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(54) Titre : ANALOGUES DE NUCLEOSIDE A BASE BICYCLIQUE MODIFIEE PAR CARBOXAMIDINE
(54) Title: NUCLEOSIDE ANALOGS WITH CARBOXAMIDINE-MODIFIED BICYCLIC BASE

(57) Abrégé/Abstract:
Novel nucleoside analog compounds are disclosed. The novel compounds or pharmaceutically acceptable esters or salts thereof may be used in pharmaceutical compositions, and such compositions may be used to treat an infection, an infestation, a neoplasm, or an autoimmune disease. The novel compounds may also be used to modulate aspects of the immune system, including modulation of Type 1 and Type 2 activity.

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(54) Title: NUCLEOSIDE ANALOGS WITH CARBOXAMIDINE-MODIFIED BICYCLIC BASE

(57) Abstract: Novel nucleoside analog compounds are disclosed. The novel compounds or pharmaceutically acceptable esters or salts thereof may be used in pharmaceutical compositions, and such compositions may be used to treat an infection, an infestation, a neoplasm, or an autoimmune disease. The novel compounds may also be used to modulate aspects of the immune system, including modulation of Type 1 and Type 2 activity.

NUCLEOSIDE ANALOGS WITH CARBOXAMIDINE-MODIFIED BICYCLIC BASE

This application claims priority to U.S. provisional application number 60/182676 filed February 15, 2000, and US utility application serial no. 09/595365 filed June 16, 2000, each of which are incorporated herein by reference in their entirety.

Field of the Invention

The present invention relates to the field of nucleoside analogs and methods of use.

Background of the Invention

Mammalian immune systems contain two major classes of lymphocytes: B lymphocytes (B cells), which originate in the bone marrow; and T lymphocytes (T cells) that originate in the thymus. B cells are largely responsible for humoral immunity (i.e., antibody production), while T cells are largely responsible for cell-mediated immunity.

T cells are generally considered to fall into two subclasses, helper T cells and cytotoxic T cells. Helper T cells activate other lymphocytes, including B cells and cytotoxic T cells, and macrophages, by releasing soluble protein mediators called cytokines that are involved in cell-mediated immunity. As used herein, lymphokines are a subset of cytokines.

Helper T cells are also generally considered to fall into two subclasses, Type 1 and Type 2. Type 1 cells produce interleukin 2 (IL-2), tumor necrosis factor (TNFα) and interferon gamma (IFNγ), and are responsible primarily for cell-mediated immunity such as delayed type hypersensitivity and antiviral immunity. In contrast, Type 2 cells produce interleukins, IL4, IL-5, IL-6, IL-9, IL-10 and IL-13, and are primarily involved in assisting humoral immune responses such as those seen in response to allergens, e.g. IgE and lgG4 antibody isotype switching (Mosmann, 1989, *Annu Rev Immunol,* 7:145-173).

As used herein, the terms Type 1 and Type 2 "responses" are meant to include the entire range of effects resulting from induction of Type 1 and Type 2 lymphocytes, respectively. Among other things, such responses include variation in production of the corresponding cytokines through transcription, translation, secretion and possibly other mechanisms, increased proliferation of the corresponding lymphocytes, and other effects associated with increased production of cytokines, including motility effects.

Previous applications (09/462714, 09/291097, 09/291093, 09/471513, 60/164365, 60/164366, 60/172097, 60/175111), each of which is incorporated herein by reference, relate to aspects of our recent discoveries involving the effect of various nucleosides (which are defined herein to include derivatives and analogs of native nucleosides) on selectively modulating lymphocyte responses relative to each other. Among other things, we have shown that either of Type 1 and Type 2 responses can be selectively suppressed while the other is either induced or left relatively unaffected, and either of Type 1 or Type 2 responses can be selectively induced while the other is either suppressed or left relatively unaffected. We have also discovered the surprising fact that some nucleosides effective in selectively modulating Type 1 and Type 2 responses relative to one another tend to have a bimodal effect. Among other things, some nucleosides that tend to generally suppress or induce both Type 1 and Type 2 activity at a relatively higher dose tend to selectively modulate Type 1 and Type 2 relative to each other at relatively lower doses.

The effect of other nucleoside analog compounds on selectively modulating lymphocyte responses relative to each other has not been previously studied or documented. We have discovered that the bimodal effect, or selective modulation of Type 1 and Type 2 responses relative to one another, also occurs after administration of other nucleoside analog compounds, such as pro-drug forms of the compounds.

There are many barriers to overcome in developing biologically active compounds into clinically useful agents. Many potent biologically active compounds never become clinically useful agents because of their undesirable biopharmaceutical properties which include low bioavailability due to low permeability through biological barriers, such as the blood brain barrier (BBB) and the intestinal barrier. Although many factors affect the bioavailability of a drug, the undesirable physicochemical properties (e.g., charge, lipophilicity, hydrogen bonding potential, size) of many drugs is probably one of the most commonly encountered factors that hinder the permeation of drugs through biological barriers. Therefore, optimization of the physicochemical characteristics (charge, lipophilicity, hydrogen bonding potential, size) of a drug is probably the most likely general strategy to facilitate the transport of drugs through such membrane barriers.

To optimize the physicochemical properties of drugs, one possible strategy is that of prodrugs. (H. Bundgaard, *Design of Prodrugs*, Elsevier, Amsterdam, **1985**; N. Bodor, L.

Prokai, W. M. Wu, H. Farag, S. Jonalagadda, M. Kawamura, J. Simpkins, *Science*, 257, 1698-1700, **1992**; H. E. Taylor, K. B. Sloan, *J. Pharm. Sci*, 87, 5-20, **1998**). The term prodrug is used to describe an agent, which must undergo chemical or enzymatic transformation to the active or parent drug after administration, so that the metabolic product or parent drug can subsequently exhibit the desired pharmacological response. By derivatizing certain polar functional groups in small organic molecules transiently and bioreversibly, the undesirable physicochemical characteristics (e.g., charge, hydrogen bonding potential) of these groups have been "masked" without permanently altering the pharmacological properties of the molecules. This strategy has been very successfully used in cases where the prodrug derivatization involves converting a carboxyl or a hydroxyl functional group into an ester, which can be readily hydrolyzed in vivo either chemically, or enzymatically. The promising prodrug concept, we anticipate that the introduction of other moieties in the parent drug would increase the bioavailabilty, adsorption and antiviral effects.

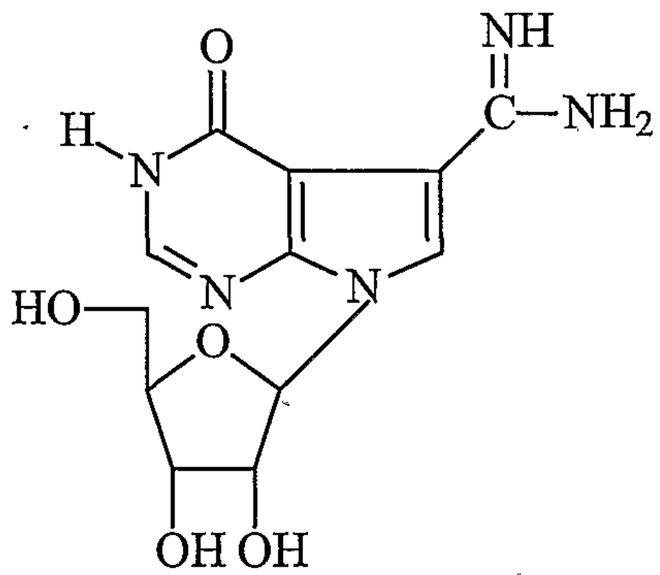
These discoveries are especially significant because modern treatment strategies for many of the above-listed diseases have limited effectiveness, significant side effects, or both. Treatment of autoimmune disease, for example, is frequently limited to palliative measures, removal of toxic antibodies (as in myasthenia gravis), and administration of hazardous drugs including corticosteroids, chloroquine derivatives, and antimetabolic or antitumor drugs, and drugs such as cyclosporines that target immune system cells.

Although numerous immunomodulatory compounds are known in the art, all or all of them suffer from one or more disadvantages. Therefore, there is still a need to provide improved methods and compositions for immunomodulatory compounds.

Summary of the Invention

The present invention is directed to novel nucleoside analog compounds and related compounds, such as prodrugs, their therapeutic uses and synthesis.

In one aspect of the invention, the nucleoside analog compounds have a general structure according to Formula 1, wherein the chemical configuration may be as the L-configuration or the D-configuration.:



Formula 1
ICN - 10776

In yet another aspect of the invention, a pharmaceutical composition comprises a therapeutically effective amount of compound according to Formula 1, or a pharmaceutically acceptable ester or salt thereof admixed with at least one pharmaceutically acceptable carrier.

In yet another aspect of the invention, a pharmaceutical composition comprises a pro-drug form of a compound according to Formula, or a pharmaceutically acceptable ester or salt thereof admixed with at least one pharmaceutically acceptable carrier.

In a further aspect of the invention, a compound according to Formula 1 or Formula 2 are used in the treatment of any condition which responds positively to administration of the compound, and according to any formulation and protocol which achieves the positive response. Among other things, it is contemplated that compounds of Formula 1 or Formula 2 may be used to treat an infection, an infestation, a cancer, tumor or other neoplasm, giant cell arteritis, or an autoimmune disease.

Brief Description of the Drawing

Figures 1A-1F are graphs depicting dose effect of contemplated compounds on cytokine and proliferative responses in activated human T-cells.

Figure 2 is an autoradiograph showing various RNA expressions in cells in response to treatment with contemplated compounds.

Figure 3 is a graph depicting the number of specific cytokine producing cells in response to treatment with contemplated compounds.

Figures 4A-4F are graphs depicting temporal effects of contemplated compounds on viability, cytokine responses, and proliferation of activated human T cells.

Figures 5A –5B are graphs depicting comparative effects of contemplated compounds on cytokine production in activated peripheral T cells from normal donors and rheumatoid arthritis patients.

Figure 6 is an autoradiograph showing various RNA expressions in cells in response to treatment with contemplated compounds.

Figure 7 is a graph depicting the influence of contemplated compounds on activated cytokine responses in CD4+ and CD8+ T cell subsets.

Figure 8 is a graph depicting the effect of contemplated compounds on the generation of murine Th1 cells.

Figure 9A is a graph depicting ear swelling measurements.

Figure 9B is an autoradiograph showing various RNA expressions in cells in response to treatment with contemplated compounds.

Figure 10A is another graph depicting ear swelling measurements.

Figure 9B is another autoradiograph showing various RNA expressions in cells in response to treatment with contemplated compounds.

Detailed Description

Where the following terms are used in this specification, they are used as defined below.

The terms "nucleoside" and "nucleoside analog compound" are interchangeable and refer to a compound composed of any pentose or modified pentose moiety attached to a specific position of a heterocycle, aromatic heterocycle or to the natural position of a purine (9-position) or pyrimidine (1-position) or to the equivalent position in an analog.

The term "nucleotide" refers to a phosphate ester substituted on the 5'-position of a nucleoside.

The term "heterocycle" refers to a monovalent saturated or unsaturated carbocyclic radical having at least one hetero atom, such as N, O or S, within the ring each available position of which can be optionally substituted, independently, with, e.g., hydroxy, oxo, amino, imino, lower alkyl, bromo, chloro and/or cyano. Included within this class of substituents are purines, pyrimidines.

The term "purine" refers to nitrogenous bicyclic heterocycles.

The term "pyrimidine" refers to nitrogenous monocyclic heterocycles.

The term "D-nucleosides" refers to the nucleoside compounds that have a D-ribose sugar moiety.

The term "L-nucleosides" refers to the nucleoside compounds that have an L-ribose sugar moiety.

The terms "L-configuration" and "D-configuration" are used throughout the present invention to describe the chemical configuration of the ribofuranosyl moiety of the compounds that is linked to the pyrrolo-pyrimidone portion of the molecule.

The term "C-nucleosides" is used throughout the specification to describe the linkage type that formed between the ribose sugar moiety and the heterocyclic base. In C-nucleosides, the linkage originates from the C-1 position of the ribose sugar moiety and joins the carbon of the heterocyclic base. The linkage that forms in C-nucleosides is carbon-to-carbon type.

The term "N-nucleosides" is used throughout the specification to describe the linkage type that formed between the ribose sugar moiety and the heterocyclic base. In N-nucleosides, the linkage originates from the C-1 position of the ribose sugar moiety and joins the nitrogen of the heterocyclic base. The linkage that forms in N-nucleosides is carbon to nitrogen type.

The term "protecting group" refers to a chemical group that is added to, oxygen or nitrogen atom to prevent its further reaction during the course of derivatization of other moieties in the molecule in which the oxygen or nitrogen is located. A wide variety of oxygen and nitrogen protecting groups are known to those skilled in the art of organic synthesis.

The term "lower alkyl" refers to methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, i-butyl or n-hexyl. This term is further exemplified to a cyclic, branched or straight chain from one to six carbon atoms.

The term "aryl" refers to a monovalent unsaturated aromatic carbocyclic radical having a single ring (e.g., phenyl) or two condensed rings (e.g., naphthyl), which can optionally be substituted with hydroxyl, lower alky, chloro, and/or cyano.

The term "heterocycle" refers to a monovalent saturated or unsaturated carbocyclic radical having at least one hetero atom, such as N, O, S, Se or P, within the ring, each available position of which can be optionally substituted or unsubstituted, independently, with hydroxy, oxo, amino, imino, lower alkyl, bromo, chloro, and/or cyano.

