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PROGRESS REPORT No.2

"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 C8-196

GRANT NUMBER 936-5544-G-00-8048-00

SUBMITTED BY

SANIT MAKONKAWKEYOON, Ph.D

PROJECT LEADER

DEPARTMENT OF CLINICAL IMMUNOLOGY

FACULTY OF ASSOCIATED MEDICAL SCIENCES

CHIANG MAI UNIVERSITY

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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 our studies, 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 study of the quantity and functional intactness of T lymphocytes producing IL2 and monocytes producing IL1, by examination of the function of T helper and T suppressor cells, and particularly their role in the regulation of IL2 and IFN-γ gone 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 IL2 LEVEL

1. Production of IL2.

PBML, 1×10^6 cells/ml were stimulated with 2 ug/ml PHA-P or 10 ug/ml PPD (Connaught International Division, Ontario, Canada) or 50 ug/ml PPD (Our own laboratory made PPD) or 20 ug/ml *M.leprae*. The culture was incubated at 37 ^{o}C 5% CO_2 18 hours for mitogen and 48 hours for antigens. Cell cultures were harvested and centrifuged, supernatant added with 20 mg/ml of α-methyl-D-mannoside, filtered sterile and then stored at -70 ^{o}C for IL2 level assay.

Resting PBML was PBML after isolating from Ficoll-Hypaque, it was incubated in 10% FBS-CRPMI 1640 medium at 37 ^{o}C, 5% CO_2 for 24 hours. The resting culture was washed once with RPMI 1640 media before further used in the experiment.

2. Assay of IL2 Activity.

Samples were diluted in serial two fold dilution 1:2, 1:4, 1:8, 1:16 to 1:256 in 100 ul in each well of a 96 wells tissue culture plate. CTLL-2 or 3-day Con A blasts cell suspension containing 8×10^3 or 3×10^4 cells/100 ul respectively, were added into each well, mixed, and incubated 24 hours. ^{3}H-TdR containing 0.2 uCi/50 ul were added into each well and incubated for 18 hours. Cultures were harvested onto glass microfiber filters and ^{3}H-TdR incorporation was determined by a liquid scintillation counter (LS 3801 Beckman).

DETERMINATION OF THE NUMBER OF IL2-PRODUCING CELLS.

1. Preparation of stimulated PBML.

PBML, 5×10^6 cells/ml was stimulated with 2 ug/ml PHA-P (Wellcome) for 4 hours in a CO_2 incubator, washed twice and then resuspended with 10% FBS-CRPMI medium to a concentration of 2.5×10^6 cells/ml.

2. Preparation of 3-day Con A-Blasts.

Inbred strain of Balb/c mice at the age of 6-8 weeks old were killed by cervical dislocation. Spleens were removed, crushed, and passed through stainless steel mesh into RPMI medium. After erythrocytes in the spleen cell suspension were lysed with hypotonic ammonium chloride buffer solution, spleen cells were washed twice and resuspended in 10% FBS-CRPMI medium. Spleen cell suspension at 1.0×10^6 cells/ml were cultured with 5 ug/ml Con A for 3 days, washed three times and readjusted to 3×10^7 cells/ml.

3. Preparation and Seeding of Culture Plates.

Soft agar gel technique was used for the determination of IL2-producing cells. Each plastic petri dish (35x10 mm) contains lower agar layer consisting of 1 ml 0.5% agarose in 10% FBS-CRPMI medium, and an upper layer consisting of 0.7 ml of 0.4% agarose in 10% FBS-CRPMI medium, 0.2 ml of responder cells (IL2-dependent cells) and 0.1 ml of effector cells. In controls, the upper layer contains only effector or responder cells. All cultures in the plastic petri dishes were incubated in 5% CO_2 at 37 ^{o}C for 5 days. The number of IL2-producing cells surrounded by IL2 responsive cells was enumerated under a dissecting microscope.

CELLULAR REQUIREMENT FOR GENERATION OF IL2 AND DEVELOPMENT OF IL2 -PRODUCING CELLS.

1. Isolation and Purification of Monocytes.

PBML, 2 x 10^{6}/ml, was added onto a 10 x 35 mm. plastic plates. After incubation at 37 ^{o}C, 5% CO_2 for 2 hours, non-adherent cells were washed out three times with warm (37 ^{o}C) RPMI 1640 medium. The adherent cells on the plastic plates were more than 90% positive for nonspecific esterase stain (+ve NSE).

2. Isolation and Purification of T and non-T Cells.

Non-adherent cells, from PBML after adhering of adherent cells to plastic plate, was rosetted with AET-SRBC overnight at 4 ^{o}C. The rosetted and non-rosetted cells were separated by Ficoll-Hypaque gradient centrifugation. The AET-SRBC in the bottom layer was lysed by hypotonic ammonium chloride solution. Both T and non-T cells were washed twice with RPMI 1640 medium

and then resuspended in 10% FBS-CRPMI 1640 medium. More than 95% of the T cells and less than 5% of the non-T cells showed positive rosette formation when tested with AET-SRBC.

3. Cells Mixing Culture.

In each study, PBML, T cells, non-T cells, adherent cells and T cells + adherent cells were mixed at the concentration of 1 x 10^6 cells/ml containing 2 ug/ml PHA-P. After 4 hours incubation, cell cultures were harvested and washed twice and then assayed for IL2-producing cells. However, For IL2 level determination, cell cultures will be incubated for 18 hours and supernatant fluid assayed for IL2 level.

ISOLATION AND SEPARATION OF MONOCYTES BY PERCOLL SOLUTION.

1. Determination of Percoll Concentration in the Upper Layer.

Nine parts of Percoll (Pharmacia) solution was added to 1 part (v/v) of 10x concentrated sterilized physiological saline or normal saline solution (NSS) to yield 100% Percoll solution. PBML was mixed and resuspended in 63% Percoll in a volume of 2 ml then overlayered with 2 ml of various concentrations of 9%, 12%, 15%, 18%, 21% and 24% Percoll solution. All tubes were overlayered with RPMI 1640 medium, then centrifuged at 300xg for 30 min. at room temperature. Cells at the interface between Percoll and RPMI 1640 medium was carefully removed with sterilized pasteur pipette, counted and stained with nonspecific esterase stain.

2. Determination of Percoll Concentration in the Lower Layer.

PBML was mixed and resuspended in various concentrations of Percoll, 45%, 51%, 57%, 63%, 69% and 75% in a volume of 2 ml, overlayered with 12% Percoll solution and then with RPMI 1640 medium. All tubes were centrifuged and all further steps of procedure were performed as above.

PREPARATION OF PURIFIED PROTEIN DERIVATIVE (PPD) OF M.TUBERCULOSIS

1. Preparation of PPD.

M.tuberculosis H37Rv was grown in Long's synthetic medium for 6 weeks. The bacteria was killed by 100 ^{o}C steam for 3 hours. Culture fluid was passed twice through sterile gauze. The cell free culture fluid was added with 4% trichloroacetic acid (TCA) and precipitate washed twice with 1% TCA and once with 0.3% KH_2PO_4. The precipitate was dissolved with 1/125 of starting volume of M/6 Na_2HPO_4 containing 2.5% NaCl. The solution was centrifuged at 3200 rpm for 2 hours. Supernatant fluid was collected and filtered through a 0.45 μ membrane filter. Proteins in supernatant fluid was precipitated with saturated ammonium sulfate solution pH 7.2. The precipitate was washed 5 times with 1% TCA and then dissolved with M/30 phosphate buffer. This purified protein derivative (PPD) solution was steriled by filtration and the protein content was determined by Biruet and Lowry methods.

2. Determination of Optimal Concentration of PPD for Stimulation of IL2 Production.

PBML 1×10^6/ml in 10% FBS-CRPMI 1640 media was incubated with 0, 5, 10, 20, 50, 100 and 200 ug/ml of PPD at 37 ^oC, 5% CO_2 for 48 hours. Culture fluids were harvested and collected by centrifugation and stored at -20 ^oC for IL2 assay.

ISOLATION AND SEPARATION OF T CELL SUBPOPULATION.

1. Determination of Complement Dilution for Complement Mediated Cell Cytotoxicity.

PBML 1×10^6/100 ul was added with 5 ul of either OKT3, OKT4 or OKT8 sera (Ortho Pharmaceutical, USA.). Rabbit complement in various dilutions was added in a volume of 100 ul. The cell suspension was incubated at 37 ^oC for 30 min and the viability of cells determined by trypan blue exclusion technique.

2. Determination of OKT4 Serum for Complement Mediated Cell Cytotoxicity.

Human T cells 1×10^6 or 2×10^6 cells/100 ul was added with various amounts of OKT4 serum and 100 ul of 1:5 rabbit complement. After 1 hour of incubation at 37 ^oC, 5% CO_2, the cells was washed once with PBS and the viability determined by the trypan blue exclusion technique.

STUDY OF IL2 AND IFN-γ GENE EXPRESSION IN NORMAL SUBJECTS.

