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PROGRESS REPORT No.3

"REGULATION OF LYMPHOKINE GENE EXPRESSION AND *IN VITRO* IMMUNOTHERAPEUTIC EFFECT OF INTERLEUKIN 1 AND 2 ON MONOCYTES AND LYMPHOCYTES IN LEPROSY"

A RESEARCH PROJECT

CDR RESEARCH PROPOSAL CB-196

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SUBMITTED BY

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Project Title : Regulation of lymphokine gene expression and *in vitro* immunotherapeutic effect of interleukin 1 and 2 on monocytes and lymphocytes in leprosy.

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BACKGROUND/INTRODUCTIONS

Leprosy is a chronic infectious disease caused by a bacterial species, *Mycobacterium leprae*. This disease has a wide distribution throughout the world. It is estimated that there are approximately 15 million leprosy cases in this world. In Thailand there are approximately 200,000 leprosy patients among a population of 50 million with about 500 new cases annually.

It is very interesting that one single species of *M.leprae* is able to cause wide variation of disease forms in different persons, depending upon the infected individual's immune status (Rees, 1969). The clinical course of leprosy is, therefore, dependent on a continuing host-parasite relationship. Leprosy patients are classified into 6 immunopathologic groups (Ridley and Jopling, 1966); polar tuberculoid (TT), borderline tuberculoid (BT), borderline (BB), borderline lepromatous (BL) and lepromatous (LL), which is subdivided into subpolar lepromatous (LLs) and polar lepromatous (LLp).

It is generally accepted that the different clinical forms of leprosy are clearly associated with varying host defense and cellular immunity to *M.leprae* (Jopling, 1984). However, the immunologic defect(s) leading to the reduction or absence of cellular immunity to *M.leprae* in lepromatous leprosy are not yet fully understood. Early studies of patients with lepromatous leprosy suggested that there is a generalized anergy or unresponsiveness for cell-mediated immunity to various antigens. Other investigators suggested that in the early stages,

lepromatous leprosy, while unresponsive to M.leprae antigens, appears quite capable of manifesting normal response to other skin test antigens such as coccidioidin, histoplasmin, and PPD (Turk and Bryceson, 1971; Myrvang et al., 1973; Mendes et al., 1974).

Evidence for suppression of the immune response by suppressor T lymphocytes or monocytes in lepromatous leprosy patients has been demonstrated (Mehra et al., 1979, 1980, 1984). However, other attempts to find disease-related suppression in lepromatous leprosy have yielded conflicting results (Bjune 1979; Nath and Singh, 1980; Bullock et al., 1982; Stoner et al., 1982). Leprosy-specific nonresponsiveness in vitro was shown to result from a lack of IL2 production and can be overcome by the addition of exogenous IL2 (Haregewoin et al., 1983). It was also shown that peripheral blood lymphocytes of lepromatous leprosy patients failed to produce IFN-γ upon exposure to M.leprae and this deficiency was restored by the addition of purified human IL2 to the in vitro lymphocyte cultures (Nogueira et al., 1983).

In this study, the immunoregulatory mechanisms in leprosy patients will be evaluated at the cellular level by measuring levels of IL1 from monocytes and levels of IL2 from T lymphocytes. The numbers of IL1-producing monocytes and IL2-producing T lymphocytes will also be evaluated. The function of suppressor T lymphocytes and helper T lymphocytes from leprosy patients will be studied by HMI and CMI assays. Molecular analysis of the genes controlling IL2 and IFN-γ production will also be evaluated from the same patients and normal controls.

The active collaboration between a molecular biology laboratory in Jerusalem and a cellular immunology laboratory in Chiang Mai provides a bridge between cellular and molecular analysis of this ancient disease. The chance to obtain scientifically meaningful results from this active collaboration can be rated extremely high. The results of this project are expected to provide novel insight into immunoregulatory mechanisms in leprosy.

OBJECTIVES

OVERALL AIM AND SPECIFIC OBJECTIVES.

1. Overall Aim. The aim of this research proposal is to elucidate the abnormality in cell-mediated immunity (CMI) in human leprosy by studying the quantity and functional intactness of T lymphocytes producing IL2 and monocytes producing IL1, by examining the function of T helper and T suppressor cells, particularly their role in the regulation of IL2 and IFN-γ gene expression in this disease.

2. Specific Objectives. The specific objectives of this proposed research is as follows:

2.1 To investigate the level and numbers of IL1-producing monocytes from various types of leprosy and normal subjects.

2.2 To determine the level and numbers of IL2-producing T lymphocytes from different types of leprosy and normal subjects.

2.3 To assess the effect, *in vitro*, of exogenous IL1 and/or IL2 on defective IL1-producing monocytes and defective IL2-producing T lymphocytes of various types of leprosy and normal subjects.

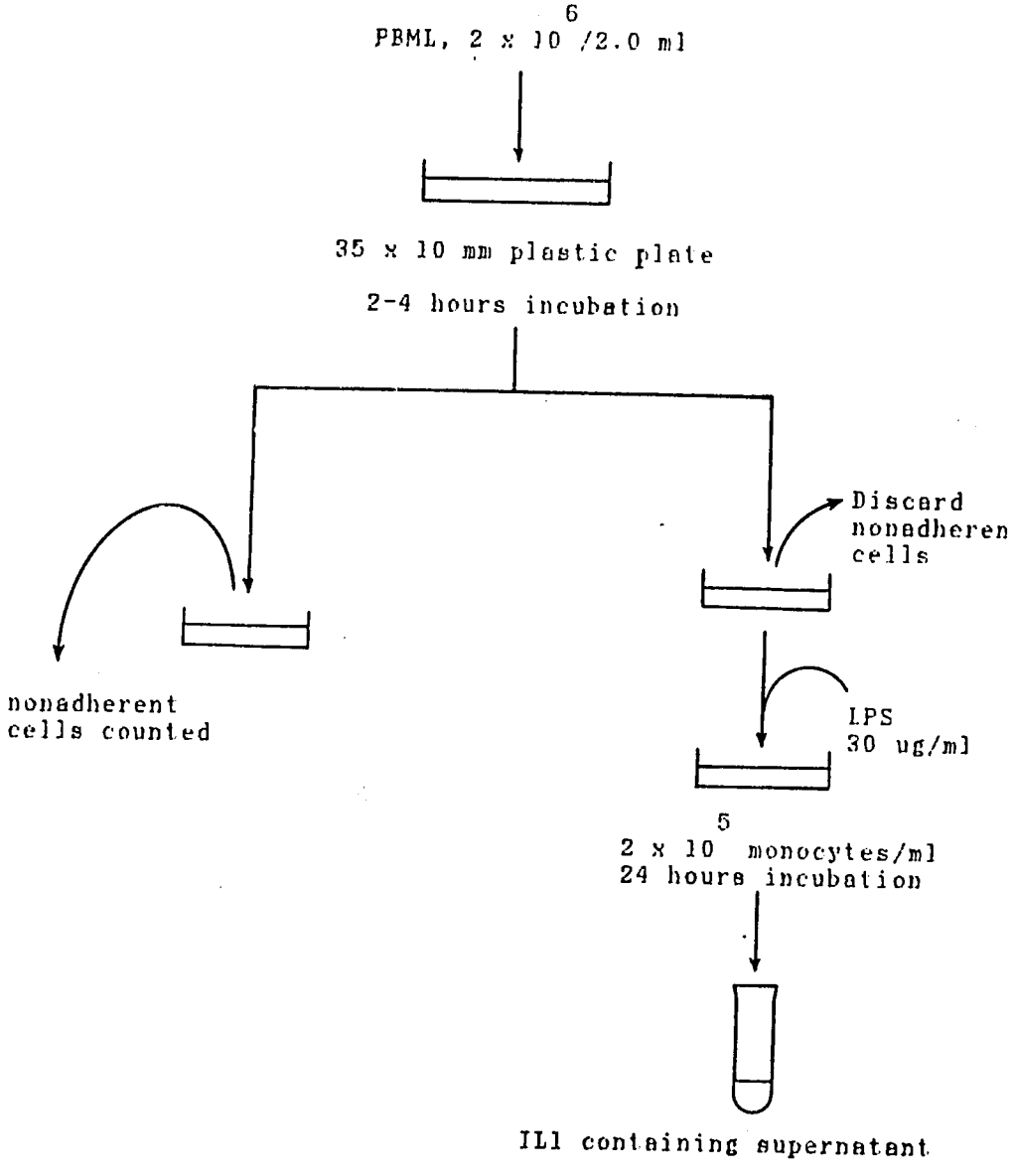
2.4 To evaluate the function of helper and suppressor T lymphocytes of different types of leprosy and normal subjects.

2.5 To investigate IL2 and IFN-γ gene regulation in different types of leprosy and normal subjects and the relation between suppressor T cells and expression of IL2 and IFN-γ genes.

MATERIALS AND METHODS

PRODUCTION AND DETERMINATION OF IL1 LEVEL

1. Production of IL1.



2. Assay of IL1 Activity

Samples for IL1 assay

↓ - Dilution

Dilution of samples

(1:2, 1:4, 1:8, 1:16 and 1:32)

↓ Thymocyte suspension 100 ul of 3×10^{7} /ml

PHA-P 30 ul of 20 ug/ml

Incubated for 72 hours

↓ ^{3}H-TdR 25 ul of 8 uCi/ml

Incubated 2-4 hours

↓ - Cell harvester

Liquid Scintillation Counter for CPM

↓

CPM or Units of IL1

PRODUCTION AND DETERMINATION OF IL2 LEVEL

1. Production of IL2.

PMBL, 1×10^6 cells/ml

↓ — 2 ug/ml PHA-P or

— 50 ug/ml PPD

Incubated at 37 °C 5% CO_2

18 hours for mitogen and

48 hours for antigen

↓ — Centrifuged

IL2 containing supernatant

↓ — α-methyl-D-mannoside

Stored at -70 °C

for IL2 assay

2. Assay of IL2 Activity.

Samples for IL2 assay

↓ — Dilution

Dilution of samples

(1:2, 1:4, 1:8, 1:16 and 1:32)

↓ — 3-day Con A blasts

Incubated 24 hours

↓ — ^{3}H-TdR

Incubated for 18 hours

↓ — Cell harvester

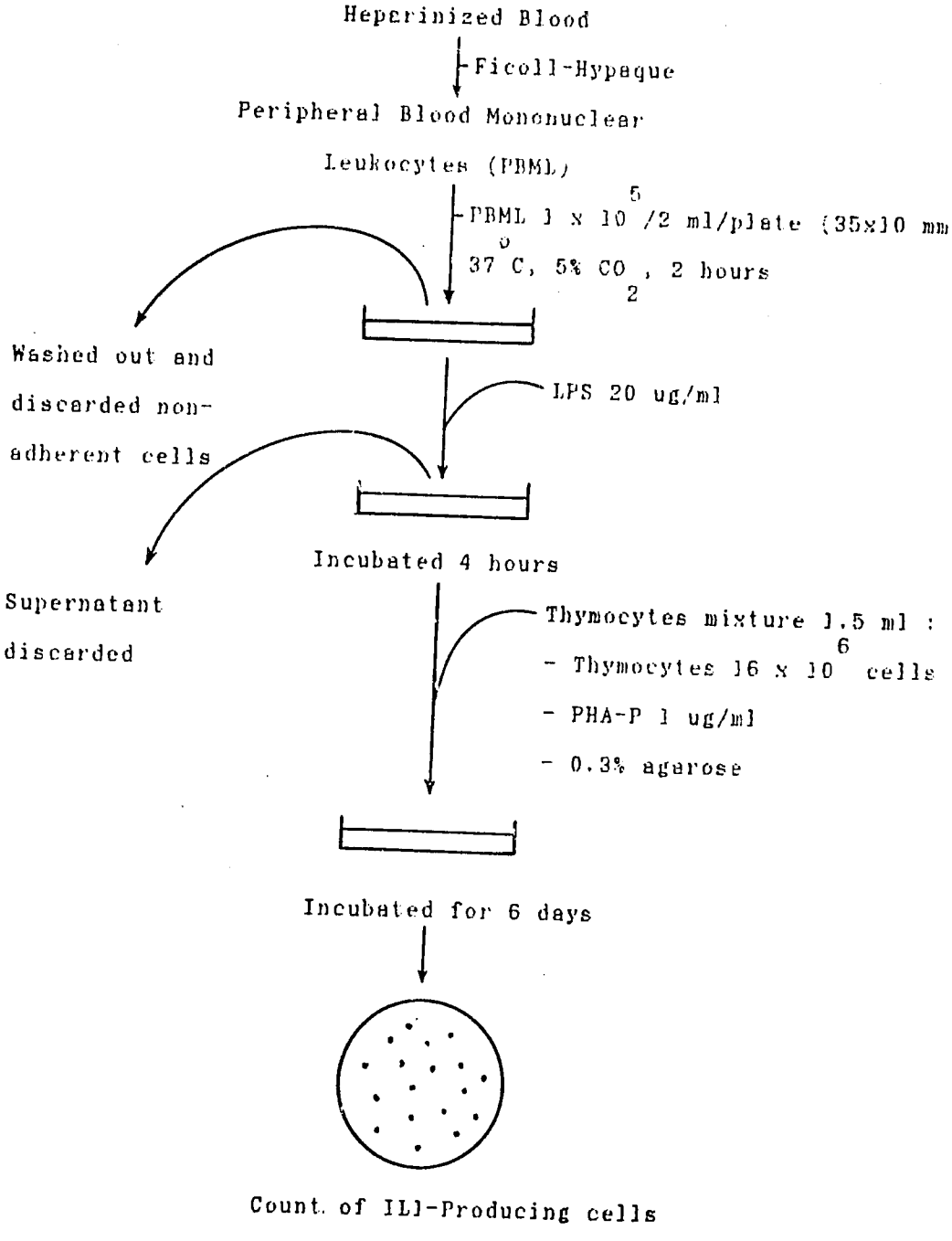
Liquid Scintillation

Counter for CPM

↓

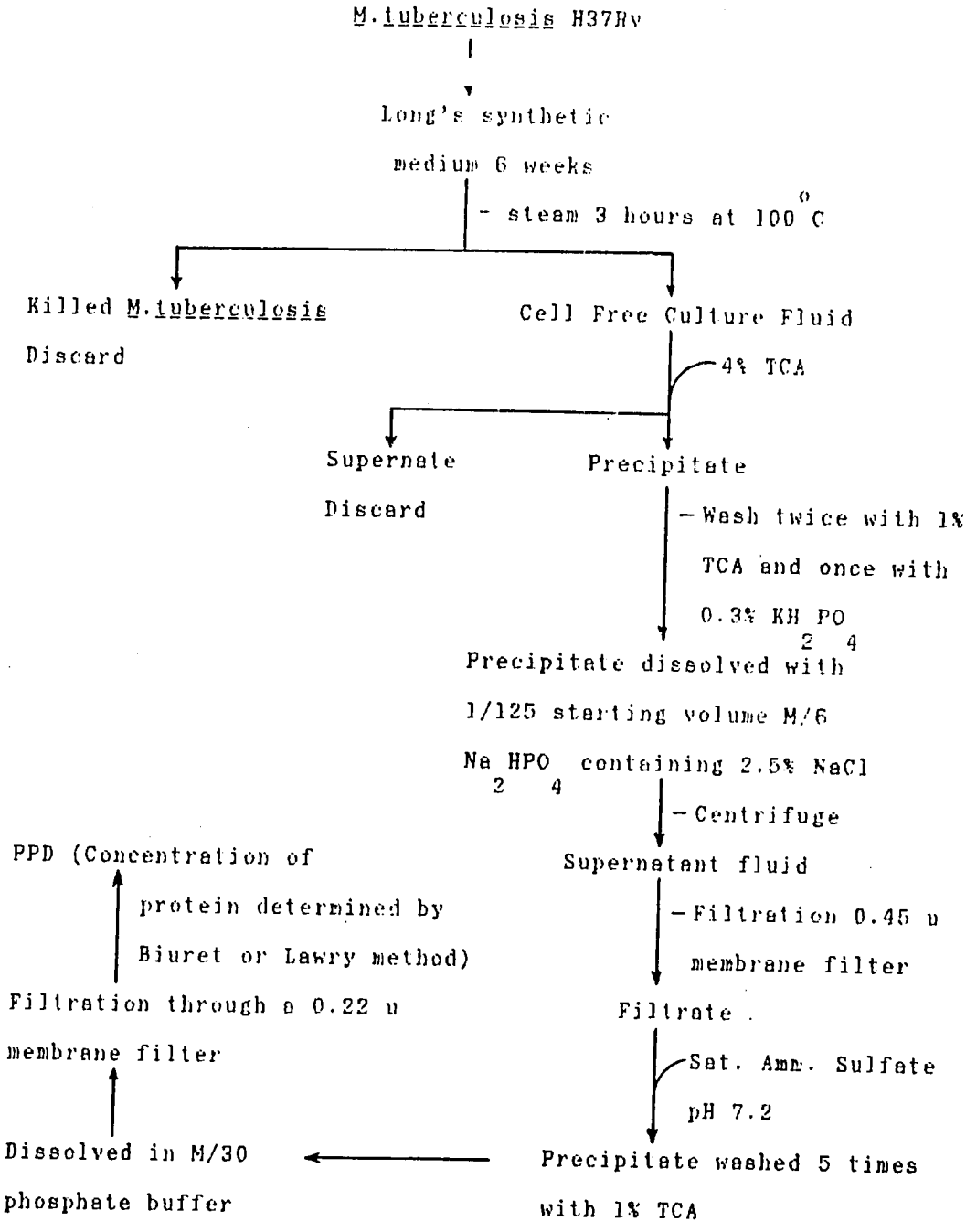
CPM or Units of IL2

DETERMINATION OF THE NUMBER OF IL1-PRODUCING CELLS

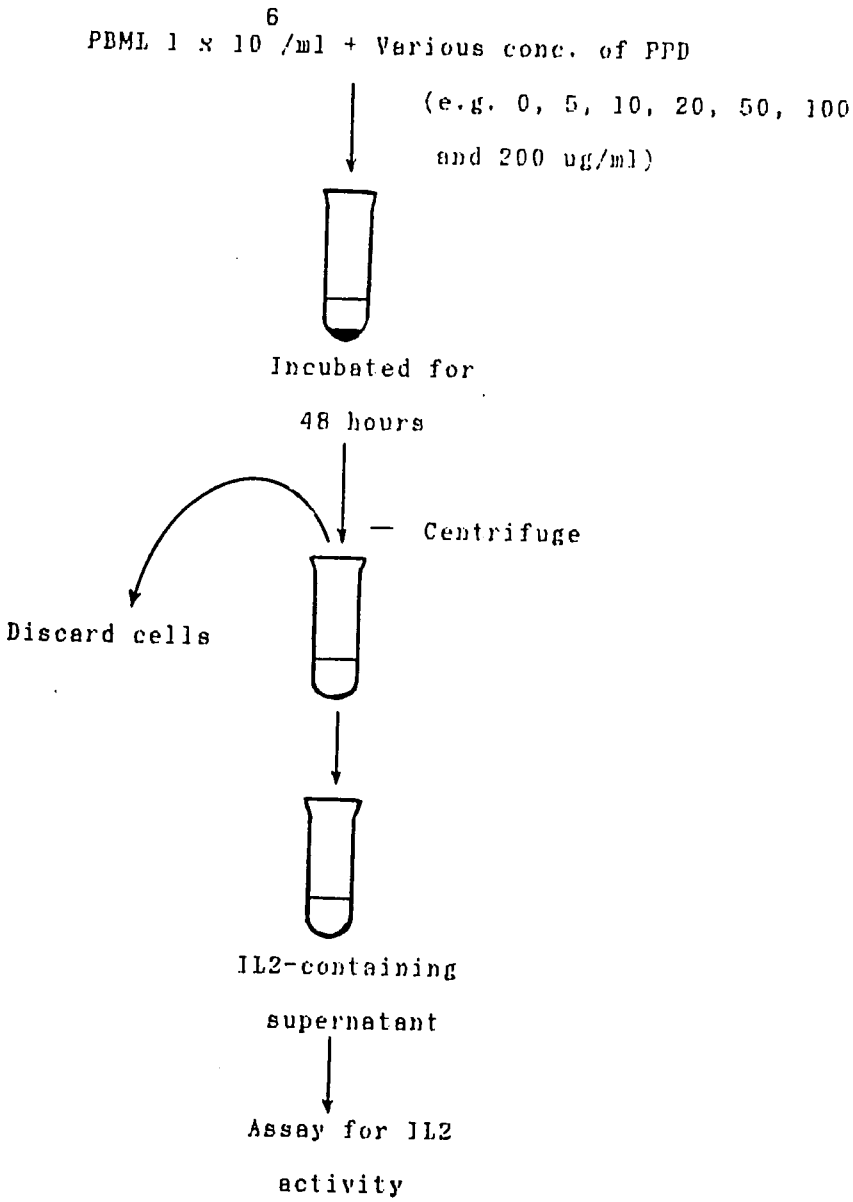


PREPARATION AND EVALUATION OF PPD FROM M.TUBERCULOSIS

1. Preparation of PPD.



2. Evaluation of PPD.



EFFECTS OF INDOMETHACIN ON SUPPRESSION OF MYCOBACTERIUM LEPRAE ON IL2 PRODUCTION IN LEPROSY PATIENTS.

