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(54) Titre : IMMUNOTHERAPIE DE RETABLISSEMENT D'IMMUNITE SUPPRIMEE
(54) Title: IMMUNOTHERAPY FOR REVERSING IMMUNE SUPPRESSION

(57) Abrégé/Abstract:
A method for overcoming immune suppression includes the steps of inducing production of naïve T cells and restoring T cell immunity. A method of vaccine immunotherapy includes the steps of inducing production of naïve T cells and exposing the naïve T cells to endogenous or exogenous antigens at an appropriate site. Additionally, a method for unblocking immunization at a regional lymph node includes the steps of promoting differentiation and maturation of immature dendritic cells at a regional lymph node and allowing presentation of processed peptides by resulting mature dendritic cells, thus, for example, exposing tumor peptides to T cells to gain immunization of the T cells. Further, a method of treating cancer and other persistent lesions includes the steps of administering an effective amount of a natural cytokine mixture as an adjuvant to endogenous or exogenous administered antigen to the cancer or other persistent lesions; preferably the natural cytokine mixture is administered in combination with thymosin $\alpha 1$.

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(54) Title: IMMUNOTHERAPY FOR REVERSING IMMUNE SUPPRESSION

(57) Abstract: A method for overcoming immune suppression includes the steps of inducing production of naïve T cells and restoring T cell immunity. A method of vaccine immunotherapy includes the steps of inducing production of naïve T cells and exposing the naïve T cells to endogenous or exogenous antigens at an appropriate site. Additionally, a method for unblocking immunization at a regional lymph node includes the steps of promoting differentiation and maturation of immature dendritic cells at a regional lymph node and allowing presentation of processed peptides by resulting mature dendritic cells, thus, for example, exposing tumor peptides to T cells to gain immunization of the T cells. Further, a method of treating cancer and other persistent lesions includes the steps of administering an effective amount of a natural cytokine mixture as an adjuvant to endogenous or exogenous administered antigen to the cancer or other persistent lesions; preferably the natural cytokine mixture is administered in combination with thymosin α_1.

IMMUNOTHERAPY FOR REVERSING IMMUNE SUPPRESSION

BACKGROUND OF THE INVENTION

1. TECHNICAL FIELD

The present invention relates to vaccine therapy for cancer patients and patients having persistent lesions, such as infections. More specifically, the present invention relates to a vaccine immunotherapy that immunizes patients, having immune suppression, to both endogenous and exogenous tumor peptides or proteins, as well as those derived from other persistent lesions.

2. BACKGROUND ART

It has become increasingly apparent that human cancers have antigens, which, if reacted upon by the host's immune systems, lead to tumor regression. These antigens have been defined by both serological and cellular immune approaches. This has led to the definition of both B and T cell epitopes (Sahin, U, et al., Curr Opin Immunol, 9:709-715 (1997); Van der Eynde, B, et al., Curr Opin Immunol, 9:684-693 (1997); Wang, RF, et al., Immunologic Reviews, 170:85-100 (1999)). Based upon these results, it has become a goal of cancer immunotherapists to induce regressions of tumors. However, historically, successful efforts have been sporadic and generally minor in frequency and magnitude.

A fundamental problem in the effort to immunize cancer patients is that the tumor-bearing state is associated with immunosuppressive mechanisms

derived from both the tumor and the host's disturbed immune system (Kavanaugh, DY, et al., Hematol-Oncol Clinics of North Amer., 10(4):927-951 (1996)), thereby making immunization difficult, and until now, impossible on a consistent basis. Immune suppression or depletion involves a reduced capacity of the immune system to respond. Such suppression can be drug or disease induced. The condition can be drug induced by treatment, virus induced as in AIDS, or induced by a disease state such as cancer. The immune system in this condition is effectively turned off.

A variety of tumor immunization strategies have been developed. However, all of these strategies are complex and deviate significantly from the conventional immunization strategies used for infectious diseases (Weber, J Tumor Medscape Anthology, 3:2 (2000)). One such tumor immunization strategy involves Theratope®, a Sialyl T_N polysaccharide mucin antigen conjugated with keyhole limpet hemocyanine and administered with Detox® mycobacterium adjuvant and low dose cyclophosphamide (Maclean GD, et al., J Immunother Emphasis Tumor Immunol., 19(4):309-316 (1996)). However, use of this vaccine in patients with metastatic breast and ovarian cancers has yielded major clinical responses in a low percentage of patients. A major response means greater than 50% tumor reduction.

Gene therapy also has been attempted using an adenovirus construct as an expression vector for genes expressing Papilloma virus. Peptide 16 has been used for immunization for patients with cervical cancer and has yielded major clinical responses in a low percentage of patients (Borysiewickz, LK, et al., Lancet, 347:1524-1527 (1996)).

Dendritic cell mediated therapy also has been attempted, wherein dendritic cells were pulsed with oligopeptide fragments of prostate specific antigens (PSA). Prostate specific membrane antigen (PSMA) has been used in patients with metastatic prostate cancer with major clinical responses in a low percentage of patients (Sanda, MG, et al., Urology, 52:2 (1999); Murphy, GP, et al., The Prostate, 38:43-78 (1999)).

Additionally, autologous tumors have been used with low dose cyclophosphamide and BCG to immunize cancer patients with malignant melanoma. However, few clinical responses were reported (Mastrangelo MJ, et al., Seminars in Oncology, 23(6):773-781 (1996)). Another strategy attempted included using MAGE antigens with a variety of vaccine adjuvants. Again, this has yielded few, if any, responses in patients with malignant melanoma.

Several U.S. Patents to Doyle, et al., (5,503,841; 5,800,810; 6,060,068; 5,643,565; 5,100,664) disclose methods of enhancing the immune response in patients using Interleukin-2 (IL-2). This method is disclosed for use in response to infectious diseases and primarily functions using antigens known to be immunogenic. Limited applicability was demonstrated. As disclosed above, the treatment of cancer is known to require different approaches. To date, treatment with IL-2 has shown minor effects in two cancers, renal cell and malignant melanoma (response rates less than 20%). It is generally considered ineffective in squamous cell head and neck cancer, cervical cancer, and in prostate cancer. Hence, it is not approved for these uses. It would therefore not be within the skill of one in the art to apply the method of the Doyle, et al. patents to the use of small peptides in the treatment of cancer.

It is important to contrast prevention with known "classic" antigens of complex structure and high molecular weights in healthy patients versus treatment (generally unsuccessful) with tumor antigens or peptides (generally unsuccessful) in immunosuppressed patients (generally unsuccessful). The first is easy and current viral vaccines attest to their efficacy. The latter is nearly impossible on a routine basis despite 30 years of intense effort.

It is important that this invention relates to, but not exclusively to, immunizing with endogenous peptide processed and presented by dendritic cells or endogenously administered to an environment (lymph node) where dendritic cells have been prepared and can present them to T cells effectively. This goal is considered by many immunologists to be insurmountable. Peptides are much too small to be effective immunogens, their half-life is short, they are often

nonmutated self-antigens to which the patient is immunologically tolerant, and gaining a response is tantamount to inducing autoimmunity.

In several of the above strategies, cellular and/or tumoral immunity to tumor-associated antigens has been induced (Weber, J Tumor Medscape Anthology, 3:2 (2000); Maclean, GD, et al., J Immunother Emphasis Tumor Immunol, 19(4):309-316 (1996); Borysiewickz, LK, et al.; Lancet, 347:1524-1527 (1996); Sanda, MG, et al., Urology, 52:2 (1999)). This is especially so in association with tumor regression. Nevertheless, the success rate of such treatments is negligible and inconsistent (<30%).

It would therefore be useful to develop a consistent and effective method of immunizing cancer patients.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a method for overcoming immune depression by inducing production of naïve T cells and restoring T cell immunity. That is, the present invention provides an immune restoration. The present invention further provides a method of vaccine immunotherapy including the steps of inducing production of naïve T cells and exposing the naïve T cells to endogenous or exogenous antigens at an appropriate site. Additionally, the present invention provides a method for unblocking immunization at a regional lymph node by promoting differentiation and maturation of immature dendritic cells at a regional lymph node and allowing presentation of processed peptides by resulting mature dendritic cells, thus exposing tumor peptides to T cells to gain immunization of the T cells. Additionally, the present invention provides a method of treating cancer and other persistent lesions by administering an effective amount of a natural cytokine mixture as an adjuvant to endogenous or exogenously administered antigen to the cancer or other persistent lesions.

DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention are readily appreciated as the same becomes better understood by reference to the following detailed descriptions when considered in connection with the accompanying drawings, wherein:

Figure 1 is a graph showing a comparison of NCM in different media utilizing continuous versus pulsed exposure to PHA;

Figure 2 is a graph showing the effect of cell concentration with continuous exposure to PHA;

Figure 3 is a bar graph similar to Figure 1 with PHA at twice the concentration (2 micrograms per ml);

Figure 4 is a graph of thymidine uptake versus units per ml of IL-2 relating to splenocytes;

Figure 5 is a graph similar to Figure 2 related to thymocytes;

Figure 6 is a graph showing ratio to control versus *in vivo* treatments for mice with involuted thymuses treated with IL-1, IL-2 or IL combinations, NCM, or saline;

Figure 7 is a graph also showing a comparison of treatment with recombinant IL-1, IL-2, IL-1 plus IL-2, and NCM;

Figure 8 is a graph demonstrating the effect of NCM treatment *in vivo* on splenocyte and thymocyte markers;

Figure 9 is a bar graph also demonstrating the effect of NCM treatment *in vivo* on splenocyte and thymocyte markers;

Figure 10 is a graph demonstrating splenocyte and splenocyte responses to *in vitro* media, including various recombinant interleukins or NCM after treatment *in vivo* with control media or NCM;

Figure 11 is a bar graph demonstrating the splenocyte and thymocyte responses *in vitro* to media, various interleukins, or NCM *in vivo* with control media or NCM;

Figure 12 demonstrates responses in splenocyte and thymocyte *in vitro* to ConA and PHA after treatment *in vivo* with control or NCM;

Figure 13 demonstrates responses in splenocyte and thymocyte *in vitro* to ConA and PHA after treatment *in vivo* with control or NCM;

Figure 14 is a bar graph showing node size in controls, and cancer controls or IRX-2 (NCM) treated populations with squamous cell head and neck cancer (H&NSCC);

Figure 15 shows two bar graphs, the first showing T cell area and the second showing density in controls and head and neck squamous cancer controls and patients treated with NCM (IRX-2);

Figure 16 shows two bar graphs showing B cell area and follicles in the three treatment groups;

Figure 17 shows a comparison of other cells and sinus histiocytosis in the three treatment groups;

Figure 18 is a graph showing node B and T and cancer B and T fit plot:

Figure 19 shows increases of lymphocyte populations in the blood of IRX-3 treated patients induced by a 10-day treatment of NCM plus thymosin α_1;

Figure 20 shows increases in naïve T cells ($CD_{45}RA^+$) and memory T cells in the blood of IRX-3 treated patients induced by a 10-day treatment of NCM plus thymosin α_1;

Figure 21 is a bar graph of thymocyte response *in vitro* to media (open bar). rIL-1 (closed bar), rIL-2 (cross-hatched), and NCM (diagonal lines) after treatment *in vivo* with saline, thymosin α_1 (5µg/animal/day), NCM (50 units IL-2 equivalence) and thymosin α_1 (5µg/animal/day)+NCM (50 units IL-2 equivalence); and

Figure 22 is a bar graph of splenocyte responses *in vitro* to media (open bar), rIL-1 (closed bar), rIL-2 (cross-hatched) and NCM (diagonal lines) after treatment *in vivo* with saline, thymosin α_1, NCM and thymosin α_1+ NCM as in Figure 21.

DETAILED DESCRIPTION OF THE INVENTION

Generally, the present invention provides methods for treating patients utilizing vaccine immunotherapy wherein the patients are immune suppressed. By immune suppressed, it is meant that the patient has reduced cellular immunity and thus impaired capacity to respond to new antigens.

T lymphocytopenia (low T cell levels in blood) is a diagnostic characteristic of cellular immune deficiency; impaired function of existing lymphocytes is the other characteristic. There is no generally accepted (clinically approved) way to treat T lymphocytopenia. Bone marrow transplants (± thymus transplants) have been used in cases of severe combined immunodeficiency (SCID - congenital, irradiation or chemotherapy induced). Recombinant IL-2 has been tried in AIDS with some effect by much toxicity.

There are two ways to make new T cells to attempt to correct T lymphocytopenia. One way, as in rIL-2 therapy, expands T cells already in the periphery, i.e., memory T cells ($CD_{45}RO$) (blood, lymph node and spleen). The other involves processing in the thymus of new T cells from bone marrow-derived precursors. This happens naturally in children but not in adults. These new cells are called recent "thymic émigrés" and have the surface marker of "naïve" T cells, i.e., $CD_{45}RA$. NCM therapy (plus thymosin α_1) results in the production of these new T cells as well as expanding pre-existing memory T cells.

More specifically, the present invention utilizes new discoveries relating to immunization to provide an immune response to antigens that is either endogenously or exogenously administered. Such antigens in the past have been believed to be immunogenic while others used in the present invention have been previously thought to be nonimmunogenic. Examples of such antigens are EADPTGHSY (melanoma) from MAGE-1 protein, EVDPIGHLY (lung carcinoma) from MAGE-3, EVDPIGHLY (lung carcinoma) from MAGE-3, and many others (see, Bellone, et al., Immunology Today, 20(10):457-462 (1999)).

The present invention utilizes several general newly derived method steps for obtaining immunization in subjects where such immunization was previously thought to be impossible. More specifically, the present invention provides a method for overcoming severe immune depression by inducing production of naïve T cells. The term "naïve" T cells is meant to mean newly produced T cells, even in adults, wherein these T cells have not yet been exposed to antigen. Such T cells at this stage are nonspecific yet capable of becoming specific upon presentation by a mature dendritic cell having antigen, such as tumor peptides, exposed thereon. Thus, the present invention replenishes or generates new T cells. This is generally accomplished by administering a natural cytokine mixture (NCM). The NCM includes IL-1, IL-2, IL-6, IL-8, IL-10, IL-12, δIFN, TNFα, and both G- and GM-CSF plus thymosin α_1. The amount and proportions of these constituents are detailed below. Preferably, about 150-600 units of IL-2 are contained in the NCM and 1.6 mg of thymosin α_1.

Preferably, the NCM with thymosin α_1 is injected around lymphatics that drain into lymph nodes regional to a lesion, such as tumor or other persistent lesions being treated. Perilymphatic administration into the lymphatics that drain into the lymph nodes, regional to the lesion, such as a cancer, is critical. Peritumoral injection has been associated with little response, even progression, and is thus contraindicated. A 10-day injection scheme is optimal and a 20-day injection protocol, while effective clinically, tends to reduce the TH1 response and shift towards a less desirable TH2 response as measured by lymphoid infiltration into the cancer. Bilateral injections are effective. Where radical neck dissection has occurred, contralateral injection is effective.

It is preferable to block endogenous suppression of T cells, such as caused by various cancer lesions. Blocking is effected by the codelivery of low dose cyclophosphamide and a nonsteroidal anti-inflammatory drug (NSAID). The NSAID of choice is indomethacin. While indomethacin is the most effective NSAID, it is also arguably the most toxic. Celebrex® and Vioxx®, Cox II NSAIDs, are less effective. Vioxx® can be more toxic, causing gastritis in many patients. Ibuprofen was effective but the histological responses were

characteristic of a TH2 rather than TH1 mediated response, this being less desirable. Side effects of NSAIDs are to be aggressively treated with proton inhibitors and a prostaglandin E analog. Zinc and multivitamins are useful agents to help restore T cell immunity. Treatment with contrasuppression and zinc without the NCM is ineffective.

In summary, the minimum regimen is perilymphatic treatment with the NCM plus thymosin α_1 combined with contrasuppression using cyclophosphamide and an NSAID. The alternative regimen is the previously mentioned regimen further including zinc and vitamins, possibly including the addition of selenium. Preferable dosing is zinc 50 to 75 mg. A standard multivitamin can be administered. The zinc can be an available gluconate.

In order to maximize clinical response, and for the greatest increase in survival rate, the degree and type of lymphocyte infiltration is important. Granulocyte or macrophage infiltration of a 90:10 ratio is optimal. T and/or B cell infiltration preferably is diffuse and intense and not peripheral. Light infiltration of less than 20% is not associated with a robust clinical response. Tumor reduction and fragmentation in the histological samples are preferred in reflecting a good response.

Lymph node changes key to good response involve at least five aspects. Lymph node enlargement, and not just reversal of tumor induced reduction of size but overall increase in size compared to normal, is preferred. Increased T and B cell areas indicate an immunization. The present data indicate that sinus histiocytosis (SH) is an accumulation of nonactivated mature dendritic cells (CD_{68}+CD_{83}+DC) that have ingested and processed tumor antigens but are unable to mature and present these tumor peptides to naïve T cells capable of stimulating TH1 and TH2 effective cells that lead to cytotoxin T and B cells. Reversal of SH and the activation of DC is a key to the invention.

Thus, the present invention provides for unblocking immunization at a regional lymph node by promoting maturation and activation of dendritic cells in a regional lymph node and thus allowing presentation by resulting mature dendritic cells of small peptides, generally nine amino acids in length, to T cells

to gain immunization of the T cells, as discussed in greater detail below. Additionally, induction of mature dendritic cells is required. Finally, mobilization of peripheral blood T lymphocytes in T lymphocytopenic patients in the presence of induction of naïve T cells capable of responding to dendritic cells presenting endogenous tumor peptides is desired (see, Sprent, et al., Science, 293:245-248 (2001)).

In view of the above, the key mechanistic features of the present invention are the *in vivo* maturation of dendritic cells resulting in effective peptide antigen presentation. Based on the examples presented herein, increases in $CD_{45}RA$ positive naïve uncommitted T cells have been found. This leads to T and B cell clonal expansion, creating immunity in the patient. The resulting infiltration into tumors by hematogenous spread leads to robust tumor destruction. The result, as found in the data herein, is increased survival due to immunologic memory (see, Sprent, et al.).

It is predicted logically that exogenously provided synthetic or extracted tumor peptides (see, Bellone, et al.) can be delivered into the preprimed or coprimed regional or distal lymph node and yield tumor antigen specific T cells, with or without B cells. Examples are set forth below. In view of the above, it can be concluded that the action of NCM and thymosin α_1 plus other agents is useful as for any tumor antigens (synthetic and endogenous, peptides and proteins). Many of these peptides are not normally immunogenic and only when presented by matured, activated dendritic cells, will they be effective in immunizing naïve T cells. Thus, the appearance of an immune T cell means, *de facto*, that a dendritic cell has been made or allowed to work properly. Also *de facto*, dendritic cell activation and maturation are considered key factors in cancer immunodeficiency, as well as the well-known defects in T cells, such as a decreased number and function with anergy and presumed apoptosis.

Referring more specifically to the protocol and medicant delivered in accordance with the present invention, the invention utilizes the natural cytokine mixture plus thymosin α_1 to immunize patients, such as cancer patients, as well as patients with other lesions or antigen producing disease conditions. More

specifically, the present invention utilizes a method of enhancing the immune response of cancer patients to a cancer by administering an effective amount of a composition containing therein the NCM plus thymosin α_1 and a tumor-associated antigen, the NCM plus thymosin α_1 acting as an adjuvant to produce the immune response.

The tumor-associated antigen can be either an endogenously processed tumor peptide preparation resident in regional nodes of patients with cancer, or in conjunction with an exogenously administered tumor antigen preparation in or near these nodes. Tumor peptides, as well as antigens, are included herein even though peptides are not expected to be immunogenic where tumor-associated protein antigens would be more likely so since they are complete.

In the preferred embodiment, the composition of the present invention involves the administration of the NCM plus thymosin α_1 plus a tumor-associated or specific antigen, as defined below with low doses of cyclophosphamide, a cyclooxygenase inhibitor, and other similar compounds that have been shown to further increase the effects of the composition of the present invention. The NCM and thymosin α_1 combination is an adjuvant creating an immune response to antigens not otherwise found to be effectively antigenic. Moreover, this adjuvant effect has been accomplished in patients who are severely immune deficient.

To clarify and further define the above, the following definitions are provided. By "adjuvant," it is meant a composition with the ability to enhance the immune response to a particular antigen. To be effective, an adjuvant must be delivered at or near the site of antigen. Such ability is manifested by a significant increase in immune mediated protection. Enhancement of immunity is typically manifested by a significant increase (usually greater than 10-fold) in the titer of antibody raised to the antigen. Enhancement of cellular immunity can be measured by a positive skin test, cytotoxic T cell assay, ELISPOT assay for δIFN or IL-2, or T cell infiltration into the tumor as described herein.

By "tumor associated antigen," it is meant an analogous protein or peptide (which were previously shown to work by pulsing of dendritic cells *ex*

vivo) or other equivalent antigen. This can include, but is not limited to, PSMA peptides, MAGE peptides (Sahin, U, et al., Curr Opin Immunol 9:709-715 (1997); Wang, RF, et al., Immunologic Reviews 170:85-100 (1999)), Papilloma virus peptides (E6 and E7), MAGE fragments, or other similar antigens. Previously, these antigens were not considered to be effective in treating patients based either on their size, i.e., they are too small or that they were previously thought to not have the immunogenic properties (i.e., self- antigens).

NCM, a nonrecombinant cytokine mixture, is defined as set forth in U.S. Patent Nos. 5,632,983 and 5,698,194. Briefly, NCM is prepared in the continuous presence of a 4-aminoquinolone antibiotic and with the continuous or pulsed presence of a mitogen, which in the preferred embodiment, is PHA.

Pooled lymphocytes, generally from the buffy coat, free of neutrophils and erythrocytes from HIV-negative, hepatitis virus-negative multiple donors are used to produce a mixed lymphocyte response (MLR). Further, in a preferred embodiment, up to 50 donors are used each time to produce the mixture to ensure that the MLR response is constant for each preparation and to even out variation.