Human PBML of 12 normal blood donors were 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. The procedure is as follows:

1. <u>Day 1-Culture of Lymphocytes</u>.

1. Collect peripheral blood from subject in heparinized tubes. Ten to twenty ml of whole blood is sufficient. Blood samples should be processed as soon as possible on the day of collection and should be kept at room temperature to ensure optimal results.

2. Dilute blood with PBS at 1:1 (v/v) in a 50 ml centrifuge tube. Mix the sample evenly.

3. Prepare 15 ml centrifuge tubes each containing 5 ml Ficoll-Hypaque. Mark the tubes with patient identification.

4. Carefully, using a 10 ml pipette, layer 8-10 ml from the diluted blood over a bed of 5 ml Ficoll-Hypaque in each tube. Don not mix the F-H and the blood sample.

5. Centrifuge at 200 g (1000 rpm) in a cell centrifuge for 30 min at 18-20 ^{o}C without using the brake.

6. Collect the white interface layer containing the lymphocytes with a pasteur pipette and transfer to a clean 50 ml centrifuge tube. It is essential to remove all the material in a minimal volume.

7. Wash cells with at least 3 volumes of RPMI 1640 warmed to room temperature. Centrifuge at 200 g for 15 min at 18-20 ^{o}C. Do second wash with 25 ml RPMI 1640.

8. Discard supernatant. Resuspend the cells in 5 ml of complete medium warmed to 37 ^{o}C.

9. Count cells.

10. Adjust cell density to be at $4x10^6$ cells/ml. Although this cell density is optimal, the assay will give satisfactory results with as few as 0.5×10^6 cells/ml. Thus, if lower cell density is obtained, keep total volume at 5 ml.

11. Dispense 1 ml of suspension into each of five culture 10-ml tubes and treat them accordingly as follows:

Induction of mRNA:

Tube 1: Control-not induced-harvest at 18 h.

Tube 2: Add 4 ul of PHA-P at 0 time and harvest cells at 18 h.

Tube 3: Add 4 ul of PHA-P at 0 time and harvest cells at 22 h.

Tube 4: Add 4 ul of PHA-P at 0 time, superinduction with 2 ul cycloheximide (10 mg/ml) at 18 h, and harvest cells at 22 h.

Tube 5: Gamma-irradiation the cells with 1500 rad with a Co^{60} gamma cell 220 (Atomic energy of Canada Ltd. or equivalent) at 0 time, then add 4 ul PHA-P. Harvest cells at 22 h.

12. Incubate cultures at 37 ^oC in 5% CO_2 atmosphere for 18 h to 22 h.

2. <u>Day 2-Cell harvesting and mRNA extraction</u>

1. Thaw 7.5 M guanidine HCl solution kept at -20 ^oC. Harvest cells at the time indicated in step 11 of Day 1. Take out cultures from the incubator. Mix the cells in the tubes by tapping the bottom and by pipetting the suspension in and out of a sterile pastour pipette or 1 ml pipette tip.

3. Transfer the cell suspension into a sterile Eppendorf tube.

4. Centrifuge at 5000 rpm (Eppendorf centrifuge) at room temperature for 5 min.

5. Discard the supernatant using a sterile tip or vacuum. Vortex the pellet to disperse the cells.

6. Add to each tube 0.4 ml of 7.5 M guanidine HCl solution.

7. Dissolve pellet by vortexing the solution for at least 20 sec. or until no aggregates are found in the solution.

8. Add to each tube 20 ul of 2 M Potassium acetate pH 5.1.

9. Add to each tube 250 ul of absolute ethanol kept at -20 ^{o}C

10. Mix and leave at -20 ^{o}C overnight. The preparation is stable at this point and can be stored at -20 ^{o}C for longer periods if necessary.

Note: It is suggested that samples be stored at this point of the procedure until material from 2-4 subjects is available for further processing.

3. <u>Day 3</u>.

1. Centrifuge the Eppendorf tubes at 12000-14000 rpm for 30 min at 4 ^{o}C in an Eppendorf centrifuge.

2. Prepare a 60 ^{o}C water bath.

3. Thaw 37% formaldehyde solution.

4. Discard the supernatant carefully with a sterile tip. Mix on vortex until no clumps are found.

5. Dissolve pellet in 100 ul of 37% formaldehyde, as the RNA is to be tested against 2 probes (IL-2 and IFN-γ). Take care to vortex until RNA pellet dissolves completely.

6. Add 110 ul of 20x SSC, and vortex.

7. Place the tubes on polystyrene foam in 60 ^{o}C water bath for 15 min for RNA denaturation.

8. Prepare 100 ml of 10x SSC by diluting the 20X SSC solution 1:1 with sterile double distilled water.

9. Wet the 9 x 13 cm nitrocellulose paper and the GB003 blotting sheet with sterile double distilled water. The nitrocellulose sheet should be wet gradually. Change the water to 10x SSC solution. Soak for at least 10 min.

10. After RNA denaturation, dilute each sample by eight 2-fold serial dilutions with 10x SSC : [Add to each well of sterile microplate 100 ul of 10x SSC. Pipette 100 ul of the sample to first well and then serially dilute by pipetting to adjacent wells]. For each patient one will finally have 40 wells and one blot will be loaded with 80 wells (2 patients). The unused wells will serve as machine blank. In a case where 2 probes are tested, there are 210 ul in each sample.

11. Prepare the Biodot apparatus. First lay the blotting sheet, then the nitrocellulose on top. Close tightly the apparatus and connect it to the vacuum trap fully opened.

12. Transfer 80 ul from each well of the microplate to the corresponding well of the Biodot. Apply the samples to the middle of the well to ensure that no RNA adheres to well walls.

13. Once dry, wash each well with 100 ul of 10x SSC and let it dry for 1-2 min.

14. Withdraw the nitrocellulose sheet and air dry between a folded Whatman #3 sheet. Bake at 80 ^{o}C in a vacuum oven for 2 hrs.

15. Repeat steps 10-14 to prepare a sec

16. Wrap the nitrocellulose in Whatm sheets together to be sent to Israel betwe by registered airmail. [NB: This material i

17. The following steps will be carri group:

- Pre-hybridization of the nitrocellul
- Hybridization
- Autoradiography
- Scanning for density of dot blots
- Analysis of results in the computer
- Statistical analysis

RESULTS

PRODUCTION AND DETERMINATION OF IL2 LEVEL.

We once again tried to determine the optimal concentration of *M.leprae* to stimulate IL2 production in tuberculoid leprosy patients and in normal subjects with positive PPD skin test. Figures 1,2 and 3 show very low IL2 production when 8 tuberculoid leprosy patients' PBML were stimulated with *M.leprae* antigen. Figure 4 also shows very low IL2 production when 10 BL/LL, 4 BT/TT and 4 normal PPD skin test positive PBML were stimulated with *M.leprae* antigen while the same leprosy patients and normal controls produce high level of IL2 when stimulated with PHA-P as shown in figure 5. The finding of present experiments are very similar to our previous experiments.

ISOLATION AND SEPARATION OF MONOCYTES BY PERCOLL SOLUTION.

Figure 6 shows optimal concentration of Percoll in the upper layer for monocyte separation when PBML is mixed with 63% Percoll in the lower layer. The optimal concentration of Percoll in the lower layer for monocyte separation is illustrated in figure 7. Although highly purified monocyte suspension is obtained by Percoll separation, However, the percent yield is very low and a lot of dead cells were observed.

CELLULAR REQUIREMENT FOR GENERATION OF IL2 AND DEVELOPMENT OF IL2-PRODUCING CELLS.

Cellular requirement for generation of IL2 and development of IL2-producing cells are studied by cell culture and cell mixing cultures by either autologous or heterologous cell mixing. Figures 8 and 9 show levels of IL2 production when PBML, monocytes, T cells, non-T cell, T cells + autologous monocytes, T cells + heterologous monocytes, and non-T cells + autologous monocytes in normal subjects when stimulated with PHA-P. Figures 10, 11 and 12 depict the numbers of IL2-producing cells from various cell types and cell mixing culture when stimulated with PHA-P. It is generally observed that T cells + autologous monocytes will give rise to a maximum number of IL2-produucing cells, while purified T cell and non-T cells will yield low number of IL2-producing cells.

PREPARATION OF PURIFIED PROTEIN DERIVATIVE (PPD) OF M.TUBERCULOSIS.

Scientists in many places around the world utilize PPD both *in vivo* and *in vitro* studies. *In vivo* skin test with PPD is the normal practice in public health programs and it is very easy to obtain PPD for this purpose. However, *in vitro* studies utilizing PPD encounter problems because many laboratories are not able to find good and appropriate PPD for their study purpose. We have a lot of difficulty in testing and finding suitable commerial PPD. We finally decided to produce our own PPD. After trying various procedures we have now a simple but effective technique to produce PPD as mentioned in the meterials and

methods. Figure 17 shows the optimal concentration of PPD for stimulation of IL2 production.