EXPERIMENTAL DESIGN:

A) Normal Subjects.

1. PBML + *M.leprae* + Indomethacin -------> IL2
   (normal)

2. PBML + *M.leprae* -------> IL2
   (normal)

3. PBML + Indomethacin -------> IL2
   (normal) + PPD + Indomethacin -------> IL2

4. PBML + PPD + Indomethacin -----> IL2
   (normal)

B) Leprosy Patients.

1. PBML + *M.leprae* + Indomethacin --------> IL2
   (BL/LL or BT/TT)

2. PBML + *M.leprae* -------> IL2
   (BL/LL or BT/TT)

3. PBML + Indomethacin -------> IL2
   (BL/LL or BT/TT)

4. PBML + PPD + Indomethacin -------> IL2
   (BL/LL or BT/TT)

RESULTS

PRODUCTION AND DETERMINATION OF IL1 LEVEL

Level of IL1 from 2×10^5 /ml monocytes of normal subjects were determined. The level of IL1 was compared with the numbers of IL1-producing monocytes from the same subject. There is high correlation between IL1 level and numbers of IL1-producing monocytes.

DETERMINATION OF THE NUMBER OF IL1-PRODUCING CELLS

1. Optimal Concentration of PBML.

The optimal concentration of PBML in producing maximal numbers of IL1-producing cells was determined using 0.5×10^5, 1×10^5, 2×10^5 and 4×10^5 PBML/plate for surface adherance. Optimal concentration of PBML was 1×10^5 /plate as shown in figure 1.

2. Optimal Concentration of thymocytes.

The optimal concentration of thymocytes as a responder cells was determined using 4×10^6, 8×10^6, 12×10^6, 16×10^6 and 20×10^6 thymocytes/plate. Optimal concentration of thymocytes at 16×10^6 cells/ml was choosen for this study as illustrated in figure 2.

3. Optimal Concentration of LPS.

Optimal concentration of LPS for IL1-producing cells stimulation was determined using concentration of LPS at 0, 5, 10, 20, 40 and 60 ug/ml PBML. After 4 hours of incubation in 5% CO_2 at 37 ^oC, adhering monocytes was washed twice with 10% FCS-RPMI media and 16×10^6 thymocytes in 1.5 ml of 0.3% agarose containing 1 ug/ml PHA-P was added. Optimal concentration of LPS at 20 ug/ml was obtained as depicted in figure 3.

4. Optimal Stimulation Time of LPS.

Optimal stimulation time of LPS for IL1-producing cells was determined by incubation of 20 ug/ml LPS with monocytes for 0, 1, 2, 4, 6 and 8 hours. The optimal stimulation time of LPS was 4 hours as shown in figure 4.

5. Optimal Concentration of PHA-P.

Optimal concentration of PHA-P for co-stimulation of IL1-producing cells was determined using 0, 0.5, 1, 2 and 4 ug/ml in 1.5 ml. of 0.3% agarose containing 16×10^6 thymocytes. Optimal concentration of PHA-P at 1 ug/ml was obtained as shown in figure 5.

6. Optimal Incubation Period for IL1-Producing Cells.

Optimal incubation period for IL1-producing cells to develop visible clusters or colonies of responder cells was determined by incubation period of 1, 2, 3, 4, 5, 6 and 7 days after overlayering of monocytes with mixtures of thymocytes, agarose and PHA-P. The optimal incubation period for maximum development of IL1-producing cells was 5 days as shown in figure 6.

PREPARATION AND EVALUATION OF PPD FROM M.TUBERCULOSIS.

Three lots of PPD were prepared from cultured filtrate of M.tuberculosis using procedure as summarized in materials and method. All PPD preparations were evaluated for in vitro stimulation of IL2 production from normal subjects' PBML (skin test positive to PPD). All three lots of PPD preparation showed very good stimulation of IL2 production from all normal subjects as shown in figures 7, 8 and 9.

EFFECTS OF INDOMETHACIN ON SUPPRESSION OF MYCOBACTERIUM LEPRAE ON IL2 PRODUCTION IN LEPROSY PATIENTS.

Results from Experimental Design

1. PBML + *M.leprae* -----> Low IL2 production
   (normal)

2. PBML + *M.leprae* + Indomethacin -----> Enhancement of IL2 production
   (normal)

3. PBML + *M.leprae* -----> Low IL2 production
   (BT/TT)

4. PBML + *M.leprae* + Indomethacin -----> Enhancement of IL2 production
   (BT/TT)

5. PBML + *M.leprae* -----> Low IL2 production
   (BL/LL)

6. PBML + *M.leprae* + Indomethacin -----> Still low IL2 production (no enhancement of IL2 production)
   (BL/LL)

7. PBML + Indomethacin -----> Low IL2 production
   (normal)

8. PBML + Indomethacin -----> Low IL2 production
   (BT/TT)

9. PBML + Indomethacin -----> Low IL2 production
   (BL/LL)

10. PBML + PPD -----> High IL2 production
    (normal)

11. PBML + PPD -----> Low IL2 production
    (BT/TT)

12. PBML + PPD -----> Low IL2 production
    (BL/LL)

13. PBML + PPD + Indomethacin -----> Increase IL2 production
    (normal)

14. PBML + PPD + Indomethacin -----> Increase IL2 production
    (BT/TT)

15. PBML + PPD + Indomethacin -----> Increase IL2 production
    (BL/LL)

NOTE : All normal subjects are skin test positive to PPD.

Summary of the experimental results were shown in figures 10-20.

DISCUSSION AND CONCLUSION

Method for determination of the number of IL1-producing cells has been set up in our laboratory. Optimal concentrations of PBML, thymocytes, LPS and PHA-P for maximal development of IL1-producing cells was determined. Optimal stimulation period of LPS and optimal incubation period for maximum development of IL1-producing cells was also determined. The number of IL1-producing cells in PBML of normal subjects are being investigated.

Three lots of PPD were prepared in our laboratory. All PPD are able to stimulate IL2 production from PBML of skin test positive to PPD in normal subjects. The activities of our PPD are equivalent to the good commercial PPD.

We investigated the effect of indomethacin to the suppressive activity of M.leprae on IL2 production. Indomethacin was able to lower suppressive activity of M.leprae on IL2 production of PBML from normal or BT/TT leprosy patients. However, indomethacin was not be able to lower the suppressive activity of M.leprae on IL2 production of PBML from BL/LL leprosy patients. Indomethacin alone has no effect on IL2 production from PBML of normal or leprosy patients.

WORKPLAN FOR THE NEXT PERIOD

The relationship of IL1 levels and numbers of IL1-producing cells in normal subjects and various types of leprosy patients

will be investigated. The function of T cell subpopulations will be studied both in CMI and HMI responses in various types of leprosy patients compared to normal subjects. The *in vitro* effects of recombinant IL1 and/or IL2 on the defective IL1-producing monocytes and the defective IL2-producing T lymphocytes of leprosy patients will be investigated. PBML from various types of leprosy patients and normal subjects will be tested for their IL2 and IFN-γ gene expression in response to PHA-P, in untreated cells and in cells treated with cycloheximide or γ-irradiation.

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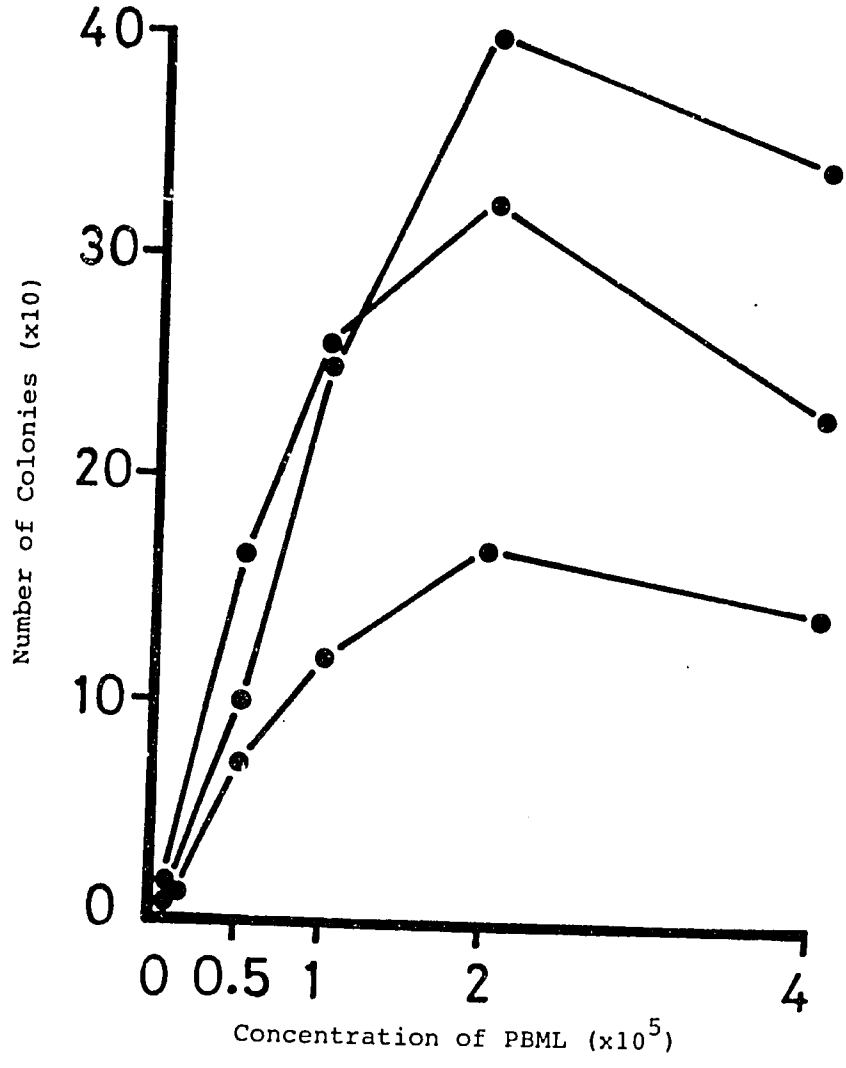

Figure 1. Determination of optimal concentration of PBML for maximal numbers of IL1-producing cells.

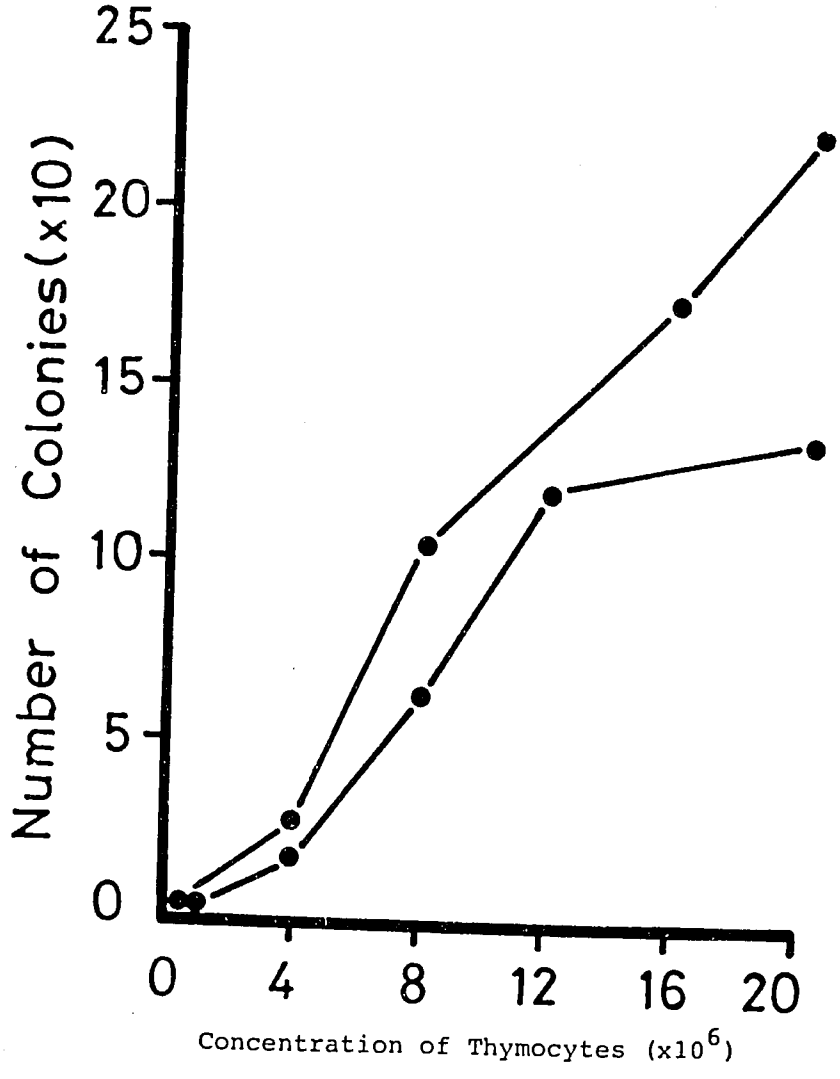

Figure 2. Determination of optimal concentration of mouse thymocytes for maximal numbers of IL1-producing cells.

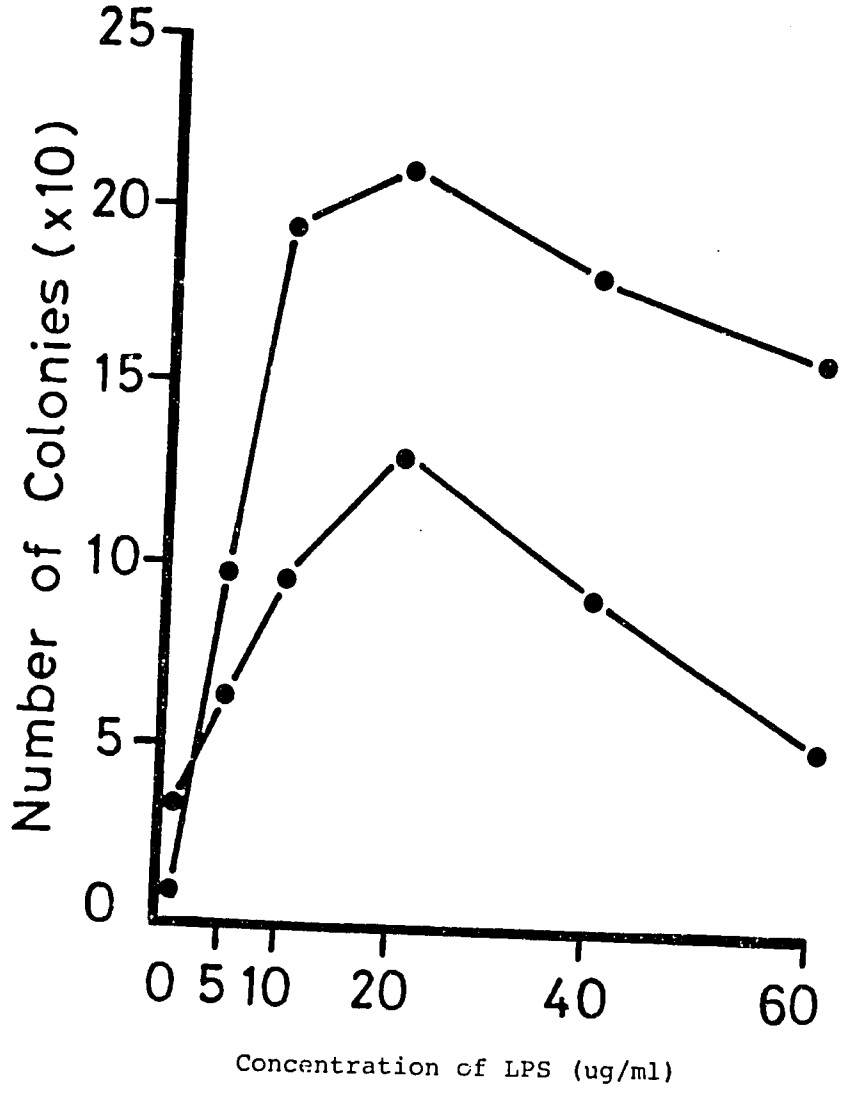

Figure 3. Determination of optimal concentration of LPS for maximal stimulation of IL1-producing cells.

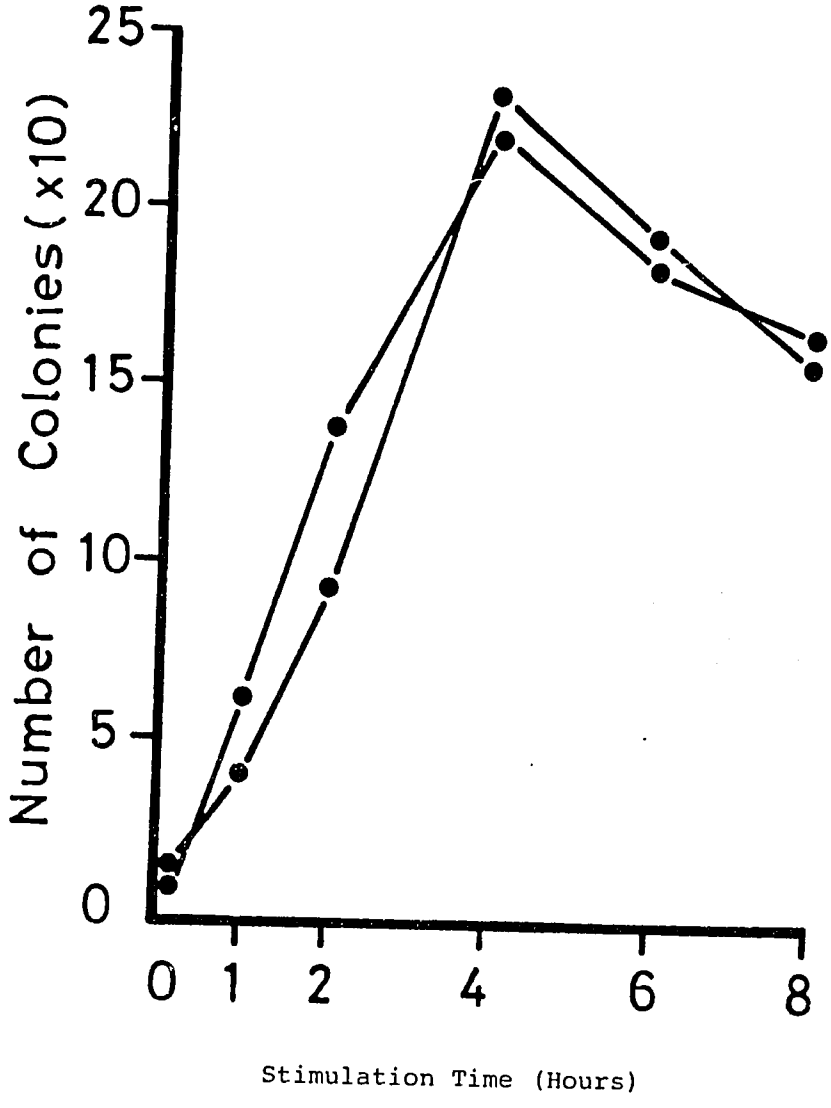

Figure 4. Determination of optimal stimulation period of LPS for maximal stimulation of IL1-producing cells.