In an alternative embodiment, autologous lymphocytes are used to generate the NCM. In these cases, the patient does have to be virus-free. Further, if autologous lymphocytes are used, they can be returned to the patient as needed. In an alternative embodiment, animals can be the cell source for veterinary uses.

The lymphocytes are cultured in the presence of immobilized mitogens in a tissue culture vessel. In a preferred embodiment, the mitogen is immobilized on surface activated cell culture flasks for selection of cell subsets (AIS MICROCELLECTOR™ T-25 plates) as described in the manufacturer's instructions. However, other methods of immobilizing mitogens on the surface of the culture vessel such as methods incorporating other "panning" techniques or coupling to sepharose 4B beads could be used as are well known in the art of cell isolation. The use of immobilizing cells for selection is well known in the art.

The mitogens are generally selected from lectins and monoclonal

antibodies that stimulate lymphocytes to produce cytokines. In a preferred embodiment, phytohemagglutinin (PHA) or OKT3 (Orthoclone®, Ortho Pharmaceuticals) are used. Other lectins such as concanavalin A (ConA) or pokeweed mitogen that stimulate B cells can be used. Monoclonal antibodies to T cell receptors such as CD_2, CD_{28}, CD_{45} can be used as mitogens. Anti- CD_{28} and CD_{45} antibodies are reported to be hyperproducers of IL-2 (Deans, et al., (1989); June, et al., (1989). Further, antilymphocyte globulin (ALG) has mitogenic activity for T cells. In addition, combinations of mitogens can be used to activate a combination of lymphocyte subpopulations. PHA is used in the preferred embodiment and is coated at a starting concentration of about 25 μg/ml.

The lymphocytes are incubated for 24 to 28 hours in a serum-free media with continuous exposure to the mitogen, i.e., no washings. In a preferred embodiment, the media is either X vivo-10 or X vivo-15 media (Whittaker). This is a serum-free and FDA-approved media for IL-2/LAK infusions in patients as set forth in the manufacturer's brochure. Serum-free media capable of supporting human lymphocyte proliferation, such as RPMI-1640 (Sigma), can be also used.

The media also contains a 4-aminoquinolone antibiotic. In the preferred embodiment, the antibiotic is Ciprofloxacin. The antibiotic is used to maintain sterility and to hyperproduce lymphokines. Ciprofloxacin and related antibiotics have been reported to increase IL-2 and other cytokines in the presence of the soluble mitogen and serum (Riesenbeck, et al. (1994)). They have not been reported to be effective in the absence of serum. Ciprofloxacin is used in the preferred embodiment at a concentration of from about 20 to about 200 μg/ml and more preferably, at a concentration of about 80 μg/ml.

The supernatant is removed and is the source of the NCM of the present invention. The supernatant is free of the mitogen as shown in Example 1. In animal and initial human studies. it does not have to be concentrated.

Human serum albumin (HSA) can be added to stabilize the NCM in the supernatant. HSA is used instead of serum albumin from a nonhuman source

because HSA has been approved by the FDA for human use.

A cytokine profile of the supernatant is established utilizing the following assays. The interleukin content of the supernatant is confirmed by bioassay for IL-2 and by ELISA's for other interleukins, CSFs, TNFs, and IFNs. Sterility is tested and endotoxin measured by limulus lysate assay. Specifically, the following assays and kits are used in a preferred embodiment: INF-γ ELISA (ENDOGEN), IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, GM-CSF, G-CSF, and TNF-α ELISAs (R&D Systems). The IL-2 bioassay of Gillis, et al., 1978, is expressed as units/ml compared to a known standard of IL-2 (Schiapparelli Biosystems, Inc., Fairfield, NJ).

In the preferred embodiment, wherein PHA is used as the mitogen, the cytokine profile for the supernatant has a profile of:

CYTOKINE	AMOUNT
IL-1	10-2000 pg/ml
IL-2	100-500 units/ml
IL-6	250-10,000 pg/ml
IL-8	12,000-100,000 pg/ml
IL-12	100-10,000 pg/ml
IFN-γ	50-15,000 pg/ml
TNF-α	50-15,000 pg/ml
CSF-G	50-1,500 pg/ml
CSF-GM	10-1,500 pg/ml
IL-3/IL-4/IL-7	Trace Amounts

Immobilization of the mitogen produces a higher yield of NCM than does pulse techniques. For example, production of interleukins by a pulse technique with PHA in serum-free media yielded IL-2 at 0-20 units/ml media (U.S. Patent Nos. 4,390,623 and 4,464,355). However, the present method allows an increased production with a pulse technique by adding a 4-aminoquinolone

antibiotic to the serum-free media to hyperinduce interleukin and yielded about 8-140 units/ml of IL-2. As predicted by the animal studies, this preparation, characterized as a natural interleukin mixture (NIM), at 200 units IL-2/dose, increased T lymphocyte counts in blood of lymphopenic patients with head and neck cancer (Hadden, et al. (1994)). This result has not been reported at doses greater than 5,000 times the amount of IL-2 in NCM. Thus, it is important to note that the dose IL-2 equivalent for NCM is used as an index of its potency and is not meant to imply that the total biological activity of NCM is that of only IL-2.

In the preferred embodiment of the present invention, utilizing continuous exposure to the mitogen by immobilization and the presence of a 4-aminoquinolone antibiotic, the NCM that is generated generally contains IL-2 at 100-353 units/ml (an index of the potency of the preparation). In the less preferred embodiment, the invention can be practiced with the continuous presence of 4-aminoquinolone antibiotic and a pulsed presence of the mitogen, producing NIM. This combination produces a level of cytokines greater than the other prior art methods with a pulsed mitogen only, but does not produce the levels seen with the preferred embodiment of the present invention.

The invention can be also practiced with a natural nonrecombinant interleukin mixture (NIM) that is produced with the continuous presence of 4-aminoquionlone antibiotic but with only a pulsed presence of a mitogen such as PHA. Other immunomodulating natural nonrecombinant cytokine preparations such as an NIM preparation, also can be used in the present invention. The various preparations are compared by IL-2 content, and the dosage is referred to as IL-2 equivalents.

Thymic peptides are used in the present invention coadministered with the immunomodulator-cytokine preparations. Thymosin α_1 (T-α_1), or its analogs and fragments, is used in the preferred embodiment of the present invention. In addition, other thymic peptides, such as thymosin α_{11} and prothymosin and their analogs can be used. Thymic peptides, analogs, and fragments that contain the thymosin α_1 sequence can be also used.

An analog will be generally at least 70% homologous over any portion that is functionally relevant. In more preferred embodiments, the homology will be at least 80% and can approach 95% homology to the thymic peptide, particularly the thymosin α_1 sequence. The amino acid sequence of an analog may differ from that of the thymic peptide when at least one residue is deleted, inserted, or substituted. Differences in glycosylation can provide analogs. Analogs as set forth in U.S. Patent Nos. 4,116,951; 4,353,821; 4,466,918; 4,470,926; 4,612,365; and 4,910,296 are examples of such analogs and can be used in the present invention.

A partially characterized NCM has been previously shown to be effective in promoting T cell development and function in aged, immunosuppressed mice. Thymosin α_1 also protected T cell development and function in aged immunosuppressed mice and the combination of NCM plus thymosin α_1 was dramatic in its action to produce new T cells in the spleen (U.S. Patent No. 5,698,194). Upon administering this NCM to immunosuppressed patients with head and neck cancer, it is demonstrated in this application for the first time that the mobilization of T lymphocytes in the blood of cancer patients treated with the NCM produces an increase in immature, naïve T cells bearing both CD_2 and $CD_{45}RA$. This is one of the first demonstrations that adult humans can generate naïve T cells. It is described in this application that NCM plus thymosin α_1 produces increased "naïve" T cells in irradiated patients resistant to NCM treatment. Previous references (Mackall, et al., New Eng J Medicine 332:143-149 (1995); and a review by Mackall, Stem Cells 18:10-18 (2000)) discuss the inability to generate new T cells in adults but not children, and discuss the problem of trying to replenish T cells following cancer chemotherapy and/or radiotherapy. In general, there is the dogma that new T cells are not generated in the adult human. However, following bone marrow transplantation for intense chemotherapy, there has been evidence that new T cells can be generated in the adult. No molecular therapy to date has been able to achieve this, although an increase in lymphocyte counts have been achieved with prolonged and intense therapy with recombinant interleukin-2 in patients infected by HIV.

These have not been clearly demonstrated to be thymus-derived T cells and are presumably an expansion of pre-existing peripheral T cells.

Previously, Cortesina, et al., employed a natural IL-2, perilymphatically, in patients with head and neck cancer and induced several tumor regressions (Cortesina G, et al., Cancer 62:2482-2485 (1988)) with some tumor infiltration with leukocytes (Valente, G, et al., Modern Pathol 3(6):702-708 (1990)). Untreatable recurrences occurred and the response was termed nonspecific and without memory and thus nonimmunologic (Cortesina, G, et al., Br J Cancer 69:572-577 (1994)). The repeated attempts to confirm the initial observations with recombinant IL-2 were substantially unsuccessful (Hadden, J.W., Int'l J Immunopharmacol, 11/12:629-644 (1997)).

The method of the present invention involves using NCM plus thymosin α_1 with local perilymphatic injections or other injections that are known to those of skill in the art to provide sufficient localization of the immunotherapy compound. In the preferred embodiment, the injections take place in the neck, but can be applied in other locations as required by the disease to be treated. This treatment induced clinical regressions in a high percentage of patients who also showed improved, recurrence-free survival (Hadden, JW, et al., Arch Otolaryngol Head Neck Surg 120:395-403 (1994); Meneses A, et al., Arch Pathol Lab Med 122:447-454 (1998); Barrera, J, et al., Arch Otolaryngol Head Neck Surg, 126:345-351 (2000); Whiteside, et al., Cancer Res 53:564-5662 (1993)), observed that in head and neck cancer, tumoral injection of recombinant interleukin-2 produced a T cell lymphocyte infiltrate, but without significant clinical responses. Peritumoral injection of Multikine (Celsci website) in combination with perilymphatic injection in up to 150 patients resulted in significant tumor responses, i.e., greater than 50% tumor reduction in only 11 patients, making their response rate less than 10% in contrast to the high degree of response observed in the present studies, 40%. In addition, they noted 50% nonresponders where Applicant has observed only 20%.

Applicant has observed that peritumoral and intratumoral injection can be associated with progression of disease even in patients who initially have had a

positive response to the NCM protocol, thus undoing its benefit. Peritumoral injection is thus contraindicated and is excluded as part of the present invention. This has led Applicant to the interpretation that the tumor is not the site of immunization and the present application presents documentation that the regional lymph node is the site of immunization. Then, unpublished analysis of regional lymph nodes revealed data that indicated that the regional lymph node is the site of immunization to postulated tumor antigens (Figures 14-18). With the identification of a number of different tumor antigens, it has been a conundrum over the last decade that given the presence of such antigens, they have not been employed effectively in immunization protocols. Sporadic positive examples have been reported, but in the main, the data are negative. The problem of antigen presentation has been focused on in the last decade and the dendritic cell has emerged as a critical player in the presentation of small peptides derived from tumors (DeLaugh, et al., Curr Opin in Immunol 12:583-588 (2000); Buchereau, et al., Ann Rev of Immunol 18:767-811 (2000); Albert, et al., Nature 392:86-89 (1998)).

In brief, in order for tumor antigens to be properly antigenic, they must arrive from an apoptotic rather than a necrotic tumor cell (Albert reference in Nature). They need to be captured by immature dendritic cells that have the morphology of large histocytes. These immature dendritic cells process (endocytosis, phagocytosis and digestion) and evolve into mature dendritic cells that display peptide fragments (generally nine amino acids) of the digested antigen in the MHC groove for presentation to T cells. T cells, in order to respond, must have antigen presented to them in the MHC groove plus various costimulatory signals (Banchereau and DeLaugh).

Investigators, such as Murphy, et al. (1999), have utilized dendritic cells generated in culture and then pulsed with tumor antigens and have achieved a small degree of success in immunizing patients against prostate specific membrane antigen peptides. Unfortunately, this approach of pulsing dendritic cells is cumbersome and has been rather inefficient. Herein, Applicant has shown that the cells present in the lymph node sinuses, which accumulate in

cancer, are cells of the lineage of dendritic cells. Following the *in vivo* treatment with the NCM protocol, these cells disappear and antigen ultimately then becomes immunogenic for T cells. They are able then to respond to the tumor. Therefore, a critical aspect of this invention is being able to generate a microenvironment in the regional lymph node that allows effective antigen processing and presentation. The immunization derives T cells able to traffic to the lesion and destroy tumors. This is *de facto* demonstration of adequate antigen processing by dendritic cells. Additionally, none of the patients treated with NCM developed distant metastasis that is expected in up to 15% clinically and up to 50% pathologically. This indicates that a systemic immunity rather than merely a local immunity has been induced by the treatment. This is a drastic improvement over the compositions in the prior art, because the prior art compositions, at best, were inconsistently effective against metastatic disease. The ability of the composition of the present invention to create systemic immunity allows more effective and efficient treatment of a patient.

The literature (Hadden, JW, Int'l J Immunopharmacol 11/12:629-544 (1997); Hadden, JW, Immunology and Immunotherapy of Breast Cancer: An Update, Int'l J Immunopharmacol 21:79-101 (1999)) has indicated that for both SCC and adenocarcinomas, the two major types of cancer, regional lymph nodes reflect abnormalities related to the tumor, including sinus histiocytosis, lymphoid depletion and often the presence of anergic tumor- associated lymphocytes (capable of reacting to tumor cells with *ex vivo* expansion and recovery using IL-2). Then, with metastases, lymphoid depletion and depressed function occur. Additionally, uninvolved cervical lymph nodes of such patients have shown a reduction in average size and an increase in sinus histiocytosis associated with head and neck cancers. (See Figures 14 through 17).

The composition of the present invention involves the natural cytokine mixture plus either endogenous or exogenous tumor-associated antigen. Additionally, low doses of cyclophosphamide, cyclooxygenase inhibitors, zinc, and other similar compounds have been shown to further increase the effects of the composition of the present invention.

Immunization for treatment of patients with cellular immune deficiencies associated with cancer, HIV infection, aging, renal transplants, and other such deficiencies can be achieved with the composition of the present invention.

Administration and protocols for treatment follow.

Delivery of gene products/synthetic antigens:

The compounds of the present invention (including NCM) and exogenous antigens are administered and dosed to achieve optimal immunization, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, and body weight. The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve immunization, including but not limited to, tumor reduction, fragmentation and infiltration, survival rate or more rapid recovery, or improvement or elimination of symptoms.

In the method of the present invention, the compounds of the present invention can be administered in various ways. It should be noted that they can be administered as the compound or as a pharmaceutically acceptable salt and can be administered alone or as an active ingredient in combination with pharmaceutically acceptable carriers, diluents, adjuvants, and vehicles. The compounds can be administered intra or subcutaneously, or peri or intralymphatically, intranodally or intrasplenically or intramuscularly, intraperitoneally, and intrathorasically. Implants of the compounds also can be useful. The patient being treated is a warm-blooded animal, and in particular, mammals including man. The pharmaceutically acceptable carriers, diluents, adjuvants, and vehicles as well as implant carriers generally refer to inert, nontoxic solid or liquid fillers, diluents, or encapsulating material not reacting with the active ingredients of the invention.

The doses can be single doses or multiple doses over a period of several days.

When administering the compound of the present invention parenterally, it is generally formulated in a unit dosage injectable form (solution, suspension,

emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol (PEG), and the like), suitable mixtures thereof, and vegetable oils. It is notable that PEG induces a chemically modified (NCE) cytokine preparation (Pegylation).

Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Nonaqueous vehicles such as cottonseed oil, sesame oil, olive oil, soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl myristate, can be also used as solvent systems for compound compositions. Additionally, various additives that enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it is desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used would have to be compatible with the compounds.

Peptides may be polymerized or conjugated to carriers such as ovalbumen or human serum albumen as is well known in the art.

Sterile injectable solutions can be prepared by incorporating the compounds utilized in practicing the present invention in the required amount of the appropriate solvent with various of the other ingredients, as desired.

A pharmacological formulation of the present invention can be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicles, additives, and diluents; or the

compounds utilized in the present invention can be administered parenterally to the patient in the form of slow-release subcutaneous implants or targeted delivery systems such as monoclonal antibodies, vectored delivery, iontophoretic, polymer matrices, liposomes, and microspheres. Examples of delivery systems useful in the present invention include: U.S. Patent Nos. 5,225,182; 5,169,383; 5,167,616; 4,959,217; 4,925,678; 4,487,603; 4,486,194; 4,447,233; 4,447,224; 4,439,196; and 4,475,196. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

Generally, the initial dose of NCM may be administered either simultaneously with thymosin α_1 or by administering one drug followed by the other, generally, and preferably, on the same day. The NCM is administered at low doses (200-500 units) of IL-2 equivalence as it is important not to use high doses (>1000 units/dose) as effect is lost and toxicity increases.

More specifically, the foregoing provides a protocol for using NCM as an adjuvant to immunize cancer patients against tumor antigens, either autologous or as defined proteins or peptides.

The antigen preparations to be used	In Cancer
1) PSMA peptides - obtained commercially	Prostate
2) MAGE 1 & 3 & MAGE fragments & NY ESO-1 obtained from the Ludwig Inst of Immunol	Melanoma H&NSCC
3) Papilloma virus E6 & E7 obtained commercially	Cervical SCC

The route of antigen plus NCM+thymosin α_1 administration is preferentially the neck because it is accessible and it contains greater than 30% of the body's lymph nodes and systemic immunity can be envisioned to result.

Low dose cyclophosphamide has been used to augment cellular immunity and decrease suppression by lymphocytes in mice and patients with cancer (Berd, D, Prog in Clin Biol Res 288:449-458 (1989); Berd, D, et al., Canc Res 47:3317-3321 (1987)) and it has been employed in effective immunotherapy of cancer patients (Weber, J. Medscape Anthology 3:2 (2000); Murphy, GP, et al.,

The Prostate 38:43-78 (1999); Hadden, JW, et al., Arch Otolaryngol Head Neck Surg 120:395-403 (1994)).

Zinc deficiency is associated with improved cellular immunity and treatment with zinc is immunorestorative in mice (Hadden, JW, Int'l J Immunopharmacol 17:696-701 (1995); Saha, A. et al., Int'l J Immunopharmacol 17:729-734 (1995)).

A cyclooxygenase inhibitor (COXi) such as indomethacin is used. Cancers produce prostaglandins and induce host macrophage production of prostaglandins (Hadden, JW, The Immunopharmacology of Head and Neck Cancer: An Update, Int'l J Immunopharmacol 11/12:629-644 (1997)). Since prostaglandins are known to be immunosuppressive for T cells, inhibition of PG synthesis with cyclooxygenase inhibitors is appropriate.

Recombinant Protein Purification

Marshak, et al., Strategies for Protein Purification and Characterization, a Laboratory Course Manual, CSHL Press (1996).

Dose and Frequency of Antigens

1-1000 µg, preferably 10-50 are used. The form of antigen is soluble (partially polymerized or conjugated to carrier, only if necessary).

Schedule:	Day 1, Day 12, Day 21
(Pre-Rx)	Day 12, Day 21, Day 31
Site of Injection:	local injection, i.e., neck injections

Expected responses are tumor reduction and tumor pathological changes reduction, fragmentation, lymphoid infiltration). Humoral immunity to antigen (RAI or ELISA) is expected, as well as cellular immunity to antigen (intracutaneous skin test *in vitro* lymphocyte proliferation or ELISPOT ASSAY).

Oligopeptides such as PSMA, MAGE fragments, E6 and E7 peptides would not normally be immunogenic without conjugation to carrier or pulsed on to dendritic cells. Thus, effective immunization would not be expected to occur. Even with effective immunization, tumor regression would be considered surprising by this method, particularly at a distance as with prostate and cervix. Regression of metastatic disease is always a surprising event with

immunotherapy. Degree and frequency of clinical responses are factors in the effectiveness and thus the novelty of this approach. Diagnostic skin tests are another guide to more effective immunization. Patients can be pretreated with IRX-2 (NCM) and thymosin α_1 to induce better responses (increase NCM and PHA skin tests and lymphocyte counts and reversal of lymph node abnormalities).

This creates an adjuvant strategy:

1) Combining immunorestoration and adjuvancy;

2) Making peptides and proteins immunogenic;

3) Obtaining the degree of immune response to effect tumor regression at a distance; and

4) Extending to all forms of tumor antigens and haptens including peptides and/or carbohydrates.

It can extend to areas of applicability as in AIDS virus vaccine in HIV positive patients; other difficult to manage situations; renal transplants, aged, etc.

Patients are HLA matched for MHC restricted peptides and skin tested for one or more tumor peptides prior to consideration of the protocol. 100 µg of one or more tumor peptides are perilymphatically administered in the neck with NCM plus thymosin α_1 using the NCM protocol as discussed below on days 1 and 10 of the NCM series. The combination is repeated on day 21. In addition to tumor response and histology, immune reaction to the peptides is monitored by repeat skin test or by other means known in the art.

The following examples demonstrate the utility of the present invention to provide immune restoration and adjuvant effect of NCM plus thymosin α_1. As an introduction to the examples, reference is made to U.S. Patent No. 5,632,983 ('983 Patent), the patent having the same inventor as the inventor herein. The '983 Patent presents data resulting from *in vivo* treatments on thymocyte (Figure 21) and splenocyte (Figure 22). The data represent *in vitro* response to stimulation with media control (open bars), rIL-1 (solid bars), rIL-2 (cross-hatched), and NCM (diagonal lines). Thymosin α_1 and NCM alone increase

many of the responses in both the central lymphoid organs. The combination produced dramatic and highly significant increases for all four responses. This data was only presented *in vivo* in mice.

EXAMPLE 1

All steps relating to cell culture are performed under sterile conditions. General methods of cellular immunology not described herein are performed as described in general references for cellular immunology techniques such as Mishell and Shiigi (Selected Methods in Cellular Immunology (1981)) and as are known in the art.

