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(54) Title: USE OF A PHARMACEUTICAL COMPOSITION COMPRISING AN EFFECTIVE AMOUNT OF INTERLEUKIN-10, AN ANALOG AND/OR AN AGONIST OF INTERLEUKIN-10

(57) Abstract

The invention relates to a use of Interleukin-10 (IL-10) or analogs or agonists thereof, for the manufacture of a medicament for the treatment or prevention of diseases involving: i) Interleukin-5 production by T-lymphocytes, or; ii) activation of T-lymphocytes through the B7/CD28 or ICAM-1/LFA-1 pathway, or; iii) activation of the procoagulant activity of monocytes; iv) severe infectious diseases involving Tumour Necrosis Factor (TNF).

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USE OF A PHARMACEUTICAL COMPOSITION COMPRISING AN EFFECTIVE AMOUNT OF INTERLEUKIN-10, AN ANALOG AND/OR AN AGONIST OF INTERLEUKIN-10

The present invention concerns the use of a pharmaceutical composition comprising an effective amount of interleukin-10, an analog and/or an agonist of interleukin-10, for the manufacture of a medicament intended for treating mammals, particularly humans, in order to cure or prevent a disease chosen among the group consisting of the diseases induced by an excessive amount of interleukin-5, an excessive T-cell stimulation through B7/CD28 and/or ICAM-1/LFA-I molecular interactions and/or an excessive monocyte procoagulant activity.

Human IL-10 is a cytokine which, in its mature form, has 160 amino acids and contains 2 intramolecular disulphide bridges. It also contains a glycosylation site, but is not glycosylated.

IL-10 is produced in the body by a number of different cell types. Cells involved in the immune system, namely lymphocytes T and B and monocytes/ macrophages represent important sources of IL-10. Outside the immune system, keratinocytes and certain tumour cells are also capable of producing IL-10. The factors controlling IL-10 synthesis are not completely understood.

IL-10, originally known as Cytokine Synthesis Inhibitory Factor (CSIF) exerces its biological effects on a number of different target cells.

Its immunosuppressive and anti-inflammatory properties are mainly linked to the effects of this cytokine on monocytes and macrophages (i.e. inhibition

of their production of TH1 cytokines) although it has recently been found that IL-10 can also act directly on T-lymphocytes. The inhibition, by IL-10, of TH1 cytokine production (IL-2, IFN-γ) has mainly been observed in systems where T-lymphocyte activation depends on monocytes and macrophages acting as Antigen Presenting Cells (APC). IL-10 acts mainly indirectly on T-lymphocytes by interfering with the activation signals presented to them by the APC. It is thought that inhibition of the expression of the Class II Major Histocompatibility Complex (MHC) on the APC surface may be one of the mechanisms involved.

For example, International Patent Application WO 91/00349 describes IL-10 (CSIF) as a mammalian cytokine inhibiting TH1 lymphocytes, controlling cell-mediated immune responses. Uses of IL-10 in controlling diseases associated with MHC-linked immune responses are described.

International Patent Application WO 93/17698 describes use of IL-10 for suppressing Graft-vs-Host disease or tissue rejection. The main mechanism of action of IL-10 in this setting is described as being the down-regulation of MHC Class II expression on monocytes. Down-regulation of ICAM-1 and B7 expression on monocytes is explicitly ruled out in this document as a mechanism of action of IL-10. Consequently, the use of IL-10 in diseases involving activation of T-lymphocytes by the B7/CD28 and ICAM-1/LFA-1 pathway are not described or suggested.

International Patent Application WO 93/02693, published on 18th February 1993, (prior art according to A.54(3) EPC) describes the use of IL-10 or an analog or agonist or antogonist thereof, for the treatment or prevention of Septic Shock and Toxic Shock. According to this document, IL-10 is

administered either simultaneously with, or after, exposure to the LPS or superantigen. No suggestion is made that administration of IL-10 prior to endotoxin or superantigen exposure can prevent morbidity and mortality associated with gram-negative or gram-positive sepsis.

International Patent Application WO 93/19770 describes compositions suitable for use in the treatment of acute or chronic inflammation, which comprise IL-10 in association with IL-4. Use of IL-10 alone is not described.

The technical problem underlying the present invention was :

i) to identify an efficient means of treating and preventing septic shock, preferably before exposure to endotoxin or superantigen ;

ii) to identify a treatment for diseases involving activation of the coagulation system by induction of tissue factor expression at the surface of monocytes ;

iii) to identify, in clinical situations involving alloreactive responses, a means of blocking all T-lymphocyte activation pathways to allow treatment of patients presenting signs of rejection resistant to standard treatments, and ;

iv) to identify a treatment for diseases involving excessive IL-5 production by T-lymphocytes.

The solution to the above technical problems has now been discovered, by the present inventors, to reside in the administration of IL-10, either alone or in combination with other treatments. This solution is based on the discovery, by the present inventors, that :

i) IL-10 is an efficient inhibitor of TNF-α production by LPS-stimulated monocytes ;

ii) IL-10 prevents the induction of monocyte procoagulant activity ;

iii) IL-10 inhibits B7 and ICAM-1 expression on monocytes, and ;

iv) IL-10 inhibits IL-5 production by T-lymphocytes.

The invention relates to pharmaceutical compositions comprising an effective amount of IL-10, an analog or an agonist of IL-10, for the treatment of conditions involving septic shock, over-production of IL-5 by T-lymphocytes ; excessive T-cell stimulation by B7/CD28 or ICAM-1/LFA-1 interactions or monocyte procoagulant activity. The invention also relates to methods of preventing or controlling or suppressing these conditions in an individual, said method comprising the step of administering to the individual the above-described pharmaceutical compositions.

By "IL-10" is meant pure mammalian, particularly human, interleukin 10 as purified from natural sources or preferably as produced by expression in a suitable host of a recombinant DNA sequence encoding interleukin 10. The IL-10 of this invention also includes the viral form of interleukin 10, chimeric proteins composed of sequences of human and viral IL-10 origin, provided that the properties of IL-10 as required by the present invention are maintained. "IL-10" also englobes analogs and peptides of interleukin 10 as described, for example, in PCT publication WO 91/00349 as a cytokine synthesis inhibitory factor.

As suitable recombinant hosts, mention is made of eukaryotic cells transfected by retroviral vectors carrying the appropriate IL-10 encoding sequence(s) or a baculoviral system. Retroviral vectors are introduced in mammalian cells (for example, CHO

cells). The recombinant proteins obtained in the supernatant is similar to the native protein expressed in mammals. Moreoever, retroviral particles carrying the IL-10 gene are produced in the supernatant of packaging cells transfected by these constructions. These particles can be used to infect mammalian cells, and thereby to induce the expression of IL-10 by these cells. Beculoviral vectors are used to produce larger quantities of recombinant protein, compared to retroviral vectors. Although this system of expression yields recombinant protein with glycosylation and disulfide-bridge, it induces altered posttranslational modifications and different glycosylations.

As used herein, "effective amount" of IL-10 means an amount sufficient at least to ameliorate or prevent a symptom of one of the aforementioned conditions. The effective amount for a particular patient may vary depending on such factors as the state of the condition being treated, the overall health of the patient, the method of administration, the severity of side-effects, and the like.

Analogs and/or agonists of IL-10 may be molecules which mimic IL-10 interaction with its receptors. Such may be analogs or fragments of IL-10, or antibodies against ligand binding side epitopes of the IL-10 receptors, or anti-idiotypic antibodies against particular antibodies which bind to receptor-interacting portions of IL-10.

Antibodies can be raised to the IL-10 cytokine, fragments, and analogs, both in their naturally occuring forms and in their recombinant forms. Additionally, antibodies can be raised to IL-10 in either its active forms or in its inactive forms, the difference being that antibodies to the active cytokine are more likely to recognize epitopes which

are only present in the active conformation. Anti-idiotypic antibodies are also contemplated in these methods, and could be potential IL-10 agonists.

Generally, IL-10 is administered as a pharmaceutical composition comprising an effect amount of IL-10 and a pharmaceutical carrier. A pharmaceutical carrier can be any compatible non-toxic substance suitable for delivering the compositions of the invention to a patient. Generally, compositions useful for parenteral administration of such drugs are well known, see e.g. : Remington's Pharmaceutical Science, 15th Ed. (Mack Publishing Company, Easton, PA 1980, U.S.A.). Alternatively, compositions of the invention can be introduced into a patient's body by implantable or injectable drug delivery system, see e.g. : URQUHART et al. (1984) Ann. Rev. Pharmacol. Toxicol. 24 : 199-236, Lewis, ed. Controlled Release of Pesticides and Pharmaceuticals (Plenum Press, New York, 1981) ; U.S. patent 3,773,919, and U.S. patent 3,270,960,

When administered parenterally, the IL-10 is formulated in a unit dosage injection form (e.g., solution, suspension or emulsion) in association with a pharmaceutical carrier. Examples of such carriers are normal saline, Ringer's solution, dextrose solution, and Hank's solution. Nonaqueous carriers, such as fixed oils and ethyl oleate, can also be used. A preferred carrier is 5 % dextrose/saline. The carrier can contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The IL-10 is preferably formulated in a purified form, substantially free of aggregates and other proteins, at a concentration in the range of about 10,000 - 100,000 U/ml. (A standard unit [U] of activity is the

amount of IL-10 necessary to produce a half-maximal response by the MC/9 mast cell line [THOMPSON-SNIPES et al (1991) J. of Exp. Med. 173:507-510].) The IL-10 can be administered in most cases by continuous infusion to the patient, so that an amount in the range of about 100,000 - 100,000,000 U is delivered per day (i.e., about 1,500 - 150,000 U/kg/day). The daily infusion rate can be varied based on monitoring of side-effects and blood cell counts of the patent. However, in some cases (e. g., for preventing side-effects from anti-CD3 treatment), Il-10 is preferably given as a single injection of about 50,000 - 5,000,000 U.

For the prevention and/or treatment of allograft rejection in accordance with this invention, it is possible to use either IL-10 administration, incubation of cells with IL-10, or IL-10 gene transfer. It is believed that such gene transfer can be carried out in a conventional manner, preferably in one of the following ways:

1) The gene coding for IL-10 is transferred in vitro into cells of the donor, which cells are subsequently injected into the patient in order to induce chimerism and allograft tolerance. The transfer can be performed using different vectors, such as retroviral vectors (Anderson (1992) Science 256 : 808-813 ; Rosenberg et al (1990) New England Journal of Medecine 323 : 570-578).

2) The gene coding for IL-10 is transferred in vitro into autologous and alloreactive lymphocytes isolated from the patient. These modified cells are subsequently reinjected into the patient in order to induce allograft tolerance. The transfer can be performed using different vectors, such as retroviral vectors.

3) The gene coding for IL-10 is transferred into the allografted cells by perfusion of the graft with a medium containing IL-10 viral particles. The transfer can be performed using different vectors, such as vectors derived from adenovirus (Rosenfeld et al (1991) Science 252 : 431-434).

I - SEVERE INFECTIOUS DISEASES :

Endotoxin or lipopolysacharide (LPS) from gram-negative bacteria is a major causative agent in the pathogenesis of septic shock (1). A shock-like state can indeed be induced experimentally by a single injection of LPS into animals. These toxic effects of LPS are mostly related to macrophage activation, leading to the release of multiple inflammatory mediators. Among these mediators, tumor necrosis factor (TNF) appears to play a crucial role, as indicated by the prevention of LPS toxicity by the administration of neutralizing anti-TNF antibodies (2-5).

IL-10 is effective in inhibiting TNF release and associated toxicity in a mouse model of septic shock. IL-10 can therefore be used to prevent the morbidity and the mortality associated with gram-negative sepsis, either alone or in combination with conventional therapeuties (26).

It has been demonstrated by the inventors that pretreatment with IL-10 prevents LPS-induced toxicity. These results indicate that IL-10 can be used preventively in situations where bacterial sepsis and related septic shock are likely to be encountered, for example before surgery, particularly field surgery, and other situations where bacterial infection is likely. In these cases, according to the invention,

IL-10 is administered before potential exposure to the bacterial micro-organism.