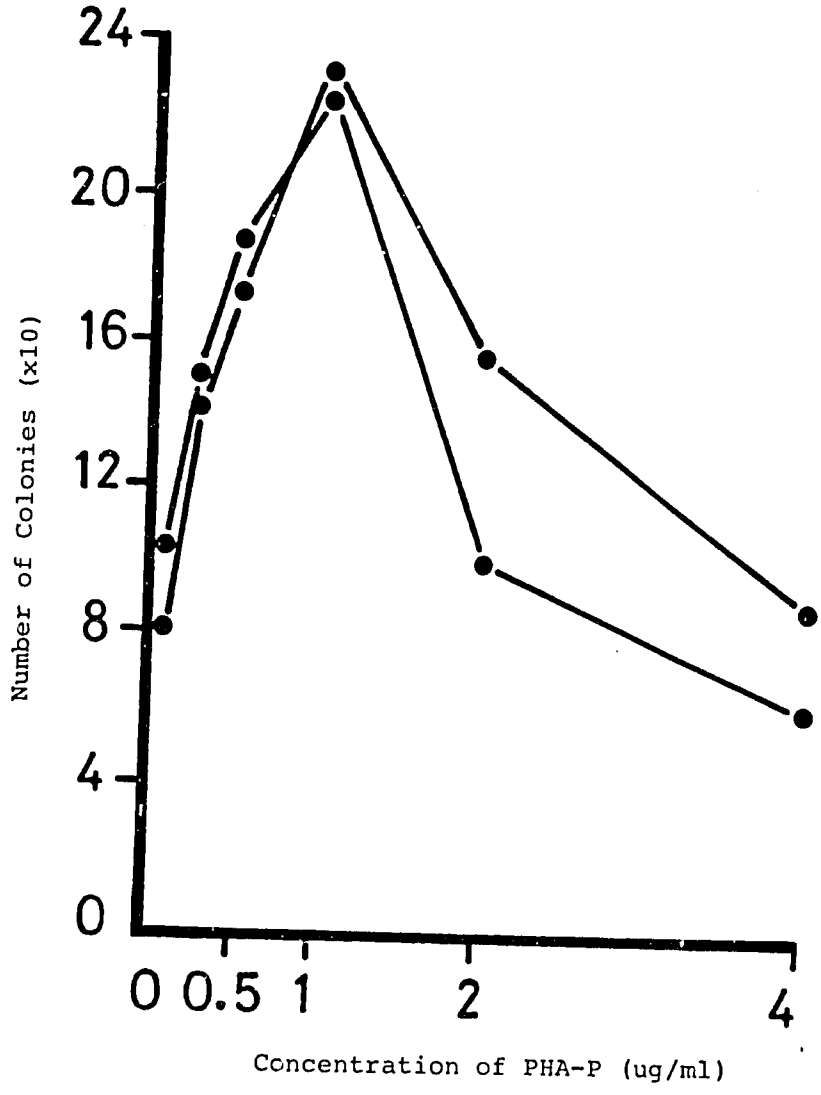

Figure 5. Determination of optimal concentration of PHA-P for maximal costimulation of IL1-producing cells.

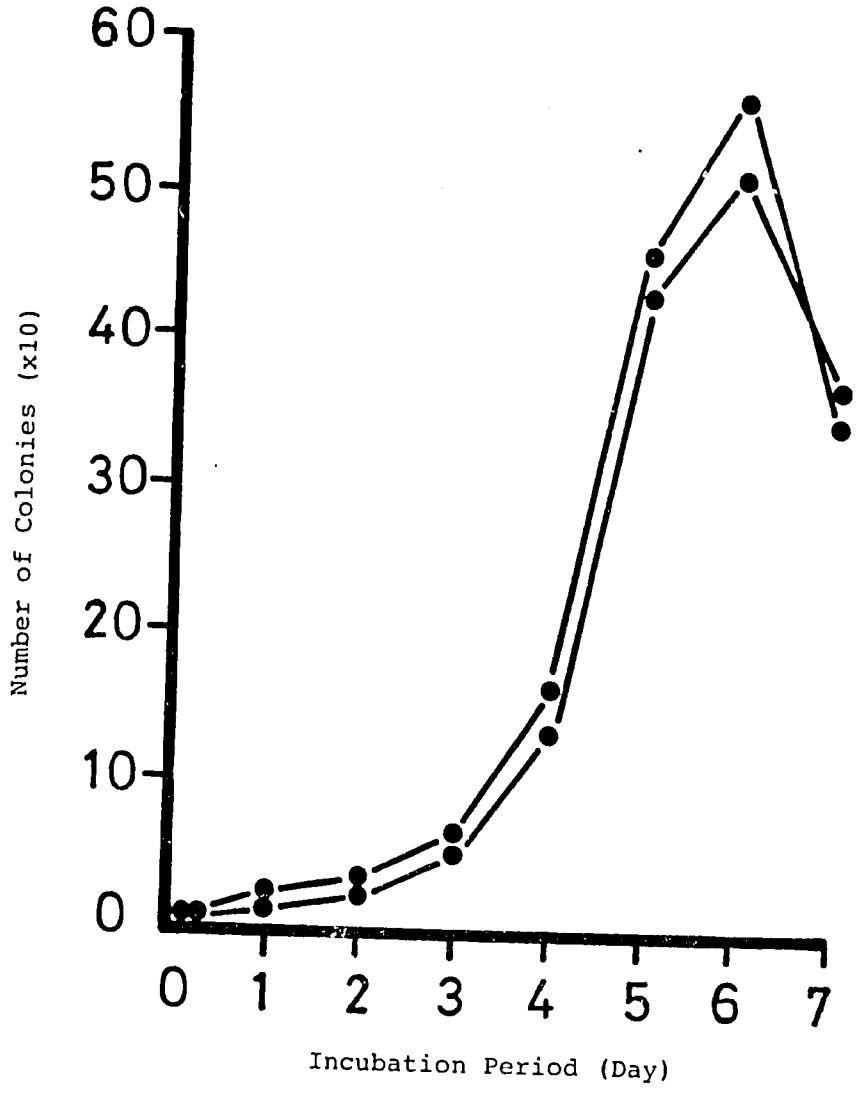

Figure 6. Determination of optimal incubation period for maximal development of IL1-producing cells.

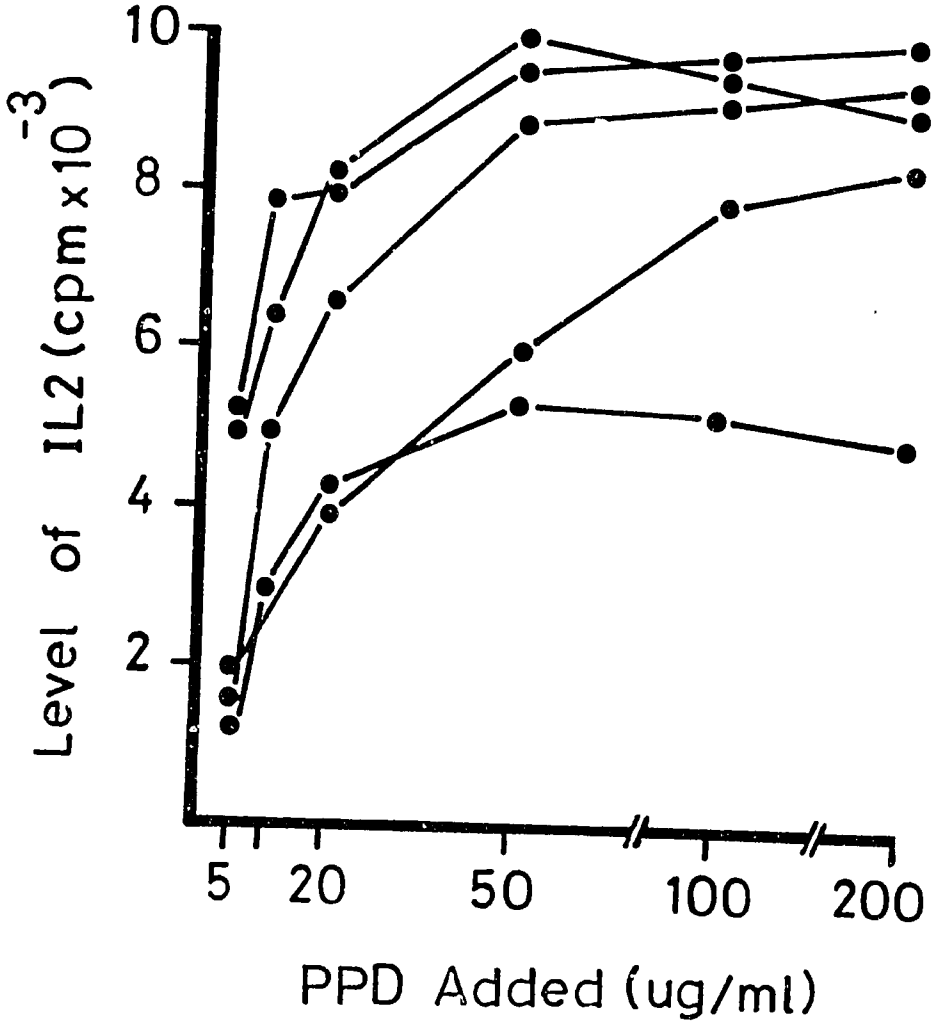

Figure 7. Evaluation of PPD lot 1 for stimulation of IL2 production from PBML of normal subjects (skin test positive to PPD).

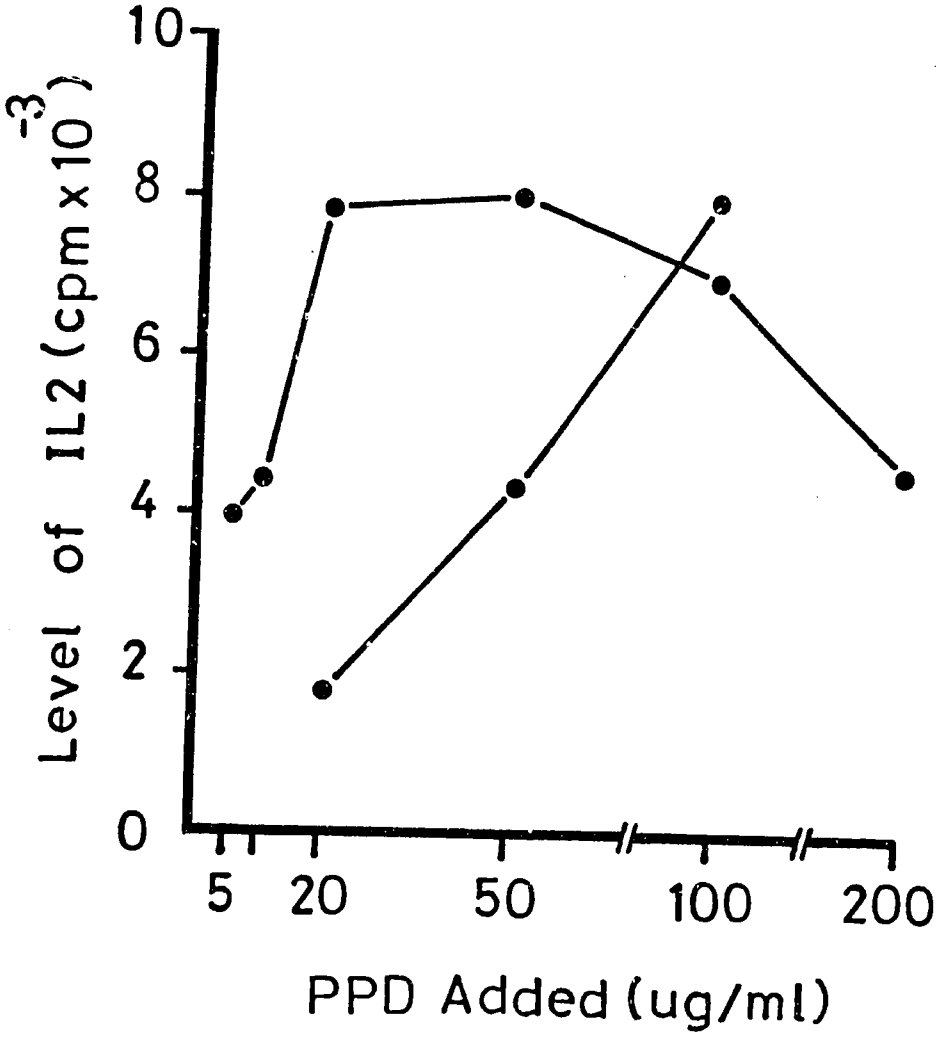

Figure 8. Evaluation of PPD lot 2 for stimulation of IL2 production from PBML of normal subjects (skin test positive to PPD).

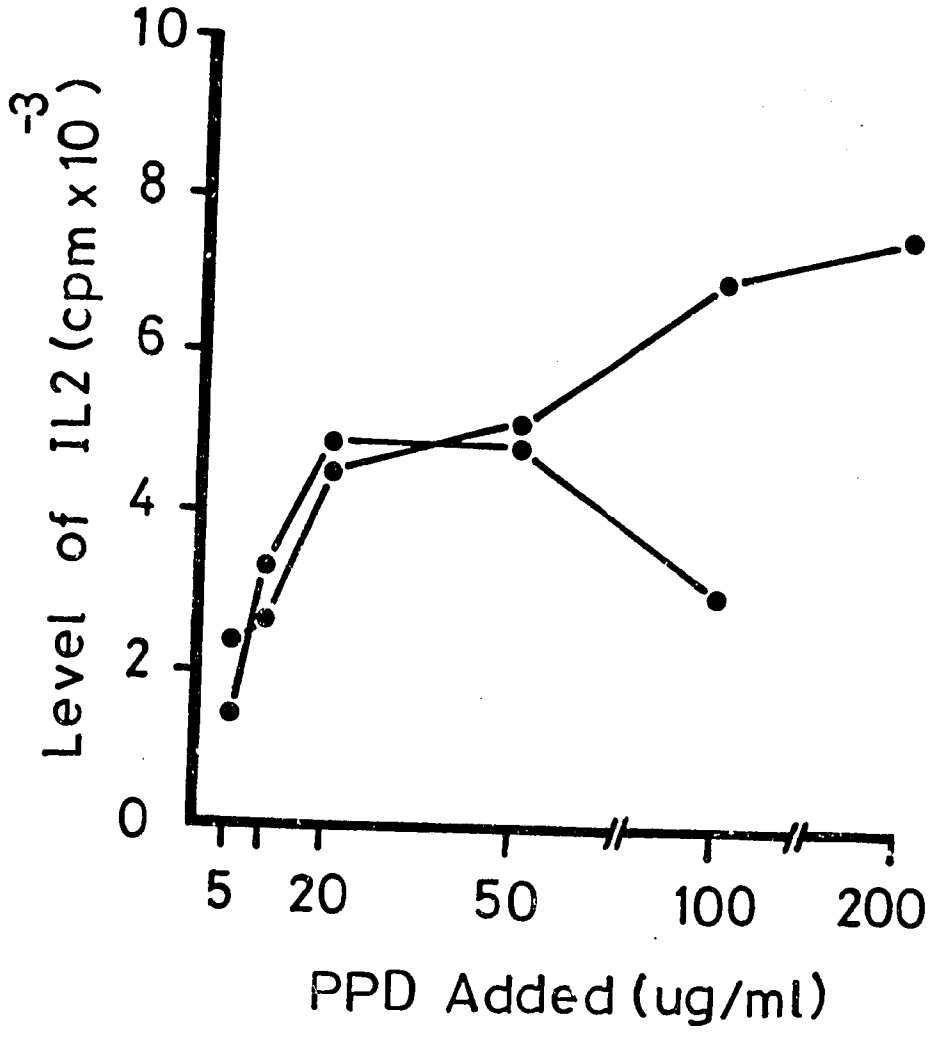

Figure 9. Evaluation of PPD lot 3 for stimulation of IL2 production from PBML of normal subjects (skin test positive to PPD).

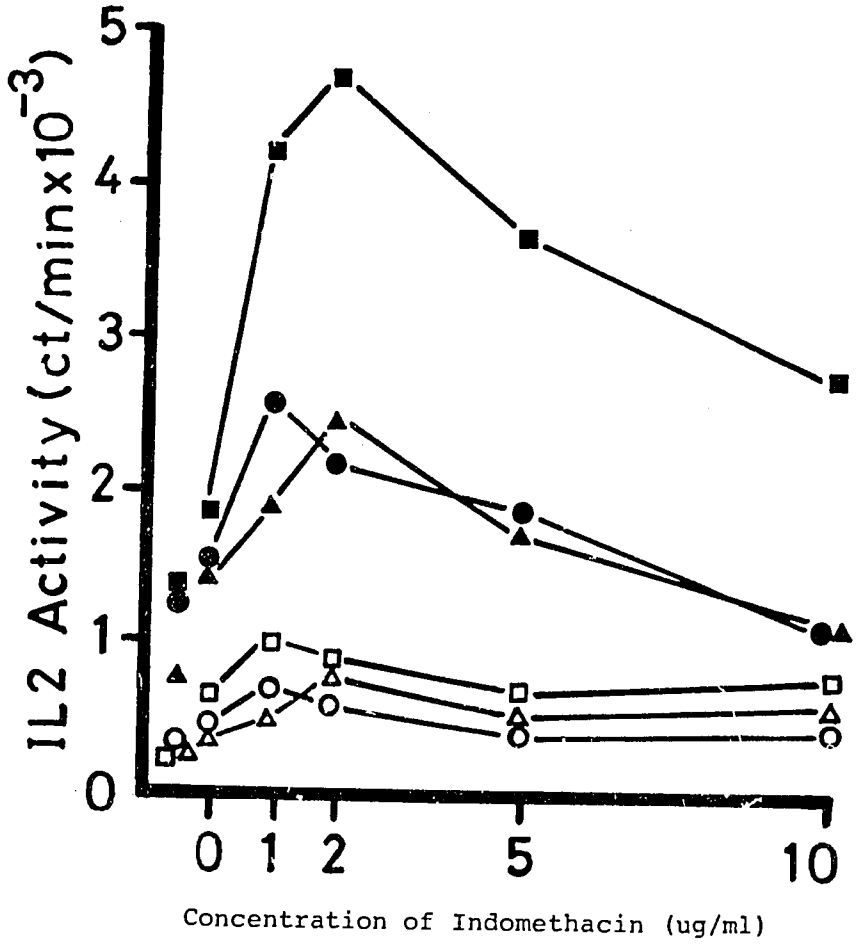

Figure 10. Effect of indomethacin on the suppressive activity of M.leprae on IL2 production of PBML from normal subjects (M.leprae 20 ug/culture).

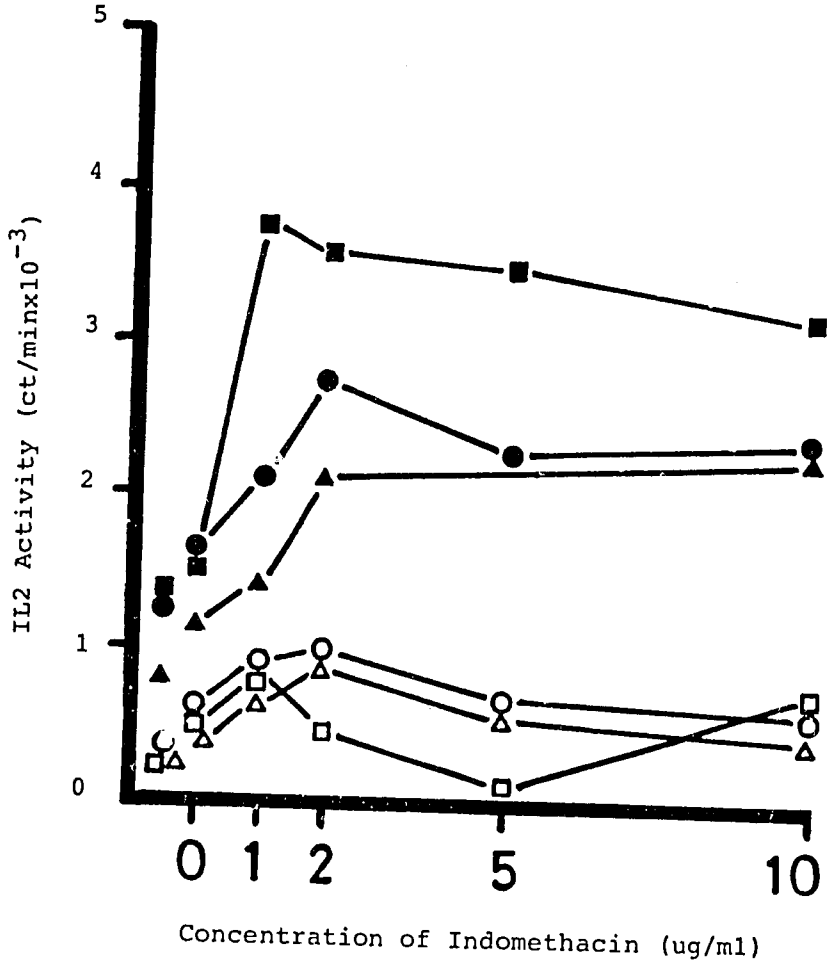

Figure 11. Effect of indomethacin on the suppressive activity of M.leprae on IL2 production of PBML from normal subjects (M.leprae 50 ug/culture)

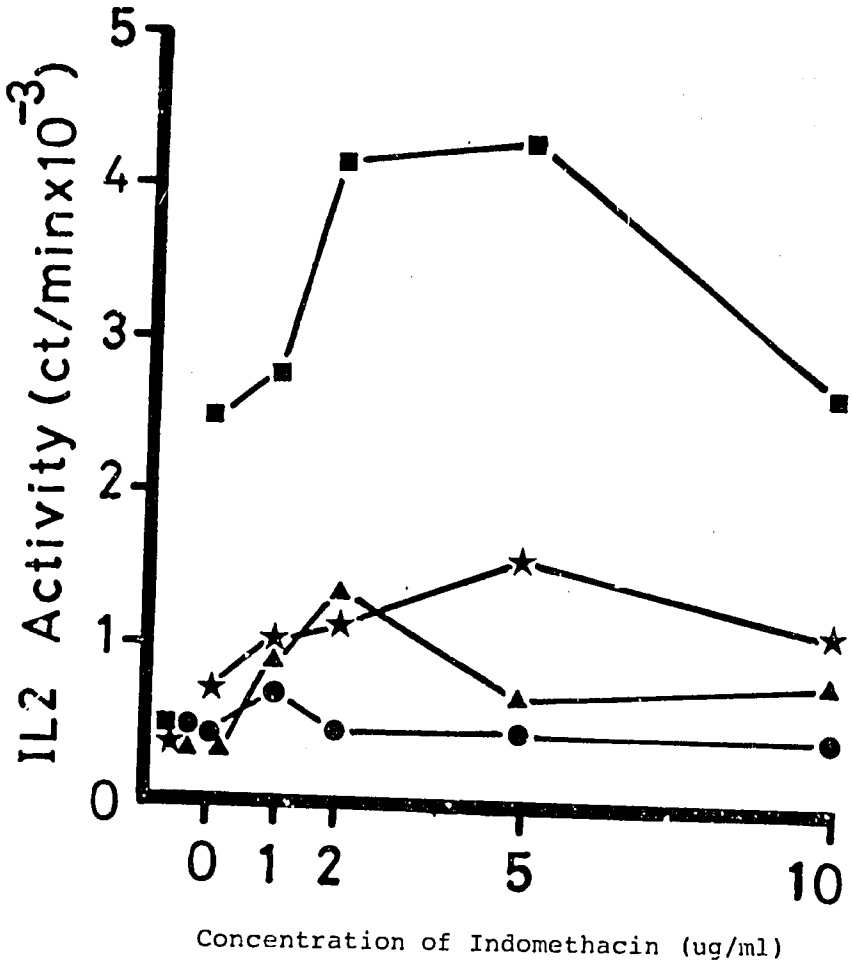

Figure 12. Effect of indomethacin on the suppressive activity of M.leprae on IL2 production of PBML from BT/TT leprosy patients (M.leprae 20 ug/culture).