Preparation of natural cytokine mixture (NCM)

The buffy coat white cells of human blood from multiple HIV-negative hepatitis virus-negative donors is collected. In an alternative embodiment, animals are the cell source for veterinary uses. The cells from the donors are pooled and layered on ficoll hypaque gradients (Pharmacia) to yield lymphocytes free of neutrophils and erythrocytes. Alternative methods could be used that would result in the same starting lymphocyte population as are known in the art.

The lymphocytes are washed and distributed in X vivo-10 media (Whittaker Bioproducts) to surface activated cell culture flasks for selection of cell subsets (MICROCELLECTOR™ T-25 Cell Culture Flasks), which contain immobilized stimulants, i.e., mitogens like PHA. In one set of experiments, X vivo-15 and X vivo-20 media were used as indicated. The immobilization process for the stimulants is as described by the manufacturer for immobilizing various substances for panning procedures, i.e., separating cells, in the flasks. Alternatively, the lymphocytes are exposed to stimulants e.g., PHA for 2-4 hours then washed three times.

The cells are incubated for 24 to 48 hours in X vivo-10 media with 80 µg/ml Ciprofloxacin (Miles Lab) at 37° in a CO_2/air incubator. Alternatively, RPMI 1640 media could be used (Webb, et al. (1973)). Generally, the HSA is used at 0.1 to 0.5% (weight by volume). Following incubation, the supernatants

are poured off and collected. Human serum albumin (HSA) may be added to stabilize further the interleukins if HSA-free media is used for generations. The supernatants are store at 4°C to -70°.

Characterization of Supernatants

The pooled supernatants are characterized by measuring the cytokine content by bioassay for IL-2 and ELISAs for the remaining interleukins: IL-1, IL-15, CSFs, TNFs, and IFNs. Sterility is tested by culture in thioglycolate broth and endotoxin measured by limulus lysate assay as is known in the art.

Standardization of supernatant of cytokine content:

Each supernatant is standardized either by concentration or amount administered so that comparisons can be made.

Removal of contaminants from supernatant:

DNA and virus exclusion, if used, employ such techniques as ultrafiltration, column chromatography, virus retentive filters, ethanol fractionation, polyethylene glycol/bentonite precipitation, gamma irradiation, and/or solvent/detergent treatment as has been used for intravenous gamma globulin and monoclonal antibodies (e.g., IGIV News Update brochure).

Model

The model of hydrocortisone induced thymic involution in aged mice was used unless otherwise indicated (Hadden JW, et al., Int'l J Immunopharmacol 17:821-828 (1995)).

Laboratory Animals

Female BALB/c (Life Science, St. Petersburg, FL) aged retired breeder mice (8-9 months) whose thymuses had begun to involute were employed in *in vivo* tests. Mice were weight matched and randomly pooled in groups of five. Animals were fed standard laboratory diets with drinking water ad lib. All mice, with exception of a control group, were treated intraperitoneally (i.p.) with hydrocortisone (5 mg/mouse in 0.1 ml 0.9% sodium chloride) for two consecutive days to induce a chemical thymectomy and reduction of spleen weight.

Hydrocortisone-treated adult mice show acute thymic involution (less than 30% of control) and reduction in spleen size (less than 80% of control) at two days with progressive recovery to ten days.

Experimental Design

Each treatment group had five animals and each experiment was repeated two to five times. Treatment was initiated intraperitoneally (i.p.) on day 3 and continued once per day for a total of five days. Treatment groups were injected with one of the following *in vivo* treatments as indicated in the text:

- 1. pyrogen free saline (controls);
- 2. recombinant interleukin-1 (rIL-1; 4 ng);
- 3. recombinant interleukin-2 (rIL-2; 50 units);
- 4. rIL-1+rIL-2 (4 ng+50 units, respectively);
- 5. natural cytokine mixture (NCM; 50 units IL-2 equivalence)

On day 8, the mice were weighed, sacrificed by cervical dislocation, and their spleens and thymuses removed and weighed. The organs were minced, the residual erythrocytes were lysed using ammonium chloride (Mishell and Shiigi (1981)), and the cells counted.

The proliferative response of the cells to various substances was then determined. A sample of cells was prepared for cell culture at 37°C, 5% CO_2 in RPMI 1640 medium with 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and 2-mercaptoethanol ($2x10^{-5}$ M). The cells were plated in 0.2 ml microwell plates in quadruplicate at a concentration of $1.5x10^6$ /ml and incubated for 72 hours with one of the following as indicated in the text:

- 1. control diluent (complete RPMI 1640 medium);
- 2. rIL-1 (1 ng/ml);
- 3. rIL-2 (2 Units/ml);
- 4. NCM (2 Units/ml of IL-2 equivalence);
- 5. concanavalin A (ConA; 1.5 μg/ml);
- 6. phytohemagglutinin (PHA; 0.5 μg/ml)

The culture was terminated to measure DNA synthesis, and thereby cell proliferation, with an 18-hour pulse of tritiated thymidine (3H-Thymidine; New

England Nuclear, Boston, MA; specific activity 6.7 Ci/mM), harvested with a multiple automatic sample harvester and processed for liquid scintillation counting. Marker studies were also performed as described by Hadden, et al., (1992). The results were expressed as arithmetic mean of cpm from three samples for each animal. In order to simplify the representation of data obtained with different animals, the results with the different animals were pooled and calculated together and in some cases are expressed as ratio to control and others as means+brackets for standard error of the mean (SEM).

Statistical Analysis

Student's T test was used to analyze data as appropriate.

Results

The objective was to find a way to stimulate lymphocytes to produce high levels of interleukin-2 in the absence of serum and in a way that did not yield significant quantities of PHA in the supernatant. To do this, the PHA was immobilized on surface activated cell culture flasks for selection of cell subsets (AIS MICROCELLECTOR™ T-25 plates) as described in the manufacturer's instructions for "panning" cell separation or pulsed into the cells followed by washing (pulse technique).

Media employed in these experiments was X vivo-10 (Whittaker) and is approved for administration to humans by the U.S. Food and Drug Administration for interleukin-2 lymphokine activated killer (LAK) cell protocols. Serum-free media capable of supporting human lymphocyte proliferation like minimal essential media (MEM) or RPMI-1640 (Sigma) could also be used.

Initial experiments indicated that PHA (HA-16, Murex Diagnostics Ltd., Dartford, UK) could be immobilized by the technique described by the manufacturer and that under appropriate optimal conditions of cell number of $7.5\text{-}15x10^6$ /ml, time of exposure of 24 hours to 48 hours, and PHA concentration of 25 or 50 µg/ml a high yield of IL-2 in the serum-free supernatant could be obtained. The yield was superior to the pulse technique employing brief exposures to PHA (NI) followed by washing and subsequent culture with Ciprofloxacin (NIM) in serum-free media (Table I). Therefore, this

flask procedure is used to generate the NCM mixture.

TABLE I

IL content of supernatant/ml

PHA brief exposure (NI) 2-20 units
PHA brief exposure 8-140 units & Ciprofloxacin (NIM) (80 µg/ml)
PHA flask immobilization 100-353 units & Ciprofloxacin (80 µg/ml)

IL-2 content was measured in the supernatant using the CTLL IL-2 dependent cell line by the methods described by Gillis et al. (1978). IL-2 was quantitated in international units against a known standard containing 640 units (Pharmacia AB).

The cell-free supernatants from flasks incubated without cells were tested on human lymphocytes to determine if residual PHA was present in sufficient quantities to produce a proliferative response. Any residual PHA greater than 0.01 µg/ml would give such a response. In the absence of cells, small amounts of PHA were observed in the supernatant at 40 to 48 hours; however, when PHA (25 µg/ml) was used for only 24 hours, these levels were negligible. Twenty-four hours incubation was thus considered optimal.

A comparison of X vivo-10, X vivo-15, and X vivo-20 (Whittaker) and MEM in the present invention was undertaken and shown in Figures 1-3. X vivo-10 and X vivo-15 are approved for administration to humans by the U.S. Food and Drug Administration for IL-2 lymphokine activated killer (LAK) cell protocols. Generation of NCM was compared in different media utilizing continuous versus pulsed exposure to PHA at 1 µg/ml (Figure 1). The effect of cell concentration was explored with continuous exposure to PHA at 1 µg/ml (Figure 2) and PHA at 2 µg/ml (Figure 3). The optimal combination of these factors was found to be continuous exposure by immobilization in X vivo-10 at cell concentrations of 2.5 or $5.0x10^6$ /ml with PHA at 2 µg/ml or at $5x10^6$ cells/ml with PHA at 1 µg/ml. Because the per cell yield is most efficient at $2.5x10^6$ cells/ml, that concentration with PHA at 2 µg/ml is chosen as the optimal.

Preliminary experiments, in tubes rather than flasks, were performed to determine the parameters for Ciprofloxacin and two other 4-aminoquinolone

antibiotics (Norfloxacin and Ofloxacin) to enhance cytokine production from human leukocytes following exposure to PHA. Table II shows that 80 µl/ml of each of these 4-aminoquinolone antibiotics enhanced production of IL-1, IL-2, IL-6, IFN-γ, TNF-α, and G-CSF. IL-8 production was maximal. IL-3, IL-4, and IL-7 were undetectable under these circumstances in all supernatants. These results indicate that under these serum-free conditions, all 4-aminoquinolones tested at 80 µg/ml enhanced PHA induced cytokine production under serum-free conditions.

TABLE II

	PHA	**Ciprofloxacin**	**Norfloxacin**	**Ofloxacin**
	Alone	**& PHA**	**& PHA**	**& PHA**
IL-1-β	81	1080	783	810
IL-2	ND	120	32	82
IL-6	1665	>3000	>3000	>3000
IL-8	18000	>18000	>18000	>18000
IFN-γ	ND	750	210	380
TNF-α	54	1935	1500	4000
GM-CSF	114	4.5	4.5	72
G-CSF	41	555	800	630

Units for cytokines other than IL-2 are pg/ml and for IL-2 international unit/ml.

It was also determined that a monoclonal antibody, OKT-3 (Ortho), which induces T lymphocytes to proliferate and produce interleukins could be employed as a stimulant under these conditions. Table III shows that OKT-3 induced cytokines similar to those induced by PHA plus Ciprofloxacin with cells incubated in flasks as set forth in Example 1. IL-3, 4, 5, and 7 were not detected with either set of stimulants. OKT-3 produced a small additive effect for several ILs when joined with PHA and Ciprofloxacin (CIPRO).

TABLE III

CIPRO OKT-3 + CIPRO + PHA + PHA OKT-3

IL-1-β	1080 1530 1125
IL-2	120 340 ND
IFN-γ	750 4660 11280
IL-6	>3000 >3000 1980
IL-8	>18000 >18000 >18000
TNF-α	1935 2700 2500
GM-CSF	4.5 12 75
G-CSF	555 375 ND

Units of interleukins other than IL-2 are pg/ml and for IL-2 international units/ml. ND not done.

In order to show the superiority of the NCM over rIL-1 *in vitro*, mouse splenocytes and thymocytes were cultured with MEM and rIL-2 at comparable levels of IL-2 as determined by bioassay and DNA synthesis measured by tritiated thymidine incorporation. NCM induces greater proliferation of splenocytes (Figure 4) and thymocytes (Figure 5) than rIL-2 based on IL-2 content.

In a series of experiments as set forth in Figures 6 and 7, mice with involuted thymuses were treated *in vivo* with rIL-1, rIL-2, combinations of these factors, NCM or saline (controls). The spleens and thymuses were removed, the cells tested for cell proliferation responses against the interleukins (IL-1, IL-2), NCM and the mitogen ConA. The results are expressed as ratio to the saline treated control. *In vivo* treatment with rIL-1, rIL-2, and their combination (rIL-1 and rIL-2) had no significant effect to increase proliferative responses of splenocytes (Figure 6) or of thymocytes (Figure 7) to *in vitro* stimulation with IL-1, IL-2, NCM or ConA. NCM treatment *in vivo* augmented significantly both splenocytes and thymocytes to all 4 stimuli. These results are consistent with an enhanced sensitivity of these cells to stimulation and/or an increase in the number of responsive cells.

Figures 8 and 9 demonstrate the effect of NCM treatment *in vivo* on

splenocyte and thymocyte markers. Nonmature T cells are indicated by -- and may represent T lymphocyte precursors particularly in the thymus. NCM increased proportionately this population in spleen and thymus. Immature T cells are indicated by ++ and this population is proportionately decreased in thymus by NCM treatment. Mature T cells are indicated by CD4+ and CD8+. NCM increased the proportions of mature T cells in thymus and their number in spleen. These results are consistent with an effect of NCM to increase T cell precursors and to promote their development to mature T cells in thymus.

Figures 10 and 11 demonstrate the splenocyte and thymocyte responses *in vitro* to media (RPMI), rIL-1 (IL-1), rIL-2 (IL-2), or NCM after treatment *in vivo* with control media or NCM in the hydrocortisone model. The mice were treated as described hereinabove. These data demonstrate that NCM augments background splenocyte responses, splenocyte responses to IL-1 and IL-2, but not NCM and background thymocyte responses and thymocyte responses to IL-1, IL-2, and NCM.

Figures 12 and 13 demonstrate the splenocyte and thymocyte response *in vitro* to ConA and PHA after treatment *in vivo* with control media or NCM. The mice were treated as described hereinabove.

The *in vitro* studies demonstrate the superiority of NCM over rIL-2 at equivalent doses in sensitizing splenocytes and thymocytes to proliferation signals. The effects on thymocytes reflect promotion of differentiation as well. The NCM composition, but not rIL-1, rIL-2, nor their combination, potently promotes *in vivo* T lymphocyte function (IL responses) and development (mitogen responses and cell markers) that is therapeutically relevant in any therapeutic measures requiring stimulation of the immune system or restoring even partial functioning of a damaged or defective immune system. For example, chemotherapeutic agents can damage cells, including T lymphocytes, involved in the immune response. The present invention, by stimulating the T lymphocyte functioning and development, can restore, either partially or entirely, this feature of the immune system if damaged.

EXAMPLE 2

There is shown that local perilymphatic injections in the neck having NCM plus low dose cyclophosphamide, indomethacin, and zinc induced clinical regressions in a high percentage of patients with squamous cell head and neck cancer (H&NSCC) (Hadden JW, et al., Arch Otolaryngol Head Neck Surg 120:395-403 (1994); Meneses A, et al., Arch Pathol Lab Med 122:447-454 (1998); Barrera J, et al., Arch Otolaryngol Head Neck Surg 126:345-351 (2000)) with evidence of improved, recurrence-free survival. Overall, including minor response (25%-50%) tumor shrinkage and reduction of tumor in pathological specimens, over 90% responded and the majority had greater than 50% tumor reduction.

These responses were speculated to be mediated by immune regression since both B and T lymphocytes were observed infiltrating the tumors. The therapy was not associated with significant toxicity.

Several unpublished observations serve to document this speculation and lead to the present invention.

1) Treatment of lymphocytopenic cancer patients with the combination of NCM has resulted in marked lymphocyte mobilization; where analyzed, these patients showed increases in $CD_{45}RA$ positive T cells (i.e., naïve T cells (Table I)).

2) Intratumoral or peritumoral injection of NCM in patients with H&NSCC resulted in either reversing immunotherapy-induced tumor regression or in progression of the tumor. The tumor is thus not the site of immunization.

3) Analysis of regional lymph nodes revealed unpublished data that indicate that the regional lymph node is the site of immunization to postulated tumor antigens (see Figures 14 to 18).

4) None of these patients treated with NCM developed metastasis expected in 15% clinically and up to 50% pathologically, indicating systemic immunity rather than merely local immunity had been induced.

5) Patients were pre-tested with a skin test to 0.1 ml of NCM prior to treatment. More than 90% of those with a positive skin test (70.3 mm at 24

hours) had robust clinical and pathological response. Patients with negative skin tests had weak or no response. This skin testing appears to select good responders.

Major increases were observed in T lymphocyte counts (CD_2) 752→1020 in these T lymphocytopenic patients (T cell counts 752 versus normal = 1600). Importantly, there was a corresponding increase in "naïve" $CD_{45}RA$ positive T cells (532→782). As mentioned previously, these increases are generally not thought to occur in adults, particularly with a pharmacological therapy like NCM. These cells presumably are recent thymic émigrés and could be considered a major new capacity for responding to new antigens like tumor antigens. The pre-existing $CD_{45}RA$ positive cells were not responding to the tumor antigens and may well be incapable of doing so due to the tumor-induced immune suppression (anergy).

The literature (Hadden JW, Intl J Immunopharmacol 11/12:629-644 (1997); Hadden JW, Intl J Immunopharmacol 21:79-101 (1999)) indicates that for both SCC and adenocarcinomas, the two major types of cancer, regional lymph nodes reflect abnormalities related to the tumor, including sinus histiocytosis, lymphoid depletion, and often, the presence of tumor-associated lymphocytes capable of reacting to tumor cells (with IL-2). With metastasis, lymphoid depletion and depressed function occur. An unpublished analysis of uninvolved cervical lymph nodes 10 H&NSCC and 10 controls showed reduction in average size and an increase in sinus histiocytosis associated with H&NSCC (Figures 14 - 17).

TABLE IV

Treatment of Lymphocyte Phase Patients with H&NSCC with NCM - Increases in Naïve T Cells in Blood (#/mm^3)

	NAÏVE T CELL MARKER			PAN T CELL MARKER		
Patient #	PRE	POST	INCREASE	PRE	POST	INCREASE
1	479	778	+299	704	1171	+467
2	938	1309	+371	1364	1249	-115
3	98	139	+41	146	178	+32
4	341	438	+97	655	590	-65
5	567	652	+97	453	643	+190
6	658	1058	+400	1118	1714	+569
7	642	1101	+459	822	1601	+779
MEAN	532	782	+250	752	1020	+269

Following treatment with one cycle of the NCM (IRX-2) protocol (Hadden JW, et al., Arch Otolaryngol Head Neck Surg 120:395-403 (1994); Meneses A, et al., Arch Pathol Lab Med 122:447-454 (1998); Barrera J, et al., Arch Otolaryngol Head Neck Surg 126:345-351 (2000)), the uninvolved cervical lymph nodes showed the changes indicated in Figures 14 to 17. Compared to the regional lymph nodes of patients with H&NSCC not treated with NCM, these nodes showed a significant increase in size, T cell area and density, and decreases in number of germinal centers and sinus histiocytosis and congestion. The lymph nodes of treated patients were all stimulated and were larger than control nodes with increased T cell area and density. These nodes were thus not only restored to normal, but also showed evidence of T cell predominance, a known positive correlate with survival in H&NSCC (Hadden JW, Intl J Immunopharmacol 11/12:629-644 (1997)).

Importantly, when the lymph node changes related to B and T cell areas were correlated with the changes in their tumors reflecting T and B cell infiltration, a high degree of correlation was obtained for T cells ($p.<.01$) and B cells ($<.01$) and overall lymphoid presence ($p.<.001$) (Figure 18). These changes correlate with tumor reduction by pathological and clinical criteria. These findings indicate that the tumor reactions are directly and positively

correlated with lymph node changes and that the tumor reaction reflects the lymph node changes as the dependent variable. These findings, taken into conjunction with knowledge about how the immune system works in general (Roitt, I, et al., Male D Immunology, JB Lippincott Co, Phila, PA (1989)), and following tumor transfection with a cytokine gene (Maass G, et al., Proc Natl Acad Sci USA 92:5540-5542 (1995)), indicate that the NCM protocol immunizes these patients to yet unidentified tumor antigens at the level of the lymph nodes.

No one has previously presented evidence for lymph node changes reflecting immunization with autologous tumor antigens. This constitutes a good starting point for trying to induce immunization with previously ineffective or poorly effective tumor antigens in an effect to yield regression of distant metastases.

EXAMPLE 3

Two patients were treated with lymphoma of the head and neck. The patients included were those with head and neck cancer who agreed to participate in the protocol. The following scheme was followed:

Before treatment, the patients were skin-tested with NCM 0.1 ml subcutaneously in the forearm, the region was marked, and 24 hours later, the test was read. The test was considered positive if the induction and erythema was equal or larger than 3 mm.

Each cycle of NCM was for 21 days, as follows:

Day 1	Low dose cyclophosphamide (300 mg/m^2 i.v.)
Days 1-21	Indomethacin 25 mg p.o. 3 times daily Zinc sulfate 50 mg p.o. once daily
Days 3-12	NCM 200 units 5 as 1 ml subcutaneously perilymphatic in the neck

Case #1

The patient was a 23-year-old male who presented with a prior history of 3 months of the presence of a tumor on the left submaxillary region, with no other symptoms. In the emergency room, he was found to have lymph adenopathy of the left submaxillary triangle of approximately 6.5 cm in diameter

of a hard consistency, partially fixed at deep levels. The rest of the physical exam was normal. The incisional biopsy showed Hodgkin's lymphoma. The lesion was staged ECIIA. A one-cycle treatment of NCM was given, obtaining a minor response, as the adenopathy reduced in size by 1 cm in diameter. The biopsy report obtained after NCM treatment showed 60% of the lesion showed normal lymphocytic infiltration, and the rest of the neoplasia (40%) showed necrosis. No viable tumor cells were found.

Following this, the patient received radiation treatment in the neck of 3600 rads. The patient is currently free of disease.

Case #2

The patient is an 82-year-old male who presented with a two-month history of a painful mid-neck tumor mass as well as a 10-kg loss of weight. On physical exam, the patient presented with tumor on the right palatine tonsil, which was enlarged to approximately 4x3 cm, with an ulcer in the center of the tonsil. On the neck, a right submaxillary lymph node measured approximately 2x2 cm and a lymph node mass at level II and III of approximately 5x5 cm. The rest of the exam was normal. The incisional biopsy of the tonsil and one of the neck's lymph nodes demonstrated defined non-Hodgkin's lymphoma mixed, of intermediate grade.

The patient was subjected to two cycles of NCM at the end of which a 1 cm reduction in the diameter of the tonsil and neck adenopathy was observed. The pathological report post-NCM treatment showed live tumor 20%, fragmented and necrotic 30%, and normal lymphocyte infiltration 50%.