The term "monocyclic" refers to a monovalent saturated carbocyclic radical having at least one hetero atom, such as O, N, S, Se or P, within the ring, each available position of which can be optionally substituted, independently, with a sugar moiety or any other groups like bromo, chloro and/or cyano, so that the monocyclic ring system eventually aromatized [e.g., Thymidine].

The terms "immunomodulator" and "modulator" are herein used interchangeably and refers to natural or synthetic products capable of modifying the normal or aberrant immune system through stimulation or suppression.

The term "effective amount" refers to the amount of a compound of formula (1) that will restore immune function to normal levels, or increase immune function above normal levels in order to eliminate infection.

The compounds of Formula 1 may have multiple asymmetric centers. Accordingly, they may be prepared in either optically active form or as a racemic mixture. The scope of the

invention as described and claimed encompasses the individual optical isomers and non-racemic mixtures thereof as well as the racemic forms of the compounds of Formula 1.

The term "α" and "β" indicate the specific stereochemical configuration of a substituent at an asymmetric carbon atom in a chemical structure as drawn.

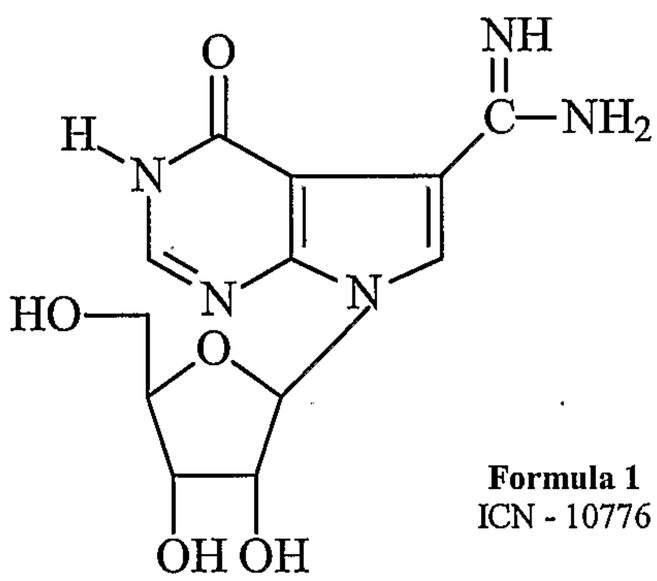
The term "enantiomers" refers to a pair of stereoisomers that are non-superimposable mirror images of each other. A mixture of a pair of enantiomers, in a 1:1 ratio, is a "racemic" mixture.

The term "isomers" refers to different compounds that have the same formula. "Stereoisomers" are isomers that differ only in the way the atoms are arranged in space.

"Pharmaceutically acceptable salts" may be any salts derived from inorganic and organic acids or bases.

Compounds

The nucleoside analog compounds of the present invention are generally described by Formula 1:



Formula 1
ICN - 10776

wherein the chemical configuration may be the L-configuration or the D-configuration, and wherein contemplated compounds may be in any appropriate salt form (*e.g.*, an HCl salt). It should further be especially appreciated that contemplated compounds also include prodrug forms of the compound according to Formula 1. Particularly contemplated prodrug forms will advantageously achieve at least one of increased specificity towards a target organ or target

cell, metabolic stability in compartments other than the target cell or organ, reduced toxicity, prolonged serum half-life time, increased uptake into specific cells and/or compartments, and enhanced physicochemical properties (increased solubility, neutralization or introduction of electrical charge, increased/decreased polarity and/or hydrophobicity), etc. Furthermore, it should be appreciated that all esters and salt of contemplated compounds are also considered appropriate.

There are numerous prodrug forms of nucleoside drugs known in the art, and contemplated prodrug forms may include modifications on the sugar moiety and/or the base. Depending on the particular compound and target, especially contemplated modifications may include formation of a tri-O-acetyl derivative of contemplated compounds, esterification of the 5'OH group on the sugar moiety to form a 5'-retinoyl derivative or derivative with alternative acids (synthesis as described in C. Sergheraert, C. Pierlot, A. Tartar, Y. Henin, M. Lemaitre, *J. Med. Chem.*, 36, 826-830, **1993**). Alternatively, coumarin or aminoacid esters may be prepared from contemplated compounds. For specific delivery of drugs to the liver and the biliary system the endogenous bile acid transport system is an attractive candidate. Consequently, cholic acid esters may be prepared from contemplated compounds.

Where contemplated compounds are nucleotides (*i.e.*, include a 5'-phosphate group), protected 5'-monophosphate derivatives (*e.g.*, cyclic and non-cyclic mono-, di-, and triesters) may be prepared, and particularly include amino acid phosphoramidates, salicylate phosphonic esters, and phosphonic esters with lipophilic compounds (modofoed and unmodified alkyl, alkenyl, cholersterol, etc). Other possible prodrugs include the possible combinations of the groups shown in PCT patent application WO 98/39342, WO 98/39343, WO 98/39344 and WO 99/45016. Prodrugs of contemplated compounds may also be obtained by derivatizing the amidine functionality, and especially preferred derivatives include substituted amides that are coupled to the carboxamide group of contemplated compounds. Further contemplated prodrug forms of nucleosides and their analogs are described in U.S. Patent Application No. 09/594,410 filed 06/16/2000, which is incorporated by reference herein.

Uses

It is contemplated that compounds according to Formula 1 will be used to treat a wide variety of conditions, and in fact any condition which responds positively to administration of

contemplated compounds. Among other things it is specifically contemplated that compounds of the invention may be used to treat an infection, an infestation, a cancer or tumor or an autoimmune disease. It is further contemplated that the compounds of the invention may be used to target conditions or diseases in specific organs of a patient, such as the liver or heart.

Infections contemplated to be treated with the compounds of the present invention include respiratory syncytial virus (RSV), hepatitis B virus (HBV), hepatitis C virus (HCV), herpes simplex type 1 and 2, herpes genitalis, herpes keratitis, herpes encephalitis, herpes zoster, human immunodeficiency virus (HIV), influenza A virus, hantann virus (hemorrhagic fever), human papilloma virus (HPV), measles, and fungus.

Infestations contemplated to be treated with the compounds of the present invention include protozoan infestations, as well as helminth and other parasitic infestations.

Cancers or tumors contemplated to be treated include those caused by a virus, and the effect may involve inhibiting the transformation of virus-infected cells to a neoplastic state, inhibiting the spread of viruses from transformed cells to other normal cells and/or arresting the growth of virus-transformed cells.

Autoimmune and other diseases contemplated to be treated include arthritis, psoriasis, bowel disease, juvenile diabetes, lupus, multiple sclerosis, gout and gouty arthritis, rheumatoid arthritis, rejection of transplantation, giant cell arteritis, allergy and asthma.

Still other contemplated uses of the compounds according to the present invention include use as intermediates in the chemical synthesis of other nucleoside or nucleotide analogs that are, in turn, useful as therapeutic agents or for other purposes.

In yet another aspect, a method of treating a mammal comprises administering a therapeutically and/or prophylactically effective amount of a pharmaceutical containing a compound of the present invention. In this aspect the effect may relate to modulation of some portion of the mammal's immune system, especially modulation of lymphokines profiles of Type 1 and Type 2 with respect to one another. Where modulation of Type 1 and Type 2 lymphokines occurs, it is particularly contemplated that the modulation may include suppression of both Type 1 and Type 2, or suppression of Type 1 and stimulation of Type 2.

For example, many autoimmune diseases (*e.g.*, rheumatoid arthritis, multiple sclerosis, diabetes) have been shown to correlate with a polarized type 1 cytokine expression. Contemplated compounds have been shown to be effective to induce type 2 cytokine expression, to reduce type 1 cytokine expression, and to reduce proliferation of activated T-cells (*infra*). Consequently, it should be appreciated that, among other things, contemplated compounds may advantageously be employed to treat diseases that are correlated with an increased type 1 cytokine expression, or diseases that are correlated with an decreased type 2 cytokine expression, or diseases that are correlated with an increased type 1 and decreased type 2 cytokine expression. Thus, particularly contemplated treatments using contemplated compounds include type 1 cytokine-mediated inflammatory responses and autoimmune diseases. Consequently, it should be appreciated that contemplated compounds may be employed as immunomodulators, and particularly as type 1 cytokine suppressants and/or as type 2 cytokine stimulant.

In general, the most preferred uses according to the present invention are those in which the active compounds are relatively less cytotoxic to the non-target host cells and relatively more active against the target. In this respect, it may also be advantageous that L-nucleosides may have increased stability over D-nucleosides, which could lead to better pharmacokinetics. This result may attain because L-nucleosides may not be recognized by enzymes, and therefore may have longer half-lives.

Administration

It is contemplated that compounds according to the present invention will be administered in any appropriate pharmaceutical formulation, and under any appropriate protocol. Thus, administration may take place orally, parenterally (including subcutaneous injections, intravenous, intramuscularly, by intrasternal injection or infusion techniques), by inhalation spray, or rectally, topically and so forth, and in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles.

By way of example, it is contemplated that compounds according to the present invention can be formulated in admixture with a pharmaceutically acceptable carrier. For example, the compounds of the present invention can be administered orally as pharmacologically acceptable salts. Because the compounds of the present invention are

mostly water soluble, they can be administered intravenously in physiological saline solution (e.g., buffered to a pH of about 7.2 to 7.5). Conventional buffers such as phosphates, bicarbonates or citrates can be used for this purpose. Of course, one of ordinary skill in the art may modify the formulations within the teachings of the specification to provide numerous formulations for a particular route of administration without rendering the compositions of the present invention unstable or compromising their therapeutic activity. In particular, the modification of the present compounds to render them more soluble in water or other vehicle, for example, may be easily accomplished by minor modifications (salt formulation, esterification, *etc.*) that are well within the ordinary skill in the art. It is also well within the ordinary skill of the art to modify the route of administration and dosage regimen of a particular compound in order to manage the pharmacokinetics of the present compounds for maximum beneficial effect in patients.

In certain pharmaceutical dosage forms, the pro-drug form of the compounds, especially including acylated (acetylated or other) derivatives, pyridine esters and various salt forms of the present compounds are preferred and can be administered in a method of treatment of a condition of a patient.

In addition, compounds according to the present invention may be administered alone or in combination with other agents for the treatment of the above infections or conditions. Combination therapies according to the present invention comprise the administration of at least one compound of the present invention or a functional derivative thereof and at least one other pharmaceutically active ingredient. The active ingredient(s) and pharmaceutically active agents may be administered separately or together and when administered separately this may occur simultaneously or separately in any order. The amounts of the active ingredient(s) and pharmaceutically active agent(s) and the relative timings of administration will be selected in order to achieve the desired combined therapeutic effect. Preferably, the combination therapy involves the administration of one compound of the present invention or a physiologically functional derivative thereof and one of the agents mentioned herein below.

Examples of other drugs or active ingredients contemplated to be effective in combination with a contemplated compounds are anti-viral agents such as interferon, including but not limited to interferon α and γ, Ribavirin, acyclovir, and AZT™; anti-fungal agents such as tolnaftate, Fungizone™, Lotrimin™, Mycelex™, Nystatin and Amphoteracin; anti-parasitics

such as Mintezol™, Niclocide™, Vermox™, and Flagyl™, bowel agents such as Immodium™, Lomotil™ and Phazyme™; anti-tumor agents such as interferon α and γ, Adriamycin™, Cytoxan™, Imuran™, Methotrexate, Mithracin™, Tiazofurin™, Taxol™; dermatologic agents such as Aclovate™, Cyclocort™, Denorex™, Florone™, Oxsoralen™, coal tar and salicylic acid; migraine preparations such as ergotamine compounds; steroids and immunosuppresants not listed above, including cyclosporins, Diprosone™, hydrocortisone, mycophenolic acid, Arava™ (Leflunomide); Floron™, Lidex™, Topicort and Valisone; and metabolic agents such as insulin, and other drugs which may not nicely fit into the above categories, including cytokines such as IL2, IL4, IL6, IL8, IL10 and IL12. Especially preferred primary drugs are AZT, 3TC, 8-substituted guanosine analogs, 2,3-dideoxynucleosides, interleukin II, interferons such as IαB-interferons, tucaresol, levamisole, isoprinosine and cyclolignans.

Examples of such further therapeutic agents include agents that are effective for the modulation of immune system or associated conditions such as AZT, 3TC, 8-substituted guanosine analogs, 2', 3'-dideoxynucleosides, interleukin II, interferons, such as α-interferon, tucaresol, levamisole, isoprinosine and cyclolignans. Certain compounds according to the present invention may be effective for enhancing the biological activity of certain agents according to the present invention by reducing the metabolism or inactivation of other compounds and as such, are co-administered for this intended effect.

With respect to dosage, one of ordinary skill in the art will recognize that a therapeuti-cally effective amount will vary with the infection or condition to be treated, its severity, the treatment regimen to be employed, the pharmacokinetics of the agent used, as well as the patient (animal or human) treated. It is contemplated that various alternative dosages are also appropriate, including dosages between 0.5 mg/kg and 0.1 mg/kg and less, but also dosages between 0.5 and 2.0mg/kg (*e.g.*, 1.25mg/kg) and more. It is further contemplated that while treatment success may be achieved with some disease conditions at relatively low plasma concentrations of contemplated compounds, other disease conditions may require relatively high dosages.. It is contemplated, however, that an appropriate regimen will be developed by administering a small amount, and then increasing the amount until the side effects become unduly adverse, or the intended effect is achieved.

Administration of the active compound may range from continuous (intravenous drip) to several oral administrations per day (for example, Q.I.D.) and may include oral, topical, parenteral, intramuscular, intravenous, sub-cutaneous, transdermal (which may include a penetration enhancement agent), buccal and suppository administration, among other routes of administration.