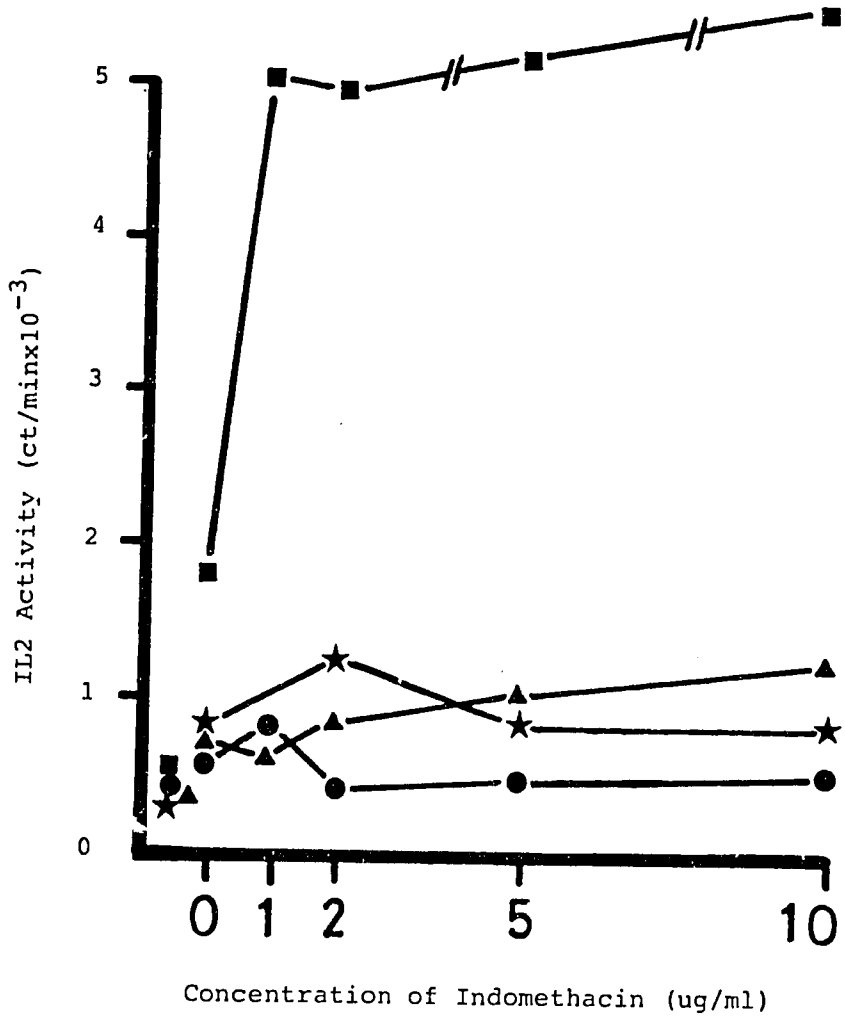

Figure 13. Effect of indomethacin on the suppressive activity of M.leprae on IL2 production of PBML from BT/TT leprosy patients (M.leprae 50 ug/culture).

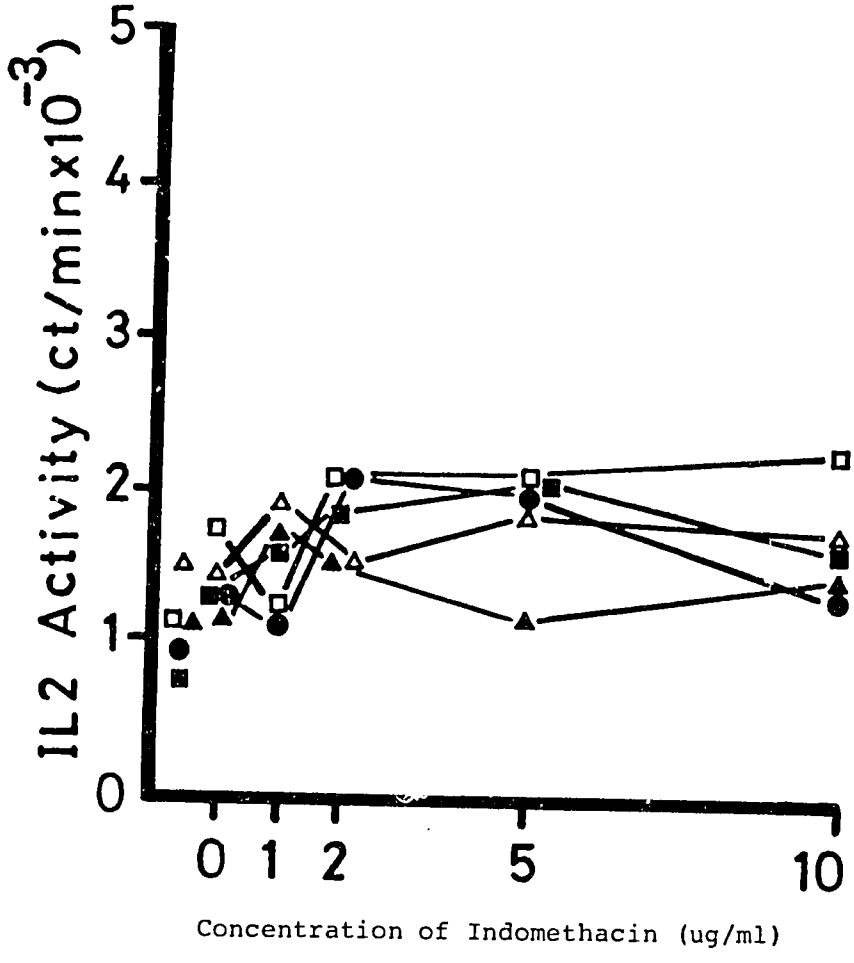

Figure 14. Effect of indomethacin on the suppressive activity of M.leprae on IL2 production of PBML from BL/LL leprosy patients (M.leprae 20 ug/culture).

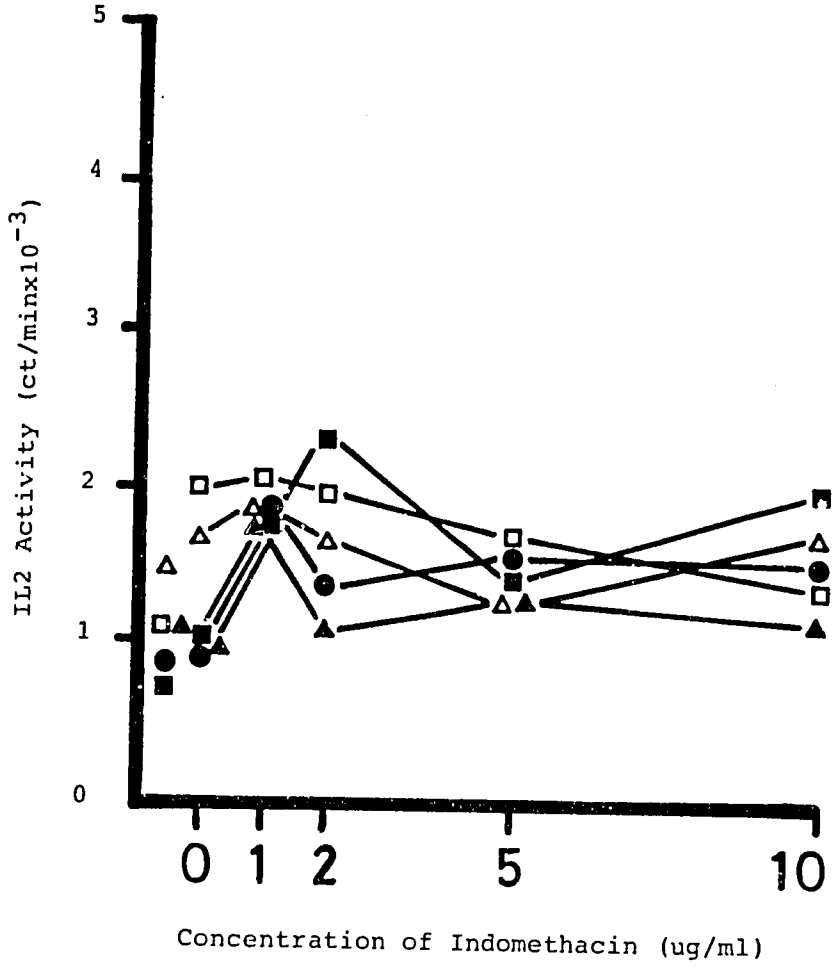

Figure 15. Effect of indomethacin on the suppressive activity of M.leprae on IL2 production of PBML from BL/LL leprosy patients (M.leprae 50 ug/culture).

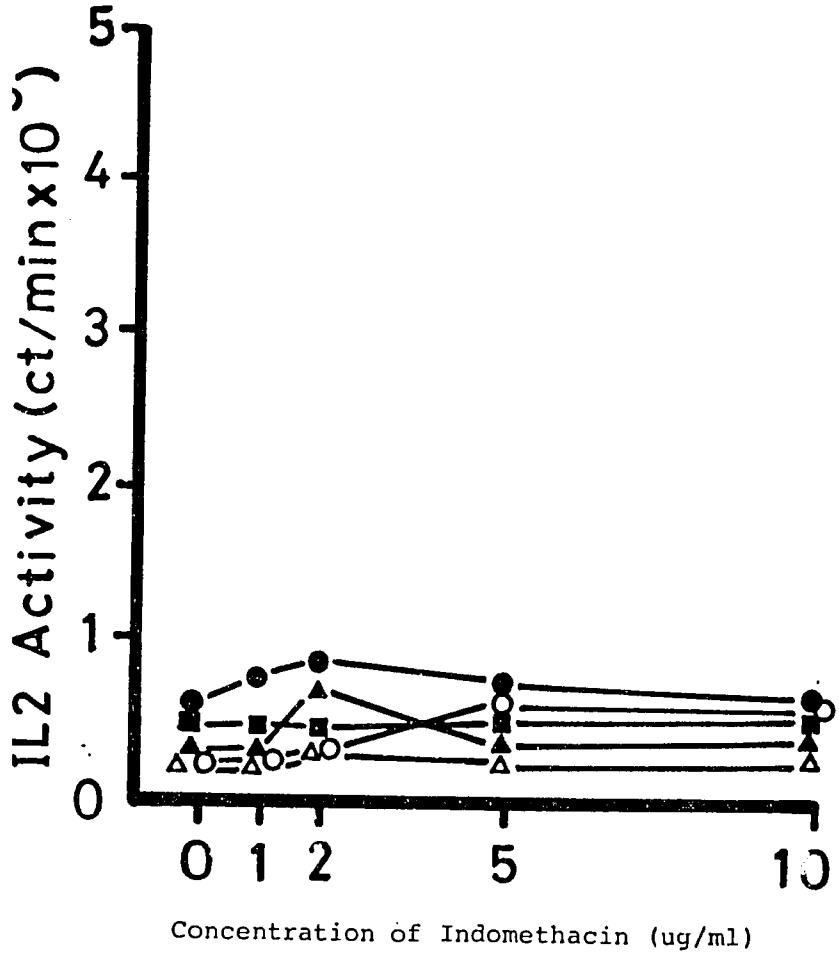

Figure 16. Effect of indomethacin on the IL2 production from PBML of normal subjects.

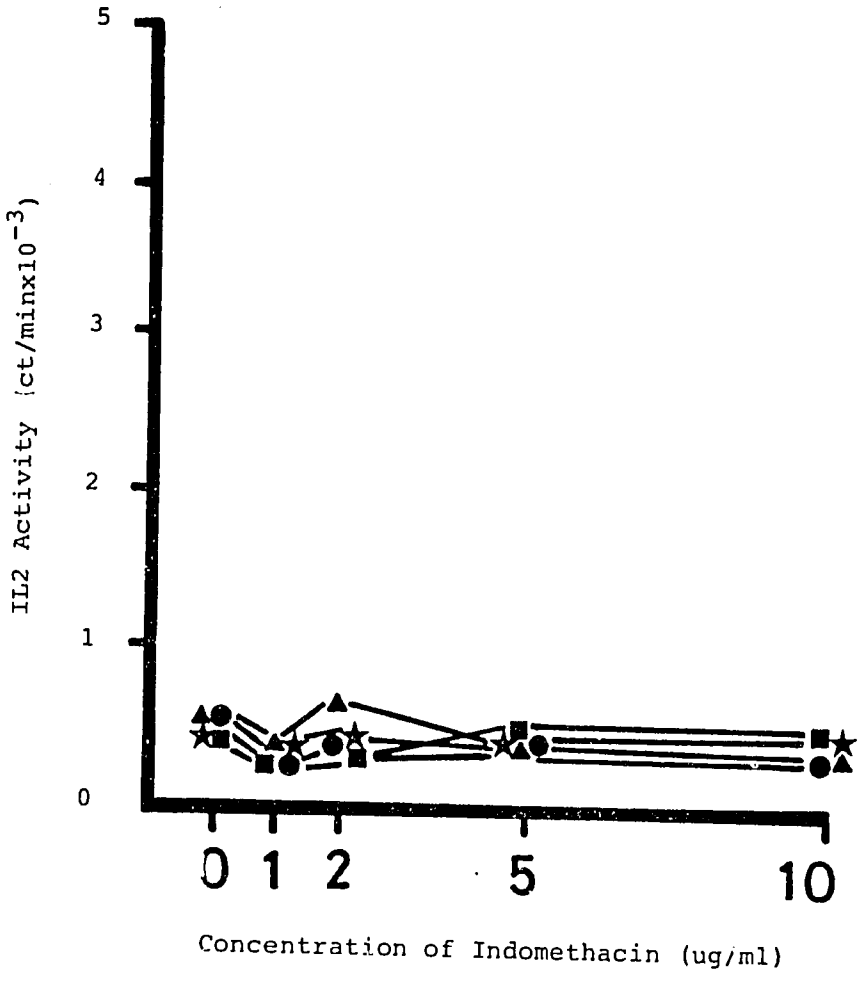

Figure 17. Effect of indomethacin on the IL2 production from PBML of BT/TT leprosy patients' PBML.

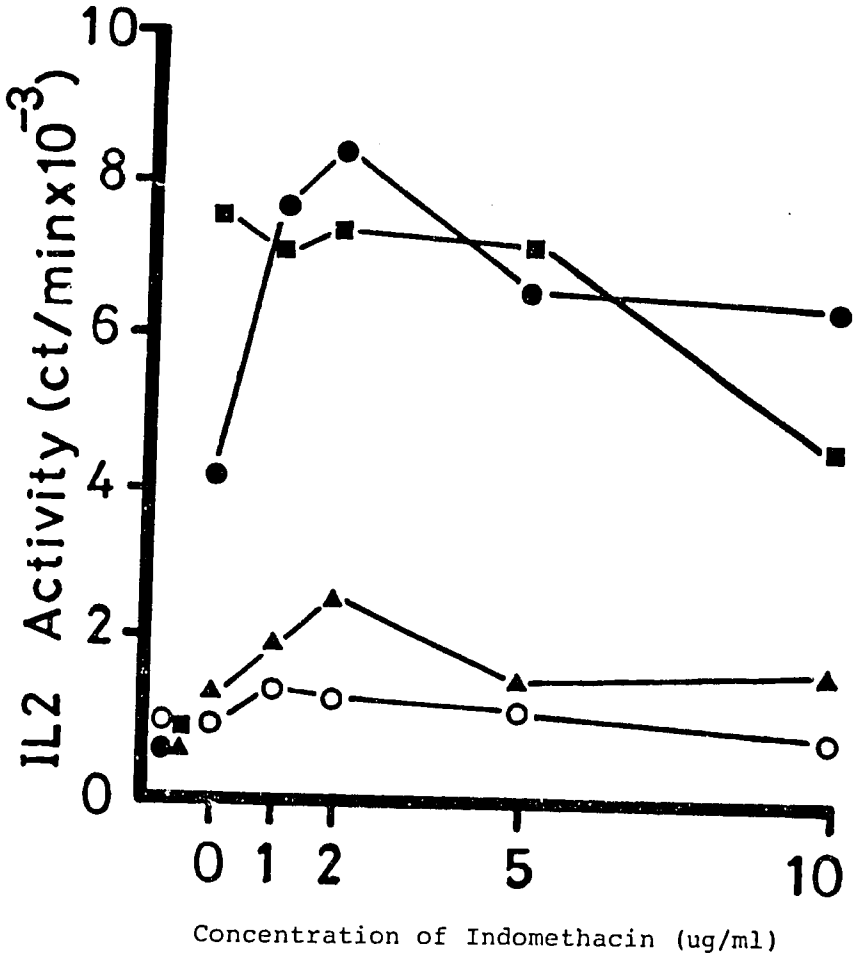

Figure 18. Effect of indomethacin on the IL2 production from PBML of BL/LL leprosy patients when stimulated with PPD.