The patient was given chemotherapy (CHOP) for six cycles and later external radiotherapy (RT) at a total dose of 4600 rads. He recurred at eight months post RT with adenomegaly at the occipital level. The patient died three months later with evidence of neck disease.

EXAMPLE 4

Ten patients with untreated early stage cervical cancer, clinically staged IB1, IB2, and IIA were treated with local, perilymphatic injections NCM as IRX-2

(10 daily injections) followed by radical hysterectomy at day 21. One day before starting IRX-2, patients received a single i.v. dose of cyclophosphamide at 300mg/m^2. Oral indomethacin or ibuprofen and zinc sulfate were administered from days 1 to 21. The clinical and pathological response, toxicity and disease-free survival were evaluated.

All patients completed NCM treatment and were evaluated for response and toxicity. Clinical response was seen in 50% of patients (3 partial response (PR), 2 minor response (MR) (>25%<50%reduction)). Seven patients underwent surgery. Pathologically, tumor reduction associated with tumor fragmentation was found in five cases. There was a rather heterogeneous pattern of cell types infiltrating the tumor that included lymphocytes, plasma cells, neutrophils, macrophages and eosinophils. Treatment was well-tolerated except for severe pain and minor bleeding during injection and gastric intolerance to indomethacin. After 24 months of follow-up, nine patients are disease-free.

This previously unpublished study shows that peritumoral NCM induces immune-mediated tumor response in early stage untreated cervical carcinoma.

EXAMPLE 5

Two patients with liver metastasis from primary hepatocellular carcinoma were treated with intrasplenic NCM (1 or injections). The protocol was otherwise as previously described for the H&NSCC, cervical, or lymphoma cases. One patient with advanced hepatocellular carcinoma had a partial response confirmed by tomography. No histology is available. The other had a partial response confirmed by surgery. Histological exam showed tumor reduction, fragmentation, and lymphoid infiltration.

EXAMPLE 6

Four patients with squamous cell carcinoma of the penis (human Papilloma virus associated) were treated with the NCM protocol as described above. All four had partial responses clinically and the surgical specimen showed tumor reduction and fragmentation and lymphoid infiltration

characteristic of the H&NSCC cancer patients.

EXAMPLE 7

Mice were immunized with PMSA peptides conjugated to ovalbumen 100 µg at 3 sites (days 1, 14, and 21) with alum (1:1 Vol) as adjuvant (5@) or NCM (20 units IL-2 equivalence) (5@). Animals were skin tested at day 28 with ovalbumen (100 µg) (2@) or peptides (100µg) (3@). Two animals treated with ovalbumen plus NCM without peptides responded to ovalbumen with positive skin tests. Two animals treated with ovalbumen plus alum did not respond. Two of three animals treated with ovalbumen plus peptides and NCM responded. None of the animals treated with ovalbumen plus peptides and alum responded. Thus, NCM was a superior adjuvant to alum for both tumor peptides and ovalbumen as antigens.

EXAMPLE 8

Phase I/II Study: NCM an NCM+Thymosin α_1 in Lymphocytopenic Patients

Following radiotherapy patients show marked decline of total lymphocyte counts, CD_3 and T lymphocytes including both CD_4 and CD_8 subsets: (CD_4 drops more than CD_8 so that CD_4/CD_8 ratio drops from 2 to close to 1). During 18 months follow-up these levels did not recover (Wolf, et al. (1985)). Following NCM treatment of T lymphocytopenic patients prior to surgery, the lymphocyte counts increased significantly (Verastegui, et al. (1999)).

A series of post radiotherapy T lymphopenic patients were treated with IRX-2 (NCM) (7 patients) or IRX-2 (NCM) and Thymosin α_1 (7 patients). At onset, both groups had mean lymphocyte counts of 800. Patients were treated daily for 10 days with perilymphatic injections in the neck or axilla (to avoid irradiated area) with 1 ml IRX-2 (approximately 150 units IL-2 by ELISA, 640 by bioassay) or IRX-2 plus thymosin α_1, 1.6 mg/1ml. Lymphocyte counts and various mononuclear cell subsets ($CD_{2,3,4,8,16,19,25}$ CD_{45} RO RA and 56) were analyzed by FACS at day 0 and approximately at day 12. The patients treated with IRX-2 showed no change in mean lymphocyte counts at day 12 (800→700) and no change in:

* T cells and T cell subsets counts (not shown)
* B cell counts (CD_{19} - not shown)
* macrophages (CD_{16} - not shown)
* non-T, non-B lymphocyte counts: (259---->265)
* CD_{45} RA counts: (279---->290)

Overall the seven patients treated with NCM+thymosin α_1 showed increases in:

* total lymphocyte counts 800---->914 p = NS
* non-T, non-B cells 261---->451 p<.05
* CD_{45} RA 221---->443 p<.05

Of the seven patients treated with NCM+thymosin α_1, four showed marked increases in mean lymphocyte counts, non-T, non-B lymphocytes counts and $CD_{45}RA$ and $CD_{45}RO$ counts without significant changes in T cells, B cells or macrophages (Figures 19 and 20). The non-T, non-B cells were CD_{56} negative (not NK cells) and correlated well with $CD_{45}RA$ positive.

Four patients showed increases in TLC following treatment and these patients were studied for CD_2 and were for a prolonged period. They give a clearer picture of maturation process.

At the onset, almost one-third of peripheral blood lymphocytes are non-T, non-B. With treatment, these data show a progressive increase early (10 to 12) days of 450 lymphocytes comprising three populations of T cells: approximately ½ of these are $CD_2^+CD_3CD_{45}RA^+CD_4^+$ or CD_8^+, i.e., naïve mature T cells; approximately ¼ of the remaining are non-T and non-B, but $CD_{45}RA^+$ and ¾ of the remaining CD_2^++CD_3^-. These latter are immature T cells. Between two weeks and three-plus months, the TLC increases persist, CD_3 and αBTcr (one patient only) progressively increase as do both CD_4 and CD_8 signifying a progressive maturation to fully mature T cells. Correspondingly, $CD_{45}RO^+$ cells increase indicating that new memory cells have been produced, meaning they have seen antigen and have been immunized.

$CD_2^+CD_3CD_{45}RA^+CD_4^+$ or CD_8^+ lymphocytes over a 6-week period

Three patients showed no net gain in total lymphocyte counts (TLC) comparing early treatments with late results (1-3 months) TLC 967---->933,

however, several observations were of note:

	All three showed
increases in $CD_{45}RA$	+173
decrease in Non-T Non-B	-159
increases in CD_3	+101
increase in B	+75
No increase in CD_4 and CD_8	---
α BTcr increase (1 patient)	+155

These changes signify an internal shift from almost half null cells (meaning non-T, non-B markers) towards early T cells CD_3+, $CD_{45}RA$+, α BTcrR+, $CD_4^-CD_8^-$. The shift involves approximately 170 cells/mm^2 or approximately 20% of the cells; if B cells are included, it involves 25% of the cells. This major internal shift has never been seen before in association with immunotherapy. It signifies a major abnormal circulating population of immature cells (committed at least, in part, to the T cell lineage, perhaps to both, 170-T:75-B) present in these cancer patients. The treatment of NCM + thymosin α_1 induced a major shift towards more mature T cells, yet they lack CD_4 and CD_8 characteristic of mature T cells. The presence of null cells has been noted in cancer and other immunodeficiencies, yet the induction of immature $CD_4^-CD_8^-$ T cells in the circulation has not been observed before.

These data document the induction and maturation of new T cells as a result of treatment with NCM and thymosin α_1. Intravascular transitional T cells progressed to maturity, i.e.:

$CD_2^+CD_{45}RA^+CD_3^-$---->$CD_2^+CD_{45}RA^+CD_3^+TCR^+CD_4^-CD_8^-$------->

$CD_{2,3}^+CD_4^+$ and $CD_{2,3}^+CD_8^+$subsets.

Where these cells are maturing is a matter of speculation. Normally these events are thought to occur in the thymus. In general, these patients are thought to have involuted thymuses incapable of making new T cells. The composition and method of the present invention apparently induces an increase and mobilization of bone marrow T cell precursors (perhaps to a lesser degree, early B cells) that are either trafficking in and out of the thymus or are differentiating extrathymically. The progressive appearance of memory cells is

important in indicating that these new $CD_{45}RA^{+}$ naïve T cells are transitioning to $CD_{45}RO$ memory cells as a response to antigen exposure.

According to the definition of adjuvant to be used in a treatment of infectious pathogens or tumors, these features are requisite:

1. the presence and the generation of "naïve" cells capable of reacting to antigen if T lymphocytopenia is present;

2. the presence of endogenous or exogenously administered peptides capable of being presented to T cells by mature dendritic cells; and

3. the action of adjuvant plus antigen in an environment capable of generating immunity, such as the regional lymph node, to yield a robust immunity particularly of the TH_1 type. This cellular immunity or T cell immunity is considered central in the resistance to most pathogens and tumors.

NCM is capable of, in the strategy with low dose cyclophosphamide and an NSAID such as indomethacin, creating lymph node changes (including dendritic cell maturation) leading to an immunization to cancer and immune rejection characterized by tumor reduction and fragmentation and a heavy lymphoid infiltration. The combination with NCM + thymosin α_1 is expected to be even more active.

In the examples below, NCM was employed in combination with thymosin α_1 plus low dose cyclophosphamide and indomethacin to treat recurrent head and neck squamous cell cancer (H&NSCC). It is notable that recurrent cancers of this type following intense immunosuppression by X-irradiation would be considered by the cancer immunotherapy community not amenable to any form of immunotherapy. While NCM was effective to palliate recurrent H&NSCC in several patients, a cure was not considered a possibility.

The next set of examples describe reactions to endogenous antigens associated with tumor and/or chronic/latent infections.

EXAMPLE 9

Patient was a 68-year-old female smoker who was treated for stage II SCC ($T_2N_0M_0$) of the tongue with partial glossectomy. Ten months later, the patient had a 1x1 cm local recurrence at the base of the residual tongue. The

patient was treated with the NCM protocol as described above containing 250 units of IL-2 by ELISA, however, in addition, 1.6 mg of Thymosin α_1 (Zadaxin/Thymalfin) was administered with each of 10 perilymphatic injections of NCM. The patient showed an increase of lymphocyte count from 600 to 900 /mm^3 with the appearance of approximately 300 $CD_2^+CD_3CD_8^+CD_{45}RA^+$ naïve T lymphocytes. No toxicity was observed. The tumor underwent a complete clinical regression with no further treatment. Histological examination of a locally respected specimen showed no tumor cells and a marked lymphoid infiltration. This immune regression exemplifies the antitumor adjuvant potential for the NCM + thymosin α_1 mixture in combination with endogenous tumor peptides.

Three additional examples show the action of NCM + thymosin α_1 to act as an adjuvant of endogenous pathogen-related antigens.

EXAMPLE 10

Patient was a 30-year-old female with cervical cancer treated with irradiation. The patient had a long history (>3 years) of condyloma accuminata (venereal warts) indicative of Papilloma virus infection. The persistent lymphocytopenia following x-ray therapy prompted use of NCM in combination with thymosin α_1 (250 units IL-2 + 1.6 mg respectively) for 10 daily injections in the axilla (without low dose cyclophosphamide and indomethacin). Three to four weeks following the initiation of treatment, the condyloma accuminata regressed completely and did not recur. Lymphocyte counts rose from a low 800 to 1300 mm^3 over a four-week period. No other treatment was given.

NCM + thymosin α_1 is interpreted to have induced immunity to HPV and thus regression of the venereal warts.

EXAMPLE 11

Patient is a 56-year-old male with Stage IV H&NSCC gum cancer treated successfully with the NCM protocol plus surgery (maxillectomy and radiotherapy). Due to persistent lymphocytopenia (lymphocyte count of 275/mm^3) and oral thrush, the patient was treated with NCM + thymosin α_1 (250 IL-2 equivalence + 1.6 mg respectively) for ten daily perilymphatic injections in the axilla. His lymphocyte count rose to a high of 1500 mm^3. Three weeks

following the initiation of therapy, he developed a maxillary sialadenitis typical of mumps infection. This resolved spontaneously without other treatment. The patient also had a reduction in oral thrush (candida infection). In this circumstance, NCM + thymosin α_1 is interpreted to eradicate and thus to act as an adjuvant for another viral antigen, as well as the fungal antigen.

EXAMPLE 12

A 66-year-old female with H&NSCC cancer of the tongue was treated. As a result of the radiotherapy, the patient suffered for 1½ years persistent oral thrush (Candidiasis) and lymphocytopenia. The patient was treated with 10 daily perilymphatic injections in the neck with the combination of NCM + thymosin α_1 as in the above examples. Following the treatment, the patient's lymphocyte count rose from 800 to 1,200/mm^3 and the oral Candidiasis resolved completely without other treatments and did not recur. The combination of NCM + thymosin α_1 is interpreted to be an adjuvant to induce immunity to *Candida albicans* antigens leading to the resolution of a chronic parasitization by this fungus.

The above four examples exemplify how NCM + thymosin α_1 can be administered with exogenous tumor, viral, or fungal antigens to induce immunity and resolution of the condition. In the case of the tumor antigen, contrasuppression with low dose cyclophosphamide was necessary to interfere with tumor-induced immune-suppression to effect an immunization. These preceding examples predict that the combination will similarly be an adjuvant with exogenously administered antigens in a classical adjuvant protocol, i.e., mixed with tumor or pathogen, antigen, or peptides in either the prevention or treatment of cancer or infection.

The novelty of the foregoing is based on the following three points:

1. Lymphocyte counts do not rise or only slightly rise following radiotherapy over 18 months of observation (Wolf, et al., Arch Otolaryngol 111:716-725 (1985));

2. No one has observed a progressive increase in the circulation of $CD_2^+CD_3^-CD_{45}RA^+$--->CD_2^+ $CD_3^-CD_{45}RA^+CD_4^-CD_8^-$------->
$CD_2^+CD_3CD_{45}RA^+CD_4^+$ or CD_8^+ lymphocytes over a 6-week period

following any 2-week treatment period including bone marrow with or without thymus transplantation. This progression of events is thought to occur in the thymus and not to occur in adults as seen here; and

3. Complete resolution of cancer, two viral infections (one as a benign tumor, venereal warts) and a fungal infection within three weeks following initiation of treatment (as would be expected timewise for an immune response under the circumstances) is completely unexpected.

Throughout this application, various publications, including United States patents, are referenced by author and year, and patents, by number. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology that has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the described invention, the invention can be practiced otherwise than as specifically described.

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CLAIMS

1. A method for unblocking immunization at a regional lymph node by:

promoting maturation and activation of dendritic cells in a regional lymph node; and

allowing presentation by resulting mature dendritic cells of antigen to T cells to gain immunization of the T cells to the antigen.

2. A method according to claim 1, wherein said promoting step is further defined as administering a natural cytokine mixture (NCM) + thymosin α_1 perilymphatically into lymphatics that drain into lymph nodes regional to a lesion to be treated.

3. A method according to claim 2, wherein the lesion is cancerous or an other persistent lesion.

4. A method according to claim 3, wherein the presented lesion is infectious.

5. A method according to claim 1, wherein the antigen is an endogenous antigen.

6. A method according to claim 1, wherein the antigen is an exogenous antigen.

7. A method according to claim 2, wherein said administering step is further defined as injecting the NCM + thymosin α_1 perilymphatically, intranodally, intralymphatically, intrasplenically, subcutaneously, intramuscularly, or intracutaneously.

8. A method of inducing immunization to cancer or persistent lesions by:

administering an effective amount of an exogenous antigen and an adjuvant consisting of a NCM + thymosin α_1.

9. A method according to claim 7, wherein said administering step is further defined as administering an NCM including IL-1, IL-2, IL-6, IL-8, IL-10, IL-12, δIFN, FGM-CSF + thymosin α_1.

10. A method according to claim 8, wherein said administering step is further defined as injecting the NCM + thymosin α_1 perilymphatically, intranodally, intralymphatically, intrasplenically, subcutaneously, intramuscularly, or intracutaneously.

11. A method for overcoming T cell depletion by inducing production of naïve T cells and restoring T cell immune response.

12. A method according to claim 11, wherein said inducing step is further defined as administering a NCM + thymosin α_1.

13. A method according to claim 11, wherein said administering step is further defined as injecting the NCM + thymosin α_1 perilymphatically, intranodally, intralymphatically, intrasplenically, subcutaneously, intramuscularly, or intracutaneously.

14. A method according to claim 12, wherein said administering step is further defined as injecting an NCM including IL-1, IL-2, IL-6, IL-8, δIFN, and thymosin α_1.

15. A method according to claim 14, wherein said administering step including administering about 150-600 units of IL-2 per injection of the NCM.

16. A method according to claim 11, wherein said blocking and inducing steps are further defined as codelivering cyclophosphamide and a nonsteroidal anti-inflammatory drug (NSAID).

17. A method of treating a cancer or other persistent lesion in a severely immune suppressed patient by administering an effective amount of a NCM as an adjuvant to endogenous or exogenously administered antigen from the cancer or persistent lesion.

18. A method according to claim 14, wherein said administering step is further defined as injecting an NCM including IL-1, IL-2, IL-6, IL-8, TNFα, and δIFN + thymosin α_1.

19. A method according to claim 18, wherein said administering step is further defined as injecting an NCM including IL-1, IL-2, IL-6, IL-8, TNFα, and δIFN + thymosin α_1.

20. A method according to claim 17, further including the steps of blocking endogenous suppression of T cells directly or indirectly by the endogenous lesion being treated.

21. A method according to claim 17, wherein said blocking and inducing steps are further defined as codelivering cyclophosphamide and a nonsteroidal anti-inflammatory drug (NSAID).

22. A method according to claim 21, wherein the NSAIDs are selected from the group including indomethacin, Ibuprofen, Vioxx, Celebrex, and other related treated compounds.

23. A method of vaccine immunotherapy including the steps of:

inducing production of naïve T cells; and

exposing the naïve T cells to endogenous or exogenous antigens.

24. A method according to claim 23, wherein said exposing step is further defined as exposing the naïve T cells to endogenously processed peptide preparation resident in regional nodes of a patient who possesses a lesion.

25. A method according to claim 24, wherein the lesion is cancerous or infectious.

26. A method according to claim 23, wherein said exposing step is further defined as administering an exogenously produced antigen.

27. A method according to claim 23, wherein said antigen is otherwise nonimmunogenic peptide.

28. A method according to claim 23, wherein said exposing step is further defined as immunizing the naïve T cells with matured peptide presenting dendritic cells at a lymph node distal from a lesion to be treated.

29. A method of treating lymphocytopenia by administering an effective amount of a NCM.

30. A method of inducing immunity by:

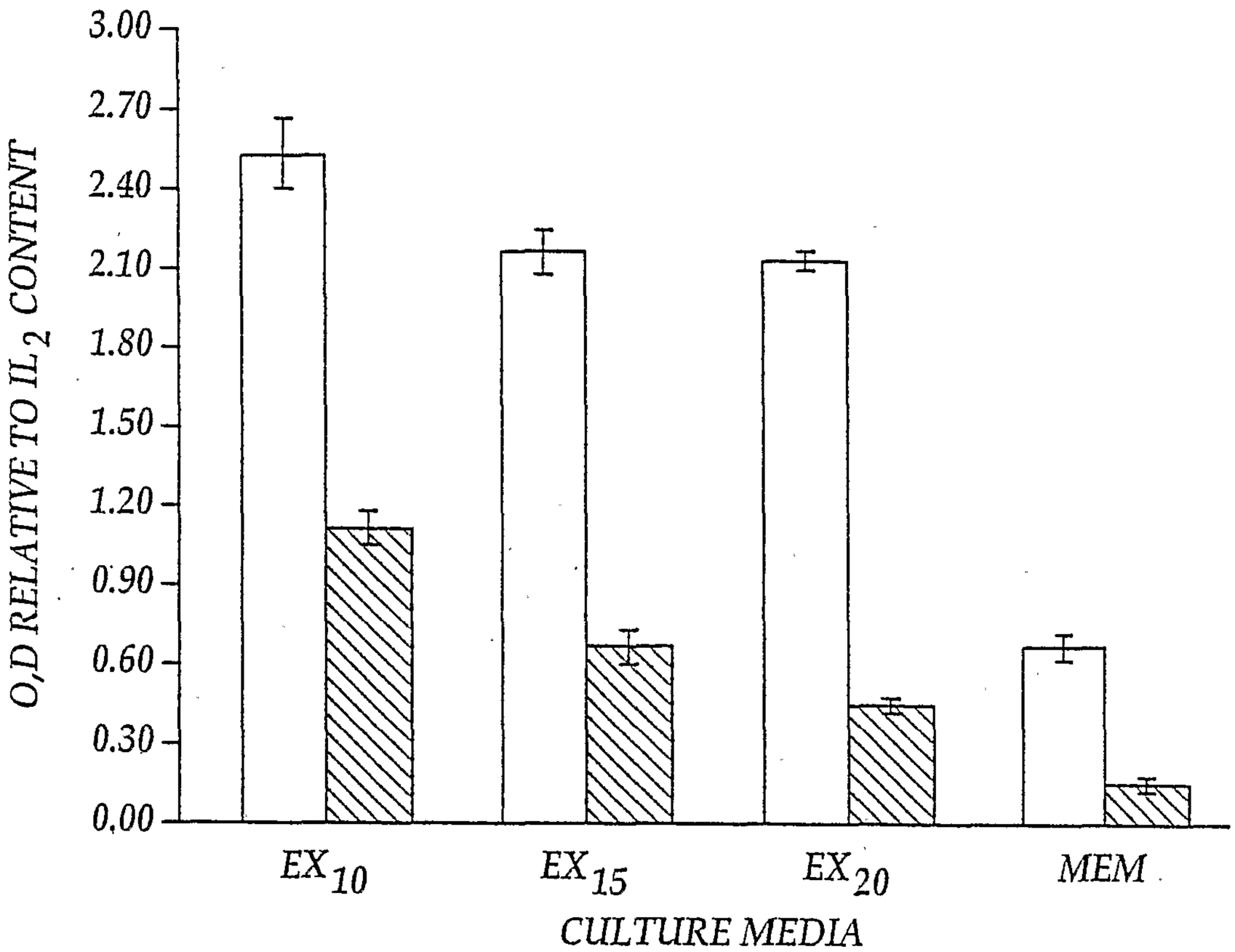
inducing *in vivo* maturation of dendritic cells resulting in effective antigen presentation to naïve uncommitted T cells, leading to clonal expansion of T and B cells, thereby creating immunity in a patient.