IL-10 can also be used to prevent cerebral malaria (neuromalaria), since :

a) adhesion molecules (especially ICAM-1) expression is playing a major role in the induction of cytoadherence of infected erythrocytes to the brain microvascular endothelium ;

b) the expression of these adhesion molecules is stimulated by TNF which is induced by malaria antigen and ;

c) malaria antigen-induced TNF production can also be inhibited by IL-10 administration. In this case, IL-10 can thus inhibit the development of the pathological entity, either alone or in combination.

IL-10 can also be used according to this aspect of the invention, in the treatment or prevention of toxic shock syndrome. This syndrome is related to the T cell and monocyte activation induced by toxins from gram-positive bacteria (e. g., Staphylocci). IL-10 efficiently blocks this process and can therefore be used therapeutically for this purpose, either alone or in combination with conventional therapies (26, 27).

II - <u>INHIBITION OF MONOCYTE PROCOAGULANT ACTIVITY BY IL-10</u> :

The present inventors have demonstrated, for the first time, that IL-10 inhibits monocyte procoagulant activity.

Therefore, IL-10 can be used effectively in acute diseases caracterized by activation of coagulation system involving the induction of tissue factor expression at the surface of monocytes, such as septic shock, meningococcemia, promonocytic leukemia, first

dose reactions induced by anti-CD3 monoclonal antibodies, etc... and all diseases characterised by thrombotic processes such as antiphospholipid syndrome, hemolytic uremia syndrome, allograft rejection, disseminated intravascular coagulation, and high-risk surgery.

In these applications, IL-10 can be used as sole active principal or in combination with other treatments acting on the extrinsic pathway of the coagulation system. Preferably, IL-10 is the sole cytokine present in the pharmaceutical composition.

Expression of Tissue Factor at the surface of human monocytes, leading to induction of procoagulant activity, can be induced by bacterial LPS (resulting in septic shock), by anti-CD3 monoclonal antibodies, or by inflammatory mediators such as TNF or IL-1.

Since the induction by LPS of monocyte procoagulant is cytokine-independent, the inhibitory effect of IL-10 on LPS-induced monocyte procoagulant activity is not linked to and could not be anticipated by the observations in the prior art that IL-10 blocks cytokine synthesis in monocytes.

According to a preferred embodiment of this aspect of the invention, IL-10 is used in the preparation of a medicament for treatment or prevention of anti-CD3-induced shock syndrome. IL-10 has a dual function in this application and acts both by inhibiting cytokine synthesis (TNF-α and IL-1) by monocytes and lymphocytes, and by inhibiting the procoagulant activity.

In addition, since anti-CD3 antibody (OKT3) therapy in kidney transplant recipients triggers activation of the coagulation system, resulting sometimes in graft thrombosis, IL-10 can be administered prophylactically before the first

injecion of OKT3 in allograft recipients, either alone or in combination with conventional therapies.

III - INHIBITION BY IL-10 OF T-LYMPHOCYTE ACTIVATION THROUGH THE B7/CD28 OR ICAM-1/LFA-1 PATHWAY :

Contrary to the reports in documents published to date (see for example, WO 93/17698 supra ; Ann. Rev. Immunol. 11 : 165-190, 1993), the present inventors have found that IL-10 inhibits not only Class II MHC expression but also B7 and ICAM-1 expression on human monocytes. This finding is also surprising in view of the observations by DING et al (J. Immunol., 151, 1224-1234, August 1993) who report, in mice, that IL-10 inhibits B7 up-regulation on mouse macrophages, but did not modify the expression of ICAM-1 on these cells.

B7 and ICAM-1 (Intercellular Adhesion Molecule-1) are "accessory molecules" expressed on the membrane of Antigen Presenting Cells (APC's), which interact with their counterpart receptors (CD28 and LFA-1, respectively) on the surface of the T-lymphocyte, and which are crucial for the co-stimulation of the T-lymphocyte. Therefore, IL-10 inhibits T-lymphocyte activation by interfering with both the MHCII/T cell receptors interaction, and the B7/CD28, and ICAM-1/LFA-1 interactions. The latter effects are especially relevant to transplantation immunity. Indeed, antigen-presenting cells (APC) deficient in these costimulatory molecules induce T cell unresponsiveness (T cell anergy) in vitro. Moreover, in vivo inhibition of ICAM-1/LFA-1 interactions by injection of monoclonal antibodies or in vivo inhibition of B7/CD28 interactions by CTLA4-Ig fusion protein prevent allograft rejection in vivo. The

inventors have also obtained evidence that IL-10 inhibits within the T lymphocyte the B7/CD28 activation pathway. The dual action of IL-10 on this pathway (at the APC and at the T lymphocyte level) indicates that IL-10 can be administered to promote tolerance to allografts.

These observations indicate that IL-10 can be used in the treatment or prevention of diseases involving activation of T-lymphocytes through the B7/CD28 or ICAM-1/LFA-1 pathway, particularly diseases associated with alloreactive responses, such as allograft rejection and Graft versus Host Disease. Diseases involving Host versus Graft reactions are particularly preferred.

According to the invention, IL-10 may be used either alone or in combination with cellular suspensions (blood cells, spleen cells, bone marrow cells, bone marrow stem cells...) from the donor to prevent or treat rejection of solid allograft. IL-10 can be injected in the graft before transplantation in order to inhibit donor passenger leucocytes and/or in the recipient by parenteral route. IL-10 can also be added to donor cell suspensions before their infusion in the recipient in order to induce transplantation tolerance.

The properties of IL-10 described above also indicate that IL-10 can be used in the prevention or treatment of acute graft versus host disease after bone marrow transplantation. In the prevention protocols, IL-10 is added to donor bone marrow cells before their infusion in the recipient or injected in the recipient after the bone marrow transplantation.

Since, contrary to previous findings, IL-10 has now been found to block the ICAM-1/LFA-1 pathway as well as the B7/CD28 pathway, known to be cyclosporin-A

resistant, IL-10 can either be used as sole active principal or in association with cyclosporin-A or other related immunosuppressive agents such as FK 506 or rapamycin. Therefore, it may be advantageous to add IL-10 to conventional treatments in order to prevent or treat allograft rejection. IL-10 may be particularly useful in patients in whom conventional treatments, including cyclosporin-A fail to prevent rejection.

IL-10 presents further advantages in comparison to standard treatments, for example it can prevent or treat allograft rejection when administered for only a short time, without requiring continuous administration. Also, as will be demonstrated below, the inventors found that IL-10 inhibits IL-5 production by T-lymphocytes. Since eosinophils are induced by IL-5 and are involved in some forms of graft rejection, the beneficial effect of IL-10 in transplantation settings will also be related to the inhibition of IL-5 production. Furthermore, IL-10 might also be used in association with anti-CD3 mAb. In this latter setting, IL-10 will enhance the immunosuppressive effect of the mAb and also prevent its side effects related to cytokine release.

IV - IL-10 INHIBITS IL-5 PRODUCTION BY T-LYMPHOCYTES :

IL-10, which is a cytokine produced by TH2 lymphocytes, has been described to date as a potent inhibitor of TH1 cytokines. There have been no reports of IL-10 as an inhibitor of TH2 cytokines.

Surprisingly, the present inventors have found that the inhibitory effect of IL-10 within T-cells does not specifically affect the production of TH1 cytokines by these cells and that IL-10 is indeed able

to inhibit the production of IL-5, a cytokine typically produced by TH2 lymphocytes. These results, obtained using peripheral blood T-cells, are particularly unexpected since results of similar experiments on T-cells clones (a laboratory model of T-lymphocytes which may present artefacts) showed that IL-10 did not block IL-5 production (DE WAAL MALEFYT et al, 1993, J. Immunol. 150 : 4754-4865).

The inventors also found that the inhibition of IL-5 production by IL-10 is dependent upon the costimulation of the T lymphocytes. Indeed, IL-10 inhibits production of IL-5 by T cells when these cells are costimulated by B7/CD28 signaling, but not when they are stimulated by PMA and A23187 calcium ionophore.

These observations indicate that IL-10 can be of therapeutic value in diseases involving excessive IL-5 production by T-lymphocytes.

Since IL-5 plays a key role in diseases associated with eosinophilia, IL-10 is of therapeutic value in eosinophilic diseases (for which no satisfactory treatments are available) such as atopic diseases including allergic rhinitis and bronchial asthma, atopic dermatitis and other cutaneous diseases associated with eosinophil infiltration (including chronic eczema, erythema multiforme, dermatitis herpetiformis, mastocytosis, bullous disorders), Crohn's disease, broncho-pulmonary aspergillosis, spanish toxic oil syndrome, myalgia-eosinophilia syndrome induced by L-tryptophane preparations, certain drug allergic reactions, tropical eosinophilia, helminthic diseases, Schulman syndrome (eosinophilic fasciitis), Churg-Strauss syndrome and other vasculitides with eosinophilia, Loeffler

syndrome, chronic eosinophilic pneumonia, eosinophilic gastroenteritis, and the hypereosinophilic syndrome.

In these diseases, rIL-10 should preferably be given either alone or in combination with interferon-alpha or interferon-gamma. Also, since hyper-eosinophilic syndromes are characterised by pulmonary involvement with interstitial infiltrates, an appropriate route of IL-10 administration is through the use of aerosols, as well as the routes mentioned earlier. Topical applications including ointments are also suitable.

The inventors have also demonstrated that endogenous IL-10, produced by the TH2 lymphocytes, controls Il-5 production by these same cells. Neutralizing anti-IL-10 monoclonal antibody increases IL-5 release. Therefore, according to the invention, anti-IL-10 antibodies can be used in therapy or prevention of diseases involving insufficient production of IL-5 from T-lymphocytes.

Different aspects of the invention are illustrated in the figures :

- **Figure 1 :** Construction of the murine IL-10 retroviral vector. Plasmid pTG1H-mIL-10 was derived from the Harvey murine sarcoma virus (Ha-MuSV) cloned in pBR322 (pCO6-HX). The entire coding region of v-ras^H (SacII-XhoI) was removed and replaced by the 560 bp fragment coding for the murine IL-10 cDNA. Ha-MuSV sequences are shown in bold lines ; pBR322, in broken lines. Restriction endonucleases sites : H, HindIII ; S, SacII ; X, XhoI ; B, BamHI. Black box : coding sequences of IL-10 ; open box : coding sequences of v-ras^H ; and cross-hatched boxes ; Ha-MuSV LTRs ;

- **Figure 2** : Time course of rectal temperature (mean ± SEM) in BALB/c mice (6 to 21 per group)

injected with either medium alone (0), LPS (100 µg i.v.) without pretreatment (●), LPS (100 µg i.v.) after pretreatment with 1000 U IL-10 (▽), or LPS (100 µg i.v.) after pretreatment with supernatant from mock-transfected CHO-K1 cells (▼) ;

- **Figure 3** : Lethality after injection of 500 µg LPS in three groups of BALB/c mice :

(●) mice with no pretreatment (n = 15) ;

(▽) mice pretreated i.p. with 1000 U IL-10 (n = 15) ;

(▼) mice pretreated i.p. with supernatant from mock-transfected CHO-K1 cells (n = 10).

Two tailed p value has been determined with the Fisher's exact test.

- **Figure 4** : Inhibition, by rIL-10, of IL-5 production by human resting T cells activated by anti-CD3 mAb in conjunction with B7/CD32 transfectants.

- **Figure 5** : Influence of rIL-10 on the secretion of cytokines by T-cells stimulated by PMA and anti-CD28 mAb.

- **Figure 6** : Systemic administration of rIL-10 inhibits draining lymph node enlargement induced by subcutaneous injection of allogeneic cells. 2.5×10^6 spleen cells from BALB/c (syngeneic) or A/J (allogeneic) mice were injected subcutaneously in the right rear footpad of BALB/c mice. From day 0 to day 5, mice receiving allogeneic cells were injected thrice daily with either 1000 U rIL-10 or control vehicle. On day 5, enlargement of right lymph node was measured by the weight difference (delta mg) with controlateral lymph node. * : $p < 0.001$ vs syngeneic ; ** : $p < 0.05$ vs allogeneic and $p < 0.01$ vs allo + contr.