To prepare the pharmaceutical compositions according to the present invention, a therapeutically effective amount of one or more of the compounds according to the present invention is preferably intimately admixed with a pharmaceutically acceptable carrier according to conventional pharmaceutical compounding techniques to produce a dose. A carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral or parenteral. In preparing pharmaceutical compositions in oral dosage form, any of the usual pharmaceutical media may be used. Thus, for liquid oral preparations such as suspensions, elixirs and solutions, suitable carriers and additives including water, glycols, oils, alcohols, flavouring agents, preservatives, colouring agents and the like may be used. For solid oral preparations such as powders, tablets, capsules, and for solid preparations such as suppositories, suitable carriers and additives including starches, sugar carrier, such as dextrose, mannitol, lactose and related carriers, diluents, granulating agents, lubricants, binders, disintegrating agents and the like may be used. If desired, the tablets or capsules may be enteric-coated or sustained release by standard techniques.

For parenteral formulations, the carrier will usually comprise sterile water or aqueous sodium chloride solution, though other ingredients including those that aid dispersion may be included. Of course, where sterile water is to be used and maintained as sterile, the compositions and carriers must also be sterilized. Injectable suspensions may also be prepared, in which case appropriate liquid carriers, suspending agents and the like may be employed.

Examples

The following examples are provided to illustrate some of the biological effects of contemplated compounds. In particular, the experimental data indicate that contemplated compounds inhibit activation-induced IFNγ, IL-2 and TNFα secretion and T cell proliferation but enhanced IL-4 and IL-5 production in human T cells in a dose-dependent manner.

The biological effect mediated by contemplated compounds on peripheral T cells peaked at 48h, was seen with stimulation either by PMA/ionomycin or PHA, was observed at cytokine protein and mRNA levels in normal individuals and rheumatoid arthritis patients, and enhanced the number of Type 2 while diminishing the number of Type 1 cytokine-producing cells.

Moreover the induction of a Type 2 cytokine bias by contemplated compounds was concurrent with a diminution in mRNA expression of inducible nitric oxide synthase, c-myc, IL-6 and IL-1b (suggesting an anti-inflammatory effect), and was more dramatic in the CD4+ population of human T cells. Furthermore contemplated compounds induced a Th2 cytokine bias in murine lymph node-derived Th1 cells in vitro, and (at 0.3 and 0.6mg/kg) administered i.p. in BALB/c mice, inhibited two Type 1 cytokine-mediated acute inflammatory responses, contact hypersensitivity to dinitrofluorobenzene and Staphylococcal enterotoxin B-induced inflammatory responses.

These *in vivo* effects were associated with augmented IL-10 and decreased IFNγ mRNA expression in lymphoid organs. Collectively these data indicate that contemplated compounds can functionally induce a Th2 cytokine bias *in vitro* and *in vivo*.

Compounds

Exemplary contemplated compound 7-β-D-ribofuranosyl-4-oxopyrrolo[2,3-d]pyrimidine-5-carboxamidine was synthesized as described previously (Synthesis and cytokine modulation properties of pyrrolo[2, 3-d]-4-pyrimidone nucleosides. *J Med Chem. 2000 Jun 29;43(13):2566-74.*). Both compounds have a molecular weight of 309.28 and are soluble in aqueous solution.

Preparation of human T-cells and activation *in vitro*

Peripheral blood mononuclear cells were isolated from healthy donors or rheumatoid arthritis patients by density gradient centrifugation followed by T cell enrichment using Lymphokwik (One Lambda, Canoga Park CA). Contaminating monocytes were removed by adherence to plastic. Purified T cells were > 99% CD2+ , <1% HLA-DR+ and < 5% CD25+ and were maintained in RPMI-AP5 (RPMI-1640 medium containing 20 mM HEPES buffer, pH 7.4, 5% autologous plasma, 1% L-glutamine, 1% penicillin/streptomycin and 0.05% 2-mercaptoethanol).

For determination of cytokine protein levels, T-cells (1 x 10^6 cells in a volume of 1 ml) were activated by the addition of 10 ng PMA plus 0.5 μg ionomycin (both from Calbiochem, La Jolla, CA) and incubated in 24 well plates in the presence of 0 to 20 μM nucleoside for up to 48 h at 37°C and 5% CO_2 in a humidified incubator. Following activation, supernatants were analysed for cell-derived cytokine production. For proliferation and viability studies, the protocol as above was modified to a 96 well-plate format using 0.2 x 10^6 cells in a volume of 0.2 ml and activation with 2ng PMA and 0.1 μg ionomycin. In separate experiments, 5 x 10^6 T cells in 2ml were activated with 20 ng PMA plus 1 μg ionomycin. Here total RNA was isolated from T cells following 6 - 24h incubation and analyzed by RT -PCR to determine mRNA levels of various cytokines and inflammatory mediators. Also in separate experiments, human T cells were purified further (using cell enrichment reagents from Stem Cell Technologies, Vancouver, BC) to give pure populations of CD4+ (< 1% CD8+ using RosetteSep human CD4+ T cell isolation reagent), and CD8+ (< 1% CD4+ using RosetteSep human CD4+ T cell isolation reagent) T cell subsets, after which 1 x 10^6 cells per ml were activated with PMA and ionomycin, as in the total T cell experiments.

Extracellular cytokine analyses

Human cytokine levels were determined in cell supernatants, following appropriate dilution, using ELISA kits specific for IL-2, IFNg, TNFa, IL-4 and IL-5 (Biosource International, Camarillo, CA). Murine cytokine levels were determined using ELISA kits specific for murine IFNg and IL-4 (R and D Systems, Minneapolis, MN). All ELISA results were expressed as pg/ml. Some data are shown as percentage of activated control, calculated as the ratio of activated T cell cytokine level in the presence of test nucleoside over the cytokine level of untreated activated T cells x 100 %. Zero effect on cytokine levels by test nucleosides would give a percentage of activated control value of 100 %. Alternatively data were shown as percentage change from activated control ([(test pg/ml – activated control pg/ml)/activated control pg/ml] x 100%). Zero effect on cytokine levels by test nucleosides would be 0 %.

ELISA spot assay

ELISA spot plates (Whatman Polyfiltronics, Rockland, MA) were coated with the capture antibody in sterile PBS overnight. Mouse monoclonal antibody (mAb) anti-human

interferon-g (IFNg, clone MD1, Biosource) was used at 4 mg/ml for IFNg. Anti-IL-2 capture antibody (4 μg/ml, clone 5334.21, R&D Systems) was used for IL-2, anti-IL-4 capture antibody (clone 8D4-8, Pharmingen, San Diego, CA) was used at 5 μg/ml for IL-4, and anti-IL-5 capture antibody (5 μg/ml, clone TRFK-5, Pharmingen) was used for IL-5. After washing 2 times with sterile PBS, the plates were blocked for 1.5 h with sterile PBS containing 1% BSA and washed 3 times with sterile PBS afterwards. 0.2 x 10^6 PBMC in 200 ml of RPMI medium (containing 1% pen-strep, 1% glutamine and 10% FCS) were placed in each well with 4 μg/ml PHA with or without the nucleoside (10 μM), and cultured for 24 h (IL-2) or 48 h (IL-4, IL-5, IFNγ) at 37 oC in 5% CO_2. After washing, biotinylated anti-lymphokine detection antibodies were added overnight, anti-human IFNγ (4 μg/ml, Biosource), anti-IL-2 (3 μg/ml, R and D Systems), anti-IL-4 (2 μg/ml, Pharmingen), and anti-IL-5 (2 μg/ml, Pharmingen). To assess binding of biotinylated antibodies streptavidin-horseradish peroxidase (1 : 2000 in PBS 0.025% Tween for 1.5 h at room temperature, Vector, Burlingame, CA) was used. The plates were developed using 400 μl AEC (Sigma, St. Louis, MO, 10 mg dissolved in 1 ml dimethyl formamide) mixed in 12 ml 0.1 M sodium acetate buffer, pH 5.0, plus 6 ml H_20_2. The resulting spots were counted on a computer assisted ELISA spot image analyzer (ImmunoSpotTM Image Analyzer, Cellular Technology, Ltd., Cleveland, OH), which is designed to detect ELISA spots using predetermined criteria.

Proliferation and viability assay

T cell proliferative responses were assessed by measuring [3H]-thymidine (1μCi, ICN, Irvine, CA) incorporation for the last 16h of each assay. Cells were harvested onto filters and DNA synthesis was measured following scintillation counting on a Wallac Betaplate counter (Perkin-Elmer/Wallac, Gaithersburg, MD). Viability was assessed by propidium iodide (5 μg/ml) exclusion in untreated and nucleoside-treated human T cells stained with FITC-CD3 (Becton Dickinson, San Jose, CA). The viability of CD3+ cells following addition of propidium iodide (Roche Molecular Biochemicals, Indianapolis, IN) was confirmed by flow cytometry (FACScan, Becton Dickinson).

Contact hypersensitivity (CHS)

Reactivity to the contact allergen, DNFB, was determined, in BALB/c mice, as previously described (Ishii, N., K. Takahashi, H. Nakajima, S. Tanaka, P.W. Askenase.

1994. DNFB contact sensitivity (CS) in BALB/c and C3H/He mice. *J. Invest. Dermatol. 102:321*). Briefly, mice were sensitized by application of 20µl of 0.3% DNFB in acetone : olive oil, 4 : 1 onto the shaved abdomens of naive mice. For optimal elicitation of CHS, the mice were challenged on both sides of each ear with 20µl of 0.12% DNFB, five days after sensitization. Unsensitized mice were also challenged and used as controls in each experiment. After 24h, ear thickness measurements were taken and response to DNFB was assessed by subtracting post-challenge from pre-challenge values. Where indicated, 7-β-D-ribofuranosyl-4-oxopyrrolo[2,3-d]pyrimidine-5-carboxamidine, at a dose of 6.2 µg in 50µl PBS (0.3 mg/kg) or 12.4 µg in 100µl PBS (0.6 mg/kg), was administered by i.p. injection at the time of challenge with DNFB. These doses of 7-β-D-ribofuranosyl-4-oxopyrrolo[2,3-d]pyrimidine-5-carboxamidine gave maximal effect in preliminary optimization studies. Following final ear thickness measurements, mice were sacrificed by cervical dislocation and axillary/lateral axillary lymph nodes were removed. Following isolation of total cellular RNA from isolated lymph node cells, RT-PCR and Southern Blot analyses were performed to monitor for mouse IFNg, IL-2, and IL-10 mRNA levels.

Staphylococcal enterotoxin B treatment in vivo

SEB was injected i.p. at a dose of 50 µg per mouse at day 0 into three groups of 4 mice. One group was injected with 6.2µg 7-β-D-ribofuranosyl-4-oxopyrrolo[2,3-d]pyrimidine-5-carboxamidine (0.3 mg/kg) and one group with 12.4µg 7-β-D-ribofuranosyl-4-oxopyrrolo[2,3-d]pyrimidine-5-carboxamidine (0.6 mg/kg) both in 50µl PBS i.p., 1h prior to SEB injection. The 0.6 mg/kg dose of 7-β-D-ribofuranosyl-4-oxopyrrolo[2,3-d]pyrimidine-5-carboxamidine gave maximal effect in preliminary optimization studies. 24h later all mice were anesthetized with an appropriate dose of the inhalation anesthetic, Penthrane (Abbott Labs, N. Chicago, IL) and exanguinated by cardiac puncture to obtain whole blood and spleens were removed. Splenocyte suspensions were prepared from individual spleens following removal of contaminating red cells with ACK lysing buffer (0.15M NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM Na_2EDTA adjusted to pH 7.2 - 7.4 and filtered). Following isolation of total cellular RNA from isolated splenocytes, RT-PCR and Southern Blot analyses were performed to monitor for mouse IFNγ, IL-2, and IL-10 and iNOS mRNA levels. Serum was obtained from clotted blood and used for determinations of nitric oxide production. Nitric oxide production was evaluated by measuring its stable end products,

nitrite and nitrate. Total nitrite/nitrate levels were determined following reduction of nitrate to nitrite through a nitrate reductase enzyme reaction followed by a colorometric assay (Sigma) based on the reduction of total nitrite by Griess reagent to a purple azo-compound.

Analysis of cytokine mRNA

Total cellular RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD). The cDNA synthesis reaction was performed using oligo (dT)12-18 primer and Superscript II (Life Technologies) reverse transcriptase. The PCR reaction (GeneAmp PCR kit, Perkin-Elmer, Foster City, CA) consisted of a 50 μl mixture containing cDNA, dNTPs (each at 200 μM), 0.5 μM of each primer pair and 1.25 unit of Taq polymerase. The primers for human IL-2, IL-10, IL-4, IL-6, IL-1b, IFNg, c-myc, IL-2R, CD40L (Stratagene, La Jolla, CA), iNOS (Clontech, Palo Alto, CA) and pHE7 ribosomal gene were used. Typical amplification conditions included a 5 minute denaturation at 94^0C and a 5 minute annealing at 60^0C followed by 35 cycles of 1.5 min at 72^0C, 45 sec at 94^0C and 45 sec at 60^0C, with a final extension time of 10 min at 72^0C. The primers for mouse IL-2, IFNγ, and β-actin were obtained from Stratagene and mouse IL-10 from Clontech. Amplification conditions for the Stratagene mouse cytokine primers were a 5 min denaturation at 94^0C and a 5 min annealing at 60^0C followed by 35 cycles of 1.5 min at 72^0C, 45 sec at 94^0C and 45 sec at 60^0C, with a final extension time of 10 min at 72^0C. For IL-10, the PCR conditions were a 5 minute denaturation at 94^0C followed by 35 cycles of 45 sec at 94^0C, 45 sec at 60^0C and 2 min at 72^0C, with a final extension time of 7 min at 72^0C.

