticoid hormones (GCH) are also critical regulators of T-cell development. In particular, dexamethasone (DEX), a synthetic GCH which by itself induces apoptosis in T-cell hybridomas and in normal T lymphocytes, can inhibit AICD. This inhibition may be due to a number of events including prevention of activation-induced expression of FasL.

To study the role of GCH in the regulation of lymphocyte apoptosis, we attempted to define the molecular events which are induced by DEX and modulate apoptosis in T cells. We report here that GCH induce the expression of GILZ gene (GILZ, for: Glucocorticoid Induced Leucine Zipper), coding for a novel member of the leucine zipper family. Our results indicate that GILZ gene is induced in thymocytes and peripheral T cells by DEX treatment. Furthermore, we show that GILZ expression selectively protects T cells from apoptosis induced by treatment with anti-CD3 mAb but not by treatment with other apoptotic stimuli. This effect correlates with inhibition of anti-CD3-induced upregulation of Fas and FasL systems (Figure 1). To evaluate whether GILZ is involved in T cell activation, we examined IL-2 receptor expression and IL-2 production after stimulation of GILZ-transfected clones with cross-linked anti-CD3 mAb. Results show
that GILZ-transfected clones express low or undetectable IL-2Rα and low levels of IL-2.

These data suggest that GCH-induced GILZ expression, by downregulation of IL-2/IL-2R and Fas/FasL system, could be one of the mechanisms by which T cells escape to AICD.

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References

11 Cytokines and cancer
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We report here about the proliferative effect of the cytokine nerve growth factor (NGF) upon MCF-7 human breast cancer cell line. The effect of NGF is comparable to that of other members of the cytokine/growth factor family, such as epidermal growth factor, insulin-like growth factor-II, prolactin, as well as to the effects of estradiol. The proliferative effect of NGF is probably mediated by its high affinity receptor p140<sup>trka</sup>, which belongs to the family of the tyrosine kinase receptors and is expressed in MCF-7 cells. We conclude that NGF may be involved in the cytokine/growth factor regulation of breast cancer growth.

A number of substances of the cytokine/growth factor family promote cancer development and growth. Their proliferative effects are mediated by specific receptors belonging to different superfamilies. Growth factors may reciprocally influence the effects of other substances of the family on cell proliferation, as well as of peptide and steroid hormones. Thus, cytokines play a relevant role in hormone-dependent cancer growth.1

Breast cancer is the most common cause of tumour-related death among women in western countries.2,3 Breast cancer growth is promoted by estrogen,4 epidermal growth factor (EGF),5 insulin-like growth factors (IGF) I and II,6 as well as by the lactogenic hormone prolactin (PRL).7 However, each single factor does not seem sufficient to promote cell transformation and growth per se, but co-participation of a number of them is necessary. Among cytokines and growth factors, nerve growth factor (NGF) plays a role in neuronal growth and repair,8 and has been shown to participate to physiological regulation of tissue functions other than in the nervous system.9 For example, NGF has been regarded as a paracrine factor mediating the cross-talk between stromal and epithelial cells in sex steroid responsive tissues.10 In addition, NGF receptor proteins are expressed in human prostate cancer10 and melanoma cell lines.11

We have studied the possible impact of NGF on the proliferation of the estradiol-responsive human breast cancer cell line MCF-7, and the reciprocal role to estradiol-17-β (E<sub>2</sub>), PRL, EGF, and IGF-II. In addition, we have evaluated the expression of NGF high affinity receptor p140<sup>trka</sup> and the NGF low affinity receptor p75 in the same cell line.

MCF-7 cells were incubated for seven days with graded concentration of E<sub>2</sub>, ovine PRL, EGF, IGF-II, or NGF. All the substances promoted MCF-7 cell growth in a concentration-dependent manner. Maximal effects were observed at day 6 of incubation. The relative EC<sub>50</sub>s were 24.2 ng/mL for PRL, 10.77 ng/mL for EGF, 5 ng/mL for IGF-II, and 7.12 ng/mL for NGF, respectively. The most powerful among substances were IGF-II and NGF (Figure 1, panel A). The expression of the NGF high affinity receptor p140<sup>trka</sup>, as assessed by western blot analysis, was significantly higher in MCF-7 cells than in MCF-10 cells, a line derived from human normal breast tissue (Figure 1, panel B). On the other hand, immunohistochemical analysis for the NGF p75 low affinity receptor was negative in both cell lines.

In conclusion, it appears that NGF is able to stimulate MCF-7 cell growth to an extent similar to oth-
er breast cancer growth factors. The effect of NGF could be mediated by its high affinity receptor, which are present in MCF-7 cells. In addition it is plausible to hypothesize that the expression of the p140trka NGF receptor is related to the state of differentiation of human breast cells. Finally, we speculate that NGF is among factors of relevance in differentiation and growth processes related to breast cancer.

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References
4. Welsh CW. Host factors affecting the growth of carcinogen induced rat mammary carcinomas: a review


12 Procoagulant activity of tumor cells and its modulation by different cytokines

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We have studied the procoagulant activity of different human tumor cell populations and the effects of IFNa, IFNγ and TNFα on this activity. Tumor cells cultured in vitro possessed a tissue factor-like procoagulant activity, while cells isolated from human tumor tissues showed a factor VII independent activity with the characteristics of cancer procoagulant. IFNa, IFNγ and TNFα were able to increase the procoagulant activity in some tumor cell populations, but not in others. These data demonstrate that tumor cells may promote blood clotting through different mechanisms and that the investigated cytokines may influence the expression of such activities.

The interactions of tumors with the hemostatic system are considered important in the pathophysiology of hemorrhagic and/or thrombotic complications of patients with cancer and may play a role even in tumor growth and dissemination.1-3 Generation of thrombin through tumor cell procoagulant activity is a key event in such interactions.4 Cytokines are peiotropic mediators of inflammation and immunity that can deeply affect the hemostatic balance5-9 and have therapeutic poten-
TNF is known to induce the expression of tissue factor in endothelial cells, while IFN-γ was shown to amplify endothelial cell responses to TNF, but it is not known whether they can influence the procoagulant activity of cancer cells.

In the present investigation we studied different human tumor cell lines (Table 1) and cells isolated from 22 human tumor tissues (Table 2).

### Table 1. Effect of human tumor cells (2x10^6/mL) cultured in vitro on the recalcification time of different human plasma substrates.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Normal plasma</th>
<th>Factor VII deficient plasma</th>
<th>Factor X deficient plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1402</td>
<td>69-85</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Me 7110/2</td>
<td>34-50</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Hep G2</td>
<td>48-63</td>
<td>232-300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>GLC1</td>
<td>88-107</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>42-49</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Lung adenocarcinoma</td>
<td>38-47</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>HBSS</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
</tbody>
</table>

*Range of values obtained with duplicate measurements in 3 different experiments.

### Table 2. Effect of cells (2x10^6/mL) isolated from human tumor tissues on the recalcification time of different human plasma substrates.

<table>
<thead>
<tr>
<th>Tumors</th>
<th>Normal plasma</th>
<th>Factor VII deficient plasma</th>
<th>Factor X deficient plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td>17-69</td>
<td>35-78</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Breast</td>
<td>25-58</td>
<td>45-79</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Stomach</td>
<td>46-89</td>
<td>81-102</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Lung</td>
<td>28-58</td>
<td>40-83</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Esophagus</td>
<td>24-29</td>
<td>53-58</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Liver</td>
<td>41-47</td>
<td>65-69</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Kidney</td>
<td>28-35</td>
<td>40-46</td>
<td>&gt;300</td>
</tr>
<tr>
<td>HBSS</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
</tbody>
</table>

*Range of values obtained with duplicate measurements in 3 different experiments.

Tumor cells cultured in vitro were able to shorten significantly the recalcification time of human plasma and the effect was not present in factor VII or factor X deficient plasma (Table 1). Concanavalin A, a tissue factor inhibitor, abolished the activity almost completely, while the cysteine protease inhibitors iodoacetamide or HgCl₂ had a small inhibiting effect. These data suggest that such activity was due to tissue factor.

On the contrary, tumor cells isolated from tumor tissues possessed a high procoagulant activity, which was present in both normal plasma and in factor VII deficient plasma, but not in factor X deficient plasma (Table 2). The activity was slightly inhibited by concanavalin A, while iodoacetamide and HgCl₂ had a potent inhibiting effect. Therefore, cells derived from disaggregated tumor tissues promote blood clotting through a direct activation of factor X with the characteristics of cancer procoagulant. In subsequent experiments we studied the effects of IFN-α, IFN-γ and TNFα on the procoagulant activity of two tumor cell lines cultured in vitro (mesothelioma and adenocarcinoma) and of cells dissociated from 6 human tumor tissues. Short-term (3 h) incubation of tumor cells cultured in vitro with cytokines (5x10^5/mL) did not modify their procoagulant activity. After longer incubation (48 h in culture) IFN-γ induced a significant increase in the procoagulant activity of mesothelioma cells, while IFN-γ induced an increase in the procoagulant activity of lung adenocarcinoma cells. Furthermore, short-term incubation of cells isolated from tumor tissues with IFN-γ or TNFα resulted in a significant increase of procoagulant activity, while IFNα had no effect.

In conclusion, our data demonstrate that tumor cells possess different procoagulant activities and that IFNα, IFN-γ and TNFα may influence their expression.

### References

10. Mulder AB, Zwaveling JH, Smid WM, et al. Augment-

13 The role of apoptotic, anti-apoptotic molecules in CD30+ cutaneous lymphomas

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This study analyses the expression pattern of apoptotic and anti-apoptotic molecules in CD30+ primary cutaneous T-cell lymphoproliferative disorders.

The expression of the CD30, a member of the tumor necrosis factor receptor (TNF-R) Superfamily, identifies a subtype of large cell non-Hodgkin’s lymphomas (ALCLs), with anaplastic cytology, and a spectrum of primary cutaneous T-cell lymphoproliferative disorders (CD30+ PCTCLDs), including anaplastic large cell lymphoma (ALCL), lymphomatoid papulosis (LyP) and borderline lesions between LyP and ALCLs. Primary cutaneous CD30+ LCLs pursue a better clinical course than morphologically similar, but lymph-node based cases. Likewise, LyP and borderline cases have a very favorable outcome, with complete spontaneous regression of the cutaneous lesions. The presence of apoptotic cells in CD30+ PCTCLDs has been morphologically described, and apoptosis may be one of the mechanisms responsible for the clinical regression of the skin lesions.

The lymphoma growth may be functionally regulated by receptors of the TNF-R Superfamily, which binding with specific ligands (cytokines) triggers various functional processes, i.e. CD95-mediated apoptosis. In normal and neoplastic haematopoietic cells, CD95 seems to have an antagonistic role with respect to other anti-apoptotic factors, as the bcl-2 oncoprotein. We analyzed 25 cases of CD30+ PCTCLDs in order to: a) document in situ the apoptotic rate; b) to evaluate the expresional and distributional pattern of bcl-2 and various members of TNF-R Superfamily, only CD95 is uniformly expressed in CD30+ PCTCLDs, suggesting its role in the functional mechanisms of spontaneous regression of the skin lesions; conversely, bcl-2 seems to have an antiapoptotic function also in CD30+ PCTCLDs.

However, the abundance of reactive tumor infiltrating lymphocytes (TILs), strong reactivity for TIA1 in both atypical CD30+ cells and TILs may suggest that local production of cytotoxic molecules can mediate self-destruction of the cutaneous lesions.

References

14 Reduction of growth ability and tumorigenicity of IL-4 transfected TS/A adenocarcinoma line is mediated by increased adhesion molecule expression

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TS/A-IL4 cell line is a mouse mammary adenocarcinoma engineered by retroviral infection to release...
IL4.\textsuperscript{1,2} Gene transfection causes rejection of tumor cell growth in Balb/c mice, in part due to IL4 induced eosinophil recruitment during the early phase of tumor growth after in vivo implantation. TS/A adenocarcinoma is an heterogeneous cell line, constituted by two cell histotypes, one with a fibroblast and the other with a spheroid-epithelial shape.\textsuperscript{3}

The aim of the present study was to analyse the rearrangement of the cell heterogeneity, the growth ability and in vitro adaptation of TS/A and TS/A-IL4 lines on different substrates and to analyse the mechanism of in vivo growth rejection at advanced stages of tumour growth. The study was conducted by analysis of cell cycle and of the expression of ICAM-1,\textsuperscript{4,5} CD71 and E-cadherin\textsuperscript{6} by flow cytometry. Histology analysis of tumour parenchyma of primary tumour of both lines in vivo and the study of cell growth on laminin, fibronectin, collagens I and IV and matrigel compared to plastics in vitro was done as well. At microscope examination, following in vitro cultivation on plastics, TS/A and TS/A-IL4 cell lines, show that the two cell types are differently represented; even though TS/A-IL4 reaches confluence, it grows in vitro more slowly than TS/A line and shows a marked increase of the more differentiated cell type, expressing less CD71 receptor (mean channel 1.9 vs 4.4) and more adhesion molecules such as ICAM-1 (71.1\% vs 7.4\%), E cadherin (42\% vs 17.4\%) and resting cells (G0/G1 75.6\% vs 25.8\%; S-G2M 16\% vs 70.4\%). Interestingly, large TS/A cells of the parental line, that do not express ICAM-1 in standard experimental conditions, when challenged with conditioned medium containing IL4 (169±19 pg/mL) result 60\% CD54\textsuperscript{+}.

Table 1. Comparison of in vitro and in vivo growth of TS/A and TS/A-IL4 cells.

<table>
<thead>
<tr>
<th></th>
<th>TS/A</th>
<th>TS/A-IL4</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro growth\textsuperscript{a} (x106cell/ml)</td>
<td>3.18±0.22</td>
<td>1.44±0.39*</td>
</tr>
<tr>
<td>Large/small ratio</td>
<td>1.47±0.22</td>
<td>0.55±0.06*</td>
</tr>
<tr>
<td>CD54*</td>
<td>33.6±3</td>
<td>51.9±1.1*</td>
</tr>
<tr>
<td>In vivo growth\textsuperscript{b} (mg)</td>
<td>128.7±24.8</td>
<td>4.16±0**</td>
</tr>
</tbody>
</table>

\textsuperscript{a}equal amount of cells were seeded on plastic plates and harvested at 72 h; \textsuperscript{b} equal amount of cells were i.m. injected and primary tumor weight measured on day 10 (75\% tumour takes); \textsuperscript{\*p=0.01} Values statistically different from TS/A, Computerized analysis Instat 2.

The ratio between large and small cells and the ICAM-1 overall expression of TS/A and TS/A-IL4 adenocarcinoma are subjected to marked modifications depending on the substrate on which the two lines are forced to grow with no relationship with the IL4 release per cell unit, which in turn is much more dependent on the substrate on which cells are grown (collagen I vs other substrates). In vivo late tumor rejection is dependent upon a marked increase of fibroblast-derived collagen\textsuperscript{7} and extracellular matrix proteins and an increased degranulation of mast cells. The co-injection of 105 TS/A and 105 TS/A-IL4 cells induces the growth of a tumour in all the injected mice, but clearly and statistically smaller than that obtained with TS/A alone. The overall result is that gene modified TS/A-IL4 line shows marked changes of behaviour, greater than those expected by the simple induction of IL4 release. The stimulation to IL4 production by tumour cells induced by type I collagen suggests that tumour cells in vivo may have a strong stimulus to IL4 production, with induction of tumour rejection, in any tissue in which type I collagen is present.

Acknowledgements

G. Forni for having provided TS/A cell lines and Ms. M. Vadori for the helpful contribution to in vitro studies. M. Magnarin and M.E. Carotenuto are supported by research grants from Cassa di Risparmio-Fondazione di Trieste.

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References


15 Effects of nerve growth factor and retinoic acid on enhanced vascular endothelial growth factor mRNA and protein levels, and proliferation of the human glioma cell line U373 MG

Laurence Lempereur, Giovanna Zappalà, Renato Bernardini*
The expression of vascular endothelial growth factor (VEGF) is related to brain tumor angiogenesis. Glioblastoma, the most common and most malignant brain tumor in humans, is characterized by the presence of multifocal necrosis and vascular proliferation. We report here that U373 MG human glioma cells do not express VEGF mRNA constitutively, and that cell overconfluence eventually enhances its expression. We show that VEGF mRNA expression is down-regulated in vitro by NGF and retinoic acid, suggesting that both substances may play a role as inhibitors of angiogenesis in human brain tumor of glial origin.

Neovascularization is essential for solid tumor growth. The progression from a benign, avascular astrocytoma to a malignant, highly vascularized glioblastoma multiforme is thought to be regulated by tumor cell-produced angiogenic factors. The expression of vascular endothelial growth factor (VEGF) has been shown to be the major regulator of tumor angiogenesis in vivo. VEGF expression is up-regulated in different subset of glioblastoma cells in vivo. Several factors and mechanisms regulate VEGF gene expression at the transcriptional and/or stabilization levels, among these, hypoxia, hypoglycemia, different cytokines and growth factors. Both retinoic acid (RA) and NGF exert effects on proliferation, differentiation and gene expression of specific cells. NGF is a pleiotropic cytokine essential to development and survival of neuronal cells, and also induces growth and differentiation of non-neuronal cells. In addition, RA has been shown to regulate both expression of NGF receptors and sensitivity in the rat tumor cell line PC12 to NGF8.

Thus, it appeared of interest to investigate: 1) whether VEGF mRNA was constitutively expressed in U373 MG human glioma cell line; and 2) whether combined treatment with NGF and RA could modulate enhanced expression of VEGF and proliferation in the same cell line.

Levels of VEGF mRNA have been analyzed by means of a specific quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Competitive amplification of the cDNA corresponding to VEGF mRNA was performed in the presence of an internal competitor designed to amplify with a set of VEGF primers. Analysis of VEGF mRNA was performed in U373 MG cell cultures at different stages of growth (day 2, 4, 6, 8, 10, and 12 of culture). VEGF mRNA level increased proportionally to cell density, reached a maximum at day 10, in overconfluent cultures, and decreased after day 11 (Figure 1).

In other experiments performed in subconfluent cultures, we assessed the time-related effect of NGF (10 ng/mL) upon VEGF mRNA levels. A significant reduction of the level of VEGF mRNA occurred after 4 h incubation with NGF, whereas minimal levels were reached after 16 h incubation, and lasted up to 48 h (Figure 1). VEGF mRNA levels were decreased after 24 h treatment with graded concentrations of NGF (range: 0.1-100 ng/mL). The effect of NGF was concentration-dependent. Minimal effective concentration of NGF was 0.1 ng/mL. The effect of NGF reached a plateau at the concentration of 1 ng/mL. Incubation with 1 mM RA (all trans) resulted in a significant decrease of VEGF mRNA levels in U373 MG cells. The effects of treatments with NGF and retinoic acid observed at the mRNA level, have been assessed on intracellular and secreted VEGF protein. Modulation of VEGF protein expression was appreciated by western blot analysis after 72 h of treatment.

The possible antiproliferative effects of RA and NGF were studied in U373 MG human glioma cell cultures incubated for 7 days with different concentrations of both substances. RA significantly inhibited U373 MG cell proliferation after 7 day treatment. On the other hand, the effect of NGF on U373 MG human glioma cells was biphasic.

Our results indicate that U373 MG human glioma cells do not express VEGF mRNA constitutively, and that its expression is eventually enhanced in overconfluent cultures. Beside their mild antiproliferative effects, NGF and RA negatively modulate VEGF mRNA and protein expression in U373 MG human glioma cells in vitro. Finally, we speculate that treat-
ments with NGF and retinoic acid could be proposed for their efficacy in the antiangiogenic treatment of human gliomas.

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References

The interaction between nitric oxide and prostaglandins has been studied in different experimental models of acute inflammation in the rat. The results indicate that the modulation of NO pathway results in a parallel modulation of prostaglandin biosynthesis.

Nitric oxide (NO) can be considered as a modulator of both acute and chronic inflammation. It has been suggested that the role of NO in inflammation may depend on its own ability to increase vascular permeability and edema formation possibly by increasing local blood flow. Another possible mechanism of action of NO as pro-inflammatory mediator could be related to the modulation of prostaglandin (PG) generation. We have studied the effect of either the inhibition or stimulation of the NO pathway on PG biosynthesis in several models of acute inflammation in the rat.

Arachidonic acid-induced paw edema was dose-dependently increased (Figure 1) by the NO-donor 3-morpholinosydnonimine hydrochloride (SIN-1) and this increase was correlated to the amount of 6-keto-PGF1α present in the oedematous fluid. In LPS-treated rats (6 mg/kg/i.p.) doses of arachidonic acid (150 and 300 nmol/paw) inactive in untreated animals, produced a remarkable edema which was well correlated to the amount of 6-keto-PGF1α. Both the edema and prostaglandin generation induced by 300 nmol arachidonic acid were reduced (52 and 65%) by the NO synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 100 nmol/paw) or (45 and 49%) by the NO scavenger hemoglobin (Hb, 30 mmol/paw) whereas it was not modified by the soluble guanylate cyclase inhibitor methylene blue (Mb, 3 mmol/paw). The substrate for NO biosynthesis L-arginine (15 mmol/paw) increased by 62% the edema and by 33% the 6-keto-PGF1α generation.

In carrageenin-induced paw edema, either the severity of the edema or the PGE2 amount recovered from the oedematous fluid were both reduced by L-NAME (49 and 53%) or Hb (41 and 40%), increased by L-arginine (43 and 4%) and unaffected by Mb.

More recently we have investigated the correlation between endogenous NO generation and prostaglandin E2 (PGE2) biosynthesis in rat carrageenin pleurisy. L-NAME (1-3-10 mg/kg/s.c., 1 hr prior carrageenin injection) inhibited the amount of NO2- + NO3- (NOx) in the pleural exudate by 20, 40, 55% and PGE2 by 10, 41, 74%. Conversely L-arginine (300 mg/kg/s.c., 1 hr prior carrageenin injection) increased NOx by 39% and PGE2 by 78%. The NO scavenger Hb (3 mg/site), injected in the pleural cavity concomitantly with carrageenin, produced a parallel inhibition of the exudate amounts of NOx (65%) and PGE2 (71%). Mb (2 mg/site) did not affect either NOx or PGE2 generation.

These results suggest that in acute inflammation the modulation of NO pathway results in a parallel

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Second session
INFLAMMATION

16 Interaction between nitric oxide and prostaglandins in inflammation
LIDIA SAUTEBIN, ARMANDO IALENTI, ANGELA IANARO, MASSIMO DI ROSA
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The modulation of prostaglandin biosynthesis. Thus the interaction between cyclooxygenase and NO pathway may represent an important mechanism for the modulation of the inflammatory response.

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References

The adhesion of human neutrophils to endothelial cells: the role of GAS6
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GAS6 is a ligand for the transmembrane tyrosine kinase receptors Axl, Rse, and Mer and is highly expressed by vascular endothelial cells. The aim of the work was to evaluate the GAS6 effect on human neutrophil adhesion to vascular endothelial cells in vitro. The results, obtained by using recombinant human GAS6 and soluble forms of Axl and Rse, point to a modulatory role of GAS6 in vascular endothelium adhesiveness.

GAS6, a protein encoded by the growth-arrest-specific gene 6, is a ligand for the transmembrane tyrosine kinase receptors Axl, Rse and Mer and displays 44% identity with serum protein SL. Despite the many efforts to characterize it, GAS6’s biological activity is still poorly understood. In NIH 3T3 cells, the initial identification of GAS6 as a protein expressed in response to growth arrest suggested that it may function as a negative regulator of cell proliferation. Subsequent observation that hyperexpression of Axl and Rse has transforming capacity in several cell types suggested that GAS6 acts as a growth factor. However, this activity was always weak or limited to potentiation of other activating stimuli. The observation that both GAS6 and Axl are highly expressed by vascular endothelial cells prompted our evaluation of the GAS6 effect on endothelium adhesiveness.

Experiments were performed using fluorescein-labeled human neutrophils (PMNs) and endothelial cells isolated from human umbilical vein (HUVEC). Adhesion was measured by computerized microimaging fluorescence analysis. HUVEC treatment with GAS6 significantly inhibited PMN adhesion evoked by IL-1β, PAF, PMA, thrombin and TNF-α (activating both on PMNs and HUVEC), but not that induced by IL-8 and FMLP (acting only on HUVEC). GAS6 did not affect adhesion to resting HUVEC. The GAS6 effect was performed on HUVEC and not PMN, because it did not inhibit PMN adhesion to serum-coated plastic wells. Moreover, inhibition was still detectable when HUVEC were pretreated with GAS6 and washed before PMN seeding. Confocal microscopy experiments showed that in resting HUVEC GAS6 displays a perimembrane distribution that is lost after PAF treatment. To evaluate the effect of endogenous GAS6 in resting HUVEC, soluble forms of Axl or Rse were added in the adhesion assays. These experiments showed that both Axl and Rse substantially potentiated PMN adhesion to resting HUVEC, which suggests that endogenously produced GAS6 works as an antiadhesive molecule. This hypothesis prompted our evaluation of the role played by endogenous GAS6 in experimental conditions mimicking ischemia/reperfusion, that increases endothelium adhesiveness. HUVEC were treated with 95% N₂ plus 5% CO₂ for 120 min, moved to room air and used in PMN adhesion assays in the presence and absence of soluble AXL or Rse. Hypoxic treatment substantially potentiated PMN adhesion
Importantly, i.v. administration of glucocorticoids reduced by hypoxia. These results further support the role played by endogenous GAS6 in vascular endothelium adhesiveness.

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References

18 Glucocorticoids, lipocortin 1, and neutrophil activation
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Lipocortin 1 is an anti-inflammatory protein induced by glucocorticoids which inhibits activation of phospholipase A2. On the basis of experimental evidence we propose that lipocortin 1 is an endogenous inhibitor of neutrophil activation in addition to its antiglycophospholipase properties. A lipocortin 1 N-terminus peptide (Ac2-26) mimics the inhibitory effects of the protein and exerts anti-inflammatory activity in vivo.

Lipocortin 1 (LC1, also termed annexin 1) belongs to a superfamily of related proteins with a high homology at the level of the primary sequence, and which all share capacity of binding for acidic phospholipids and calcium ions. There are at the moment at least 13 members of the lipocortin (or annexin) family, however LC1, and also lipocortin S, seems to be the most relevant to the physiology-pharmacology of inflammation.

Almost two decades ago LC1 was discovered and characterized by its ability to suppress eicosanoid generation via an effect upon phospholipase A2. The purified protein mimicked the action of glucocorticoid hormones and, indeed, endogenous LC1 appeared to be responsible for several aspects of the anti-inflammatory effect of these potent drugs. The action upon arachidonate and eicosanoid release in vitro was accompanied by an inhibitory effect in experimental models of inflammation in vivo. More recently it has been shown that LC1 exerts potent inhibitory effects upon neutrophil accumulation in experimental models where endogenous eicosanoids were not involved: it appeared, then, that LC1 was somehow affecting neutrophil responsiveness. In these models, the anti-migratory action of desamethasone (DEX) was also abrogated by passive immunisation of pouch with neutralising anti-LC1 antisera. This line of research was boosted by the discovery that a peptide spanning most of the LC1 N-terminus (peptide Ac2-26) was also a potent inhibitor of IL-1α, IL-8 and substance P (SP)-induced neutrophil accumulation. Quantification of neutrophil adhesion to endothelial monolayers in static conditions showed that peptide Ac2-26 displayed inhibitory action when adhesion was obtained by neutrophil stimulation but not when it was the result of stimulation of the endothelial monolayers (with histamine or PAF). These data identify the neutrophil as a novel target for LC1 and peptide Ac2-26 and are in agreement with the observation made in vivo, that these agents had to interact with circulating neutrophils (i.e. prior to their extravasation) to reduce their migration in experimental inflammation; hence, LC1 and peptide Ac2-26 are effective when given systematically, but not locally at the site of inflammation.

The hamster cheek pouch microcirculation model was used to investigate the mechanism of action of LC1 and peptide Ac2-26 on neutrophil migration. Treatment of hamsters with an anti-inflammatory dose of DEX affected neither SP- nor formyl-Leu-Met-Phe (FMLP)-induced leukocyte rolling onto the endothelium, nor the extent of leukocyte adhesion induced by either inflammatory stimulus. However, treatment with the steroid altered the fate of the adherent leukocytes. Adherent leukocyte will occasionally detach and return to the blood stream rather than emigrate through the endothelium. This is a physiological phenomenon (which indicates the existence of a negative control system) which brings about the detachment of some 15-20% cells after application onto the microvasculature of chemoattractants like SP, FMLP or PAF. Treatment of hamsters with DEX greatly increased the rate of detachment (up to 70%); in addition, those leukocytes which eventually emigrated through the endothelial cell junctions, required much more time to complete the diapedesis process. These two effects of DEX upon the fate of adherent leukocyte were abolished by an anti-LC1 antibody. Importantly, i.v. administration of high doses of peptide Ac2-26 also pro-
longed the time required by adherent leukocytes to begin the diapedesis process.

These studies show that the progression of leukocyte adhesion to migration is affected by LC1. Since this protein is the most abundant lipocortin in human neutrophils (2–4% of cytosolic proteins are molecules of LC1), potential alterations in localization of endogenous LC1 in relation to the state of neutrophil activation were sought with a series of in vitro experiments which focused on neutrophil adhesion to endothelial cell monolayers. Adherent neutrophils, but not cells in suspension, externalized LC1 on the plasma membrane in an extensirre manner, such that ≈60% of the total intracellular LC1 were lost in post-adherent neutrophils. We then searched a functional role for the externalized protein. Neutralising anti-LC1 monoclonal antibodies did not alter the extent of neutrophil adhesion to endothelial monolayers, however, they potentiated neutrophil transmigration through endothelial monolayers grown onto Transwell™ filters. This prompted to propose that endogenous LC1 is an autocrine inhibitor which controls neutrophil emigration through the vessel wall in physiopathological situations. Upon neutrophil adhesion onto the endothelium a specific signalling occurs which leads to externalization of LC1 on the plasma membrane: at this site the protein acts to impede migration, and to actually cause neutrophil detachment.

In conclusion, under physiological conditions, neutrophils do not extravasate and this may be due by the tonic inhibitory action exerted by endogenous LC1. It is tempting to propose that in the absence of inflammation the autocrine LC1 pathway is responsible for keeping the cells inside the vessels even after casual contacts with the endothelial wall. Once the inflammatory insult is received, a cascade of pro-inflammatory processes is activated including de novo synthesis of chemokines specific for leukocyte subsets; this overcomes the inhibitory action that LC1 and other endogenous mediators oppose, and neutrophils will extravasate. Due to the release of multipotent cytokines such as IL-1 and IL-6, endogenous glucocorticoid hormone levels are increased. This will produce i) a potentiation of endogenous anti-inflammatory pathways (for example increasing cell-associated LC1 level) and ii) an inhibition of endogenous pro-inflammatory pathways (for example by inhibiting cytokine, chemokine and adhesion molecule synthesis). The endpoint will be a suppression of further leukocyte extravasation. The cellular response is then blocked, and mechanisms of repair can become activated to remove the inflammatory insult and the signs of the host response (cell apoptosis, macrophage phagocytosis, etc.) to eventually bring the injured tissue back to its physiological and pre-inflammatory condition. Finally, the entire system can be switched towards a predominance of anti-inflammatory pathways at earlier stages by administration of exogenous LC1, LC1 mimetics (such as peptide Ac2-26) or DEX. Future studies will tell whether a better knowledge of this endogenous inhibitory pathway can be used to develop novel anti-inflammatory drugs.

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References

19 Leukocytes, inflammation and atherosclerosis
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The interactions between leukocytes and intrinsic vascular wall cells characterize many inflammatory reactions and contribute importantly to the pathogenesis of many vascular diseases. A growing body of evidence suggests that atherosclerosis may be initiated and/or perpetuated by a chronic inflammatory process in the arterial wall possibly initiated by oxidation of LDLs that would lead to recruitment of monocytes and, subsequently, of specific T lymphocytes into the intima. The presence of costimulatory signals in the atherosclerotic plaques suggests that
the infiltrated T cells are activated locally but the specificity of such immune cells has not been established yet. Furthermore, T cell clones obtained from atherosclerotic lesions are heterogeneous with regard to antigen receptor gene organization, indicating that they are derived from several progenitors and respond to different antigenic epitopes. The latter could be either self-modified proteins, like oxLDL, or microorganism such as Chlamydia p. or cytomegalovirus. The protective or harmful role of the specific immune response in progression of atherosclerosis has still to be clarified.

Inflammatory mechanisms could possibly be involved in plaques rupture and thrombosis since they may promote either synthesis or lysis of the fibrous cap.

In fact, a systemic inflammatory response is associated with acute coronary syndromes and their prognosis while quiescent atherosclerosis is not associated with systemic signs of acute inflammation. Accordingly, activated T-lymphocytes (specific immune response?) are frequently found in peripheral blood of patients with acute coronary syndromes, at variance with chronic atherosclerotic diseases. The role of the specific immune response in progression and complication of atherosclerosis is presently under study in many laboratories. We have recently investigated the temporal relation between acute inflammation and the specific immune response in acute coronary syndromes and their relation to the clinical prognosis. It is not known, yet, whether the clinical events are actually initiated and/or influenced by the immune/inflammatory response, nor the specific target of the immune response in acute atherosclerotic complications. Even considering the immune response as a consequence of the disease, it may still be important as therapeutic guidance for its prognostic value. But, a causal role for the immune system in the pathogenesis and/or in the clinical outcome of acute atherosclerotic syndromes would open new puissant therapeutic approaches for the main cause of death in the modern era. In this view, the beneficial or harmful effect of the immune response needs to be clarified by experimental studies.

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References
Cardiol 1998; 31:1217-25.