ISOLATION AND SEPARATION OF T CELL SUBPOPULATION.

Table 1 shows determination of complement dilution for maximum killing of monoclonal antibodies coated T lymphocytes. The dilution of 1:5 of rabbit complement yield acceptable killing of T cells. Table 2 shows optimal concentration of OKT4 serum for maximum complement mediated cell cytotoxicity of T lymphocytes.

STUDY OF IL2 AND IFN-γ GENE EXPRESSION IN NORMAL SUBJECTS.

Figure 13 and 14 illustrate IL2 mRNA level of 12 normal PBML culture at various conditions. Treatment of PBML with cycloheximide induces large amounts of IL2 mRNA. When PBML was treated with PHA-P for 22 hours or with γ-irradiation, moderate increase of IL2 mRNA level was observed. Figure 15 shows the increase of IFN-γ mRNA when treated PBML with cycloheximide or γ-irradiation or PHA-P for 18 or 22 hours. Figure 16 summarizes the average level of IL2 mRNA and IFN-γ mRNA from 8 normal subjects' PBML.

DISCUSSION AND CONCLUSION

We have confirmed our previous finding that M.leprae antigen is not able to stimulate IL2 production from PBML of BL/LL, BT/TT leprosy patients and normal subjects (skin test +ve PPD), However, when PHA-P is used as stimulating agent in these subjects, IL2 is effectively produced in all groups of these subjects.

Very high purity of monocytes is obtained when PBML mixed with 63% Percoll in lower layer and overlayed with 12% Percoll in the upper layer. However, the yield of monocytes is poor and percent viability is low.

The cellular requirement for generation of IL2 and development of IL2-producing cells are evaluated by cell mixing cultures. Purified monocytes and non-T cells produce very low level of IL2 while purified T cells gives rise moderate level of IL2. However, PBML, T cells + autologous monocytes, and T cells + heterologous monocyte produce high level of IL2. PBML is stimulated with PHA-P producing moderate numbers of IL2-producing cells while pure T cells, non-T cells produce very low numbers of IL2-producing cells. However, when T cells are mixed with autologous monocytes, very high numbers of IL2-producing cells are observed.

The problem of finding or purchasing the appropriate PPD for stimulation of IL2 production has been solved by our own preparation and isolation of PPD in our laboratory. Our own PPD preparation shows very good stimulation of PBML to produce high level of IL2. We think that our PPD will be very useful to

scientists who are working with scientific experiments using PPD as a study agent.

We have set up the appropriate conditions to eliminate helper or suppressor T cells by complement mediate cell cytotoxicity. We are now studying the functions of T cell subpopulations both in HMI and CMI responses.

One of my co-investigator, Dr.Kriangsak Praputpittaya, has been trained in Dr.Raymond Kaempfer's laboratory at The Hebrew University of Jerusalem, Israel. The training included basic and advanced techniques to start research on gene expression. Techniques involved in *in vitro* culture conditions for measuring both function of the IL2 and IFN-γ genes in peripheral blood mononuclear leukocytes and also the activation of suppressor T cells circuits that may regulate the expression of the two genes in leprosy. We are now beginning to set up all these techniques in Chiang Mai.

WORKPLAN FOR THE NEXT PERIOD

The relationship of IL2 levels and numbers of IL2-producing cells in normal subjects and various types of leprosy patients will be evaluated. 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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TABLE 1 : TITRATION OF COMPLEMENT DILUTION FOR COMPLEMENT MEDIATED CELL CYTOTOXICITY OF T LYMPHOCYTES.

MONOCLONAL ANTIBODY	WITHOUT COMPLEMENT	% LYSIS COMPLEMENT DILUTION 1:2	1:5	1:10	1:20
EXP.1					
OKT3	4	ND	48.5	32	22
OKT4	2	ND	28	32.5	11.5
OKT8	3	ND	23.5	20	22
EXP.2					
OKT4	3	3	21	ND	ND
EXP.3					
OKT4	6	5	33	ND	ND

TABLE 2 : TITRATION OF OKT4 CONCENTRATION FOR COMPLEMENT MEDIATED CELL CYTOTOXICITY OF T LYMPHOCYTES.

EXPERIMENT	WITHOUT COMPLEMENT	% LYSIS AMOUNT OF OKT4(ul) 5	10	20	40	60
T cell 1 x 10^6 cells						
1	10	10	35	36	42.5	44
2	14	40	39	ND	ND	ND
T cell 2 x 10^6 cells						
1	6	21	33	ND	ND	ND
2	9	27	35	36.5	40.5	ND
3	10	35	36	35	42.5	44

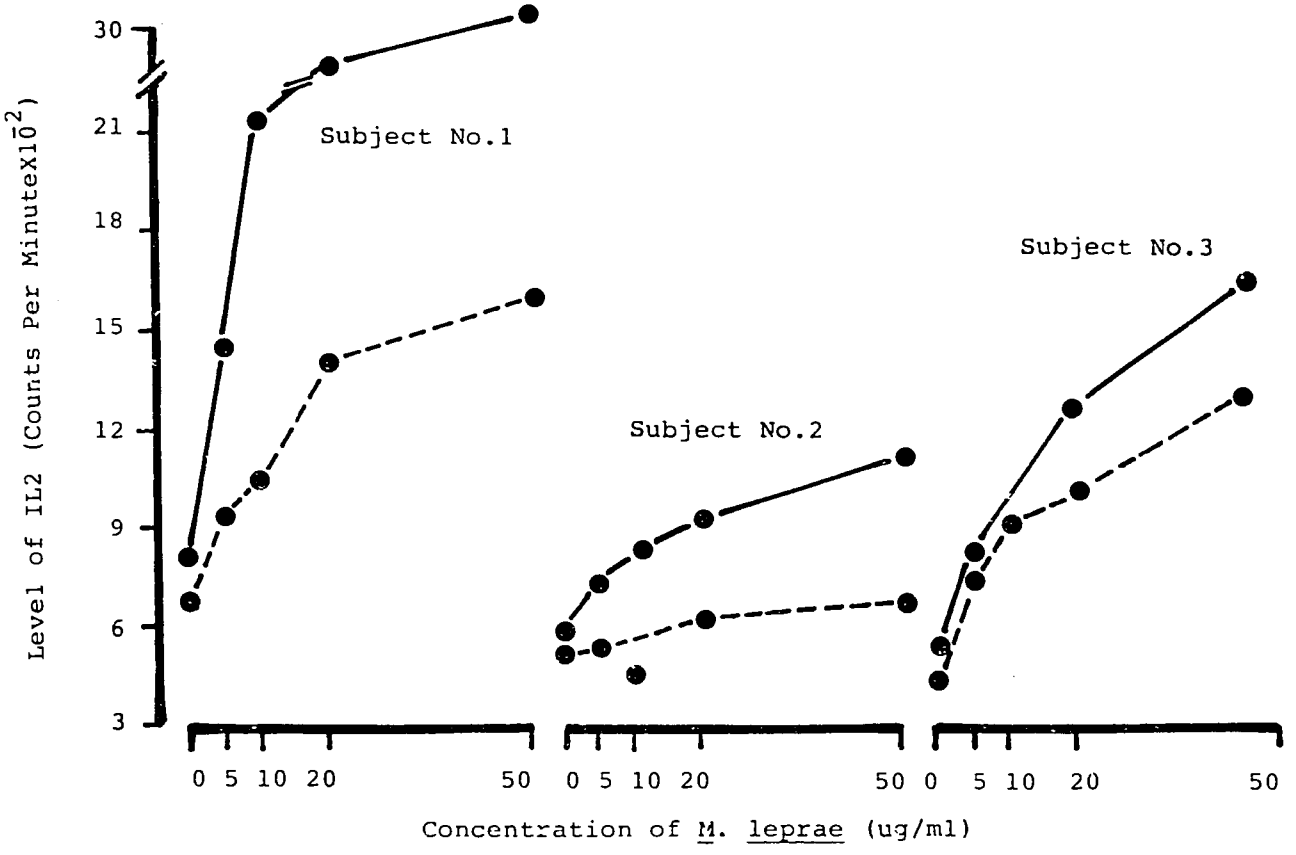

Figure 1. Determination of optimal concentration of M. leprae for stimulation of IL2 production from PBML(— =resting PBML; - - - = nonresting PBML of tuberculoid leprosy patients).