For each gene product, the optimum cDNA dilution was determined experimentally and was defined as the cDNA dilution that would achieve a detectable concentration that was well below saturating conditions. PCR products were separated on 2% agarose containing ethidium bromide and immobilized to Hybond N+ membrane (Amersham Pharmacia, Piscataway, NJ) overnight using 0.4 M NaOH and 0.2M NaCl. Blots were hybridized with 32P-γATP labeled oligonucleotide probes generated from the original primers (Stratagene) or from specific probes designed to be complimentary to a central region within individual PCR products (human iNOS, mouse IL-10 and human pHE7). Equivalent loading was assessed following hybridization with a probe generated from pHE7 sense primer. Washed blots were then analyzed using a PhosphorImager (Biorad, Richmond, CA). Relative changes in cytokine or other test mRNA were presented as densitometric readings and normalized for

any variations in input RNA by determining the densitometric ratio of mRNA of interest relative to mRNA of the housekeeping gene, pHE7.

Generation of Th1 and Th2 T cells

Eight to ten BALB/c or C57BL/6 mice were sacrificed by cervical dislocation, axillary/lateral axillary and popliteal lymph nodes were removed, lymph node cell suspensions were prepared from each animal and pooled. Murine CD4+ T cells were isolated from pooled lymph node preparations of each mouse strain using StemSep murine T cell enrichment reagent (Stem Cell Technologies). The generation of Th1 and Th2 cells was performed using a protocol similar to that described previously (Austrup, F., D. Vestweber, E. Borges, M. Lohning, R. Brauer, U. Herz, H. Renz, R. Hallmann, A. Scheffold, A. Radbruch, A. Hamann. 1997. P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. *Nature. 385:81*). Briefly, cells were activated on 24 well tissue culture plates coated with an optimal amount (1mg per well) of anti-CD3 mAb (clone 145-2C11, Pharmingen) in the presence of the indicated recombinant murine cytokines and cytokine Abs (all from Pharmingen) (IL-2 alone, 50U/ml; Th1: IL-2, 50U/ml; IL-12, 1000U/ml; IFNg with and without 10mM ICN 10776 and Th2: IL-2, 50U/ml; IL-4, 10ng/ml; anti-IFNg (clone XMG1.2) 10mg/ml) and cultured for 4 days. Cells were transferred to fresh plates without medium change and incubated for a further 48h then washed three times and fresh medium added, incubated overnight then restimulated for 5h with PMA (10ng) and ionomycin (0.5mg). Cell free supernatants were then taken for ELISA determination of murine IL-4 and IFNg levels. Cells which were not restimulated were negative for IL-4 and IFNγ, indicating that the cytokine added during culture was not carried over.

Statistical analysis

Trend analysis was performed using one -way or two-way ANOVA analysis. Statistical significance, where relevant, was assessed using the Student-Newman-Keuls multiple comparison method.

Results

The dose effect of ICN 10776 on cytokine and proliferative responses by activated human T cells

We examined the Type 1 and Type 2 cytokine profile, as well as the influence on T cell proliferative responses, induced by a 48h incubation of 7-β-D-ribofuranosyl-4-oxopyrrolo[2,3-d]pyrimidine-5-carboxamidine (ICN 10776) with activated peripheral human T cells. In the dose range 0.2 to 20μM, ICN 10776 enhanced PMA-ionomycin (PMA-ION)-induced levels of the Type 2 cytokines, IL-4 and IL-5, in a dose-dependent manner with a peak enhancement at 10μM of 166% and 77% respectively (Fig. 1B, 1D). The data shown are from a representative of five donors. In addition, in the same dose range and also in a dose-dependent manner, ICN 10776 dramatically suppressed PMA-ION-induced levels of the Type 1 cytokines, IL-2, IFNγ and TNFα with a peak suppression of 62%, 72% and 78% respectively at 20μM (Fig. 1A, 1C, 1E), as well as substantially inhibiting T cell proliferation with a peak inhibition of 99% at 20μM (Fig. 1F). A second proliferation assay was also performed using a colorimetric assay (MTT cell proliferation kit I, Roche Molecular Biochemicals) based on the conversion of the tetrazolium salt, MTT by mitochondrial dehydrogenases to a formazan dye (detectable at 540 nm). Using this assay ICN 10776 also dramatically inhibited T cell proliferation (data not shown) demonstrating that the effect by ICN 10776 was not due to interference of thymidine uptake as has been demonstrated for other nucleoside analogues. Similar effects on cytokine modulation were seen in T cells and PBMCs stimulated with PHA (data not shown).

In a second set of experiments, T cells were activated with PMA-ION alone or in the presence of 2, 5 or 10 μM ICN 10776 or its L-enantiomer, ICN 17465, for 6h or 24h prior to RNA isolation and RT-PCR analyses to determine levels of Type 1 and Type 2 cytokine mRNA. We observed that with increasing doses of ICN 10776 there was an increase in IL-4 mRNA and a concomitant decrease in IFNγ and TNFα mRNA (Fig. 2, showing data from a representative of three donors). Interestingly, no similar modulation of the activated levels of these cytokines were observed with the L-enantiomer of ICN 10776, ICN 17465, demonstrating an absolute requirement for a D-ribose sugar moiety for bioactivity (Fig. 2).

In a third set of experiments, PBMCs were activated with PHA alone or in the presence of 10 μM ICN 10776 or its L-enantiomer, ICN 17465, for 24 - 48h on specific cytokine antibody bound ELISA spot plates. The number of specific cytokine producing cells were then determined as described in Materials and Methods. A dramatic fall in the number of IL-2 and IFNγ-producing cells were observed in the presence of ICN 10776 but not the L-

enantiomer, ICN 17465 (Fig. 3). In contrast ICN 10776, but not the L-enantiomer, ICN 17465 induced a substantial increase in the number of both IL-4 and IL-5 producing cells (Fig. 3).

Altogether these data show that ICN 10776 reduces both the production of Type 1 cytokines and T cell proliferation while elevating levels of Type 2 cytokines in a dose-related manner. The effects of ICN 10776 on cytokine responses were observed at the protein and mRNA levels and affected the number of specific cytokine producing cells. Moreover the induction of a Type 2 cytokine bias required D-ribosylation as the L-enantiomer of this nucleoside did not show similar bioactivity.

The temporal effect of ICN 10776 on viability, cytokine responses and proliferation of activated human T cells

We determined whether the suppression of Type 1 cytokine expression and the inhibition of T cell proliferative responses were merely the product of T cell toxicity by ICN 10776. If toxicity was responsible for these observations then viability of T cells would be influenced by both incubation time and dose of ICN 10776. Using propidium iodide exclusion analyses we observed that viability did decrease in resting $CD3^+$ T cells from 95% to 85% following 48h incubation with 20μM 10776 (Fig. 4A, 4B). In PMA-ION activated $CD3^+$ T cells without nucleoside treatment, a temporal decrease in viability was observed from 91 ± 1% at 8h to 88 ± 2% at 24h to 83 ± 1% at 48h however no significant difference to these numbers was found in the presence of nucleoside across the dose range 0.2 to 20μM of ICN 10776 (Fig. 4C). These data suggest that the small decrease in viability was induced presumably by activation-induced apoptosis of T cells and not a direct toxic effect by the nucleoside. Moreover, the temporal effect of incubation with ICN 10776 did show a dose-dependent decrease in T cell proliferation (Fig. 4D) and IFNγ secretion (Fig. 4F) but with a peak suppression at 48h. Likewise, the peak dose-dependent increase in IL-4 was also observed at 48h (Fig. 4E). These data showed that the biologic effects observed following treatment with ICN 10776 were not the result of in vitro toxicity and had a peak effect after 48h incubation. The decline in effect after 48h may be related to metabolism of the nucleoside to inactive degradation products via dephosphorylation or deribosylation.

Comparative effects of ICN 10776 on TNFα, IFNγ and IL-4 production in activated peripheral T cells from normal donors and rheumatoid arthritis patients

It has been previously shown that *in vitro* differentiation of peripheral T cells towards a Type 2 phenotype is impaired in rheumatoid arthritis (RA) patients when compared to normal donors (Berg, D.J., M.W. Leach, R. Kuhn, K. Rajewski, W. Muller, N.J. Davidson, D. Rennick. 1995. Interleukin 10 but not interleukin 4 is a natural suppressant of cutaneous inflammatory responses. *J. Exp. Med. 182:99*). Here we compared the effect of ICN 10776 on the activated cytokine profiles from PMA-ION-activated peripheral T cells of 16 normal donors and 16 RA patients. We observed that the Type 2 cytokine bias induced by 10μM ICN 10776 occurred in activated peripheral T cells from both normal donors and RA patients, as determined by reduced levels of secreted IFNγ (43% and 63% respectively, $p = 0.0008$ that ICN 10776 < than untreated group and ICN 17465 group) and heightened IL-4 production (266% and 192% respectively, $p < 0.0001$ that ICN 10776 > than untreated group and ICN 17465 group) (Fig. 5A, left panel). Although cytokine responses were significantly greater in the normals group ($p<0.0001$), there was no significant difference in the magnitude of Type 2 cytokine bias induced by ICN 10776 in either group (Fig. 5A, left panel). No similar effect on the activated cytokine profile in either group was observed following exposure of peripheral T cells to 10μM ICN 17465, the L-enantiomer of ICN 10776 (Fig. 5A, right panel). In separate experiments, the activated cytokine mRNA profiles of PMA-ION-stimulated peripheral T cells from both normal donors and RA patients in the presence and absence of 10μM ICN 10776 and ICN 17465 were determined. As seen with the secreted cytokine protein data above, ICN 10776, but not ICN 17465, elevated IL-4 mRNA levels and suppressed IFNγ and TNFα in peripheral T cells from both subject groups (Fig. 5B). Therefore the induction of a Type 2 cytokine bias both at protein and mRNA levels by ICN 10776 represents a general phenomenon in peripheral blood T cells and appears to be not impaired in peripheral T cells from RA patients.

The influence of ICN 10776-mediated cytokine responses on pro- and anti-inflammatory mediators

As a Type 1 cytokine bias generally induces pro-inflammatory responses whereas a Type 2 cytokine bias usually leads to anti-inflammatory responses, we determined the mRNA levels of Type 1 and Type 2 cytokines in activated human T cells and compared the

expression of these with mRNA levels of certain critical anti-inflammatory or pro-inflammatory mediators in cells from the same donor. We observed that ICN 10776, in addition to its influence on Type 1 (suppression of IFNγ, TNFα and IL-2) and Type 2 cytokines (enhancement of IL-4) also induced enhancement of IL-10, an anti-inflammatory cytokine, and suppressed levels of two pro-inflammatory cytokines, IL-6 and IL-1β. In addition, concomitant reduction in the expression of inducible nitric oxide synthase (iNOS), an enzyme responsible for the generation of the pro-inflammatory mediator, nitric oxide was also observed (Fig. 6). Furthermore, an anti-proliferative effect by ICN 10776 was evident due to the suppression of the proto-oncogene, c-myc, a gene which is upregulated in proliferating lymphocytes (Fig. 6). These data show that the heightened Type 2 cytokine response and diminished Type 1 cytokine response elicited by ICN 10776 treatment results in an increased anti-inflammatory environment as well as a marked anti-proliferative effect in peripheral T cells.

The influence of ICN 10776 on activated cytokine responses in $CD4^+$ and $CD8^+$ T cell subsets

The T helper ($CD4^+$) and cytotoxic ($CD8^+$) T cell subsets both play important roles in the immune response to antigens, whether foreign or self. Both subsets can be differentiated to produce either Type 1 or Type 2 cytokines. As $CD4^+$ and $CD8^+$ T cells play distinct roles both in the pathogenesis of autoimmunity and protective response to pathogens, it was important to distinguish any differential effects of the induction of a Type 2 cytokine bias by ICN 10776 in these two T cells subsets. We thus compared the ICN 10776-mediated activated cytokine response in isolated $CD4^+$ and $CD8^+$ T cells and total T cells from the same donor. Secreted IL-4 levels were significantly enhanced in all T cell groups following 48h stimulation with PMA-ION in the presence of ICN 10776, but not in the presence of 17465, when compared to untreated activated controls ($p < 0.0001$ that ICN 10776 > untreated and ICN 17465) (Fig. 7). In addition, IFNγ levels were signifiacntly reduced in all three T cell groups compared to activated control or ICN 17465-treated groups ($p < 0.0001$). Surprisingly, the ICN 10776-mediated enhancement of IL-4 was most dramatic in $CD4^+$ T cells (994% increase over activated control compared to 228% and 320% in $CD8^+$ T cells and total T cells respectively, $p < 0.03$). Furthermore, the suppression of IFNγ by ICN 10776, although significant in all three T cell groups, was again far more dramatic in $CD4^+$ T cells

(81% decrease over activated control compared to 37% and 52% decrease in both $CD8^+$ T cells and total T cells respectively, $p < 0.05$) (Fig. 7). No similar suppressive effect was observed in all three T cell groups following treatment with ICN 17465. These data show that $CD4^+$ T cells were the most susceptible to cytokine modulation by ICN 10776. It also suggests that $CD8^+$ T cells may be more resistant to the ICN 10776-mediated effects and may actually provide a regulatory signal to $CD4^+$ T cells which dampens the Type 2 cytokine bias induced by ICN 10776.