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Neutrophil inflammatory responses.
Role of nonsteroidal anti-inflammatory drugs
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Here we described the results of our experimental approach to identify therapeutic targets for pharmacologic control of the development of tissue injury during neutrophil inflammatory responses. Sulphasalaminamide as well as salicylic acid derivatives represent starting point for future progresses in this field.

It is well known that neutrophils represent the hallmark of the inflammatory responses of a variety of diseases ranging from chronic obstructive pulmonary disease, cystic fibrosis, adult respiratory distress syndrome to ulcerative colitis, certain glomerulo-nephritides, rheumatoid arthritis and vasculitides. In these conditions, locally recruited neutrophils can promote the development of tissue injury, which, when persistent, can lead to the irreversible destruction of normal tissue architecture with consequent organ impairment. In the last two decades, major advances have been achieved in understanding the mechanisms of neutrophil-mediated tissue damage but, in spite of these progresses, effective therapeutic approaches to control tissue injury in neutrophilic inflammation are still lacking. The major pathogenetic steps which might be identified as therapeutic targets are represented by the extravascular recruitment of neutrophils at sites of inflammation, the activation of recruited cells with consequent production of oxidants and release of proteases, the relative imbalance between the generated oxidants and local anti-oxidant defences and between released proteases and natural anti-protease systems. Although some of the classical non-

teroidal anti-inflammatory drugs have been shown to interfere with various neutrophil responses, there are no rational and experimental bases for raising a histoprotective approach with these drugs. We studied a panel of these drugs on neutrophil functions. In particular we found that some drugs, i.e. sulphasalaminamide derivatives (nimesulide, dapsone, sulpyrine) and salicylic acid derivatives (5-aminosalicylic acid) are capable of efficiently reducing the recovery of hypochlorous acid (HOCI) generated by neutrophils in the pericellular microenvironment. Owing to this activity, these drugs were found to prevent the oxidative inactivation of alpha-1-antitrypsin, the major natural inhibitor of neutrophil serine proteases, such as elastase and proteinase. Moreover, among these drugs, nimesulide displayed the ability to down-regulate the level of neutrophil activation also by synergistically interacting with endogenous mediators such as adenosine and prostaglandin E2. The ability of this drug to curb neutrophil activation has been found to be particularly relevant as far as production of oxidants and release of enzymes are concerned. These effects are conceivably related to the drug ability to increase the intracellular cAMP levels in neutrophils, an effect which was also found to account for the drug ability to reduce the trans-endothelial migration of these phagocytes. Presently, nimesulide is thought to affect cAMP levels by inhibiting phosphodiesterase type IV in neutrophils. Therefore, sulphanilamide derivatives and 5-aminosalicylic acid, have the potential to reduce the bioavailability of neutrophil-derived HOCI, in turn favoring the alpha-1-antitrypsin-dependent control of neutrophil proteolytic activity. This can limit the oxidant/anti-oxidant and protease/anti-protease imbalances at sites of neutrophilic inflammation. In this regards, 5-aminosalicylic acid is well-known to be effective in ulcerative colitis. Finally, owing to the herein described activities, nimesulide can be considered a prototype for the development of new drugs able to control neutrophil activation and in turn neutrophil histotoxicity in inflammation.

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References
4. Ottonello L, Dapino P, Sciocco MC, Balbi A, Bevilacqua M, Dallegrì F. Sulphonamides as anti-inflamma-
Phagocytosis and killing properties evaluated on whole blood from mice by a radiochemical method

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We describe a radiochemical method proposed for the study of phagocytosis and killing properties in mice using whole blood rather than isolated phagocytes. A comparison between the two methods was made by measuring the immunodepression produced by similar doses of cyclophosphamide. Depression in whole blood phagocytes was quite similar to that in elicited PMN.

Current methods for the evaluation of phagocytosis and killing properties normally employ elicited polymorphonuclear leukocytes (PMN).

The drawbacks of the use of elicited PMN are well known. On the other hand, when information on resting PMN is needed, a separation from gradient is inevitable. The process requires gradient separation and several osmotic shock treatments, that may impair the cell physiology and generate artifacts. Moreover, all procedures require large volumes of blood, which is troublesome and sometimes even impossible, particularly in small animals and human newborns. In these conditions, phagocytosis and killing properties can be tested only on whole blood. However methods using whole blood do not permit the simultaneous measurement of these parameters, and although they are not time consuming or elaborate, they are not completely accurate, due to subjectivity-related variations.

The object of this study was to evaluate phagocytosis and killing properties of murine PMN by a radiochemical method on whole blood. The immunodepressant used was cyclophosphamide (Cy), Endoxan Astra 500, preliminarily dissolved in distilled water, and then diluted in saline. Ninety-six hours before the experiment, 25 male CD1 mice were injected intraperitoneally (i.p.) with 50 mg/kg of Cy, and another 25 mice received 100 mg/kg of Cy. Controls received saline solution. Whole blood samples were collected from the orbital sinus: 10 mL of each sample were used for total leukocyte count and 200 mL were put in heparinized tubes for testing. Total leukocyte counts were performed by Coulter Counter. A volume of 10 mL was diluted 1:100 (v/v) in Coulter Isoton II (Coulter Electronics Ltd., Luton UK). Leukocyte differentials were determined in blood smears after Hemofast staining. Phagocytic peritoneal exudates (PEC) were obtained 18 hr after i.p. injection of 1.2% (w/v) sodium caseinate at dose of 1.5 mL per mouse. The peritoneal cavity of each animal was washed out twice with 2 mL of PBS (Ca++ and Mg++ free). Phagocytes were counted, centrifuged at 1200 rpm for 10 min and suspended at a concentration of 4x10^6 PMN/mL at pH 7.4 in RPMI 1640 with Heps. Candida albicans cell suspension was prepared from a broth cultured (Sabouraud Dextrose Broth-Difco) for 3-4 days (cell were in east form), centrifuged, resuspended in RPMI 1640 with Heps (25 mM), counted to 2x10^7/mL and opsonized with omongous serum for 20 min. The viability of the cells was investigated by trypan blue exclusion.

Candida albicans and PMNs were mixed in 2 ml sterile polytubes, each containing 1x10^7 Candida organisms and 2x10^6 PMNs (1:5 cell ratio) suspended in a total volume of 0.5 mL of medium containing 5% serum. Whole blood samples were mixed with Candida albicans organisms, diluted in order to obtain the same cell ratio (1:5), after counting total leukocytes in all blood samples. Control tubes without PMNs or whole blood containing 1x10^7 Candida organisms suspended in 0.5 mL medium with 5% serum were also set up. All tubes were incubated at 37°C under constant-end-over-end rotation (30 rpm) for 40 min. Phagocytosis was measured by transferring a single 100 mL aliquot from each tube into a microtitre well containing 0.2 mCi of 3H-uridine in 110 mL of medium. Intracellular killing was measured by transferring a 100 mL aliquot from each tube into a microtitre well containing 0.2 mCi of 3H-uridine in 10 mL of medium, 625 mg of sodium deoxycolate in 50 mL of distilled water and 5 mg of deoxyribonuclease (DNAse) in 50 m of medium.

Phagocytosis and killing properties were measured in the same microtitre plate incubated for 60 min at 37°C to allow the microorganisms to incorporate uridine. Cell-associated radioactivity was then collected into glass fibre discs with a Skatron Harvester, placed in toluene-PPO scintillation fluid (Lumac, Landgraaf, The Netherlands) and counted in a LKB 1612 Rack-Beta counter.

Results were expressed as phagocytic and killing indexes calculated as the average number (for 100 PMNs) of PMNs phagocytosed and killed by Candida albicans cells. Values were given as mean ± standard deviation. Statistical analysis was performed with the student’s t-test paired data.

Figure 1 shows the results obtained with the traditional radiochemical method on elicited PMN, and with a modified version of the test on mice’s whole blood.

A quite similar decrease in phagocytosis properties of mice treated with 50 and 100 mg/kg cyclophos-
Phosphamide was observed when using elicited PMNs and whole blood (-21.6 and -21.3%, for 50 mg Cy, -29.6 and -33.3% for 100 mg Cy, respectively). These data are confirmed when killing properties are investigated. Indeed the treatment with 50 and 100 mg/kg Cyclophosphamide resulted in a decrease of 32.7% and 51.7% when employing elicited PMNs and of 42.8% and 59.5% when employing the whole blood.

We have found that the radiochemical method of Bridges et al. can be applied on whole blood.

Avoiding the separation step, the leukocyte function is tested under better physiological conditions.

A number of factors such as optimal culture conditions and soluble products from monocytes, macrophages, and serum factors contribute to the creation of a physiological microenvironment, essential for phagocyte physiology, that enables to fully express the proliferating potential from stimulated cells. The importance of these factors is neglected or underestimated when isolated cells are used.

Finally, it can be concluded that the radiochemical method applied to whole blood is quick, simple, requires a minimum amount of blood and reduces the handling of effector cells to the minimum.

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References

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Monoclonal lym-1 antibody-dependent cytolysis by human neutrophils exposed to GM-CSF: role of Fcγ receptors and adhesion molecules
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Normal human neutrophils, incubated with the anti-target monoclonal Lym-1 antibody, were found to lyse lymphoblastoid Raji target cells in the presence of GM-CSF. The cytolysis process involves FcγR type II without co-operation with FcγR type III and requires the interaction between CD11b/CD18 integrins and CEAC-like CD66b glycoproteins.

The ability of normal neutrophils to exert antibody-dependent cellular cytotoxicity (ADCC) towards certain human tumor cells is well documented. In particular using heterologous human anti-target monoclonal antibodies, neutrophils have been shown to lyse lymphoma and leukemia cell lines efficiently. More recently human neutrophils, incubated with
selected murine anti-target monoclonal antibodies (mAb), were found to mediate consistent lysis of melanoma and neuroblastoma cells. and no lysis of lymphoma and leukemia targets. In the present paper, using a particular (Lym-1) mAb towards B lymphoblastoid Raji target cells, GM-CSF was found to promote neutrophil-mediated ADCC. The lysis was competed inhibited by adding the anti-FcγRII mAb IV. On the contrary, neutrophil ADCC was unaffected by the anti-FcγRIII mAb 3G8. These results suggest that neutrophil cytolysis involves FcγRII without cooperation with FcγRIII. Moreover, the role of CD11/CD18 integrins was tested using a panel of mAbs. The lysis was inhibited by the anti-CD18 mAb MEM-48, whereas none of the anti-CD11 mAbs used (MEM-25, 3.9, KB90) had inhibitory activity. On the other hand neutrophil ADCC was efficiently inhibited by the presence of a mAb specific for CE1-like and glycophosphatidylinositol-isoinositol (GPI)-linked glycoproteins (CD66b). In parallel experiments, using an immunofluorescence staining procedure, mAb-induced cross-linking of CD66b caused redistribution of CD11b on the neutrophil surface with distinct areas of CD11b clustering. This CD11b clustering was efficiently inhibited by D-mannose which, as mentioned above, efficiently prevents CD66b-lectin-like physical interaction on the neutrophil surface. Such a type of interaction is presumably instrumental for neutrophil Lym-1 ADCC activity in that: a) the lysis was efficiently inhibited by D-mannose which, as mentioned above, efficiently prevents CD66b-CD11b/CD18 integrins and CE1-like CD66b glycoproteins to undergo lectin-like physical interaction on the neutrophil surface. Therefore, the present results prove the absolute requirement for FcγRII in GM-CSF-stimulated Lym-1-dependent neutrophil cytolysis, and, on the other hand, define the crucial role of the interaction between CD66b and CD11b/CD18 during the expression of the cell lytic potential.

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References

23 Increased in vitro phagocytosis of Candida albicans induced by aconitum root extracts
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Alkaloids extracted from the root of rancunculaceous plants Aconitum napellus L. and Aconitum lycostenum L. are described as putative enhancers of phagocytosis.

Aconitine and lycocotnine, the major alkaloid compounds, are credited as Na+ channel activations and competitive antagonist of nicotine receptors. However, these products are extremely toxic and the currently available literature describes marginal and controversial biological effects on a number of immunological functions, including phagocytosis. Moreover, phagocytosis assays in vitro are quite delicate, and no reports on this matter still exist with the use of robust and well controlled in vitro methods such as flow cytometry (FCM).

The aim of this study was first to identify, purify and chromatographically characterise the A. napellus and A. lycostenum extracts on different plant fractions. Subsequently, purified and standardized A. napellus and A. lycostenum alcoholic extracts were studied for their effects on the in vitro ingestion of fluorescent Candida albicans on a direct, whole blood, dual color fluorescence test by FCM. A. napellus and A. lycostenum extracts from plants collected in different areas have a different chromatogram profile, and the major compound peak is usually accompanied by a number of related substances.

We used the ingestion assay described by Saresella et al. The FCM test was optimized as follows: a Candida to PMN ratio of 2.2:1 was chosen, with standardization of this proportion in every instance; use of heparinized whole blood; incubation time 1 hour; red cell lysis by ammonium chloride; adding of stop
solution (cold 0.04 % EDTA in PBS); counterstain of extracellular Candida with ethidium bromide; FCM analysis (FACS Calibur).

The total percent ingestion of PMN and monocytes was calculated by selective gating. Moreover, the fractions of either cells with only internalized or internalized and adherent Candida were calculated, assuming the control sample as 100%.

The final extract concentrations tested were 1 µg/ml for A. napellus and A. lycoctonum, respectively. We focused on the 1 µg/ml the final concentration, because it was the highest not causing appreciable cell apoptosis or death in vitro.

Our results show that in presence of A. napellus and A. lycoctonum extracts at 1 µg/mL, both PMN and monocytes undergo a slight but rather reproducible increase in their phagocytic activity, as measured as the fraction of cell internalizing fluorescent Candida blastospores. Monocytes show a higher ingestion efficiency in this in vitro model. Some differences are evident among the various extracts from plants collected in different areas, thus stressing possible local differences in plant maturation and metabolism.

The FCM assay we used proved to be highly robust and reproducible, thus enabling us to detect subtle differences in this in vitro assay. The possible effects of A. napellus and A. lycoctonum extracts in vivo are still to be elucidated, especially as far as their marked toxicity is concerned. We wonder if the in vitro concentrations that proved effective in enhancing the phagocytic capabilities of PMN and Monocytes can be obtained in an in vivo model.

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References

24 Phagocytosis of immune complexes accelerates neutrophil apoptosis. Role of Fcy receptors

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We evaluated the effect of immune-complex phagocytosis on neutrophil apoptosis. Our results show that immune complexes accelerate the rate of spontaneous apoptosis by a FcγRII-dependent mechanism in part unrelated to the generation of reactive oxygen species.

Circulating neutrophils are end cells already programmed for undergoing spontaneous cell death, i.e. apoptosis, through a process susceptible of modulation by environmental influences. Some cytokines such as GM-CSF, IL-6, IL-2, TNF have been indeed shown to delay the process. On the other hand, the effect of triggering cell effector functions is largely unknown. We used human albumin/IgG immune-complexes to activate FcγR-mediated phagocytosis in purified normal human neutrophils. Neutrophil apoptosis was evaluated by light microscopic examination, flow-cytometric analysis of propidium iodide-stained cells, flow-cytometric determination of annexin-V expression and electrophoresis of DNA. Immune-complexes (25 µg/mL) were found to stimulate apoptosis efficiently as detected at 12 hrs. The percentage of apoptotic neutrophils was primary reduced by the monoclonal antibody (mAb) against FcγRII (IV.3). A slight reduction of apoptosis was observed in the presence of the mAb against FcγRIII 3G8. Immune-complexes are well-known activators of neutrophil oxidative metabolism, and reactive oxygen species (ROS) are presently considered the major mediators of neutrophil apoptosis. Nevertheless, whereas spontaneous apoptosis was completely inhibited by neutrophil co-incubation with catalase (hydrogen peroxide scavenger), the apoptosis in presence of immune-complexes was only partially prevented by catalase. Furthermore, neutrophils from a patient with chronic granulomatous disease (CGD), congenitally incapable of producing ROS, showed a trivial level of spontaneous apoptosis, but underwent a nearly ten-fold increment in the apoptosis rate when incubated with immune-complexes. These data suggest that: a) spontaneous neutrophil apoptosis is strictly dependent on ROS generation; b) immune-complex- and FcγRII-induced apoptosis appears to involve both ROS-dependent and independent pathways. Consistent with the intervention of a ROS-independent pathway in neutrophil apoptosis accelerated by immune complexes, apoptosis but not oxidative burst was inhibited by the anti-FcγRII mAb IV.3. In fact, neutrophil superoxide and hydrogen peroxide production triggered by immune-complexes was inhibited only in the presence of both the anti-FcγRII mAb IV.3 and the anti-FcγRIII mAb 3G8, whereas neutrophil incubation with only one of the two mAb was ineffective. On concluding, the present results suggest that the activation of neutrophil effector functions, i.e. the phagocytosis of immune complexes, uncovers a FcγRII-mediated apoptosis-inducing pathway that is in part independent from the generation of ROS.
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References


25
Endogenous catecholamine metabolism in human lymphocytes
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Evidence is presented that human peripheral blood mononuclear cells contain catecholamines and their metabolites. In vitro, pharmacological inhibition of tyrosine hydroxylase or monoamine oxidase affects intracellular dopamine, norepinephrine and their metabolites, suggesting intracellular synthesis and degradation. Incubation with monoamine uptake blockers enhances dopamine and norepinephrine in the incubation medium, suggesting the presence of release and uptake mechanisms. The present results indicate the existence of a catecholamine life cycle in human mononuclear cells.

During the last decades, the role of the autonomic nervous system in the modulation of immune function has become increasingly evident.1 In lymphoid organs sympathetic nerve endings lie close to immunocytes and release sympathetic neurohormones. Leukocytes express receptors for catecholamines (CTs) and neuropeptides and these mediators profoundly affect leukocyte function. In this context, an emerging area of investigation concerns the ability of leukocytes to produce and utilize sym pathetic factors to regulate their own activity. Endogenous CTs and a CT synthetic pathway have been reported in lymphocytes, thus the existence of an adrenergic autocrine and/or paracrine regulatory loop has been hypothesized.2-4 However, the life cycle of CTs in lymphocytes has not been systematically investigated, so far. We have thus studied CTs and their metabolites in human lymphocytes to obtain evidence for the existence of enzymatic pathways responsible for their synthesis and degradation. In addition, we have examined whether CTs are released in and taken up from the extracellular fluid. Experiments were performed on lymphocytes isolated by density-gradient centrifugation from venous blood obtained from healthy donors. Cells were resuspended at the final concentration of 1×10⁶/ml in Iscove’s medium and incubated with the various drugs at 37°C for 1 h. Cells were then centrifuged and the supernatant was removed for CT assay. The cell pellet was extracted by addition of 1 mL of HClO₄. 0.1 N and 20 µL of digitonin 10 µg/mL. After a final centrifugation, the supernatant was recovered and assayed for CTs and their metabolites. The assay was carried out by high performance liquid chromatography with electrochemical detection.5 Lymphocytes contained significant amounts of dopamine (DA), norepinephrine (NE) and epinephrine (E) and of several of their metabolites. Intracellular metabolites included the DA metabolites DL-3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylglycol and the E metabolite metanephrine. Table 1 reports the levels of CTs and of their metabolites in control conditions and after incubation with the tyrosine-3-monooxygenase inhibitor α-methyl-p-tyrosine (α-Me-Tyr) or with the monoamine oxidase inhibitor pargyline. These drugs significantly affected the levels of DA, NE and E and of several of their metabolites. DA, NE and E were also found in the incubation medium, in concentrations ranging between 97.3 and 190.4 pg/mL. A possible contamination by plasma CTs or eventual leakage from damaged cells are unlikely, since cells were accurately washed during preparation and their viability was always >95%. Ten µM desipramine or 0.1 µM GBR 12909, two monoamine uptake blockers, significantly enhanced DA and NE in the incubation medium by 45-68% and 550-750%, respectively. We conclude that human lymphocytes are able to synthesize and breakdown CTs. Synthesized CTs are released in the extracellular environment and can be taken up by mechanisms similar to the neuronal monoamine transporters. DA seems to act mainly as NE precursor, while NE is likely to be the end product of the synthetic pathway. E seems to be for the most part of extracellular origin, but it may undergo degradation intracellularly. Evidence is available that CTs can regulate a number of leukocyte functions1 and it has been shown that lymphocyte CTs correlate with cAMP production.6 The demon-
Lymphocytes play an important role in the formation and evolution of coronary atherosclerotic plaques. In particular, T lymphocytes are found in large numbers in human atherosclerotic plaques, indicating that immune and inflammatory mechanisms are important factors in the pathogenesis of atherosclerosis. The finding that T lymphocytes, isolated from endarterectomy specimens, have a polyclonal origin supports the hypothesis that these leukocytes would be recruited in an activated state from peripheral blood. Recent data have also implicated T lymphocytes in the pathogenetic mechanism of unstable angina. Lymphocytes are equipped to eradicate noxious agents that disturb the body’s equilibrium, but when their cellular activity is excessive, the results are harmful. The role of catecholamines on lymphocyte activation in this field, may be more important than previously assumed. To clarify this aspects, we investigated the role of β-adrenergoreceptor agonists involved in the cell activation. To assess the relative efficacy of dobutamine during echocardiography in T-lymphocytes activation in 18 patients suffering from coronary artery diseases. Dobutamine exhibits significant effect on increase T-lymphocyte cytotoxic cells.

Recent studies suggest the role of T-lymphocyte activation on coronary artery diseases. This study was designed to investigate whether β-adrenergoreceptor agonists are involved in the cell activation. To assess the relative efficacy of dobutamine during echocardiography in T-lymphocytes activation in 18 patients suffering from coronary artery diseases. Dobutamine exhibits significant effect on increase T-lymphocyte cytotoxic cells.

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References
2. Josefsson E, Bergquist J, Ekman R, Tarkowski A. Catecholamines are synthesized by mouse lymphocytes and regulate function of these cells by induction of apoptosis. Immunology 1996; 88:140-6.

Table 1. CTs and their metabolites in human peripheral blood mononuclear cells in control conditions and after incubation with 10 μM α-Me-Tyr or 10 μM pargyline. Control values are mean±SEM of 5-10 observations and are expressed as 10⁻¹⁰ mol/mL. *p<0.05, **p<0.01 vs control.

<table>
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<tr>
<th>Control</th>
<th>α-Me-Tyr</th>
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<tr>
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<td>Metanephrine</td>
<td>890.0±88.6</td>
<td>400.5±99.3**</td>
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Meeting supplement
Figure 1. T-Lymphocytes CD8+/CD11b+ increase during ecocardiography with dobutamine. Whole blood were taken at baseline (B) at peak stress (P) and after recovery (R).

en at baseline (B) at peak stress (P) and after recovery (R). Lymphocyte were stained in whole blood and phenotyped with the following monoclonal antibodies ratio CD3, CD4, CD8, CD11b CD16, CD56 using a double fluorescence in flow cytometry. There was no difference in the group I for leukocytes and lymphocytes on their subset at all time of analysis. In the group II an increase of lymphocytes x 10^6/ul was reported (B=1863±646, P=2487±1149, R = 2523± 1168, p=NS). Group II pts showed a higher expression of CD8+ lymphocytes x10^6/ul (B=492±85, P=887±238, R=884±129, p<0.05), CD11b+ (B=453±69, P=1058±205, R=996±208, p < 0.02) and CD16+ (B=280±46, P=762±155, R=670±137, p < 0.009). The double fluorescence evaluation demonstrated an increase of cytotoxic lymphocytes CD8+/CD11b+ x 10^6/ul (B=248±124, P=522±364, R=531±247, p<0.01) and CD8+/CD16+ x10^6/ul (B=112±35, P=318±146, R=310±124, p=0.03). No difference in lymphocytes subsets was found between pts with (n=5) and without (n=5) an ischemic response to DOB. This drug exhibits significant effect on increase T-lymphocyte cytotoxic cells. The same results in a patient without spleen, suggests a bone marrow origin of these cells induced by dobutamine. The data reported here underline the role of interaction between catecholamine and lymphocyte when studying the immune system in coronary artery disease.

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References

27 Induction of antibacterial activity by α-D-oligogalacturonides in Lunularia cruciata (L.) Dum. (BRIOPHYTA). Could this be an immune response in non-vascular plants?

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In this paper we report findings of antibiotic activity in vitro by liverwort Lunularia cruciata induced by α-D-oligogalacturonides (OGs). The results show that the OGs induce the antibiotic activity against Gram negative strains in the liverwort tested. We suggest that the production of antibiotic substances can be elicited by different factors, such as products of synthesis or degradation of the biotic component of the soil or...
by OGs (in axenic culture) that can mimic the effect of natural elicitors.

Plants lack a circulating adaptive immune system to protect themselves against attack. They have evolved other mechanisms of antimicrobial defence which are either constitutive or inducible. In this respect the most investigated taxa are the angiosperms. Their primary cell wall plays an important role in determining the resistance to pathogen attack; in fact, from its complex polysaccharides, oligosaccharides are released which act as elicitors of defence substances such as phytoalexins. No data are available about non-vascular plants such as Bryophyta.

Our previous findings demonstrate that the acetonic extract from *L. cruciata* has antibacterial activity and modulates human PMN activity. An OG mixture influences the morphogenesis in the same liverwort *in vitro*. The aim of this study was to determine a possible in vitro induction of antimicrobial activity in a thalloid liverwort *L. cruciata* (Bryophyta) by a mixture of OGS. These were obtained as reported elsewhere and enzyme purification (endopoly-galacturonidase) was carried out to electrophoretic homogeneity from Aspergillus niger pectinase (SIGMA, P4802) according to Cervone.

*Lunularia cruciata* (L.) DUM was collected in the botanical gardens of Naples University, where a voucher specimen is deposited and authenticated by Professor R. Castaldo Cobianchi (Department of Plant Biology, University of Naples). Gemmae of liverworts were surface sterilized and cultured, as reported elsewhere in modified Mohr medium, with or without 6 mg/L of OG mixture (expressed as mg equivalents of galacturonic acid) in Na-acetate buffer 20 mM, pH 5. Thirty days after planting, gametophytes were utilized for the preparation of the extracts and tested as reported elsewhere. The extracts were tested against 5 bacterial strains purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA): *Escherichia coli* (ATCC 11229), *Salmonella typhi* (ATCC 19430), *Staphylococcus aureus* (ATCC 13709), *Proteus mirabilis* (ATCC 7002), *Pseudomonas aeruginosa* (ATCC 27853), *Streptococcus faecalis* (ATCC 14428). Bacterial strains were grown on MH (Mueller-Hinton) agar plates and suspended in MH broth. The MIC and MBC (minimum inhibiting concentration and minimum bactericidal concentration) values against bacterial strains were performed using the Ericson and Sherris broth-dilution method (MH broth), as reported elsewhere. The MIC values were also determined for tetracycline hydrochloride (Pharmacia, Milan, Italy), benzyl penicillin sodium (Cynamid, Catania) and cefotaxime sodium (Roussel Pharma, Milan, Italy), in MH broth using the standard method. The extracts from *L. cruciata*, axenically grown in control medium, never inhibited bacterial growth. Similarly, the extract from the liverwort grown in Mohr medium and mixed with phosphate buffer and OG solution showed no antibiotic activity after growth. The extract from the liverwort grown in medium containing OGS showed antibacterial activity against *Proteus mirabilis* (256 mg/mL), *Escherichia coli* (256 mg/mL), *Pseudomonas aeruginosa* (512 mg/mL) and *Salmonella typhi* (512 mg/mL), while *Staphylococcus aureus* and *Streptococcus faecalis* showed no sensitivity. All strains were sensitive to reference antibiotics (Table 1). The extracts had no bactericidal activity even at the highest concentration used.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Cefotax</th>
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<th>Tetrac</th>
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<td>Salmonella typhi</td>
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<td>Staphylococcus aureus</td>
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<td>0.03</td>
<td>0.1</td>
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<tr>
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<td>2</td>
<td>R 4</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>512</td>
<td>16</td>
<td>R 32</td>
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<tr>
<td>Streptococcus faecalis</td>
<td>R R 2</td>
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</table>

Darvill and Albersheim showed that the environment affects the production of defence substances in plants through the action of oligosaccharide elicitors. Our data clearly demonstrate that the liverwort grown in the presence of OGS has antimicrobial activity. We suggest that, as bacterial or fungal products (degradation or secretion) or degradation products from infected plants induce the production of antimicrobial compounds in field-grown liverwort, likewise the OGS elicit the production of antimicrobial molecules in the axenic-cultured liverwort. In addition, the antibiotic activity found in the presence of the OGS shows a comparable intensity to that observed in a field-grown liverwort in previous experiments.

The extracts from wild plants inhibited the growth of all bacterial strains tested, while the antimicrobial compounds produced by axenic cultured plants in the presence of the OGS inhibited only 4 of the 6 strains. Therefore, we hypothesize that the antimicrobial substances produced by the liverwort grown in the presence of OGS are more specific.

In conclusion, just as in higher plants, our data suggest that an immune response mediated by oligosaccarins could be present in bryophytes.

**Correspondence**
We have investigated the effect of catechin and its derivatives, (+)-3-propionylcatechin and (-)-3-O-valeryl catechin, on human phagocytic activity. All compounds induce a decrease of whole blood chemiluminescence (CL) emission. In presence of phorbol myristate acetate (PMA), the two derivatives are more active than native catechin. The increased lipophilic characteristics of catechin derivatives could explain their higher activity.

Flavonoids are phenolic components widespread in the plant kingdom. They are present in plant extracts which have been used for centuries in oriental medicine for the treatment of several illness. The pharmacological properties of flavonoids have now been extensively reviewed. They exhibit many biological activities such as anticarcinogenic, anti-inflammatory, antibacterial and antioxidant. Most of their properties are related to the location of hydroxyl groups in the flavane skeleton and a fundamental role have those in 3'-4' -position of the B ring. In the present study we have investigated the activity of catechin and its derivatives, (+)-3-propionylcatechin and (-)-3-O-valeryl catechin, on CL emission by human peripheral whole blood (WB) phagocytes, namely polymorphonuclear leukocytes (PMNs). The catechin derivatives were prepared as reported by Nicolosi et al. (Patent M196A002727, 1996).

The luminol-dependent CL assay is a sensitive indicator of phagocyte respiratory burst, characterized by an increase of reactive oxygen intermediate (ROI) production, including superoxide and hydroxyl radicals that induce phlogistic effects.

Samples of blood from six healthy donors, were anticoagulated with heparin and underwent CL assay according to De Sole et al., using an automatic luminometer. The reaction mixtures were made up in 4-ml propylene vials. Each vial contained 100 µL of 1 mM luminol (5-amino-2,3-dihydro-1,4-phthalaldehyde), 100 µL of 1.5 µM PMA, 100 µL of diluted WB and sufficient modified Krebs-Ringer phosphate medium to yield a final volume of 1 ml. The CL assay was performed in the absence or in the presence of various concentrations of test compounds. The reaction temperature was maintained at 37 °C and the light emission was recorded for 90 min. All measurements were performed in triplicate. The effect of catechins on phagocyte viability was also determined by the trypan blue exclusion test at 30, 60 and 90 min incubation. The CL emission was evaluated as total integral counts (cpm/PMN). The results are expressed as % inhibition. The data analysis was carried out with the Student’s t test. Values of p≤0.05 were regarded as significant.

The results show that leukocytes were healthy after exposure to all the compounds studied, independently by the concentrations used. Figure 1a shows the inhibitory effects of the three catechins on whole blood resting PMN light emission. Catechins (0.1-100 µM) significantly (p≤0.05-0.001) inhibit CL emission in concentration-dependent fashion. The maximal activity was at 100 µM. Figure 1b shows the inhibitory effects of the three catechins on PMA-stimulated whole blood phagocytes. The simultaneous presence of PMA and catechins induced an overlapping and not significant decrease of CL emission.
until reaching 1 µM concentration. Instead, the greatest differences between catechins tested occurred at 10 µM. At this concentration, the most active was the 3-O-valerylcatechin. However, the two catechin derivatives were more active than native catechin (p ≤ 0.01-0.001). They were also more potent than ascorbic acid (1 µM) used as a positive antioxidant control (data not shown).

Our data demonstrate that the two catechin derivatives are very powerful antioxidant, more potent than the native catechin and ascorbic acid. In particular, the 3-O-valerylcatechin is the most active. Since the inhibitory effect of the catechin derivatives is more evident in presence of PMA, they could inhibit phagocyte oxidative metabolism by altering the PKC system.

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References
Although organ transplantation is one of the most remarkable achievements of modern medicine, we still face important challenges in the management of transplanted patients. The strategies for immunosuppression must be refined and improved. Chronic rejection, quality of life and late sequelae of a non-specific pharmacological immunosuppression remain areas of particular concern. The goal of immunosuppressive therapy is to suppress the patient's immune response to the allograft while preserving a sufficient degree of immunity to avoid opportunistic infections and malignancies.

Liver transplantation today is the therapy of choice for many patients with chronic, advanced, irreversible liver disease and also for patients with fulminant hepatic failure. Less than 50% of liver transplanted patients nowadays experiences graft acute rejection, but the drugs available have a narrow therapeutic window, and patients frequently suffer serious complications from overimmunosuppression or experience acute and/or chronic rejection as a result of underimmunosuppression.

The introduction of cyclosporine in the early 80s represented the cornerstone of the solid organ transplantation clinical success. After Borel's description of cyclosporine effect on human immune system, Calne and Coll in 1978 used this drug alone and in combination with steroids in primary immunosuppressive protocols. Cyclosporine inhibits the gene transcription for IL-2, γ-interferon, IL-3, IL-4 and other genes required for the proliferation and differentiation of B and T-lymphocytes. The drug is not cytotoxic and spares T-suppressor lines. The selective immunosuppressive activity of cyclosporine has significantly reduced rejection rates and improved patient and graft survival after solid organ transplantation. However the efficacy and safety prophylaxes are influenced by the inter- and intra-patient variability in drug pharmacokinetics and pharmakodinamics.