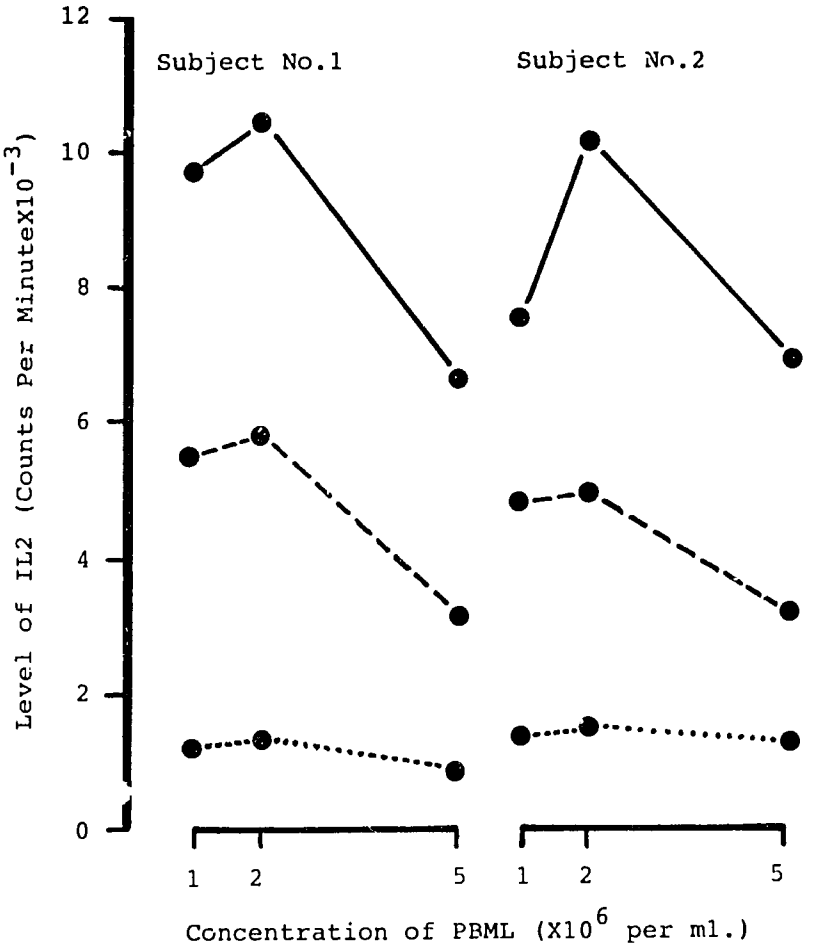

Figure 2. Determination of optimal concentration of PBML for IL2 production stimulated with 20 ug M. leprae (—— =resting PBML; — — =nonresting PBML; and ····· =unstimulated nonresting PBML of tuberculoid leprosy patients).

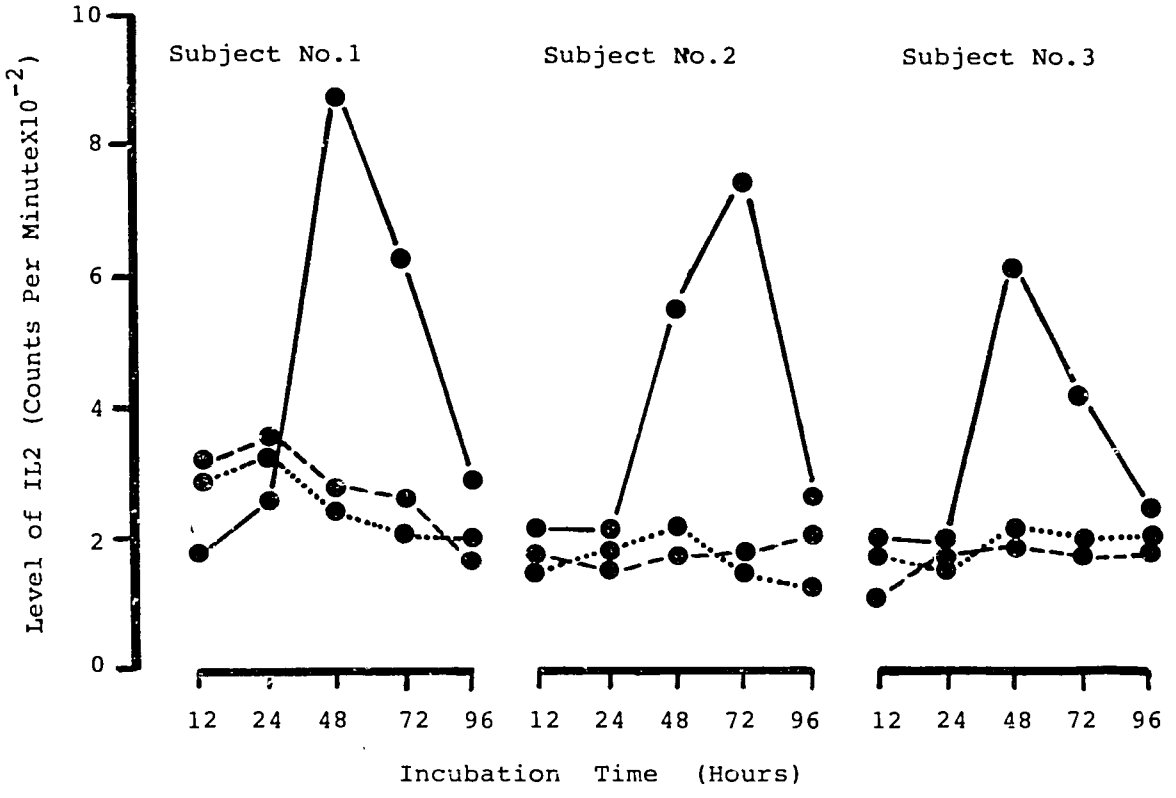

Figure 3. Determination of optimal incubation time for IL2 production from $2x10^6$ PBML stimulated with 20 ug M. leprae (·····=unstimulated PBML; — — =stimulated PBML; and — =stimulated resting PBML of tuberculoid leprosy patients).

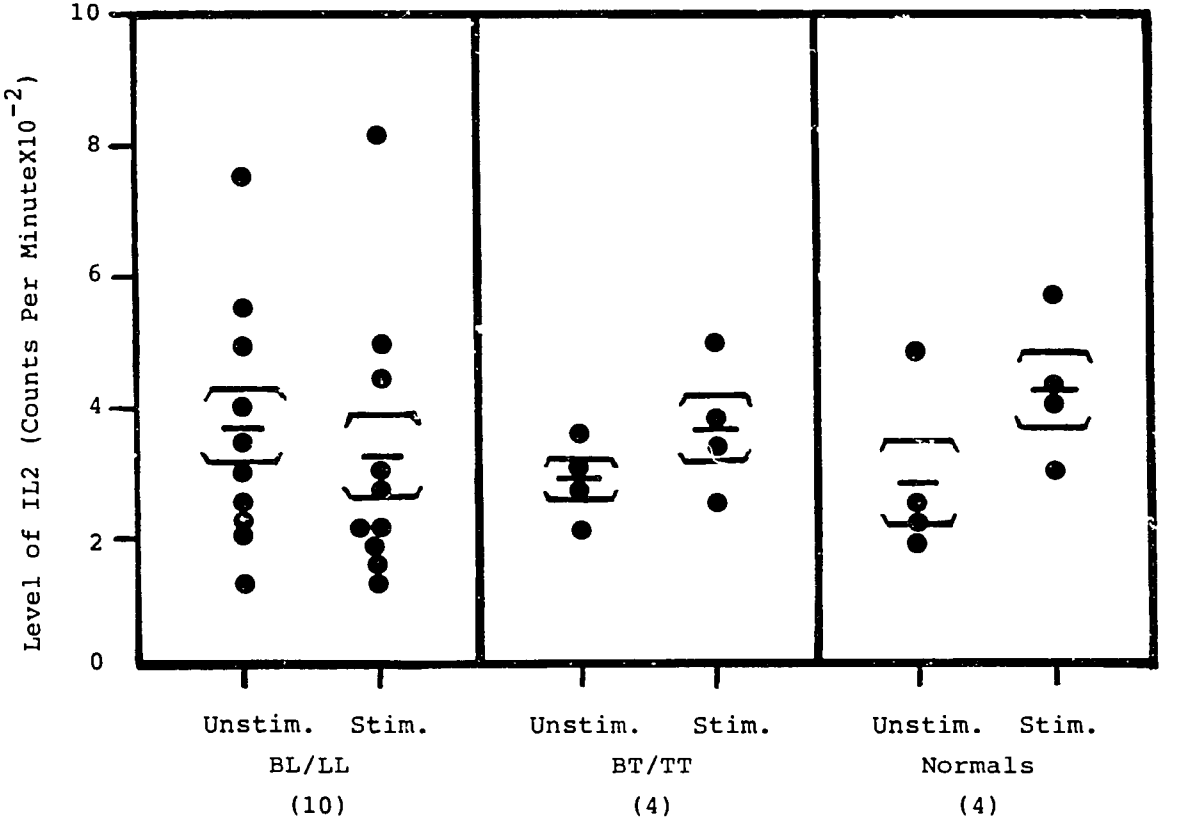

Figure 4. Determination of IL2 production from resting PBML of various subjects when stimulated with 20 ug M. leprae (all normal subjects are skin test positive for PPD).