EXAMPLES

EXAMPLE 1 : IL-10 cDNA's AND VECTORS USED IN THESE EXAMPLES

Unless otherwise indicated, the cDNA's used in the examples are the following :

IL-10 cDNA's :

The cloning of mIL-10 (mouse IL-10) cDNA is described in detail in example 2 as well as in the article entitled "Interleukin-10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia", by C. Gérard et al., Journal of Experimental Medicine 177 : 547-550, 1993. The sequence of this cDNA is identical to the published sequence (patent application WO 91/00349, page 29, lines 15-26), except for one nucleotide located #247 where the present inventors found a "C" and not a "T" as described in WO 91/00349.

The cloning of hIL-10 (human IL-10) cDNA is described in the paper entitled "Interleukin-10 inhibits the induction of monocyte procoagulant activity by bacterial lipopolysaccharide", by O. Pradier et al., European Journal of Immunology 23 : 2700-2703, 1993. The sequence of this cDNA is identical to the published sequence (patent application WO 91/00349).

VIL-10 (viral IL-10) DNA (Viera P. et al., P.N.A.S. 88 : 1172-1176, 1991) has been cloned by performing polymerase chain reaction (PCR) using specific oligonucleotides for the viral IL-10 DNA from the Epstein-Barr virus (EBV). The target DNA used in

the PCR was extracted from peripheral blood mononuclear cells of an EBV-infected patient.

The vectors used in the examples, unless otherwise indicated, are the following :

Retroviral vectors :

Construction of the murine IL-10 retroviral vector is shown in figure 1, and is also described by Gérard et al., J. Exp. Med. 177 : 547-550, 1993.

The same vector is used to insert the human and viral IL-10 cDNA's described above.

A second retroviral vector (pCO7-FX) is derived from the sequences of both Harvey Murine Sarcoma Virus and Friend Murine Leukemia Virus. This vector allows higher expression and higher viral titer compared to tne first vector described above. It has been published by Feldman et al. in Journal of Virology 63 : 5469-5474, 1989. To construct the IL-10 constructions, which may be designated pTG2F-IL10, the IL-10 cDNA's were inserted in the SacII and XhoI sites of the pCO7-FX vector.

mIL-10 and hIL-10 expression using Baculovirus system :

The same cDNA's as those described above have been inserted in the NheI and BamHI cloning sites of the pBlueBac2 transfer vector available commercially (Invitrogen). In these constructions, the cDNA's are under the control of the polyhedrin promoter. By transfecting them, recombinant viruses able to infect insect cells were obtained, from which recombinant IL-10 was prepared and purified.

EXAMPLE 2 : IL-10 REDUCES THE RELEASE OF TNF-α AND PREVENTS LETHALITY IN EXPERIMENTAL ENDOTOXEMIA

MATERIALS AND METHODS

Animals. 10-15 week-old BALB/c mice were from the KUL Proef Dieren Centrum (Leuven, Belgium).

Reagents. LPS from E. coli (serotype 055:B5) was from Sigma Chemical Co. (St. Louis MO, U.S.A.). JESS-2A5 mAb, a neutralizing rat anti-mouse IL-10 IgG1 mAb, was from Tim Mosmann (Department of Immunology, University of Alberta, Edmonton, Canada) (9). LO-DNP mAb, a rat IgG1 antibody used as control, was from H. Bazin (Experimental Immunology Unit, Université Catholique de Louvain, Brussels, Belgium).

Mouse recombinant IL-10: cloning and expression. Specific oligonucleotides for the murine IL-10 cDNA were synthesized according to the IL-10 sequence (10). Restriction sites were included at their 5' end for subcloning: HincII/SacII for the sense primer 5'-CTCCATCATGCCTGGCTCA-3' (nucleotide 69-87) and SmaI/XhoI for the antisense primer 5'-TACACACTGCAGGTGTTTTAGC-3' (nucleotide 608-629). Total RNA was prepared from the spleen of mice injected with the hamster anti-CD3 mAb 145-2C11 as a nonspecific stimulator of cytokine transcription (11). 1 μg RNA was reverse transcribed using the antisense oligonucleotide (1 μg) as primer and 200 U Mo-MuLV reverse transcriptase (RT) (Promega Corp, Madison, WI, U.S.A.) in RT buffer (50mM KCl, 20mM Tris HCl, pH 8.3, 2.5mM $MgCl_2$, 0.1 mg/ml acetylated BSA, dNTPs 2.5 mM each, RNasin 20 U [Promega Corp.]) in a final volume of 20 μl. To this were added 2.5 U Taq DNA polymerase, and 1 μg of each sense/antisense primer, in the same

buffer, and in a total volume of 100 µl. A PCR was performed using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, U.S.A.) for 35 cycles: 1 min denaturation at 94°C, 2 min annealing at 55°C, and 3 min extension at 72°C. A band with the 560-bp predicted size was obtained. The specificity of the amplified cDNA was validated by its restriction pattern. Sequencing was also performed following the dideoxy-nucleotide chain termination method (12). Nucleotides 322, 465-468, 522 and 523 were different from those published (10), but the amino acids remained unchanged. The SacII-XhoI restriction fragment, containing the murine IL-10 cDNA, was inserted in the Harvey murine sarcoma virus-derived retroviral vector pCO6-HX in place of the coding sequence of p21 ras (13). In this construction, called "pTG1H-mIL10", the IL-10 cDNA is the only functional gene. A similar vector, with no inserted IL-10 gene, was used as a negative (mock) control. To derive individual colonies of cells expressing high levels of IL-10, pTG1H-mIL10 was cotransfected with the plasmid pSV2-neo, which contains the neomycin resistance gene as a selectable marker. Transfection was performed in the CHO-K1 (Chinese hamster ovarian K1) cell line using a modified calcium phosphate method, except that no carrier DNA was present (14). Selection for transfectants was initiated by adding 400 µg/ml G418 (Geneticin; Gibco, Paisley, Scotland). 10 days later, individual resistant colonies were isolated and tested for IL-10 expression using a specific enzyme-linked immunosorbent assay (Pharmingen, San Diego, CA, U.S.A.). IL-10 concentrations were determined by reference to a standard curve of recombinant IL-10 expressed in COS cell supernatant (470 U/ml) (Pharmingen). The supernatant collected after 24 h

culture of a colony with high IL-10 level (200 U/ml) was concentrated to 5,000 U/IL-10/ml on an ultrafiltration membrane (Amicon Corp, Danvers, MA, U.S.A.). Control (mock) supernatant from CHO-K1 cells transfected with the control plasmid and with pSV2-neo was selected, collected, and concentrated similarly. The endotoxin content of the injected preparations was below 1 ng/ml.

Determination of Serum TNF Levels. Blood samples were obtained from individual mice by retroorbital puncture. Serum levels of TNF were estimated by a cytotoxicity assay on actinomycin-D-treated WEHI-164 clone 13 cells (15). Results were expressed in pg/ml in reference to the cytotoxic activity of a standardized (NBSB, UK) preparation of recombinant murine TNF expressed in E. coli (16). This preparation had a specific biological activity of 2.25×10^8 IU/mg, with 1 IU representing about 4 pg murine TNF. IL-10 was found not to be interfering with this bioassay.

Experimental protocol. Mice were injected intravenously with 100 μg of LPS 30 min after an intraperitoneal administration of either CHO-IL-10 or mock supernatants. Serum TNF levels were determined 1.5, 3, and 6 h after LPS challenge, whereas rectal temperatures were measured at regular intervals with a digital thermometer. In another series of experiments, the effects were evaluated of IL-10 on the lethality induced by a single dose of 500 μg LPS.

Statistics. TNF levels and temperatures were compared using the Mann-Whitney U test. Lethality data were analyzed with the Fisher's exact test.

RESULTS AND DISCUSSION

To assess the in vivo effects of IL-10 administration on LPS-induced TNF release, three different doses of IL-10 (10, 100, or 1,000 U) were given intraperitoneally 30 min before the intravenous injection of 100 μg LPS. Since previous studies showed that TNF levels peaked 1.5 H after LPS challenge, blood samples were taken at that time (17). IL-10 pretreatment resulted in a significant reduction in the amounts of TNF released in the circulation after LPS challenge, as shown in Table 1, representing one out of three experiments which gave similar results. This in vivo effect of IL-10 was already observed with doses as low as 10 U ($p < 0.01$ as compared with LPS after pretreatment with control medium). As 1,000 U had a more pronounced and reproducible activity, this dose was chosen for further studies. The specificity of the effect of the IL-10 preparation was assessed by pretreating mice with a supernatant from mock-transfected CHO-K1 cells. As shown in Table 1, this control preparation had no effect on TNF release. TNF serum levels returned to baseline value 3 h after LPS challenge in all groups of mice, indicating that IL-10 did not merely delay the release of TNF (Table 1).

Table 1. Effects of various doses of IL-10 on the systemic release of TNF induced by LPS

Pretreatment	Challenge	TNF serum levels (pg/ml) 1.5h	3h
medium	-	< 10	< 10
medium	LPS	5268 ± 1118	< 10
mock	LPS	5720 ± 1283	< 10
IL-10 (10 U)	LPS	1216 ± 469*	ND
IL-10 (100 U)	LPS	1846 ± 1295**	ND
IL-10 (1000 U)	LPS	416 ± 70***	< 10
IL-10 (1000 U)	-	< 10	ND

BALB/c mice (n = 5 in each group) were injected i.p. with the indicated doses of IL-10, 30 min before i.v. injection of 100 μg LPS. As control, mice were pretreated with either the medium used for culturing CHO-K1 cells, or the mock supernatant. Serum levels of biologically active TNF (mean ± SEM) were determined 1.5h and 3h after LPS challenge.

*$p < 0.01$, **$p < 0.05$, and ***$p < 0.005$, as compared to mice pretreated either with control medium or with mock supernatant before LPS challenge.

As a first approach to evaluate in vivo the effect of IL-10 on LPS-induced toxicity, rectal temperature was monitored for 24 h after challenge with 100 µg LPS. Indeed, LPS caused a profound hypothermia which is probably related to TNF release (18). The data presented in Fig. 2 indicate that IL-10 pretreatment efficiently prevented LPS-induced hypothermia ($p < 0.0001$ at 4 h, and $p < 0.005$ at 6 h, as compared with either LPS alone or LPS after pretreatment with mock supernatant), whereas control supernatant from mock-transfected CHO-K1 cells had no significant effect. A substantial variation in the recovery from hypothermia was seen among the animals injected with 100 µg LPS, either given alone or after pretreatment with mock supernatant. Added as an additional control of the specificity for the effect observed after pretreatment with CHO-IL-10 supernatant was 1 mg of the neutralizing anti-mouse IL-10 JESS-2A5 mAb or of a control rat IgG1 LO-DNP mAb to the IL-10 preparation. The anti-IL-10 completely abrogated the protective effect of IL-10 on hypothermia whereas the control rat mAb had no effect.

To evaluate the effect of IL-10 pretreatment on LPS-induced lethality, mice were challenged with 500 µg LPS, a dose which is lethal within 72 h in 50 % of the animals. All mice pretreated with 1,000 U IL-10 survived the LPS injection, whereas pretreatment with supernatant from mock-transfected CHO-K1 cells did not modify the LPS-induced lethality (Fig.3).

Taken together, these data indicate that pretreatment with IL-10 prevents the toxicity of LPS in a murine model of endotoxin shock. The beneficial effect of IL-10 could be due at least in part to the reduction of TNF synthesis or release. Indeed, a similar level of protection against LPS-induced

lethality has been obtained by the use of neutralizing anti-TNF antibodies (2-5). In vitro data suggest that IL-10 might also inhibit or prevent the synthesis and/or release of other monocyte/macrophage-derived cytokines involved in the pathogenesis of septic shock, especially IL-1(1, 6, 19, 20).

IL-10 can therefore be added to the potential immunointervention strategies for the prevention and/or treatment of septic shock, which already include anti-TNF mAb (2-5), IL-1 receptor antagonist (21-23), differenciation factor/leukemia inhibitory factor (24), and G-CSF (25). Because of its deactivation effect on macrophages, IL-10 therapy could be of particular interest, possibly in combination with some of these other biological agents.