The drug absorption represents the Achilles' heel in cyclosporine pharmakokinetics, mainly after liver transplantation: the gut absorption is bile-dependent and the metabolism is related to liver function. The main adverse events related to cyclosporine are: nephrotoxicity, neurotoxicity, hypertension and a diabetogenic effect. Gingival hyperplasia and hirsutism are cause of cosmetic discomfort. Due to the narrow therapeutic window, cyclosporine trough blood levels have to be strictly monitored by a specific radioimmunoassay, and maintained between 200-400 ng/ml during the first postoperative month and between 100 and 200 ng/mL after the first year.

Now a new formulation of cyclosporine is available. The drug is suspended in a microemulsion, simulating a mixed micellar phase. The new formulation of cyclosporine does not require digestion, and the absorption is less dependent on bile and pancreatic juices presence.

The new cyclosporine formulation revealed a more favorable pharmacokinetics: a shorter T1/2, higher Cmax, greater bioavailability and a better correlation between AUC and trough levels. Due to these characteristics, the intravenous formulation is near to be completely abandoned after liver transplantation, with benefits in the safety profile.

Corticosteroids were the first immunosuppressive agents used in solid organ transplant therapy and are still used in many immunosuppressive regimens in low dose prophylactic regimens and high dose therapy as first-line treatment for acute rejection. The precise mechanisms of action have not been fully elucidated. Steroids inhibit antibody and complement binding and reduce the synthesis of cytokines as IL-2 and γ-interferon. The well-known complications associated to steroid administration have led to modifications in immunosuppressive regimens aimed at reducing or eliminating steroid therapy, above all in pediatric patients because of the suppression of pituitary hypotalamic axis resulting in growth retardation, and in viral patients in order to prevent the reactivation of the preexisting disease.

Azathioprine has been a mainstay of immunosuppression induction after liver transplantation for more than 30 years and is already employed in triple immunosuppressive regimens in combination with cyclosporine and steroids. Azathioprine is an antimetabolite that acts as a purine analog and alters the RNA synthesis preventing mitosis and proliferation. Myelosuppression remains the main adverse effect of azathioprine therapy and leukopenia is most frequently observed. Daily doses of 1-2 mg/kg are used and adjusted in accordance with the patient's white-cells total count.

Polyclonal antithymocytes antibodies (ATG) are still used in many liver transplant centers to induce primary immunosuppression by a quadruple therapeutic regimen (plus cyclosporine, steroids and azathioprine). These antibodies were introduced in clinical transplantation in 1967. The mechanism of action is a complement-mediated lysis of lymphocytes. The effect of these antibodies is limited to a short course (5-10 days) in the early postoperative period just to permit a more gradual introduction of...
the other immunosuppressive agents.

Monoclonal antibodies (OKT3) are preferred to ATG in reversing steroid-resistant acute rejection after liver transplantation. Monoclonal antibodies are produced by human hybridomas composed of mouse myeloma and splenic lymphocytes. These antibodies are directed against CD3 receptor of T-lymphocytes, inhibiting the lymphocytes to recognize the antigen. Many clinical trials have demonstrated that the induction therapy with OKT3 is not related to a lower rejection rate during the first postoperative month compared to the standard therapy, but the potential formation of anti-OKT3 antibodies renders these agents ineffective for rescue use. Many studies, however, confirm a significant higher morbidity following repeated OKT3 courses in terms of higher incidence of viral infections (CMV, EBV) and lymphoproliferative diseases.

Tacrolimus (FK506) is a macrolide antibiotic isolated from Streptomyces Tsukubaensis that shares many characteristics with cyclosporine. The mechanism of action is quite similar to cyclosporine and also the toxic effects are similar. Hirsutism and gingival hyperplasia were never described after tacrolimus administration. This drug is well absorbed after oral administration and the absorption is not bile dependent. Tacrolimus pharmacokinetics is not affected by the clamping or re-clamping of the T-tube as is common for temporary bile drainage after liver transplantation. Many trials have demonstrated that oral tacrolimus is able to achieve effective blood trough levels without the need of intravenous administration. The current daily dosages are 0.1 mg/kg via nasogastric tube as starting dose and the through levels are to be maintained between 10 and 15 ng/mL during the first postoperative month and then below 10 ng/mL. Tacrolimus is effective in preventing liver allograft rejection and also in rescuing acute steroid-resistant rejections and early chronic rejections. Multicenter clinical trials that compare cyclosporine versus tacrolimus as primary immunosuppressant after liver transplantation are now ongoing. New immunosuppressive molecules have advanced from the laboratory to clinical trials.

Sirolimus (Rapamycin) is an antifungal agent that unexpectedly showed the capability to inhibit the lymphocytes growth signal from cytokines receptors. The molecule does not have the toxic effects of cyclosporine and tacrolimus. Trials are ongoing on sirolimus associated to tacrolimus or cyclosporine.

Mycophenolate Mofetil (MMF) is an antimetabolite that has found extensive applications in renal transplantation. Its mechanism of action is based on the prevention of clonal expansion inhibiting the synthesis of purine nucleotides. Very recent clinical trials indicate that MMF could be promising associated to tacrolimus and permits a rapid withdrawal from steroids.

New interesting biological drugs are under clinical investigation: anti-CD25 monoclonal antibodies that inhibit the IL-2 receptor revealed a high efficacy in preventing liver allograft rejection but a remarkable safety and a long half-life. They raise challenging questions about the possibility to immunosuppress patients without organ specific toxicity and with a margin of safety that makes infections infrequent, by capturing the endogenous regulatory pathways of the host immune system and the possibility to eliminate the patient noncompliance by an intravenous monoclonal therapy every few weeks.

Remaining at the present time there are some unmet needs for immunosuppression after liver transplantation that are the main aims of the majority of current clinical trials:
- cyclosporine/tacrolimus at nontoxic doses, without increased rejections;
- decrease or eliminate steroids, without increased rejections;
- decrease or eliminate myelotoxic drugs;
- decrease or eliminate the OKT3 rescue use;
- reach a reproducible, not bidependent absorption and metabolism;
- minimal need for drug level monitoring, avoiding under-and over-immunosuppression;
- acceptable compliance;
- reduce the risk of viral hepatitis reactivation on the new liver graft;
- reduce overall yearly cost of care.

These agents in combination with the currently available immunosuppressants, could represent the bridge to tolerance induction that will remain the principal aim of transplant researchers at the beginning of the new millennium.

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References
In the past 2 decades, progressive improvements in the results of organ transplantation, as a therapeutic strategy for patients with end-stage organ disease, have been achieved, due to greater insight into the immunobiology of graft rejection. It is now known that T cells play a central role in the specific immune response of acute cellular rejection. Strategies to prevent T cell activation or effector function are thus all potentially useful for immunosuppression.

Heart transplantation. The delicate balance between desired benefit of immune-altering drugs and their adverse effects has constantly challenged transplant doctors. The struggle for optimal balance continues today, since the ideal immunosuppressive regimen is yet to be defined. Initially, prednisone and azathioprine were the primary agents available for use in cardiac transplant recipients. Maintenance prednisone doses were high, leading to significant infectious complications, as well as to the late development of side effects. Anti-thymocyte globulin was later added to the immunosuppressive regimen in the mid-1970s, allowing for some reduction in steroid use.

The addition of cyclosporin in 1980 appears to have had a beneficial impact on morbidity and mortality in cardiac transplant recipients. Early experimental work by Calne and others demonstrated enhanced survival in orthotopic pig heart transplants with cyclosporine. Similar results were found in man, combining cyclosporine with conventional immunosuppression. In recent years, the use of anti-T-cell antibodies as a therapeutic means to controlling rejection or inducing immunosuppression in solid organ recipients has become commonplace. Both polyclonal and monoclonal preparations, as RATG or OKT3, have been used in cardiac transplantation, with decrease in incidence of acute rejection. The development of new molecules represents the continuation of a clear trend toward more specific immunosuppression. None of the new small molecule drugs depends on reduction on the number of lymphocytes for its efficacy. The actions of these drugs are directed primarily to T and B cells rather than to all white blood cells. FK506 displays similar, but more potent, immunosuppressive properties to cyclosporin, inhibiting cell-mediated and humoral immune responses. It inhibits the activity of calcineurin, the production of IL-2 and other cytokines. In heart transplantation, FK506 can decrease the frequency of acute rejection episodes and low-dose administration allows a lower infection rate without an increase in rejection. Rapamycin has been demonstrated to have remarkable activity in inhibiting allograft rejection in animal models of transplantation. It belongs to the class of macrocyclic immunosuppressive drugs that are bioactive only when bound to immunophilins. Rapamycin acts at a later stage in T-cell cycle progression by blocking cytokine-mediated signal transduction pathways. Daily rapamycin dosing has been shown to inhibit arterial intimal thickening caused by both alloimmune and mechanical injury.

Lung transplantation. The current used standard triple-drug immunosuppressive therapy with cyclosporin A, azathioprine and steroid fails to prevent rejection episodes, both acute and chronic, in most lung transplanted patients. The incidence of both is significantly higher in lung transplantation than for other solid organ graft. Two main causes of death affect lung transplanted patients. There are infections in the first year and bronchiolitis obliterans after a year. The new micromulsion formulation of cyclosporin A increases overall exposure of patient and graft. The improved pharmacokinetic has been demonstrated in lung transplanted patients, including patients with cystic fibrosis. In one study comparing cyclosporin A and FK506 in lung transplantation a better survival cureve at two years in the FK506 group vs cyclosporin A was demonstrated. Moreover, there was a reduction in acute rejection episodes in FK506 treated patients in respect to CyA group (p=0.007). The incidence of bronchiolitis obliterans was significantly lower than in CyA group (p=0.025).

Recently, a new molecule named Mycophenolate Mofetil has been used in the therapy of transplanted patients. The initial experience in lung transplantation is still to small but fewer episodes of acute rejection without significant increase in infection has been reported. Finally, Rapamycin (Sirolimus), a structural drug similar to FK506, but with different mode of action, has been introduced as immunosuppressant. The studies are limited, but it decreased acute rejection with a not significant increase of infections. This make the drug interesting for an use after lung transplantation.

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References
2. Kormos RL, Herlan DB, Armitage JM et al. Mono-

Many factors complicate immunosuppressive therapy with cyclosporine and macrolide immunosuppressants:
- Narrow therapeutic index
- Severe toxicity in case of blood concentrations above the target range
- Rejection in case of blood concentrations below the target range
- Highly variable pharmacokinetics

Drug metabolism and p-glycoprotein mediated anti-transport are largely responsible for the low and highly variable oral bioavailability of cyclosporine and the macrolide immunosuppressants. These are influenced by genetic variability (10-fold), coadministered drugs, food, age, disease, race, and possibly, gender. It can be expected that inhibition of p-glycoproteins and CYP3A enzymes in the small intestine will result in a higher and less variable oral bioavailability.

Liver dysfunction, such as cholestasis, and interactions with CYP3A inhibitors lead to accumulation of metabolites in blood. The immunosuppressive activity of metabolites is generally less than 10%. The only metabolites, for which an association with toxicity has been described are the cyclized cyclosporine metabolites. However, a cause-effect relationship has to be established. Tacrolimus metabolites significantly cross-react with the antibody used for immunoassay, during therapeutic monitoring. The extent of cross-reactivity does not parallel immunosuppressive activity. During episodes associated with accumulation of metabolites, results of the immunoassays may be misleading. For cyclosporine, relatively specific immunoassays are available. Therapeutic drug monitoring has proved useful to provide information about the adequacy of the dosage regimen or the likelihood of toxicity. Today, concentration-controlled dosing of cyclosporin A and tacrolimus is accepted throughout the world. Therapeutic drug monitoring is recommended for mycophenolate mofetil and it is expected to be necessary for sirolimus.

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References
Effect of donor bone marrow cells infusion after intestinal transplantation in swine

ANDREA VIEZZOLI, ANTONELLO PLEGGI, MARIO ALESSIANI, PIERO FERRARI, FULBERTO FAYER, TADEJA PINTAR, LAURA BABBATO, FRANCESCA ABBIATI, MAURIZIO BONFICHI, MARCO SCANDONE, ELOISA ARRUSTINI, PATRIZIA MORBINO, PAOLO DIONIGI, ARIS ZONTA

Department of Surgery, Genetics, Anesthesiology, Hematology, Pathology, University of Pavia and IRCCS Policlinico San Matteo, Pavia, Italy

Combined donor bone marrow cells (DBMC) and solid organ transplantation has been proposed as a means to augment chimerism and to facilitate the development of donor-specific tolerance. In this study we evaluate the effect of combined DBMC and small bowel transplantation (SBTx) in a pig model. Under full immunosuppressive regimen, DBMC infusion does not affect survival after SBTx in pigs. There is an increased risk of immunological reactions (GVHD, chronic rejection) in DBMC infusion-treated animals after discontinuation of immunosuppression.

The multilineage bone marrow-derived (passenger) leukocytes of donor origin, which include pluripotent stem cells and dendritic cells, are detected as circulating mononuclear cells in the recipient during the first few days after organ transplantation (microchimerism). Therefore, these leukocytes home to the recipient’s lymphoid organs and are largely replaced in the graft by similar cells of the recipient. It has been postulated that the bi-directional traffic between the two co-existing donor and recipient cell populations – called systemic mixed allogeneic chimerism – is the basis for graft acceptance as well as the initial step towards the evolution of transplantation tolerance.

One of the strategies suggested to prove this theory is to increase microchimerism with infusion of unaltered HLA incompatible bone marrow cells as an adjunct to whole organ transplantation, without recipient preparation or deviation from generic immunosuppression. Clinical trials to determine the effect of donor bone-marrow cells (DBMC) infusion after liver or kidney transplantation are underway. Purpose of this pre-clinical study is to assess the safety and efficacy of DBMC infusion after intestinal transplantation in swine.

Our study demonstrates that the approach of DBMC infusion after intestinal transplantation does not affect survival under full immunosuppressive regimen. In this study, DBMC infusion was not responsible of increased risk of ACR or infection. After IS discontinuation, the immunological reactions differ between the two groups with or without DBMC infusion. Acute rejection is the rule in the untreated animals (group 2) while GVHD affect survival of most of the animals treated with DBMC infusion (group 3).

Twenty-nine outbred, nonrelated piglets underwent total orthotopic, in continuity, SBTx from equivalent donors perfused through the aorta with Ringer solution. The animals were divided in 3 experimental groups according to treatment (Table 1). Mycophenolate Mofetil (MMF) was given orally at the dose of 10 mg/kg b.i.d. Immunosuppression (IS) was discontinued on the 60th postoperative day. Group 3 animals were treated with 5.28±2.01×10^8 unmodified DBM cells/kg, infused on 3rd postoperative day. No animals received any kind of preconditioning treatment. Fresh DBM cells were isolated from vertebral bodies harvested from the donor and stored at 4°C, until the E.V. administration.

One animal in group 1, two in group 2 and two in group 3 died of technical complications and were excluded from further studies. Group 1 pigs died of acute cellular rejection (ACR). No episodes of moderate or severe ACR (monitored by weekly mucosal biopsies) have been diagnosed, both in groups 2 and 3, during IS. In group 2, two animal died within 60 days for sepsis and 8 were alive when IS was discontinued. Thereafter, all but one died of moderate to severe ACR within one month. The survivor was sacrificed after 6 months while in good conditions. In group 3, three animals died within 60 days for infections (2) and unknown cause (1), and 8 survived for more than 60 days. However, in this latter group, after IS discontinuation 4 of the 8 animals developed a fatal GVHD and died within a few days while two others died of chronic rejection within one month. The remaining two pigs had a normal development and growth until the sacrifice at 6 months. Results are summarized in Table 1.

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### Table 1. The study groups with a summary of the results.

<table>
<thead>
<tr>
<th>group No.</th>
<th>IS</th>
<th>DBM</th>
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<th>Median survival (days)</th>
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consensus panel. Ther Drug Monit 10995; 17: 676-680.
trolled by immunosuppression. The degree of chimerism achieved in both groups is under evaluation.

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References

33 Effects of thyroid hormone modulation on rat liver injury associated with anoxia, oxidative stress and cold storage
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The effects of thyroid hormone modulation on rat liver injury associated with anoxia, oxidative stress and cold storage were investigated. In isolated hepatocytes anoxia and t-BuOOH caused a progressive and time-dependent loss of cell viability, which was greater in hepatocytes from thyroxine(T4)-pretreated rats. On the contrary, the administration of the antithyroid drug 6-propylthiouracil (PTU) increased the liver concentration of reduced glutathione (GSH), reduced the liver injury and improved the liver function after cold storage.

Since brain-dead patients present reduction of circulating thyroid hormones,1,2 some authors have recommended the administration of triiodothyronine (T3) or thyroxine (T4) to brain-dead potential organ donors to improve the outcome of transplantation.1,3 Nevertheless, experimental and clinical studies indicate that the use of T3 therapy is controversial and there is concern that iatrogenic hyperthyroidism may endanger donor organs.4,5 Aim of the study was to investigate, by in vitro experiments, the effects of thyroid hormone modulation on rat liver injury associated with anoxia, oxidative stress and cold storage, unavoidable processes during liver transplantation. Oxidative stress was evaluated because post-ischemic reperfusion is associated with the production of reactive oxygen species.

L-thyroxine (50 µg/100 g b.w.) was administered i.p. to male Wistar rats for 4 days consecutively and experiments were performed on the fifth day. Serum T4 concentration was 9.8±2.3 nmol/dL in controls, 4.4±0.9 pmol/mL in T4-pretreated rats (p<0.01); serum T3 was 2.9±1.4 pmol/ml in controls, 4.4±0.9 pmol/mL in T4-pretreated rats (p<0.01); 2.1±0.3 pmol/mL in PTU-pretreated rats (p<0.001). Similarly, when hepatocytes were exposed to t-BuOOH, there was a time-dependent loss of cell viability. Again, the rate of cell-killing was higher in hepatocytes from T4-pretreated rats (Figure 1B). The cold storage experiments showed that after 20 hours at 0-1°C, livers from PTU-pretreated rats were less damaged and had a better energetic status, as shown, respectively, by the LDH released and the bile production. After the reperfusion that followed the
cold storage, liver tissue concentration of GSH was significantly higher in PTU-pretreated rats than in euthyroid controls (20.6±0.4 versus 15.2±2.3 nmol/mg protein, p<0.05), suggesting that GSH may have a protective role during reperfusion.

In conclusion, the results of this study show that T4 administration increases hepatocyte susceptibility to anoxia and oxidative stress, while PTU administration reduces liver necrosis and improves liver function after cold storage, which may favour graft survival. These results might generate new thoughts in the management of brain-dead organ donors.

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References
Fourth session  
BONE MARROW TRANSPLANTATION

34  
Allogeneic bone marrow transplant as adoptive immunotherapy  
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Convincing evidence that donor-derived effector cells have an effective antileukemic activity comes from clinical studies performed in patients submitted to allogeneic bone marrow transplant (allo-BMT). The ability of this last procedure to eradicate leukemia is caused by both the conditioning regimen and the graft versus leukemia (GvL) reaction. This last, still poorly defined, is probably mediated by MHC-unrestricted natural killer LAK cells or by MHC-restricted T-cells. The existence of a GvL reaction determined by the allogeneic graft is based on: a) studies on patients, relapsed after allo-BMT, who have achieved a new complete remission (CR) after the withdrawal of cyclosporine A, b) the demonstration that graft versus host disease (GvHD) is accompanied by a lower risk of relapse, c) the proved high relapsed rate in patients who have received a syngeneic or a T-cell depleted allogeneic graft. GvL is particularly evident in cases with chronic granulocytic leukemia (CGL) relapsed after an allo-BMT. In these patients the use of cytotoxic drugs or of alpha interferon are almost always ineffective and the execution of a second allo-BMT is associated with a high morbidity and mortality rate. On the contrary, in these cases donor leukocyte infusions (DLI) result in a remission rate of 70±1. The procedure is especially effective in patients with chronic phase CGL, in those with advanced disease response rates are lower. Most studies have documented that in cases with acute myelogenous leukemia (AML) the probability of achieving a new CR is higher if DLI is performed after having reached a second CR by chemotherapy. DLI have also been successfully performed in acute lymphoblastic leukemia (ALL), in non-Hodgkin lymphoma (NHL) and in multiple myeloma (MM). It is believed that the GvHD after adoptive immunotherapy is caused by a median number of alloreactive > 1 x 10^8/kg recipient weight contained in DLI. These last can induce CR if the T-cell content is above 1 x 10^7/kg. The use of a lower T-cell dose is accompanied by less GvHD. Clinically the first sign of response is a fall in white blood cell count with the disappearance of immature myeloid cells. Response may be associated with a reduction of bone marrow cellularity, but a spontaneous recovery usually occurs. However marrow aplasia is one of the major toxicities of DLI, occurring in almost 50% of cases. The other frequent complication is GvHD, that has an incidence of 90%. DLI mortality rate is about 20%. Factors predictive of response to DLI are: the alloreactivity in the graft-versus-host direction, the phase of CGL or the tumor cell burden, the still debated potentiative effect of alpha interferon therapy on DLI and the time interval from allo-BMT to DLI.

A more specific strategy for increasing the antitumor response of a hematopoietic stem cell allograft would be to induce a selective reaction against a defined tumor-specific antigen. The possibility to transfer a tumor antigen-specific T cell immunity in patients with multiple myeloma is the promising ground for future clinical trial. Immunoglobulin heavy and light chain variable regions combine to create the unique recognition site for antibodies. Determinants of these same regions can be themselves recognized as antigens or idiotypes. Therefore MM and B cell lymphoma will express idiotypic determinants that, being unique to each tumor, could be employed as tumor specific antigens. Up to now the clinical effectiveness of adoptive transfer of T cell immunity with allo-BMT is still to be proved. Some points can however be made: 1) the idiotypes of myeloma cells can form an immunogenic vaccine able to elicit a specific immune response in healthy donors, 2) a myeloma idiotype-specific T cell response may be transferred from the donor to the recipient, 3) this T cell response was not blocked by circulating myeloma cells or by immunosuppression performed after allo-BMT, 4) GvHD severity is not increased by this immunotherapeutic maneuver.

Donor-selected effector cells, durably engrafting in allogeneic marrow chimeras, also represent an adoptive cell therapy for EBV-dependent lymphoproliferative disorders and for CMV-associated severe infections complicating allo-BMT.

The aim of this report is to summarize these experiences, to try to answer the many questions remained insolved, to point out future directions for these innovative therapeutic approaches.

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References  


35 Approaches to prevent acute GvHD following allogeneic bone marrow transplantation
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Acute graft-versus-host-disease (GvHD) is still one of the major hazard in allogeneic bone marrow transplantation resulting from reactivity of donor immunocompetent cells versus host tissues. The major target organs are skin, liver, gastrointestinal tract and lymphoid tissues. Acute GvHD represents the end effect of complex interactions among donor T cells, donor and recipient antigen presenting cells, cytokines.1 Billingham defined in 1966 conditions favouring the development of GvHD: a) the graft must contain immunocompetent cells; b) the recipient must have minor or major transplantation antigens lacking in the marrow donor; c) the host must be incapable of rejecting the grafted marrow. According to these criteria GvHD can develop in various clinical settings when tissues like blood products, bone marrow or solid organs are shipped in an immunocompromised patient. The incidence and severity of acute GvHD following allogeneic bone marrow transplantation increases with greater genetic disparity between the donor and the recipient and with the number of donor T cells transfused. The International Bone Marrow Registry identified HLA match, age, female donor sex, post-transplant immunosuppressive therapy as prognostic factors predictive of GvHD development.

Prophylaxis
Strategies for acute GvHD prevention are based on attempts to eliminate donor T-cells or block their activation.

T-cell depletion. Depletion of marrow donor T-lymphocytes is the most effective method to prevent GvHD (2). Depletion of 3 logs of T-cells significantly reduces incidence and severity of GvHD. T-cell depletion, however, is associated with a greater rate of leukemia relapse and rejection. Because of these disparate effects, disease free survival has not been improved with T-cell depletion in recipients of HLA-identical transplants.

Immunoglobulin high dose and protective environments. The administration of high dose immunoglobulin has an immunosuppressive effect which may be of value in preventing acute GvHD. Reduction in intestinal flora and the use of protective environments have reduced GvHD particularly in aplastic anaemia. The benefits in acute leukemia patients are doubtful.

Immunosuppression. The conventional approach for GvHD prevention is the in vivo immunosuppression. Currently used agents are methylprednisolone (MP), cyclosporine A (CyA), methotrexate MTX), tacrolimus (FK 506). The different mechanism of action (Table 1) provides the rationale for the use of these drugs in combination.

Cyclosporine A, Methotrexate, Methylprednisolone. Without post-transplant immunosuppressive therapy, over 60% of bone marrow recipient transplants from an HLA sibling develops moderate to severe acute GvHD. Post-transplant immunosuppressive therapy with CyA or MTX reduces the incidence to 45%. The combination MTX and CyA further reduces the incidence to approximately 25% with improvement in treatment-related mortality.3,4

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucocorticoids</td>
<td>Reduce the number of T lymphocytes, prevent the synthesis of IL1, may impair some APCs functions</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>Interrupts the IL2 synthesis and prevents the second stage of individual T-cell activation.</td>
</tr>
<tr>
<td>Methotrexate:</td>
<td>Inhibits the activated T-cell clonal expansion</td>
</tr>
<tr>
<td>Tacrolimus (FK506)</td>
<td>Operates by a mechanism of action similar to CyA, but with a more powerful immuno suppressive effect</td>
</tr>
</tbody>
</table>

In a series of adult patients affected with hematological malignancies submitted in our Institution to allogeneic bone marrow transplantation, 68 received CyA and MP given at the dose of 3 mg/kg iv infusion over 12 hours and 0.5 mg/kg iv respectively, while 39 patients received CyA at the above reported dosage, associated with a short course of MTX (10 mg/m² at
day + 1 followed by 8 mg/m² at day +3 +6 +11). The association CyA/MTX induced a significant reduction of acute GvHD; the incidence of acute GvHD was particularly high in transplant from HLA phenotypically identical unrelated donors (Table 2).

Table 2. Incidence of GvHD according to prophylaxis: personal data.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CyA+MP (%)</th>
<th>CyA+MTX+MP (%)</th>
<th>CyA+MTX+MP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>67</td>
<td>39</td>
<td>25</td>
</tr>
<tr>
<td>Age(years)</td>
<td>33(16-55)</td>
<td>34(17-55)</td>
<td>30(17-51)</td>
</tr>
<tr>
<td>Female donor</td>
<td>34(51%)</td>
<td>28(72%)</td>
<td>10(40%)</td>
</tr>
<tr>
<td>Pregnancies</td>
<td>16(24%)</td>
<td>11(28%)</td>
<td>1(4%)</td>
</tr>
<tr>
<td>Acute GvHD</td>
<td>40(60%)</td>
<td>19(49%)</td>
<td>19(76%)</td>
</tr>
<tr>
<td>Grade 2</td>
<td>17(25%)</td>
<td>2(5%) P &lt; 0.01</td>
<td>6(24%)</td>
</tr>
<tr>
<td>Grade 3/4</td>
<td>10(15%)</td>
<td>6(15%)</td>
<td>4(16%)</td>
</tr>
<tr>
<td>Chronic GvHD</td>
<td>34(60%)</td>
<td>16(44%)</td>
<td>6(40%)</td>
</tr>
<tr>
<td>Relapse</td>
<td>19(29%)</td>
<td>10(26%)</td>
<td>2(8%)</td>
</tr>
</tbody>
</table>

CyA : 3 mg/kg day iv from day – 1 to take, than 6 mg/kg day orally until day + 100. MP: 0.5 mg/Kg from day + 4 to day +30 and taper. MTX*: 10 mg/m² day iv + 1, than 8 mg/m² days +3,+6,+11. MTX: 15 mg/m² day iv day + 1, than 10 mg/m² days +3,+6,+11.

New drugs. Tacrolimus – it is an immunosuppressive agent that has been proven effective in the treatment of GvHD after allogeneic bone marrow transplantation. In a recent prospective randomized trial comparing CyA plus MTX with FK506 plus MTX, the association FK 506/MTX gives promising results.5

Mycophenolate mofetil (MFM) – it is a powerful new drug which has been proved to be effective in preventing graft rejection in solid organ transplantation. MFM was evaluated either alone or combined with CyA for preventing GvHD in dogs. Data support the notion of synergism between MFM and CyA.6 Preliminary results from Bornhauser demonstrate the feasibility and lack of toxicity of the drug combination in humans.7

Innovative strategies and experimental approaches for preventing GvHD. Prophylaxis of GvHD with current immunosuppressive regimens are only partially successful. T-cell depletion efficiently prevents GvHD.2 This method, however, is associated with impaired engraftment, loss of graft versus leukemia effect, increased frequency of infections or leukemia relapse. Some authors have tried to develop strategies allowing the conditional destruction of alloreactive cells responsible for GvHD, preserving a competent T-cell pool of donor origin.

The use of engineered T-cells. Experimental data provide proof for a therapeutic strategy of GvHD prevention using genetically engineered T-cells: Cohen developed a therapeutic strategy based on the selective destruction of donor alloreactive T-cells using the herpes simplex virus thymidine kinase (HSV-TK) suicide gene system.8,9

Working on the cytokine profiles. The recent proposal that a Th1 to Th2 shift of T-helper cells may impair cell mediated immune response suggested that one potential prophylaxis for GvHD might be the inhibition of Th1 cytokine production by the administration of Th2 cytokines.10 Some authors treated donor mice in vivo with a combination of rHuIL1 and murine rIL4; by this approach they were able to generate CD4 enriched splenic populations with a Th2 cytokine pattern. Subsequent transplantation of these cells inhibited the secretion of the inflammatory cytokine.11 These and other evidences support the concept that the balance in Th1 and Th2 cytokines is critical for the development or prevention of acute GvHD.1

Treatment

Methylprednisolone, ATG, monoclonal antibodies against T-cell, CyA, FK 506, PUVA, antibodies against IL2 receptors, thalidomide have been used to treat patients affected with acute GvHD. Corticosteroids, anti-T-cell antibodies, anti-IL2 receptor antibodies, anti-TNF antibodies; attempts have been performed with high dose of N-acetyl-cysteine.12,13

Table 3. Treatment of acute GvHD (grade =>2)

- Methylprednisolone 2 mg/kg daily for 6 days tapering of 20% every three days. Patient continues cyclosporine A prophylaxis iv.
- For patients with severe GvHD: 500 mg of methylprednisolone iv daily for 3 days, than 250 mg for 3 days, than 125 mg for 3 days. If no response: patients receive ATG 10-15 mg/Kg daily for 5 doses or N-acetyl-cysteine 50 mg/kg continuous infusion over 14 days with cyclosporine A and MP.

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References

11. Fowler DH, Kurasawa K, Husebekk A, et al. Cells of the intestine. Many inflammatory cytokines tion of the graft against the keratocytes of the epi cGVHD is related to the activation of T-cell populatio and steroids. In patients not responding to this conven
tional therapy, alternative treatments are represen
ted by the use thalidomide, total lymphoid irradiation, PUVA therapy, etc. T-cell depletion of the donor graft is an approach to prevent the disease, but it is associated to an increase in the engraftment failure or delay. Immunosuppressive therapy, on the other hand, is loaded with an augmented risk of relapse of the primitive disease and of development of a second tumor. The immunopathogenesis of cGVHD is related to the activation of T-cell population of the graft against the keratocytes of the epidermis, the cells of bile canaliculi and the glandular cells of the intestine. Many inflammatory cytokines play also a critical role both in the induction and progression of cGVHD. Some investigators consider cGVHD as a late expression of the alloreactivity causing acute GVHD (aGVHD), while others believe cGVHD to be an immune disfunction able to generate autoreactive clones. ECP is a relatively new therapeutical option to face up to the cGVDH. This strategy points not to induce immunosuppression, but to stimulate the resources of the immune system of the host in order to inhibits the activation of the alloimmune T-lymphocytes, inducing a new advantageous balance between the host and the graft’s cells. ECP is based on the biological effects of ultraviolet light-A on mononuclear cells collected by apheresis in presence of 8-methoxy-psoralen (8-MOP) and the reinflation of the treated cells to the patients. In recent years the historical Edelson’s ECP technique has been improved by the French groups with a relevant amelioration in terms of safety, reproducibility and tolerability.

We report our experience in the treatment of 12 patients affected with cGVHD resistant to conventional treatment. From December ‘96 to May ‘98 8 male and 4 female patients (mean age 24, 6 ys, range 7-48) were enrolled in the study.