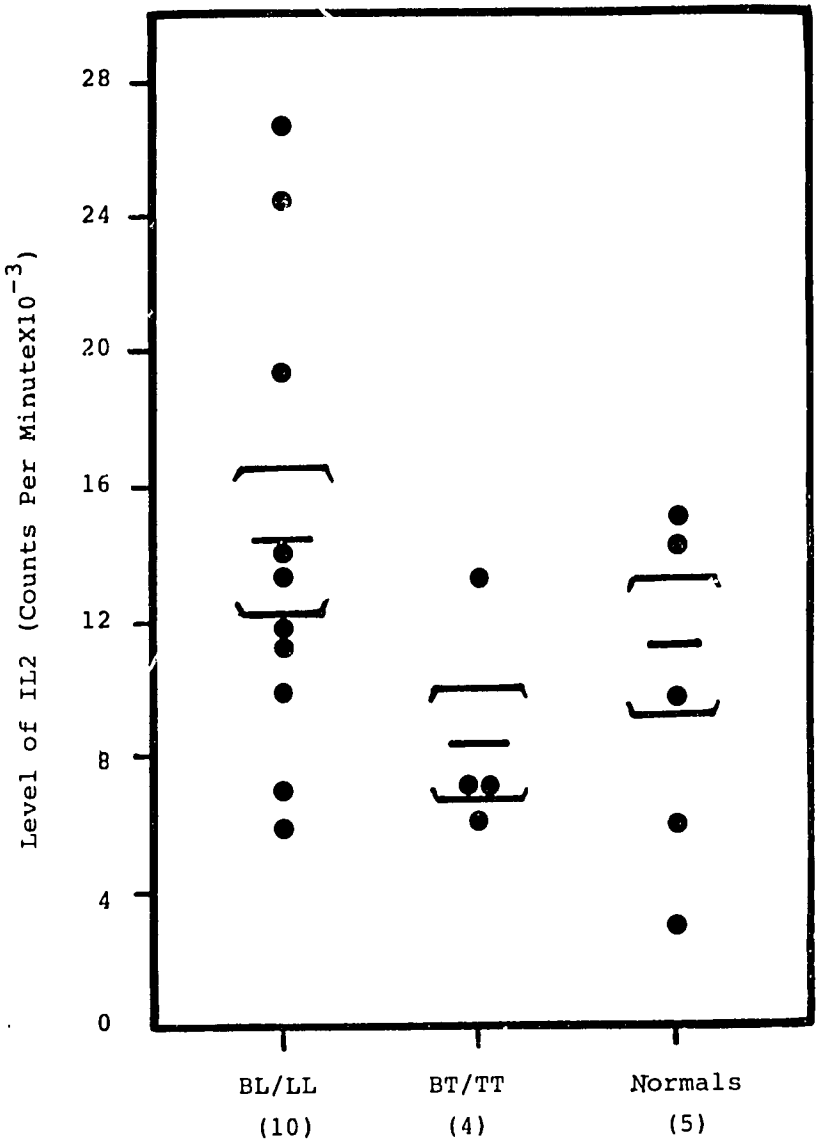

Figure 5.. Determination of IL2 production from nonresting PBML of various subjects when stimulated with PHA-P.

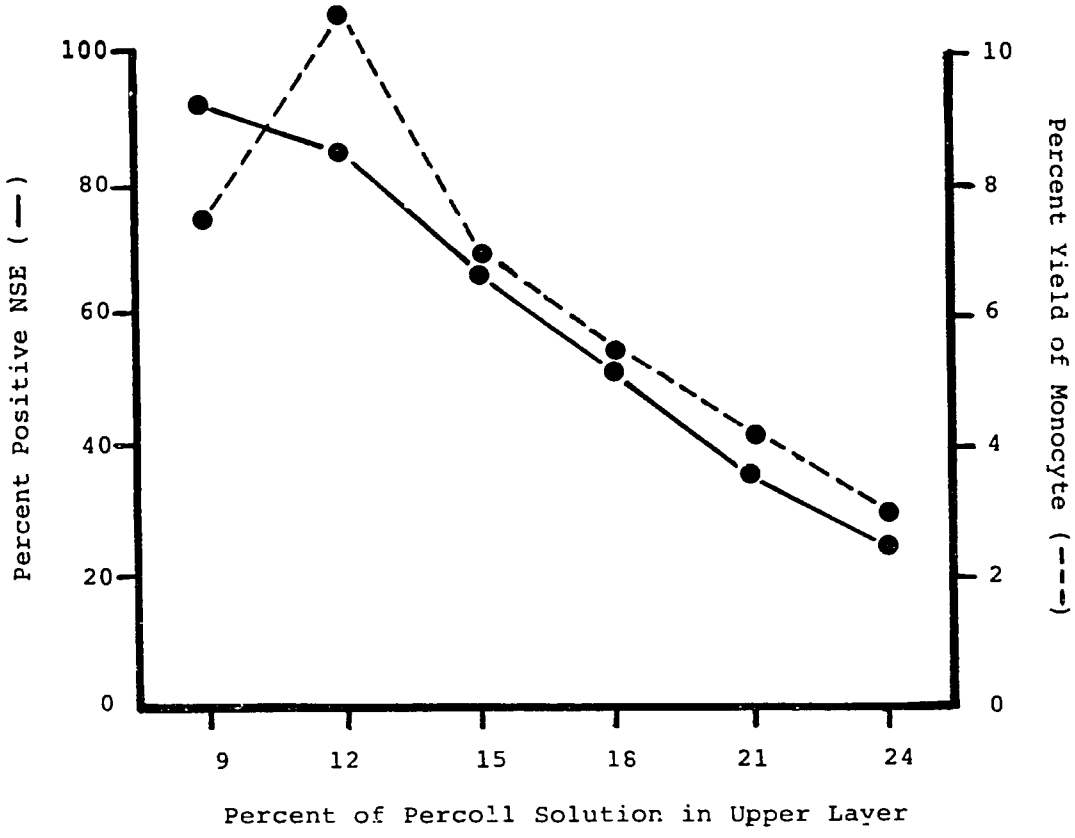

Figure 6. Determination of Percoll concentration in the upper layer for monocyte separation when PBML mixed with 63% Percoll in the lower layer (2 subjects).

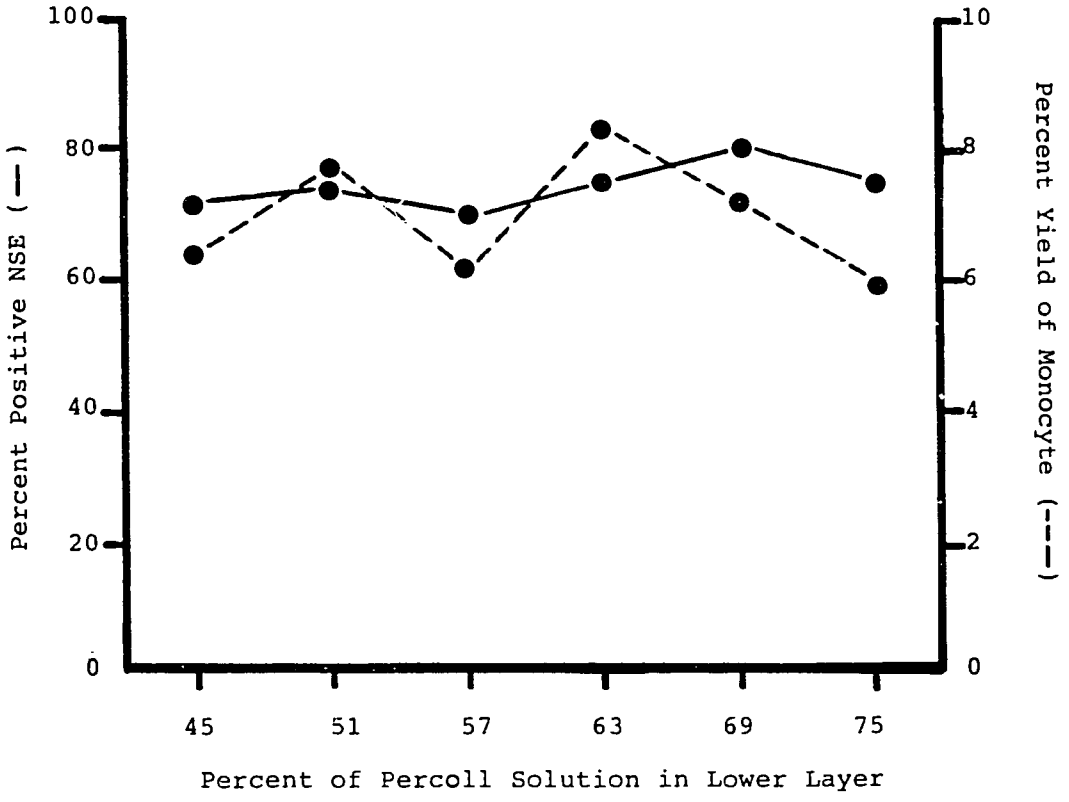

Figure 7. Determination of Percoll concentration in the lower layer for monocyte separation when the upper layer consisting 15% Percoll.