The effect of ICN 10776 on generation of murine Th1 cells

We determined whether ICN 10776 could induce a Type 2 cytokine bias even under conditions, which normally generate Th1 T cells in mouse lymphoid T cells. BALB/c and C57BL/6 lymph node T cells were isolated and Th1 cells were generated following initial incubation in the presence of plate bound anti-CD3 Ab and IL-2, IFNγ and IL-12 as described previously (Austrup, F., D. Vestweber, E. Borges, M. Lohning, R. Brauer, U. Herz, H. Renz, R. Hallmann, A. Scheffold, A. Radbruch, A. Hamann. 1997. P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. *Nature. 385:81*). These Th1 cells upon restimulation secreted low levels of IL-4 and substantial amounts of IFNγ when compared to anti-CD3 Ab and IL-2 treatment alone (Fig. 8). Co-incubation with ICN 10776 in the first incubation period however induced these murine T cells to produce the elevated levels of IL-4 and low levels of IFNγ similar to those observed in Th2 cells (generated following restimulation in T cells treated with anti-CD3 Ab and IL-2, IL-4 and anti- IFNγ) (Fig. 8). These observations were consistent in both mouse strains. Collectively these data show that ICN 10776 can induce the generation of Th2 cells even in conditions, which are conducive to generating Th1 cells.

Impaired contact hypersensitivity responses following ICN 10776 administration is associated with elevated IL-10 expression and reduced IL-2 and IFNγ expression in mouse lymph node cells

Contact hypersensitivity (CHS) responses were elicited in DNFB-primed BALB/c mice following ear challenge with 0.2% DNFB as described previously (Ishii, N., K. Takahashi, H. Nakajima, S. Tanaka, P.W. Askenase. 1994. DNFB contact sensitivity (CS) in BALB/c and C3H/He mice. *J. Invest. Dermatol. 102:321*). Fig. 9A shows the ear swelling

measurements, following challenge, determined in DNFB-primed and naive mice, and in DNFB-primed mice treated with 0.3mg/kg and 0.6mg/kg ICN 10776. In this representative experiment, i.p. injection of ICN 10776, at the time of challenge, greatly impaired CHS responses to DNFB in a dose dependent manner as shown by a mean decrease in ear thickness of 42% with a 0.3mg/kg dose and 68% with a 0.6mg/kg dose at 24h post-challenge (inhibition of CHS was calculated following subtraction of responses in unprimed mice challenged with DNFB (naive)). No substantial changes in post-challenge ear thickness were observed in DNFB-primed mice challenged with acetone : olive oil or in mice primed with acetone : olive oil and challenged with DNFB. In addition, as shown in Fig. 9B, mRNA expression of murine IL-10 was greatly enhanced but IL-2 and IFNγ mRNA levels were substantially reduced from lymph node cells in ICN 10776-treated, DNFB-primed mice when compared to DNFB-primed mice alone. Collectively, these data demonstrate that the reduction of *in vivo* CHS responses in BALB/c mice by ICN 10776 was associated with elevation of IL-10, the principal regulatory cytokine in CHS responses to DNFB, and suppression of IL-2 and IFNγ, both important mediators of CHS responses.

Impaired SEB-induced inflammatory responses as assessed by nitric oxide release following ICN 10776 administration in vivo is associated with elevated IL-10 expression and reduced IFNγ expression in mouse splenocytes

SEB-induced inflammatory responses were elicited in BALB/c mice following ip injection with 50μg SEB as described previously (Tam, R.C., K. Ramasamy, J. Bard, B. Pai, C. Lim, D.R. Averett. 2000. The ribavirin analog ICN 17261 demonstrates reduced toxicity and antiviral effects with retention of both immunomodulatory activity and reduction of hepatitis-induced serum alanine aminotransferase levels. *Antimicrob. Agents. Chemother. 44:1276*). Fig. 10A shows the total serum nitrite measurements, following SEB challenge, determined in mice treated with 0, 0.3mg/kg or 0.6mg/kg ICN 10776. In this representative experiment, i.p. administration of ICN 10776, at the time of challenge, greatly impaired inflammatory responses to SEB in a dose dependent manner as shown by a mean decrease in serum nitrite levels of 54% with a 0.3mg/kg dose and 78% with a 0.6mg/kg dose at 24h post-challenge. In addition, as shown in Fig. 10B, mRNA expression of murine IL-10 was greatly enhanced but IFNγ mRNA levels were substantially reduced from splenocytes in ICN 10776-treated, SEB-challenged mice when compared to SEB-challenged mice alone. Collectively,

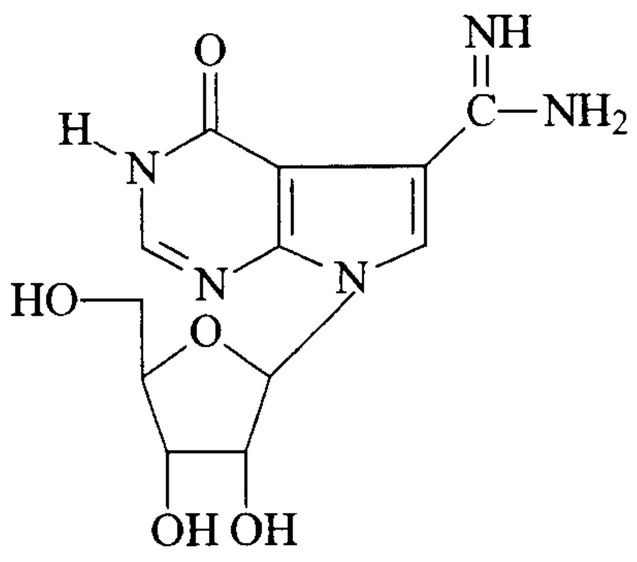
these data demonstrate that the reduction of *in vivo* SEB-induced inflammatory responses in BALB/c mice by ICN 10776 was associated with elevation of the anti-inflammatory cytokine, IL-10, a principal regulatory cytokine in SEB-induced inflammatory, and suppression of IFNγ, an important mediator of SEB-induced inflammatory responses.

AMENDED CLAIMS

[received by the International Bureau on 7 August 2001 (07.08.01);
original claim 1 amended; remaining claims unchanged (2 pages)]

1. A method of treatment of a condition in a patient comprising:

administering a compound according to Formula 1, wherein the condition is an autoimmune disease or an inflammatory disease, and wherein the chemical configuration may be in an L-configuration or a D-configuration.



Formula 1

2. The method of claim 1, wherein the condition is an autoimmune disease.

3. The method of claim 1, wherein the autoimmune disease is selected from the group consisting of rheumatoid arthritis, multiple sclerosis, and diabetes.

4. The method of claim 1, wherein the autoimmune disease is rheumatoid arthritis.

5. The method of claim 1, wherein the autoimmune disease is multiple sclerosis.

6. The method of claim 1, wherein the autoimmune disease is diabetes.

7. The method of claim 1, wherein the condition is an inflammatory disease.

8. The method of claim 1, wherein the inflammatory disease is a contact dermatitis.

9. The method of claim 7 wherein the inflammatory disease comprises an expression of a type 1 cytokine at a level higher than a level of a type 1 cytokine in a healthy person.

10. The method of claim 9 wherein the type 1 cytokine is interleukin-2.

11. The method of claim 9 wherein the type 1 cytokine is tumor necrosis factor alpha.

12. The method of claim 1 wherein the step of administering comprises *in vivo* administration.

13. The method of claim 1 wherein the step of administering comprises oral administration.

14. The method of claim 1 wherein the step of administering comprises injection of the L-ribonucleoside.

15. The method of claim 1 wherein the step of administering comprises administering the compound in a dose between 0.1mg per kg of body weight of the patient and 1.0mg per kg of body weight of the patient.

16. The method of claim 2 wherein the autoimmune disease comprises an expression of a type 2 cytokine at a level higher than a level of a type 2 cytokine in a healthy person.