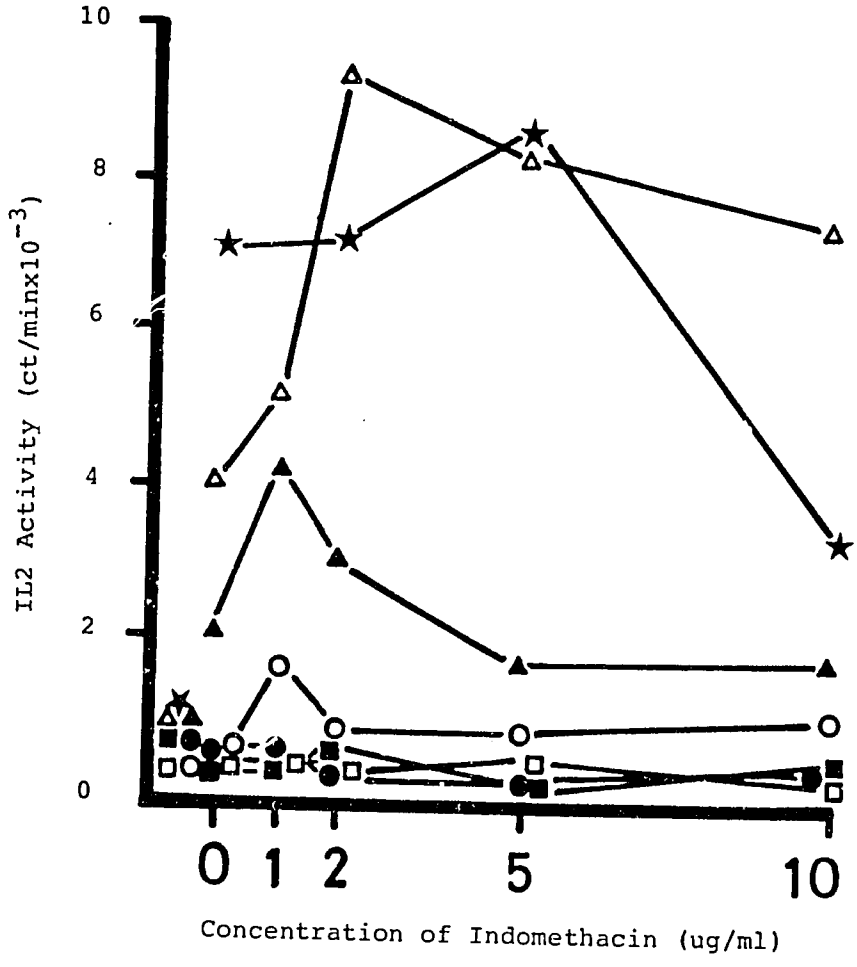

Figure 19. Effect of indomethacin on the IL2 production from PBML of BT/TT leprosy patients when stimulated with PPD.

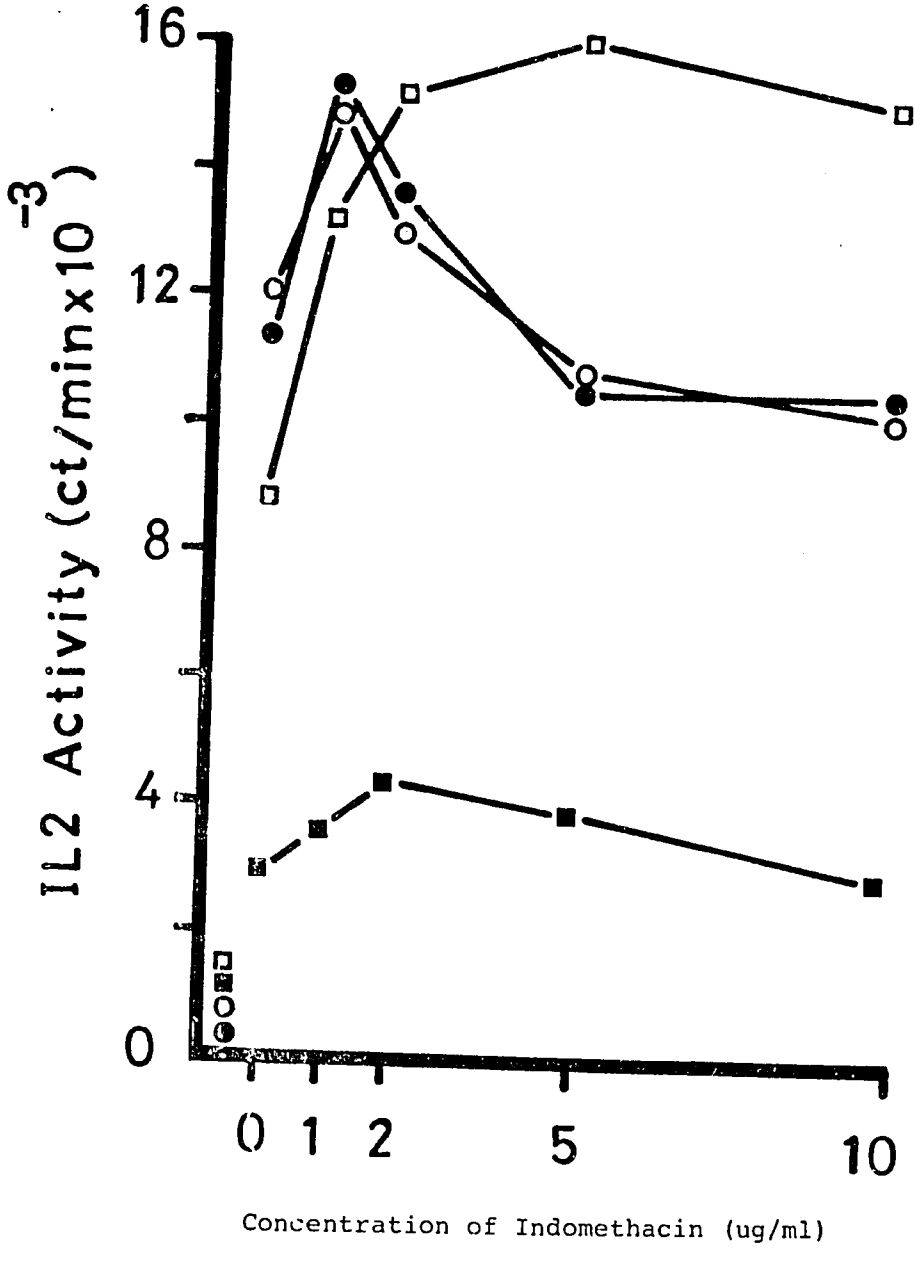

Figure 20. Effect of indomethacin on the IL2 production from PBML of normal subjects when stimulated with PPD.

Regulation of Lypmhokine Gene Expression and In Vitro Immunotherapeutic Effect of Interleukin 1 and 2 on Monocytes and Lymphocytes in Leprosy

INTERIM SCIENTIFIC REPORT

December 31, 1989

Prepared by

Dr. Raymond Kaempfer

Department of Molecular Virology
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Raymond Kaempfer

Objectives

The aims of this project are:

1. To train, at The Hebrew University of Jerusalem, personnel from the team of Dr. Sanit Makowkawkeyoon at the University of Chiang Mai, Thailand, in techniques of molecular biology to permit performance of those steps that must be done in Chiang Mai for the analysis of regulated human interleukin-2 (IL-2) and interferon-gamma (IFN-γ) gene expression in primary peripheral blood mononuclear cells (PBMC) from patients with leprosy.

2. To analyze the dynamics of regulated expression of IL-2 and IFN-γ genes in PBMC from leprous patients of various types, in order to obtain a more precise insight into the molecular basis for the immunodeficiency exhibited in this disease. This research requires the culture of PBMC, under precisely defined conditions, and the extraction of RNA from such cells in Chiang Mai, followed by quantitative hybridization analysis and data processing in Jerusalem.

3. To follow changes in gene expression accompanying conversion of a patient from one leprous state to another.

4. To obtain insight into the role of suppressor cells in inhibiting the function of IL-2 and IFN-γ genes in cell populations from patients with various forms of leprosy.

Abstract

1. Training of a Thai scientist in Jerusalem has been completed successfully during July, 1989. See previous Report.

2. Analysis of patient material has begun in Chiang Mai, with first series of samples from normal donors, to verify that procedures used are correct and that data on gene expression of high standard can be obtained upon processing in Jerusalem. This point has now been reached. Analysis of patients with leprosy is starting at this time, with the expectation that the pace of advancement will increase very strongly as all technical barriers have now been removed. The Jerusalem end of the analytical process is firmly in place.

3. The Israeli principal investigator visited the Chiang Mai laboratory in December, 1989 to obtain first-hand impressions of the experimental progress as well as the leprous patient material available for this study. A detailed report of this visit forms part of this Report and is attached as Appendix I.

3. In Jerusalem, a study of the regulated expression of IL-2 and IFN-Y gene expression in patients with end-stage renal disease has been completed. The interesting and unexpected result is that subjects on hemodialysis show a complete loss of inducibility of the IL-2 gene, concomitant with lower inducibility of IFN-Y mRNA. In subjects on peritoneal dialysis, by contrast, expression of IL-2 mRNA is as vigorous as for normal donors, while IFN-Y mRNA is even more strongly inducible. The defect in IL-2 gene expression in subjects on hemodialysis, occurring most likely at transcription, may underly

their impaired immune function. A manuscript detailing these findings forms part of this Report and is attached as Appendix II. This, and our earlier study showing defective expression of the IL-2 gene in Down syndrome (see June, 1989 Report), yield valuable insight for our present study of dynamics of IL-2 and IFN-Y gene expression in the various forms of leprosy.

4. In Jerusalem as well as in Chiang Mai, very good progress was made in understanding the role of suppressor cells in general and in leprosy in particular. The visit to Chiang Mai helped us to focus together on a subject of great interest today, as detailed in Appendix I. We expect that work in both laboratories on suppression will now benefit from techniques and approaches used in the collaborating group. One of our mss. on the subject (in press) is attached as Appendix III.

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REPORT ON THE VISIT OF DR. RAYMOND KAEMPFER

TO CHIANG MAI UNIVERSITY MEDICAL SCHOOL

WITH DR. SANIT MAKONKAWKEYOON

December 16-21, 1989

AID US-ISRAEL CDR PROJECT NO. C8-196

Regulation of Lymphokine Gene Expression and In Vitro Immunotherapeutic Effect of Interleukin 1 and 2 on Monocytes and Lymphocytes in Leprosy

Signed:

Raymond Kaempfer — Prof. Raymond Kaempfer

Sanit Makonkawkeyoon — Prof. Sanit Makonkawkeyoon

Chiang Mai, Northern Thailand, December 21, 1989

Dr. Raymond Kaempfer (RK), Israeli Co-Principal Investigator on this project, just completed a site visit to the laboratory of his Thai partner at Chiang Mai University Medical Center, Dr. Sanit Makonkawkeyoon (SM). This visit included four full days of discussion with SM and his team, some experimental work, meetings with Drs. Vicharn Vithayasai (Co-Investigator at Chiang Mai) and Dr. Sanong Chaiyarusmee, Dean of the Faculty of Associated Medical Sciences, and an extensive guided tour of patients displaying various forms and stages of leprosy at the McKean Leprosy Rehabilitation Center in Chiang Mai. Dr. Kaempfer also gave a 2-hr seminar, entitled: *Regulation of Human Interleukin-2 and Interferon-Gamma Gene Expression and Its Disturbance in Diseases.*

We summarize here the essence of the scientific discussions, in which took part Dr. Raymond Kaempfer, Dr. Sanit Makonkawkeyoon, Dr. Vicharn Vithayasai, Dr. Kriangsak Praputpittaya, Dr. Niwat Maneekaru, Ms. Charkrit Hirunpetaharat, Ms. Luksana Makonkawkeyoon and Ms. Aramsri Sriburi.

A. Molecular Biology

1. The cell culture system that uses 10 ml of peripheral blood from a patient to study the regulated expression of IL-2 and IFN-gamma genes in 5 different conditions, chosen to reveal inducibility of these genes as well as their appropriate, or aberrant, regulation as a novel and sensitive indicator of immune responsiveness, has been set up successfully in SM's laboratory, through the work of Dr. Kriangsak Praputpittaya who was trained for a month in RK's laboratory in Jerusalem in June, 1989. A problem

arose when RK's team performed IL-2 and IFN-gamma gene hybridization analysis of dot blots prepared in Chiang Mai: it turned out that the dot blot apparatus was not functioning as expected, due to an erroneous exposure to the autoclave, leading to malformation of the pressure plates. By using the dye, methylene blue as marker, we were able to solve the problem on the spot, by increasing the number of support filter sheets until leakage from individual wells became insignificant. We believe that the apparatus, when used under modified conditions, will yield satisfactory results. If not, it was agreed that SM should replace the machine with a new one to be sent from Israel as soon as possible.

2. A cross-section of various patients at McKean's Center will serve as initial clinical material for screening the performance of IL-2 and IFN-gamma genes in leprosy. By using cycloheximide to inhibit protein synthesis, the extent of post-transcriptional repression of both genes that is exerted by a labile protein (see original proposal), can be assessed. By exposing the cells of a given donor to low doses of gamma-irradiation before induction of gene expression, the extent of down-regulation by suppressor cells can be measured. These methods are set up in Chiang Mai, and it should be expected that a representative first set of data on 4-5 patients from each stage of leprosy, ranging from tuberculoid (TT) to lepromatous (LL) through borderline leprosy states, should be available very soon. Age-matched normal donors will be included in each batch of analyses, to ensure an appropriately sized control group for statistical analysis. Gene expression is quantitated and analyzed in RK's laboratory using filters mailed from Chiang Mai; a data base will be made in Jerusalem using a special program designed to facilitate analysis of individual patients as well as of groups.

Gene expression is expressed as standard units of mRNA per cell, making it possible to compare the results of analyses from different donors.

3. Dr. Kriangsak Praputittaya will teach another person in SM's team the know-how needed for this project, to ensure continuity.

4. RK's team has studied the basic regulation of human IL-1 gene expression and adapted this knowledge to conditions useful for small samples of peripheral blood mononuclear cells (PBMC) from 10 ml of blood. It will thus be possible, at a later stage, to ask directly how the IL-1-beta gene is functioning in various forms of leprosy.

5. We will focus on the predictive value of the gene expression test, in terms of telling ahead of time how a patient is likely to develop in the course of the disease. Encouraging results in RK's laboratory show that patients with bladder carcinoma respond well to repeated BCG treatment if their IL-2 and IFN-gamma gene expression patterns become close to normal, whereas if these are aberrant, the tumor relapses between 6 and 12 months later.

6. After the initial cross-section screening of different patients in various phases of the disease, individuals will be followed up through stages of change. This can be done at McKean where patients often convert from lepromatous (LL) or borderline leprosy states (BL) to ENL (erythema nudosum leprosum, a painful nerve disorder). Close contact with physicians at McKean is thus essential and RK's visit there served this purpose well.

7. For selected patients, a third dot blot analysis will be performed to a control probe, e.g., alpha-actin, available in RK's laboratory. This and the IL-2, IFN-gamma and IL-1-beta probes are all resident in transcription plasmids constructed by RK's team to generate radioactive riboprobes for this analysis.

8. An growing data base with clinical details on patients and cellular immunological parameters will be prepared in Chiang Mai in English, for transmission to Jerusalem.

9. We expect to have material ready for publication by the end of 1990.

B. Cell Biology

1. RK's team has made considerable advances in proving that the expression of both IL-2 and IFN-gamma genes is controlled by the action of suppressor cells. Using immunomagnetic beads coated with monoclonal antibodies directed against specific cell surface markers, it was possible to demonstrate that distinct subsets are involved in expression and in suppression. Suppressor cells are CD8, Leu8, or Leu15-positive, while cells that express the genes for IL-2 and IFN-gamma in general lack this marker. The ability to suppress IL-2 and IFN-gamma mRNA accumulation is induced in the course of mitogenic stimulation, concomitant with the induction of IL-2 and IFN-gamma gene expression. A slight lag before suppression strongly sets in allows sufficient escape expression to generate IL-2 and IFN-gamma mRNA signals which are then promptly shut off. We are thus dealing here with two very highly regulated genes subject to down-regulation by suppressor cells, by as much as 99%. The

seminar covered these experiments. Moreover, RK's team has found that the ability to express or suppress IL-2 and IFN-gamma genes is not stably associated with a given cell subset as defined by surface markers, but is subject to dynamic change in the course of an immune response. The balance between active suppressor cells and cells active in expression of IL-2 or IFN-gamma genes is critical, as explained in our original research proposal.

2. SM reviewed the cellular immunology of leprosy conducted by his team. This included good bioassays developed for IL-2 as well as IL-1. Production of both cytokines was shown to be defective in all states of leprosy examined. Antiviral activity assay for IFN production has now also been set up, and levels of IFN are commensurately reduced in leprous patients of all types.

3. SM's team has developed a fortuitous method to separate suppressor cells from those that produce IL-2. This is based on the use of Con A rosetting (paper in press). This method is far cheaper, be it less specific, than those relying on monoclonal antibodies and thus offers important advantages. This method will be tested in RK's laboratory to examine if it is useful in the preparation of functional suppressor cells that act on IL-2 and IFN-gamma gene expression. Such a technique would greatly advance the elucidation of the mechanism by which suppressor cells inhibit the expression of IL-2 and IFN-gamma genes, a subject of intense investigation in RK's laboratory.

4. SM's team has used standard cell separation methods (nylon wool, adherence) to separate T cells from monocytes and macrophages and has found earlier that both subsets show lower

activity in leprosy, when analyzed for the ability to produce IL-2. More recent data support the interpretation that the primary defect is in the T cell population. It is here that the role of suppressor cells is thought to be most crucial, since IL-2 gene expression occurs exclusively in such cells.

5. SM's team has published a stimulating paper showing that the causative agent of leprosy, *Mycobacterium leprae*, acts to induce suppression of IL-2 production in whole PBMC populations from normal donors. The WHO already has put suspensions of *M. leprae* into trial in India and the Maldive Islands, as potential vaccine against leprosy. The findings in SM's laboratory would suggest that caution should be used here, as the antigen may act as immunosuppressant instead.

6. The findings in section 5 above are of direct interest to studies of suppressor cells in RK's laboratory because the antigen, *M. leprae*, could serve as a general inducer of suppressor cell activity and thus enhance research on the mechanism underlying this induction. This antigen will thus be obtained from Dr. P.J. Brennan in Colorado who was commissioned by NIH to distribute the agent to investigators. The point to emphasize here is that *M. leprae* would thus be used as a tool to study the molecular basis of immune failure in leprosy on one hand, and to study mechanisms of suppression in general on the other.

C. Summary

Significant progress has been made in both laboratories on the two main lines of research that form the mainstays of this project.

On one hand, molecular biology of IL-2 and IFN-gamma gene expression in patients with leprosy is now well under way and expected to yield to experimentation within a few months. Second, basic research on suppressor cells and their role in the expression of IL-2 and IFN-gamma genes is moving well in both laboratories, allowing us to exchange strategies as well as materials. There are few laboratories worldwide that are actively engaged in such basic research on suppressor cells, using tools of molecular biology. We had not realized, when we wrote up our proposal, to what extent our two laboratories dovetail in their efforts in this field.

We foresee further developments in both lines of research beyond the immediate next stages. Patients will be followed up to generate patterns of regulated gene expression in the course of progression of leprosy. In our cellular work, we will ask how recombinant IL-2 and IL-1 affect the activity of these genes, upon *in vitro* cultivation of cells from leprosy patients in the presence of these cytokines.

ขอเชิญฟังบรรยายพิเศษ

"Regulation of Human Interleukin-2 and Interferon-gamma gene Expression and Its Disturbance in Diseases"

โดย

Professor Raymond Kaempfer, MD., Ph.D.
Department of Molecular Virology
Faculty of Medicine
The Hebrew University of Jerusalem
Jerusalem
Israel

ณ ห้องประชุมภาควิชาภูมิคุ้มกันวิทยาคลินิก
ชั้น 5 ตึกเทคนิคการแพทย์ มหาวิทยาลัยเชียงใหม่

วันที่ 19 ธันวาคม 2532 เวลา 13.30-15.30 น.

REGULATION OF INTERLEUKIN-2 AND INTERFERON-γ GENE EXPRESSION IN RENAL FAILURE

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Summary Regulated expression of interleukin-2 (IL-2) and interferon-γ (IFN-γ) genes, induced in cultured peripheral blood mononuclear cells from patients with end-stage renal disease on hemodialysis (HD) or peritoneal dialysis (PD), was compared to that of normal donors. HD subjects showed a complete loss of inducibility of the IL-2 gene, concomitant with decreased inducibility of IFN-γ mRNA. In PD subjects, by contrast, expression of IL-2 mRNA was as vigorous as in normal donors, while IFN-γ mRNA was even more strongly inducible. The defect in IL-2 gene expression in HD subjects, occurring most likely at transcription, may underly their impaired immune function.