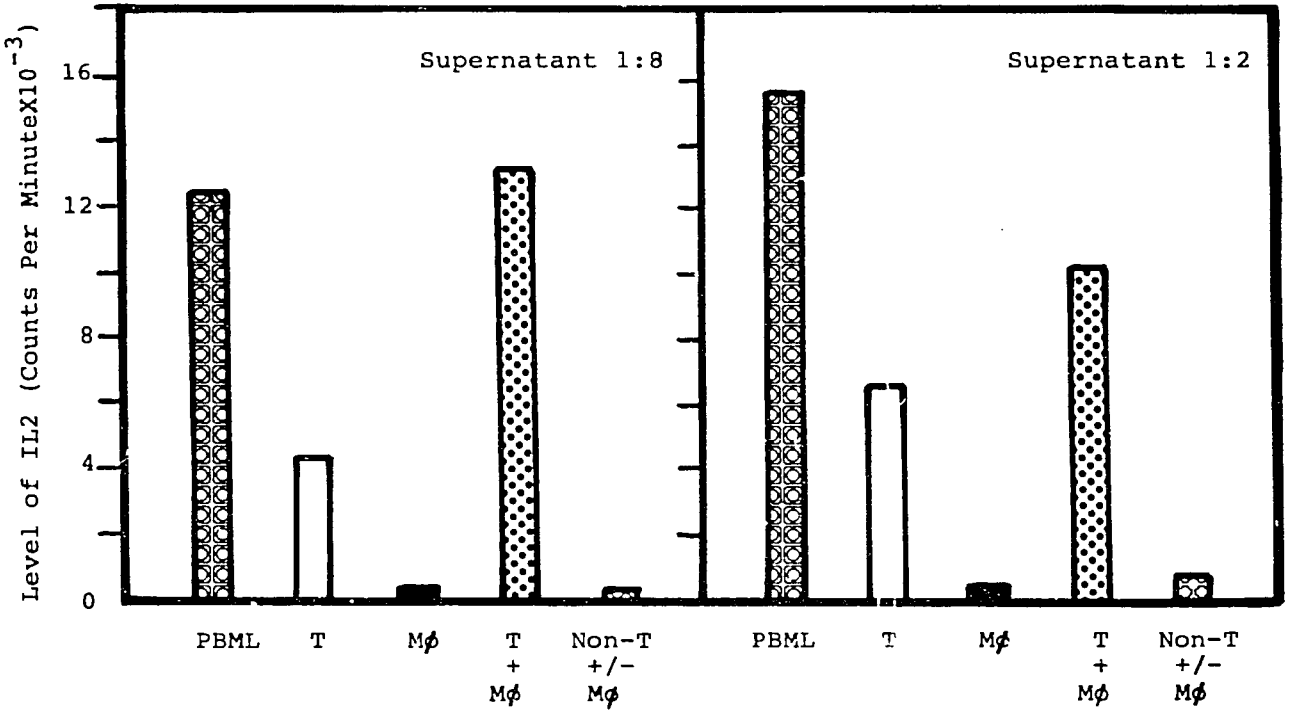

Figure 8. Production of IL2 by various cell types and cell mixing culture.

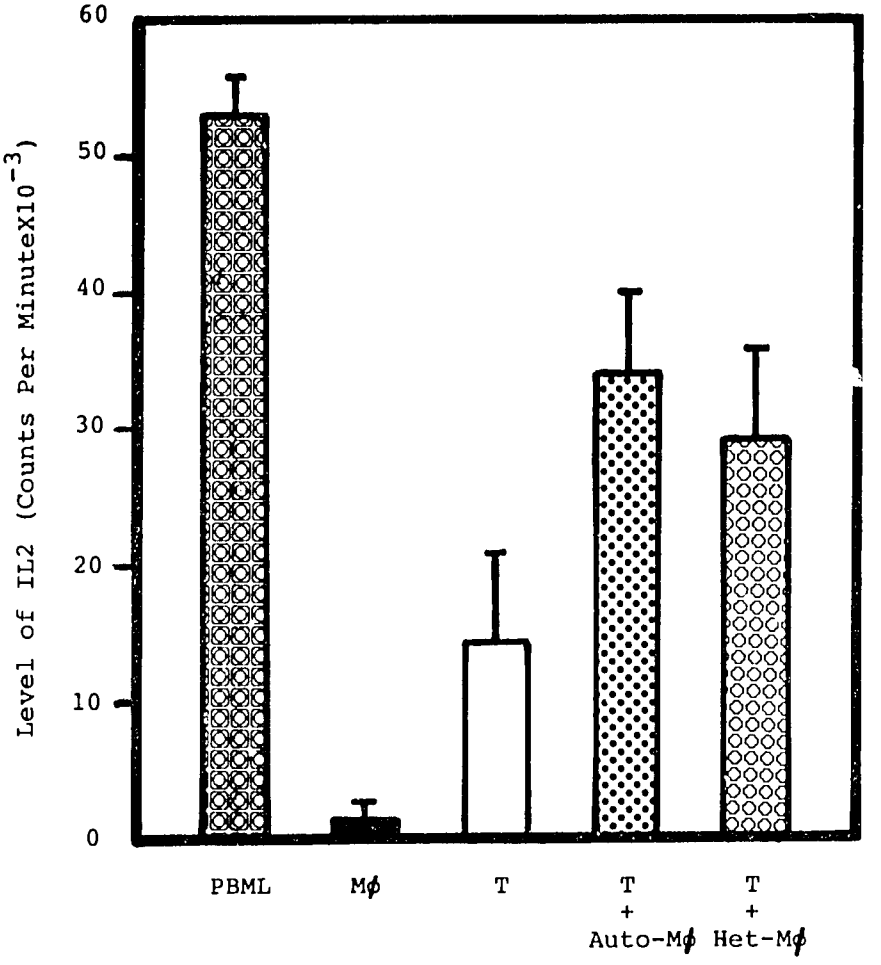

Figure 9. Production of IL2 by various cell types and cell mixing culture (average of 4 subjects).

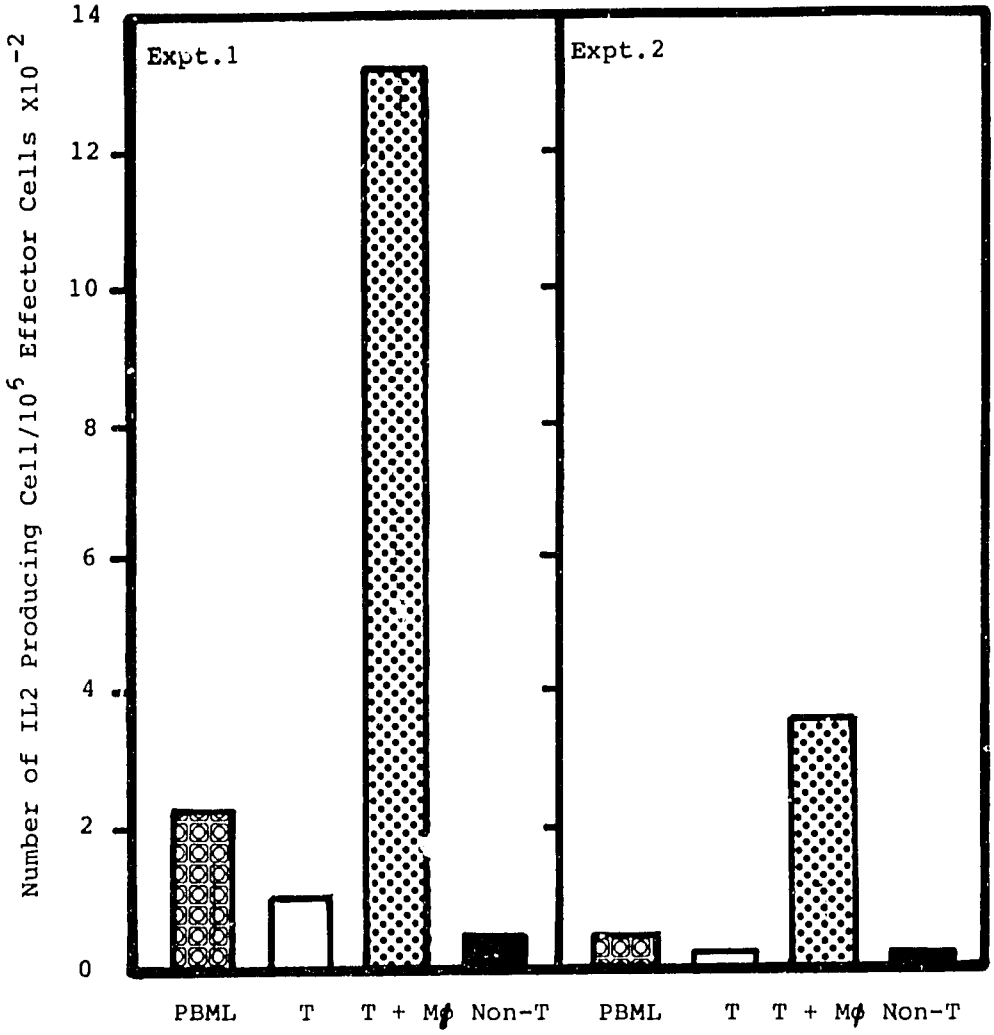

Figure 10. Numbers of IL2 producing cells from various cell types and cell mixing culture (2 subjects).