EXAMPLE 3 : USE OF IL-10 IN PROTHROMBOTIC STATES : INHIBITION OF PROCOAGULANT ACTIVITY OF MONOCYTES

LPS has been shown to induce the expression of a procoagulant activity (PCA) due mainly to the expression of tissue factor (TF) on monocytes and endothelial cells. Inflammatory mediators, such as TNF or interleukin 1 (IL-1), as well as LPS, could also induce on endothelial cells the expression of TF. Therefore, the effects of IL-10 on this prothrombotic mechanism was investigated, using cultured human umbilical vein cells (HUVEC) and peripheral blood mononuclear cells (PBMC).

PCA and TF expression induced on monocytes by LPS (1 µg/ml) was neutralized by more than 80% when human recombinant IL-10 (1 to 2.5 U/ml) was preincubated overnight with the PBMC prior to the adjunction of LPS. At least 6 hours of monocytes-preincubation with

IL-10 was necessary to obtain maximal PCA inhibition. No influence of IL-10 on LPS-induced tissue factor expression by EC was found.

The human recombinant IL-10 (hIL-10) was obtained as follows. Total RNA from human PBMC stimulated in vitro with an anti-CD3 monoclonal antibody, OKT3, was isolated and reverse transcribed using Mo-MuLV reverse transcriptase. A polymerase chain reaction (PCR) was performed using specific oligonucleotides for the human IL-10 cDNA: sense primer 5'-AAGGCATGCACAGCTCAGCACTGCTC-3' (nucleotides 26-51) and anti-sense primer 5'-CCACCCTGATGTCTCAGTTTCGTATC-3' (nucleotides 555-580). A PCR product with the predicted size of 554 and the predicted restriction pattern was cloned in a retroviral vector and cotransfected in CHO-K1 cells with the plasmid pSV2-neo which contains the neomycin resistance gene. After 10 days in selective medium (400 µg/ml G418), individual resistant colonies were isolated, and their supernatants collected after 24 hours culture were tested for their ability to inhibit the OKT3-induced IFN-γ production by human PBMC.

Control (mock) supernatant from CHO-K1 cells transfected with the control plasmid and with pSV2-neo has been selected and collected similarly.

Bacterial LPS. LPS from E.coli 055.B5 was obtained from Sigma (St Louis, Mo).

Isolation of peripheral blood mononuclear cells (PBMC) :

PBMC from healthy volunteers were prepared from blood anticoagulated with citrate phosphate dextrose adenine (CPDA). After centrifugation of buffy coats on a Ficoll Hypaque gradient followed by washing with

Hank's balanced salt solution (HBSS) without calcium and magnesium, PBMC were cultured at $3*10^6$/ml in one ml culture medium consisting of RPMI 1640 supplemented with Hepes (20 mM), glutamine, and 10% fetal calf serum (FCS). Final PBMC preparations typically contained 22% to 30% monocytes, 70% to 78% lymphocytes, less than 1% neutrophils and less than 20.000 platelets/ml as estimated with a STK-S cell counter and cell identification using May Grünwald Giemsa staining. All reagents and the culture medium contained less than 10 pg/ml endotoxin as measured using a Limulus Amoebocytes Lysate assay.

Preparation of endothelial cells :

Human umbilical vein endothelial cells (HUVEC) were cultured using M199 medium supplemented with 20% pooled human serum, essential amino-acids, endothelial cell growth factor (ECGF - 40 µg/ml), heparin (100 µg/ml), penicillin and streptomycin. Cells from the second passage were transfered into 96 wells plates and used at confluence after 24 h in culture medium without ECGF and heparin.

Procoagulant activity (PCA) assays :

PCA on the surface of PBMC stimulated for 6 h with 1 µg/ml LPS was determined by a single stage clotting assay. The number of monocytes among the PBMC suspension was adjusted to $5*10^5$ monocytes/ml. This cell suspension was then incubated 1 min with 100 µl of 25 mM $CaCl_2$ before the initiation of clotting by the addition of 100 µl pooled normal citrated plasma. Clotting time was recorded with a KC10 apparatus and interpolated into U/ml of PCA by reference to a standard curve generated with serial dilutions of a

commercial thromboplastin. One unit corresponds to the amount of thromboplastin giving a normal clotting time of 12.4 sec. To determine the role of tissue factor/factor VII pathway in the PCA, normal human plasma was replaced in some experiments by a factor VII-deficient plasma or by normal plasma pre-incubated for 30 min with 12D10, a neutralizing mouse anti factor VII MoAb, or the cells were incubated with a neutralizing mouse IgG_1 anti-tissue factor MoAb (4507).

PCA induced at the HUVEC surface :

This was determined after 6 hours of incubation with 10 µg/ml LPS. Briefly, the generation of thrombin at the surface of HUVEC was measured after sequential addition of calcium (100 µl, 30 mM $CaCl_2$, 2 minutes at 37°C), normal human citrated plasma (45 seconds, 37°C) and a chromogenic substrat (S2238, 0.7 mM, 100 µl, 20 min at 37°C). The reaction was stopped with acetic-acid (50 µl) and the absorbance read at 405 mm. The amount of thrombin generated in this system was calculated using a standard curve obtained with purified thrombin and was expressed per $1*10^5$ HUVEC.

Flow cytometry analysis for tissue factor expression :

The expression of TF molecules at the surface of cultured PBMC was quantified by flow cytometry after staining with a fluoresceinated mouse IgG1 anti-tissue factor MoAb. After culture, cells were washed with PBS supplemented with 0.5% BSA, and $6*10^5$ cells were incubated for 1 h at 4°C with the anti-tissue factor MoAb or with an irrelevant IgG1 MoAb as control. After washing and fixation with 1% formalin, cells were

analysed with a FACScan flow cytometer. Monocytes were gated using side scatter and forward light scatter properties. In this gate more than 70% cells expressed the TF antigen and 95% of cells bearing the TF antigen coexpress the CD14 molecule. Flow cytometry standardisation microbeads coated with known amounts of fluorescein were used to establish a calibration curve allowing the determination of mean equivalents of soluble fluorescence (MESF).

Determination of tissue factor mRNA expression by reverse PCR.

RESULTS

IL-10 inhibits LPS-induced PCA on PBMC

Incubation of PBMC with 1 μg/ml LPS results in the appearance of high levels of PCA. Preliminary studies confirm that induction by LPS of PCA on PBMC peaked after 6 hours. So this period was chosen in further experiments. No PCA was generated when factor VII deficient plasma was used in the clotting assay. Futhermore PCA observed with normal plasma could be inhibited by more than 90% both by addition to the cells of a neutralizing anti tissue factor MoAb before the PCA assay and by the preincubation of the indicator plasma with an anti-factor VII MoAb indicating that increased PCA in this setup was due to tissue factor. We then preincubated PBMC overnight (18 h) with IL-10, before stimulation for 6 hours with LPS and measurement of PCA. This resulted in a dose-dependent decrease in PCA with maximal inhibition ranging from 75 to 90% in various experiments being achieved with 1.5 to 2.5 U/ml IL-10. This was not due to toxic effects of IL-10 supernatant as cell viability determined by trypan blue exclusion was more

than 90% after culture. Control supernatant from Mock transfected CHO cells has no significant effect on LPS induced PCA. Preincubation of PBMC with IL-10 alone did not or in some experiments mederatly diminished basal PCA.

IL-10 inhibits the LPS-induced tissue factor expression on monocytes :

PBMC incubated for 6 hours with LPS display large amounts of tissue factor on their surface. Almost all cells positive for TF staining were monocytes as indicated by size and forward scatter properties as well as by double staining experiments with an anti-CD14 MoAb. This staining was specific since no cells were stained with an isotype-matched fluoresceinated control MoAb. Overnight preincubation of PBMC with IL-10 before LPS addition resulted in a nearly complete prevention of the increased tissue factor expression on monocytes. The dose response of this phenomenon closely paralleled that of the PCA inhibition by IL-10.

Preincubation is required for IL-10 mediated inhibition of LPS-induced PCA and TF expression on PBMC :

In previous experiments, IL-10 was incubated for 18 hours with PBMC before the 6 hours challenge with LPS. Incubation with IL-10 for 6 to 24 h before LPS addition also leads to optimal prevention both of PCA and of TF expression on monocytes. The inhibitory effects of IL-10 steadily diminished when preincubation was reduced to 3 and 1.5 h. No or minimal inhibition (<20%) of PCA or TF expression was seen when IL-10 was added together with LPS.

IL-10 fails to inhibit LPS-induced PCA on endothelial cells :

As shown in table 1, incubation of HUVEC with LPS for 6 hours resulted in a large induction of both PCA as well as the ability to generate thrombin on their membranes. addition to HUVEC of IL-10, in dose ranging from 0.2 to20U/ml and with preincubation times from 0 to 18 h did not modify PCA or thrombin generation induced by LPS.

These results show that hIL-10 inhibits the LPS-induced monocytes procoagulant activity. This direct anticoagulant effect, associated with the hIL-10's ability to suppress inflammatory mediator release, suggests that exogenous administration of hIL-10 could provide a new therapeutic strategy for septic shock.

EXAMPLE 4 : INTERLEUKIN-10 MODULATES THE RELEASE OF CYTOKINES AND REDUCES THE SHOCK SYNDROME INDUCED BY ANTI-CD3 MONOCLONAL ANTIBODY IN MICE

IL-10 decreases the secretion of interferon-γ and TNF-α by activated TH1 cells and macrophages. The ability of IL-10 to inhibit cytokine release, as well as the toxicity induced by activating anti-CD3 monoclonal antibodies, was therefore investigated.

Recombinant human IL-10 (hIL-10) was applied to PBMC simulated in vitro with OKT3. Supernatants of unstimulated and of OKT3-stimulated PBMC contained 150 pg/ml and 8000 pg/ml of TNF-α (measured by IRMA) respectively. hIL-10 decreased TNF-α secretion in a dose-dependent manner, with the highest hIL-10 concentration resulting in a 85% inhibition of TNF secretion. hIL-10 was also able to supress by more than 90% the release of INF-γ induced by OKT3.

The effect was also investigated of recombinant murine IL-10 (mIL-10) on TNF-α release and hypothermia induced in BALB/c mice by in vivo injection of 10 μg of the anti-mouse CD3, 145-2C11 mAb. TNF-α serum levels rose from undetectable (<50 IU/ml) to (mean±sem) 795±150 IU/ml 90 min after injection of 145-2C11 mAb. Intraperitoneal injection of 1000 U of mIL-10 30 min before anti-CD3 challenge allowed for a significant reduction of serum TNF-α levels (at 90 min: 327±36 IU/ml, $p<0.05$ as compared to mice injected with 145-2C11 alone). In parallel, the (mean±sem) 5.8±1.5°C decrease in rectal temperature observed 8 hours after anti-CD3 injection was reduced to 1.2±0.4°C by mIL-10 pretreatment ($p<0.05$).

MATERIALS AND METHODS

Monoclonal antibodies and recombinant mouse IL-10 :

The hamster mAb 145-2C11 directed against the mouse CD3 complex was produced as ascites in nude mice. The neutralizing anti-mouse IL-10 mAb JES5-2A5 was a kind gift of Dr T. Mosmann (University of Alberta, Edmonton, Canada). Murine recombinant IL-10 (mIL-10) was obtained as culture supernatants from CHO-K1 cells stably transfected with the corresponding cDNA, as previously described. Supernatants collected from mock-transfected cells were used as control. The endotoxin levels of the mAb preparations and CHO-K1 cell supernatants were below 1 ng/ml as determined by a Limulus amoebocyte lysate assay.

Cloning and expression of recombinant human IL-10

As described in example 2.

Lymphokine assays :

Serum levels of TNF were measured according to the method of example 1. IFN-γ was quantitated by two-site ELISA using Db-1 and F1 rat anti-mouse IFN-γ mAbs. IL-10 serum levels were determined by ELISA using the JES5-2A5 and SXC1 mAbs purchased from Pharmingen. IL-6 serum levels were measured using the Il-6 dependent 7TD1 cell line and the hexosaminidase method. The lower limits of detection of TNF, IFN-γ, IL-10 and IL-6 were 2 U/ml, 5 U/ml, 2 U/ml and 5 pg/ml respectively.