**36**

Extracorporeal photochemotherapy in the treatment of chronic graft versus host disease


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Graft-versus-host disease (GVHD) continues to be a major complication of allogeneic transplantation of haematopoietic stem cells (HSC), which may occur even when the donor is a compatible sibling. GVHD has been traditionally subdivided into two syndromes: acute GVHD (aGVHD) and chronic GVHD (cGVHD). cGVHD, in general, occurs more than 100 days after transplantation and the clinical pattern is quite different from that seen in aGVHD: mesenchymal rather than epithelial tissues are more commonly involved and cGVHD mimic various connective tissue disorders such as progressive systemic sclerosis and Sjögren’s syndrome. The incidence of cGVHD ranges from 30 to 60% and paediatric patients are less commonly involved than adults. The most important risk factors predicting the development of cGVHD are previous aGVHD, advanced recipient age, use of non T-cell depleted BM and alloimmune female donors for male recipients. cGVHD is categorized as either limited (localized skin involvement and/or hepatic dysfunction) or extensive and patients with extensive cGVHD have a less favorable prognosis. Other factors associated with a higher probability of death are a low platelet count and a profound state of immunodeficiency, resulting in an increased incidence of infectious complications (i.e. infections from capsulated bacteria, disseminated fungal infections etc.). Moreover, it must be emphasized that the impact of GVHD is especially important in children, where the growing organism is particularly vulnerable to the consequences and long-term side effects of GVHD itself. Several approaches have been proposed for the treatment of patients with cGVHD, the most widely used being the combination of cyclosporine and steroids. In patients not responding to this conventional therapy, alternative treatments are represented by the use thalidomide, total lymphoid irradiation, PUVA therapy, etc. T-cell depletion of the donor graft is an approach to prevent the disease, but it is associated to an increase in the engraftment failure or delay. Immunosuppressive therapy, on the other hand, is loaded with an augmented risk of relapse of the primitive disease and of development of a second tumor. The immunopathogenesis of cGVHD is related to the activation of T-cell population of the graft against the keratocytes of the epidermis, the cells of bile canaliculi and the glandular cells of the intestine. Many inflammatory cytokines play also a critical role both in the induction and progression of cGVHD. Some investigators consider cGVHD as a late expression of the alloreactivity causing acute GVHD (aGVHD), while others believe cGVHD to be an immune disfunction able to generate autoreactive clones. ECP is a relatively new therapeutical option to face up to the cGVDH. This strategy points not to induce immunosuppression, but to stimulate the resources of the immune system of the host in order to inhibit the activation of the alloimmune T-lymphocytes, inducing a new advantageous balance between the host and the graft’s cells. ECP is based on the biological effects of ultraviolet light-A on mononuclear cells collected by apheresis in presence of 8-methoxy-psoralen (8-MOP) and the reinflation of the treated cells to the patients. In recent years the historical Edelson’s ECP technique has been improved by the French groups with a relevant amelioration in terms of safety, reproducibility and tolerability.

We report our experience in the treatment of 12 patients affected with cGVHD resistant to conventional treatment. From December ’96 to May ’98 8 male and 4 female patients (mean age 24, 6 ys, range 7-48) were enrolled in the study.
ECP consisted of 3 distinct steps:

1. collection of mononuclear cells by a third generation cell separator (Spectra Cobe), processing 2 blood volumes, with the aim to collect a final volume of MNC, always less than 150 mL, with an hematocrit value not exceeding 5%. The procedure time was setted at 180 min as a maximum.

2. processing: the buffy-coat was adjusted to a constant volume of 300 mL by addition of normal saline and of 3 mL of 8-MOP to obtain a final concentration of 200 ng/mL of the drug. The so diluted buffy-coat was transferred into a special UV-A permeable bag and UV-A irradiation was performed at 2 J/cm².

3. reinfusion: the 8-MOP photoactivated MNC was finally reinfused to the patient within 30 minutes.

Treatment schedule consisted of 2 consecutive ECP (1 cycle) weekly x 3 courses, than 2 consecutive ECP monthly. At a mean follow up of 6.8 months (range 1-19), 174 ECP procedures were completed. The mean of total nucleated cells collected was 6.8 x10⁹ (range 0.65-23.8), with a mean MNC percentage of 85% (41.4-98) in a mean final volume of 115.5 mL (37-160). According to a score from -3 to +3 (considering 0 value as stable disease) adopted in our Institution, 11/12 patients had a positive score (range +1-+7), moreover four patients tapered and four patients stopped the immunosuppressive therapy. No relevant side effects were documented, except for sporadic headache, fever and chills. No infectious episodes occurred during the treatment. ECP proved to be safe, reproducible and well tolerated. No relevant procedure-related complications were recorded; the mild side effects observed did not affect the treatment schedule. The highly reproducibility of the technique in all the steps described will permit the comparison of the results among different centers. The clinical results demonstrate that ECP is a new interesting therapeutic option in the treatment of GVHD not responsive to standard therapy. Moreover, ECP should be considered as first-line therapy with the aim to limit the dosage of conventional immunosuppressive drugs.

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References


37 Hematopoietic growth factors in bone marrow transplantation

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In patients undergoing autologous or allogeneic bone marrow transplantation (BMT) hematopoietic growth factors (CSFs) have been used both in vivo in order to speed the hematologic recovery posttransplant and to mobilize peripheral blood progenitor cells (PBPC) for collection by apheresis and in vitro in the attempt to improve the quality of the bone marrow cells infused. Moreover CSFs have allowed the shift from BMT to peripheral blood stem and progenitor cell therapy (PBPCT). Their role have been explored first in the autologous and then in the allogeneic setting. CSFs have permitted high dose therapy to be administered to patients uneligible for conventional auto-BMT. The in vitro expansion of PBPC with a cocktail of CSFs will further increase the number of patients who will enjoy this treatment schedule. As hematologic recovery after allo-BMT is rapidly achieved with the use of CSFs, they might allow transplantation across major donor-recipient tissue-type disparities.

Autologous bone marrow transplantation. In auto-BMT, performed for various malignant hematologic disorders, CSFs have been commonly employed to hasten hematologic reconstitution after the infusion of cryopreserved progenitor cells. Most randomized controlled clinical trials1 have pointed out that GM-CSF as well as G-CSF are able to shorten the period of neutropenia, decrease the incidence of febrile episodes and the use of antibiotics and shorten hospital stay. No effect have been seen on platelet recovery and red blood cell or platelet transfusion requirements. The consistent message from all studies2 is that the time to neutrophil engraftment (absolute neutrophil count >500/µL) is indeed shortened by around 5-7 days. This result is obtained with GM- and G-CSF, independently of the mode of administration (intravenous or subcutaneous) and of the
source of growth factor (E. coli or yeast derived). The issue of when to start growth factor treatment is still debated. Some studies used G- and GM-CSF 24 hours posttransplant others four and eight days after auto-BMT. The optimal dose of CSFs has not been clearly defined. GM-CSF is usually given at 250-500 µg/m², while G-CSF at 10-30 µg/m². CSFs should be continued until there is a sustained adequate neutrophil recovery, although there is no consensus as to what the neutrophil absolute count should be. One approach is to keep on administering CSFs until neutrophil absolute count is >500/µL on two consecutive days, even if in cases with an ongoing sepsis it is better to achieve a much higher count. G-CSF is thought to have fewer adverse effects than GM-CSF, though there are few data supporting this statement. This last CSF, however, may have additional efficacy against fungal infections. Therefore various clinical trials are testing the efficacy of this CSF in association with Amphotericin-B in invasive fungal infections.

In all the studies already reported CSFs had no effect on the major endpoints of early mortality, complete remission (CR) and overall survival. Considering the CR rate, the fact that functional cytokine receptors are expressed on acute myeloid leukemia (AML) and myeloma (MM) cells raised some concern about their possible stimulatory effects on these neoplastic cells in vitro and the possible risk of neoplastic cells outgrowth or therapy with these factors. In AML however this possibility as yet has not appeared as a problem with an appreciable clinical impact; in MM it remains to be proved.

Peripheral blood progenitor cell transplantation. In 1971 it was shown that peripheral blood leucocyte from normal subjects could give rise in vitro to granulocytic and monocytic cell colonies. Later on, it was suggested that the transfusion of these colony-forming cells (CFC) might be as effective as bone marrow in hemopoietic reconstitution after high dose chemotherapy. In 1976 it was shown that the number of circulating stem cells increased dramatically after chemotherapy given for solid tumors. Therefore pivotal studies were carried out, convincingly showing that these cells realized into the circulation by chemotherapy alone or by chemotherapy plus CSFs, or by CSFs alone were not only able to cause rapid engraftment, but also a sustained long-term hemopoiesis. In the autologous setting the advantages of employing peripheral blood rather than bone marrow consist in: 1) avoidance of anesthesia and hospitalization, required for marrow harvest, 2) harvesting stem cells even when the marrow is infiltrated by the tumor or when it is avascular because of radiation or chemoradio-chemotherapy, 3) possible collection of a larger number of stem cells, as several blood volumes can be processed repeatedly. Initial results were obtained from patients with AML, Burkitt’s lymphoma in remission and with refractory marrow-infiltrating lymphoma. In all of them autologous blood progenitor cells (PBSC) had been harvested in the steady state with 7 to 10 alternate-day 4-hour apheresis procedure, that yielded 7 to 8x10⁶ mononuclear cells (MNC)/kg and 8 to 20x10⁶ CFU-GM/kg recipient weight. Afterwards it was observed that the time to hemopoietic recovery and the number of apheresis procedures could be reduced if PBSC collection was performed after high dose chemotherapy. A 14-fold increase in the mean CFU-GM content of blood was found to happen two days after recovering of white blood cell (WBC) count first exceeded 1x10⁹/L in cases with non-Hodgkin’s lymphoma (NHL), MM, breast and ovarian cancer receiving high dose cyclophosphamide (4 to 7 gm/m²). The addition of CSFs to chemotherapy, or the use of CSFs alone further optimized the autologous progenitor cell yields. The number of apheresis procedures necessary to achieve the target dose of PBSC, the toxicity of chemotherapy mobilizing regimen and the time to hemopoietic reconstitution were all shortened. Many studies, carried out to optimize the apheresis procedure, demonstrated that beginning PBSC harvest when the WBC first exceeded 1x10⁹/L was probably misleading and incorrect and perhaps resulted in a large number of unnecessary apheresis procedures. In fact in earlier studies CSFs were discontinued when WBC count reached 1x10⁹/L. Later on, by monitoring PBSC levels, it was documented that at first their value increased simultaneously with WBC counts, but peak levels lasted only one to two days. In these studies peak blood CFU-GM or CD34+ cell concentrations were observed either: a) two days after the WBC first exceeded 2x10⁹/L, b) one to two days after the WBC first exceeded 10x10⁹/L c) when the recovering WBC count reached 5 to 10x10⁹/L. Therefore this last is considered the optimal time to begin PBSC collections after chemotherapy. Anyway the progenitor cell dose was directly influenced by the extent of prior chemotherapy, the phase of WBC recovery post-chemotherapy and the degree of marrow infiltration by the tumor.

A great number of studies have documented that mobilized blood CD34+ cells are different from those present in steady state marrow, as they are much richer in early progenitors. Blood CD34+ cells contain higher proportion of CD38- and HLA-DR- cells, express lower c-kit and CD71 levels and retain less rhodamine 123. A dose of CFU-GM of 20-50x10⁶ or 2 to 5x10⁶ CD34+ cells per kilogram of recipient weight is always associated with a rapid and sustained return of hemopoiesis. Below 2 to 2.5x10⁶ CD34+/kg of recipient weight, engraftment may still occur even if delayed and platelet recovery in particular may be seriously affected.

Only two agents G- and GM-CSF are currently employed in autologous PBSC mobilization. Usually G- and GM-CSF are started 24 hours after the end of chemotherapy and are continued till the last apheresis collection has been completed. G-CSF is
Given at a dose of 10 µg/kg daily by a single subcutaneous injection, while GM-CSF is administered at a dose of 250 µg/m² intravenously. Adverse reaction to both drugs include bone pain, myalgias, headache, and fatigue. GM-CSF, especially at higher dose, may cause fevers, edema, pericardial and pleural effusion. This last agent can rarely determine a central venous catheter-associated thrombosis. G-CSF was found to be much more effective than GM-CSF in expanding the circulating progenitor cell pool. G-CSF mobilized PBSCs significantly decreased the duration of posttransplant thrombocytopenia, while the use of CSFs posttransplant shortened the period of neutropenia but did not affect platelet recovery.

Auto-PBSC have been extensively employed in AML, NHL, MM and solid tumors expecting that their contamination in tumor cells would be lower than that of the marrow graft. The use of highly sensitive immunohistochemical and molecular techniques has however unequivocally demonstrated that malignant cells are present in 20% to 30% of PBSC collections from patients with NHL and solid tumors. Despite this datum a recent study has shown a significantly better disease-free survival rates after autologous PBSC versus marrow transplants. Tumor cell contamination might be decreased by positive CD34 selection and/or by purging techniques.

In the allogeneic setting PBSC harvests were initially performed when donors did not tolerate general anesthesia, donated a second graft after the failure of the primary one or after relapse of the malignant disease, and were syngeneic twins. These earliest PBSC transplants demonstrated the feasibility of the procedure that was associated with a rapid and durable engraftment and with a low incidence of severe graft versus host (GvH) reactions in the recipient, and with the lack of significant toxicity in the donor. Use of higher G-CSF dose was accompanied by a higher CD34+ yield and by a more rapid rise in the platelet count, while the period of neutropenia was unaffected. In the earliest studies the incidence of acute GvHD was only 40%, despite the fact that the PBSC graft contained a quantity of CD3+ and CD56+ cells tenfold more than those present in an ordinary marrow harvest. In more recent studies the incidence of acute GvHD is still fixed at 40%. On the contrary all centers performing allo-PBSC transplants report an incidence of severe chronic GvHD of 70%. This datum has recently been confirmed by the Seattle transplantation team, that compared the occurrence of this complication in PBSC versus marrow recipients. The risk of developing chronic GvHD was significantly higher after allo-PBSC than after allo-BMT. For this reason, in order to obtain a less contamination of the PBSC graft by CD3+ lymphocytes, CD34+ positive selection with immunoadsorption or with biotin-avidin systems was performed. By this way CD3+ cell content of the graft is 2-3 logs lowered.

Recently, a very exciting datum was obtained in haplo-identical three-loci incompatible transplants. In this setting the addition of T-cell-depleted G-CSF-mobilized PBSCs to T-cell-depleted marrow may ensure engraftment by increasing the number of progenitor cells contained in the inoculum. This approach was used in 17 patients. One case had graft failure, one died of grade IV acute GvHD. No other case had grade >II acute GvHD. Nine patients died of transplant related mortality, 2 relapsed and 6 were alive and event-free at a median follow-up of 230 days.

In healthy donor the kinetics of progenitor cell mobilization is consistent predictable and reproducible from study to study. G-CSF is given daily and cause a raise in CD34+ cells, beginning in the day of the third dose. CD34+ cells reach the highest value with the fourth dose then peak on day 5 or 6. Their percentage in peripheral blood increases from less than 0.05% to 1% to 2%, achieving the proportion present in steady state marrow. A strict direct correlation between G-CSF dose and CD34+ cell yield has been observed. Nowadays most transplant teams use G-CSF at a daily dose of 10 µg/kg subcutaneously for five days. Apheresis is carried out on day 5, on days 5 and 6, or on days 4, 5 and 6, depending on how much CD34+ cells are needed.

G-CSF adverse effects include bone pain, headaches, fatigue, myalgias, all responsive to analgesics. Moreover the drug causes a seven-fold raise of the WBC and granulocyte count over baseline and a reduction of platelet count for a sustained period of time. Low platelets are also due to their loss during the MNC apheresis procedure. Minor increases in liver enzymes have also been observed, but they return to normal values when G-CSF is discontinued. Up to now no long-term hematopoietic consequences, ie myeloproliferative disorders, possibly induced by G-CSF have been noted in healthy donors after a 2 to 5 years follow-up.

Allogeneic bone marrow transplantation. G-CSF and GM-CSF have been employed after allo-BMT from HLA-identical siblings, from matched unrelated donors and also from mismatched related transplants with a reduction in the rate of hematologic recovery. The incidence and the severity of acute GvHD was not increased. Moreover, GM-CSF has been successfully used in many studies to treat graft failure that occurs posttransplant. The survival of these patients is poor and although these studies are uncontrolled, they showed survival rates in cases who received GM-CSF that are equivalent to those of historical control patients who successfully engrafted prior to the introduction of the CSFs.

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The first signal delivered through the T-cell receptor (TCR) and antigen itself is responsible for the specificity of immune response. The second, or co-stimulatory signal, is not inherited or acquired bone marrow failure, severe immunodeficiencies, hemoglobinopathies and selected errors of metabolism. Fortunately, only 25-30% of patients who need an allogeneic transplant of HSC have an HLA-compatible relative and the probability of finding a well-matched unrelated donor is no greater than 40-50%; the median time for locating a non-family donor being 4-6 months. Alternative approaches to overcome the problem of identifying a donor in short time are represented by the use of one-antigen disparate unrelated donor and, particularly, by the use of HLA-partially matched family donor. However, in these cases, the use of unmanipulated HSC is associated with an unacceptably high incidence of severe graft-versus-host disease (GVHD), which causes the death of a great proportion of patients. The use of peripheral blood cytokine-mobilized HSC after procedures of T-cell depletion has been demonstrated to be associated with a high chance (>95%) of donor hematopoietic engraftment without development of significant GVHD. In fact, the engraftment of donor hematopoiesis is considered to be a dynamic phenomenon depending on competition between both immunocompetent and progenitor cells of donor and recipient, this technique offers a unique possibility for enormously increasing the number of donor HSC infused, this promoting the establishment of permanent donor chimera. Unfortunately, the infusion of T-cell depleted HSC, patients experience a period of profound immunodeficiency lasting for at least 4-6 months. In fact, immunological recovery after transplantation of HSC is considered to be dependent on two distinct phenomena. In the early post-transplant period (3-6 months), there is an expansion of mature donor-derived lymphocytes transferred with the graft; thereafter, naïve lymphocytes derived from the differentiation of donor HSC colonise the lymphoid organs and sustain the late immune response of recipients. Patients given T-cell depleted transplant cannot benefit from the former contribution of adaptively transferred donor-derived lymphocytes to the immunological reconstitution and this explains why they are exposed to a markedly higher incidence of infectious complications and of leukaemia recurrence as compared to recipients of unmanipulated transplants. A possible strategy to improve the process of immune recovery is to infuse donor T-lymphocytes selectively rendered non-reactive towards alloantigens of the recipient, but maintaining the capacity to generate an immune response against viruses, fungi and leukemia cells.

In this regard, the manipulation of co-stimulatory molecules is a promising field of investigation. In fact, full activation of T-cell requires two distinct, but synergistic signals. The first signal delivered through the interaction between T-cell receptor (TCR) and antigen itself is responsible for the specificity of immune response. The second, or co-stimulatory signal, is not

### References


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**Induction of unresponsiveness towards alloantigens**

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Allogeneic transplant of hematopoietic stem cells (HSC) is increasingly being used for the treatment of patients affected by hematological malignancies,
antigen specific, is delivered through the interaction between the T lymphocyte and the antigen-presenting cell (APC) and is of crucial importance for activation of T-cells. In fact, in the absence of co-stimulatory signals, the interaction of the TCR with its specific ligand causes long-term antigen-specific T-cell unresponsiveness (anergy) or programmed cell death (apoptosis). It is conceivable that several T- and APC-molecules contribute to the delivery of co-stimulatory signals, but, for the time being, the B7:CD28 and CD40:CD40 ligand pathways seem to play a key role for T-cell activation. Drugs, monoclonal antibodies and other proteins (i.e. CTL4-Ig) able to block these pathways have been demonstrated to prevent T-cell activation in response to alloantigens and to induce a state of anergy. In particular, our group documented that the in vitro addition of a combination of blocking anti-B7 monoclonal antibodies and cyclosporine-A to a mixed lymphocyte culture (MLC) was able to generate a state of alloantigen-specific T-cell anergy in the majority of responding cells. Moreover, anergic cell populations generated by this approach displayed a remarkable suppressor activity when a very small number of them was added to a primary or secondary MLR, performed among the same or different individuals. Further experiments demonstrated that alloantigen-specific anergic cells were not impaired in their capacity to react toward virus antigens and leukemia cells. Results obtained so far suggest the usefulness of this approach for planning strategies aimed at accelerating the process of immune reconstitution, without augmenting the risk of severe GVHD, by means of donor T-cell add-back after T-cell depleted transplant of HSC from HLA-partially matched donor. These encouraging in vitro data on the possibility of inducing a state of alloantigen-specific tolerance could also open an exciting era on the in vivo use of HSC and solid organ transplant through the major histocompatibility complex barrier.

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References


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In vivo and in vitro antioxidants therapy to induce tolerance overcoming the HLA barrier

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Bone marrow transplantation (BMT) has become an important therapeutic modality for a wide range of immunologic, hematologic, metabolic and neoplastic disorders for which no alternative treatment exists. However, the success of allogeneic BMT is limited by the restricted donor pool and severe complications during the post transplant period. Attempts to increase the donor pool by selecting increasingly disparate donors, including matched unrelated or haploidentical individuals, are associated with increased incidence and severity of acute and chronic GVHD (Ferrara et al., 1991). Graft versus host disease (GVHD) and long-lasting immunodeficiency are major complications and may lead to increased morbidity and mortality. GVHD is a complex pathophysiologic event resulting from the cooperative interaction of multiple effector cell populations resident in the donor graft and persistent in the host after conditioning. GVHD occurs when the host is immunocompromized and therefore unable to reject the allogenic cells in the graft. Donor origin T cells play an important role in the induction of this process. In the HLA genotypically identical BMT, anti-host alloreactive T cells are directed against minor histocompatibility (mH) antigens presented by the host. These antigens are rec-
ognized in the context of molecules encoded by the major histocompatibility complex (MHC). For matched sibling donors, the frequency of donor precursor helper T lymphocytes directed against host-specific alloantigens is predictive for the subsequent development of acute GVHD. GVHD may also develop following transplantation of solid organs that contain significant numbers of T cells, e.g. the lung or small bowel. Because solid organs are transplanted across MHC barriers, GVHD in such patients may be directed against allogeneic Major Histocompatibility Complex molecules.

The acute phase of GVHD is initiated by mature alloreactive T cells of donor origin and results in the destruction of a number of target organs, including skin, gut, and liver.

Although, GVHD can be prevented or ameliorated by non specific immunosuppressive therapy or T-cell depletion, these measures are associated with increased infection and relapse rates, and in the case of T-cell depletion increased graft failure and lymphoproliferative diseases. Neither immunosuppressive therapy nor T-cell depletion specifically target the initial recognition of host alloantigens. An approach that specifically prevents donors T cell recognition of host alloantigens would preserve T cell control of both pathogens and tumor cells. In both animal and human model systems antigen specific T-cell tolerance or anergy is performed by T cell receptor (TCR) signaling in the absence of costimulation. One critical costimulatory pathway involved in the prevention of anergy is mediated by ligation of CD28 on T cell by its ligands B7-1 (CD80) or B7-2 (CD86) expressed on antigen presenting cells (APC) (Blazar et al., 1994). Following a TCR signal, ligation of CD28 results in cytokine secretion, in particular Interleukine-2 (IL2), and up regulation of α, β, and γ chains of IL2 receptor. Complete blockade of B7-family mediate costimulation, but not of major histocompatibility complex recognition or adhesion, induces host alloantigen-specific anergy by reducing cytokine production below threshold levels necessary for common γ chain signaling. The associated reduction of alloreactive precursor helper T lymphocytes frequency below that predictive for GVHD, without depletion of either non allospecific T cells or hematopoietic progenitors, has been proposed for clinical trials of haplomismatched allogeneic BMT (Gribben et al., 1996).

The activation of T cells involves multiple, rapidly occurring intracellular biochemical changes, including the rise of intracytoplasmic free calcium and the activation of protein kinase C and tyrosin kinases, the activation of mechanisms of gene transcription for cytokines as IL2, tumor necrosis factor (TNF-α), interferon α, and γ and their receptors.

Free radicals, particularly reactive oxygen compounds such as superoxide anion, hydroxyl radicals and the non radical hydrogen peroxide, can be stim-ulated by the cytokine TNF-α and are responsible for some of the effects produced by TNF-α. Scavengers of oxygen radicals can prevent this effect. In T cells many studies have demonstrated the importance of the intracellular redox status in functions such activation, proliferation, and differentiation. The cellular redox status is determined by the production and removal of reactive oxygen species (ROS), the obligatory by-products of aerobic metabolism which, when present in excess, damage cellular macromolecules including DNA, protein and lipids. To combat oxidative damage, cells have evolved a variety of antioxidant defense systems. Of particular interest is the small molecule antioxidant glutathione (GSH) the most prevalent intracellular thiol ubiquitous to all cells types that is pivotal in protection cells from lipid peroxidation. GSH is cysteine containing tripeptide (γ-glutamyl-cysteinyl-glycine) that is found in eukaryotic cells at millimolar concentrations. GSH may exist in two forms, reduced (GSH) and oxyzided (GSSG), a the aminoacid precursor of GSH biosynthesis, L-cysteine, similarly has an oxidized derivative. Intacelular GSH is involved in numerous metabolic pathways including cell protection against oxidative injury and alkylating agents. Adequate concentrations of GSH are required for a variety of immune functions and a variety of T cell functions including the binding, internalization and degradation of IL-2, activation induced death, and protection against gamma irradiation are influenced by GSH intracellular concentrations. GSH is therefore critical for the maintenance of T cell function, decreased levels of total and reduced GSH in CD4+ lymphocytes as in common variable immunodeficiency, are associated with activation of TNF system and may play a immunophogenic role of oxidative stress (Aukrust et al., 1995).

N-acetyl-cysteine (NAC) is a thiol antioxidant precursor GSH, used in human therapy usually as mucolytic agent, an antidote in acute poisoning, and proposed as anti HIV agent (Roederer et al., 1993). Apoptosis induced by TNF-α, which can stimulate ROS production, is inhibited by treatment with NAC. Moreover, NAC has been reported to inhibit the CD80 expression and the proliferation of normal and neoplastic B cells after in vitro activation by CD40 ligand (Colombo et al., 1994).

Activated cells and proinflammation cytokines may contribute to the damage to GVHD target tissues by a production of free radicals or oxidants agents. Our group described a clinical experience with use of NAC in four patients with severe acute GVHD resistant to conventional therapy with cyclosporine and methylprednisolone. At least three of the patients received a second transplant following relapse of the underlying disease, and no postgrafting immunosuppression was given to maximize a graft versus leukemia effect. When GVHD developed and was unresponsive to cycloparsine alone or in combination with methylprednisolone, patients were given NAC at a dose of 150
mg/kg followed by 50 mg/kg over a period of 4 hours for 3 weeks. All patients continued cyclosporine at 3 mg/kg and tapering dose of methylprednisolone. Two of the four patients had complete responses and two had partial regression of GVHD symptoms. Concurrent flow cytometric analysis revealed that NAC therapy was associated with decreased CD80, CD25 expression and CD8+ cells. Our preliminary considerations are that NAC therapy may be useful as a scavenger of free radicals in treating some effects of GVHD. The use of NAC during the standard therapy of GVHD may be useful in advance disease, when low performance status of patients represents the most important limitation for evaluation of real efficacy of NAC in inducing a tolerance status after BMT.

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**References**


**40 New aspects of immune responses against mycobacteria: immunopharmacology studies**

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Host’s resistance against tuberculosis relies on classical MHC-dependent immunity and on CD1-restrictive responses against non-peptide antigens (e.g. mycolic acid) presented by cytokine-activated monocytes (CAM) to CD3+, CD4-/CD8- (i.e., double-negative, DN) T cells. A number of antitubercular or anti-HIV agents do not impair the in vitro induction of CD1b molecule by GM-CSF on normal monocytes, or the functional activity of DNT cells. However, azidothymidine reduces CAM number, whereas Rifampin increases CD1b expression. These results point out that immunopharmacological analysis of chemotherapeutic agents should include the CD1-dependent immune system.

CD3+ lymphocytes bearing the αβ T-cell receptor (TCR), predominantly of CD4+ phenotype, play a significant role in resistance against mycobacteria.1,2 Effector CD4+ T cells show a Th1-like response pattern, following sensitization with mycobacteria-derived peptides presented by antigen-presenting cells in association with class II major histocompatibility complex (MHC).3 However, more recently it was found that a non-classical, MHC-independent system is additionally involved in T cell responses against mycobacteria. In this case the human antigen-presenting molecules are the group I, non-polymorphic CD1 proteins,3 expressed by cytokine-activated macrophages (CAM).4 The antigens presented by the CD1 system are non-peptide macromolecules, including mycolic acids, lipoarabinomannan and other lipid structures associated with the mycobacterial cell wall.4 In this system, the majority of responder cells are CD3+, CD4-, CD8- (double negative, DN) T cells showing γδ or αβ TCR.4 These cells proliferate and generate cytotoxic clones following interaction with mycobacterial glycolipids, presented by CD1b+ monocytes preactivated with granulocyte/macrophage-colony stimulating factor (GM-CSF), alone or in combination with interleukin-4 (IL-4).4

HIV-positive subjects are particularly susceptible to mycobacterial pathogens,5,6 and provide a substantial contribution to the progressive increase of the number of infected cases. It is conceivable that positive or negative effects can be afforded by chemotherapy on host’s resistance against mycobacteria.7,8 Therefore, studies have been performed on the immuno-pharmacological profile of antitubercular or anti-HIV agents concerning CD1-dependent immunity.

Plastic-adherent mononuclear cells (AMNC) of peripheral blood of healthy donors were incubated
with GM-CSF (10 IU/ml) + IL-4 (200 IU/mL), alone (control CAM), or in the presence of 3'-azido-3'-deoxythymidine (AZT 0.4, 2, 10 µg/mL), isoniazid (1, 5, 25 µg/mL), ethanbutol (1, 5, 25 µg/mL), rifampin (RFP 2, 10, 50 µg/mL) or thioacetazone (0.4, 2, 10 µg/mL). On day 3 AMNC proliferation was tested as H3-thymidine uptake and the percentage of CD1b+ cells was evaluated by FACS analysis as previously described.9,10 The results show that all drugs did not depress substantially AMNC proliferation, except for AZT, that afforded 50% and 70% reduction at 0.4 and 10 µg/mL, respectively. Therefore AZT appears to down-regulate the presentation potential of the system. This is in line with the antiproliferative activity of the agent, that is known to produce granulocytopenia and anemia in patients,11 and to inhibit macrophage functions in vitro.12 FACS analysis showed that: (a) limited or no expression of CD1b molecule is detectable on the membrane of AMNC not treated with GM-CSF; (b) marked expression of CD1b can be found on the membrane of AMNC treated with GM-CSF alone or in combination with IL-4. CD1b induction is not inhibited by cotreatment with all drugs tested. In the case of RFP, substantial increase of CD1b is detectable in samples treated with 2 or 10 µg/mL, over those of controls treated with the cytokines alone. Northern blot analysis of total RNA, hybridized with a CD1b cDNA probe,10 showed the presence of a transcript of approximately 2.0 Kb in AMNC stimulated with GM-CSF + IL4, but not in untreated AMNC (Figure 1, lanes 1 and 4, respectively). Additional treatment of AMNC with RFP (10 or 50 µg/mL, lanes 2 and 3) substantially increased the level of CD1b mRNA with respect to that of controls. Hybridization of a similar blot containing the same samples with a GAPDH probe resulted in comparable hybridization signals in all lanes, indicating that equal amounts of RNA were loaded (Figure 1). Integrity of RNA samples was confirmed by ethidium bromide staining of the gel before blotting (data not shown). Since RFP was found to be devoid of cytostatic effects on CAM, it is anticipated that RFP could up-regulate the presentation potential of the CD1 system. However, no data are presently available to explain the effects of RFP on CD1b gene expression.

In conclusion, the present report points out that immunopharmacology and immunotoxicology studies of anti-infectious agents should be performed analyzing not only classical immune responses, but also the CD1-restricted cell-mediated immunological functions.

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**References**

Metastatic renal cell cancer (mRCC) displays inherent resistance to chemotherapy and median survival is usually less than a year. Immunomodulatory treatment with recombinant interleukin-2 (rIL-2) and/or interferon-α (rIFNα) has provided new opportunities for these patients. The optimal dose and schedule of immunotherapy has yet to be determined. We herein report the effectiveness of very-low dose immunotherapy cycles chronically repeated during a long-term follow-up.

In our Department, an ongoing, open, non-randomized phase 2 trial is taking place with the purpose of evaluating response, toxicity and immunological effects of very-low dose immunotherapy in mRCC patients. No selection criteria has been used to enrol patients. Patients' characteristics are shown in Table 1. According to the protocol, rIL-2 is administered s.c. on days 1 and 2 (1 million IU/m² every 12 hours), followed by 1 million IU/m² on days 3-5 of each week; concomitantly, rIFNα is also given (1.8 million IU/m²) i.m. on days 3 and 5. No therapy was administered on days 6 and 7. Four-week treatment cycles are repeated indefinitely at 4-month intervals both in responding and in progressing patients.

So far, 241 immunotherapy cycles have been given to 50 mRCC patients; out of 41 patients valuable for treatment response, only one achieved a complete remission. Five patients exhibited a partial remission, and five showed a stable disease. In spite of the therapy, a progressive disease was seen in 30 subjects. Treatment-related toxicity was limited to WHO grades 1 and 2 only. The overall survival rate of the present series is 48% at 36 months.
Table 2. Median values (cells/ml) of eosinophils, lymphocytes, and lymphocyte subsets before and after the early six cycles of very low dose immunotherapy in mRCC patients. Values of soluble IL-2 receptor (sIL-2R) are also shown (number of cases in brackets).

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<th>Cycle 3</th>
<th>Cycle 4</th>
<th>Cycle 5</th>
<th>Cycle 6</th>
<th>Cycle 7</th>
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<th>Between</th>
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* calculated by General Linear Model procedure, SPSS; 1 Null Hypothesis H0: Is there a significant variation within-cycle over the whole follow up (within-cycles analysis)?; 2 Null Hypothesis H0: Is there a significant cycle effect on the within-cycle variation (within-cycles analysis)?; 3 Null Hypothesis H0: Is there a significant cycle effect on pre-treatment values vs the pre-treatment values of cycle 1 (between-cycles analysis)?; P<0.05 post/pre treatment ratio vs post/pre treatment ratio of cycle 1 (within-cycles analysis); P<0.05 pre-treatment value vs pre-treatment value of cycle 1 (between-cycles analysis).

References


42 Adoptive immunotherapy (AI) with tumor infiltrating lymphocytes (TIL) and recombinant interleukin-2 (rIL-2) in advanced non small cell lung cancer

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AI of NSCLC has been described as a new approach for the integrated treatment of advanced lung cancer, in association with surgery. The use of in vitro expanded TIL allowed the control of cancer progression in locally advanced tumors, even in the presence of residual disease, but was largely ineffective against distant metastases. The use of a combination of AI and post-operative chemotherapy seems to be ineffective in the control of distant metastases and results in a inhibition of the efficient immune response mediated by AI.