Introduction

Interleukin-2 (IL-2) is the essential growth factor for all types of T cells,[1] induced upon antigenic or mitogenic stimulation[2] by helper T lymphocytes.[3] IL-2 is able to induce cytotoxic T cell activity[4] and to activate natural killer cells.[5] The strength of an immune response is determined to a large extent by the amount of IL-2 elicited by a stimulus.[6] Interferon-γ (IFN-γ) is also an essential immunoregulatory protein[7] with antiviral activity[8] that activates macrophages[9] as well as natural killer cells[10] and exhibits potent anti-tumor activity in synergy with the tumor necrosis factors.[11]

Expression of human IL-2 and IFN-γ genes is induced by mitogen or antigen. This process requires synthesis of new RNA molecules and results in the appearance of a wave of mRNA.[12-15] The amplitude of the IL-2 and IFN-γ mRNA waves can be superinduced extensively in the presence of inhibitors of translation such as cycloheximide (CHX), yet the shape of each wave remains unchanged.[13-16] A corresponding superinduction of IL-2 and IFN-γ protein is observed upon the removal of CHX.[13,15] These findings support the view that expression of IL-2 and IFN-γ genes is

down-regulated strongly through a mechanism involving a labile protein component. This mechanism acts after transcription of the genes has occurred.[14,15]

A second mechanism regulating expression of IL-2 and IFN-γ genes involves suppressor cells. A transient activation of T cells, able to suppress IL-2 and IFN-γ gene expression, occurs concomitant with the induction of expression of these genes in human tonsil cell populations. Expression of IL-2[17] and IFN-γ[15] mRNA and protein can be superinduced by low doses of γ-irradiation, a treatment thought to prevent the activation of suppressor T cells. The effect of γ-irradiation is also exerted after transcription.[15]

Humoral and cellular immune responses are impaired in chronic renal failure.[18] Uremic patients are more susceptible to bacterial infections[19] and malignancies,[20] show delayed cutaneous hypersensitivity to various antigens,[21] a diminished response to immunization,[22] lymphocytopenia[23] and prolonged graft survival.[24] Defects in cellular immunity include a reduction in number of circulating B and T lymphocytes,[25] a reduced helper to suppressor T cell ratio,[26] and significantly impaired E-rosetting and blast transformation.[21] While hemodialysis (HD) does not restore these defects,[18] patients on peritoneal dialysis (PD) exhibit a better immune response.[18,25,27]

Thus far, few specific immune functions have been analyzed in uremic patients.[18] To obtain more precise insight into the molecular basis underlying defective immune cell function in end-stage renal disease, we have followed the dynamics of IL-2 and IFN-γ gene expression and their control. We report here that expression of these genes differs strikingly in HD and PD subjects.

Patients and Methods

Patients

Patients with end-stage renal disease: 13 on HD for 56.8±31.7 months

(11-120), aged 57±16.7 yr (22-78), serum creatinine on the day of study, 10.89±2.33; 13 on PD [either intermittent peritoneal dialysis (IPD, n=9) or continuous ambulatory peritoneal dialysis (CAPD, n=4)] for 25.2±24.7 months (7-95), aged 61±13.4 yr (28-76), serum creatinine on the day of study, 10.05±2.74. Blood was collected before dialysis. Thirty-two normal donors, serum creatinine, approx. 1. M/F ratio was about 1 in each group.

Laboratory Methods

Cell culture and induction of gene expression. From 10-30 ml of peripheral blood per donor, mononuclear cells (PBMC) were isolated by standard methods, washed and cultured at a density of 0.5-4x10^6 cells/ml in 5 ml of culture medium containing 2% fetal calf serum. For each subject, 1-ml cell cultures were incubated: (A) for 18 hr in the absence of inducer; (B) for 18 hr with PHA; (C) for 22 hr with PHA; (D) for 22 hr with PHA, but with CHX (20 μg/ml) from 18 to 22 hr; (E) for 22 hr with PHA, but after γ-irradiation of the cells with a dose of 1,500 rad.

Plasmids and hybridisation probes. Human IL-2 DNA (p3-16 from T. Taniguchi)[28] and IFN-γ cDNA (pBR327γ0 from R. Devos and W. Fiers)[29] were placed under the phage T7 promoter in pGEM-3 (Promega Biotec) to generate anti-sense RNA transcripts labeled with [α-^{32}P]ATP.[30] In total RNA isolated from PBMC induced with phytohemagglutinin (PHA),[15] IL-2 and IFN-γ antisense RNA transcripts hybridise to RNA migrating at 1,000 and 1,300 nt, respectively, as expected for mRNA, comprising over 85% of the total hybridisation signal; remaining hybridisation is to larger, precursor forms of IL-2 and IFN-γ mRNA. Sense RNA, generated from the SP6 promoter, gave no detectable hybridisation.

Quantitation of specific mRNA in cultured cells. Cells from each culture were collected and lysed in 7.5 M guanidinium-HCl.[31] RNA was precipitated overnight in ethanol at -20°C, dissolved into formaldehyde and incubated for 15 min at 60°C. Eight serial 2-fold dilutions, made in 10 x saline sodium

citrate, were applied in duplicate to nitrocellulose sheets, using a 96-well dot blot apparatus. After baking at 80°C in a vacuum oven, sheets were hybridised separately with ^{32}P-labeled RNA probes for IL-2 and IFN-γ, respectively. Each hybridisation included a strip of nitrocellulose containing serially diluted standard RNA, purified from human tonsil cells induced with PHA for 24 hr.[12] Exposed autoradiograms were scanned at 630 nm in an elisa reader. For each dilution series, hybridisation intensities were subjected to linear regression analysis. The slope is proportional to the mRNA concentration for the gene being probed. This value is expressed as standard units of mRNA/cell. Significance of the difference of means was determined using the paired, two-tailed *t* test.[32]

Results

To allow sensitive and quantitative measurement of specific RNA expressed in microcultures of PBMC derived from 1 ml of peripheral blood, we devised an adaptation of a method[31] for detecting gene expression. It is thus possible to analyze the dynamics of IL-2 and IFN-γ gene expression starting with as little as 10 ml of peripheral blood. Hybridisation intensity, measured by determination of the slope of linear regression analysis of serial dilutions of each RNA sample in dot blots, is linear not only with amount of RNA but also with cell number and can be detected over a 200-fold range. Specific mRNA content for a culture can be expressed in terms of standard units of mRNA per cell, allowing direct comparison of results obtained with individual subjects.

To facilitate analysis of regulated expression of IL-2 and IFN-γ genes in PBMC derived from subjects with renal failure, we defined a set of conditions that measure not only the induced expression of these genes, but also the intactness of mechanisms that control their expression. Five different conditions of induction were chosen (see Methods), based on earlier studies of the regulation of IL-2 and IFN-γ gene expression in mononuclear cells from tonsils or peripheral blood. A wave of IL-2 and of

IFN-γ mRNA is induced by exposure to PHA, reaching a maximum for both genes in the 18-22 hr time range.[13,15] The amplitude of the waves of mRNA can be superinduced in the presence of inhibitors of translation, for example, CHX.[13,15] Expression of IL-2[17] and IFN-γ genes[15] into mRNA and active protein can also be superinduced by exposing cells, before mitogenic stimulation, to low doses of γ-irradiation that prevent activation of suppressor T cells. These conditions of induction are designed to determine if IL-2 and IFN-γ genes are inducible and if the two major post-transcriptional mechanisms regulating their expression, involving a CHX-sensitive component on one hand and suppressor T cells on the other (see Introduction), are functioning normally.

Expression of IL-2 and IFN-γ genes in cells derived from 32 normal donors is shown in Fig. 1. mRNA for both genes is induced by PHA (cf. A and B or C), expression increasing between 18 and 22 hr (cf. B and C), and responds to superinduction by CHX (cf. D and C). The IFN-γ gene is, however, significantly more sensitive to superinduction by γ-irradiation (cf. E and C).

Induction of IL-2 and IFN-γ genes in cells derived from 13 HD subjects is analyzed in Fig. 2. Comparison of Figs. 1 and 2 reveals a complete lack of inducibility of the IL-2 gene in cells from HD individuals, even when CHX is present. The IFN-γ gene, by contrast, is inducible in HD subjects, although to a lower extent than in normal donors, and is responsive to superinduction by CHX and, more weakly, by γ-irradiation. Cells from HD subjects thus show a severe impairment in IL-2 gene expression and decreased activity of the IFN-γ gene.

Figure 3 shows that in cells derived from 13 PD subjects, by contrast, IL-2 and IFN-γ genes remain inducible. mRNA for both genes is superinducible by CHX and the IFN-γ gene also responds to γ-irradiation.

Comparison of Figs. 1 and 3 reveals that on average, twofold lower levels of IFN-γ mRNA are expressed in PD subjects; IL-2 gene expression is reduced even less.

Activity of PBMC was also examined by measuring incorporation of ^{3}H-labeled thymidine induced over 24 hr by PHA. A stimulation index of 28.5±7.6 was obtained for HD subjects, significantly lower ($p < 0.05$) than that for PD subjects (51.6±8) and normal donors (51.4±4.3). On the other hand, CD4/CD8 ratios for the HD and PD patient populations were indistinguishable (1.19±0.05 and 1.22±0.07, respectively), yet well below the normal value of 1.8.[33]

Figure 4 depicts the index of induction and superinduction of IL-2 and IFN-γ mRNA, respectively, in the population of normal donors, HD subjects, and PD subjects. Index of induction is defined as the ratio of specific mRNA levels expressed after culture in the presence of PHA for 18 hr (P) or 22 hr (Q) and in its absence. Index of superinduction R is defined as the ratio of specific mRNA levels induced after culture in the presence of CHX for 4 hr and in its absence. Index of superinduction S is defined as the ratio of specific mRNA levels induced in cells after γ-irradiation and induced directly. Induction or superinduction occurs only if the index exceeds 1.

Comparison of IL-2 and IFN-γ gene expression in Fig. 4 shows that in all subjects, induction of IFN-γ mRNA is twofold greater (P and Q). Both genes are superinduced to a similar extent in the presence of CHX (R). Though in normal donors IFN-γ mRNA is superinduced significantly upon γ-irradiation (S), that is not the case for IL-2 mRNA. The most pronounced differences in expression of IL-2 and IFN-γ genes, however, concern HD and PD subjects.

As seen from Fig. 4, index of induction P and Q for the IL-2 gene do not exceed 1 in HD subjects, but are essentially normal in PD subjects. In HD

subjects, the IL-2 gene can be superinduced by CHX, but not to a statistically significant extent (R). In PD subjects, on the other hand, the index of superinduction of IL-2 mRNA by CHX is at least equal to that seen in normal donors.

A clear difference between HD and PD subjects is revealed also by analysis of the inducibility of the IFN-γ gene (Fig. 4). Whilst IFN-γ mRNA is inducible in all conditions studied, index of induction P and Q are distinctly lower than normal in HD individuals, yet higher than normal, by almost twofold, in PD individuals. IFN-γ mRNA is superinduced by CHX to a normal extent in HD subjects, yet more vigorously in PD subjects (R). In all 3 groups, the IFN-γ gene responds similarly to γ-irradiation, but superinduction is significant only for normal donors.

Discussion

Using a sensitive and quantitative assay for specific mRNA, capable of measuring mRNA levels expressed in cells from as little as I ml of peripheral blood, we have analyzed the regulated expression of IL-2 and IFN-γ mRNA in PBMC from subjects with end-stage renal disease, on either HD or PD, and compared it to that of normal donors. HD subjects exhibit a striking loss of inducibility of the IL-2 gene, concomitant with decreased inducibility of IFN-γ mRNA. In PD subjects, by contrast, expression of IL-2 mRNA is as vigorous as in normal donors, while IFN-γ mRNA is even more strongly inducible. These anomalies in gene activity may underly impaired immune function in subjects with chronic renal failure on HD, as opposed to a far more normal response in those on PD.

Patients on PD (or CAPD) show better immune responsiveness than those on HD.[27] Lectin-induced blast transformation of lymphocytes is equal in CAPD patients and normal donors, but lower in HD subjects.[34] IFN-γ production and T cell function improve progressively with CAPD

treatment.[25] These biological responses are, however, the cumulative result of a sequence of events. Determination of mRNA, on the other hand, gives dynamic information about gene function and is more direct and specific. Here, we have quantitated mRNA levels for IL-2 and IFN-γ, two key immunoregulatory proteins, in cells subjected to a plurality of culture conditions, allowing assessment not only of inducibility of the genes but also of appropriate control of their expression by post-transcriptional and suppressor T cell-dependent mechanisms. Since IL-2 and IFN-γ mRNA are expressed transiently, aberrations in gene expression are most readily detected early in the induction process. By comparing the performance of IL-2 and IFN-γ genes, as we have done, aberrant expression of either is detected with greater sensitivity.

Expression of both genes is down-regulated by a mechanism involving a labile protein whose neutralization, in the presence of CHX, leads, in both normal donors and PD subjects, to extensive superinduction of mRNA levels expressed upon mitogenic stimulation (Figs. 1 and 3). In HD subjects, the IFN-γ gene also responds with a significant superinduction, but IL-2 gene expression is not restored to normal values when CHX is present (cf. Figs. 1 and 2). It is unlikely that the CHX-sensitive mechanism has become CHX-resistant in HD subjects. Excessive repression of the IL-2 gene by the CHX-sensitive mechanism thus cannot explain the lack of expression.

Expression of IL-2 and IFN-γ genes is also controlled by the action of suppressor T cells. Although a decrease in the CD4 (helper) over CD8 (cytotoxic/suppressor) cell subset ratio, reported in chronic renal failure,[26] is confirmed in this study, HD and PD subjects exhibited no difference in CD4/CD8 ratio. In PBMC from normal donors, expression of IFN-γ mRNA, but not that of IL-2 mRNA, is superinduced by exposure of cells to γ-irradiation prior to induction (Figs. 1 and 4). Apparently, the IFN-γ gene is far more sensitive than the IL-2 gene to down-regulation by suppressor

T cells. Hence, if enhanced suppressor T cell activity were to occur in HD, a selective inhibition of IFN-γ gene expression would have been expected. This is not the case. Instead, expression of the IL-2 gene is selectively impaired and cannot be restored, even in part, by γ-irradiation (Figs. 2 and 4). The failure of IL-2 gene expression in HD is, therefore, not readily explained by enhanced suppressor T cell activity.

In PBMC from normal donors, induction of IFN-γ mRNA is about twofold greater than that of IL-2 mRNA (Fig. 4). Induction of these mRNA species requires *de novo* transcription[13,15] which is apparently more vigorous for the IFN-γ gene. Induction of IFN-γ mRNA is greatly enhanced in PD subjects, when compared to normal donors (Fig. 4). In HD subjects, on the other hand, induction of the IFN-γ gene is lower than in normal donors. The complete lack of inducibility of the IL-2 gene in HD subjects is indicative of reduced transcriptional activity. Although quantitation of transcription rates would require 50 times more PBMC than were used here,[15] our findings indicate that the impairment in IL-2 gene expression in HD subjects is not caused by excessive downregulation by suppressor T cells, nor by the CHX-sensitive, post-transcriptional mechanism, but most likely occurs at transcription.

It is not clear how uremia leads to impaired immune function. Uremic serum is thought to have a predominant role,[35] through toxicity of accumulated substances.[27,36] These sera, or their dialysate,[37] contain an activity that inhibits lectin-induced blast transformation[36] and the graft-versus-host reaction.[38] CAPD is more effective than HD in clearing this activity.[39] As seen here, the IL-2 gene is more sensitive than the IFN-γ gene to the negative effects of uremia in HD subjects, although expression of both is reduced severely.

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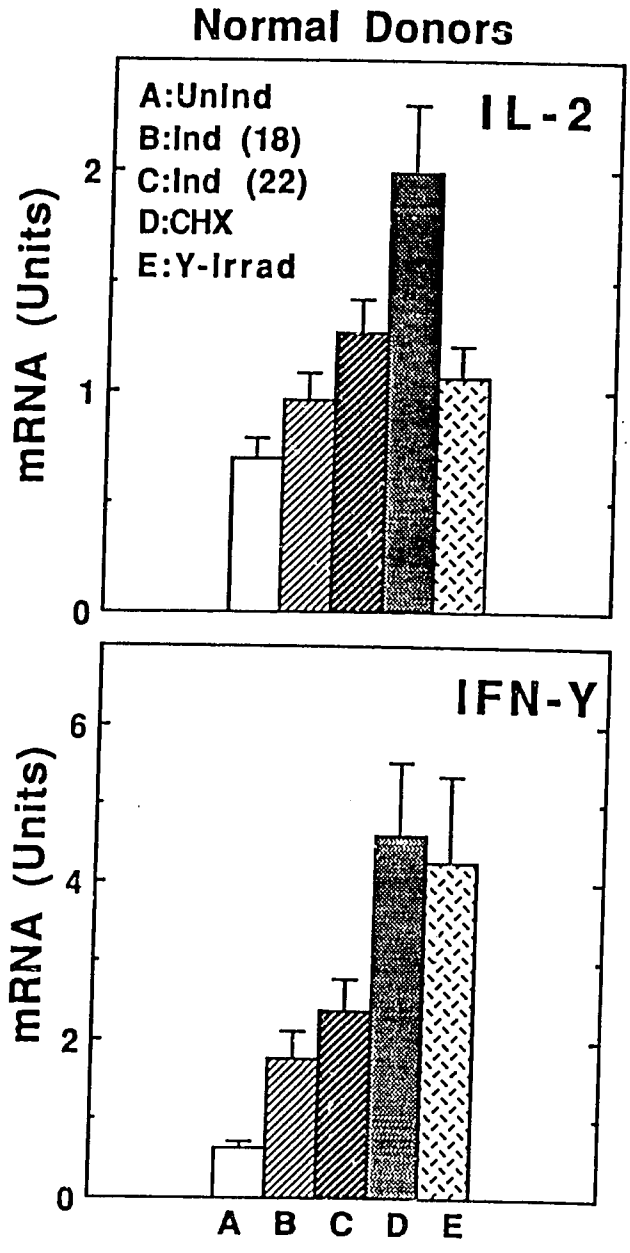

Fig 1 - Regulated expression of IL-2 and IFN-γ genes in 32 normal donors.

IL-2 and IFN-γ mRNA were quantitated, in standard units/cell, in PBMC from each donor, cultured in 5 different conditions (A-E). Mean ± SEM is depicted.

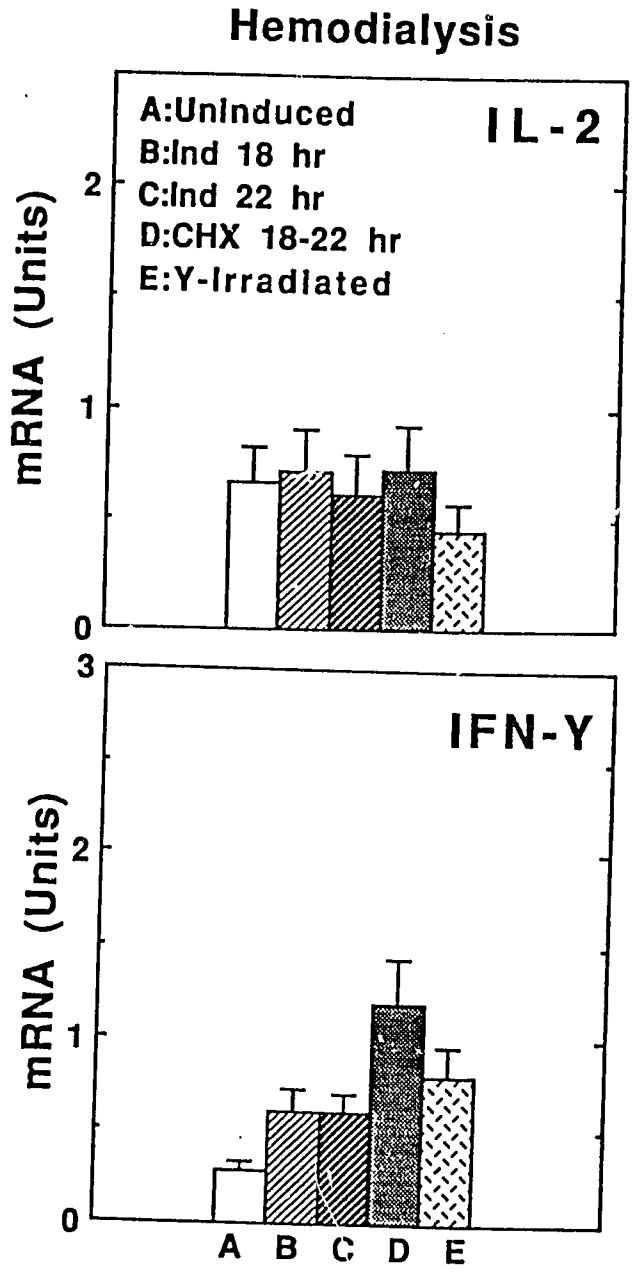

Fig 2 - Regulated expression of IL-2 and IFN-γ genes in 13 subjects with end-stage renal disease on hemodialysis.

IL-2 and IFN-γ mRNA was quantitated in standard units/cell, in PBMC from each donor, cultured in 5 different conditions (A-E). Mean ±SEM is depicted.