Experimental protocol :

Mice were injected ip with 200 μl of either 1000 U mIL-10 or mock supernatant, followed 30 min later by iv injection of 10 μg of the 145-2C11 mAb. This dose of IL-10 was chosen because it was previously found optimal in a model of endotoxemia. Rectal temperature was monitored with a digital thermometer during the next 24 h. Blood samples were obtained by retrooribtal puncture at various times after the anti-CD3 mab injection for determination of serum cytokine levels. Blood glucose levels were measured at 4 h using a standard micromethod with Glucostix strips and Glucometer II M. In order to determine the effects of mIL-10 in a lethal model of anti-CD3 mAb toxicity, mice were sensitized by an ip injection of 10 mg of D-galactosamine given 90 min before the administration of 200 μg of the mAb. In some experiments, cyclosporine A (CsA) was injected ip (50 mg/kg for each dose) 18 and 3 h before anti-CD3 challenge, a protocol previously shown to inhibit the systemic release of cytokines.

Statistics :

Comparison between groups was made using Student's test except for lethality data which were analyzed by one tailed Fischer's exact test.

RESULTS

m-IL10 inhibits the systemic release of IFN-γ and TNF but not of IL-6 after injection of 145-2C11 mAb :

The iv injection of 10 μg of the 145-2C11 mAb into BALB/c mice induced a massive release in serum of IFN-γ and TNF, which peaked at 4 h and 90 min, respectively. Administration of 1000 U of mIL10 30 min before the injection of the 145-2C11 mAb almost completely abrogated the release of IFN-γ and significantly reduced the induction of TNF without affecting the kinetics of the responses. Contrasting with its inhibitory effect on IFN-γ and TNF, mIL10 pretreatment did not significantly affect the in vivo induction of IL-6 by 145-2C11 mAb. Supernatants from mock-transfected CHO-K1 cells given with the same timing as mIL10 did not modify the release of IFN-γ and TNF, demonstrating the specificity of the effect observed with the Il-10 preparation. Furthermore, the activity of this preparation on the in vitro production of IFN-γ by spleen cells stiulated with the 145-2C11 mAb was completely neutralized by the JES5-2A5 anti-IL-10 mAb.

hIL10 differentially regulates the release of IFN-γ and IL-10 induced by the 145-2C11 mAb :

As it has recently been observed that anti-CD3 mAb also induces the secretion of IL-10 in vivo, the effect of pretreatment with exogenous IL-10 on the

release of this cytokine was determined. In order to avoid the interference of the injected IL-10 on the measurement of endogenous IL-10, these experiments were performed with hIL-10 which is not detectable and does not interfer in the ELISA for mIL-10. First, we confirmed the data indicating that hIL-10 is bioactive in mouse systems as it significantly blocked 145-2C11 mAb-induced IFN-γ and TNF release in vivo. In the same experiments, hIL-10 pretreatment did not reduce the endogenous release of IL-10, as assessed by peak serum levels of IL-10 4 h after 145-2C11 mAb injection. The resistance of IL-10 to the inhibitory effects of Il-10 contrasts with the effect of CsA which blocked equally IFN-γ, TNF and Il-10 release.

mIL-10 reduces the acute toxicity of the 145-2C11 mAb :

As previously reported, hypothermia and hypoglycemia are sensitive parameters of the shock syndrome induced by the 145-2C11 mab. We found that IL-10 significantly reduced the drop in rectal temperature consecutive to 145-2C11 mAb injection. Control experiments showed that mice pretreated with mock supernatant were not protected against anti-CD3 induced hypothermia (mean±SEM at 6 h : 31.6 ± 1.8°C vs. 32.5 ± 0.8°C in mice injected with 145-2C11 mAb alone, P=NS). On the other hand, the hypoglycemia following 145-2C11 mAb injection was not modified by mIL-10 pretreatment.

The protective potential of IL-10 was further investigated in a lethal model obtained in mice sensitized with D-Gal, 30% of D-Gal-sensitized mice died after 145-2C11 mAb and pretreatment with mIl-10 reduced this lethality to 5%.

These results show that the cytokine release and the in vivo toxicity induced by anti-CD3 mAbs are reduced by IL-10.

EXAMPLE 5 : B7/CD28 - DEPENDENT IL-5 PRODUCTION BY HUMAN RESTING T-CELLS IS INHIBITED BY IL-10

This example demonstrates the effects of rIL-10 on IL-5 production by human resting T-cells isolated from peripheral blood. IL-10 was found to inhibit IL-5 production in a dose-dependent manner in T-cells activated with either anti-CD3 mAb cross linked on B7/CD32 - transfected mouse fibroblasts, or by p.m.A in conjunction with anti-CD28 mAb.

MATERIAL AND METHODS

Reagents :

Human IL-10 (rIL-10) was expressed in a baculoviral system using the IL-10 cDNA clone described previously (Pradier, O., C. Gérard, A. Delvaux, M. Lybin, D. Abramowicz, P. Capel, T. Velu and M. Goldman, 1993, Eur. J. Immunol., 23 : 2700). The IL-10 preparation was semi-purified by ionic separation chromatography followed by gel filtration. In some experiments, we verified that the activity of this preparation was similar to that of commercially available rIL-10.

Transfectant cell lines :

The mouse fibroblast cell lines 3T6 expressing human CD32 (FcγRII) either alone or in association with the B7/BB1 antigen (referred to as B7) were obtained and maintained in culture. Transfected cells

were incubated for 5 hours with 10 µg/ml mitomycin-C, washed three times and then used in coculture with T cells.

Isolation of resting T cells from peripheral blood :

Whole PBMC were isolated from buffy coats of healthy donors by density gradient centrifugation on Lymphoprep. After three washings in Hanks' balanced salt solution (HBSS), cells were resuspended in tissue culture medium RPMI 1640 supplemented with 2mM L-glutamine, gentamicin (20 µg/ml) and 10% FCS. T lymphocytes were purified using one cycle of Lymphokwik-T treatment. The T lymphocytes were further incubated with anti-CD56, -CD19, -DR, -CD14 and anti-CD11b mAb for 30 min at 4°C, washed and incubated with goat anti-mouse lgG-coated magnetic beads. After 1 h incubation at 4°C, uncoated cells were removed using a magnet. The resulting T cells preparations contained more than 98% CD3+ CD28+ DR- T cells without detectable CD14+ monocytes. In some experiments, CD4+ T lymphocytes were further purified by magnetic immunodepletion using anti-CD8 coated Dynabeads.

T cell stimulation :

Resting T cells were incubated at 37°C and 5% CO2 in flat-bottomed 96-well plates with either PLA (1 ng/ml) and A23187 (0.1 µg/ml), or PMA (1 ng/ml) and anti-CD28 mAb (1 µg/ml), or anti-CD3 mAb (100 ng/ml) cross-linked on either CD32 or B7/CD32 transfected fibroblasts (10^4 cells/well), or phorbol 12-myristate 13-acetate (PMA, 1 ng/ml) and B7/CD32 transfected fibroblasts (10^4 cells/well) in a total volume of 200 µl. T cells were seeded at 2.10^5/well in the

experiments using transfectant cells and at 1.10^5/well in the other conditions. After different times of incubation, culture supernatants were collected and stored at -20°C until assayed for cytokine determinations.

Determinations of cytokine levels in culture supernatants :

IL-5 levels were determined by sandwich ELISA using the following anti-human-IL-5 mAbs : H30 rat anti-human IL-5 mAb IgG2b as coating mAb and mAb7 mouse anti-human IL-5 mAb IgG1 as second mAb. In preliminary experiments, we verified that rIL-10 did not interfer with the immunoenzymatic detection of rIL-5. Commercially available kits were used for determination of IL-2 and IFN-γ. Lower limits of detection were 10 pg/ml for IL-5, 1 IU/ml for Il-2, and 10 U/ml for IFN-γ.

PCR analysis of IL-5 gene expression :

RNA from purified T cells was extracted using the guanidium thiocyanate method and analysed for IL-5 mRNA and hyposanthine phosphoribosyl transferase (hprt) mRNA by a reverse PCR method. Briefly, 1 μg of RNA was reversed-transcribed using Moloney murine leukemia virus (MoMuLV) reverse transcriptase (200 U/assay). The resulting cDNA was subjected to 32 PCR cycles. Each cycle was performed at 93°C for 1 min, 53°C for 2 min and 70°C for 3 min. Il-5 and HPRT primers were synthetized according to the human cDNA sequences. For IL-5, 5' sense primer was 5'-GCTTCTGCATTTGAGTTTGCTAGCT-3' and 3' antisense primer was 5'TGGCCGTCAATGTATTTCTTTATTAAG-3'. PCR

amplification using these primers resulted in a mRNA-specific 291 bp fragment (16).

RESULTS

Resting T cells costimulated by CD28/B7 signaling secrete high IL-5 levels :

Costimulation provided by the B7 molecule is known to dramatically enhance the responses of human resting T cells to anti-CD3 mAb (reviewed in Annu. Rev. Immunol., 1993, 11 : 191, Linsley P.S. and J.A. Ledbetter, the role of the CD28 receptor during T cell responses to antigen). The production of IL-5 by resting T cells stimulated by the anti-CD3 mAb in presence of B7/CD32 transfected fibroblasts (B7/CD32 transfectants), was evaluated, using transfected fibroblasts expressing CD32 alone (CD32 transfectants) as control. The CD32 molecule (FcγRII) allows binding of the mAb to transfectants which is required for efficient crosslinking of the TCR/CD3 complex. While IL-5 was undetectable in presence of cells transfected with CD32 alone, significant levels IL-5 were produced together with IL-2 when B7/CD32 transfectants were used for costimulation. Peak levels of IL-2 were detected at 24 h while maximal IL-5 levels were achieved at 48 h. To determine whether TCR/CD3 engagement is required for IL-5 induction, T cells were stimulated with PMA together with B7/CD32 transfectants or anti-CD28 mAb. IL-5 was produced in both systems while PMA alone was inefficient to induce significant cytokine secretion. Interestingly, IL-5 production in response to PMA and calcium ionophore A23187 was always much lower than in response to PMA and B7/CD28 signaling. In these systems too, maximal IL-5 levels were higher at 48 h than at 24 h while

IL-2 was maximal at 24 h. IL-5 production by resting T cells stimulated with anti-CD3 mAb or PMA and B7/CD28 signaling was observed in 7 independent experiments with 7 different donors. As expected, CD4+ cells were the major source of IL-5 as depletion of CD8+ cells did not influence IL-5 production.

rIL-10 inhibits IL-5 production by human resting T cells costimulated by B7/CD28 signaling :

The effects of rIL-10 on IL-5 production were first analyzed in the activation system based on anti-CD3 mAb in conjunction with B7/CD32 transfectants. As shown in figure 4, rIL-10 inhibited in a dose-dependent manner the production of IL-5 in this setting. In parallel, we confirmed previous data indicating that rIL-10 inhibits IL-2 but no IFN-γ production by purified T cells. In control experiments, rIL-10 modified neither B7 expression by transfected fibroblasts nor CD28 expression by purified T cells. As IL-2 has been shown in other systems to be involved in the induction of IL-5, it was verified that the effect of rIL-10 on Il-5 was not due to IL-2 inhibition. Indeed, it was found that the addition of up to 1000 U/ml exogenous rIL-2 did not modify the inhibition of IL-5 secretion by rIL-10. Moreover, we observed that cyclosporin A (CsA) which inhibited IL-2 secretion more efficiently than rIL-10, did not reduce and even significantly enhanced IL-5 secretion.