Lung cancer is the most frequent neoplasm in developed countries.\(^1\) Despite the intensive use of surgery, chemotherapy and radiotherapy, advanced non small cell lung cancer (NSCLC) has a poor prognosis. For these reason, the need of other innovative approaches is evident. In the ‘80, Rosenberg and co-workers showed that the population of TIL, derived from both experimental and human cancers, had the capability of efficiently lyse autologous cancer cells following long term in vitro culture.\(^2,3\) Basic and clinical data demonstrated that TIL infusion could significantly interfere with the progression of melanoma, renal and ovarian carcinoma. On these bases, we started a preclinical study to evaluate the in vitro characteristics of TIL derived from NSCLC. TIL are derived in a proportion of 60% of unselected samples, are oligoclonal (on the basis of C region of T cell receptor) and very heterogeneous from a phenotypic point of view. Functional analysis demonstrated that TIL derived from NSCLC have the capability of lysing both autologous and allogeneic cancer cells.\(^4\) Interestingly, the analysis of molecular and functional characteristics of T lymphocytes derived from NSCLC draining lymph nodes indicated that these could be a very important source of effector cells for clinical studies of AI.\(^5\) Based on these results, we started two pilot studies to evaluate the potential use of AI in patients operated upon advanced NSCLC. Clinical data demonstrated that the approach is feasible in patient at stage IIia and IIib and safe. More interestingly, we demonstrated that AI is able to control the cancer progression in patients with residual disease and that long term disease free period could be obtained in treated patients.\(^6,7\) Thus, we started a randomized phase III study to evaluate the efficacy of AI (associated with radiotherapy in stage IIIB patients) in comparison with conventional chemoradiotherapy.\(^8\) Analysis of survival demonstrated that AI group had a significant increase of survival. In particular, the more significant results were obtained in patients with residual disease at stage IIIa and IIib of the disease. Stage II had no significant modification of survival rate. Interestingly, this phase III study demonstrated also that the quality of life of AI treated patients was better than that of controls. Despite the good clinical results obtained in the control of locally advanced diseases, poor results were obtained by the use of AI for the control of distant metastases. In particular, AI significantly modified the frequency of intrathoracic recurrences but was completely ineffective against extrathoracic metastases. In an attempt to control distant metastases, post-operative AI was integrated by a chemotherapeutic treatment. Briefly, patients with advanced NSCLC were operated upon, then a sample of the resected tumor was mechanically and enzymatically dissociated and infiltrating lymphocytes were expanded in culture. After 4-6 weeks of in vitro culture, pure populations of proliferating lymphocytes were obtained in 13 patients, which were e.v. infused and then treated with doses ranging from 6 to 18 millions IU fro 30 days. At the end of this period, patients were treated with three cycles of cisplatin and etoposide associated to radiotherapy (60 Gy). At 9-month follow-up, ten out of 13 patients had progressed. Thus the study was closed due to evident absence of efficacy of the analyzed schedule.

The results presented may contribute to a better understanding of AI approaches to advanced cancer. First, the association between surgery and AI is highly synergic. Second, intact functions of the immune system are necessary to mediate an efficient cancer control by AI. Along this line, previously untreated patients have better probabilities to experience a positive response than heavily pretreated ones. Third, AI followed by chemotherapy, seems to completely lose its therapeutic potential, thus suggesting that AI is able to drive an efficient and long term immunoresponse, which may significantly contribute to cancer control. At present, results obtained using the combined treatment with surgery, AI (and radiotherapy in advance cases) seems the most efficient approach for the treatment of NSCLC. Future trials, foreseeing the use of neo-adjuvant chemotherapy, could potentiate the efficacy of post-operative AI. More interestingly, the use of new tools, such as dendritic cells, cellular vaccine and gene therapy, associated to AI, could result in a more efficient control of advanced NSCLC.

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IL-2 is increasingly used to treat cancer patients. The increase in sIL-2R levels during IL-2-based immunotherapy represents a negative biological effect which may impair IL-2 efficacy. Here we provide evidence that the use of very-low doses of s.c. IL-2, despite clinically effective, cannot reduce the expected increase in sIL-2R.

Even though its real clinical benefit is still debated, today interleukin-2 (IL-2) is widely used to treat renal cell carcinoma patients, often in association with interferon-α (IFN). Originally, IL-2 was administered using chemotherapy guidelines where the maximum tolerable dose was given by intravenous bolus or continuous infusion; subsequently, Atzpodien et al. demonstrated that subcutaneous (s.c.) IL-2 is both clinically and immunologically effective.

More recently, Buzio et al. demonstrated that very-low doses of IL-2, given s.c. together with IFN, may induce persistent immunological effects and are clinically effective in controlling tumor growth.

Since it is well known that the marked increase in soluble IL-2 receptor (sIL-2R) levels during immunotherapy correlates with a poor response to treatment, probably because of the soluble receptor’s capacity to bind IL-2 and compete for it with IL-2 surface receptors (7), we wondered how sIL-2R titers vary during treatment in patients receiving very-low doses of s.c. IL-2. For this reason, we titered sIL-2R in the plasma of 13 advanced renal cell carcinoma patients (8 men and 5 women, average age: 62.5) treated according to the protocol originally proposed by Buzio et al.; briefly, after giving informed consent, patients were given s.c. IL-2 at the dose of 1x10^6 IU/m^2 every 12 hours on days 1 and 2, followed by 0.5x10^6 IU/m^2 twice daily on days 3-5 of each treatment weeks, together with IFN, given i.m. at 1.81x10^6 MU/m^2 on day 3 and 5 of each week; a treatment cycle consisted of 4 consecutive weeks and was repeated at 4-month intervals.

Plasma samples were obtained from each patient the morning before the start of immunotherapy and 24 hours after the completion of each cycle (again, in the morning); sIL-2R levels were titered using a commercial enzyme-linked immunosorbant assay kit (T-Cell Diagnostic Inc., Cambridge, UK).

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References

As shown in Figure 1, baseline sIL-2R titers did not vary during time, while they significantly increased following IL-2 and IFN administration, thus suggesting that even very-low doses of IL-2 given s.c. induce sIL-2R in cancer patients.

In conclusion, both immunostimulatory and immunosuppressive events occur during immunotherapy of cancer; in particular, the marked increase in sIL-2R in advanced human malignancies.

Alpha interferon determines the inhibition of the soluble receptor capacity of binding exogenous IL-2, thus leaving IL-2 free to interact with (and thus activate) its cellular targets, i.e., CD4+, CD8+ lymphocytes and NK cells. The use of very-low doses of s.c. IL-2 allows to obtain persistent immunological and antitumor effects, with no significant toxicity; unfortunately, the increased sIL-2R levels we documented in our patients, suggest that such a negative biological effect cannot be eliminated using lower IL-2 doses.

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References

The worldwide prevalence of hepatitis C virus (HCV) infection is currently based on epidemiologic studies of blood donors and it is most probably underestimated. A proof of this has been recently given by the results of the study of Guadagnino et al. which showed that 3.6% in 1352 unselected subjects recruited by systematic sampling among the general population. The HCV prevalence increments ranged from 1.3% among individuals aging less than 30 years to 33.1% among individuals older than 60 years. No significant clinical data were available and about 5% of them had elevated ALT. We may speculate that about 10% of them could be estimated to have some degree of liver disease and consequently we can foresee without being too much pessimistic that 3-5% of the overall Italian population might have some form of liver disease associated with HCV infection. The rate of HCV infection is rapidly declining in the young population and about 2/3 of the anti-HCV positive individuals are older than 60 years. The reason of such a significant decrease is most probably the use of disposable syringes for intramuscular injections. In fact HCV is thought to have been spread in the past via intramuscular injections using boiled glass-syringes. According to these observations we can not believe that it is cost-effective to treat all HCV infected individuals with alpha interferon which may cause important adverse effects and worsen the quality of life during treatment. Therefore one important issue is to identify the patient who is worth to treat. Alpha interferon (IFN) decreases serum alanine aminotransferase (ALT) and serum viral nucleic acid levels improving liver histology in approximately 10-40% of cases. Long term responders to interferon who recover from hepatitis and virus infection are unlikely to develop cirrhosis and hepatocellular carcinoma (HCC). Alpha interferon determines the inhibition of viral replication, reduces hepatic necrosis, inflammation and fibrosis, but it has also antiproliferative activity. The question is whether alpha interferon is able to prevent or delay the progression to cancer in patients whose liver disease has already progressed to the cirrhotic stage. Preliminary studies were performed in anti-HCV positive patients only and from restricted areas of the world and did not always analyse important variables which may influence the HCC incidence such as: stage of cirrhosis, grade of intrahepatic inflammation, aetiology of viral hepatitis (HCV versus HBV) and years of illness. On the other hand only subgroups of patients might benefit from treatment. To overcome these problems we studied whether IFN has an independent effect on the HCC attack rate in a larger series of patients from
The study inclusion criteria were: 1) HBsAg and/or anti-HCV positive; 2) histologic evidence of chronic hepatitis and cirrhosis; 3) untreated patients with at least 3 years follow-up (defined as the interval from the first diagnosis of cirrhosis until the date of the last visit in hospital); 4) Treated patients with at least 3 MIO I.U. IFN thrice weekly for 3 months). The response to therapy was defined as: Sustained Response = persistent normalization of serum transaminases during the whole post-treatment follow-up for HCV patients and in addition loss of serum HBeAg and/or HBV-DNA for HBV patients and IgM anti-HD for HDV patients; temporary response = serum ALT elevation after initial normalization during treatment; no response = persistence of elevated ALT levels with none of the characteristics listed above. Firstly we studied the HCC attack rate in 925 consecutive patients with chronic viral hepatitis and Child A cirrhosis, 537 (58%) untreated and 388 (42%) IFN treated. Anti-HCV (692) and HBsAg (223) patients were tested yearly by ultrasound scanning and serum alpha-1-fetoprotein. HCC diagnosis was confirmed by histology. We identified prognostic HCC risk factors by multivariate Cox-regression analysis: age (p<0.001), sex (p<0.001) and portal hypertension (p<0.001) were significant risk factors whereas liver inflammation (p=0.21) and iron storage (p=0.18) were borderline. In the second step of our study we matched treated and untreated patients according to prognostic factors including the individual follow-up time from the histological diagnosis of cirrhosis and conditional logistic regression was used to analyze the independent treatment effect. The relative (untreated vs. (red) HCC risk of untreated vs. treated patients was 1.99 (p<0.03) for the overall population in all patients, 1.14 (p=0.004) for in anti-HCV positive and 6.28 (p<0.007) for in anti-HCV positive/anti-HBc negative patients whereas it was not significant in HBsAg positive patients. The effect of treatment was analyzed independently of the type of biochemical response to IFN because of the low number of sustained responders after matching. To study the effect of alpha interferon on the development of HCC we had to define a minimum treatment duration in order to allow IFN to exhibit its effect on liver disease. Our decision was influenced by the current consensus9 that patients should be treated for at least 3 months before stopping therapy in non-responder patients. The analysis of the independent effect of alpha-interferon on the basis of group matching for the variables sex, age, signs of portal hypertension, intrahepatic inflammation, stage of cirrhosis, storage and follow-up time showed a 2-fold lower HCC risk in IFN-treated versus untreated patients. The subgroup analysis showed that the effect was stronger in anti-HCV positive patients (risk ratio of 3) and the stratification of HCV infected patients for HBV infection (anti-HBc positive) showed that treated patients, infected by HCV alone had more than 6 folds lower HCC risk than untreated patients. The effect of treatment was not significant in HBsAg carriers and in anti-HCV/anti-HBc positive patients indicating that a short term IFN therapy does not appear to modify the oncogenetic risk of HBV infected patients. These data support the hypothesis that HBV has oncogenetic potentials not only in HBsAg carriers and in fact integrated HBV DNA sequences can be found also in HBsAg negative, anti-HBc positive individuals. On the contrary IFN therapy does not appear to modify the oncogenetic potential of HCV which appears to be mostly mediated by hepatic inflammation and regeneration. The positive effect of treatment appears to be independent of the sustained response to therapy and it which could be due to the anti-proliferative activity of alpha interferon. Even if we could not analyse the independent effect of sustained response to therapy because of the low number of cases after group matching we found that in spite of the wide range of therapy schedules a median weekly dose of 9 MIO I.U. with cumulative dose of 276 MIO I.U. for at least 6 months induced a sustained response in 15% of cases. The finding suggests that in the single patient cirrhosis at histology without signs of decompensation can not be considered a major predictive factor of non-responsiveness of chronic viral hepatitis to IFN.

In conclusion we may speculate that it appears cost effective to treat patients with more advanced forms of chronic viral hepatitis and also patients with Child A cirrhosis where we observed IFN treated for chronic viral hepatitis and matched for prognostic HCC risk factors and follow-up time that therapy reduced the HCC risk of the overall population by 2 fold, and by more than 6 fold in the subgroup of HCV infected patients without HBV markers. This hypothesis needs to be validated in future studies. P prospective clinical trials. should be performed to test whether patients with chronic hepatitis and cirrhosis have a reduced HCC and mortality rate when they receive prolonged or repeated courses of interferon-alpha. The In such trials patients should be stratified for HBV markers and the influence of prolonged or repeated therapy on HCC development and mortality should be studied.

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References
The production of interferon has shown that therapy with IFNs may prevent hepatocellular carcinoma in HCV-related liver cirrhosis. J Hepatol 1996; 24:141-7.

In all our patients suffering from chronic hepatitis HCV related genotype 1b no responder to interferon-α. In these patients we administered interferon-β (IFN-β) 6 million 3/time weeks for 3 months. Titers of hepatitis C virus (HCV) RNA and HCV genotypes were determined. In the present study was investigate the effects of IFN-β on liver function parameters, serum hepatitis C viremia, serum IFN-β, hepatic growth factor (HGF), CD8 cytotoxic lymphocytes and IFN receptors on monocytes in patients with HCV genotype 1b. The clinical utility and specificity of these parameters were also underlined. Before therapy, all sera showed elevated ALT, were HCV RNA positive, infected by HCV genotype 1b, and high viral burden in serum. Changes in ALT level in both CAH and CPH during IFN-β treatment are shown in Table 1, the level significantly in all patients at 3 and 6 months after 3 months of stop therapy. Seven patients not respond to therapy and in five patients to have a negative HCV-RNA after therapy. In Figure 1 the effects of IFN-β therapy on plasma level of HGF were demonstrated. Various epidemiological analyses have identified several clinical and serological features, that predict a high likelihood of a long-term response to therapy with interferon. The most important factors identified were an age of less than 45 years, the duration of hepatitis less than 5 years, the absence of cirrhosis, low concentration of iron in liver tissue, low level of HCV-RNA in serum, genotype 2 or 3 as opposed to genotype 1, and low level of genetic diversity of HCV (so-called quasi species). In all our patients suffering from chronic hepatitis HCV genotype 1b we demonstrated an additional factor to influence the IFN response.
The plasma level of IFN-β is higher in non-responders patients compared to responders, in our preliminary results the level of IFN-β > 2 IU/ML is correlated to poor response to treatment, a this regard may be necessary to extended the analysis to confirm our preliminary data. Our data suggest that IFN-β retreatment act to immune system and is clinical efficient in genotype 1b HCV chronic hepatitis. These drug could be represent a valid alternative in achieving an efficient antiviral effects in patients unresponsive to IFN-α.

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**References**

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**Table 1. Clinical characteristics of treated patients.**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Chronic hepatitis</th>
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<tr>
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<tr>
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<tr>
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<td>ASMA</td>
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</tr>
<tr>
<td>HIV</td>
<td>neg</td>
</tr>
</tbody>
</table>

* CAH=Chronic Active Hepatitis; CPH=Chronic Persistent Hepatitis.

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**Cyclosporin A treatment in rheumatoid arthritis**
CARLOMAURO MONTECUCCO, ROBERTO CAPORALI
Servizio di Reumatologia; IRCCS S. Matteo, Pavia, Italy

Cyclosporin A is a potentially useful drug for the treatment of RA both in early and advanced disease. It appears to be as effective as other disease modifying anti-rheumatic drugs (DMARDs) but it seems superior in slowing radiological progression of the disease. In severe, refractory patients its use in combination with other DMARDs appears effective in some controlled studies. Careful patient selection and strict adherence to monitoring guidelines can minimize toxicity related to treatment.

Rheumatoid arthritis (RA) is a chronic disease with variable course that often leads to functional decline, work disability and increase in mortality rate. Current therapy generally consists of a nonsteroidal anti-inflammatory drug and a second-line agent, or disease-modifying anti-rheumatic drug (DMARD). However, this second-line therapy remains unsatisfactory in that it is frequently only partial effective, and many patients discontinue therapy because of drug toxicity and/or loss of efficacy.

Cyclosporin A (CyA) has been used in RA since the late 1970s. The rationale for its use in RA refers to its mechanism of action, leading to interleukine 2 release inhibition and consequently suppression of T cell activation, inhibition of interleukine 1 and tumour necrosis factor alpha release from macrophages.

CyA used as a single drug. In early open studies, CyA was used at 10-mg/kg/day, but in subsequent trials, the starting dose was scaled down to < 5 mg/kg/day and modulated according to strict guidelines related to both efficacy and or toxicity. These initial studies showed that CyA provides a clinically relevant bene-
fit in patients with refractory, longstanding disease. Yocum and Torley have summarized the eight open trials and nine controlled studies of CyA in RA with similar conclusions.² In 1993, Wells and Tugwell published a meta-analysis of five studies of CyA in RA.³ The authors included in their analysis studies in which CyA was compared to placebo, D-penicillamine and azathioprine. CyA showed a clear benefit over placebo and improvement in all clinical parameters was noted. When compared with conventional DMARDs, the efficacy of CyA was similar. As for the laboratory parameters evaluated, CyA showed little effect on ESR values, but an average 40% improvement in CRP over a period of 6 months. The lack of effect of CyA on ESR was noted in almost all studies and probably reflects the inability of the drug to affect certain acute-phase reactants. So, from a practical point of view, ESR cannot be used as a reliable guide for response to therapy in clinical practice.

Symptoms amelioration is only one of the objectives in RA treatment; the effects of any DMARDs on joint damage appear to be particularly important, because less progression should mean real disease control.⁴ Moreover, a slower rate of progression is thought to alter the progression of disability. The conclusions that can be drawn from these observations concern the possibility of altering the course of joint damage by starting DMARD’s treatment at a very early stage and by monitoring the patient also with an accurate radiological assessment. Pasero et al studied the effect on progressive joint damage in early RA by performing an open trial in which CyA was compared with other commonly used DMARDs.⁵ CyA led to a significant decrease in progression and in the appearance of new erosions over a period of 12 months; these data were confirmed at 24 months. Moreover, there was a lower dropout rate for severe side effects in the CyA group than in the DMARD group, even though the number of patients experiencing adverse effects was higher in the CyA treated group.

Combination therapy with CyA. The use of combinations of different DMARDs is one of the approaches to improve the effectiveness of treatment in non-
responsive RA patients. While the results of uncontrolled clinical studies have suggested that combinations were more effective than single DMARD, most controlled clinical trials using a variety of combination therapies have shown increased toxicity without increased efficacy. Various uncontrolled clinical studies using CyA in combination with other DMARDs have been reported so far, however only a few studies have demonstrated the usefulness of this therapy. A 24-week, placebo-controlled, randomized, multicenter trial in 148 patients with RA that was partially responsive to MTX, in which the combination of low-dose CyA and weekly MTX was found to be significantly more effective than the combination of placebo and MTX was recently reported. To obtain more data regarding the continuing efficacy and safety of CyA + MTX combination therapy, a multicenter, open-label extension of the previous study was performed. Patients who had been randomized to receive CyA + MTX in the initial study continued with the same treatment for a further 24 weeks, while patients who had been randomized to receive placebo were given CyA + MTX. The clinical improvement previously observed in patients treated with CyA + MTX was maintained for 24 subsequent weeks, without serious adverse effects, and was also observed in the patients whose treatment was switched from placebo + MTX to CyA + MTX. Recently, we were involved in a 12 months, multicenter, open, prospective pilot trial in which the efficacy and tolerability of CyA associated with hydroxychloroquine (HCQ) vs CyA alone was evaluated in early active RA. As for this experience, the association seems to induce a more rapid clinical improvement with an acceptable safety profile. The main safety issue relates to the renal toxicity and the potential for induction of malignancies. Alexopolou and Ludwin have reviewed CyA nephropathy in RA and conclude that, although RA patients are at increased risk of acute reversible as well as chronic structural changes, careful patient selection and adherence to monitoring guidelines can minimize this risk. RA patients at high risk for CyA induced nephrotoxicity include patients with a reduced GFR, those receiving concurrent nephrotoxic drugs, patients treated with > than 5 mg/kg/day, patients who develop high CyA plasma levels. Data concerning the concomitant use of nonsteroidal anti-inflammatory drugs are not conclusive. To minimize renal toxicity, patients should start with a dosage of 3 mg/kg/day, administered in a twice-daily oral regimen. A rise of serum creatinine to levels > 30% of pre-treatment levels should prompt a 50% dose reduction or temporary discontinuation, according to the severity. Routine measurement of CyA levels is no essential, but may be useful in detecting drug interactions, non-compliance or poor bioavailability. The issue of relationship between CyA and malignancies still remains a matter of debate. In a review of 1,000 patients with RA who had received CyA in clinical trials, 17 cases of cancer were found; this incidence was similar to that reported with other DMARDs. The other frequently observed side effects of CyA include gastrointestinal intolerance, hypertrichosis, headaches and gingival hyperplasia. Many of these regress with dose-reduction, but are not recognized to be invariably dose related.

Safety: The main safety issue relates to the renal toxicity and the potential for induction of malignancies. Alexopolou and Ludwin have reviewed CyA nephropathy in RA and conclude that, although RA patients are at increased risk of acute reversible as well as chronic structural changes, careful patient selection and adherence to monitoring guidelines can minimize this risk. RA patients at high risk for CyA induced nephrotoxicity include patients with a reduced GFR, those receiving concurrent nephrotoxic drugs, patients treated with > than 5 mg/kg/day, patients who develop high CyA plasma levels. Data concerning the concomitant use of nonsteroidal anti-inflammatory drugs are not conclusive. To minimize renal toxicity, patients should start with a dosage of 3 mg/kg/day, administered in a twice-daily oral regimen. A rise of serum creatinine to levels > 30% of pre-treatment levels should prompt a 50% dose reduction or temporary discontinuation, according to the severity. Routine measurement of CyA levels is no essential, but may be useful in detecting drug interactions, non-compliance or poor bioavailability. The issue of relationship between CyA and malignancies still remains a matter of debate. In

<table>
<thead>
<tr>
<th>Variable</th>
<th>12 months (39 patients)</th>
<th>24 months (14 patients)</th>
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*12 months group only.

References

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47 Combination therapy with cyclosporin A, methotrexate and hydroxychloroquine in severe, refractory rheumatoid arthritis: a pilot study

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Servizio di Reumatologia, IRCCS S. Matteo, Pavia, Italy

A pilot clinical study was performed in order to evaluate the efficacy and safety of CyA in association with methotrexate and hydroxychloroquine in 39 patients with severe refractory rheumatoid arthritis was performed. Data after 12 months (39 patients) and after 12 months (14 patients) show a significant clinical improvement with an acceptable safety profile.

The use of combination of disease modifying anti rheumatic drugs (DMARDs) for the treatment of rheumatoid arthritis (RA) is not a new concept. This approach represents an attempt to enhance efficacy and maximize safety in non-responder patients or in those patients showing only a partial response to a single drug therapy. Cyclosporin A (CyA) has been used in the treatment of RA since the late 1970s; the rationale for its use refers to its mechanism of action, and in particular to its ability in suppressing T cell activation. Till now, many clinical studies have been reported showing either clinically relevant benefit and reduced radiological progression of joint damage in RA patients treated with CyA. There are also evidences from controlled studies that indicates that CyA may be useful in combination therapy in patients who exhibit suboptimal response to CyA alone or with severe disease resistant to other DMARDs therapy.

A recently reported double blind, randomised, placebo controlled study shows that the combination of methotrexate (MTX) and CyA was effective in RA patients and the results were confirmed at 48 weeks. The association of CyA and hydroxychloroquine (HCQ) has been shown to be safe and effective in a recent multicenter study in which we were involved. Thus, we have undertaken a pilot study to evaluate the efficacy and safety of the association of CyA, MTX and HCQ in patients with severe refractory RA. We treated 39 patients (37 females, mean age 52.5 years; mean disease duration 4.5 years) with active RA using CyA (Neoral® 3-mg-kg-day per os in two daily doses) associated with MTX (15 mg IM once a week) and HCQ (400 mg in two daily doses). All patients were treated also with oral corticosteroids (mean dosage at the beginning 9.7 mg-day of prednisone) and non steroidal antiinflammatory drugs. All patients have been previously treated with at least 3 DMARDs, without significant clinical improvement (MTX in all cases, HCQ in 34 cases, sulphasalazine in 21, CyA in 8, IM gold salts in 19,

<table>
<thead>
<tr>
<th>Variable</th>
<th>12 months</th>
<th>24 months</th>
<th>P (anova)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain (VAS) % reduction</td>
<td>41%</td>
<td>51%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tender joint count % reduction</td>
<td>54%</td>
<td>59%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Swollen joint count % reduction</td>
<td>38%</td>
<td>40%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HAQ functional score % reduction</td>
<td>27%</td>
<td>36%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Physician assessment % reduction</td>
<td>35%</td>
<td>42%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Patient assessment % reduction</td>
<td>30%</td>
<td>41%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C-reactive protein  % reduction</td>
<td>57%</td>
<td>66%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Daily prednisone dose % reduction</td>
<td>22%</td>
<td>25%</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Side effects requiring drug withdrawal (no.)</td>
<td>2</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Side effects requiring dose reduction (no.)</td>
<td>8</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>All side effects (no.)</td>
<td>17</td>
<td>7</td>
<td>–</td>
</tr>
</tbody>
</table>

*12 months group only.

Table 1: Outcome measures (reported as percent reduction as compared to starting values) and adverse reactions in severe refractory RA treated with CyA, MTX and HCQ combination therapy.
Laboratory assessment at baseline and at each subsequent visit included evaluation of CRP value, complete blood cell and platelet count, measurement of transaminases, bilirubin, creatinine, blood urea nitrogen, albumin and uric acid, urinalysis. Monitoring for possible side effects was done using open-ended questions to identify any new problems occurred since last visit. Duration of follow-up was 12 months for all the patients and 24 months for 14.

As shown in Table 1, a significant improvement in all the clinical variables considered and a decrease in the CRP values was detectable after 12 months of therapy. We also observed a reduction in the daily corticosteroid dose. This trend was confirmed after 24 months in 14 patients.

Four patients had adverse reactions requiring drug withdrawal (1 gastric bleeding, 1 persistent elevation of serum creatinine, 2 Varicella/Zoster infection). Other adverse reactions observed were nausea (3 patients), transient serum creatinine elevation (5), Hypertension (8), hypertricosis (3), Herpes Zoster (1), and anaemia (1); in 11 cases CyA dosage was reduced. The findings in the present study seem to confirm the efficacy of this association in severe refractory RA with a quite low incidence of severe side effects. Controlled studies are needed to obtain definitive data about clinical efficacy of this association; however the low incidence of side effects and the possibility to maintain patients in treatment for a long time appear promisingly.

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**References**


---

**Table 1**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>After 12 months</th>
<th>After 24 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>34.5</td>
<td>12.3</td>
<td>9.7</td>
</tr>
<tr>
<td>Serum Creatinine</td>
<td>1.3</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Urea</td>
<td>52</td>
<td>48</td>
<td>47</td>
</tr>
<tr>
<td>Albumin</td>
<td>40</td>
<td>35</td>
<td>34</td>
</tr>
<tr>
<td>Uric Acid</td>
<td>6.2</td>
<td>5.9</td>
<td>5.6</td>
</tr>
</tbody>
</table>

**48**

**Association of cyclosporin A and iloprost in the treatment of systemic sclerosis: outcome after one year therapy**

GILBERTO FILACI, MARCO SCUDELETTI, MAURIZIO CUTULO, CORRADO CASTAGNETO, GIUSEPPE MURDACA, FRANCESCO INDIVERI

Department of Internal Medicine, University of Genoa, Genoa, Italy

Therapy of systemic sclerosis is still debated. The usage of vasodilators, mainly acting on the microvascular district, and immunosuppressants have been supported by recent advances in the comprehension of disease pathogenesis. We present the results of a study in which 14 patients affected by systemic sclerosis were randomized for receiving treatment with Iloprost alone (group A) or the combination of Iloprost plus low dose (2.5 mg/kg/day) cyclosporin A (group B). After one year treatment amelioration of skin, microvascular and esophageal clinical score were observed in group B while no significant variations were seen in group A. No cyclosporin A side effects were observed at the used dosage. These data support the usage of Iloprost plus low dose cyclosporin A in the treatment of systemic sclerosis.

Systemic sclerosis (SSc) is a chronic inflammatory disease of connective tissue characterised by fibrosis involving skin, small vessels and internal organs. Etiology and pathogenesis of the disease are unknown. Recently, the attention of researchers has been focused on the microvascular lesions and on the abnormalities of the immune system as the main and precarious alterations leading to disease development. \(^1\) On these bases, we hypothesised that the therapeutic association of a vasodilator such as Iloprost, a synthetic analogue of Prostaglandin I2 inducing preferential vasodilatation of the microvascular district, with cyclosporin A, a very effective immunosuppressive drug, could be useful.

In fact, Iloprost demonstrated already to possess clinical efficacy in reducing the number and the severity of Raynaud's phenomena, \(^3\) although in a recent study no significant amelioration of the nailfold videocapillaroscopic score was detected. \(^4\) However, the long term outcome of Iloprost treatment on visceral organ involvement in SSc is not known. Furthermore, CyA has been reported to induce improvement of SSc skin lesions \(^5\) and esophageal function, \(^6\) although the latter effect deserves further investigation. However, CyA treatment of SSc elicited some constraints mainly due to its renal toxicity when used at doses \(\geq 3 \text{ mg/kg/day}. \) \(^2\) We hypothesised that CyA dosages \(< 3 \text{ mg/kg/day} \) could be still therapeutically effective and associated with a poor incidence of side effects. Thus, we started a double arm trial in which
14 SSc patients were randomised for receiving treatment with iloprost alone or iloprost plus CyA. Patients were affected by diffuse (11), limited (2) or sine scleroderma (1) clinical forms of SSc and were not receiving either steroid therapy or immunosuppressive drugs. Patients were equally randomized into two groups homogeneous for age, sex, and disease pattern. Group A received monthly iloprost administration (1 ng/kg/min in 6 hours i.v. infusion, for 5 consecutive days, one week per month); group B received the same iloprost regimen as group A plus oral CyA (2.5 mg/kg/day).

We utilized an already described panel of tests to evaluate cutaneous, esophageal, pulmonary, renal and cardiac functions as well as the nailfold microvascular pattern.

Table 1. Evaluation of the clinical outcome of one year long therapy of 7 SSc patients with iloprost plus CyA.

<table>
<thead>
<tr>
<th></th>
<th>Skin*</th>
<th>Capillaries*</th>
<th>Esophageal**</th>
<th>Lung</th>
<th>Kidney</th>
<th>Hearth</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>14.5±1.8°</td>
<td>5.1±1.9°</td>
<td>5.2±0.8°</td>
<td>0.8±0.3°</td>
<td>0.2±0.1°</td>
<td>0°</td>
</tr>
<tr>
<td>T12</td>
<td>11.1±1.5°</td>
<td>3.9±0.6°</td>
<td>4.4±0.5°</td>
<td>0.6±0.3°</td>
<td>0.2±0.1°</td>
<td>0°</td>
</tr>
</tbody>
</table>

* Statistically significant difference between T0 (basal time) and T12 (after 1 year therapy): P < 0.01; **: Statistically significant difference between T0 (basal time) and T12 (after 1 year therapy): P < 0.05; °: Scores were calculated as described in reference #7.

All patients complained a good tolerance of both therapeutic regimens and no withdrawal from the study were required. The most frequent iloprost side effects were headache, flush and nausea. No CyA side effects were observed.

No statistically significant differences have been detected between basal and after therapy skin, NCV, and internal organ scores in the group A. Group B showed a significant reduction of skin, NCV and esophageal scores after one year therapy (Table 1). No significant modifications were observed even in this group of patients regarding to lung and renal scores. Accordingly, group B patients referred increased skin elasticity, reduced frequency and intensity of Raynaud’s phenomena and improvement of dysphagia and pyrosis.

In conclusion, the results of our study show that one year long treatment of SSc patients with the association of low dose CyA and iloprost, but not with iloprost alone, produced beneficial effects in that the improvement of skin, microvascular and esophageal compromissions was observed.

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References

49 Effects of iloprost infusion on adhesion molecules and microcirculation in ischemic disease
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Department of Internal Medicine and Nephrology, *Department of Cardiology; University of Pavia, IRCCS S. Matteo Hospital, Pavia, Italy

The aim of this study was to assess the expression of αMβ2 integrin adhesion molecules, in patients with ischemic diseases treated with iloprost, contemporarily, to evaluate the concentration of endotelin and nitric oxide. The decrease of αMβ2 integrin after iloprost infusion was reported, Nitric oxide and endotelin are not involved in the beneficial effects of iloprost in ischemic diseases.