31. A method according to claim 29, including the further step of infiltrating into tumors by hematogenous spread leading to tumor destruction.

32. A method of inducing immunity by:

generating a microenvironment in a regional lymph node allowing effective antigen processing and presentation; and

decreasing cells of the lineage of dendritic cells in the lymph node sinuses that accumulate in a cancer patient so that antigen becomes immunogenic for T cells.



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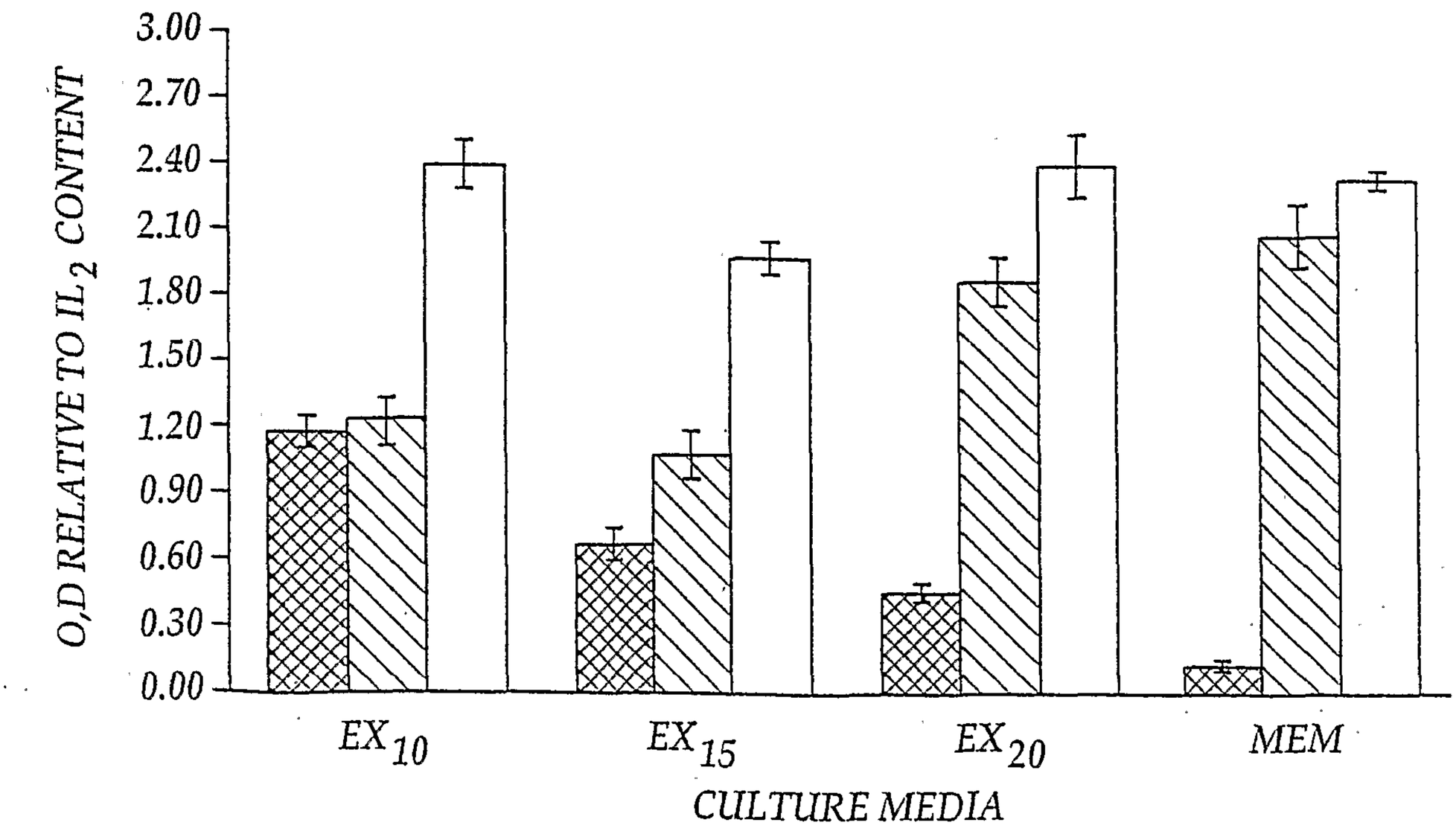

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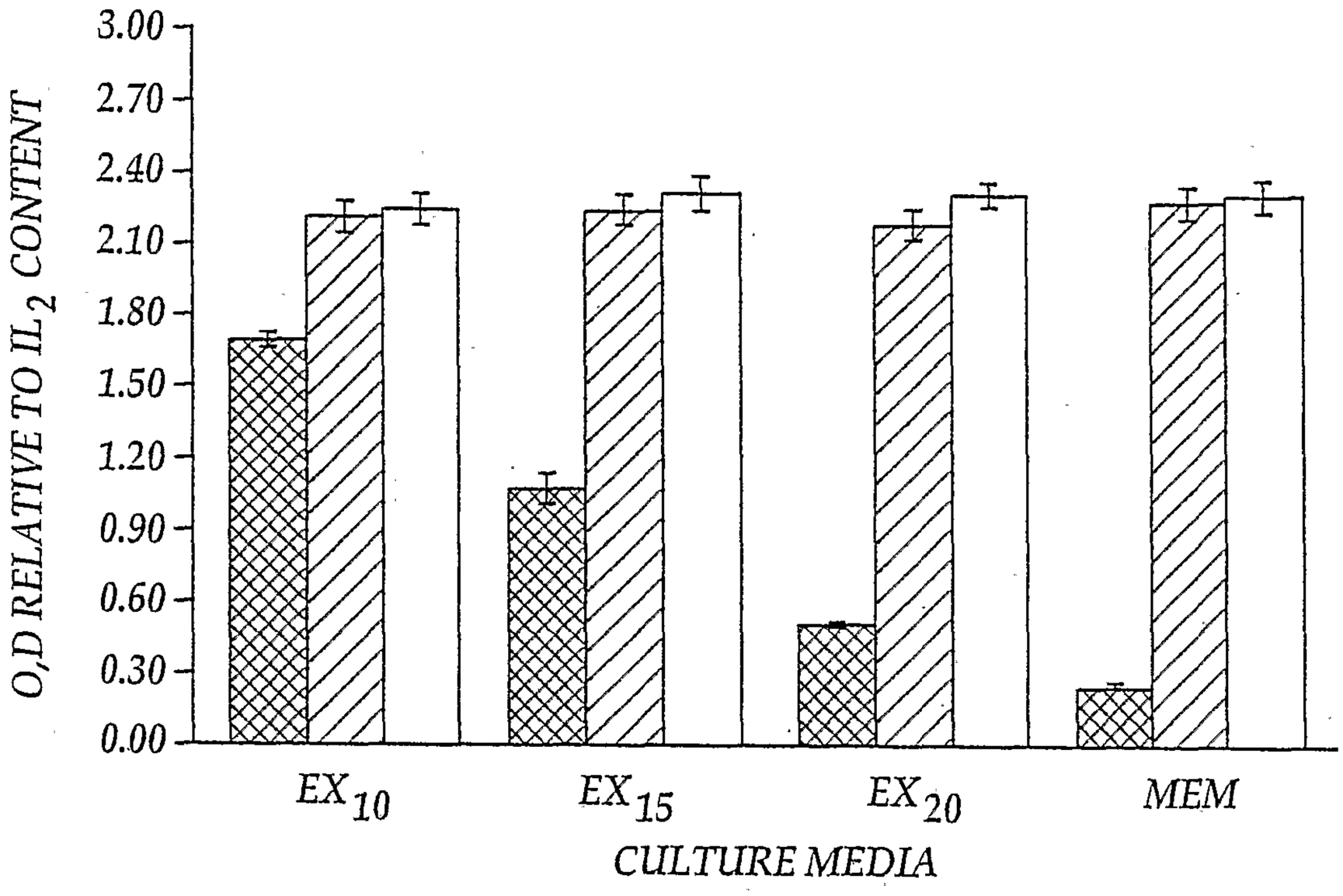

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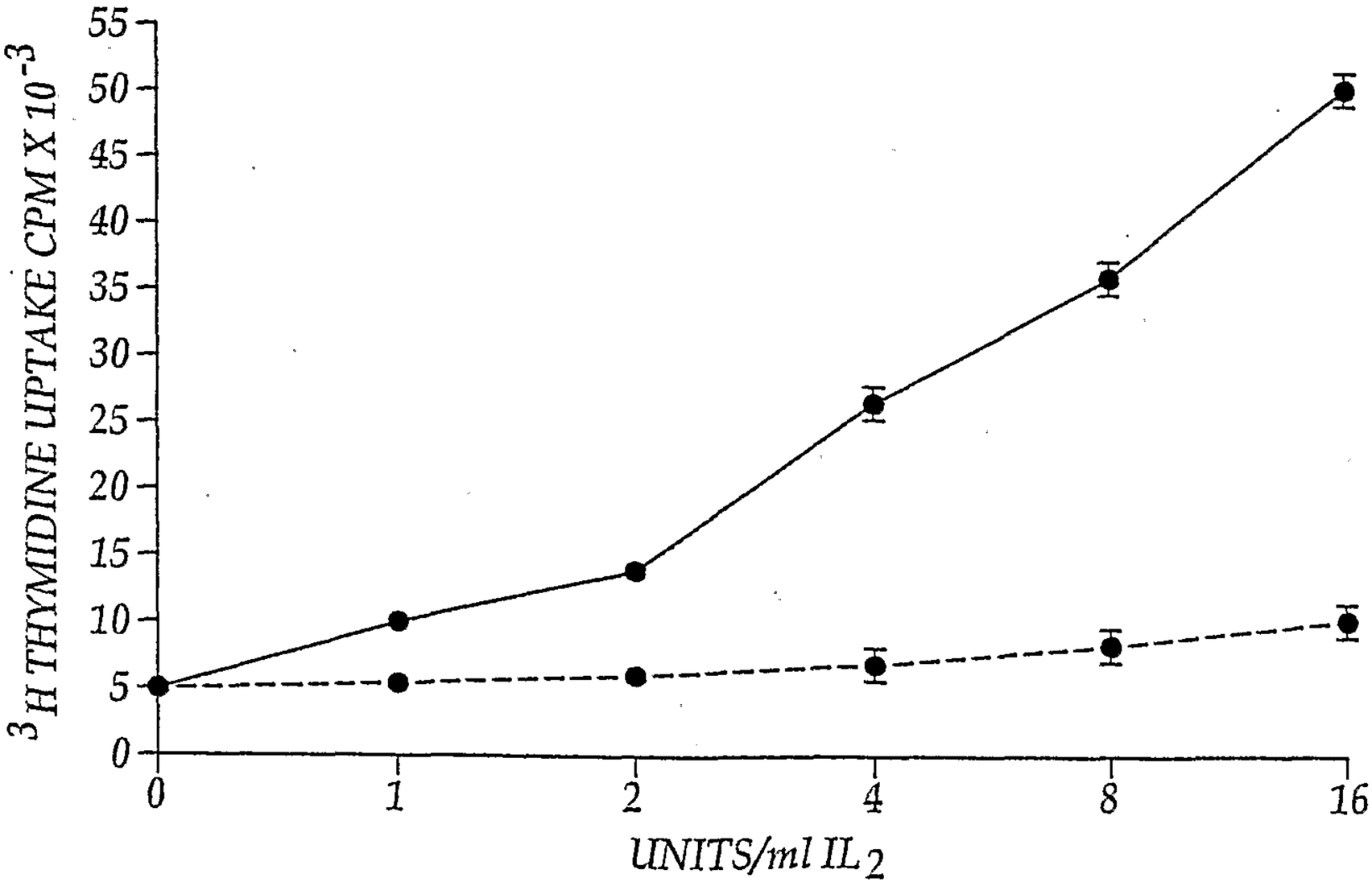

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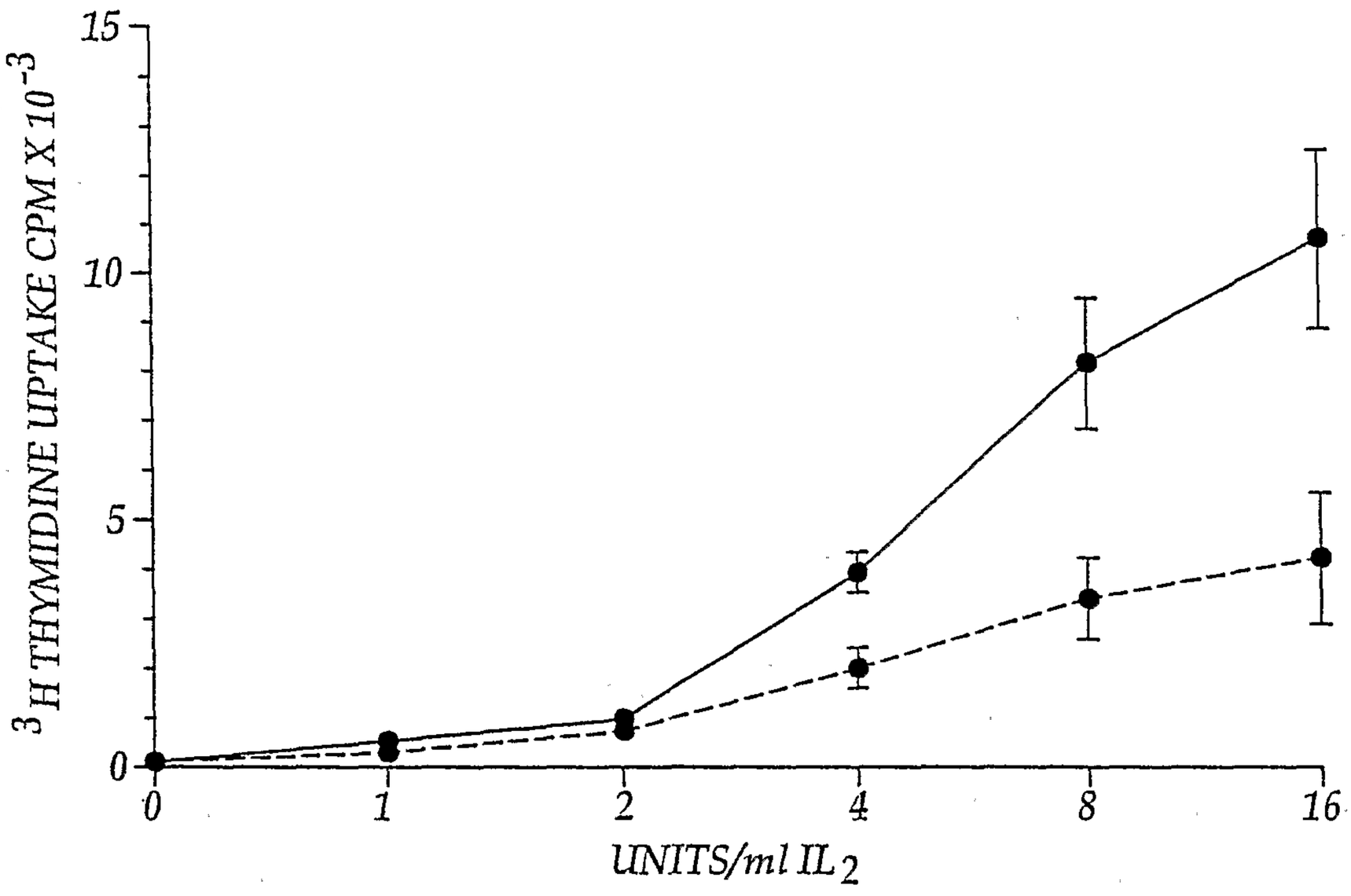

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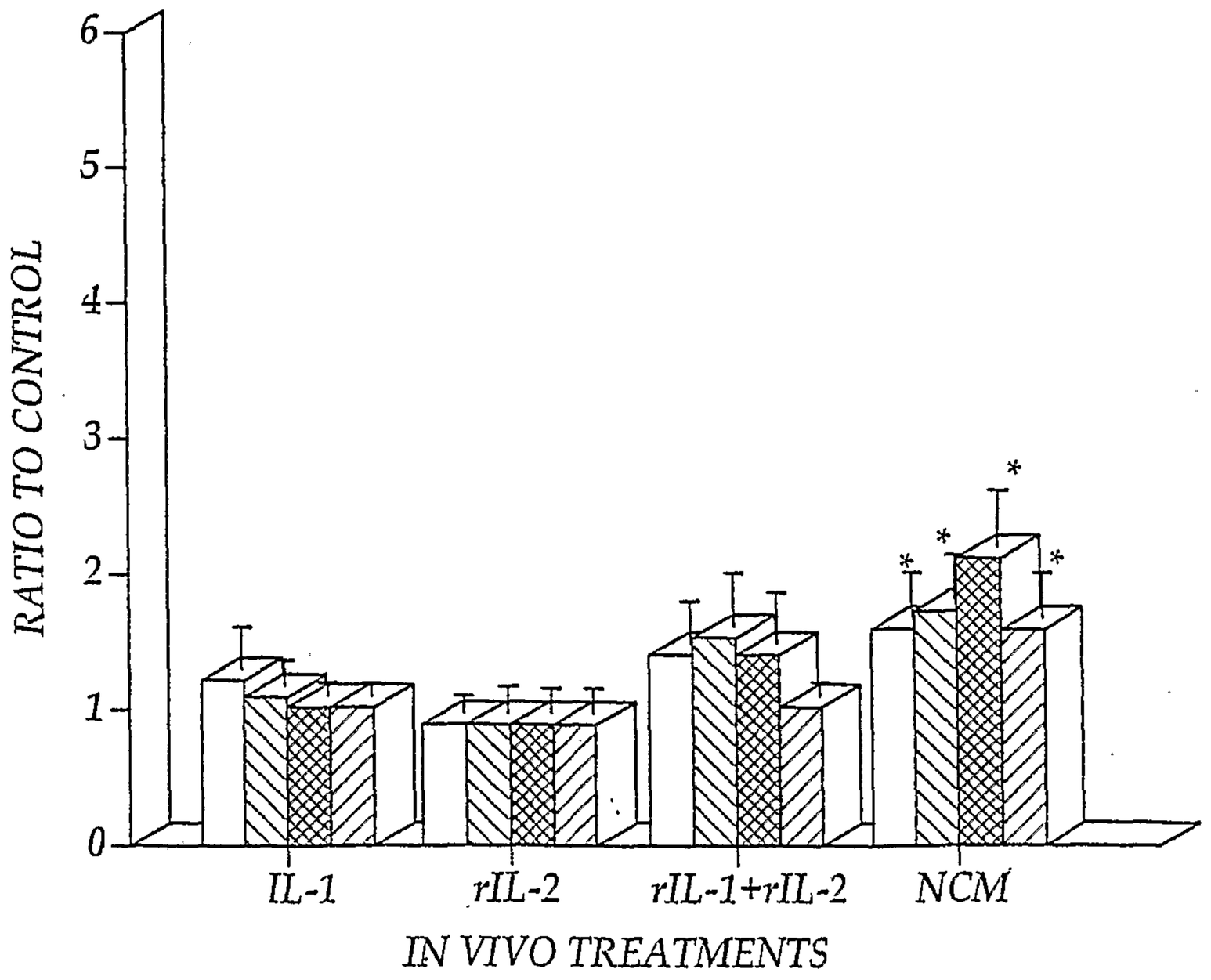

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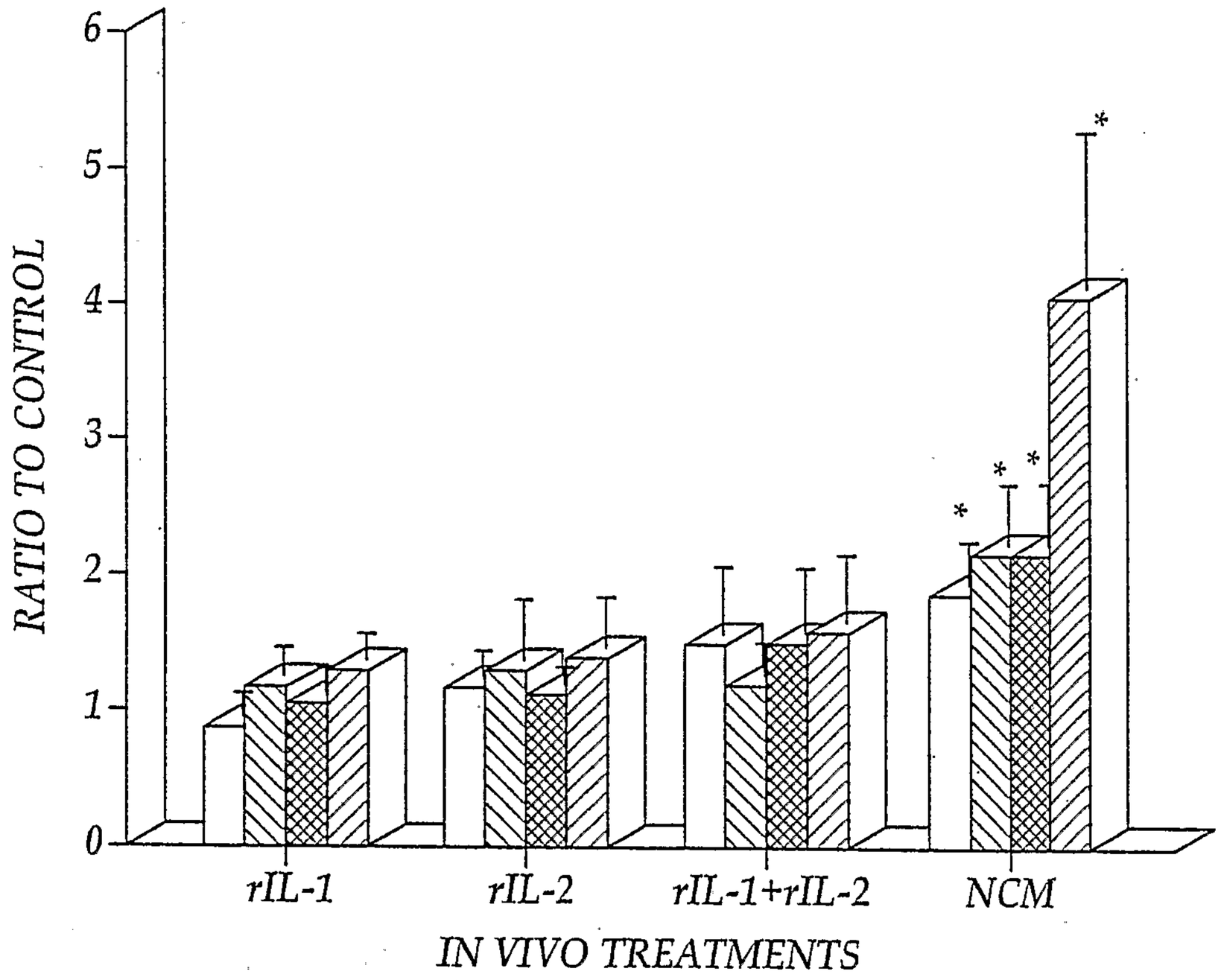