The influence of rIL-10 on the secretion of cytokines by T celles stimulated by PMA and anti-CD28 mAb was also evaluated. As illustrated in figure 5, rIL-10 inhibited in a dose-dependent manner IL-5 production under this condition. IL-2 was also

inhibited but less efficiently than IL-5 while IFN-γ secretion was not affected by rIL-10 (figure 5). Together with the date presented above, these results indicate that the effects of rIL-10 on IL-5 secretion are virtually identical whether resting T cells are activated by TCR/CD3 engagement or PmA provided that they are costimulated by B7/CD28 signaling.

rIL-10 reduces Il-5 mRNA accumulation in T cells activated by PMA and anti-CD28 mAb :

To determine whether IL-10 interfered with IL-5 gene expression, resting T cells were stimulated for 24 h with PMA and anti-CD28 mAb in absence of presence of rIL-10 (50 U/ml) before IL-5 mRNA analysis by reverse PCR. rIL-10 clearly inhibited IL-5 mRNA accumulation whereas it had no detectable effect on the expression of HPRT housekeeping gene.

rIL-10 does not inhibit IL-5 production in response to PMA and A23187 calcium ionophore :

In these experiments, the effects of IL-10 on IL-5 production by T cells stimulated with either PMA and anti-CD28 mAb or PMA and A232187 calcium ionophore were compared. As T cells from healthy individuals produced relatively low levels of IL-5 in this latter setting, cells from a patient with Omenn's syndrome (severe combined immunodeficiency with hypereosinophilia) which secreted high IL-5 levels under both conditions of stimulation were also included in this analysis. Indeed, the profile of cytokines secreted by the CD4+ cells of this patient corresponds to a TH2-like profile. Both in healthy individuals and in the patient with Omenn's syndrome, rIL-10 did not reduce and even enhanced IL-5

production induced by PMA + A23187. In contrast, rIL-10 dramatically reduced the levels of IL-5 produced in response to PMA + anti-CD28 mAb. The degree of inhibition was similar (around 70%) in healthy individuals and in the patient with Omenn's syndrome.

Endogenous IL-10 down-regulates IL-5 production by resting T cells :

As peripheral blood T cells are known to secrete IL-10 upon activation, the influence of endogenously produced IL-10 on IL-5 secretion was investigated. Because the mAb used to neutralize IL-10 mAb binds CD32 and therefore interferes with cross-linking of anti-CD3 mAb on B7/CD32 transfectants, this study was conducted on T cells activated by PMA and anti-CD28 mAb. In the 3 experiments performed, addition of anti-IL-10 mAb resulted in 114%, 48% and 64% increase in IL-5 production while the isotype-matched control mAb had no effect, indicating that IL-5 production is regulated by endogenous IL-10 in this system.

EXAMPLE 6 : IL-10 INHIBITS B7 AND ICAM-1 - EXPRESSION ON HUMAN MONOCYTES

This example demonstrates modulation by IL-10 of the expression of the accessory molecules B7 and ICAM-1 on monocytes. This property may contribute to the immunosuppressive properties of IL-10.

MATERIALS AND METHODS

Recombinant cytokines :

Recombinant IL-10 was obtained from supernatant of CHO (Chinese Hamster Ovary-K1) cells transfected with an expression vector containing the human IL-10 cDNA. Control (mock) supernatant was collected from CHO-k1 cells transfected with a similar vector without inserted IL-10 gene.

Cell preparation and culture :

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of heparinized blood from healthy donors on Lymphoprep. PBMC were cultured for 24 h at $1x10^6$/ml in RPMI 1640 medium supplemented with 5% foetal calf serum in absence or presence of 250 U/ml IFN-γ. Serial dilutions of IL-10 or mock supernatant were added to the cells or 1 hour before IFN-γ stimulation.

Flow cytometry analysis :

Cells were washed in PBS supplemented with 0.5% BSA, 10 mM NaN_3 and incubated for 30 min at 4°C with 10 μg/ml of the primary murine monoclonal antibody (mAb). Primary mAbs were F(ab)'2 fragments of the B7-24 anti-B7 mAb (18), anti-ICAM-1 mAb, anti-HLA-DR mAb or irrelevant mouse IgG mAb. The binding of these antibodies was revealed by incubation for 30 min at 4°C with FITC-coupled F(ab)'2 fragments of a rabbit antimouse immunoglobulin antiserum. After washing, cells vere again incubated with irrelevant murine IgG for 10 min at 4°C before adding phycoerythrine(PE)-conjugated anti-CD14 mAb for 30 min at 4°C. B7, ICAM-1 and HLA-DR expression was measured at the surface of CD14-positive cells using a FACScan flow cytometer. Neither IFN-γ nor IL-10 interfered with the gating of cells. Flow cytometry standardization microbeads

coated with a defined amount of fluorescein were used to establish that the mean fluorescence channels related linearly to the number of fluorescein molecules bounds per cell. This allowed the transformation of the mean fluorescence channels measured into mean equivalent of soluble fluorescence (MESF). Antigen specific mean fluorescence intensity (MESF) were calculated by substraction of non-specific fluorescence.

RESULTS

IL-10 inhibits ICAM-1 expression on monocytes :

Data indicating that IL-10 decreased the expression of MHC class II molecules (HLA-DR) on monocytes both at the basal state and after IFN-γ stimulation was first confirmed. The influence of IL-10 on ICAM-1 expression on those cells was then examined. The FACS histogram showed that IL-10 reduced the expression of ICAM-1 on resting monocytes. In the 3 experiments performed on 3 different donors, the percentage of inhibition of basal ICAM-1 expression ranged between 50 and 65%. The lack of influence of mock supernatant demonstrated the specificity of the IL-10 preparation. IFN-γ stimulation led to a clear upregulation of ICAM-1 expression on monocytes as compared to medium alone while it did not change the isotype control staining. Addition of IL-10 (3 U/ml) inhibited IFN-γ-induced ICAM-1 upregulation at the surface of monocytes. The inhibitory effect of the IL-10 preparation was dose-dependant and specific, since no inhibition was observed with the mock supernatant.

IL-10 inhibits B7 upregulation on IFN-γ stimulated monocytes :

Incubation of PBMC for 24 h with IFN-γ (250 U/ml) induced a clear upregulation of B7 expression at the surface of monocytes. The addition of IL-10 (3 U/ml) to IFN-γ markedly decreased B7 upregulation on monocytes. Neither IFN-γ nor IFN-γ + IL-10 modified the control staining observed with the FITC-conjugated anti-mouse immunoglobulin antiserum in the absence of the F (ab)'2 anti-B7. MESF values of B7 staining in 3 representative experiments on 3 different donors show that the inhibitory effect of IL-10 was dose-dependent and specific. In these 3 experiments, the percentage of inhibition of B7 expression by IL-10 ranged from 60 to 97%.

These results show that IL-10 inhibits ICAM-1 and B7 expression at the surface of monocytes. The in-vivo relevance of these observations is demonstrated in experimental models where allograft rejection was prevented by anti-ICAM-1 and anti-LFA-1 mAb, or by CTLA-4-Ig which block the B7/CD28 - CTLA-4 pathway of T-cell activation (Isobe et al., Science, 1992, 2545 : 1125 ; Lenschow et al., Science, 1992, 357 : 759).

EXAMPLE 7 : SYSTEMIC ADMINISTRATION OF rIL-10 INHIBITS EDEMA FORMATION AND IFN-γ SYNTHESIS DURING ALLOREACTIVE RESPONSE IN VIVO

In addition to inhibition of MHC expression, IL-10 down-regulates the expression of B7 molecules and of ICAM-1 on macrophages or monocytes. These properties of IL-10 suggest that it might inhibit the induction as well as the effector phase of acute cellular transplant rejection and possibly promote

allograft tolerance. As a first approach to this question, we analyzed the effects of systemic administration of rIL-10 in an in vivo model of alloreactivity induced by localized injection of allogeneic cells.

The results obtained indicate that the major effect of rIL-10 is to prevent edema formation and IFN-γ synthesis induced by alloantigenic challenge.

MATERIAL AND METHODS

Mice :

10 to 12 week-old BALB/c (H-2^d), A/J (H-2^k) and, C57/BL6 (H-2^b) were obtained from Harlan CPB (Zeist, the Netherlands), the Centre National de la Recherche Scientifique (Orléans, France) and Iffa Credo (l'Arbresle, France), respectively.

Preparation of mouse rIL-10 :

Mouse rIL-10 was cloned as previously described (Example 1) and produced in Sf9 insect cells using the baculoviral expression vector pBlueBac2 from Invitrogen Corp. (San Diego, CA). rIL-10 was affinity purified using the JES5-2A5 anti-IL-10 monoclonal antibody obtained from Pharmingen. For in vivo administration, rIL-10 was diluted in saline buffer containing 0.1% mouse serum (rIL-10 vehicle) to obtain a final concentration of 10.000 U/ml as determined by ELISA.

Lymph node enlargement assay and experimental protocol :

The right rear footpads of BALB/c mice were injected on day 1 with a suspension of $2.5x10^6$

syngeneic (BALB/c) or semi-allogeneic (A/JxBALB/c) F1 spleen cells in 0.1 ml of RPMI 1640. From day 0 to day 5, mice received every 8 h an intraperitoneal injection of either 1000 U rIL-10 or control vehicle. This dose of IL-10 was chosen because it was found effective in inhibiting cytokine release in two different in vivo models, namely endotoxin shock and polyclonal T cell activation induced by anti-CD3 monoclonal antibody. On day 5, left and right lymph nodes were surgically removed from the popliteal fossa and weighed. Results were expressed as delta values (in mg) representing the difference in weight between the right (injected side) and left (uninjected side) lymph nodes.

Enumeration of lymph node cells and histological analysis :

Cell suspensions of each lymph node were counted and lymph node cells from each group were pooled, stained with phycoerythrin-conjugated anti-CD4 (L3T4) or FITC-conjugated anti-CD8 monoclonal antibody (Lyt2) obtained from Becton Dickinson and percentages of CD4+ and CD8+ cells were determined by flow cytometry.

Analysis of IFN-γ mRNA expression by reverse PCR :

mRNAs from popliteal lymph node cells were extracted using the Micro-Fast Track™ mRNA isolation kit from Invitrogen Corp. 1 μg of each RNA sample was reverse transcribed into cDNA using oligo dT primers. PCR amplification was then performed using primers specific of IFN-γ or hypoxanthine-phosphorybosyl transferase (HPRT). Amplified products were separated

on 2% agarose gels and visualized by ethidium bromide staining.

RESULTS

rIL-10 inhibits draining lymph node swelling after subcutaneous injection of allogeneic cells :

As enlargement of draining lymph node is a good indicator of the alloreactive response induced by subcutaneous injection of allogeneic cells, we first evaluated the effects of rIL-10 administration on this parameter. As shown in figure 6, the injection of allogeneic cells in the right footpad resulted in a significant enlargement of the draining popliteal lymph node as measured by the difference in weight with the left popliteal lymph node (mean ± SD of delta values : 5.0 ± 1.8 mg). Administration of rIL-10 significantly reduced lymph node enlargement (mean ± SD of delta values : 2.8 ± 1.7 mg, $p < 0.001$ as compared with mice injected with allogeneic cells alone). This effect of rIL-10 could not be attributed to inhibition of cell recruitment as the increased cell numbers in draining lymph nodes from mice injected with allogeneic cells were not significantly reduced by rIL-10 administration (table 2). Moreover, the mean percentages of CD4+ and CD8+ cells in draining lymph nodes were similar whether or not mice received rIL-10 together with allogeneic cells (35% vs. 43% and 11% vs. 14% for CD4+ and CD8+ cells, respectively). These date suggest that the inhibition of lymph node enlargement by rIL-10 administration was mainly due to decreased edema formation. This was confirmed by histological analysis of draining lymph nodes (table 3).

TABLE 2: Cellular recruitment in draining lymph node after subcutaneous injection of allogeneic cells

Cells injected[a]	Treatment[a]	N	Number of cells/node[b]
BALB/c	none	6	$2.1 \pm 0.5 \times 10^6$
A/J	vehicle	15	$9.7 \pm 0.8 \times 10^6$
A/J	rIL-10	15	$7.8 \pm 0.8 \times 10^{6*}$

[a] Same protocol as described in legend of fig 6

[b] Data are given as mean±SEM.

(*) P<0.001 as compared with mice injected with syngeneic BALB/c cells but not significantly different from mice injected with A/J allogeneic cells and control vehicle.

TABLE 3 - rIL-10 treatment does not influence priming of alloreactive cells

Cells injected[a]	Treatment[a]	Anti-A/J activity in MLC[b] Proliferation (cpm)	IFN-γ production (U/ml)
Balb/c	None	3224±241 (590±77)	4 (<2)
A/J	Vehicle	42644±360 (2354±426)	68.3±9.7 (6.1±1.3)
A/J	IL-10	39674±1665 (1795±200)	74.3±12 (11.8±4.6)

[a] Same protocol as described in legend of figure 6

[b] On day 5 of the in vivo protocol, $5x10^5$ lymph node cells were rechallenged in vitro with the same number of irradiated A/J allogeneic spleen cells or syngeneic BALB/c spleen cells (values under brackets). Proliferative responses and IFN-γ production were measured as described in material and methods. Data are mean±SEM of 10 mice per group except for IFN-γ determinations in mice injected with syngeneic BALB/c cells (values from a pool of 5 mice).

rIL-10 administration prevents IFN-γ mRNA accumulation :

As IFN-γ production is a crucial component of the alloreactive response, we determined the effects of rIL-10 on the induction of IFN-γ mRNA in draining lymph nodes of mice injected with allogeneic cells. Date of reverse PCR experiments indicate that rIL-10 administration dramatically reduced IFN-γ mRNA accumulation in these mice without modifying the expression of β-actin mRNA, a housekeeping gene.