17. The method of claim 16 wherein the type 2 cytokine is interleukin-4.

Figure 1

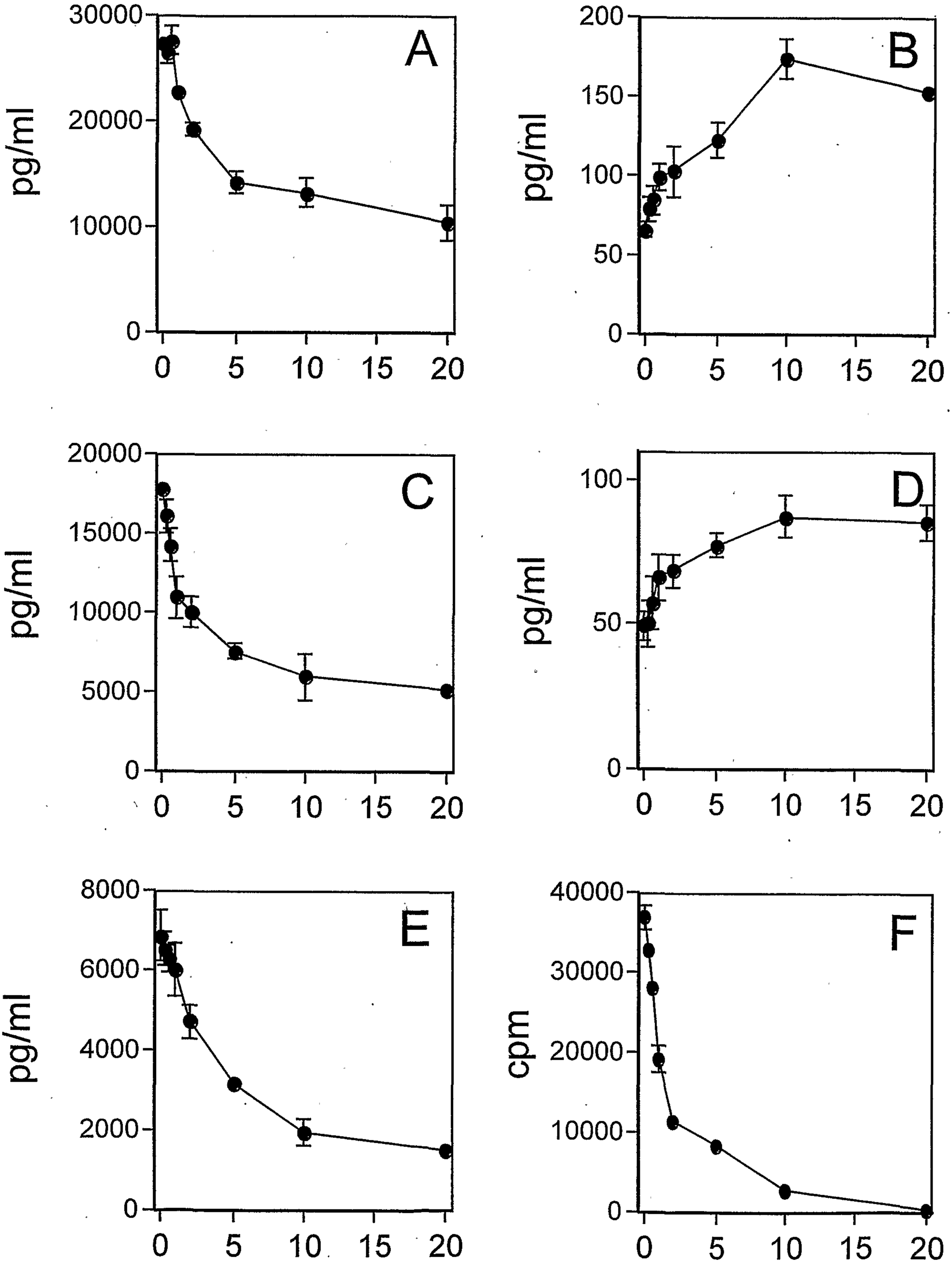


Figure 2

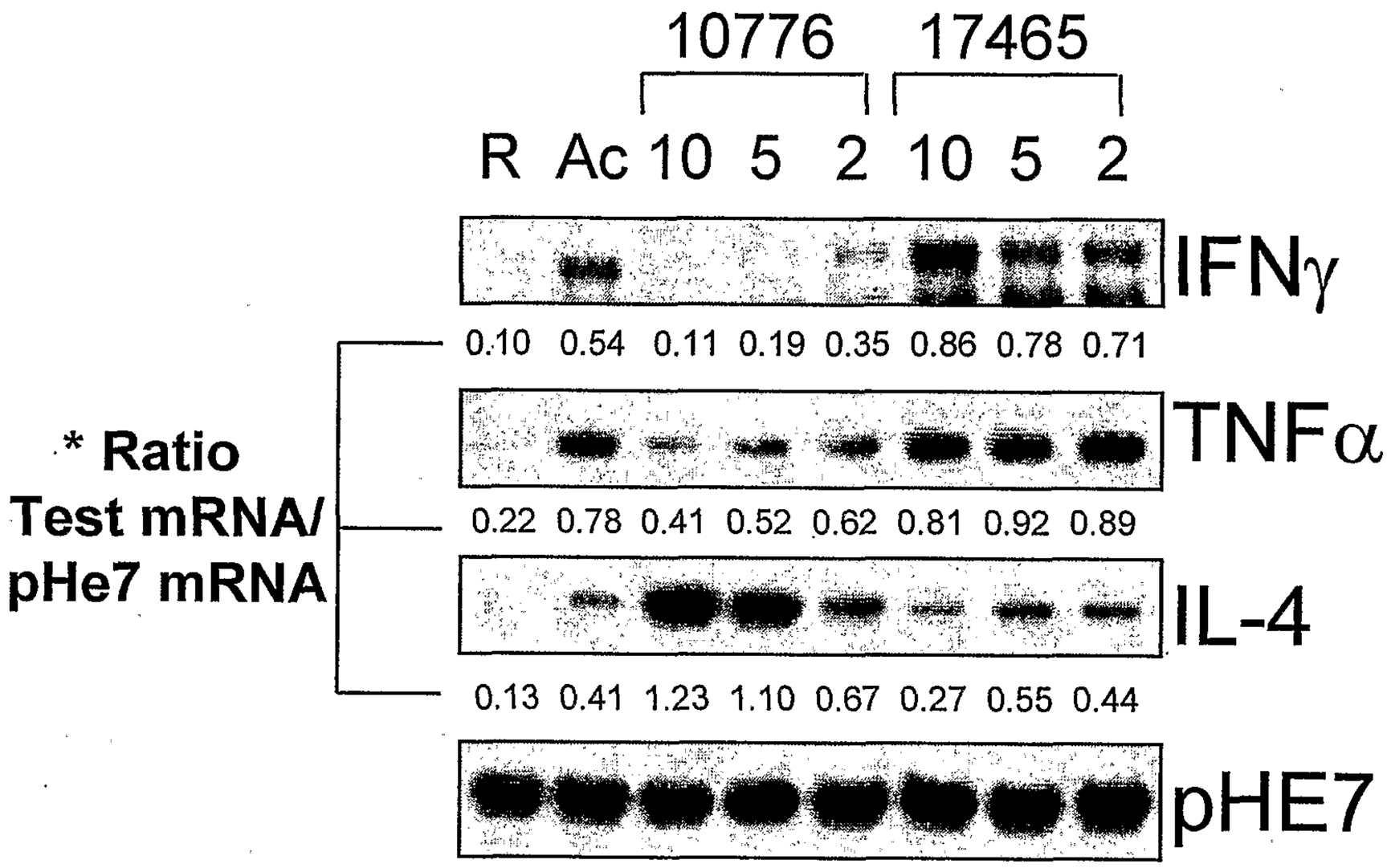

10776
17465
R Ac 10 5 2 10 5 2
IFNγ
0.10 0.54 0.11 0.19 0.35 0.86 0.78 0.71
TNFα
0.22 0.78 0.41 0.52 0.62 0.81 0.92 0.89
IL-4
0.13 0.41 1.23 1.10 0.67 0.27 0.55 0.44
pHE7
* Ratio
Test mRNA/
pHe7 mRNA

Figure 3

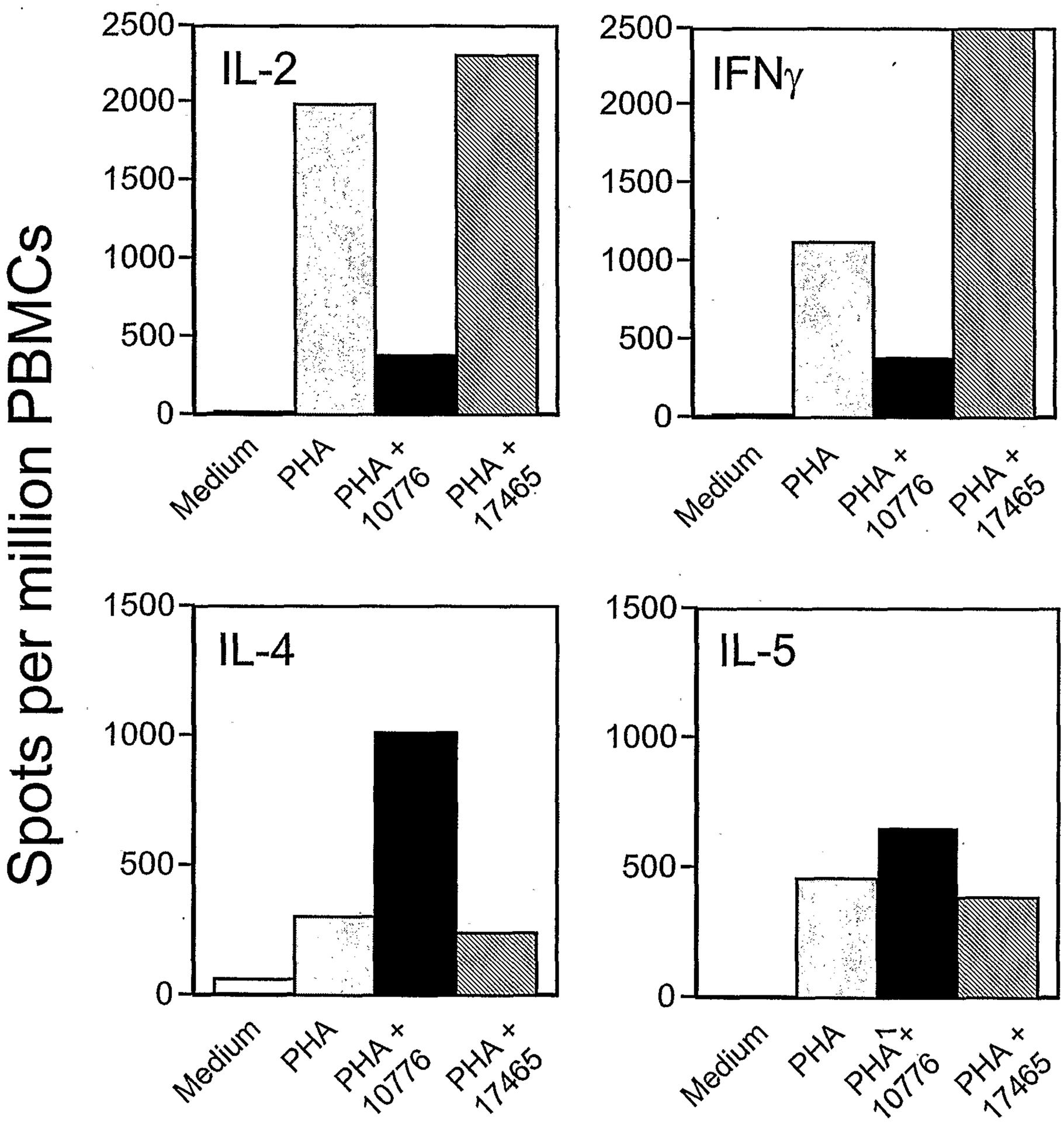
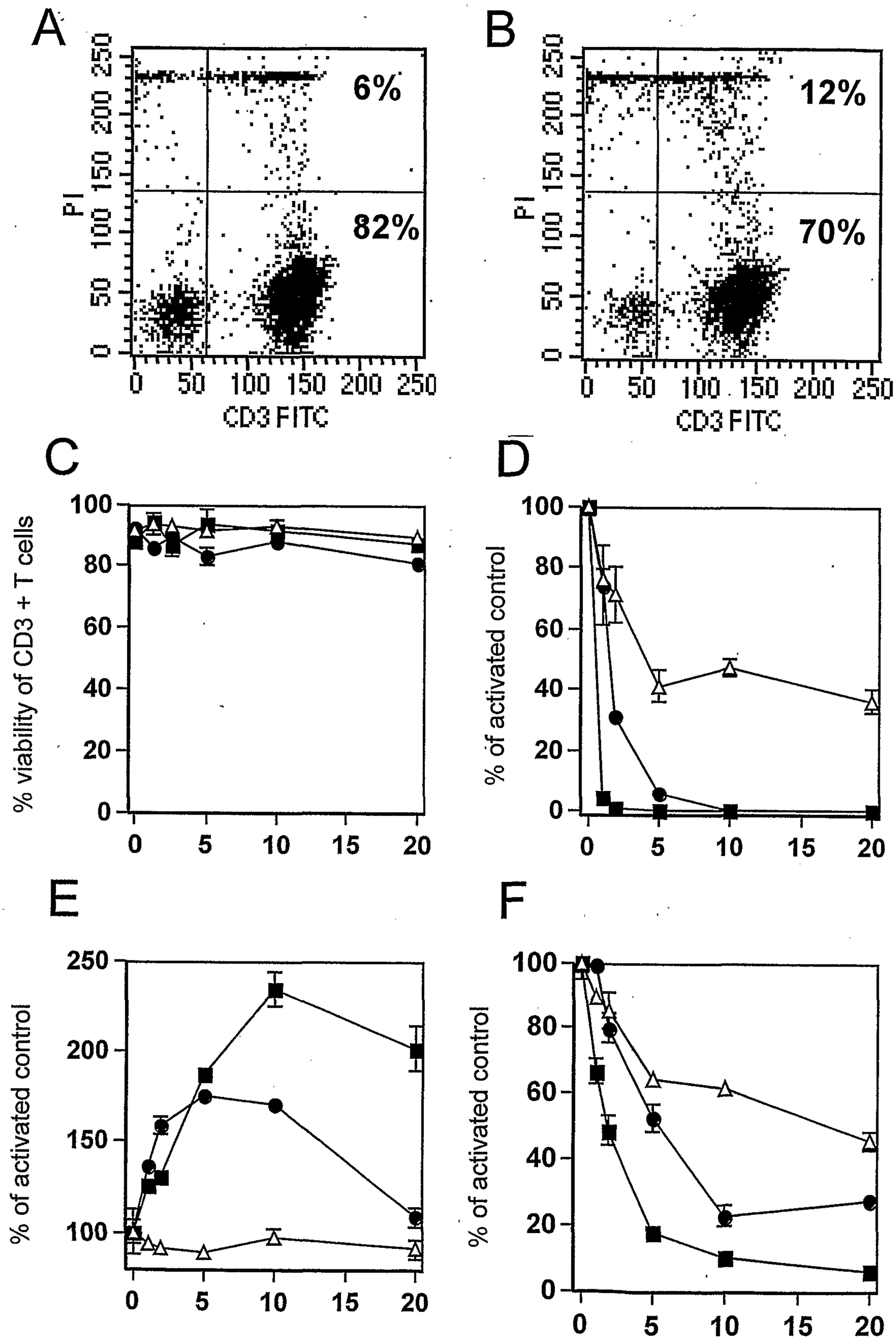
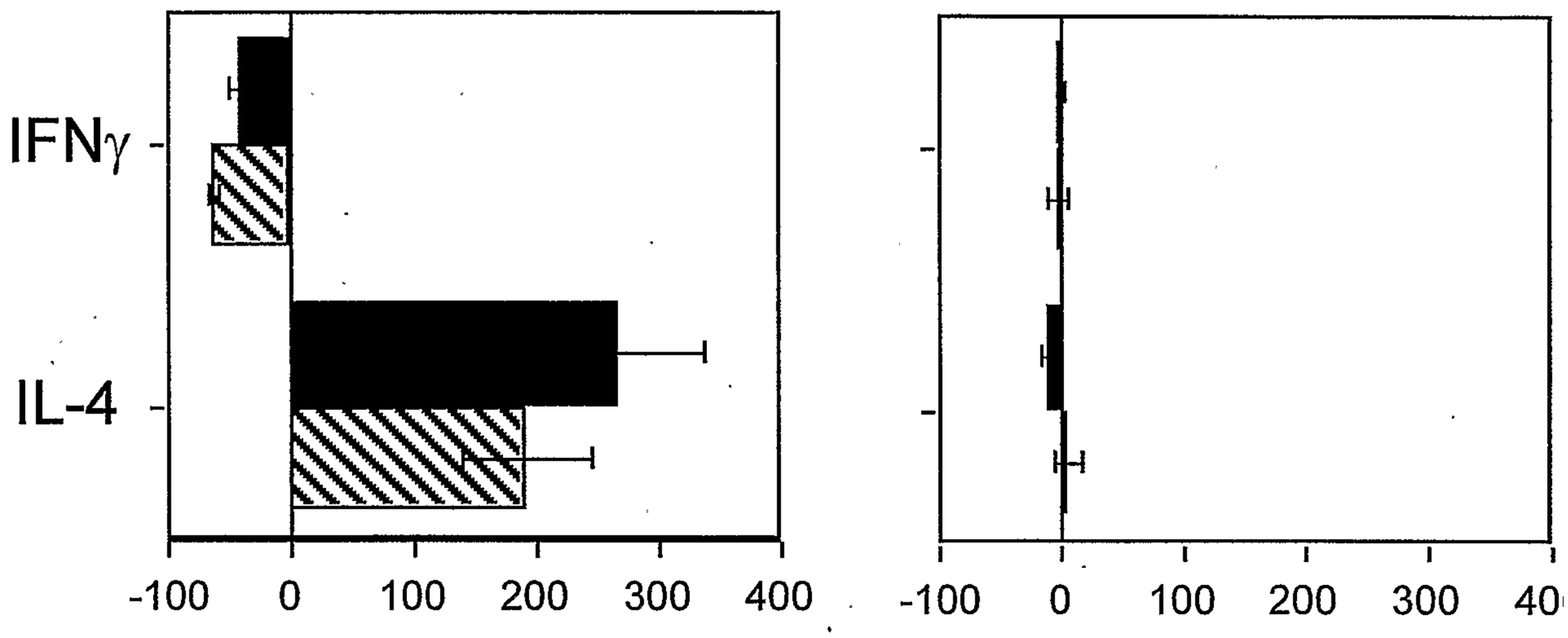

Spots per million PBMCs
IL-2
IFNγ
IL-4
IL-5
2500
2000
1500
1000
500
0
Medium
PHA
PHA + 10776
PHA + 17465

Figure 4


A
B
PI
CD3 FITC
6%
82%
12%
70%
C
D
% viability of CD3 + T cells
% of activated control
E
F
% of activated control
Nucleoside concentration (μM)

A

Figure 5


10776
17465
IFNγ
IL-4
-100
0
100
200
300
400
-100
0
100
200
300
40
% change compared to activated control