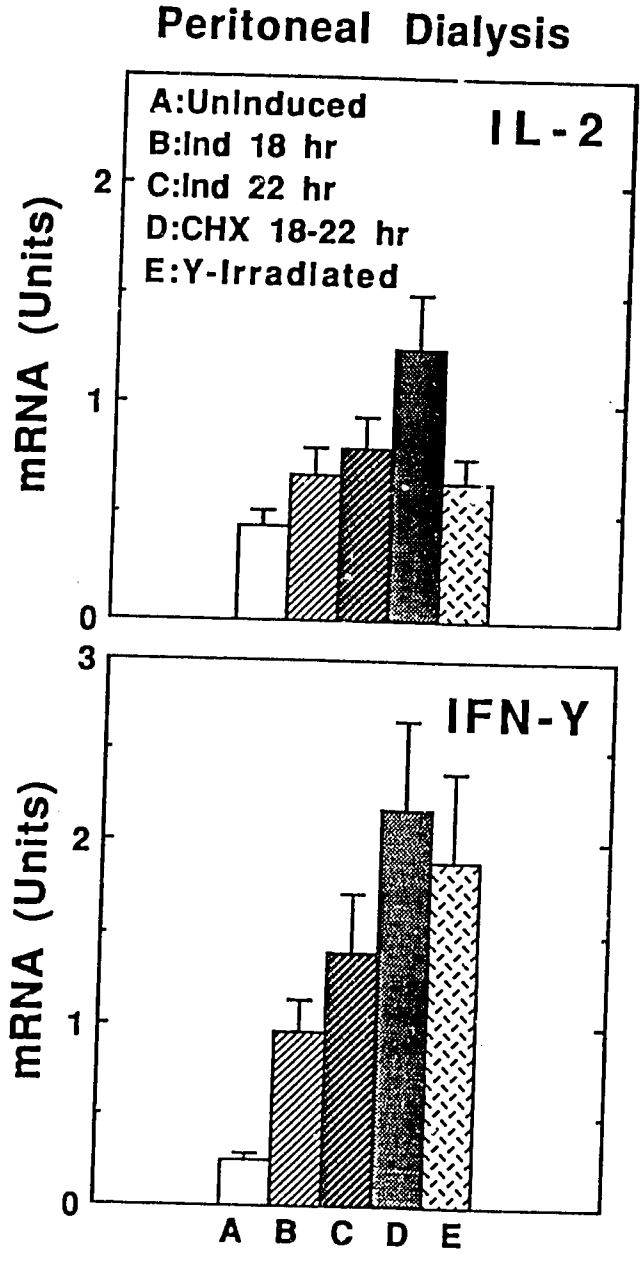

Fig 3 - Regulated expression of IL-2 and IFN-γ genes in 13 subjects with end-stage renal disease on peritoneal dialysis. IL-2 and IFN-γ mRNA was quantitated, in standard units/cell, in PBMC from each donor, cultured in 5 different conditions (A-E). Mean ± SEM is depicted.

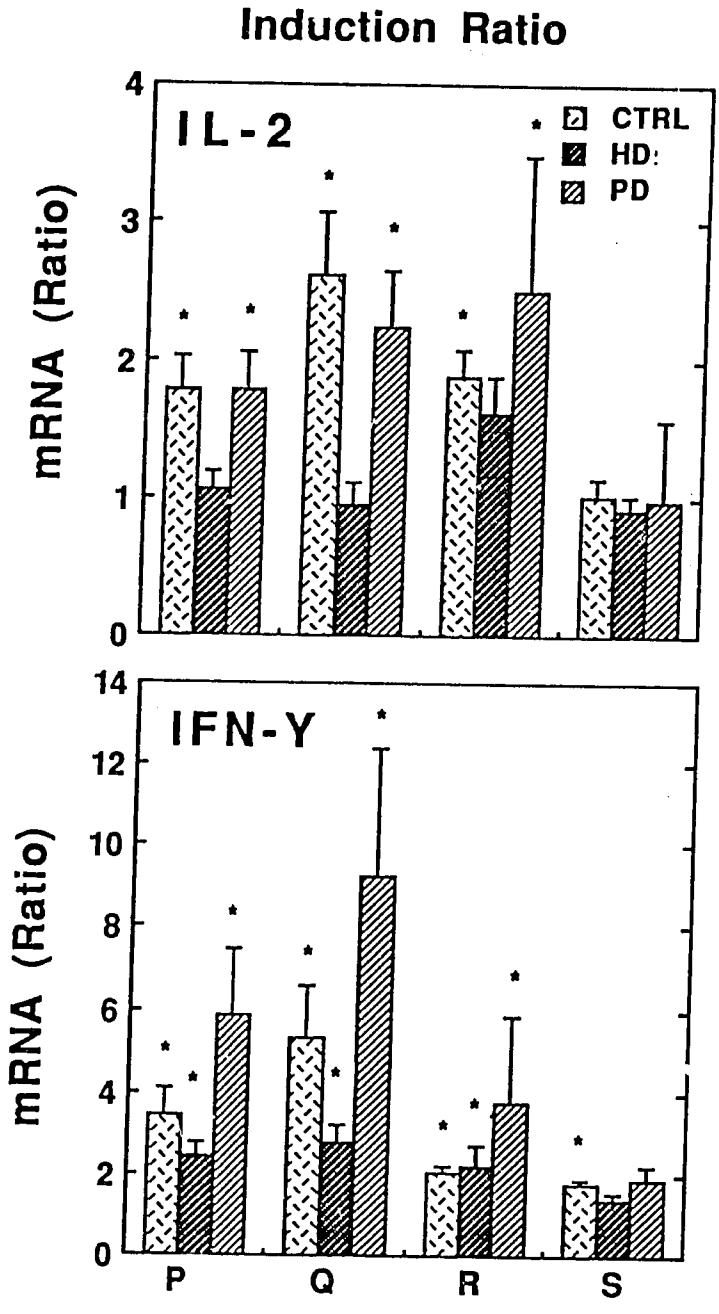

Fig 4 - Index of induction and superinduction of IL-2 and IFN-γ mRNA in PBMC from normal donors (CTRL), HD and PD subjects.

Ratios of specific RNA values from Figs. 1-3 are depicted (see Results). Mean ratio ± SEM is shown; (*) denotes $p < 0.05$.

THE POTENTIAL TO EXPRESS OR SUPPRESS HUMAN INTERLEUKIN-2 AND INTERFERON-γ GENES IS NOT RESTRICTED TO DISTINCT CELL SUBSETS

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Dynamic Changes in Function Occur in Specific T Cell Subsets

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Abstract-Cell surface markers CD4, CD8, Leu8, and Leu15 (CD11) were used to separate human lymphoid cell subsets with monoclonal antibody-coated immunomagnetic beads. We show that each of these subsets is able to suppress the induction of IL-2 and IFN-γ genes effectively. This is manifested by a pronounced superinduction of IL-2 and IFN-γ mRNA, as well as IFN-γ protein, in cell populations depleted of one of these subsets. Co-culture of cell subsets with total cell populations or depleted ones, on the other hand, leads to severe inhibition of expression of these genes. In these experiments, cells in suppressor subsets exhibit little if any, expression of IL-2 and IFN-γ genes. By contrast, depending on donor and lymphoid tissue examined (tonsils or peripheral blood mononuclear cells), CD4, CD8, Leu8, and Leu15 cell subsets are also able to express IL-2 or IFN-γ genes to high levels. Moreover, in Leu8+ cells that do not express the IFN-γ gene, extensive expression of both mRNA and protein can be elicited by inhibiting the activation of suppressor cells with γ-irradiation before induction. These results support the concept that the potential to express or suppress human IL-2 and IFN-γ genes is not restricted to distinct cell subsets. Suppression or expression can be elicited in cells carrying a given surface marker, depending on the state of the immune system in a lymphoid tissue.

INTRODUCTION

Expression of human IL-2 and IFN-γ genes is induced by antigen or mitogen (Gillis *et al.*, 1978; Perussia *et al.*, 1980). This process requires synthesis of new RNA molecules and results in the appearance of a wave of mRNA (Efrat *et al.* 1982; Efrat and Kaempfer, 1984; Kaempfer *et al.* 1987; Lebendiker *et al.*, 1987). Expression of IL-2 and IFN-γ mRNA can be superinduced by low doses of γ-irradiation, a treatment thought to prevent the activation of suppressor T cells (Lebendiker *et al.*, 1987; Kaempfer and Efrat 1986; Schwartz *et al.*, 1982). The amplitude of the waves of IL-2 and IFN-γ mRNA is increased upon γ-irradiation, yet the shape of the wave remains unchanged. This mechanism acts post-transcriptionally (Kaempfer *et al.* 1987; Lebendiker *et al.*, 1987). A corresponding superinduction of IL-2 and IFN-γ protein is observed in γ-irradiated cell cultures (Efrat and Kaempfer, 1984; Lebendiker *et al.*, 1987).

These findings support the view that expression of IL-2 and IFN-γ genes is down-regulated through a mechanism involving suppressor cells. In this study, we sought to analyze the interaction of cells active in expression of IL-2 and IFN-γ genes with cells able to suppress this expression. A powerful tool for this purpose is the use of monoclonal antibodies that define specific cell surface markers. Using immunomagnetic beads coated with an appropriate monoclonal antibody, specific subsets of cells can be isolated conveniently by positive selection (Lea *et al.*, 1985; Lea *et al.*, 1986). Individual subsets of T cells and other immune cells were shown to express specific functions. Thus, $CD4^+$ cells, defined as helper T cells, when isolated in this manner express the IL-2 gene, while $CD8^+$ cells, thought to comprise cytotoxic and suppressor T cells, do not express this gene (Leivestad *et al.*, 1989). $Leu15^+$ cells, denoted as D12, were characterized as suppressor/cytotoxic cells (Landay *et al.*, 1983; Clement *et al.*, 1984). The Leu8 marker is associated with a greater range of cells, including T cells, B cells, neutrophils and monocytes (Gatenby *et al.*, 1982).

Here, we have asked if the respective functions, to express or suppress IL-2 and IFN-γ genes, are associated with distinct subsets of

cells carrying different surface markers. Our finding is that this is not the case and that instead, cells bearing a given surface marker can express either function, depending on the state of the immune system in a lymphoid tissue.

MATERIALS AND METHODS

Cell culture and induction of gene expression

Peripheral blood mononuclear cells (PBMC) are separated from buffy coat taken from healthy donors on Ficoll Paque (Pharmacia) and washed twice with 50 ml of RPMI 1640 medium. Cells are resuspended at a density of $4x10^6$ cells/ml and incubated at 37°C in a water-saturated atmosphere of 5% CO_2 in air, in RPMI 1640 medium containing 2% fetal calf serum, 2 mM glutamine, 10 mM MEM nonspecific amino acids, 100 mM Na-pyruvate, 10 mM Hepes pH 7.2, $5x10^{-5}$ M 2-mercaptoethanol, 100 u/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml nystatin. Tonsils from otherwise healthy donors are disrupted mechanically and cultured at $4x10^6$ cells/ml in culture medium as above.. γ-Irradiation to a dose of 1,500 rad is performed, using a ^{60}Co source, before the addition of phytohemagglutinin (PHA). PHA-P (Difco) is added to 0.4% (v/v).

Monoclonal antibodies

Monoclonal mouse anti-human antibodies Leu8, Leu15 (CD11), Leu3a (CD4) were from Becton & Dickinson. T4 (CD4) and T8 (CD8) were from Dako. FITC goat anti-mouse IgG was from Coulter.

Separation of cell subsets on immunomagnetic beads

Monosized polystyrene particles with a magnetic core (Dynabeads, Dynal) were coated indirectly (via sheep anti-mouse IgG) with individual monoclonal antibodies (see above)(Lea, *et al.*, 1986). Subsets were isolated from PBMC or tonsil cells by incubating for 1 hr at 4°C with immunomagnetic beads in a ratio of 3-4 beads/cell (Lea *et al.*, 1986) and

collecting with a powerful hand-held magnet (Dynal). Efficiency of depletion was monitored by fluorescence microscopy of cell populations labeled with the appropriate monoclonal antibody and FITC goat anti-mouse IgG. Depletion exceeded 90% for Leu8, CD8, and CD4, and 75% for Leu15. Cells attached to beads and depleted cell populations were cultured immediately after separation, as described for total cell populations.

Plasmids and hybridization probes

Human IL-2 cDNA (p3-16 from T. Taniguchi) (Taniguchi *et al.*, 1983) and IFN-γ cDNA (pBR327γ0 from R. Devos and W. Fiers) (Devos *et al.*, 1982) were placed under the phage T7 promoter in pGEM-3 (Promega Biotec) to generate anti-sense RNA transcripts labeled with [α-^{32}P]ATP (Melton *et al.* 1984). In total RNA isolated from PBMC or tonsils induced with PHA (Lebendiker *et al.*, 1987), IL-2 and IFN-γ antisense RNA transcripts hybridize to RNA migrating at 1,000 and 1,300 nt, respectively, as expected for mRNA, comprising over 85% of the total hybridization signal; remaining hybridization is to larger, precursor forms of IL-2 and IFN-γ mRNA. Sense RNA, generated from the SP6 promoter, gave no detectable hybridization.

Quantitation of specific mRNA in cultured cells

Cells from each culture were collected and lysed in 7.5 M guanidinium-HCl (Cheley and Anderson, 1984). RNA, precipitated overnight in ethanol at -20°C, was dissolved into formaldehyde and incubated for 15 min at 60°C. Four serial 2-fold dilutions, made in 10x saline sodium citrate, were applied in duplicate to nitrocellulose sheets, using a 96-well dot blot apparatus. After baking in a vacuum oven at 80°C, sheets were hybridized separately with ^{32}P-labeled RNA probes for IL-2 and IFN-γ, respectively. Exposed autoradiograms were scanned at 630 nm in an ELISA reader. mRNA levels are expressed as A_{630}.

Assay for IFN-γ

IFN-γ was quantitated in culture medium by radioimmunoassay (Lebendiker *et al.*, 1987).

RESULTS

Suppression and expression of IL-2 and IFN-γ genes by Leu8 cells

Expression of IL-2 and IFN-γ mRNA in human tonsil cells is quantitated in Figs. 1A and B, respectively, for the total cell population, Leu8+ cells isolated by interaction with immunomagnetic beads coated with Leu8 monoclonal antibody, and Leu8-depleted cells. Leu8+ cells express little IL-2 or IFN-γ mRNA, but their depletion leads to a pronounced superinduction of these genes. Upon co-culture of the Leu8-depleted cells with Leu8+ cells, a strong suppression is observed, manifested by a 9-and 6-fold reduction in IL-2 and IFN-γ mRNA, respectively. Leu8+ cells thus act effectively to suppress expression of both genes, which occurs solely in the Leu8-depleted cell population.

Figure 2 depicts kinetics of IL-2 and IFN-γ mRNA expression induced by PHA in total, Leu8+, and Leu8-depleted PBMC. It is seen that here, Leu8+ cells express IL-2 (A) and IFN-γ (B) mRNA, though less than the total cell population. Leu8-depleted cells, on the other hand, express little, if any, of these mRNA species, yet when co-cultured with Leu8+ cells, act to suppress both genes. In cells from this donor, therefore, suppressive activity is not associated with the Leu8 marker.

In the experiment of Figs. 3A and B, prominent expression of waves of IL-2 and IFN-γ mRNA is observed in Leu8+ cells. Relative to the total cell population, expression occurs earlier in Leu8+ cells, reaching its maximum at a time when expression in the total cell population is still low. In Leu8+ cells from a different donor (Fig. 4), expression of IL-2 (A) and IFN-γ (B) mRNA is high at the earliest time examined, but then declines rapidly, reaching low levels at a time when expression of these

mRNA species rises strongly in the total cell population. Apparently, Leu8+ cells have the potential to suppress their own expression of IL-2 and IFN-γ genes.

Indeed, in the experiment of Fig. 5, expression of both IFN-γ mRNA and protein is lacking in Leu8+ cells, but can be elicited upon γ -irradiation, a treatment considered to inhibit activation of suppressor T cells. The level of IFN-γ mRNA in irradiated Leu8+ cells exceeds that seen in the total cell population, even after γ-irradiation. Since Leu8+ cells comprised only 16% of the total cell population, it is evident that upon γ-irradiation, they become highly active in IFN-γ gene expression.

Expression and suppression of the IFN-γ gene by Leu15 (CD11) cells

As seen in Fig. 6, the Leu15+ cell subset can express the IFN-γ gene (A) or suppress this gene (B). In Fig. 6A, a total cell population expresses little IFN-γ mRNA upon induction, when compared to the Leu15+ cell subset. This subset comprised only 2% of the total cell population, yet it expresses high levels of IFN-γ mRNA. In the experiment of Fig. 6B, by contrast, Leu15+ cells do not produce IFN-γ but actively suppress its production in the total cell population, as seen from the extensive superinduction of IFN-γ activity observed in Leu15-depleted cells. Leu15+ cells comprised only 25% of the total cell population. Hence, the extent of this superinduction, 25-fold, cannot be explained by enrichment of IFN-γ-producing cells.

Expression and suppression of IL-2 and IFN-γ genes by CD4 cells

CD4+ cells, helper T cells, are considered to be the main subset active in expression of IL-2 and IFN-γ genes (Reinherz and Schlossman, 1980a, b; 1981). In Fig. 7, waves of IL-2 (A) and IFN-γ (B) mRNA are expressed in CD4+ cells, although some expression is retained by the CD4-depleted cell population. In the donor of Fig. 8, on the other hand, CD4+ cells express neither IL-2 (A) nor IFN-γ (B) mRNA, but almost totally suppress these genes, when co-cultured with the total cell population.

Expression and suppression of IL-2 and IFN-γ genes by CD8 cells

CD8+ cells are thought to comprise cytotoxic and suppressor T cells (Reinherz and Schlossman, 1980a, b; 1981). Indeed, as seen in Fig. 9A, expression of IL-2 and IFN-γ mRNA is suppressed by co-culture of the total cell population with CD8+ cells that by themselves do not express these genes. CD8+ cells, which comprised only 17% of the total cell population, are thus effective suppressors of IL-2 and IFN-γ gene expression. Yet, in the CD8+ cell subset from a different donor, IL-2 and IFN-γ mRNA are expressed transiently (Figs. 9B and C), in a manner resembling that for Leu8+ cells in Fig. 4. Since CD8+ cells comprised only 7% of the total cell population, the observed level of expression of IL-2 and IFN-γ genes is significant.

DISCUSSION

The currently held view is that individual subsets of T cells and other immune cells express specific functions. For example, CD4+ cells are considered to be helper T cells that express the IL-2 gene, while CD8+ cells comprise cytotoxic and suppressor T cells, but do not express the IL-2 gene (Reinherz and Schlossman, 1980a, b; 1981). It is thus thought that the balance between activities of cells belonging to different subsets, as defined by surface markers, determines the nature and strength of the immune response (Lanier *et al.*, 1983). We show here that, in fact, the situation is more complex. Subsets of cells carrying a given surface marker are not narrowly restricted in function but can either express the IL-2 and IFN-γ genes, or act to suppress them. Our results thus support the concept that the function of individual cell subsets, to suppress or express these genes, is subject to dynamic change in the course of an immune response. Consequently, cell surface markers cannot be used to define cell function, at least with respect to the expression of IL-2 and IFN-γ genes.

In these experiments, CD4, CD8, Leu8, and Leu15 (CD11) cell subsets are each able to suppress the induction of IL-2 and IFN-γ genes effectively. Co-culture of Leu8, CD4, or CD8 cell subsets with total cell

populations, or depleted ones, leads to severe inhibition of expression of IL-2 and IFN-γ genes (Figs. 1, 8 and 9). In these experiments, cells of suppressor subsets exhibited little, if any, expression of IL-2 and IFN-γ mRNA. On the other hand, a pronounced superinduction of IL-2 and IFN-γ mRNA, as well as of IFN-γ protein, occurs in cell populations depleted of Leu8+ or Leu15+ cells. The results of Fig. 6B show this particularly well for IFN-γ production, which in Leu15-depleted cells is superinduced about 25-fold within 24 hr, when compared to the total cell population. Leu15+ cells thus have the potential to suppress IFN-γ gene expression by over 95%.

By contrast, depending on donor, CD8, Leu8, and Leu15 cell subsets are also able to express IL-2 or IFN-γ genes to high levels. Expression of IL-2 mRNA by CD8+ cells was also reported (Halvorsen, *et al.* 1988; Leivestad *et al.*, 1988). Expression of IL-2 and IFN-γ genes by CD4+ cells has been well established (Reinherz and Schlossman, 1980a, b; 1981). Yet, even within the CD4+ subset, there is a clear dichotomy into distinct subpopulations, defined by the 45R surface marker. CD4+ 45R- cells express the IL-2 and IFN-γ genes, while CD4+ 45R+ cells do not (Dohlsten et al., 1988). Expression of IL-2 and IFN-γ genes, therefore, cannot be defined solely by the CD4 surface marker.