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CLAIMS

1. Use of Interleukin-10 (IL-10) or analogs or agonists thereof, for the manufacture of a medicament for the treatment or prevention of diseases involving :

i) Interleukin-5 production by T-lymphocytes, or ;

ii) activation of T-lymphocytes through the B7/CD28 or ICAM-1/LFA-1 pathway, or ;

iii) activation of the procoagulant activity of monocytes ;

iv) severe infectious diseases involving Tumour Necrosis Factor (TNF) production.

2. Use of IL-10 according to claim 1 for the manufacture of a medicament for the treatment or prevention of diseases involving IL-5 overproduction by T-lymphocytes, such as diseases associated with eosinophilia.

3. Use according to claim 2, for the manufacture of a medicament for the treatment or prevention of a disease selected from the group consisting of : atopic diseases including allergic rhinitis and bronchial asthma, atopic dermatitis and other cutaneous diseases associated with eosinophil infiltration (including chronic eczema, erythema multiforme, dermatitis herpetiformis, mastocytosis, bullous disorders), Crohn's disease, broncho-pulmonary aspergillosis, spanish toxic oil syndrome, myalgia-eosinophilia syndrome induced by L-tryptophane preparations, certain drug allergic reactions, tropical eosinophilia, helminthic diseases, Schulman syndrome (eosinophilic fasciitis), Churg-Strauss syndrome and other vasculitides with eosinophilia, Loeffler

syndrome, chronic eosinophilic pneumonia, eosinophilic gastroenteritis, and the hypereosinophilic syndrome.

4. Use according to any one of claims 2 to 3, in which the IL-10 is the sole active principal in the medicament.

5. Use according to any one of claims 2 to 3, in which the IL-10 is associated, in the medicament, with interferon-α (IFN-α) and/or with interferon-γ (IFN-γ).

6. Use of IL-10 according to claim 1, for the manufacture of a medicament for the treatment or prevention of diseases involving activation of T-lymphocytes through the B7/CD28 or ICAM-1/LFA-1 pathway, such as diseases associated with alloreactive responses.

7. Use according to claim 6 for the manufacture of a medicament for the treatment or prevention of a disease selected from the group consisting of : allograft rejection and other host versus graft diseases, and Graft versus Host disease.

8. Use according to claim 6 or 7, in which the IL-10 is the sole active principal in the medicament.

9. Use according to claim 6 or 7, in which the IL-10 is associated, in the medicament with cyclosporin A, or other related immunosuppressive agents such as FK 506, rapamycin, or anti-CD3 monoclonal antibody.

10. Use of IL-10 according to claim 1, for the manufacture of a medicament for the prevention or treatment of diseases involving activation of the procoagulant activity of monocytes by induction of surface tissue factor expression.

11. Use according to claim 10, for the manufacture of a medicament for the prevention or treatment of a disease selected from the group consisting of : septic shock, meningococcemia,

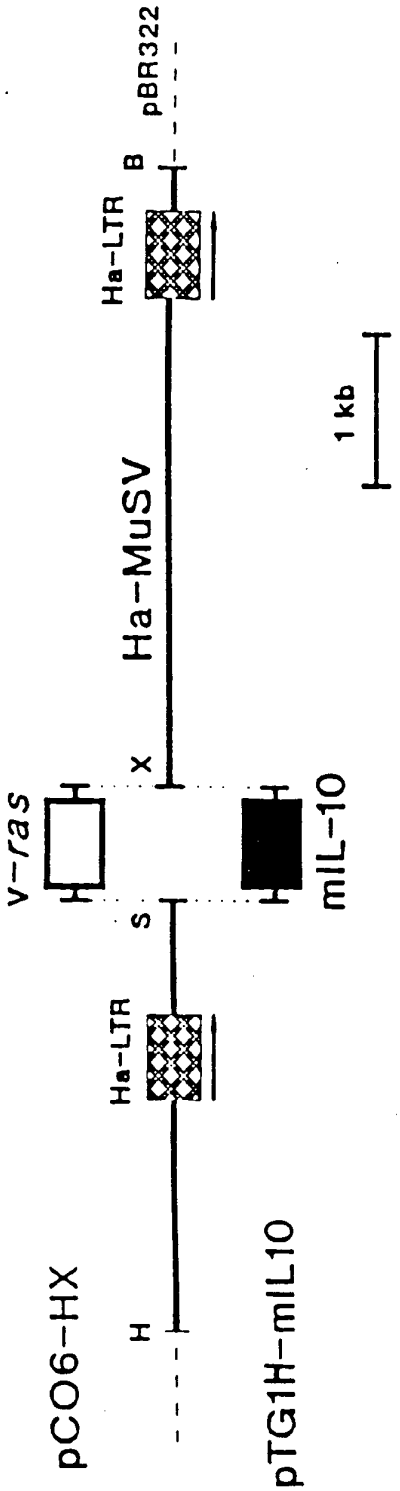
promonocytic leukaemia, reactions induced by anti-CD3 antibodies, cerebral malaria, anti-phospholipid syndrome, hemolytic uremia syndrome, disseminated intravascular coagulation and high-risk surgery.

12. Use of IL-10 according to claim 1 for the manufacture of a medicament for the prevention of severe infectious diseases involving TNF production.

13. Use according to claim 12, for the manufacture of a medicament for the prevention of diseases selected from the group consisting of septic shock induced by gram-negative bacteria and cerebral malaria.

14. Use of IL-10 according to claim 1 in which the IL-10 is recombinant human IL-10.

15. Use according to claim 14 or claim 1 in which the IL-10 is viral IL-10, an IL-10 analog, and IL-10 agonist, an IL-10 peptide or a chimeric protein derived from both cellular and viral IL-10.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B
Ha-LTR
Ha-MuSV
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X
v-ras
mIL-10
S
Ha-LTR
H
pCO6-HX
pTG1H-mIL10