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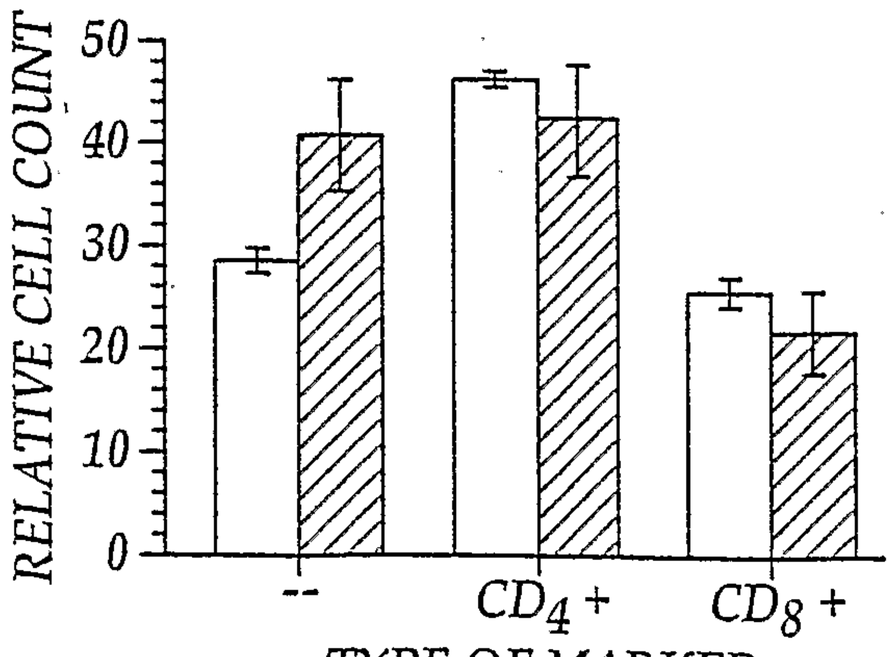

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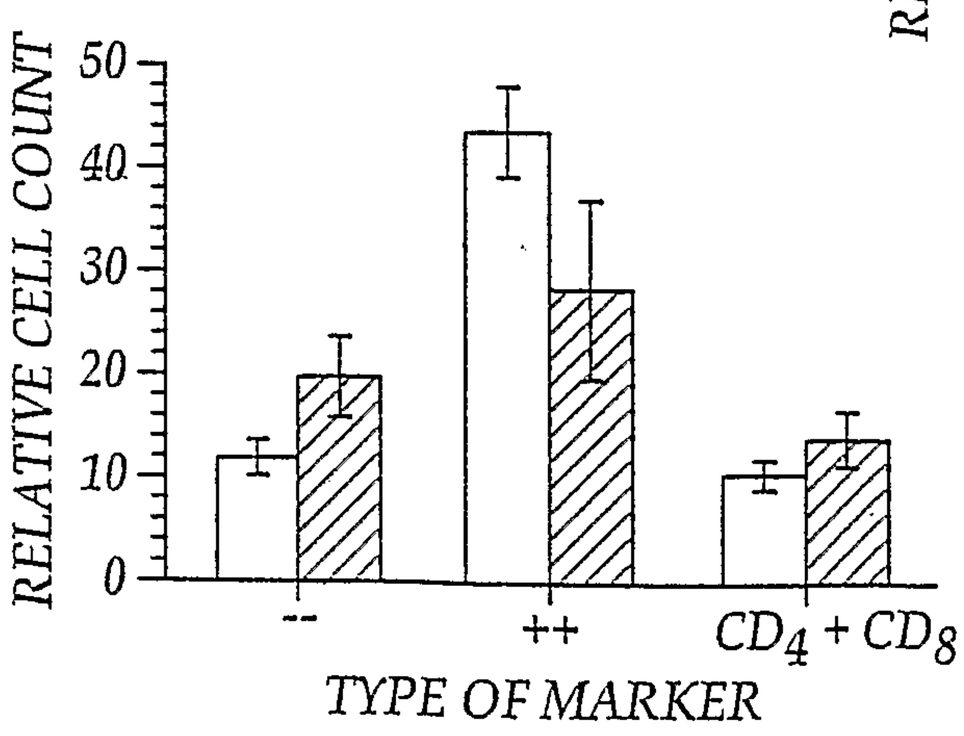

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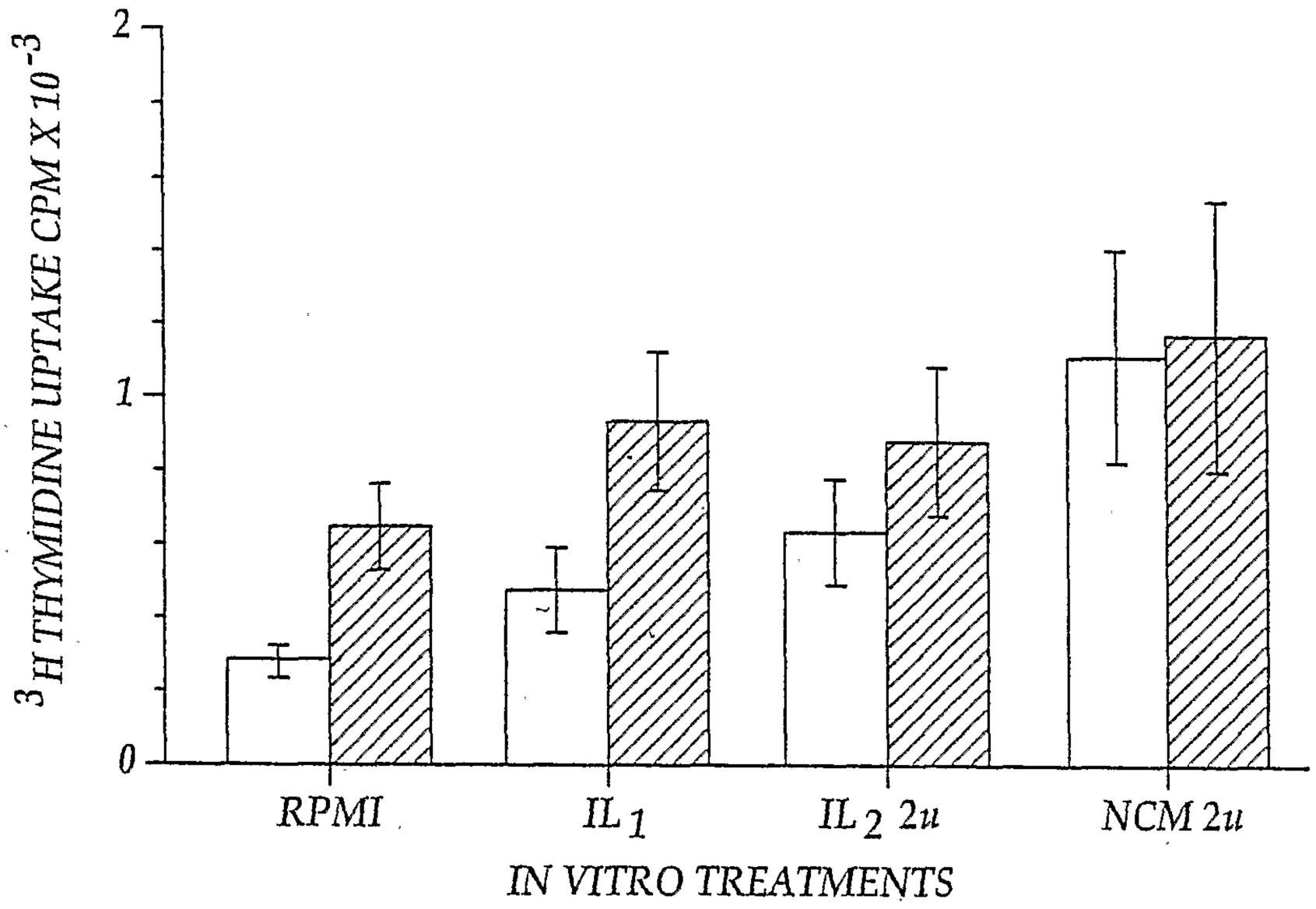

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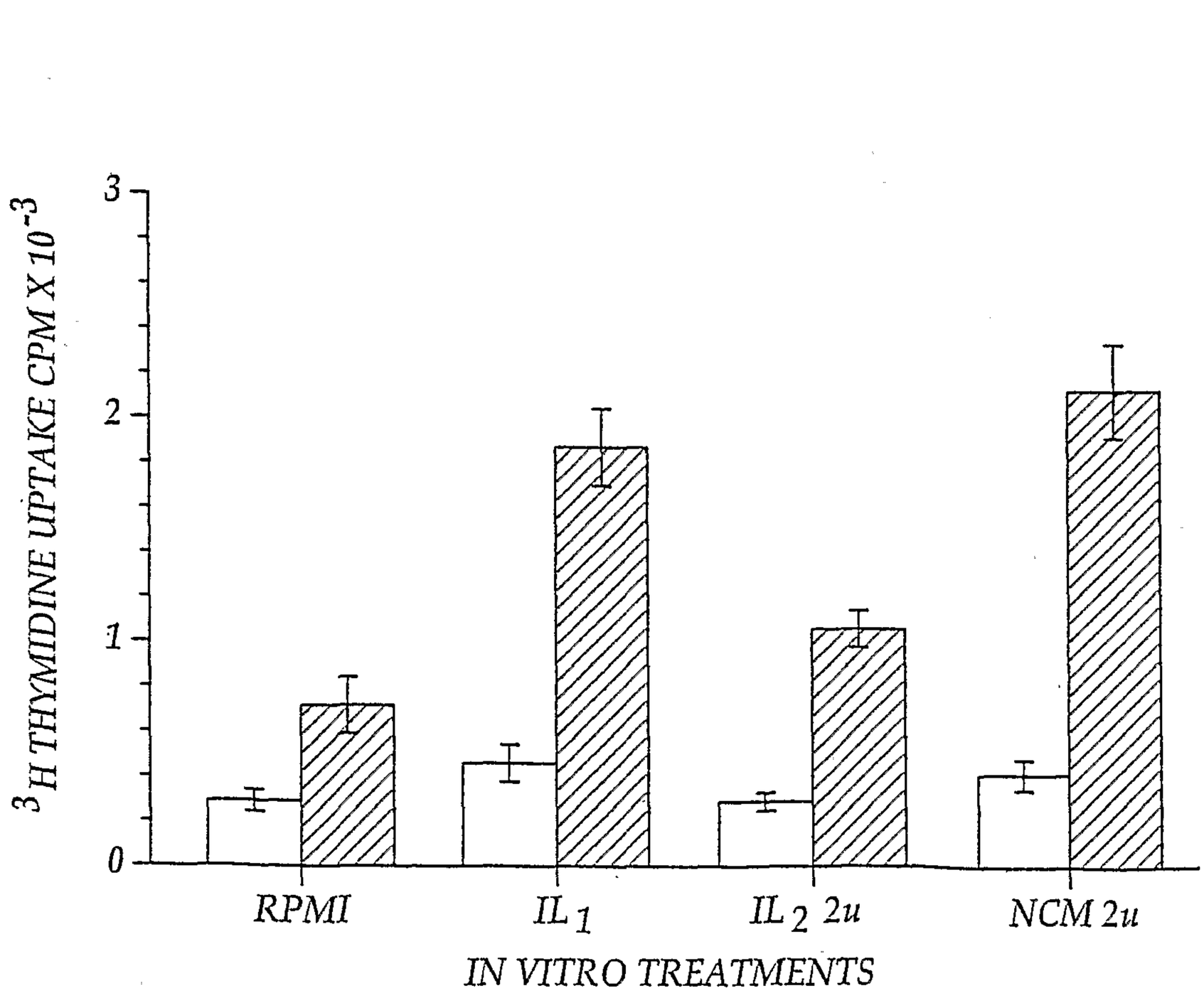

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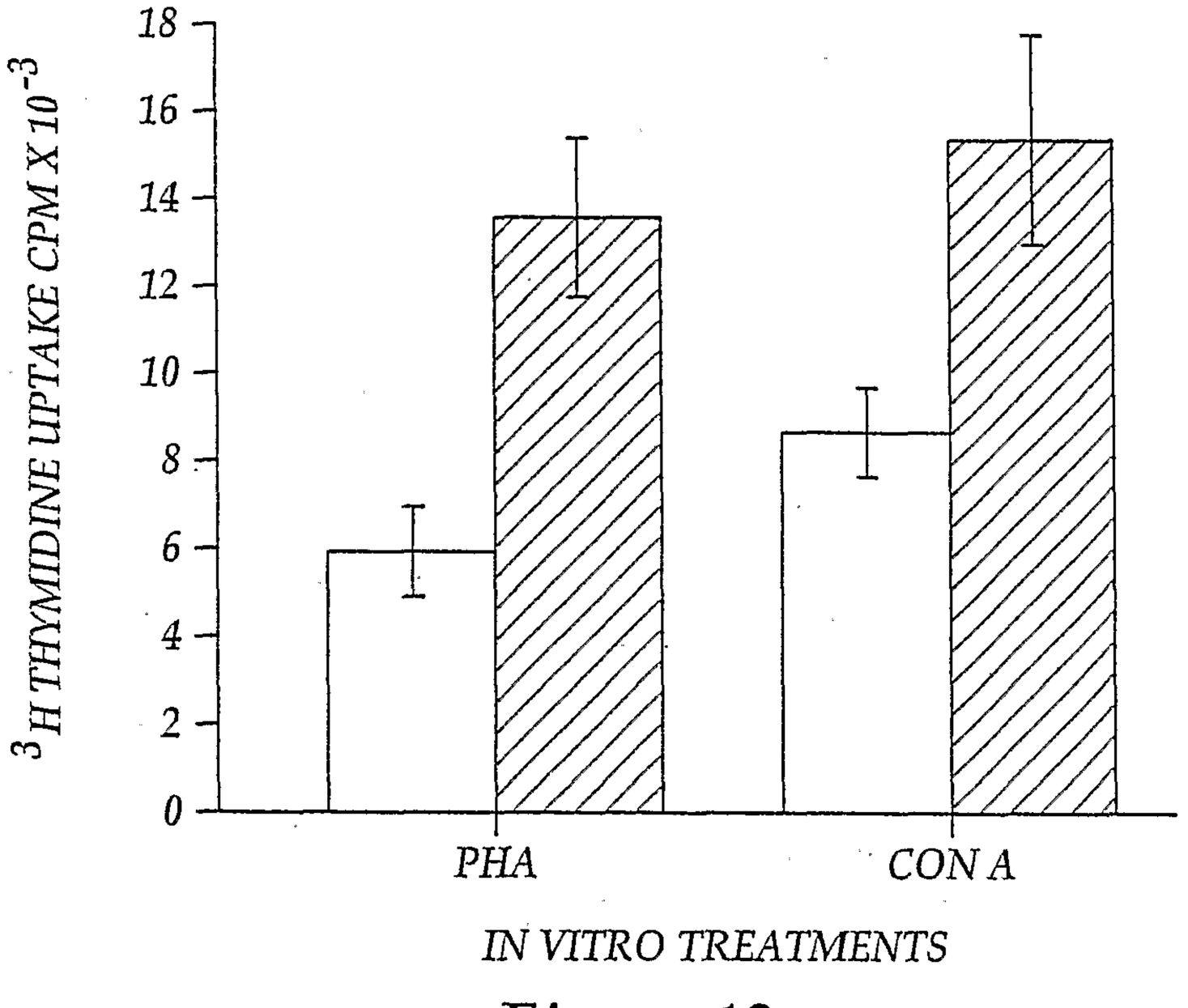

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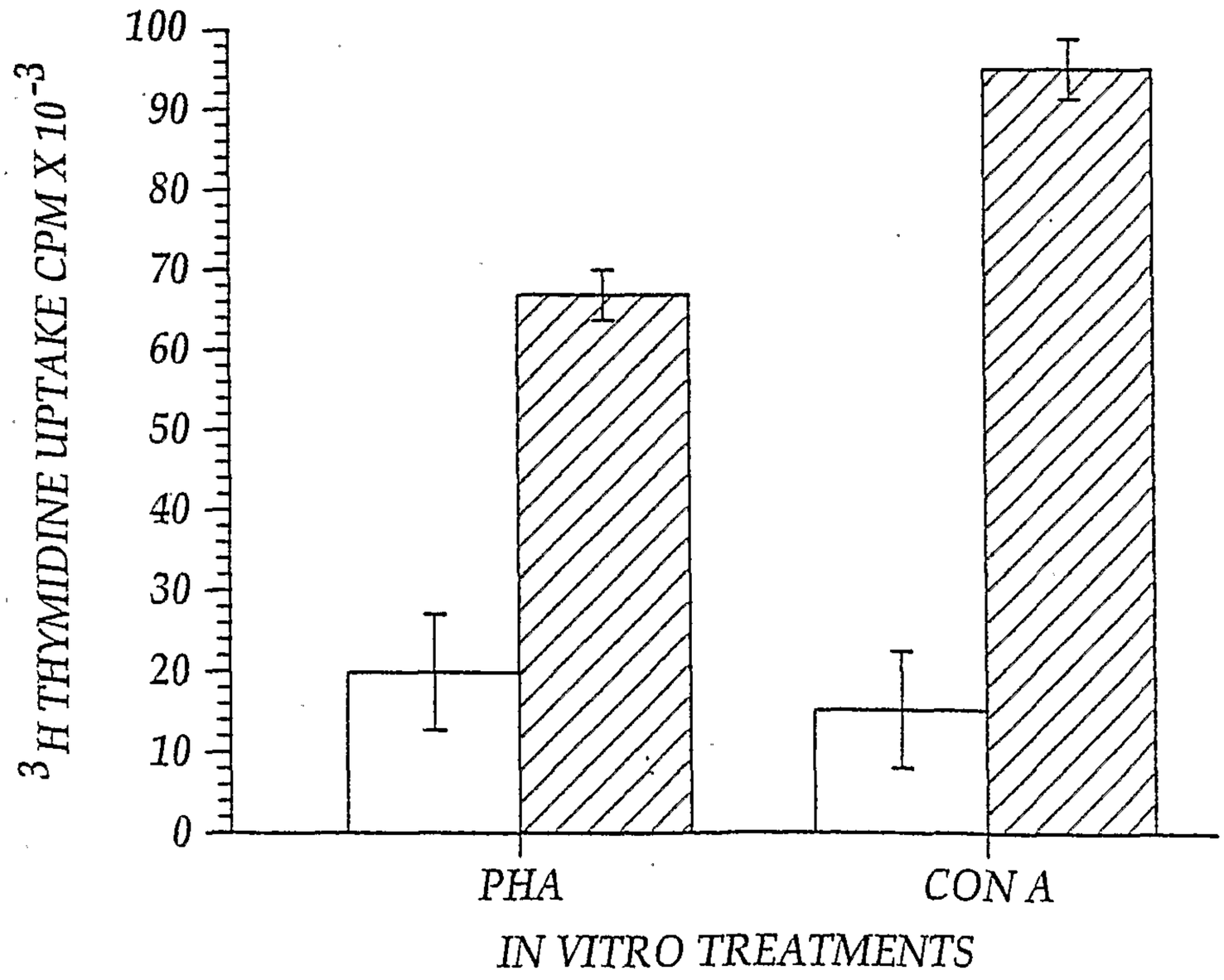