B

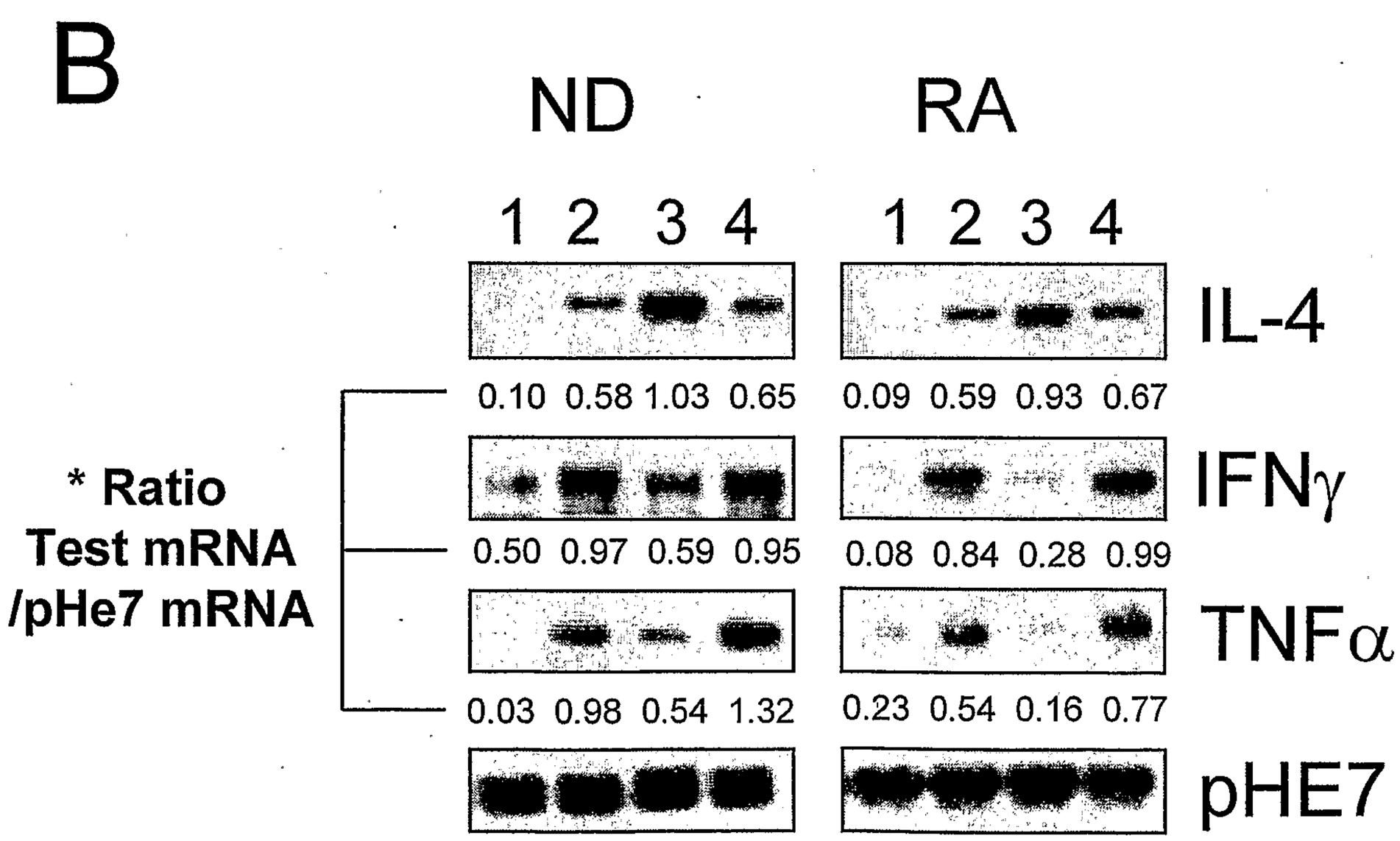
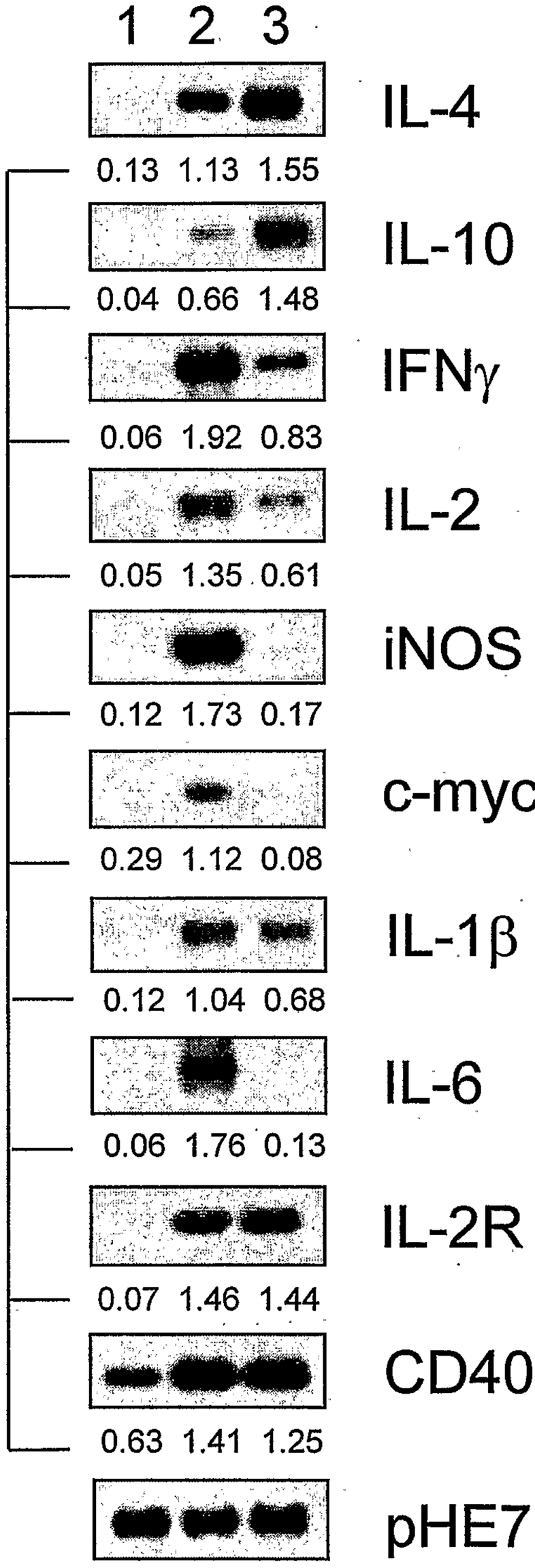

ND
RA
1 2 3 4
1 2 3 4
IL-4
0.10 0.58 1.03 0.65
0.09 0.59 0.93 0.67
IFNγ
0.50 0.97 0.59 0.95
0.08 0.84 0.28 0.99
TNFα
0.03 0.98 0.54 1.32
0.23 0.54 0.16 0.77
pHE7
* Ratio
Test mRNA
/pHe7 mRNA