In the experiment of Fig. 6A, isolated Leu15 cells were far more active in expressing IFN-γ mRNA than the total cell population, showing that expression by the Leu15+ cell subset, which comprised 2% of all cells, was suppressed in the total cell population. Likewise, in Fig. 2, expression of IL-2 and IFN-γ mRNA by Leu8+ cells was suppressed by the Leu8-depleted cell population. Moreover, in Leu8+ cells that did not express the IFN-γ gene, extensive expression could be elicited by inhibiting the activation of suppressor cells with γ-irradiation before induction (Fig. 5).

These findings demonstrate that suppression is dominant over expression of IL-2 and IFN-γ genes in conditions where both are activated. As we shall show elsewhere, concomitant with the induction of IL-2 or IFN-γ gene expression, mitogenic stimulation induces a transient activation of T cells possessing the ability to effectively suppress expression of these genes. Induction of IL-2 and IFN-γ mRNA

generally precedes the appearance of suppressor T cell activity and it is this lag that allows expression of both genes to occur before strong down-regulation is exerted. Thus, in the experiments of Figs. 4 and 9 (B and C), early expression of IL-2 and IFN-γ mRNA by $Leu8^+$ and $CD8^+$ subsets, respectively, is followed by a rapid decline in expression.

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Legends

Fig. 1. Suppression of IL-2 and IFN-γ genes by Leu8+ cells. Human tonsil cells (CTRL) were separated into Leu8+ (Leu8) and Leu8-depleted cell populations (DEP 8) as described in Materials and Methods. Cultures of $2x10^6$ cells from each population and a co-culture of $2x10^6$ cells each of Leu8+ and Leu8-depleted cells were induced with PHA. After 36 hr, IL-2 (A) and IFN-γ (B) mRNA were quantitated by hybridization to ^{32}P-labeled anti-sense RNA transcripts. Film blackening was scanned at 630 nm. Leu8+ cells comprised 28% of the total cell population.

Fig. 2. Suppression of IL-2 and IFN-γ genes by Leu8-depleted cells. PBMC (○) were separated into Leu8+ (□) and Leu8-depleted cell populations (●). These cell populations were induced with PHA alone or during co-culture of Leu8+ and Leu8-depleted cells in the ratio in which they were present in the total cell population (▲). At the times indicated, IL-2 (A) and IFN-γ (B) mRNA were quantitated as for Fig. 1. Leu8+ cells comprised 80% of the total cell population.

Fig. 3. Expression of IL-2 and IFN-γ genes by Leu 8+ cells. Aliquots of $2x10^6$ tonsil cells (○), or Leu8+ cells isolated from them (●), were induced with PHA. At the times indicated, IL-2 (A) and IFN-γ (B) mRNA were quantitated as for Fig. 1. Leu8+ cells comprised 22% of the total cell population.

Fig. 4. Prompt shutoff of expression of IL-2 and IFN-γ genes in Leu8+ cells. Aliquots of $2x10^6$ tonsil cells (○), or Leu8+ cells isolated from them (■), were induced with PHA. At the times indicated, IL-2 (A) and IFN-γ (B) mRNA were quantitated as for Fig. 1. Leu8+ cells comprised 50% of the total cell population.

Fig. 5. Superinduction of IFN-γ mRNA and protein in γ-irradiated Leu 8+ cells. Aliquots of $4x10^6$ tonsil cells (CTRL), or Leu8+ cells isolated from them (Leu8), were induced with PHA for 48 hr, directly or after exposure to 1500 rad of γ-irradiation. IFN-γ mRNA was quantitated as for Fig. 1; IFN-γ was determined by radioimmunoassay. Leu8+ cells comprised 16% of the total cell population.

Fig. 6. Expression and suppression of the IFN-γ gene by Leu15+ cells. (A) Aliquots of $4x10^6$ tonsil cells (○), or Leu15+ cells isolated from them

(●), were induced with PHA; at the times indicat ., IFN-γ mRNA was quantitated as for Fig. 1. (B) Aliquots of $4x10^6$ PBMC (○), Leu15+ cells isolated from them (●), or Leu15-depleted cells (▲) were induced with PHA; at the times indicated, IFN-γ was quantitated by radioimmunoassay. In (A) and (B), Leu15+ cells comprised 2% and 25% of the total cell population, respectively.

Fig. 7. Expression of IL-2 and IFN-γ genes by CD4+ cells. PBMC (○) were separated into CD4+ (Leu3a) (■) and CD4-depleted cell populations (□). These cell populations were induced with PHA in the numbers in which they were present in the total cell population. At the times indicated, IL-2 (A) and IFN-γ (B) mRNA were quantitated as for Fig. 1. CD4 cells comprised 11% of the total cell population.

Fig. 8. Suppression of IL-2 and IFN-γ genes by CD4+ cells. Aliquots of $2x10^6$ tonsil cells (○), or CD4+ (T4) cells separated from them (□), cultured alone or together (●), were induced with PHA. At the times indicated, IL-2 (A) and IFN-γ (B) mRNA were quantitated as for Fig. 1. CD4 cells comprised 48% of the total cell population.

Fig. 9. Suppression and expression of IL-2 and IFN-γ genes by CD8+ cells. (A): Aliquots of $2x10^6$ tonsil cells, or CD8+ cells separated from them, cultured alone or together, were induced with PHA. After 24 hr, IL-2 and IFN-γ mRNA were quantitated as for Fig. 1. CD8 cells comprised 17% of the total cell population. Bars represent, from left to right, total cell population, CD8+ cells, and both. (B) and (C): Aliquots of $2x10^6$ tonsil cells from a different donor (○), or CD8+ cells separated from them (●), were induced with PHA. At the times indicated, IL-2 (B) and IFN-γ (C) mRNA were quantitated as for Fig. 1. CD8 cells comprised 7% of the total cell population.

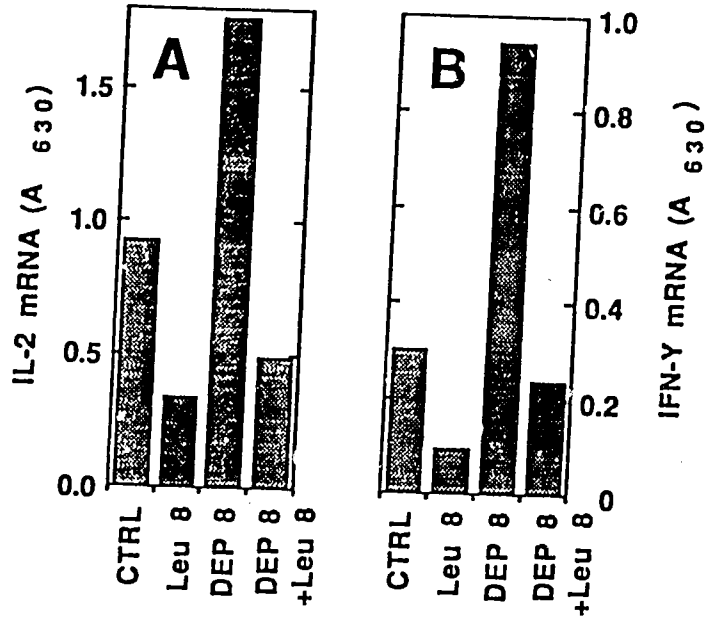
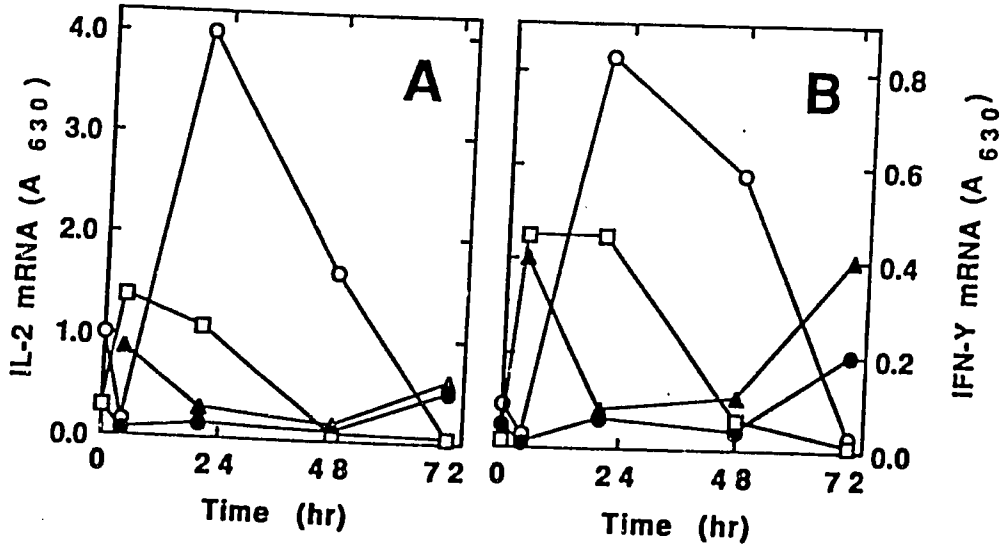


Figure 1.



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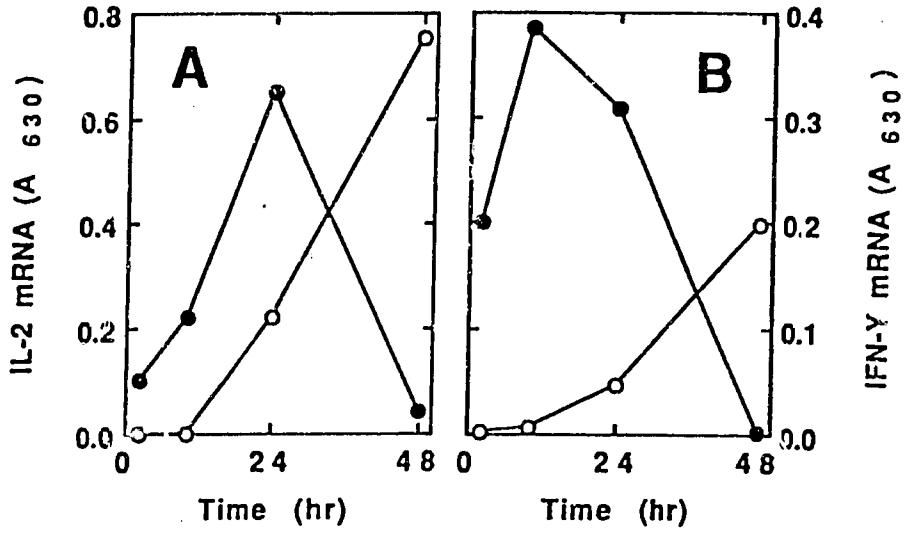

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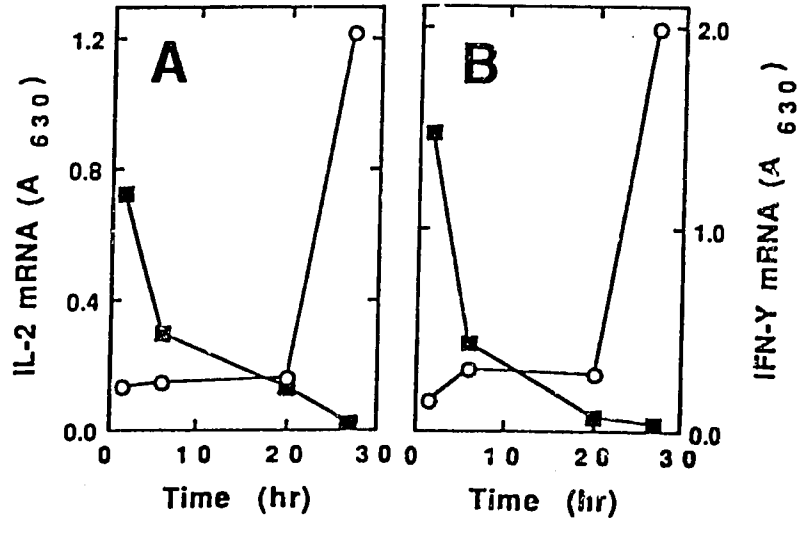

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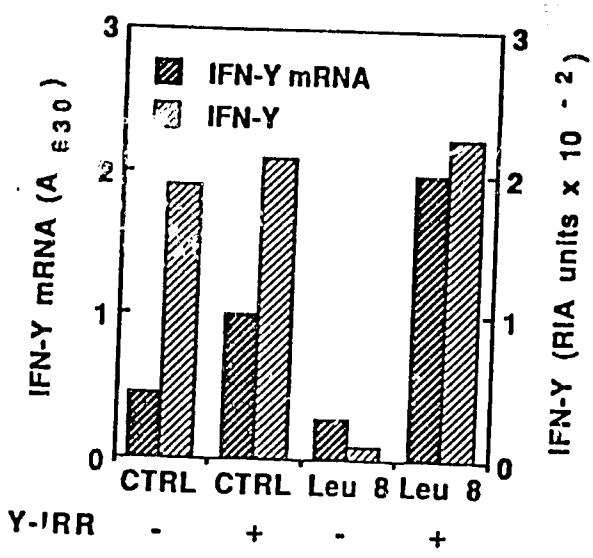

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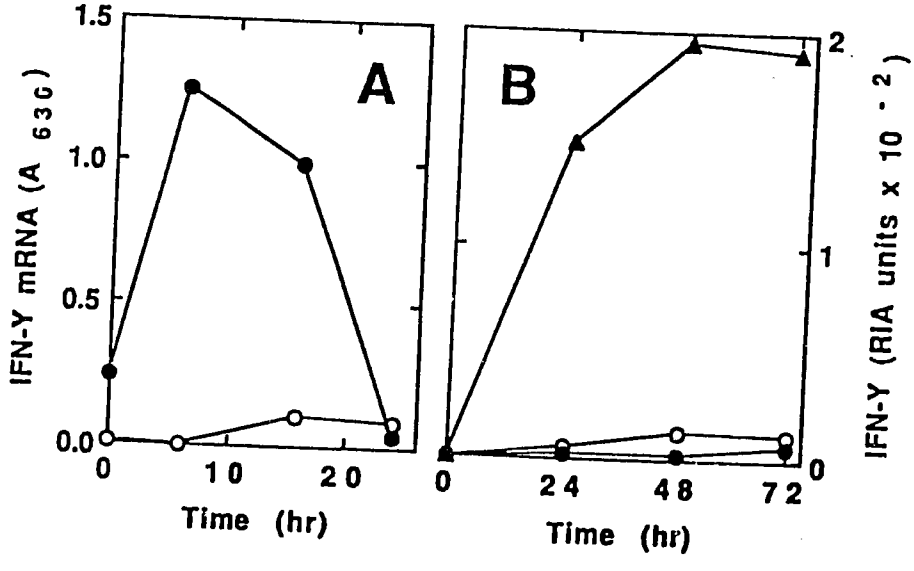

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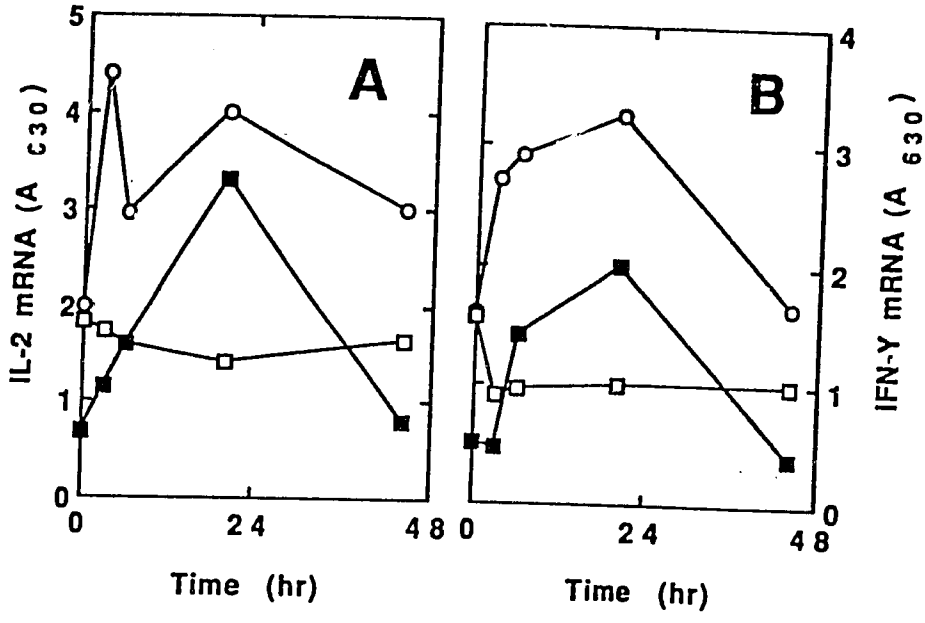

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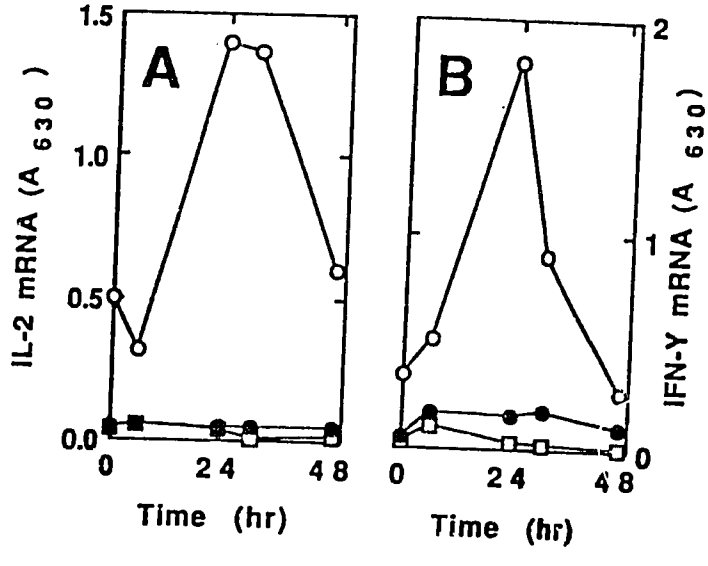

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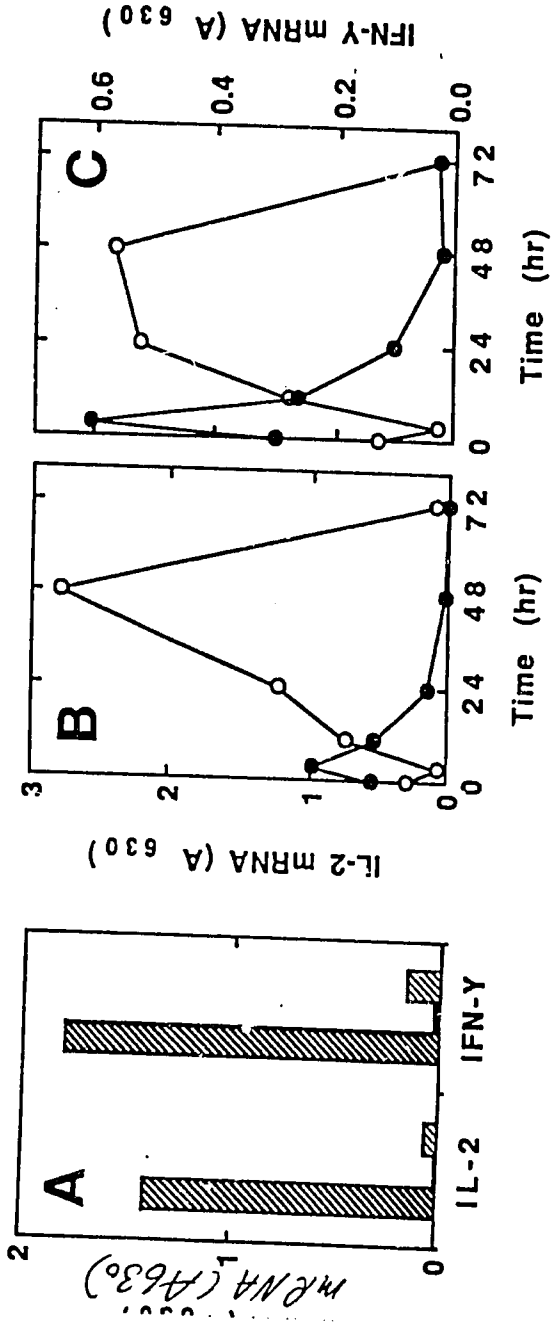
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Time (hr)
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IFN-γ mRNA (A 630)
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24
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Time (hr)

Figure 9.