Recently, the inflammation role in vascular ischemic disease are underlined.1 Phagocyte endothelial interactions with adhesion molecules play a pivotal role in cellular driven at the interface of thrombosis and inflammation.2 The activation of neutrophil and monocytes modifies the rheology, produce endothelial injury, causing vessel wall occlusion and thrombosis. β2 integrins plays a prominent role in mediating
these cell-cell and cell-extracellular matrix attachment.
We focused the attention to β2 integrin because it is
known that it binds ICAM-1 with adhesion of neu-
trrophil and monocytes to the endothelium, and it links
fibrinogen to activated factor X, culminating in rapid
fibrin formation and thus modulated fibrin formation/dissolution.1,3 In systemic sclerosis (SS)1 and crit-
ical leg ischemia (PAOD) are characterized by the pres-
ence of peripheral ischemia,4 and although PGI2 ana-
logues have been used to induce clinical response in
both the two conditions, the etiopathogenesis of
peripheral ischemia is different: whilst in PAOD, the
large artery atheroma which chronically impairs the
microcirculatory blood flow (pre-microcirculation)
inducing peripheral ischemia;6 it links fibrinogen to
activated factor X, culminating in rapid fibrin formation
and thus modulated fibrin formation/dissolution.

This study was performed to investigate in vivo
effects of iloprost therapy on expression of adhesion
cells perivascular infiltrates, followed by perivascular
fibrotic process (intra-microcirculation), seem to be
responsible for primary microcirculatory disturbances.3

Thirty patients suffering from PAOD or SS were
enrolled. Following a 2-week washout, patients
received intravenous iloprost 0.5-2.0 ng/kg/min by
tinuous infusion. Clinical and laboratory data
were performed at entry and at 6h of first day of treat-
ment. The neutrophil and monocytes of PAOD and
PSS patients, showed a significantly lower expression
of the αMβ2 integrin adhesion receptor expression
(MESF x 1000), after 6 hours of iloprost infusion,
(PMN: baseline- 92.0±24 vs 6h=38.9±12, p<0.007;
monocytes:baseline=97.6±25 vs 6h=54±12, p<0.009)

0In patients, higher baseline NO and ET1 levels
were quantified p<0.004. After 6 hrs of iloprost infu-
sion no significant modifications have been detected.
The echo-doppler evaluation showed that the per-
centage of arterial reactive dilatation was not modi-
fied neither by placebo nor by iloprost and that the
increase in blood velocity flow (indirect index of the
reactive hyperemia of the microcirculatory vasodil-
itation) lasted for a significant longer. After three
months the clinical improvement was confirmed.7,9

These data confirm other clinical observations, but
in vivo demonstrated that this drug modified expres-
sion of αMβ2 integrin of phagocytes that have a key
role in the leukocyte endothelial interactions, inflam-
mation and thrombosis. Therefore this study provides
evidence for an elective microcirculatory vasodilata-
tion induced by iloprost intravenous infusion, and
confirms that NO, mainly produced in conductance
vessel, is not involved in this microcirculatory vasodi-
litation. These data demonstrated efficacy of iloprost
in inducing endothelium recovery and long term clin-
cal benefit independently of interactions with vascular
tone modulators.

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zone@smatteo.pv.it

**References**
1. Bult H. Nitric Oxide and Atherosclerosis: possible
implications for therapy. Mol Med Today. 1996; 510-
6.
expression of CD11b/CD18 on phagocytes in ischemic
disease: a bridge between inflammation and coagula-
3. Carvalho D, Savage COS, Black CM, Pearson JD. IgG
anti Endothelial cell autoantibodies from Scleroderma
patients induce adhesion to human vascular endothe-
neutrophil and monocytes adhesion molecules in
unstable coronary artery disease. Circulation. 1993;
88, 358-63.
Effects on phagocytes in patients suffering from
ischemic diseases: in vivo evidence for down regulation
is responsible for flow-dependent dilatation of human
peripheral conduit arteries in vivo. Circulation 1995;
1; 91:1314-9.
oxide in reactive hyperemia in human forearm vessels.
Circulation 1994; 90:228-5.
8. Hislop S, De Nucci G. The mechanism and signifi-
cance of the coupled releases of endothelium-derived
relaxing factor EDRF and prostacclin (PGI2) from
endothelial cells. Wien Clin Wochenscr 1991; 103:
422-34.
Cyclosporine A nephrotoxicity is characterized mainly by tubular atrophy and interstitial fibrosis however the mechanisms of the CyA-induced nephrotoxicity is not yet understood. In this report we show that cyclosporine reduces the growth of human and porcine renal tubular cells in a dose and time dependent manner with a mechanism involving apoptosis.

Cyclosporine A is so far the most widely immunosuppressive agent employed in organ transplantation. However the improvement in the long term survival of transplant recipients has revealed several side effects of cyclosporine treatment such as nephrotoxicity. CyA nephrotoxicity is characterized by tubular atrophy, interstitial fibrosis and arteriopathy. Even though lesions have been described also with low doses of CyA, according to a large body of literature, renal injury seems largely dependent on the dose of cyclosporine. The mechanisms of cyclosporine induced renal damage are not fully understood. The tubulointerstitial lesions are not consequent to the altered hemodynamic observed in the acute cyclosporine injury since the treatment with ACE-inhibitors protects against the injury without restoring renal blood flow and GFR. Studies in rats have demonstrated that interstitial infiltration by mononuclear cells precedes interstitial fibrosis suggesting an indirect effect of cyclosporine in the induction of renal damage. However, others have shown a direct effect of cyclosporine on smooth muscle and mesangial cells. The aim of the present study was to evaluate the direct effect of cyclosporine on tubular epithelial cells and investigate the mechanism of cyclosporine-induced cell toxicity. Subconfluent human epithelial cells (HK-2)(ATCC, Bethesda, MD) and primary porcine tubular epithelial cells (PETC) were treated with cyclosporine A, untreated cells were used as controls. The effect of cyclosporine A on cell proliferation was evaluated in a dose- and time-dependent manner by counting cell number at definite incubation time with a Neubauer chamber. Cell viability was evaluated by the Trypan blue method. Analysis of cell cycle and evaluation of apoptotic cells were performed by quantifying the amount of DNA by flow cytometry using propidium iodide.

After 24-hour incubation cyclosporine A significantly reduced PETC and HK-2 cell number in a dose dependent manner with a maximal reduction at 800 ng/mL (22.5 x 10^3 ± 6.1 x 10^3 and 7.5 x 10^3 ± 2.9 x 10^3 vs 45 x 10^3 ± 8.1 x 10^3, p<0.05 and p<0.01 PETC and HK-2 respectively). The antiproliferative effect of cyclosporine A was also dependent on the time of incubation. A concentration of cyclosporine (200 ng/mL) corresponding to therapeutic plasma levels significantly decreased PETC and HK-2 cell number starting at 24-hour incubation and reaching a maximum at 72 hours (25 x 10^3 ± 5.2 x 10^3 and 18 x 10^3 ± 3.9 x 10^3 vs 48 x 10^3 ± 8.2 x 10^3, p<0.05 and p<0.01 PETC and HK-2 respectively). When PETC and HK-2 were incubated with 800 ng/mL of cyclosporine the percentage of apoptotic cells significantly increased (35±5% and 27±3.6% vs 6.6±2.3%, p<0.05 and p<0.05 PETC and HK-2, respectively). However at lower concentration no difference could be detected.

Acute and chronic nephrotoxicity are well described side effects of cyclosporine immunosuppressive therapy. The first is characterized by hemodynamic changes and may cause acute tubular necrosis whereas chronic cyclosporine A nephrotoxicity is associated with irreversible changes such as tubular atrophy and interstitial fibrosis. Although acute and chronic vasoconstriction may be very important in the induction of renal lesions by cyclosporine the exact mechanism(s) is not clear yet. In the present study we show that cyclosporine A has a direct toxic effect on renal tubular epithelial cells and this effect is dose and time dependent.

Apoptosis is a distinct form of cell death and occurs under a variety of physiological and pathological conditions. Our results demonstrates that the effect of cyclosporine is probably mediated by the induction of apoptosis confirming previous studies conducted in biopsy specimens of allografted kidneys and in T lymphocyte cell line and human peripheral blood lymphocytes.

These findings suggest that the direct effect of cyclosporine on tubular epithelial cells may be central in the pathogenesis of cyclosporine-induced nephropathy. The toxicity of cyclosporine on epithelial cells may be aggravated in vivo by the changes of renal hemodynamics induced by cyclosporine treatment.

Meeting supplement

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References

Pharmacotherapy of chronic asthma. Asthma can be defined as a chronic inflammatory disease of the airways in which many cells play a role, in particular eosinophils. In susceptible subjects, this inflammation causes symptoms that are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment and causes an associated increase in airway responsiveness to a variety of stimuli. This definition focuses on the inflammatory mechanisms involved in asthma, and thus suggesting the most approaches to therapy.1

Objectives of treatment. The key aims of therapy for asthma are to prevent or reduce symptoms, to improve the quality of life of patients and if possible to reduce or eliminate asthma-related mortality.

The National Heart Lung and Blood Institute International Asthma Consensus report (1997) proposed that the goals of therapy should include: achieving and maintaining control of symptoms, preventing exacerbations, attaining normal lung function, maintaining normal activity levels and avoiding adverse effects from asthma medication.2

Treatment strategies. The ideal current approach to the management of chronic asthma can be summarised as follows: step-wise treatment, tailor general therapy guidelines to meet individual patient needs, identify the minimal medication necessary to maintain control, treat or avoid trigger factors and associated conditions and arrange regular monitoring of the patients’ condition.3

Guidelines for chronic asthma therapy. The guidelines provide for a step-wise approach to therapy (Figure 1), moreover the treatment for asthma depends on the severity of symptoms and of functional respiratory impairment. Two main categories of drugs provide for guidelines of chronic asthma are "controllers" and "relievers"

Pharmacotherapy

Step 1. Mild Episodic Asthma. This mild episodic asthma should be avoided by avoidance of triggers stimuli and a short-acting inhaled β2-agonist to relieve symptoms. A β2-agonist short-term (e.g. salbutamol 100-200 mg) or nedocromil sodium or sodium cromoglicate may be inhaled before exercise or exposure to allergens. A need to use a short-acting β2-agonist more than three times a week, however, is an indication to move the patient to the next step of therapy, which includes regular inhaled anti-inflammatory therapy.

Step 2 - Mild Persistent Asthma. These asthmatic patients have persistent symptoms and they need regular inhaled anti-inflammatory therapy. There has a logic in line with the studies demonstrating the presence of intense inflammation in the airways of mild and newley diagnosed asthmatics.4 Therapy provide for: inhaled steroids in the lowest dose which is required to the control of symptoms and may include the new steroids such as fluticasone 125 mg or budesonide.

51 Pharmacology treatment of chronic asthma

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Asthma is an inflammatory disorder of the airways, as can be confirmed by the involvement of a wide range of inflammatory mediators. Inhaled steroids and bronchodilator drugs are the essential components of stepwise approach of therapy in patients with chronic asthma. It should be considered the possibility of associated conditions (GER, ASA intolerance, pregnancy, elderly etc.), for an accurate asthma therapy.
200 mg twice daily, otherwise nedocromil sodium or
sodium cromoglycate (4 mg four times daily or 5-20
mg four times daily respectively). In asthmatic children
is appropriate to begin with cromones (nedocromil or
cromoglycate). It is important remember that in
patients with persistent symptoms and beginning
impairment of respiratory function, it is advisable to
start at the higher doses of inhaled steroids until
control is achieved and then reduce dose slowly.

Other therapeutic options are the use of low doses of
slow-release theophylline or anti-leukotrienes drugs
even if for the latter more studies are needed before
any recommendation can be made.

Step 3 - Moderate Persistent Asthma. In patients
with moderate asthma, inhaled anti-inflammatory
therapy should be increased. The dose of inhaled
steroids should be increased up to a maximum of
800 mg of budesonide or 500 mg of fluticasone, but
if adequate control is still not achieved, it is rational
to add a long-acting bronchodilator (inhaled long-
acting β2-agonists, slow-release oral theophylline or
inhaled anticolinergics, in order of preference) especial-
ly for nocturnal symptoms, trying to establish the
best association of drugs.

Step 4 - Severe Persistent Asthma. This form of
asthma should be treated with a combination of high-
dose inhaled steroids (until 1500 mg /daily)
with additional long-acting bronchodilators at max-
imum dose. If clinical and functional control of asth-
ma cannot be achieved, it may be necessary to add
oral steroids (given as a single morning dose), possi-ly for limited period and then to return as soon
possible inhaled therapy.

Step-down. Once control of asthma has been
achieved, the dose of inhaled steroids may be
reduced in line with the recommendations of inter-
national guidelines, so that the patient is on the min-
imum therapy compatible with both control of asth-
ma and low adverse effects.

Effects of therapy on bronchial hyperresponsiveness (BHR).
BHR is a key feature of asthma, and it is a reliable
indicator of underlying airway inflammation. The
degree of BHR in asthmatic patients is most com-
monly assessed by progressively increasing the dose
of an inhaled stimulus to a level that provokes a pre-
determined fall in FEV1 and the result expressed as
the dose of the provoking agent required to produce
that fall. Comparisons of the results of such chal-
lenge tests before and after drug therapy give a mea-
sure of the effects on BHR. The control of asthma by
inhaled steroids therapy is usually accompanied by
improvement in BHR. This effect persists during many
months of regular inhaled steroid therapy. Our data
confirm that long-term inhaled therapy with budes-
onide in mild-moderate asthmatic patients allow to
reduce significantly BHR (Figure 2).

Asthma induced by gastroesophageal reflux. Although
the causal relationship between asthma symptoms
and gastroesophageal reflux (GER) is often uncertain
and should be best demonstrated by simultaneously
monitoring esophageal pH and airway function,
lifestyle and medical therapy based on clinical judg-
ment are often effective. Eating smaller and more fre-
cent meals with low-fat content, avoiding food or
drink between meals and at bedtime, eliminating alco-
hol, caffeine, peppermint, chocolate and drugs, such
as theophylline and oral β2-agonists, which potentially
relax the lower esophageal sphincter, and elevating the
head of the bed all are useful measures to reduce GER
and related pulmonary symptoms. Antireflux medical
treatment including H2-receptor antagonists that par-
tially block gastric acid secretion or better, proton
pump inhibitors that almost suppress gastric acid
secretion, has been shown effective in controlling GER
and improving asthma symptoms and lung function.
Antireflux surgery (fundoplication with laparoscopic
or open procedure) is reserved for severely symp-
omatic patients with well-documented esophagitis and
failure of the medical management. Long-term recur-
rence rates with surgery are estimated to be between 10
and 20%.

Aspirin-induced asthma. Since aspirin and non-
sterooidal anti-inflammatory drugs (NSAIDs) intoler-
ance, once developed, is present for life, patients with
known aspirin-induced and NSAIDs-sensitive asth-
ma should avoid aspirin, products containing it and non steroidal anti-inflammatory medications which inhibit cyclooxygenase. In case of acute asthma exacerbations following aspirin or NSAIDs assumption the treatment should be aggressive because these reactions can be dangerous. For patients requiring NSAIDs because of other chronic diseases, a desensitization may be conducted under the care of a specialist. Recognition of the pivotal role of 5-lipoxygenase products in the reaction of aspirin-sensitive asthmatic to aspirin indicates cysteiny1 leukotriene receptor antagonists as particularly suitable for prevention and treatment of asthma associated with aspirin and NSAIDs ingestion.9–11

Exercise-induced asthma. The occurrence of exercise-induced asthma (EIA) which is one expression of airway hyperresponsiveness often reflects an inadequate control of underlying asthma. Therefore, appropriate anti-inflammatory therapy generally results in the reduction of EIA. For patients in whom EIA is the only manifestation of asthma or EIA is still present despite apparently adequate treatment, inhalation of short-acting β2-agonists soon before exercise represents the most effective treatment for preventing asthma exacerbations. Long-acting β2-agonists are equally effective and their prolonged protective effect could be useful at times. Other anti-asthma drugs, namely sodium cromoglycate and nedocromil and to a less extent anticholinergic agents, have been shown to modulate EIA. Recently, several lines of evidence support the effectiveness of anti-leukotrienes in providing prophylaxis against EIA. Physical training and warmup exercise reduce the incidence and the severity of EIA.12,13

Asthma and pregnancy. Retrospective studies show that in 1/3 of the cases asthma worsens during pregnancy, in 1/3 it gets better and in the last cases it is not changed. The poor control of the respiratory function during pregnancy rises perinatal mortality risk, preterm births and low weight at birth, for this reasons it is strongly recommended a good control of disease during pregnancy being careful when choosing the drugs. There is no data on the chronic use of β2-agonist during pregnancy, although reassuring data has been published regarding a lack of adverse effect on utero-placental blood flow of terbutaline and salbutamol (FDA. pregnancy risk classification of drugs: class B), while formeterol is classified as a class C drug by the FDA. The best choice for inhaled steroids is beclomethasone because there has been more human experience, triamcinolone acetonide and flunisolide belong to class D and C. Oral steroid therapy in the elderly should be avoided because of the wrong common knowledge of poor reversibility of airways obstruction in these patients. Inhaled β2-agonists (short and long acting) at therapeutic dose regimens, are well tolerated and safe also in elderly patient even if a careful attention should be paid to the associate disease (for example the CID). In the elderly the evidence of a reduction of β-receptor sensitivity could be explained with the ageing of the adenil-cyclase b-receptorial system.

Antimuscarinic compounds (characterized by the fact that only 1% of the inhaled dose is absorbed in the systemic circulation) are the first choice bronchodilators in the chronic obstructive syndromes of the elderly patients.

The reduction of receptor sensitivity is not expected in muscarinic receptors and if it occurs, it is seen much later if compared to β2-agonist. For this reason these compounds could be more effective than the adrenergic drugs and moreover the two different classes of bronchodilators could be associated to achieve a better bronchodilatation response.

The inhaled steroids are helpful in the treatment of asthma in the elderly and could be associated to β2-agonist to achieve a better control of disease. Theophylline therapy in the elderly patient should be carefully evaluated because of the reduction of the drug clearance of about 20-40% to avoid the risk of a chronic over-dosage of this drug.16,17

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References
In addition, suppression of apoptosis might be a mechanism responsible for myeloid expansion in CML, as suggested by the inhibition of the apoptotic machinery induced by BCR/ABL gene product. Dendritic cells (DC) are professional antigen presenting cells (APC) capable of (i) taking up, process and present antigen; (ii) migrate selectively through tissues; (iii) interact with, stimulate and direct T-lymphocyte response. Normal DC can be easily ex vivo generated and expanded from either CD34+ cells or blood monocytes.

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder due to acquired abnormality in a pluripotential hematopoietic stem cell. CML is cytogenetically marked by the Philadelphia (Ph) chromosome, which originates from a reciprocal translocation between chromosomes 9 and 22, and molecularly marked by a chimeric BCR/ABL gene, resulting from juxtaposition of the ABL proto-oncogene on chromosome 9 with the BCR gene, normally located on chromosome 22. The BCR/ABL fusion gene expresses an 8.5-kb hybrid messenger RNA transcript giving rise to a 210-kD fusion protein (p210BCR/ABL) with increased tyrosine kinase activity, transforming activity for hematopoietic cells, and the ability to cause CML-like myelopoesis in mice. BCR/ABL signals to cause transformation through several mechanisms, including deregulation of cell proliferation by activation of mitogenic signaling pathways, and induction of anchorage independent growth. In addition, suppression of apoptosis might be a mechanism responsible for myeloid expansion in CML, as suggested by the inhibition of the apoptotic machinery induced by BCR/ABL gene product.
ditions for antigen exposure, antigen preparation or modification, etc. Oncogenes, fusion peptides, differentiation antigens as well as viral peptides represent potential targets for a DC-based immune attack. Despite leukemia-associated antigens have yet to be identified, acquired genetic abnormalities (chimeric gene products, mutated oncogenes, tumor suppressor genes) identified in hematological malignancies can be recognized by specific T cells. In fact, intracellular proteins can be processed and presented on the cell surface by MHC molecules indicating the possibility that leukemia-specific genetic abnormalities may be targets for cytotoxic T cells. Recently, normal peptide-pulsed DC have been used for the generation of cytotoxic, BCR-ABL-specific T cells. It has been shown that T cells generated from a normal donor after stimulation with autologous DC primed with a 16 mer peptide spanning the b3a2 breakpoint of BCR-ABL, lysed cells from the peripheral blood of chronic myelogenous leukemia patients.8

Since data on the generation of DC from CML CD34+ cells are still controversial, we performed a study aimed at: (i) establishing optimal culture conditions allowing DC growth from BCR/ABL positive CD34+ cells; (ii) investigating the functional properties of DC in terms of ability to stimulate autologous and allogeneic T lymphocytes proliferation in mixed lymphocyte reaction (MLR); (iii) studying the involvement of DC in the leukemic transformation.

The maximum yield of DC was achieved by adopting a biphasic culture system. From day 0 to 14, CD34+ cells were stimulated to proliferate by using a combination of SCF, IL-3, IL-6, G-CSF. Subsequently, maturation to DC was induced by switching the growth factor combination to SCF, TNF-α, GM-CSF, IL-4. Total nucleated cell expansion (on average, 121-fold) was detected on day 14 of culture, whereas the highest percentage of CD1a+ CD14- cells (24±7%) was detected on day 21 of culture. This allowed a mean output of 120±20x10^6 DC from 10 x10^6 CD34+ cells. Serum from G-CSF-mobilized normal donors and fetal bovine serum had comparable effects on CD34+ cell proliferation (fold expansion: 93±39 vs 121±37), but was less effective in supporting DC differentiation (CD1a+ CD14+ cells: 9 ± 2% vs 24±7%). CML DC expressed HLA-DR (100%), CD40 (71%), CD80 (50%) and CD86 (37%). At the functional level, CML-derived DC showed high activity for induction of pri-mary proliferative re sponses by al-logeneic T cells and for induction, after in vitro pulsing, of primary proliferative responses to KLH soluble anti gen by autologous T cells. Fluorescence in situ hybridization (FISH) and reverse transcription polymerase chain reaction (RT-PCR) for BCR/ABL demonstrated that flow sorted CD1a+CD14- cells were derived from a BCR/ABL positive CD34+ progenitor.

In conclusion, we have established an optimal culture system allowing the large scale generation of PH-positive DC. These leukemia specific DC may be very useful to study HLA class I or II restricted CD8+ or CD4+ antileukemic T-cell reactivity. CML-derived DC may be superior to unprocessed bone marrow for the generation of HLA-restricted leukemia reactive cytotoxic T lymphocytes (CTL). Such CTL may be employed as a more specific treatment for patients with a relapse of CML after bone marrow transplant, resulting in antileukemic responses without graft-versus-host disease. Finally, CML-derived DC may also be used in the context of autologous stem cell transplantation strategies.

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References

53 Dendritic cells, CD4+ lymphocytes, and interleukin 12 in an experimental model of antitumor immunotherapy

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In an experimental model using dendritic cells and synthetic tumor peptides to induce antitumor protection, interleukin 12 exerts potent immunoadjuvant effects, including regulatory functions on both dendritic cells and antigen-specific CD4+ cells. These data offer useful information for developing vaccination strategies using dendritic cells and tumor peptides in humans.

Cell-mediated immunity involving CD8+ lymphocytes is effective in mediating rejection of murine mastocytoma bearing P815AB, a tumor-associated and self antigen showing similarity to tumor-specific shared antigens in humans. Although this antigen may act as an efficient target for class I-restricted responses in suitably immunized mice, a synthetic nonapeptide encompassing the relevant class I-restricted epitopes will not activate unprimed CD8+ cells for in vivo reactivity. Using P815AB-pulsed dendritic cells (DC) and monitoring class I-restricted skin test reactivity in DC-primed mice, we have previously shown that the development of cell-mediated reactivity to P815AB requires T helper effects, such as those mediated by coinmunization with class II-restricted (helper) peptides or by the use of recombinant interleukin 12 (IL-12). The adjuvanticity of IL-12 was suggested to involve improved recognition of class II-restricted epitopes of P815AB. In the absence of helper peptide or IL-12, P815AB not only fails to initiate CD8+ cell responses in vivo and in vitro, but results in a transient state of functional unresponsiveness. The anergic state involves unresponsiveness in CD8+ cells, as detected by skin test assay in vivo and IFN-γ production in vitro, and suppression of IL-2 production by CD4+ cells. In contrast, transfer of DC exposed sequentially to interleukin 12 (IL-12) and P815AB in vitro confers CD8+ cell-mediated reactivity on prospective recipients of an intra-footpad challenge with the tumor peptide, which is accompanied by the production of high levels of IFN-γ by CD8+ cells and of IL-2 by CD4+ cells at the time of the skin test assay. Figure 1 shows the effect of sensitization with P815AB using DC exposed to IL-12 prior to pulsing with P815AB and transfer into hosts to be assayed for delayed-type hypersensitivity reaction (DTH) at two weeks. Mice assayed for DTH activity also served as a source of splenic CD8+ and CD4+ cells to be cultured in vitro for cytokine production. After restimulation with the tumor peptide in the presence of antigen presenting cells, IFN-γ and IL-2 levels were measured in culture supernatants of CD8+ and CD4+ cells, respectively. Consistent with our previous results, P815AB-specific footpad reactivity and high antigen-specific cytokine production were observed only in mice receiving DC treated with IL-12 before peptide pulsing. In this experimental setting, we have previously shown that although the efferent phase of the DTH reaction is primarily class I-restricted, there is an absolute requirement for CD4+ cells in the induction of the response. Having demonstrated the occurrence of previously undescribed class II-restricted epitopes in P815AB, we have further hypothesized that the in vivo effects of IL-12 on DC might include improved ability of these cells to present helper epitopes of P815AB to CD4+ cells.

Figure 1. Induction of T cell responses in vivo to P815AB by host transfer with DC exposed sequentially in vitro to IL-12 and P815AB. Two weeks after cell transfer, mice were assayed for delayed-type hypersensitivity reaction in vivo and also served as donors of purified CD8+ and CD4+ cells that were tested for cytokine production after restimulation in vitro with P815AB and accessory cells. Controls included mice receiving unpulsed DC exposed to IL-12 or peptide-pulsed DC not treated with IL-12. Specificity controls involved the use of the antigenically unrelated P91A peptide for both footpad challenge and restimulation in vitro of CD8+ and CD4+ cells. *Significant difference (p = 0.001) in footpad weight increase (FWI) between experimental and control footpads. **Significant difference (p < 0.001) in cytokine production with respect to controls.
lymphocytes in vivo. This would imply direct effects of IL-12 on DC, an issue that we have been recently addressing.

In conclusion, our results suggest that the combined use of DC, that can be now obtained in larger numbers, and of IL-12, an initiation cytokine for cell-mediated immunity, may open new avenues for immune therapy against tumors because of the increasing availability of synthetic peptides representing tumor-specific shared antigens in humans.

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References

IL-6 receptor antagonists carrying substitutions that abolish interaction with gp130 either at site 2 alone (site 2 antagonist), or at both sites 2 and 3 (site 2+3 antagonist). In addition, substitutions were introduced at site 1 that increased affinity for IL-6Ra. These IL-6 super-antagonists have been shown to inhibit IL-6-induced osteoclast formation in human bone marrow cultures. Therefore they might ameliorate morbidity factors (like bone destruction) caused by IL-6 in MM. When tested as growth inhibitors on a representative set of IL-6-dependent human myeloma cell lines (XG-1, XG-2, XG-4, XG-6 and U266), although site 2 antagonists were effective on 3 out of 4 of the cell lines, only the site 2+3 antagonist Sant7 showed full antagonism on the entire spectrum of cells tested. IL-6 receptor antagonists also proved to be pro-apoptotic factors for myeloma cells. Their capacity to induce cell death was directly related to the impairment of gp130 binding and to their ability to fully block intracellular signalling. In fact, the most potent inducer of apoptosis was again Sant7, which also counteracted the protective autocrine effect exerted by the endogenously produced IL-6. The super-antagonist Sant7 can therefore be considered as a promising tool for the immunotherapy of MM.

Obviously, the possibility exists that the introduction of amino acid changes may generate immunogenic variants. We have tested this possibility in transgenic mice expressing human IL-6. NSE/hIL-6 transgenic mice express hIL-6 under the control of the neuron specific enolase rat promoter. Transgenic mice of line 26 show peripheral expression of bioactive hIL-6 with measurable levels in the serum soon after birth (average 25 ng/mL). Early production of hIL-6 in these mice suggests that they might be immunologically tolerant to hIL-6, as tolerance to the transgene product has been demonstrated in several other transgenic mice models. We decided therefore to test wild-type IL-6 and IL-6 variants Sant1 and Sant7 in these animals. Sant1 contains seven aminoacid substitutions: Y31D/G35F in helix A and S118R/V121D in helix C that abolish interaction with one gp130 receptor chain (defining site 2) and cause loss of biological activity, while Q175I/S176V/Q183A are located in the C-terminus of helix D, the main binding site for the IL-6Ra receptor subunit, and improve affinity of IL-6 for IL-6Ra approximately 4.5-fold. In addition to the aforementioned seven substitutions, Sant7 contains also the substitutions Q75Y/S76K in the AB loop, a second binding site for the IL-6Ra, and L57D/F59/F60W in site 3: due to the presence of these additional mutations affinity of Sant7 for IL-6Ra increases by approximately 65-fold as compared to IL-6 and, besides having lost interaction with one gp130 molecule at site 2 (like Sant1), Sant7 has also lost interaction with the second gp130 molecule at site 3, as mentioned above.

Wild-type hIL-6, Sant1 and Sant7 were formulated in two different adjuvants, CFA or Al(OH)3, and injected at doses of 100 µg (3 injections) either IP (for the CFA formulations) or IP and ID (for the Al(OH)3 formulations); the intradermal (ID) immunization route in mice corresponds to the sub-cutaneous (SC) immunization route in humans. Groups of 5 to 10 transgenic mice were immunized in parallel with non-transgenic littermates. Antibody responses against the injected antigen were determined at day 50 (10 days after the last immunization). The results are shown in Table 1. Wild-type hIL-6 was poorly immunogenic in hIL-6 transgenic mice, with antibody titers 20- to 50-fold lower than those obtained using the same antigen and the same immunization protocol in non-transgenic mice. Sant1, on average, gave rise to antibody levels that were 20- to 50-fold higher than those achieved with wild type hIL-6; Sant7 produced an antibody response more potent than that elicited by wt hIL-6, but less intense than that elicited by Sant1 (Table 1). Immunization of non-transgenic mice with Sant1 and Sant7 gave rise to antibody responses similar to those obtained in transgenic mice (not shown).

Table 1. Antibody titers in hIL-6, Sant1 and Sant7 injected mice, cross-reactivity against hIL-6 of Sant-elicited antibodies and circulating hIL-6 levels in NSE/hIL-6 transgenic mice before and after immunization.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Immunization protocol</th>
<th>Titer against antigen</th>
<th>wt hIL-6 levels</th>
<th>hIL-6 levels Before immunizat.</th>
<th>immunizat.</th>
</tr>
</thead>
<tbody>
<tr>
<td>wthIL-6</td>
<td>CFA I.P.</td>
<td>221</td>
<td>28700</td>
<td>15430</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Al(OH)3 I.P.</td>
<td>508</td>
<td>25290</td>
<td>7080</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Al(OH)3 I.D.</td>
<td>211</td>
<td>22130</td>
<td>6170</td>
<td></td>
</tr>
<tr>
<td>Sant1</td>
<td>CFA I.P.</td>
<td>4368</td>
<td>4180</td>
<td>29670</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Al(OH)3 I.P.</td>
<td>24345</td>
<td>19157</td>
<td>25400</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Al(OH)3 I.D.</td>
<td>9690</td>
<td>7983</td>
<td>23340</td>
<td>86</td>
</tr>
<tr>
<td>Sant7</td>
<td>CFA I.P.</td>
<td>N. D.</td>
<td>N. D.</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td></td>
<td>Al(OH)3 I.P.</td>
<td>8050</td>
<td>4050</td>
<td>36800</td>
<td>2570</td>
</tr>
<tr>
<td></td>
<td>Al(OH)3 I.D.</td>
<td>7240</td>
<td>3230</td>
<td>41800</td>
<td>1310</td>
</tr>
</tbody>
</table>

N. D.: not determined. Microtiter plates were coated with purified antigen. After blocking and incubation with serial serum dilutions, plates were incubated with peroxidase-labelled anti-mouse immunoglobulins. After color development the difference of OD at 450 and 620 nm was read by an automated ELISA reader. Titers were calculated as the reciprocal of the dilution which yielded half-maximal OD reading. Averages of the various groups are shown. Serum IL-6 levels were determined with the human IL-6 Quantikine™ ELISA.