FIGURE 1

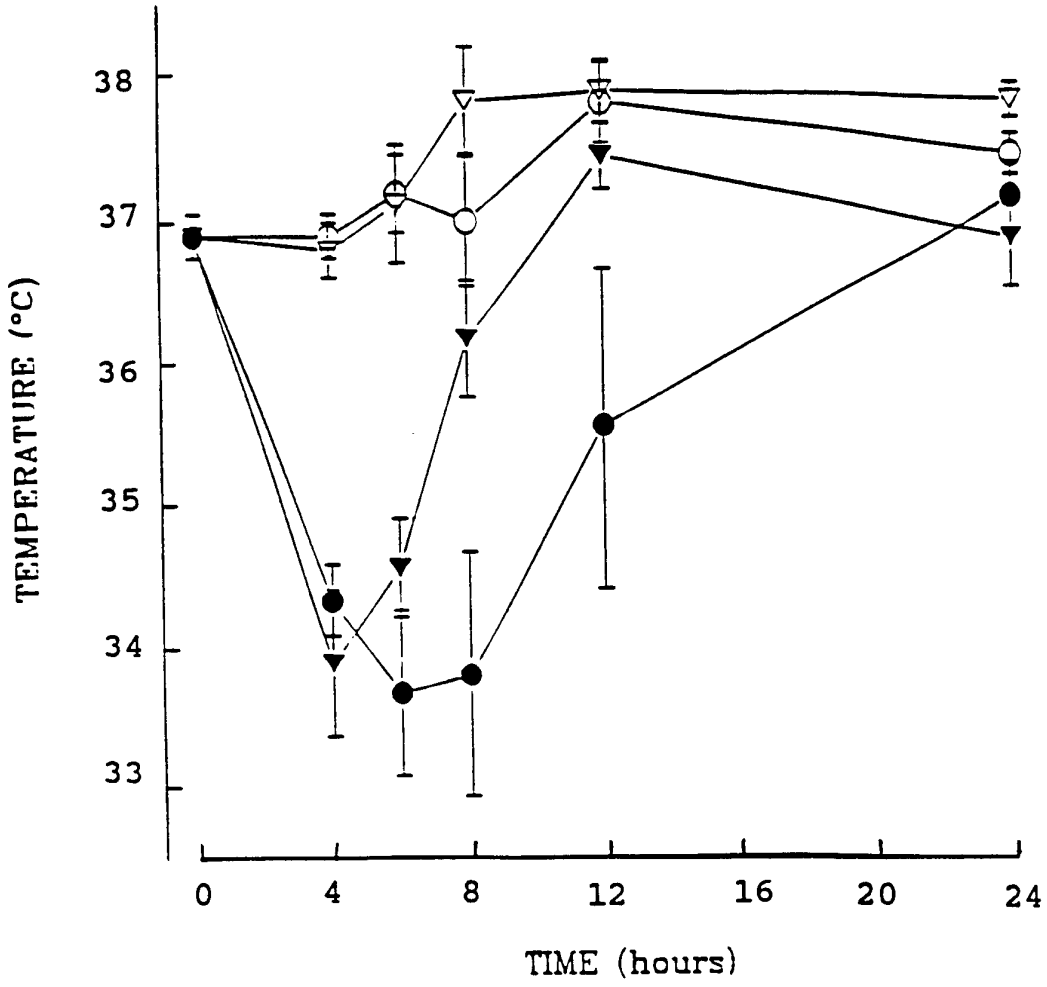


FIGURE 2

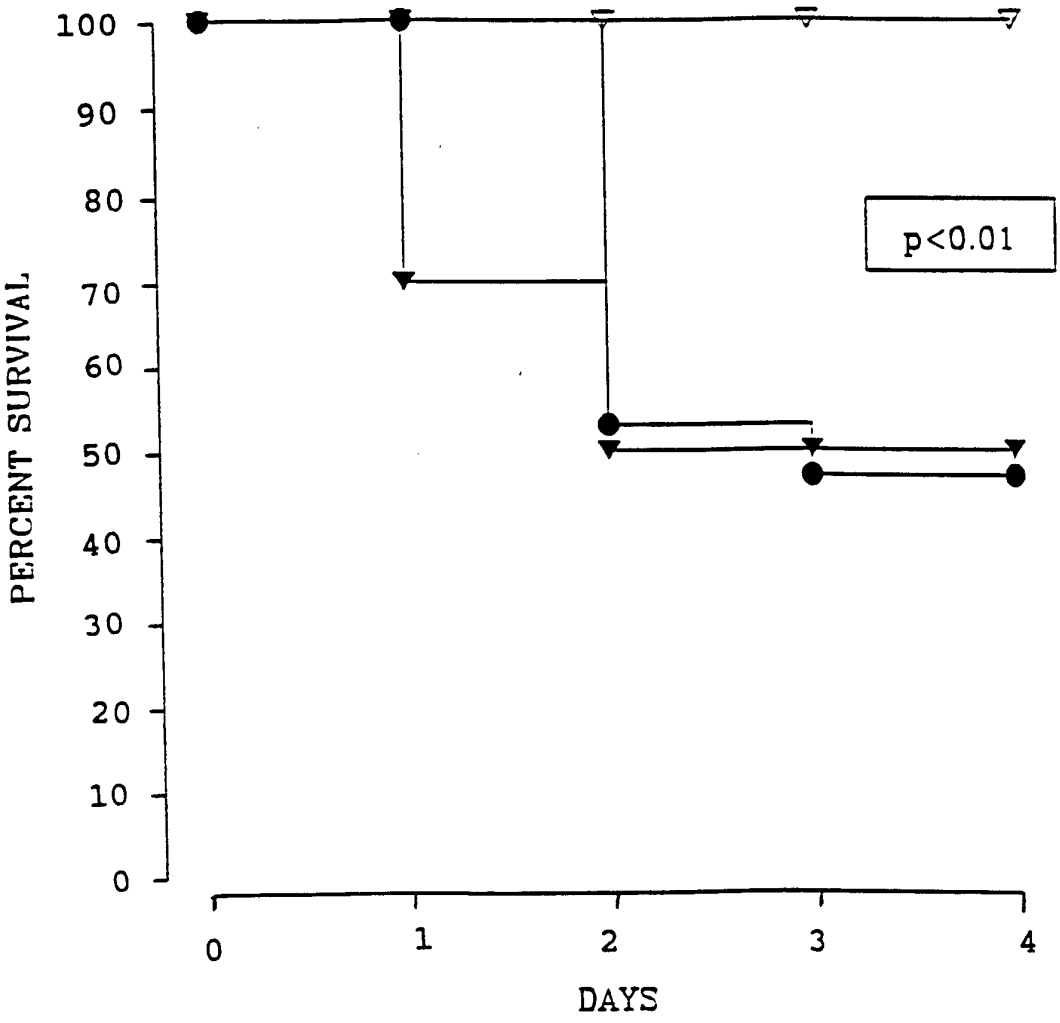

100
90
80
70
60
50
40
30
20
10
0
PERCENT SURVIVAL
p<0.01
0
1
2
3
4
DAYS

FIGURE 3

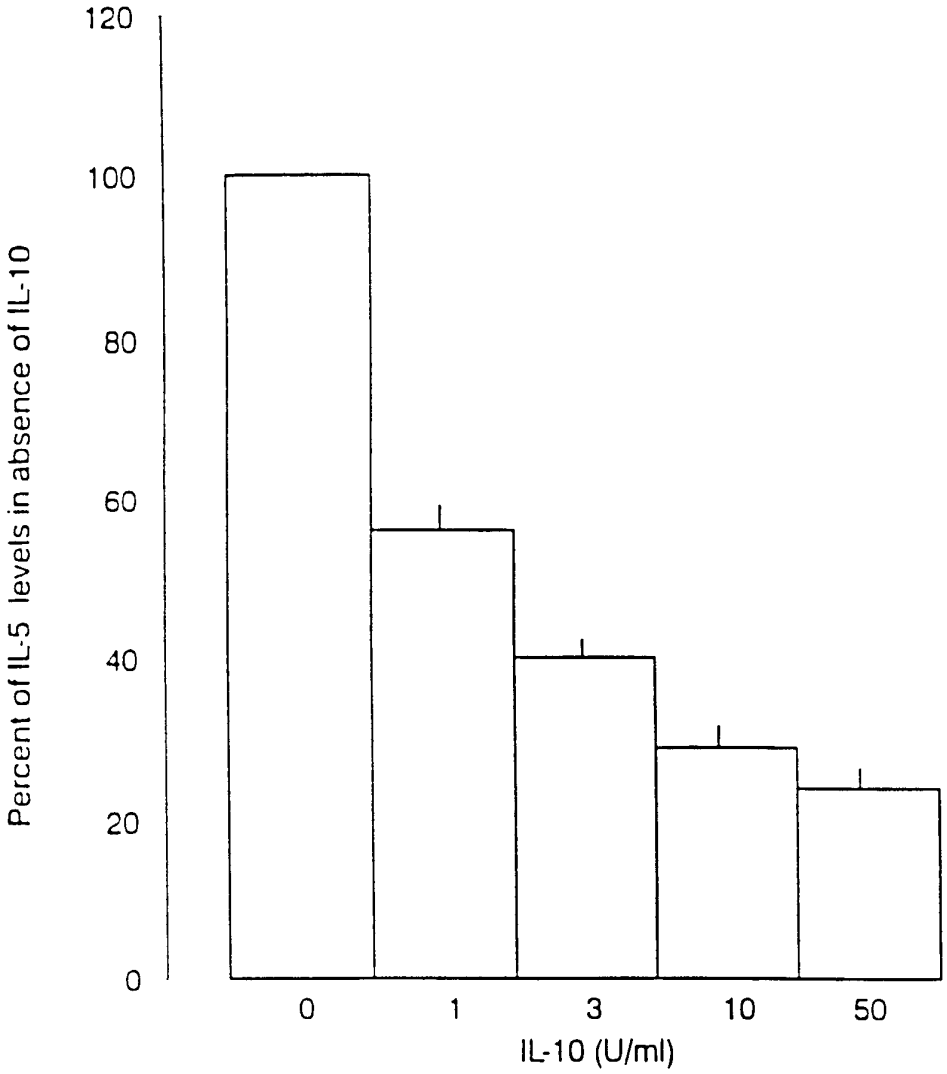
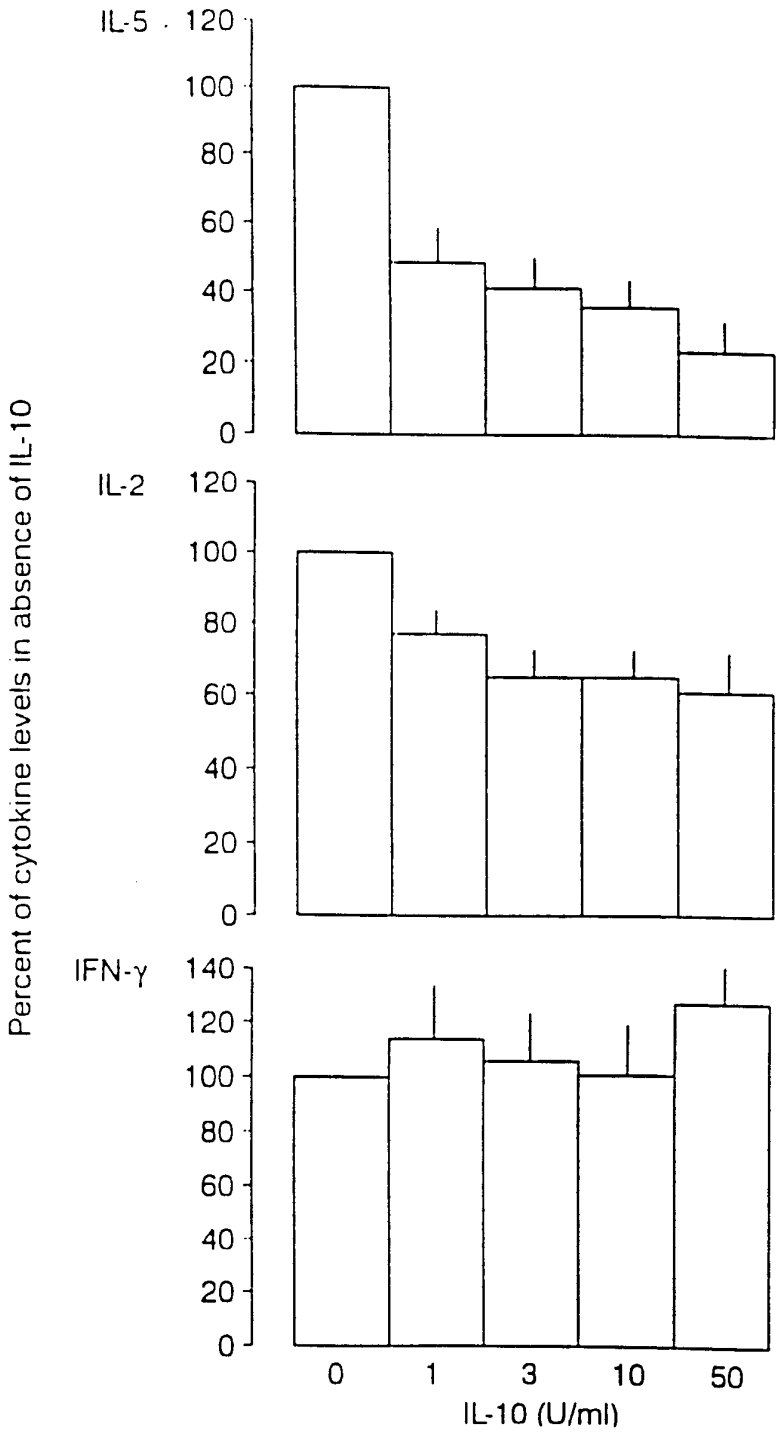

120
100
80
60
40
20
0
Percent of IL-5 levels in absence of IL-10
0
1
3
10
50
IL-10 (U/ml)

FIGURE 4


IL-5
IL-2
IFN-γ
120
100
80
60
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0
140
Percent of cytokine levels in absence of IL-10
0
1
3
10
50
IL-10 (U/ml)

FIGURE 5

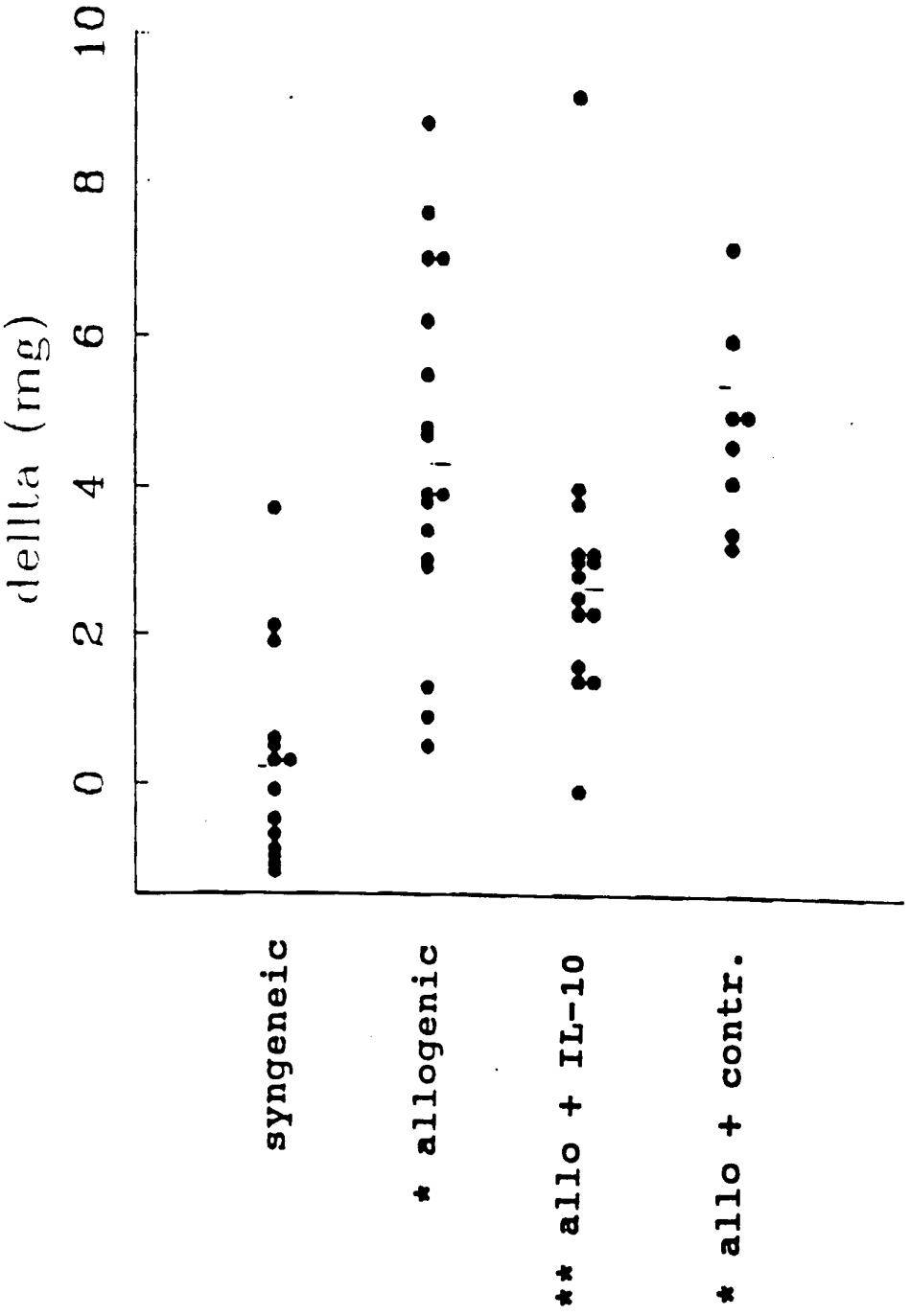

FIGURE 6