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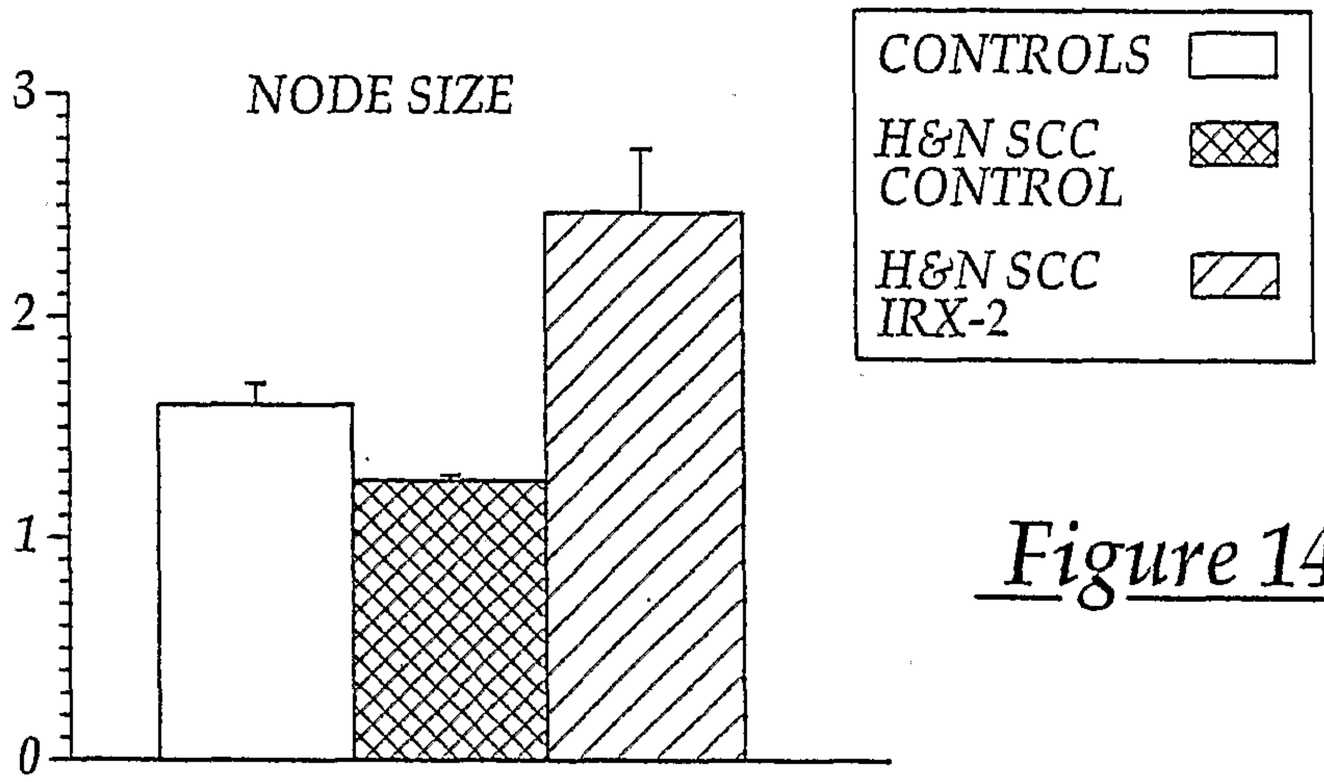

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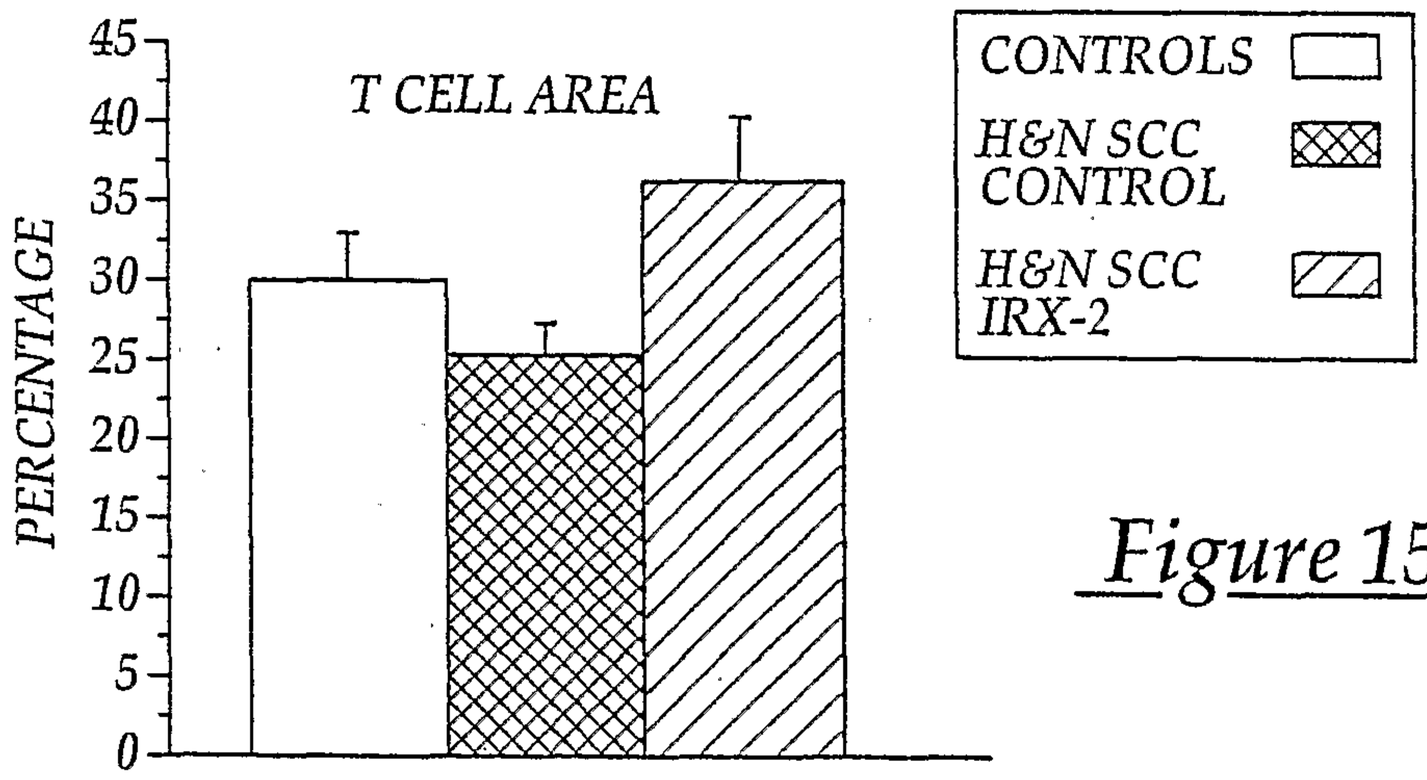