Based on the above data it would seem that IL-6 superantagonists are immunogenic (although Sant7 less that Sant1) in animals which otherwise respond poorly to immunization with wild-type IL-6. It is inter-
testing to note that IL-6 neutralizing IgG auto-antibodies can be detected in 10-15% of the population. Although their biological function is not yet clear, their presence in neutralizing concentrations in a few healthy individuals indicates that neutralization of IL-6 might be compatible with normal life, suggesting therapeutic possibilities offered by the intentional induction of a specific cytokine-autoimmunity. We tested whether antibodies raised in NSE/hIL-6 transgenic mice by immunization with IL-6 superantagonists also recognize wild-type hIL-6. When sera of transgenic mice immunized with Sant1 were tested in ELISAs in which hIL-6 were immobilized, a strong cross-reactivity was observed (Table 1), indicating that the majority of Sant1 elicited antibodies also recognized by hIL-6. When Sant7 was used for immunization the trend was the same, although less pronounced (Table 1). Natural anti-IL-6 autoantibodies detected in 10-15% of healthy humans have been shown to neutralize IL-6 biological activity and to mask IL-6 detection by common ELISA assays. We therefore investigated whether hIL-6 detection in the sera of transgenic animals was modified by this immune response. In all groups we observed a significant decrease in measurable free hIL-6 after immunization (Table 1). However, while injection of hIL-6 gave rise only to a modest decline (2- to 3-fold), immunization with Sant1 caused a remarkable drop to almost undetectable levels (Table 1), and this was greater for those sera with the higher antibody titers. When Sant7 was used for immunization, the drop in detectable hIL-6 was less pronounced (Table 1). Moreover, while circulating hIL-6 in Sant1-injected mice remained almost undetectable for at least five months after immunization, in Sant7-injected mice hIL-6 reached levels of 3,500-3,800 pg/mL roughly one month after the immunization (data not shown). It is possible that auto-antibodies to hIL-6 mask the circulating cytokine, or modify its clearance. In order to more precisely assess the amount of total hIL-6 complexes followed by gel filtration. 10 Fractions corresponding to 12-30 kD (LMW-low molecular weight), and >30 kD (HMW-high molecular weight),

Figure 1. In vitro ad in vivo neutralization of hIL-6 biological activity by sera of NSE/hIL-6 immunized mice. (A) Sera of NSE/hIL-6 transgenic mice immunized with Sant1 neutralize hIL-6 biological activity on Hep3B hepatoma cells. Human Hep3B hepatoma cells were transfected with the IL-6 inducible CRP promoter-SEAP construct (Sporeno et al., 1996), stimulated with 4 ng/ml exogenous rhIL-6 in the presence of serial dilutions of sera from mice immunized with either hIL-6 (open circles) or Sant1 (open squares) formulated in Al(OH)3 and injected IP. The activity of the reporter gene is expressed as the percentage of the reporter gene activity in cells incubated with 4 ng/ml rhIL-6 alone. Similar results (inhibition by the sera of mice immunized with Sant1, lack of inhibition by the sera of mice immunized with hIL-6) were obtained with sera from mice immunized with the antigens formulated in CFA or in Al(OH)3 with I.D. administration (data not shown). (B) In vivo neutralization of hIL-6. Serum Amyloid A (SAA) protein levels were measured before and after hIL-6 injection: the ratio between the SAA levels before and after the hIL-6 injection is indicated as an open circle for each mouse. Controls: non-immunized NSE/hIL-6 transgenic mice; Sant1: NSE/hIL-6 transgenic mice immunized with Sant1 formulated in Al(OH)3 and injected ID; Sant7: NSE/hIL-6 transgenic mice immunized with Sant7 formulated in Al(OH)3 and injected ID. Similar results were obtained for the IP immunization route or for the CFA formulations (not shown).
were collected and analyzed separately. We limited these studies solely to the sera of Sant1-immunized mice, as this was the only case in which circulating hIL-6 became almost undetectable (Table 1). Interestingly, dissociation procedures revealed the presence of hIL-6 at levels comparable to those before immunization. For instance, in the group of mice immunized with Sant1 formulated in CFA, no increase could be detected after immunization (26,980±4540 pg/mL vs. 29,670±3500 pg/mL before immunization). Similar results were obtained for the sera immunized with Sant1 formulated in Al(OH)3 (not shown). HMW fractions were used to measure anti-hIL-6 antibodies binding avidities and capacities by Scatchard plots of data obtained with 125I-hIL-6 in soluble phase assays. In addition, we calculated the percentage of anti-hIL-6 antibodies complexed with the cytokine. The avidity of anti-hIL-6 antibodies was similar in all Sant1 immunization groups and was between 7 and 20 pM. The binding capacity ranged from an average of 273 nM to 791 nM and, also taking into account individual variations, was always in large excess over circulating hIL-6 (1-1.22 nM). It has to be stressed that, even in the sera with the lowest anti-hIL-6 antibody concentration (some of the mice in the CFA immunization group) a binding capacity of 62 nM was obtained vs. 1 nM total hIL-6, which implies that less than 2% of the antibody binding capacity was saturated with the cytokore.

In order to measure if Sant-induced anti-IL-6 antibodies were able to neutralize IL-6 biological activity, human hepatoma Hep3B cells, transfected with an IL-6 inducible promoter, were stimulated with 4 ng/ml exogenous recombinant hIL-6 (rhIL-6) in serial dilutions of sera from immunized mice (Figure 1A). While sera of hIL-6 immunized animals did not modify the response to rhIL-6, sera of Sant1 immunized mice competitively blocked rhIL-6 (Figure 1A). In line with previous observations, inhibition was greater for those sera with the highest antibody titer and the greatest decrease of circulating free hIL-6. The capacity of sera from Sant7 immunized mice to competitively block rhIL-6 was approximately 10-fold lower than sera of Sant1 immunized mice (not shown). Finally, we wanted to determine whether the anti-hIL-6 antibodies elicited in NSE/hIL-6 transgenic mice by Sant immunization were also able to neutralize hIL-6 bioactivity in vivo.

We measured the in vivo response to exogenously injected hIL-6 by monitoring the increase of serum acute phase response protein serum amyloid A (SAA). Sant1, Sant7 and hIL-6 immunized animals were bled 30 days after the last injection of the antigen (pre-hIL-6) or hIL-6. 10 µg of hIL-6 were injected I.P., 9 h after post-hIL-6 injection samples were collected and analyzed (Figure 1B). In non-vaccinated animals the average levels for SAA induction were roughly 5-fold. The level of induction was not significantly different in mice immunized with hIL-6, thus confirming the in vitro results. On the contrary, SAA induction in vivo was abolished in Sant1 immunized mice (Figure 1B; p<0.0001). Again in good agreement with the in vitro neutralization results, immunization with Sant7 was less effective in blocking hIL-6 induced SAA induction: injection of hIL-6 in Sant7-immunized mice resulted in an SAA induction index which was lower than the one observed in non-vaccinated animals (p=0.0014) but higher than that observed in Sant1-immunized mice (p=0.0328).

Based on these data, it would seem that Sant1 (a site 2 antagonist) is a very potent immunogen, able to elicit a strong immune response which is cross-reactive against wild-type hIL-6 and which effectively neutralizes its bioactivity both in vitro and in vivo, whereas Sant7, a site 2+3 antagonist is a weak immunogen. The molecular basis for this apparent discrepancy is presently unknown.

The present studies suggest two potential clinical applications for the recently generated IL-6 super- antagonists: they could be used as competitive inhibitor to directly antagonize IL-6 activity in immune-suppressed individuals like MM or EBV-lymphoma patients, and in this case Sant7 would certainly be the molecule of choice, because it is fully active on all cell lines tested so far; alternatively, they could be used to elicit anti-hIL-6 autoantibodies in immune- responsive individuals like RA patients.

In this second option the different behaviour of Sant1 and Sant7 in NSE/hIL-6 transgensics suggests that the immune response could be properly modulated by using antagonists with different potency as immunogens.

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References
4. Devlin RD, Reddy SV, Savino R, Ciliberto G & Roodman GD. IL-6 mediates the effects of IL-1 or TNF, but not PTHrP or 1,25(OH)2D3 on osteoclast-like cell formation in normal human bone marrow cultures. J Bone Miner Res 1998; 13:393-9.
6. Demartis A, Bernassola F, Savino R, Melino G, Cili-
Hymenoptera venom immunotherapy

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The Hymenoptera venom allergy is cause of serious, occasionally fatal, systemic reactions. The ideal treatment, in people reporting systemic reactions to stings, is venom immunotherapy (VIT), which can completely prevent a fatal outcome and any reaction to the culprit insect’s sting. After VIT 85-95% of the patients remain protected.

Hymenoptera venom allergy would seem to be the oldest allergic form since in the tomb of the Egyptian pharaoh Menes there is a hieroglyphic describing his death in 2621 B.C. following a wasp sting.

Allergic reactions to insect stings involve 1-3% of the general population. The Hymenoptera venom allergy is cause of serious, occasionally fatal, systemic reactions. Deaths from Hymenoptera allergy far outnumber deaths from snake bites. In the U.S.A. Hymenoptera allergy cause about 40 deaths a year while data are lacking for several countries, including Italy. The most frequent clinical patterns are large local reactions exceeding 10 cm in diameter and 24 h in duration, and generalized immediate-type allergic reactions such as urticaria, angioedema, asthma and anaphylactic shock.

Hymenoptera venom allergy is most often caused by Apidae (genus Apis and Bombus) and Vespidae (subfamilies Vespinae and Polistinae). Beekeepers can normally identify the species of insect which stung them correctly, but for all other patients the insect’s identify remains unknown. The patient should try to describe the insect or to identify it from photographs. In these occasions skin tests and serological IgE will be especially valuable.

A lot of substances are found in Hymenoptera venoms: biogenic amines, basic peptides and high molecular weight proteins, mostly enzymes. The venom of Apis mellifera has been shown to consist of several allergic components: phospholipase A2, hyaluronidase, acid phosphatase, melittin and allergen C. The Vespidae venom contain: phospholipase A and B, hyaluronidase and antigen 5.

The pathophysiology of anaphylaxis from insect stings is related to activation of mast cells and basophils by the IgE-related reaction and the consequent release of chemical mediators whose effects cause the clinical symptoms. Mast cells seem particularly involved, as demonstrated by the fact that their typical marker of activation, tryptase, was found in the circulation with a precise time course after anaphylaxis from bee stings. The mediator most likely provoking anaphylactic symptoms is histamine, while the role of newly formed mediators from arachidonic acid metabolism is as yet undertermined.

As to treatment, the drug of first choice is epinephrine hydrochloride in a 1:1000 dilution (0.3-0.5 ml), administered as soon as possible by subcutaneous route, with the dose repeated every 10 minutes if necessary. Other therapeutic agents are antihistamines and high doses of intravenous corticosteroids, which especially prevent the late-phase reaction in biphasic anaphylaxis. The ideal treatment, in people reporting systemic reactions to stings, is venom immunotherapy (VIT), which can completely prevent a fatal outcome and, in as many as 90% of patients, any reaction to the culprit insect’s sting. Immunotherapy with Hymenoptera venoms was shown to be highly superior to treatment with Hymenoptera whole body extracts. The VIT consists of subcutaneously injecting the patients with a first very low, then successively increasing doses of the allergen responsible for this symptoms. Indication for venom immunotherapy is a history of systemic allergic reactions to Hymenoptera stings and documentation of IgE-mediated allergy by skin tests and/or RAST (Table 1). Only patients with severe cardiovascular or respiratory reactions are treated, but also the following factors are decisive, the risk at re-exposure and the psychological situation. A variety of conventional, clustered and rush protocols have been described. The usually recommended maintenance dose is 100 mcg of venom corresponding to about two honey bee stings and probably 5-10 vespula stings. The mode of action of VIT is not completely understood. Recently, the major interest in the mechanism of action of specific IT has focused on the role of T-lymphocytes and cytokines. During VIT with aeroallergens a shift was reported from a Th2 cytokines pattern, characterized by production of IL-4, IL-5, IL-6 and IL-13 favouring the allergic response, to a Th1 cytokines pattern in which IFN-γ inhibiting the allergic response prevails. A shift like this was also found during VIT with honeybee venom and currently seems to be most adequate model of interpreting the immunological modifications elicited by VIT.
The vast majority of patients with VIT of 3-5 years duration were still protected when restung within a year or two after stopping the treatment. Two recent studies reported results up to 7 years after stopping VIT. During this prolonged observation period and repeated re-exposure, 85-95% of the patients remain protected. The results of VIT seemed more favourable in children than in adults and in Vespula venom than in bee venom treated patients.

A problem in Hymenoptera venom allergy is related to the safety of VIT, which may cause systemic allergic reactions in up to 20-40% of patients treated. Finally, it is recommended that VIT is restarted in patients who react again systemically after stopping the treatment.

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References

7. K.J. Hunt, M.D. Valentine, A.K. Sobotka et al. A controlled trial of immunotherapy in insect hypersensitiv-

56 Cytokines and opportunistic infections

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The investigation of the different cytokines patterns: addressing cytokines immunotherapy in the opportunistic infections

Due to the expansion of opportunistic infections and the failures of chemotherapy against the relevant agents of disease, immunotherapeutic approaches to the control of opportunistic infections are being widely advocated. Numerous obstacles, however, must be overcome before a degree of consistent success in this area is achieved. The main obstacle is the poor comprehension of host-parasite relationship in the opportunistic infection.

Most of these agents are human commensals which are held in check by a number of natural and adaptive immune responses which are not oriented to the elimination of the microorganism but rather to restrict its presence (and growth) at permitted body sites. Under this situation, the pattern of cytokines is extremely complex and variable depending on the number and amount of antigen expressed and the body location where expression occurs. For instance, the cytokine pattern (primarily Th1) elicited by the primary cryptococcal infection in the respiratory tract diverges from the mixed Th0 pattern mostly seen during experimental disseminated infection. At least in vitro, there is critical dependence of cytokine pattern upon antigen dose and route of administration. Particularly low doses (<1 ng) of potent secretory mannoprotein, of C.albicans induced IL-4 production in human PBMC (a rare event in this ex vivo model) but higher doses induced IFN-γ, preferentially. The same widely accepted “dogma” whereby a classical Th1 pattern (IFN-γ and IL-2 production driven by IL-12-stimulated T cells) is protective is under dispute, for a number of powerful immunogens such as the hsp70 proteins, induction in vivo of Th1 cytokines is not followed by protection but rather by disease enhancement. Production of IL-12 by BAL cells was enhanced in mice infected by Pneumocystis carinii, and the level were consistently more elevated than in uninfected mice, yet the animals succumbed to the offender, even after exogenous pro-
vision of the putatively protective cytokines.3
Several clinical attempts to use IL-12, IFN-γ and IL-2 in opportunistic infections of humans have seldom given rise to hope, and most were totally ineffective. The only limited success occurred in particular clinical settings (e.g., the neutropenic patient upon antineoplastic chemotherapy induction) where colony growth factors, in particular GM-CSF, has provided synergistic positive effect with antifungal chemotherapy in controlling deep-seated candidiasis (several groups).

It is clear that immunotherapy with cytokines is still a long and winding road and progress in this area rests upon in-depth elucidation of the complex cytokine patterns, identification of other cytokines and receptors and the use of animal models more representative of the human disease.

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References
1. Maggi et al. manuscript in preparation

57 Blood products transmitted viral infections rather than contaminants of clotting factor concentrates impair lymphocyte activation in hemophilia

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Most hemophiliacs, cronically infused for years with plasma derived clotting concentrates to cure bleeding episodes, present anomalies of immune response attributed both to contaminants of plasma products and to transfusion transmitted viral infections. Aim of this investigation was to establish the role of transfusional requirements and of HCV or HIV infections on the expression of some activation markers (DAPIV/CD26, CD38, CD45RA, CD25, HLA-D) of circulating lymphocytes. Our results indicate that the replacement treatment with high purity or recombinant clotting factors do not affect lymphocyte activation. DAPIV/CD26 and CD45 RA expressions are significantly depressed on lymphocyte membrane surface of HIV+ patients, even in asymptomatic stages before CD4 cell depletion, whereas HCV infection seems to involve chiefly the CD45RA response.

In the past years, immunological abnormalities found in hemophilia have been attributed to contaminants present in the cryoprecipitate or lyophilized concentrates and/or to viral infections transmitted by blood product infusion.1-3 Since 1985 yr, after application of virucidal technique on plasma derivatives and, more recently in the last decade, after registration of high purified or recombinant products, the treatment of bleeding episodes can be considered safe of virus hepatitis and HIV transmission, and theoretically devoid of antigenic stimulation. CD26 with Dipeptidylaminopeptidase IV (DAPIV) activity, CD38, CD45RA, CD25, HLA-D represent surface lymphocyte antigens expressed during T cell activation. Therefore we research if these markers could be affected by the infusion of high purity plasma derive products or recombinant FVIII, taking into account the presence of HIV or HCV infections.

We considered 53 hemophiliacs aged between 3 and 70 yrs. In the year preceding our study 17 pts with mild hemophilia were never infused, 30 pts were treated with high purity products and 6 with rF VIII, total amounts of clotting factor infused were in 19 pts > 300 U/kg/yr and in 17 pts <300 U/kg/yr. 8pts were HIV+(clinical stage CDC II-III) and 45 HCV+ (included the 8 HIV+pts).

Table 1 Behaviour of lymphocyte antigens in relation to the HIV infection in hemophilia (percentages of positive circulating lymphocytes: m±sd)

<table>
<thead>
<tr>
<th>HIV-</th>
<th>HIV+</th>
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<tbody>
<tr>
<td>DAPIV</td>
<td>20±13.8*</td>
</tr>
<tr>
<td>CD26</td>
<td>22.3±11.3*</td>
</tr>
<tr>
<td>CD38</td>
<td>17.7±11.598,7±13.9*</td>
</tr>
<tr>
<td>CD45RA</td>
<td>4.2±3.2</td>
</tr>
<tr>
<td>CD25</td>
<td>3.1±2.3</td>
</tr>
<tr>
<td>HLA-D</td>
<td>21.9±12.6</td>
</tr>
</tbody>
</table>

*p<0.05.

CD26/DAPIV expression were evaluated on peripheral blood buffy coat smears by immunocytochemical and cytochemical techniques as already described.4 CD38, CD45RA, CD25, HLA-DR were detected on peripheral blood leukocytes by flow cytometry (Cytoron Absolute-ORTHO-flow cytometer) using specific monoclonal antibodies (Eurobiox, Barberino del Mugello, Italy) and expressed as percentage of positive lymphocytes. In HIV- patients the behavior of the studied markers of lymphocyte activation was not affected by the high or low amounts of clotting factor infused and it was similar in not infused patients. HIV+ patients showed significant lower percentages of positive circulating lymphocytes for DAPIV/CD26 and CD45RA than those observed in HIV-pts (Table 1). Only CD45RA expression was significant lower (p<0.05) also in HCV+ patients. In hemophilia the replacement treatment with high purity or recombinant clotting factors do not influence the lymphocyte activation. The interactions between CD26 with DAPIV activity and CD45 with protein tyrosine-phosphatase activity is considered the key for CD26 mediated T cell activation.5 In HIV+ hemophilic patients the significant...
Selective loss of CD26/DAPIV and CD45RA represents an early event of impaired T cell function, occurring before CD4 cell depletion, even in asymptomatic stages. Furthermore, the CD45RA expression seems to be chiefly affected by HCV infection.

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**References**


**Societa` Italiana di Farmacologia**

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**Meeting Supplement**

**Seventh session**

**IMMUNOTOXICOLOGY**

**58**

**Δ9-THC-induced inhibition of NK activity is mediated by CB2 receptors**

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We employed the selective cb2-antagonist, sr144528 (10 mg/kg p.o., 90 min before the cannabinoid) to analyze the role of the cb2 receptor in the inhibition of cytolytic activity of nk cells induced by an acute injection of d9-thc (15 mg/kg s.c.). Cb2-antagonist pre se did not alter nk activity whereas it partially antagonized the d9-thc decrease in cytolytic activity. These data firstly demonstrated that cb2 receptors are involved in the inhibition of nk cells function.

Besides their psychotropic effects, cannabinoids have been widely described as influencing immune function (klein et al., 1998). Their mechanism on immune system are still questioned, although the recent identification of specific functional cannabinoid receptors on immune cells, suggests that cannabinoid immunological effects could be mediated at least partly by a highly specific receptor-associated mechanism. This is supported by the expression of the cb1 receptor on leukocytes (bouaboula et al., 1993) and the cloning of a second cannabinoid receptor, designated cb2 (munro et al., 1993), that is only present at the periphery and more particularly on cells of immune origin.

To date, no specific agonist that can discriminate between cannabinoid receptor subtypes has been published. Immunological studies have been performed using cannabinoid agonists that are equipotent in their binding to cb1 and cb2. We recently reported (patrini et al., 1997) that the synthetic cannabinoid compound cp-55,940 in vivo caused inhibition of nk cytolytic function not antagonized by the selective cb1 antagonist sr141716a, demonstrating that this inhibition was not linked to cb1 receptor.

The recent discovery of the first potent and specific antagonist for cb2 receptor, sr 144528, (rinaldicarmona et al., 1998) provided a new tool to better understand the role of cb2 receptors in the modulation of immune function. The present study was therefore undertaken to survey in mice the effect of in vivo pretreatment with the cb2 antagonist sr144528 on the inhibition of nk cytolytic activity induced by acute injection of d9tetrahydrocannabinol (d9-thc).

Male swiss mice (charles river, calco, italy), 20-25 g body weight were used, fed a pellet diet (altrominrieper, bolzano, italy) with water ad libitum. Environmental conditions were standardized (22 ± 2 °c, 60% humidity and 12 h artificial lighting per day).

D9-thc (a generous gift from national institute of drug abuse, u.s.a.) Was dissolved in 1:1:18 cremophor (sigma), ethanol, saline and injected s.c. At a dose of 15 mg/kg in a volume of 0.1 ml/10g of body weight. Sr144528 (a generous gift from sanofi recherche, montpellier, france), was dissolved in 1% tween-80, 2% dmso, distilled water and administered p.o. At a dose of 10 mg/kg in a volume of 0.2 ml/10g of body weight. According to kinetic parameters of sr144528 binding after in vivo administration (rnaldi-carmona et al., 1998), the antagonist was injected 90 min before d9-thc and mice were killed 1 h after the cannabinoid injection.

The splenocytes were collected aseptically, and for determining natural killer cell activity were suspended in rpmi, 10% fcs at a concentration of 105 cells/ml. They were then incubated with 51cr labeled yac-1 (104 cells/well) in 96-well microtiter plates in an effector:target cell ratios (e:t) of 100:1 and 200:1.
Data obtained are presented as mean ± standard error and evaluated by two-way anova, followed by tukey’s test for multiple comparisons.

Fig. 1 shows the effect of the acute s.c. Injection of d9-thc on the cytotoxic activity of nk cells. The cannabinoid significantly reduced nk activity with a mean of 61% and 53% respectively for e:t ratios of 200:1 and 100:1. The antagonist sr144528 per se did not affect the nk activity at both e:t ratios. In contrast pretreatment with the cb2 antagonist lowered the inhibitory effect of the cannabinoid, the suppression of nk activity being respectively 40% and 30% for e:t ratios 200:1 and 100:1.

Although these data require future investigations on dose- and time- dependence relationships, they firstely demonstred in vivo the relevance of cb2 receptors in the d9-thc induced inhibition of nk activity and could open up future potential therapeutic application for immune diseases.

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References

59
Opioids and immune function
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Although it is well known that morphine induces a relevant immunosuppression, the potential immunosuppressive activity of morphine derived drugs commonly used in the treatment of pain (codeine, hydromorphone, oxycodone) has never been evaluated. We evaluated in the mouse the effect of the natural opiates (morphine and codeine) and synthetic derivatives (hydromorphone, oxycodone, nalorphine, naloxone and naltrrexone, and tramadol) on antinociceptive thresholds and immune parameters (splenocyte proliferation, NK activity and IL-2 production).

The effects of opioids on the immune system could be relevant for their therapeutic and non medical use. In therapy, opioids are used in acute pain, e.g. in post-operative pain; chronically they are used in malignant (cancer) and non malignant e.g. arthritic, pain. In all these conditions, the knowledge of the effects of the different opiates on immune responses could be of interest in order to chose the proper drug. In acute, post-operative pain, it would be better to have a drug that does not increase the already present immunosuppression elicited by the surgical stress; in cancer pain it would be better to use drugs that do not affect immune responses, while in non malignant pain the presence of an immunosuppressive effect of the oploid drug could be useful, e.g. in rheumatoid arthritis pain.

Morphine displays a potent immunosuppressive effect that is not dose related to the antinociceptive effect, codeine possesses a weak antinociceptive effect and limited immunosuppressive activity; nalorphine, a µ-antagonist and k-agonist, exerts a potent immunosuppressive effect, but a very weak antinociceptive activity. The pure k-antagonist nor-BNI antagonises the antinociceptive, but not the immunosuppressive effect of nalorphine.

Hydromorphone and oxycodone are potent antinociceptive drugs, devoid of the immunosuppressive effect. The pure antagonists naloxone and naltrexo-
one potentiate immune responses since they relieve the tonic immunosuppression exerted by the endogenous opioids, e.g., β-endorphin.²³

Our data indicate that the C₆ carbonyl substitution, together with the presence of a C₇ single bond potentiates the antinociceptive effect, but abolishes immunosuppression (hydromorphone and oxy-codone). The single substitution of an allyl on the piperidinic ring results in a molecule antagonist of the antinociceptive effect, that maintains the immunosuppressive effect. Molecules that carry modifications of C₆, the C₇,₈ bond and C₁₄, together with an allyl or caboxymethyl group on the piperidinic ring antagonise both the antinociceptive and the immunosuppressive effect of opiates and are themselves immunostimulant.

Tramadol is a centrally acting analgesic drug with a dual mechanism of action: binding to μ-opioid receptors and potentiation of the monoaminergic systems. After acute subcutaneous administration, tramadol induced antinociception, whereas it significantly enhanced natural killer activity and IL-2 production.⁴ After the chronic administration, the antinociceptive effect of the drug was still present, whereas the 2 immune modifications disappeared. Thus, the pharmacological profile of tramadol is totally different from that of other drugs which bind p-opioid receptors.

Consistently with the results of our studies, methadone that has a totally different structure from morphine and its analogue is at least equipotential to morphine for the analgesic response, while it is totally ineffective on the immune system after acute or chronic administration.

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References


60 Immunotoxicity of antiepileptic drugs

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Chronic administration of antiepileptic drugs can result in alterations of the immune response and/or in immunotoxic reactions. Available evidence suggests that the highest risk is associated with phenytoin and carbamazepine. It is possible that a more in-depth knowledge of the mechanism(s) involved will also contribute to clarify the central nervous system-immune system bidirectional signalling pathways.

A huge amount of clinical observations indicates the existence of an association between the use of antiepileptic drugs and immunological disturbances and/or immunotoxic reactions.¹² Although epilepsy per se might be associated with immune alterations, nevertheless a number of in vitro, in vivo and clinical data suggests that antiepileptic drugs can affect immune system functions. In particular, based on the available evidence the highest risk is associated with the use of phentoyin and carbamazepine. At present, however little information is available concerning the possible mechanism(s) involved. In vitro phenytoin inhibits DNA synthesis in human lymphocytes and reduces the percentage of active T lymphocytes (see, e.g., refs. #3 and 4) however the clinical relevance of these effects has been questioned. Recently, it has been shown that carbamazepine in the plasma therapeutic range interacts with peripheral benzodiazepine receptors (pBRs).³ Such receptors together with their ligands form the basis of a neuroimmune network that contributes to the central nervous system-immune system bidirectional signalling.⁶ Evidence has been obtained that carbamazepine affects human neutrophil function through an action on pBRs.⁷ In vitro, carbamazepine concentration-dependently inhibits FMLP- or LPS-induced chemotaxis and these effects are reversed by the pBR antagonist PK11195 and mimicked by the pBR agonist Ro 5-4864. Interestingly, neutrophils from epileptic patients on chronic carbamazepine monotherapy had impaired FMLP- and LPS-induced chemotaxis and enhanced expression of pBRs. Enhanced expression of pBRs however seems unlikely to depend on a direct interaction of carbamazepine with these receptors, since it has been reported in epilepsy patients on phenytoin and valproic acid, as well.⁸ Preliminary evidence indicates that the levels of diazepam-binding inhibitor, an endogenous ligand of the pBRs, are
enhanced and pBR density is decreased (possibly as a downregulation phenomenon) in drug-resistant patients compared to controls and drug-sensitive.9 On this basis, pBR density on leukocytes has been proposed as a peripheral marker of drug response. The pBRs however have also a direct modulatory action on the function of leukocytes6 and it has been recently shown that their ligands can affect intracellular Ca++ concentrations in human neutrophils10 (Figure 1). The present knowledge about pBRs suggests that they may a role in the immune effects of antiepileptic drugs. In addition, leukocytes pBRs and plasma DBI may represent a readily accessible model to study neurochemical changes in the central nervous system, possibly related to the therapeutic outcome.

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References


Peripheral benzodiazepine receptor-mediated modulation of intracellular calcium in human neutrophils

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Immune cells express benzodiazepine receptors of the peripheral type (pBRs) which bind several endogenous ligands such as benzodiazepine-like substances,1 porphyrins2 and bioactive peptides derived from the polypeptide diazepam-binding inhibitor (DBI).3 Since DBI is released from GABAergic nerve terminals and its fragments can be found in liquor and peripheral blood,4 it has been suggested that pBRs together with their peptide ligands may contribute to the bidirectional connections between the central nervous system and the immune system.5 However, so far the effects of DBI-derived peptides on immune cells have not been studied in detail and the sole informations available concern their ability to affect cytokine production by macrophages and monocytes.6 Because pBRs can modulate transmembrane Ca++ fluxes in astrocytes,7 we have inves-
tigated the effects of pBR ligands on cytosolic free Ca\(^{++}\) concentrations ([Ca\(^{++}\)]\(_i\)) in human neutrophils. Experiments were performed on neutrophils isolated by density gradient centrifugation from venous blood obtained from healthy donors. After incubation at 37°C for 30 min with 5 µM Fura-2/AM, cells were washed and finally resuspended at the concentration of 2x10\(^6\) /mL in PBS-HEPES supplemented with 1.8 g/L glucose, 0.25% bovine serum albumin and 1 µM CaCl\(_2\). Fluorescence measurements were performed using a Perkin-Elmer LS-50B spectrofluorimeter. Excitation of Fura-2 was performed at 340 and 380 nm and the ratio of emitted fluorescence signals at 510 nm was used to calculate the [Ca\(^{++}\)]\(_i\). Basal [Ca\(^{++}\)]\(_i\) was 142.3±22.4 nM (mean±SEM; n=14). Addition of the DBI-derived 34 amino acid fragment DBI17-50, which has a prevalent affinity for the pBRs, concentration-dependently induced a rapid and transient rise of [Ca\(^{++}\)]\(_i\) up to 319.1±105.5 nM (n=3) at the concentration of 100 µM (Figure 1). This effect was completely prevented by chelation of extracellular Ca\(^{++}\) with EGTA 5 µM (Figure 1, inset). A similar although lower effect (+151.7±33.4 nM; n=5) was induced by the selective synthetic pBR agonist Ro 5-4864 at 1 mM. The selective pBR antagonist PK 11195 at 10 mM significantly antagonized the effects of both compounds. The present data indicate that endogenous and exogenous pBR ligands act on pBRs to increase [Ca\(^{++}\)]\(_i\) in isolated human neutrophils, possibly by inducing a rapid influx of extracellular Ca\(^{++}\). Ca\(^{++}\) is one of the fundamental signal transduction elements modulating many crucial cellular processes, thus in-depth studies are required to clarify the functional significance of this effect.

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**References**


**Effect of methanandamide on lypopolysaccharide-induced hyporesponsiveness of rat aorta to phenylephrine in organ culture**

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We investigated if methanandamide, a stable analogue of the endogenous cannabinoid ligand ananandamide, affected the functional effects of NOS II induction evoked in vitro by exposing rat aortic rings to lypopolysaccharide (LPS). Endothelium-denuded aortic rings were incubated for 24 hours with methanandamide before being mounted in isolated organ baths and challenged with the a1-adrenergic agonist...
phenylephrine. The results shows that prolonged incubation with methanandamide decreased the vasoconstriction to phenylephrine, possibly through a potentiation of NO production from NOS II.

Methanandamide (METHA) is a stable analogue of anandamide, the endogenous cannabinoid ligand of CB1 receptors. Activation of these receptors has been shown to be associated with vasodilatation and hypotension in vivo; however, it is still unclear whether cannabinoids could act directly on vascular smooth muscle or if they could modulate the production and/or release of other vasodilators. For example, it has been claimed that endothelium-derived hyperpolarizing factor is anandamide or an anandamide-related compound, but this has not been further confirmed. On the other hand, it has recently been shown that anandamide is able to vasodilate juxtaglomerular afferent arterioles perfused in vitro by stimulating NO release. Cannabinoid CB1-receptors have been detected in endothelium as well as in vascular smooth muscle by reverse-transcription polymerase chain reaction, but their physiological role remains unknown. Here we used rat aorta organ culture, in which NOS II induction can be stimulated by lipopolysaccharide (LPS), to elucidate if prolonged incubation with METHA affected the functional effects of high output NO production. Wistar rats were killed by decapitation, thoracic aorta was removed, dissected in a sterile manner and cut into rings (3–5 mm diameter). Aortic rings were carefully rubbed in the inner surface to remove endothelium, then placed in a 24-well plate (one ring per well) with culture medium (Dulbecco's modified essential medium) containing 10% fetal calf serum, 100 units mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, 50 µg mL⁻¹ LPS (Escherichia Coli, serotype 055:B5), and with METHA (1 µM), METHA and the CB1 receptor antagonist SR 141716A (SR, 1 mM), dexamethasone (DEXA, 10 µM) or vehicle (ethanol, final concentration 0.1% v/v). Incubation of tissues was performed at 37°C in a 5% CO₂ incubator for a period of 24 hours. After 24 h-incubation, rings were placed in isolated organ baths and connected to isometric force transducers. The baths contained physiological solution (composition, mM: NaCl, 118; KCl, 4.6; NaHCO₃, 25; MgSO₄, 1.2; KH₂PO₄, 1.2; CaCl₂, 2.5; glucose, 10; EDTA, 0.025, pH 7.4), warmed at 37 °C and bubbled with 95% O₂-5% CO₂. The preparations were firstly challenged with phenylephrine (PE, 1 µM); when the contractile response to PE had reached a plateau, acetylcholine (3 µM) was added to the bath in order to confirm that endothelium had been successfully rubbed. After washout and incubation with the NOS inhibitor N^G-nitro-L-arginine (L-NNA, 100 µM), preparations were challenged again with 1 µM PE. Finally, cumulative concentrations of the NO-donor isosorbide dinitrate (ISDN, 100 nM–100 µM) were added on the plateau of contraction.