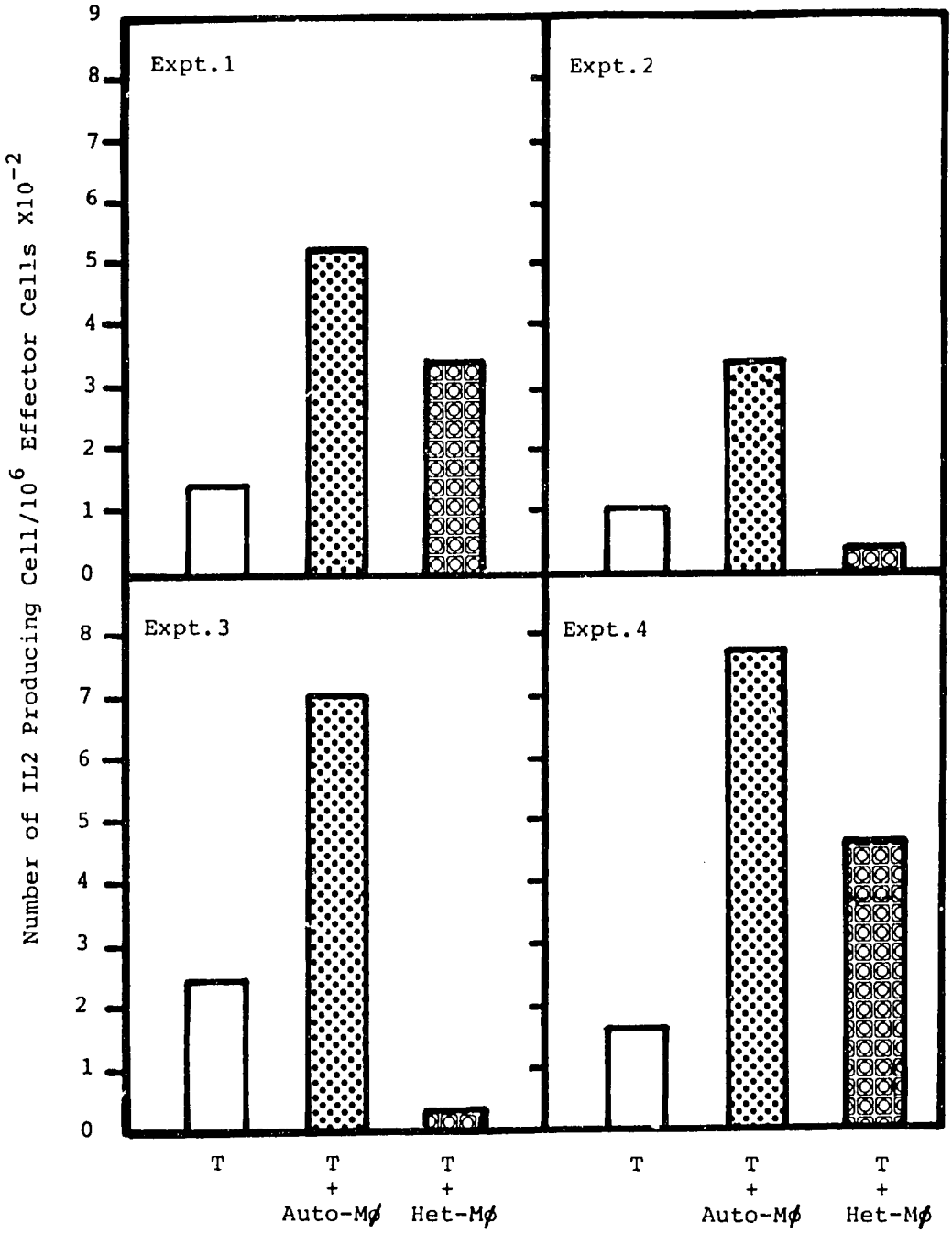

Figure 11. Numbers of IL2 Producing cells from various cell types and cell mixing culture (4 subjects).

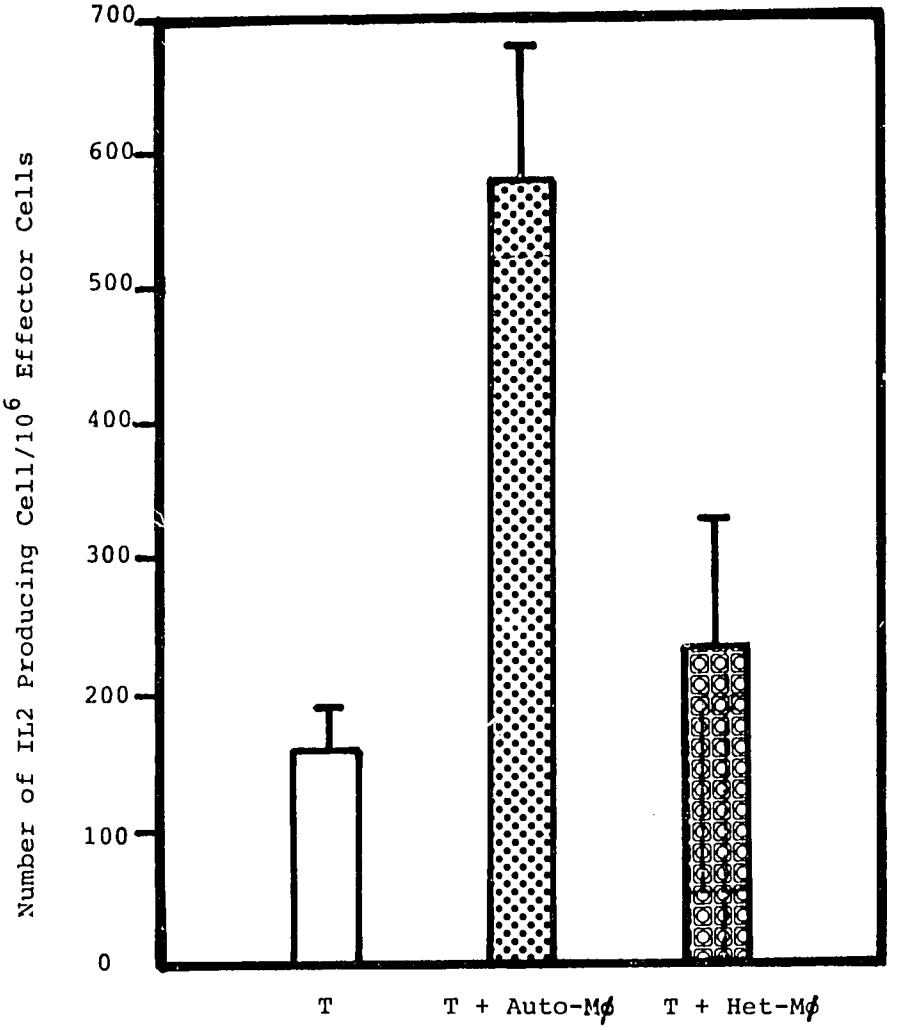

Figure 12. Numbers of IL2 Producing cells from various cell types and cell mixing culture (average of 4 normal subjects).