Figure 6



*** Ratio Test mRNA/ pHE7 mRNA**

Figure 7

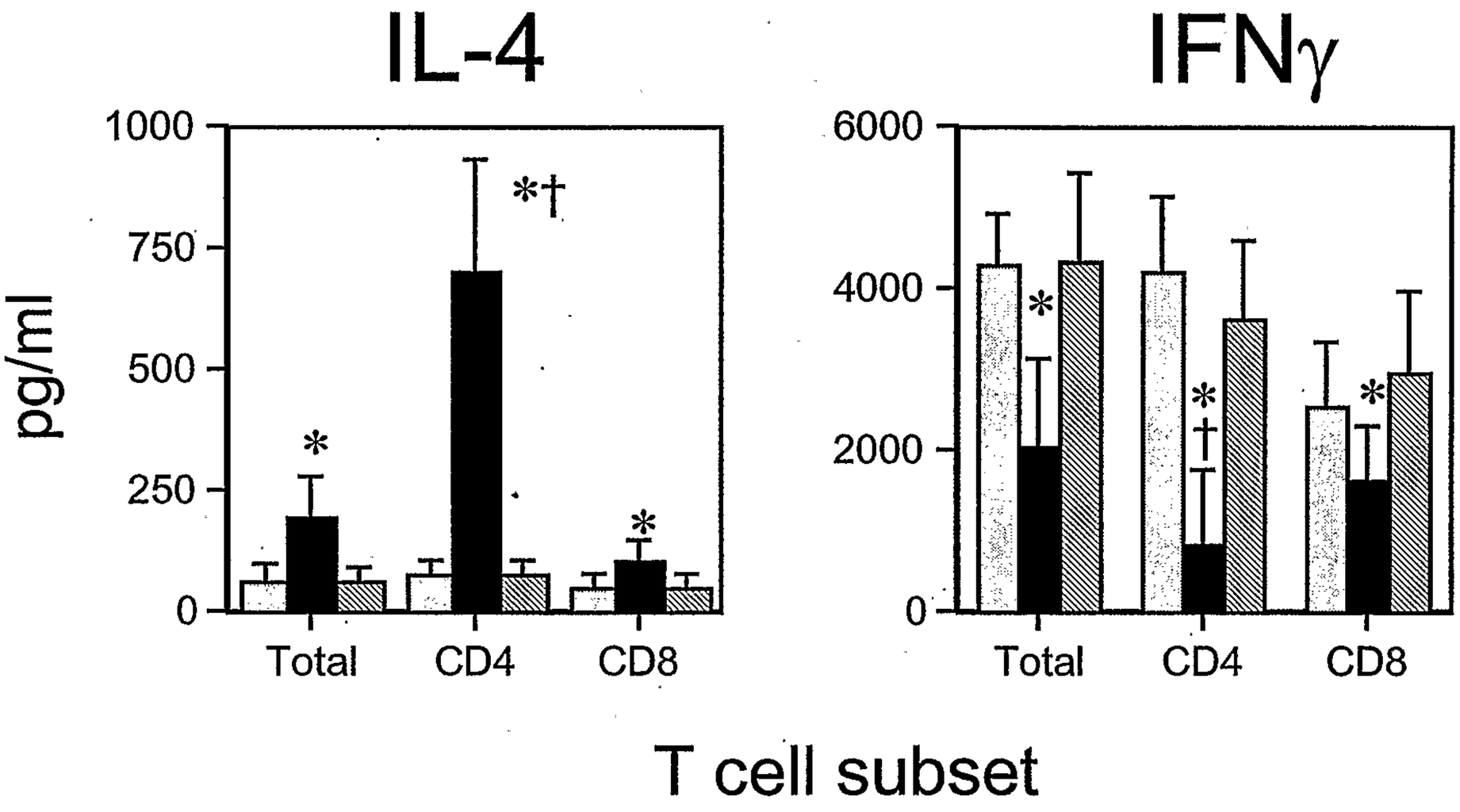


Figure 8

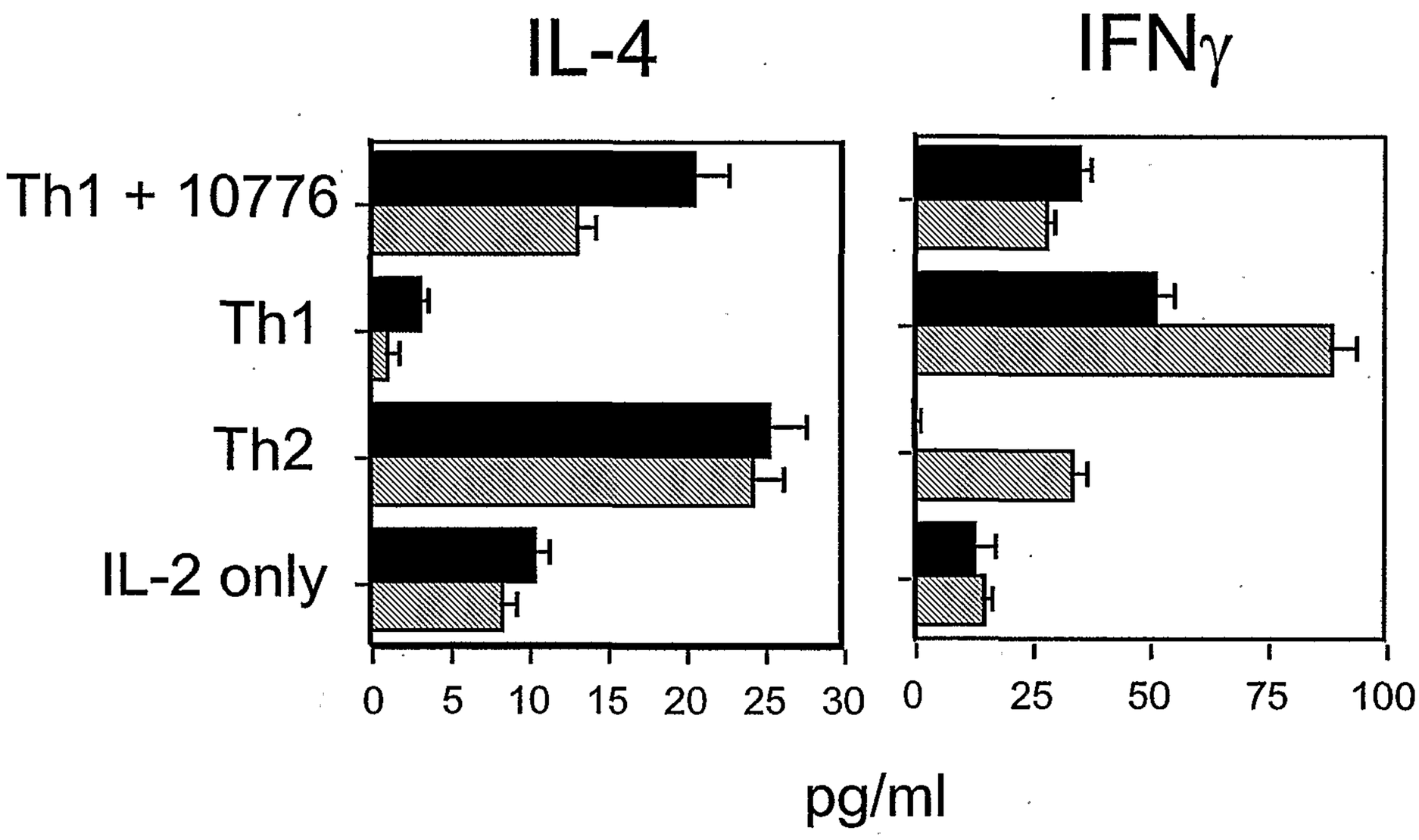
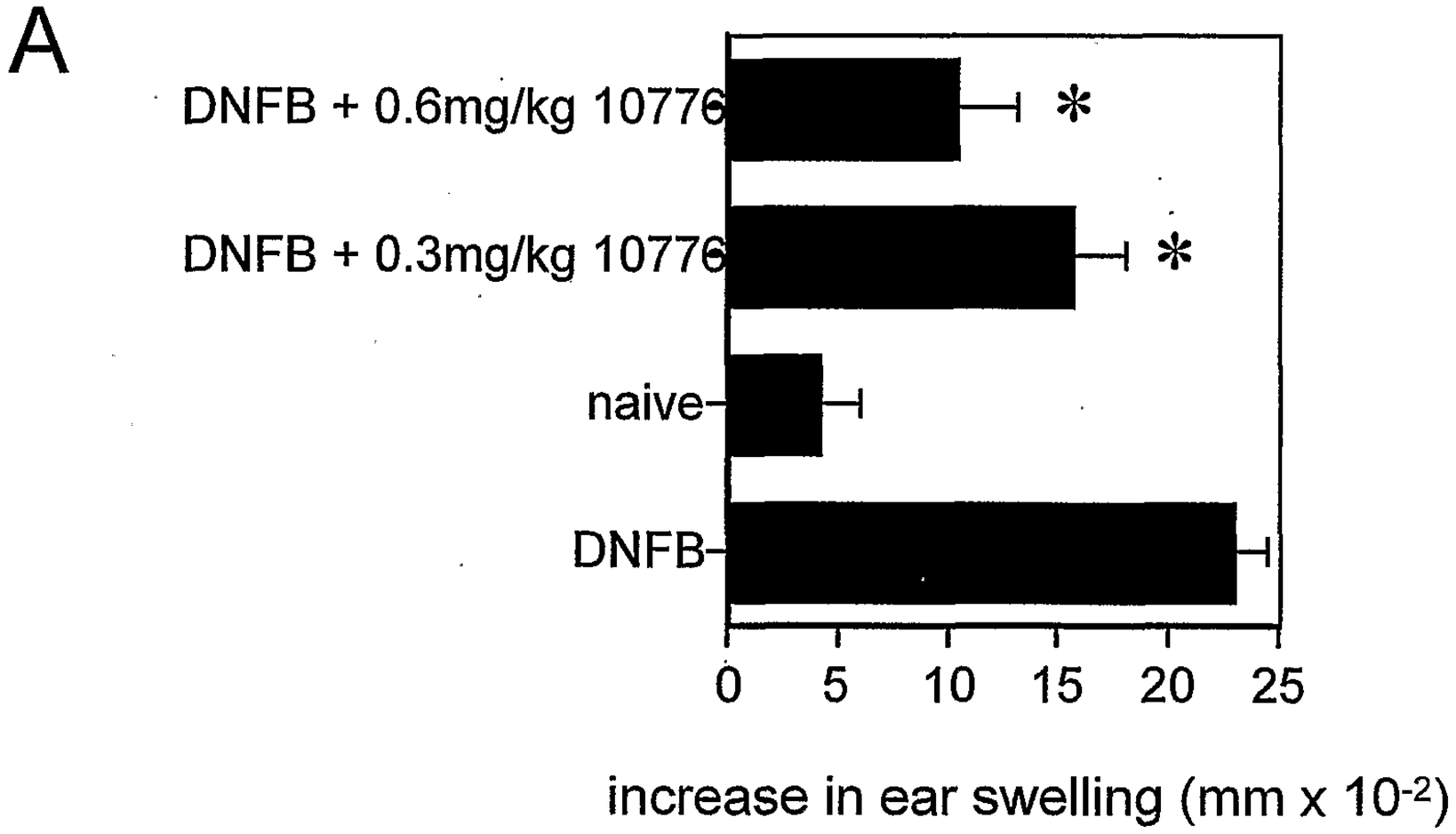


Figure 9


A
DNFB + 0.6mg/kg 10776
*
DNFB + 0.3mg/kg 10776
*
naive
DNFB
0
5
10
15
20
25
increase in ear swelling (mm x 10-2)

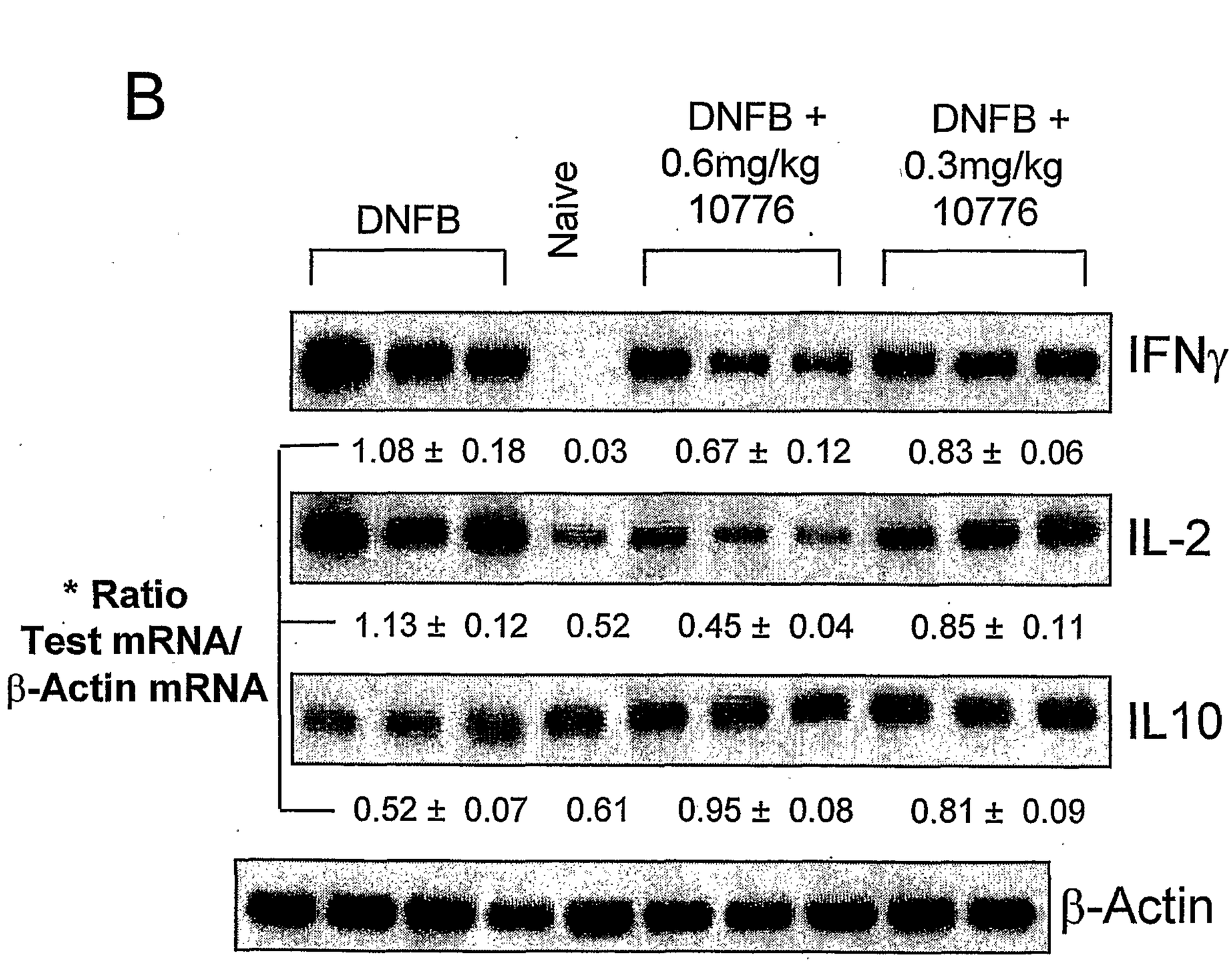
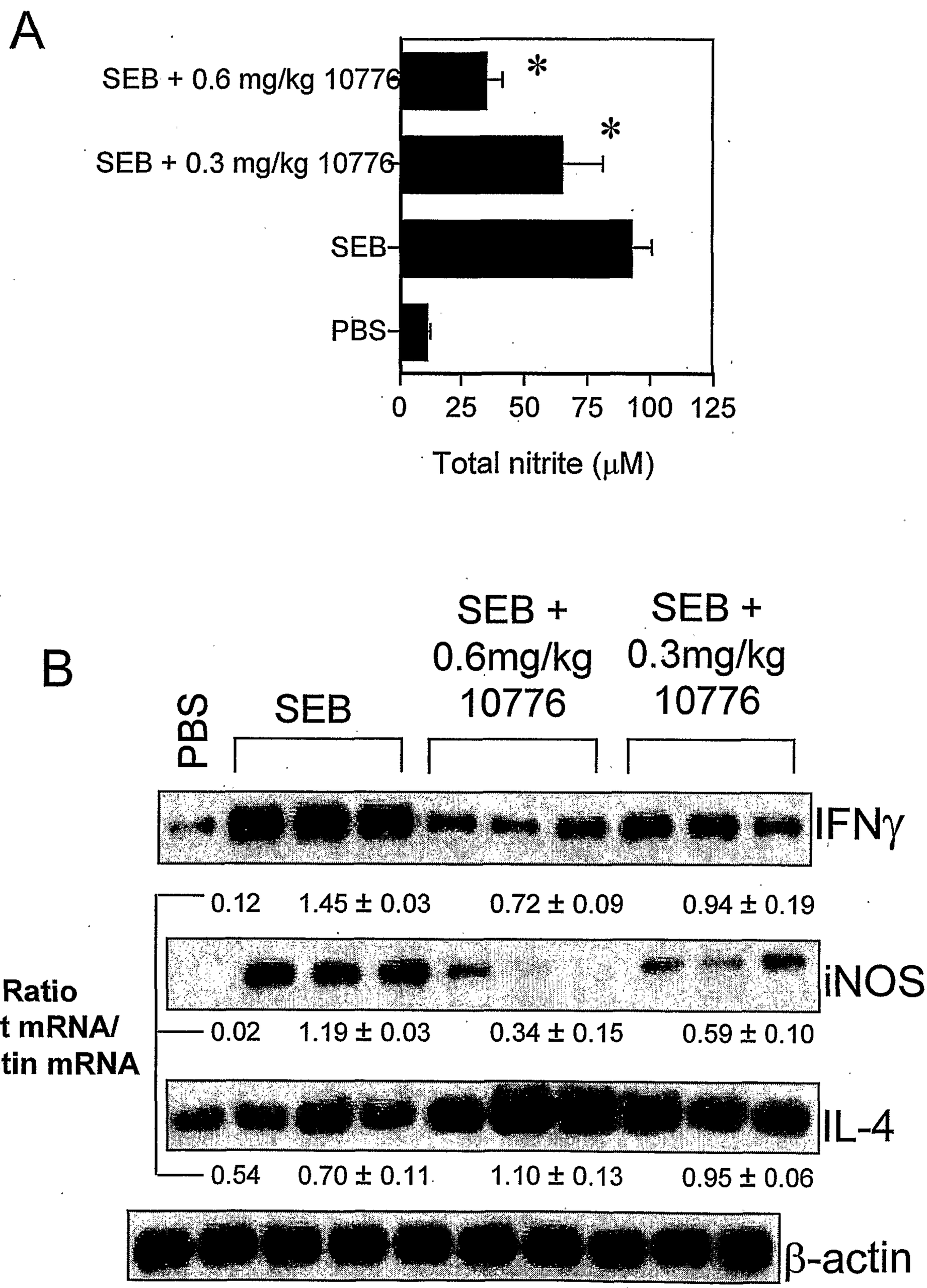

B
DNFB
Naive
DNFB + 0.6mg/kg 10776
DNFB + 0.3mg/kg 10776
IFNγ
1.08 ± 0.18
0.03
0.67 ± 0.12
0.83 ± 0.06
IL-2
1.13 ± 0.12
0.52
0.45 ± 0.04
0.85 ± 0.11
* Ratio Test mRNA/ β-Actin mRNA
IL10
0.52 ± 0.07
0.61
0.95 ± 0.08
0.81 ± 0.09
β-Actin

Figure 10


A
SEB + 0.6 mg/kg 10776
SEB + 0.3 mg/kg 10776
SEB
PBS
*
*
0
25
50
75
100
125
Total nitrite (μM)
B
PBS
SEB
0.6mg/kg 10776
0.3mg/kg 10776
IFNγ
0.12
1.45 ± 0.03
0.72 ± 0.09
0.94 ± 0.19
iNOS
0.02
1.19 ± 0.03
0.34 ± 0.15
0.59 ± 0.10
IL-4
0.54
0.70 ± 0.11
1.10 ± 0.13
0.95 ± 0.06
β-actin
* Ratio Test mRNA/ β-Actin mRNA