Figure 1. Contractile responses to phenylephrine (PE) and vasodilation to isosorbide dinitrate of isolated aortic rings after incubation with lipopolysaccharide and methanandamide (metha), methanandamide + SR 141716A (SR) or dexamethasone (Dexa). (a) contractile responses to PE in the absence of L-NNA; (b) contractile responses to PE in the presence of L-NNA; (c) relaxation to isosorbide dinitrate of rings precontracted with 1 mM PE in the presence of L-NNA. *P<0.05 vs. Control, †P<0.05, vs. Metha; one-way ANOVA.
The first contractile response to PE was (in mN mg⁻¹): 0.55±0.08 in vehicle, 0.19±0.05 in METHA, 0.65±0.14 in METHA+SR, 1.17±0.13 in DEXA (vehicle vs. METHA and vehicle vs. DEXA, p<0.05; METHA vs. METHA+SR, p<0.05; one-way ANOVA). In the presence of L-NNA, the contractile responses to PE were markedly increased in all groups and attained about 2 mN mg⁻¹ regardless of treatment. Relaxing responses to ISDN were not different among groups. The present results show that prolonged incubation of rat aorta with METHA increased the hyporesponsiveness to PE induced by LPS; this hyporesponsiveness is related to NO production since it is reversed by L-NNA. The effect of METHA seems to be related to an increased NO production and not to an increased capability of vascular smooth muscle cells to relax in response to NO, since the vasodilatation evoked by ISDN, an NO-donor, was similar in control and METHA group. Furthermore, since the vascular hyporesponsiveness was observed in endothelium-denuded aortic rings, the endothelial NOS isoform is not involved in this phenomenon. The effect of METHA on vascular hyporesponsiveness to PE induced by LPS was antagonized by SR, known as a relatively selective antagonist of CB₁, receptor, and could therefore be related to CB₁ receptor stimulation. CB₁ receptors have been so far characterized in CNS as well as in periphery. Although their role in periphery has not been elucidated, it has been suggested that they could exert modulatory effects on inflammation, affecting both cytokine production and the response of target cells to cytokines.⁶

Our data are also consistent with a stimulation of nitric oxide release by cannabinoids which has been observed in human monocytes.⁶ Interestingly, the present study shows that, in control rings, the hyporeactivity to PE was completely overcome by L-NNA, while the NOS inhibitor seemed to restore only partially the contractility of the aorta in METHA-treated rings (P < 0.05 vs. DEXA). This suggests that METHA induced an additional mechanism of hyporeactivity, which however remains to be elucidated.

In summary, these results show that prolonged incubation with METHA decreased the responsiveness to PE possibly through a potentiation of NO production from NOS II; this effect seems to be related to CB₁ receptor stimulation, since it is antagonized by SR.

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References

63 Effects of endocannabinoids on CGRP release in capsaicin-injected rats
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During neurogenic inflammation several peptides, including calcitonin gene-related peptide (CGRP), can be released by trigeminal unmyelinated C-fibers. The aim of the present study was to elucidate whether endocannabinoids could affect CGRP release and thereby neurogenic inflammation. Rats were intraperitoneally pretreated with vehicle, methanandamide (METHA) or palmitoylethanolamide (PEA), before receiving capsaicin, a potent stimulator of CGRP release from nerve endings. Plasma-EDTA samples were collected and analysed by radioimmunossay. The results show that pretreatment with either METHA or PEA prevented the CGRP rise after capsaicin injection, suggesting that they could have a modulatory role in neurogenic inflammation.

Neurogenic inflammation (NI) within the meninges has been proposed as an important event in the pathogenesis of migraine headaches.¹ NI develops following vasoactive neuropeptide release from perivascular trigeminal nerve fibers and is characterized by plasma protein extravasation, platelet aggregation, mast cell degranulation and endothelial activation. Among neuropeptides involved in NI, calcitonin gene-related peptide (CGRP) appears to be the major mediator of vasodilatation and blood flow changes occurring as a consequence of trigeminal nerve activation.² We recently reported that the duration and
Figure 1. Plasma CGRP levels in rats treated or not with methanandamide or palmitoylethanolamide 20 min before capsaicin. All treatments were carried out intraperitoneally; rats were sacrificed 1 h after capsaicin injection. Each column represents the mean (standard error is represented by vertical bar) from 8 different animals.

The severity of attacks are reduced in migraineurs who smoke cannabis; we attributed the action of cannabis on migraine attacks to a putative inhibition, occurring through cannabinoid receptor stimulation, of the release of neuropeptides involved on NI. The present experiment was therefore undertaken in order to elucidate if acutely administered endocannabinoids could affect the release of CGRP induced in rats by injecting capsaicin, an agent known as able to induce the release of neurally stored peptides. Male Sprague-Dowley rats were intraperitoneally (i.p.) pretreated with vehicle, methanandamide (METHA, a stable CB1 receptor agonist, 200 µg kg⁻¹) or palmitoylethanolamide (PEA, a CB2 receptor agonist, 600 µg kg⁻¹); after 20 min rats were given capsaicin (500 µg kg⁻¹, i.p.), a potent stimulator of CGRP release from nerve endings. After 1 hour rats were sacrificed and plasma-EDTA samples collected and analysed by radioimmunoassay. The extraction of CGRP from samples was performed as follows: 2 mL of each plasma sample was acidified to pH 3, by adding 0.5 mL HCl (2 M); after acification, samples were loaded on Amprep minicolumns (PH 500 µg, Amersham) pre-conditioned with 10 ml methanol followed by 10 ml water; after sample loading, the columns were washed with 20 ml of 0.1% trifluoroacetic acid and the recovery of CGRP was finally obtained by eluting with 4 ml of 60% acetonitrile and 0.1% trifluoroacetic acid in water. The samples were then lyophilized, resuspended in the RIA buffer and analysed for CGRP content according to the manufacturer’s instructions (RIA kit from Peninsula). Capsaicin, as expected, significantly increased the CGRP plasma concentration in vehicle-injected rats (control: 26.7±3.4 pM, n=8; capsaicin: 67.3±12.4 pM, n=8, p<0.01). Pretreatment with either METHA or PEA prevented the CGRP rise after capsaicin injection (capsaicin after METHA: 22.4±2.6 pM, n=8; capsaicin after PEA: 25.2±3.5 pM, n=8, p<0.01 vs. capsaicin after vehicle). These results seem to indicate that endocannabinoids, acting on either CB1 or CB2 receptors, inhibit CGRP release induced by capsaicin. Endocannabinoids could therefore have a modulatory role in neurogenic inflammation and thereby in migraine, which is also consistent with the antimigraine effect of cannabis, previously observed in humans.

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References

64 Immunotoxicokinetics of drugs of abuse
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We show that the observed changes in immune function of opioid treated mice are affected not only by drug dose but also by the times at which immune function is assessed after drug administration. We suggest the possible involvement of multiple receptors in the bidirectional effects described.

Many studies conducted in animals for understanding the behavioral, biochemical, and immunological effects of drugs of abuse suggest that the observed effects are associated not only with drug dose but also with the times at which the function is assessed after drug administration.1

Biphasic effects are not uncommon when dealing with immunological endpoint, and this demonstrates the importance of time of observation after drug treatment in any study concerning the effects of a drug on immunocompetence.

Several authors described biphasic effects of opio-
oids, or noticeable dispersion of the data concerning the immunomodulatory effects of these substances, as being more evident in vivo, or in the case of cells obtained from living organisms, than in experiments performed with cultured cells. Previously, we provided evidence of the biphasic effects of morphine in vivo: the rapid stimulation of the immune parameters measured was followed by their depression 24 h after drug administration in mice. These effects were not evident in case of methadone treatment.

In that study, we correlated the immunomodulatory effects of a single s.c. injection of morphine or methadone with serum concentrations and with their analgesic effects in C57BL6 mice. The effect of the morphine was shown to follow a biphasic time course during the first 24 h: indeed, an increase of immune response when analgesia and blood levels were maximal, followed by a decrease 24 h later were observed. Twenty and forty min after the in vivo morphine treatment, the ingestion of C. albicans by PMN increased significantly (41 and 21%, respectively), returning to baseline levels at 120 min; in contrast, PMN phagocytosis after 24 h was significantly reduced (-42%). A similar time course was observed for the cytostatic activity of peritoneal MC. Conversely, methadone had no effect on PMN and MC activity, which remained at base-line levels at each of the times tested.

In a different study we also showed that in vivo administration of morphine can induce a modulation of the NO biosynthesis of unstimulated and immunoactivate murine peritoneal macrophages. The study’s primary finding was that the immunomodulation induced by morphine is biphasic and time dependent. Both cytostasis and NO2− production of L1210-activated macrophages were significantly enhanced by the opioid treatment immediately after drug injection (peaking after 40 min). By contrast, morphine induced a strong inhibition of both cytostasis and NO2− production 24 h after the treatment. In an extension of these studies, we also investigated the kinetic release of cytokine at different time intervals after an acute injection of morphine and heroin in mice (i.e. 20 mg/kg).

The tested cytokines were the principal inflammatory cytokines like IL-1β, IL-2, TNF-α, and IFN-γ, known to play a crucial role in the pathogenesis of many infections and autoimmune disease, and pleiotropic antiinflammatory cytokines like IL-10 and TGF-β1 known to produce profound effects on cells involved in the immune response. For murine spleen cell preparation, spleens were aseptically removed from sacrificed mice and gently homogenized. The cells were collected, washed and adjusted at final concentration of 1x10⁶/well. Splenocytes were stimulated at optimal conditions for the induction of cytokines.

For quantitative measurement of murine IL-1β, IL-2, IL-10, TNF-α, IFN-γ and TGF-β1 in supernatants of splenocytes cultures, specific solid-phase ELISA assay, employing the multiple antibody sandwich principle, were used (Genzyme Inc., Cambridge, MA, USA). Results of a kinetic study of mitogen-stimulated release of cytokines from splenocytes of morphine treated mice are shown in Table 1. IL-1β production in morphine treated mice was first stimulated after 20 and 40 min (28.6% and 32.4% respectively) and then depressed (-87.9 at 24 hr and -38.6% at 48 hr).

When animals were exposed to morphine, a significant enhancement of IL-2 production was observed at 20 and 40 min. The enhancement was approximately 33% at 20 min and did not occur at 120 min. Conversely, at 24 hr and 48 hr a high decrease was observed (-86.7% and 57.6% respectively).

Table 1. Changes of the cytokine production in morphine-treated mice (% vs control)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>20 min</th>
<th>40 min</th>
<th>120 min</th>
<th>24 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>+28.6</td>
<td>+32.4</td>
<td>-1.4</td>
<td>-87.9</td>
<td>-38.6</td>
</tr>
<tr>
<td>IL-2</td>
<td>+32.9</td>
<td>+34.1</td>
<td>+1.4</td>
<td>-86.7</td>
<td>-57.6</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+22.6</td>
<td>+27.9</td>
<td>+2.1</td>
<td>-55.1</td>
<td>-23.4</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+16.1</td>
<td>+10.4</td>
<td>-2.9</td>
<td>-81.2</td>
<td>-23.4</td>
</tr>
<tr>
<td>IL-10</td>
<td>+3.4</td>
<td>-3.0</td>
<td>+3.5</td>
<td>+47.7</td>
<td>+38.0</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>+3.5</td>
<td>-8.5</td>
<td>-2.1</td>
<td>+57.5</td>
<td>+41.8</td>
</tr>
</tbody>
</table>

By 20 and 40 minutes, a significant enhancement of TNF-α was relieved into the supernatants (22.6% and 27.9% respectively). Thereafter, no detectable differences in TNF-α release were observed at 120 min. In contrast, a decrease at 24 and 48 hr was observed (-55.0% and 23.4% respectively).

Treatment with morphine resulted in an approximately 2-fold increase of IL-10 release at 24 hr from treatment compared to non-treated mice. The same effect, but more mitigated, was observed at 48 hr from treatment.

In the current study, we provided evidence of...
biphasic immunomodulatory effects of acute morphine treatment on pro-inflammatory cytokine release by murine cultured splenocytes. Biphasic effect have been described in the case of many neuroactive substances. 5, 6 Dual activity of α and β endorphin in the modulation of cytokines secretion has been observed. 6 It was suggested that cytokine production may be increased by the binding of the opioid peptide to non-opioid receptors, while opioid receptor inhibits cytokine release. Indeed, receptors for opioid alkaloids and for opioid peptides in immunocompetent cells are described as opioid and non-opioid.

Non opioid receptors has often been found to mediate stimulatory effects, whereas the effects mediated by opioid receptors may be stimulatory or inhibitory depending on the cell and function under investigation. 1 A recent cytofluorimetric study using the same animal model and treatment used in our study demonstrated that all cell subpopulations possess receptor binding sites for opiates and similar binding levels. 7

Different classes or different subtypes of opioid receptors can be present in the same cell. Therefore, the co-presence of different receptors on splenocytes may permit the rationalization of the dual effects on cytokine secretion demonstrated in this research. The results seem to indicate that central opioid or nonopioid receptors are involved in exogenous opioids-induced stimulatory effects while peripheral opioid or non-opioid receptors should be involved in depressive effects. Since exogenous opioids seem to exert effects throughout multiple receptor types, the use of more selective agonist and antagonists is necessary to investigate this issue.

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References

65 Immunological effects of pesticides exposure

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The evaluation of immunotoxic effects of pesticides for domestic and industrial purposes is of major concern for public health. We described the results of a study evaluating the effects of agricultural exposures to organophosphates and phenoxycetic acid on human peripheral blood lymphocytes. The observation on humans that both pesticides are able to exert short-term immunodepressive effects is of particular interest since for these chemicals an immunological mechanism of lymphomagenesis has been hypothesized.

Chemical agents may significantly interfere with the immune system in humans and a growing number of studies provided examples of immunotoxic effects in subjects exposed to industrial chemical as pesticides. Pesticides that comprise a large group of substances including insecticides, fungicides, herbicides, and rodenticides have been shown to produce differential and several effects on immune system in humans.

In 33 workers exposed to DDT neutrophil functions such as chemotaxis, adhesion and phagocytosis were found to be significantly depressed. 1 Chronic home exposure to chlordane heptachlor in 24 subjects was correlated to a significant increase in activated T-lymphocytes. 2 Immunological alterations in 27 patients exposed to chlordane were demonstrated and consisted on decreased frequencies of the suppressor-inducer phenotype CD45RA on CD4 lymphocytes and elevated light-chain frequencies on B cells. 3 Decreased percentage of CD4 and CD8 lymphocytes subsets and elevated CD26 cells were found in 12 subjects exposed to chlorpyrifos. 4 A significant decrease of T4/T8 ratio was demonstrated in 23 subjects chronically exposed to aldicarb contaminated groundwater. 5 In pentachlorophenol exposed subjects proliferative responses of blood lymphocytes to mitogens or antigen were shown to be depressed. 6

Recently, we evaluated short term immunological changes after agricultural exposure to commercial formulations of chlorophenoxy herbicides. 7 Blood samples were collected from 10 farmers (age
44.0±9.1 years) within seven days before exposure, one to 12 days after exposure, and again 50 to 70 days after exposure. The mean amount of herbicides applied was 39.1 (12-155) Kg.

Blood samples were obtained in EDTA vacutainer tubes and were analysed within two hours after phlebotomy. Complete blood profile and count was used to count lymphocyte subsets with commercial monoclonal antibodies (CD4-FITC/HLA-DR-PE; CD8-FITC/HLA-DR-PE; CD3-PE/CD16-CD56-FITC).

The PBM cells were used to measure natural killer cell mediated cytotoxicity by a 51Cr release assay. To evaluate lymphocyte proliferative response, PBM cells were stimulated with phytohemagglutinin (PHA) and concavalin A (ConA). The statistical analyses were performed using Stata software.

In comparison with values obtained before exposure, a significant reduction was found one to 12 days after exposure in the following variables (p<0.05): circulating helper (CD4) and suppressor T cells (CD8), CD8+CD4+ T-cells (CD8+ T-cells), cytotoxic T lymphocytes (CTL), natural killer cells (NK), and CD8 cells expressing the surface antigens HLA-DR (CD8-DR), and lymphoproliferative response to mitogen stimulations. All immunological values found 50-70 days after exposure were comparable with concentrations before exposure, but mitogenic proliferative responses of lymphocytes were still significantly decreased.

In an extension of the this study, we also monitored a group of 25 farmers in order to evaluate immunological changes following exposure to both organophosphates (OPs) and phenoxyacetic acid (PhAs).

Blood samples were collected before exposure and subsequently within 1-12 days following exposure to specific pesticides. The data were analysed taking into account the longitudinal feature of the study and possible confounding factors, such as concomitant exposures, age, and smoking habits.

Exposure to PhAs was associated to a highly significant (p<0.01) depression of all parameters under study: CD4+ T-cells % change – 15.3, 95% CI – 22.1; CD8+ T-cells % change – 33.9, 95% CI – 45.1; CD8− T-cells % change – 20.3; NK cell activity % change – 43.0, 95% CI – 56.2; stimulation index absolute change – 40.8, 95% CI – 52.0; – 29.7.

Following exposure to OPs, a statistically significant reduction was observed for NK cell activity (% change = – 28.9, 95% CI = – 48.9; – 21, p<0.05), and stimulation index (absolute change = – 17.8, 95% CI = – 28.8; – 6.8, p<0.05). A strong association was observed between inhibition of peripheral blood acetylcholinesterase (AChE), used as an indirect index of intensity of exposure, and reduction of both NK cell activity and stimulation index (p-values for trend < 0.002 and < 0.001, respectively).

The observation on humans that OPs and PhAs are able to elicit short-term immunodepressive effects is of particular interest since an immunological mechanism of lymphomagenesis has been hypothesized for both these chemicals. Agricultural exposure to phenoxy herbicides has been associated with an increased risk of non Hodgkin’s lymphoma, and less consistently, with an increased risk of soft tissue sarcoma.1 Excesses of non-Hodgkin’s lymphoma have been well documented in association with immunological disorders or immunosuppressive treatment suggesting that pesticides exposure might cause this type of cancer by altering the function of lymphocytes.

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References

66
Selective induction of interleukin-12 by contact allergens in reconstituted human epidermis

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We describe the development of an in vitro test to screen chemicals for allergic potential. At this purpose, we have explored the possibility to use IL-12 production
to discriminate in vitro between skin irritants and contact allergens in reconstituted human epidermis. This model was used because more closely resembles the human epidermis. The results obtained testing a limited number of chemicals (dinitrochlorobenzene, oxazolone, eugenol as allergens and sodium dodecyl sulfate and benzalkonium chloride, as irritants), suggest that the induction of IL-12 may be indeed an useful marker to discriminate contact allergens from irritants.

The two most frequent manifestations of skin toxicity are irritant contact dermatitis and allergic contact dermatitis. Depending on the country, dermatoses comprise from 20 to 70% of all occupational diseases, and between 20 and 90% of these are contact dermatitis. Whereas irritant contact dermatitis is thought not to be mediated by lymphocytes, allergic contact dermatitis represents a lymphocyte-mediated delayed type hypersensitivity reaction that requires previous sensitization by the same chemicals. Contact hypersensitivity can occur as a result of exposure to a wide variety of chemicals and certain drugs, cosmetics1,2 and various metals including nickel3 and chromium.4

In the screening of topical drugs, cosmetics and other chemicals for human use, it should be very important, both from safety and economic point of view, to have biological markers to discriminate these events that have different impact on human health. Furthermore, animal studies are not practical for large-scale screening purposes and their costs are high. As a consequence, there is a growing need for in vitro assay systems to assess chemicals for skin toxicity and, if possible, for allergic potential. Due to their anatomical location and critical role in skin inflammatory and immunological reactions, the use of the keratinocytes and skin organotypic culture as a simplified in vitro model to evaluate the potential toxicity of chemicals destined for epicutaneous application is amply justified.6

Keratinocytes have been shown to direct T cell education by production of cytokines, such as interleukin (IL)-10 and IL-12. The purpose of this work was to explore the possibility to use IL-12 production to discriminate in vitro between skin irritants and contact allergens. Functionally interleukin12, a 70kD heterodimer composed of a molecule of p35 covalently linked to a molecule of p40, is a potent immunoregulatory molecule that is critically involved in a wide range of diseases. Because of its promotion of IFN-γ production, it is required for optimal Th1 cell development.7 Furthermore, in a variety of inflammatory skin disorders, including contact hypersensitivity, Th1 cells are critically involved.

Initially, the reconstituted human epidermis consisting of well-differentiated epidermal keratinocyte layers, including a stratum corneum, was treated with a relevant skin irritant, sodium dodecyl sulfate (SLS), and a relevant contact allergen, 1-chloro-2,4-dinitrobenzene (DNCB). At different times thereafter, the expression of both IL-12p35 and IL-12p40 was assessed by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Data obtained indicate that only DNBCB induced an up-regulation of both IL-12p35 and IL-12p40, see Table 1. This up-regulation occurred as early as 3 hours after DNBC treatment. Importantly, the application of SLS or vehicles did not induce IL-12 mRNA up-regulation. Total IL-12 protein was detected in supernatants of allergen-stimulated, but not vehicle stimulated, reconstituted epidermis. To confirm these results, the effects of benzalkonium chloride, oxazolone and eugenol were assessed. At concentration that resulted in equivalent IL-1a release, only contact allergens increased IL-12 expression, confirming the previous data. These preliminary data suggest that IL-12, crucial for Th1 responses, may be indeed an useful marker to discriminate contact allergens from irritants.

Table 1. Selective induction of IL-12p40 by DNBCB.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-12p40 expression (AU)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.61±0.33 (n=4)*</td>
</tr>
<tr>
<td>SDS (1 mg/mL)</td>
<td>0.55±0.17 (n=3)</td>
</tr>
<tr>
<td>DNBCB (0.8 mg/mL)</td>
<td>2.05±0.82 (n=4)**</td>
</tr>
</tbody>
</table>

* IL-12p40 expression was normalized to the housekeeping gene GPDH. AU= arbitrary unit. b Each value represents the mean ± SD of 3-4 independent experiments (n). Statistical analysis was performed by Dunnet’s t test, with *p<0.05 vs control and °p<0.05 vs SDS.

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References

Adverse reactions to drug therapy are a major consideration in clinical medicine and are very common in everyday clinical practice.

Averse drug reactions (ADR) can be classified into 2 categories: types A and B. Type A ADR are common (80% of ADR) and occur in nonsusceptible patients. These reactions include overdosage (toxic pharmacological effects of a supratherapeutic dose of a drug), side effects (therapeutically undesirable but unavoidable pharmacological actions of a drug occurring with recommended dosage), secondary effects (events only indirectly related to the primary pharmacological action of the drug), drug interactions (unusual effects due to the simultaneous pharmacological activity of 2 or more drugs). Type B ADR are uncommon and unpredictable reactions occurring only in susceptible patients. These reactions include intolerance, idiosyncrasy and allergic (or hypersensitivity) reactions. Intolerance is a characteristic pharmacological effect by small dosage of a drug in certain persons; idiosyncrasy is a qualitatively abnormal response to a drug that is different from its pharmacological effects; it occurs only in susceptible patients and does not involve an immune mechanism, although it can resemble an immunologic reaction clinically. An allergic or hypersensitivity drug reaction may be defined as any immunologic response to a drug or its metabolites that results in an adverse reaction. Only reactions mediated by immune mechanisms should be classified as allergic. Allergic drug reactions occur in a small percentage of the population receiving the drug and can occur with low dosages of the drug, require previous exposure to the same drug or to a chemically related drug, develop rapidly after reexposure and produce clinical syndromes commonly associated with immunologic reactions.

Allergic reactions to drugs are classified according to Coomb’s I-IV. High-molecular-weight drugs, for instance antiserum and insulin, are more likely to produce allergic reactions. Low-molecular-weight drugs are, like penicillin, haptens and must first combine with carrier proteins to induce an immune response. Pseudoallergic reactions to drugs (i.e. a reaction with the same clinical manifestation as an allergic reaction, but nonimmunologic in origin) may mimic these immunological mechanism (for example, direct release of histamine by opioids or complement activation by radioactive contrast media).

Clinical manifestations of ADR can involve 1 or more organ systems. The most serious patterns are multiple organ system patterns: anaphylaxis, anaphylactoid or pseudo-allergic reaction, erythema multiiforme-Stevens-Johnson syndrome, toxic epidermal necrolysis, hypersensitivity syndrome, serum sickness-vasculitis, drug fever. The most common patterns are dermatologic patterns: urticaria-angioedema, pruritus without urticaria, morbilliform rashes, fixed drug eruption, photoallergic photosensitivity, phototoxic photosensitivity, contact dermatitis. Others patterns include hematologic patterns: eosinophilia, thrombocytopenia, and hemolytic anemia.

Evaluation of drug allergy must begin with a precise and detailed history, including clinical symptoms and their timing and duration in relation to drug exposure. Skin prick tests may be helpful for diagnosing IgE dependent drug reactions. Radioimmunoassays (for example, the radioallergosorbent test, RAST) may detect serum IgE antibodies to certain drugs (penicillin and succinyl choline). Direct challenge can confirm the relationship of a suspected drug to a given clinical syndrome; because of the potentially morbidity and mortality involved this test is generally unjustified.

As a general rule, a drug responsible for an allergic reaction should not be reused, unless there is an absolute need and no alternative drug is available. When a drug is essential in a patient with a past history of an adverse reaction to it, incremental challenge testing can be considered; it must be performed only by experienced physicians, under strict medical supervision and with resuscitative equipment available. Protocols have been developed to assist in management of patients with a history of adverse drugs reactions who again require treatment with a drug or class of drugs to which they claim they have had a previous reaction.

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References
5. Wood AJJ. Adverse reactions to drugs. In: Isselbacher
Allogenic bone marrow transplantation (BMT) is now established as a therapeutic approach toward a number of malignant and non-malignant hematological disorders. Nearly half of the patients that undergo BMT achieve long term disease-free survival but in a similar number experience significant complications, most of which involving the lung. Early pulmonary complications include infectious and non-infectious pneumonitis, idiopathic pneumonia syndrome, acute graft versus host disease (GVHD), pulmonary vascular thrombotic disease, and obstructive airway disease. Late pulmonary complications are also frequent sequelae of BMT. Apart from infections, abnormality in respiratory functions may occur as a result of prior cytotoxic chemoradiation, because of an involvement of the lung in the course of chronic GVHD or because of a development of a bronchiolitis obliterans. In all these occurrences severe functional impairment leads progressively to fatal respiratory failure. Present study was designed to assess in a cohort of 19 BMT recipients a number of bronchoalveolar lavage features (including cellular and chemokine profiles) correlate them with the presence of functional abnormalities and or respiratory symptoms, in order to identify markers possibly related with the presence of interstitial lung injury. BAL findings among BMT recipients were also compared with those obtained on a group of normal control subjects. Among chemokines interleukin 8 (IL8), Macrophage inflammatory protein-1α (MIP-1α), RANTES (regulated on activation, normal T cell expressed and secreted) and monocyte chemoattractant protein-1 (MCP-1) have been studied.

Chemokines constitute a large family of small cytokines with four conserved cysteines linked by disulfide bonds. The two subfamilies, CXC and CC chemokines, are distinguished according to the position of the first two cysteines, which are separated by one amino acid or are adjacent. Chemokines have been recently intensively studied because of their selective actions on leukocytes and their role in inflammation and immunity. Interleukin-8 is a CXC chemokine produced principally by monocytes, macrophages, T-lymphocytes and epithelial cells. Because of its potent neutrophil chemotactic effect, it is thought to be the primary regulatory molecule of acute inflammatory states. Among CC chemokines, MIP-1α is produced principally by fibroblasts, monocytes, neutrophils, eosinophils and bone marrow stromal cells. Its biological activities include prostaglandin-independent pyrogenic activity, a potential role in wound healing, monocyte chemotaxis and suppression of immature bone marrow stem and progenitor cell. RANTES (regulated on activation, normal T cell expressed and secreted) is the only CC chemokine known to be present in platelets, and has potent chemotactic and activating properties for basophils, eosinophils, and NK cells. MCP-1 has been shown to chemoattract monocytes. It seems to be involved in a number of lung inflammatory and non-inflammatory disease states characterized by the accumulation of leukocytes within the airway and the interstitium.

Twenty-four BMT recipients were initially recruited, among them 19 (11 males and 8 females, mean age 34±10 years), still disease-free at day +100, were included in this study. All patients underwent chest X ray and lung function tests on day −7 and +100 from BMT in addition on day +100 a fiberoptic bronchoscopy and a broncho-alveolar lavage was performed as previously described. In addition 6 healthy subjects (3 males and 3 females, mean age 43±5) were selected among those receiving BAL because of haemoptysis or cough of unknown origin. BAL samples were processed as follow: cells recovered after centrifugation were counted, assessed for viability and used for cytokine preparations (May Grunwald-Giemsa and Papanicolaou stains); BAL fluids (BALf) were concentrated by ultrafiltration and frozen (−80°C) until subsequent ELISA determinations. Albumin levels were assessed by nephelometry while chemokine levels (IL8, MCP-1, MIP1-α and RANTES) were assessed by commercially available ELISA kits (Quantikine, R&D Systems, MN, USA). Routine investigations on BAL samples included: microscopy and culture for bacteria and fungi, microscopy (silver staining ) for pneumocystis carinii, isolation of CMV by immunofluorescence. Diagnosis of bacterial infection was made on the basis of significant bacterial growth (105 CFU/mL) + clinical signs or symptoms. A total of 3 cases of respiratory infections were detected among BMT recipients.

Chronic GVHD (cutaneous or any other localization) was also present in 10 out of 19 BMT recipients at the time of the lavage. BMT recipients without evidence of respiratory infections were classified in asymptomatic or symptomatic according to the absence or presence of respiratory symptoms such as dyspnoea coughed, hypoxemia, ≥ chest X ray infl-
trates or functional abnormalities. BAL features in the group of BMT recipients (symptomatic + asymptomatic) and controls are summarized in Table 1. BMT patients had a significantly higher mean lymphocyte count in the BAL and the levels of all tested chemokines were higher among BMT recipients, but the difference resulted statistically significant only for IL8 and MCP-1 (Table 1 and Figure 1).

When comparing BAL results obtained from symptomatic (n=6) and asymptomatic (n=13) BMT recipients no difference was found in the mean lymphocyte number or percentage, while symptomatic subjects showed a significant increase in mean neutrophil count (Table 2). Chemokine levels varied also significantly according to the presence of symptoms. As shown in Figure 1 mean BAL IL8 and MIP-1α levels were significantly higher among symptomatic subjects than among asymptomatic ones. In addition a significant positive correlation was found between IL8 and MIP-1α levels and neutrophil counts (Spearman rank coeff. P<0.05).

In conclusion our results showed that a significant alteration in BAL cellular profile (increase in lymphocyte count) is present BMT recipients even if asymptomatic. Symptomatic subjects show a significant increase in neutrophil count and concomitantly, an increase in the levels of IL8 and MIP-1α. Therefore, the determination of BAL cellular and chemokine profile of BMT recipients seems to be of interest in monitoring lung damage and in assessing the lung injury mechanisms.

Table 1: BAL variables (cell n° and percentages; albumin and chemokine concentrations) among normal subjects (n = 6) and Allogenic Bone Marrow Transplant recipients (n=19). Means ± SD are shown.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal subjects</th>
<th>BMT recipients</th>
</tr>
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<tbody>
<tr>
<td>Macrophages %</td>
<td>93.4±2.1</td>
<td>71.2±24.6 *</td>
</tr>
<tr>
<td>n°(10⁶/mL)</td>
<td>14.6±5.1</td>
<td>8.75±5.9 *</td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td>5±1.5</td>
<td>25.5±23.3 *</td>
</tr>
<tr>
<td>n°(10⁶/mL)</td>
<td>2.2±3.46</td>
<td>7.41±24.3 *</td>
</tr>
<tr>
<td>Neutrophils %</td>
<td>1.6±1.7</td>
<td>3.53±5.8</td>
</tr>
<tr>
<td>n°(10⁶/mL)</td>
<td>0.32±0.3</td>
<td>0.46±0.7</td>
</tr>
<tr>
<td>Eosinophils %</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>n°(10⁶/mL)</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Total cells n°</td>
<td>9.9±4.3</td>
<td>16.7±28.9 *</td>
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<tr>
<td>Albumin mg/dL</td>
<td>0.1±0.06</td>
<td>0.51±0.93 *</td>
</tr>
<tr>
<td>IL8 pg/mL</td>
<td>5.07±3.8*</td>
<td>32.01±24*</td>
</tr>
<tr>
<td>MIP1alpha pg/mL</td>
<td>0.4±0.13</td>
<td>2.15±3.56</td>
</tr>
<tr>
<td>MCP1 pg/mL</td>
<td>10.57±7.41</td>
<td>55.36±38*</td>
</tr>
<tr>
<td>RANTES pg/mL</td>
<td>8.6±5.5</td>
<td>14.38±24.85</td>
</tr>
</tbody>
</table>

*Significant difference between normal subjects and allogenic BMT recipients (Mann Whitney, p≤0.05).

Table 2: BAL cell differential in asymptomatic (n=13) and symptomatic (n=6) BMT recipients.

<table>
<thead>
<tr>
<th>Cell differential</th>
<th>Asymptomatic BMT recipients</th>
<th>Symptomatic BMT recipients</th>
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</thead>
<tbody>
<tr>
<td>Macrophages %</td>
<td>69.36±29.5</td>
<td>73.75±17.2</td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td>29.91±29.5</td>
<td>18.75±14.38</td>
</tr>
<tr>
<td>Neutrophils %</td>
<td>1.01±1</td>
<td>7±7.8*</td>
</tr>
<tr>
<td>Eosinophils %</td>
<td>0.09±0.3</td>
<td>0.5±1.07</td>
</tr>
</tbody>
</table>

* Significant difference between Symptomatic and Asymptomatic BMT recipients (Mann Whitney test, p≤0.05).
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