Figure 15A

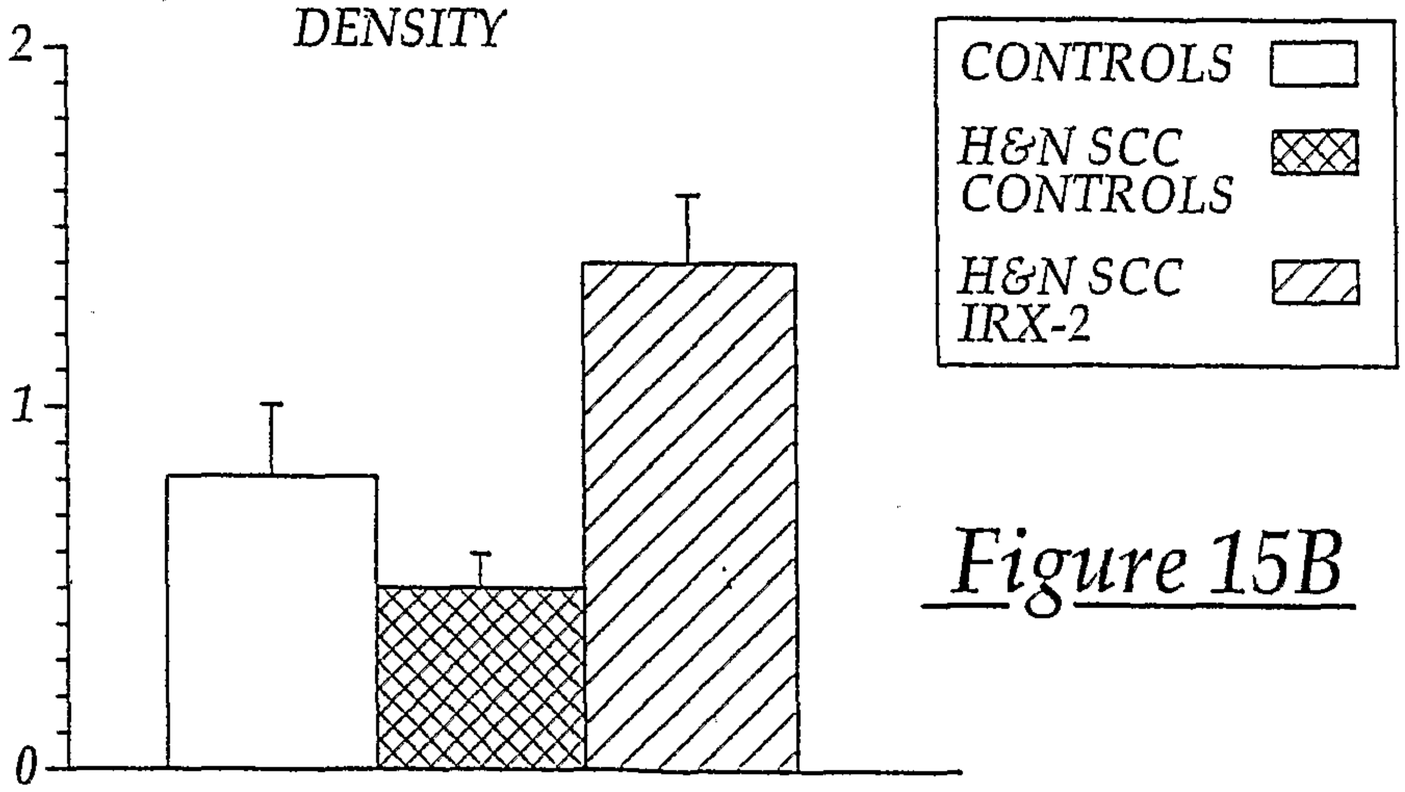


Figure 15B

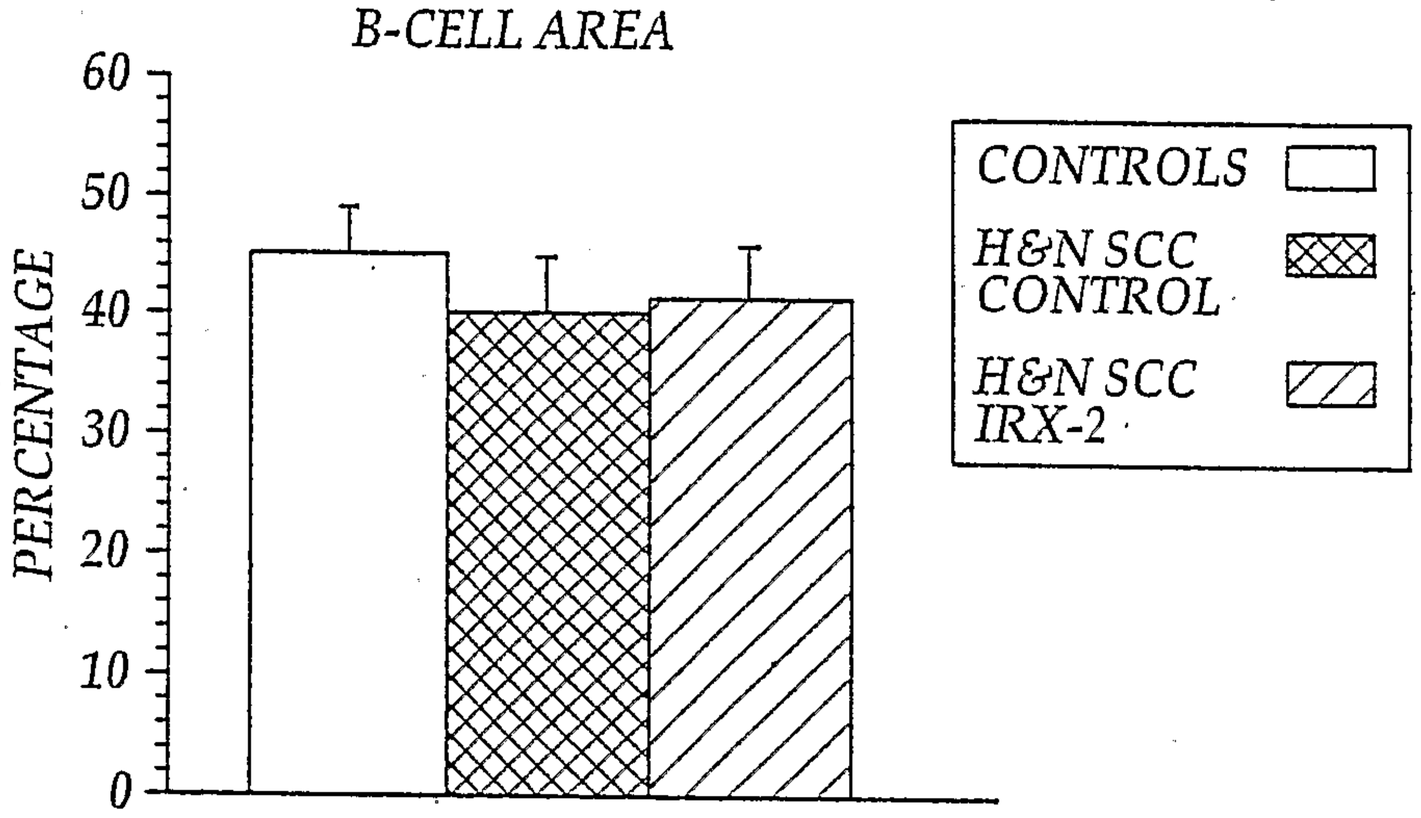


Figure 16A

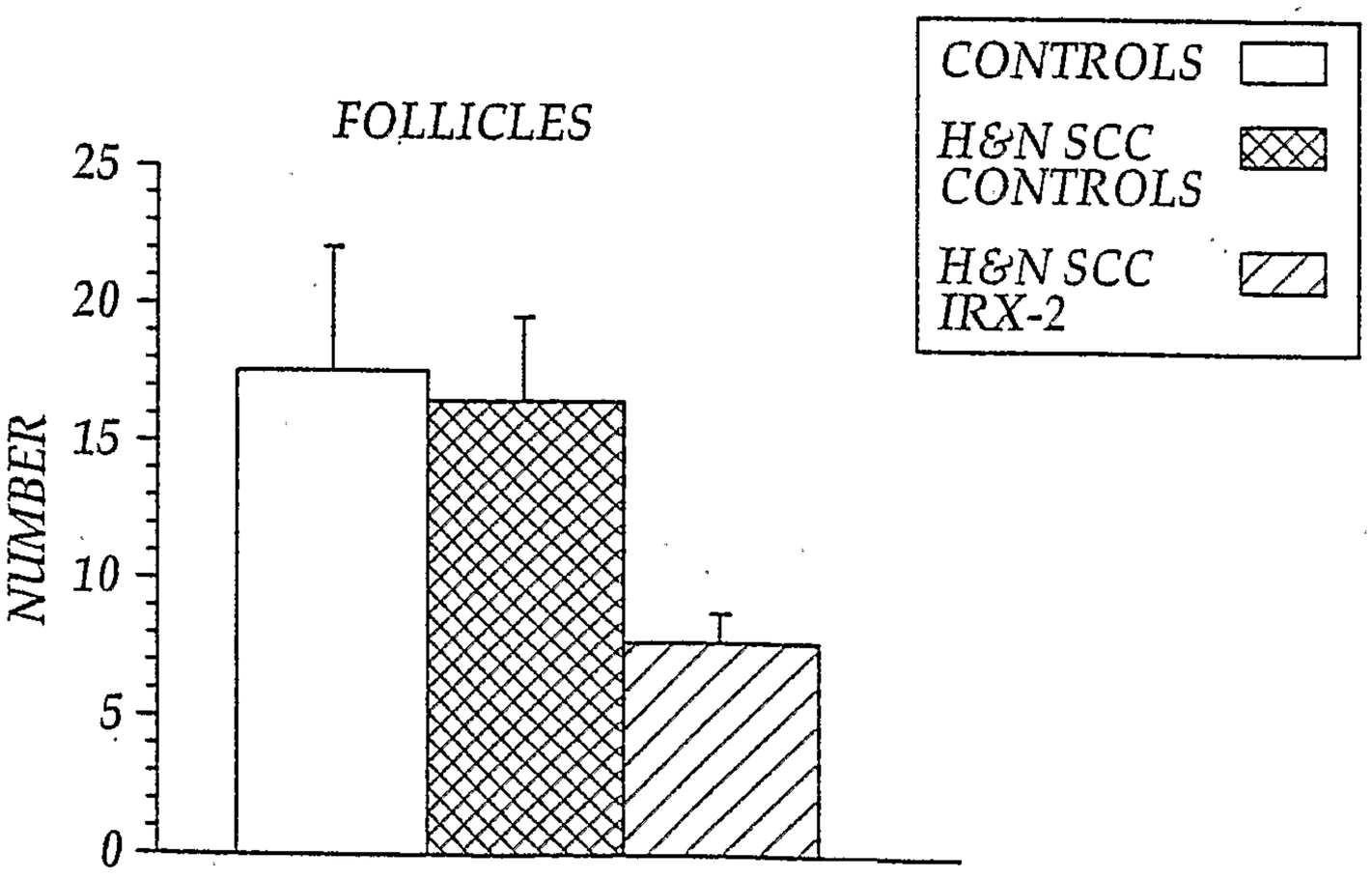


Figure 16B

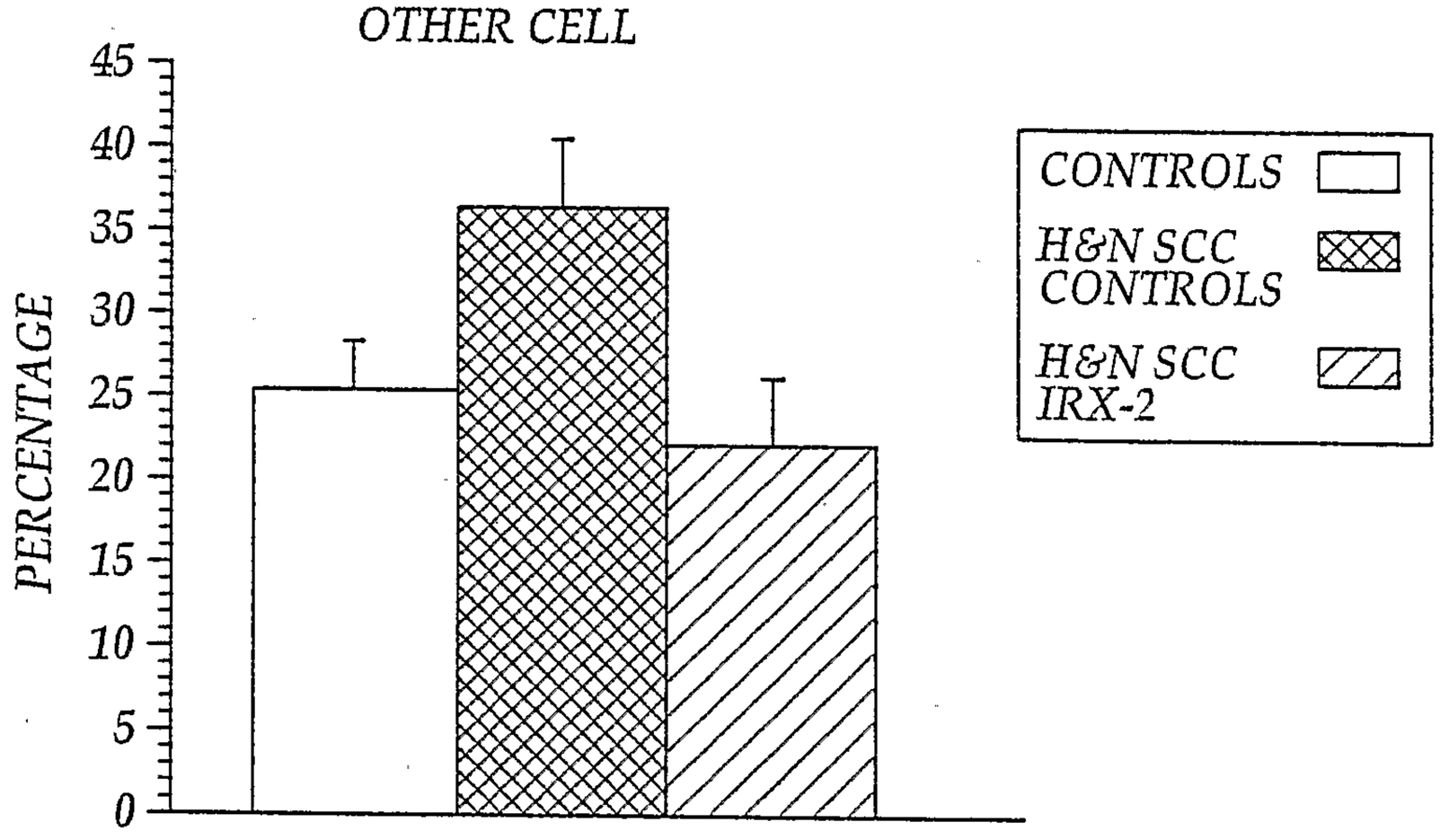


Figure 17A

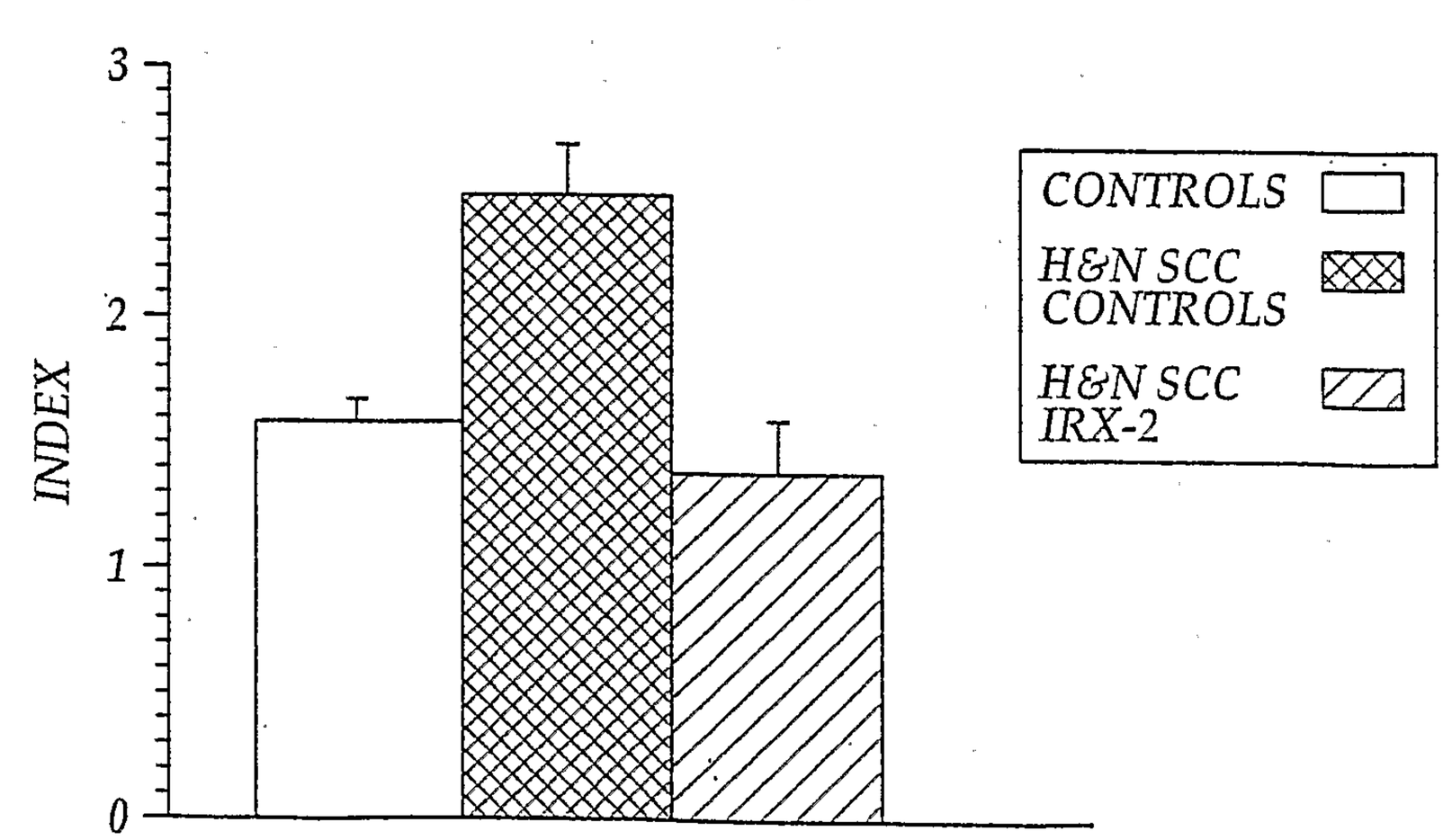
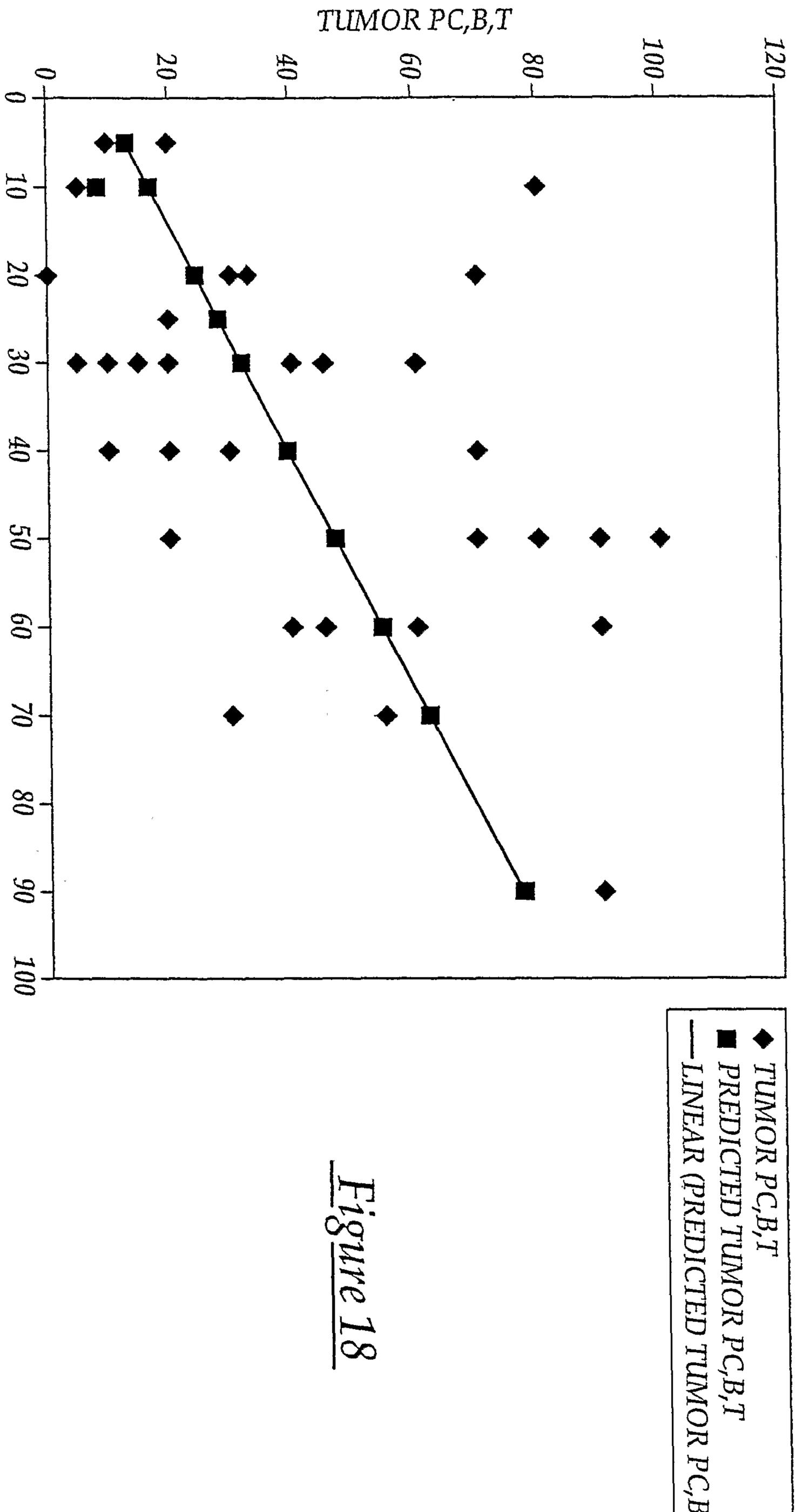


Figure 17B


TUMOR PC,B,T
NODE B,T LINE FIT PLOT
NODE B,T
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TUMOR PC,B,T
PREDICTED TUMOR PC,B,T
LINEAR (PREDICTED TUMOR PC,B,T)

Figure 18

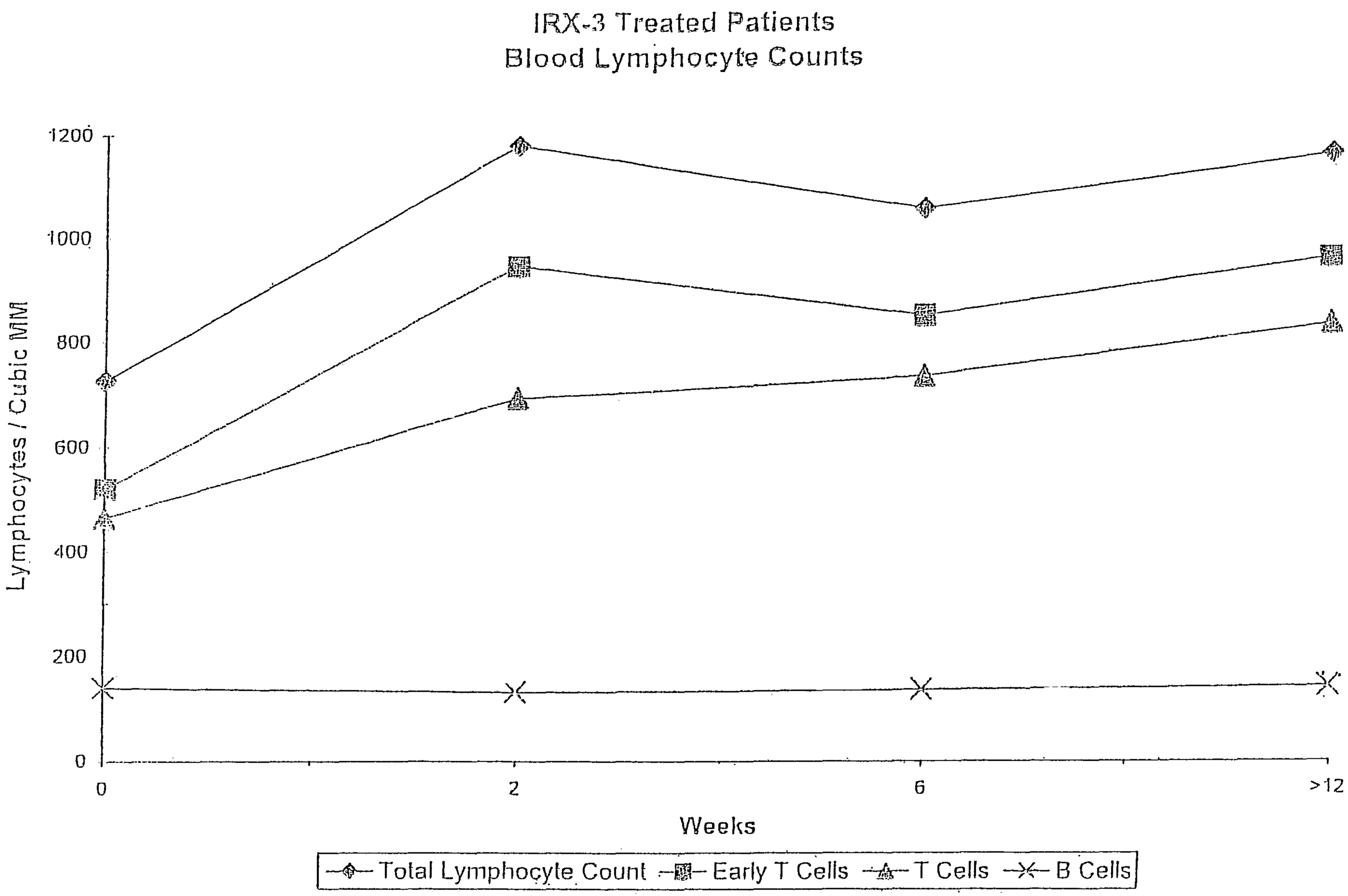
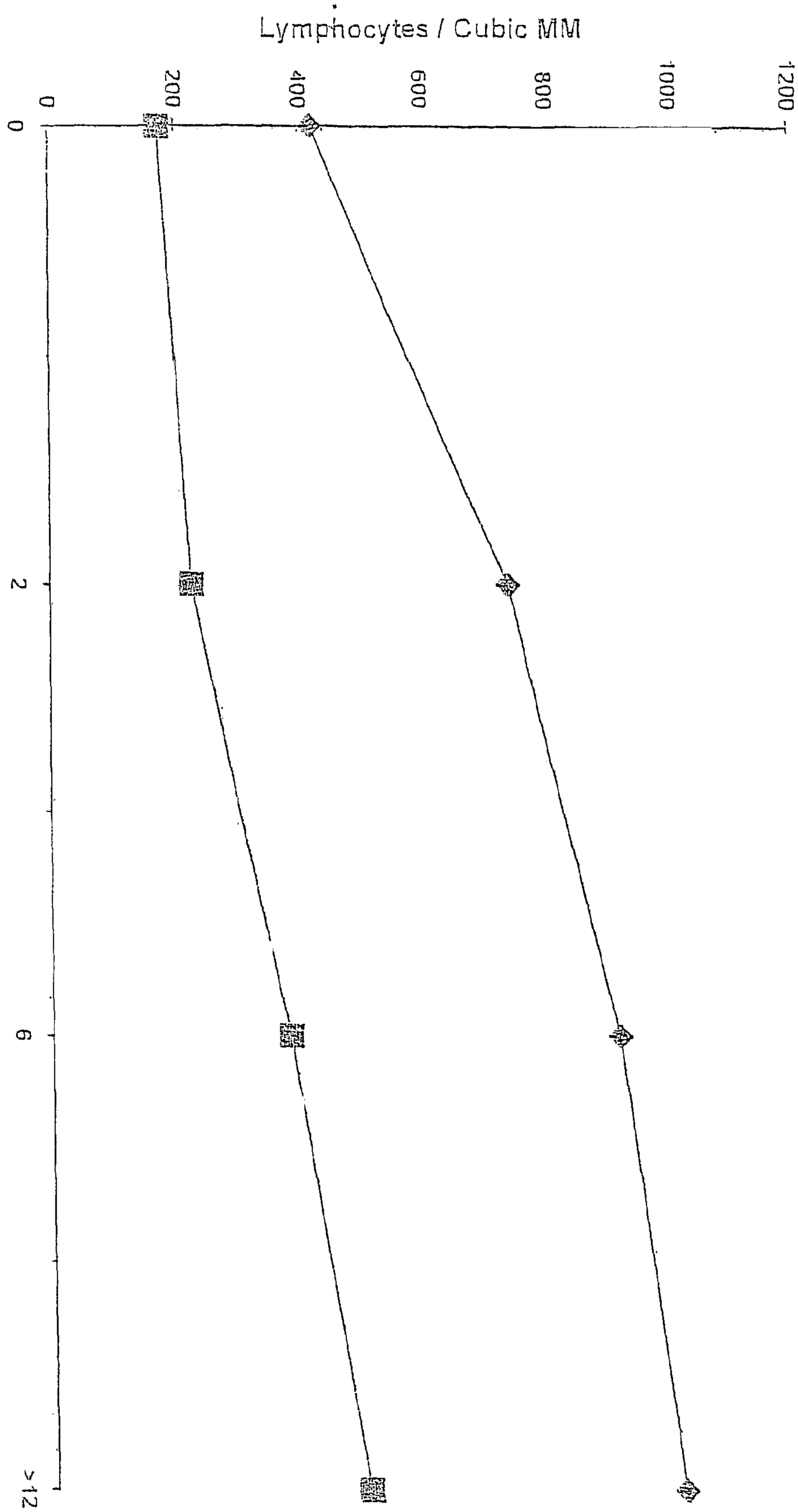


Figure 19



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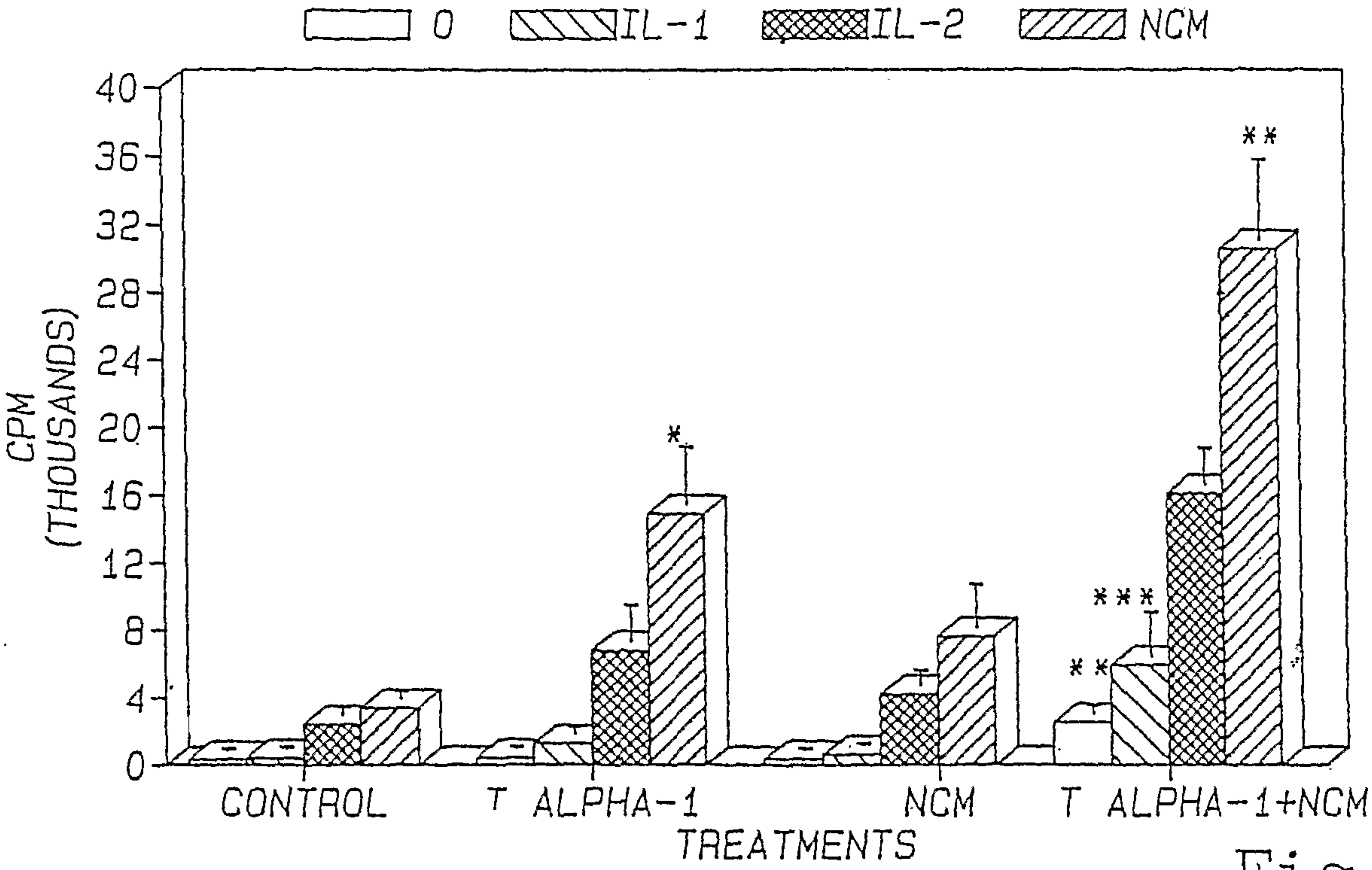
12/13

IRX-3 Treated Patients
Naive and Memory T Cells

Figure 20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IL-1
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CONTROL
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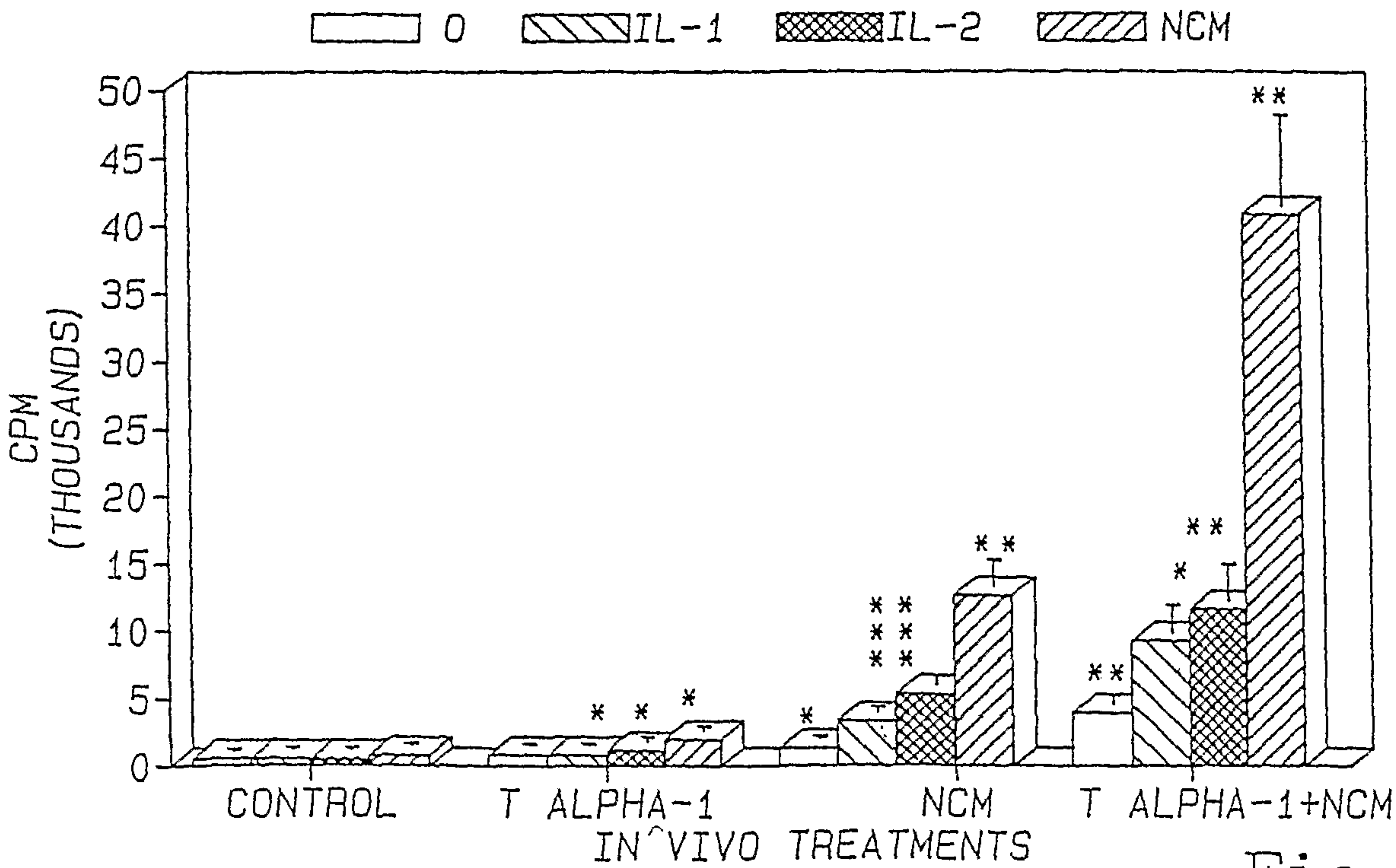
TREATMENTS

Fig- 21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CONTROL
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T ALPHA-1+NCM
IN^VIVO TREATMENTS

Fig- 22