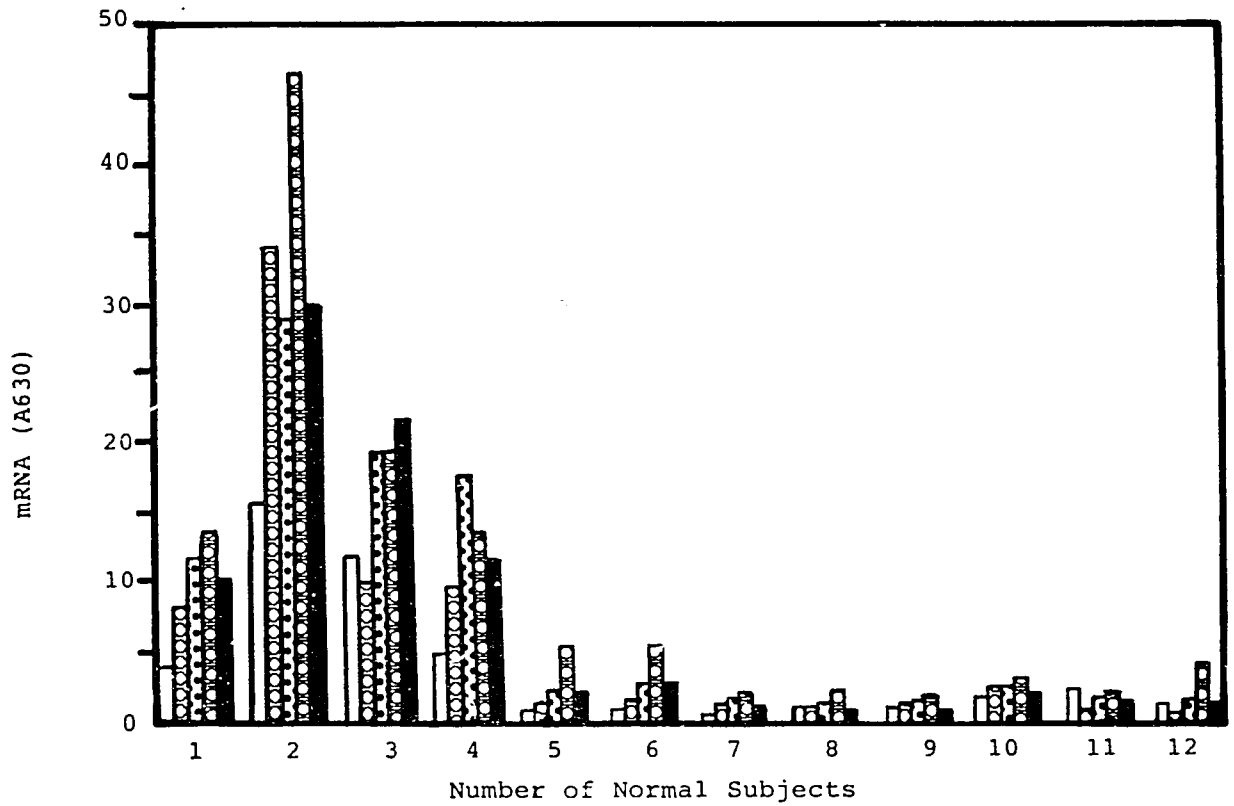

Figure 13. Determination of IL2 mRNA level of 12 normal PBML culture at various conditions (CTRL; PHA 18 h; PHA 22 h; CHX 22 h; and γ-irr).

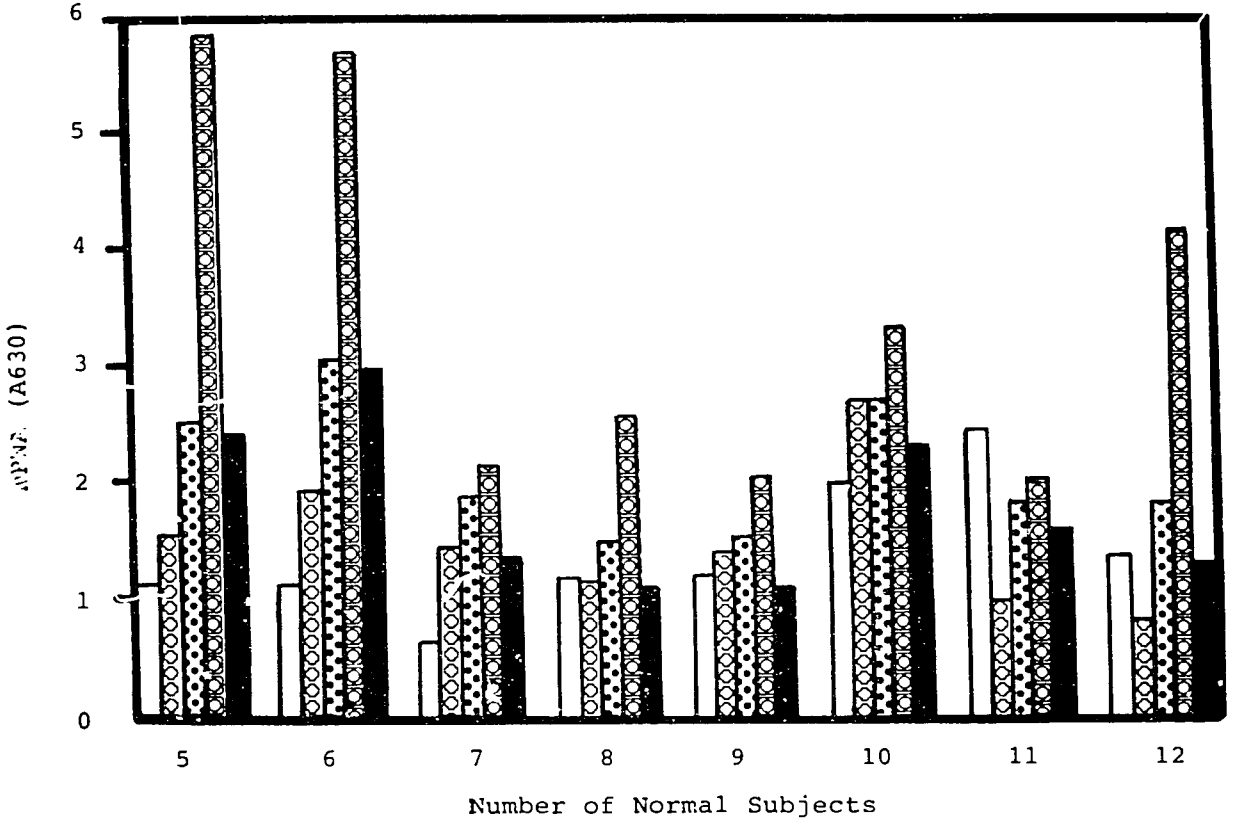

Figure 14. Determination of IL2 mRNA level of 5 normal PBML cultured at various conditions (CTRL; PHA 18 h; PHA 22 h; CHX 22 h; and γ-irr).

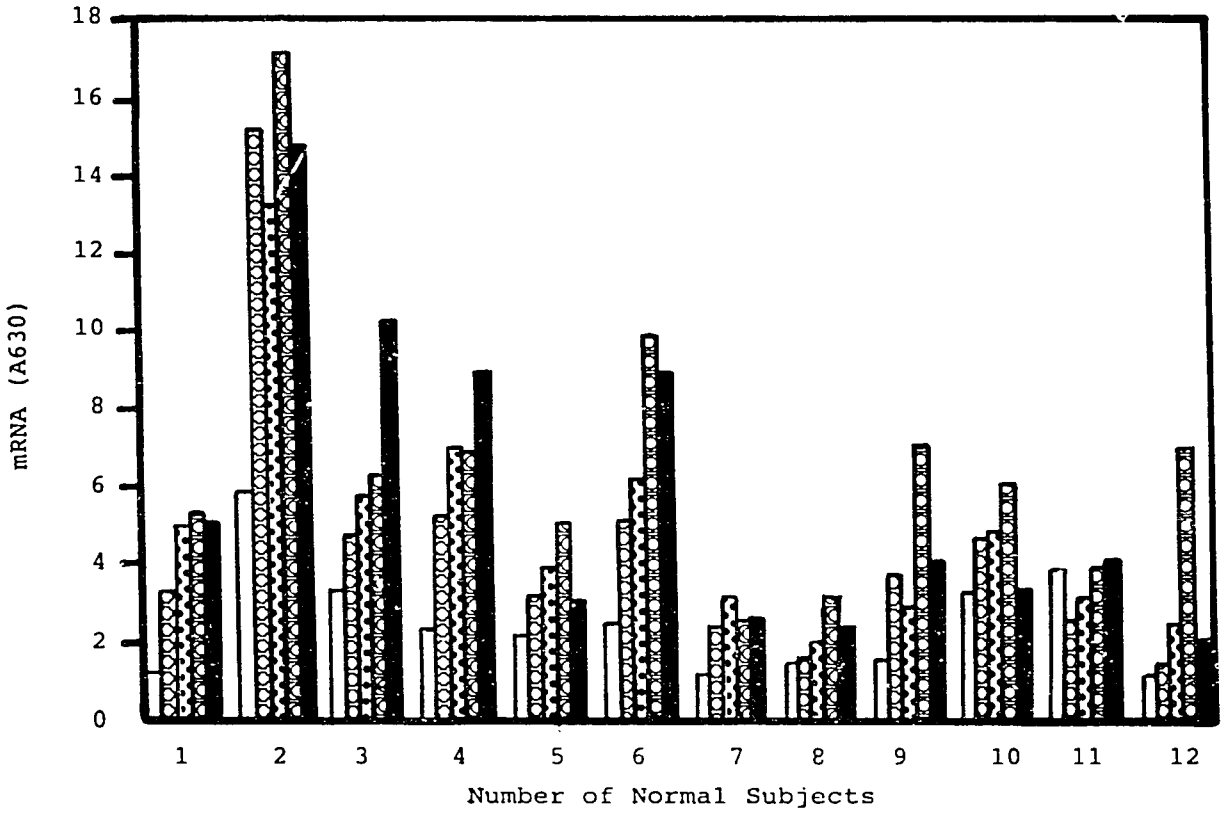

Figure 15. Determination of IFN-γ mRNA level of 12 normal PBML cultured at various conditions (CTRL; PHA 18 h; PHA 22 h; CHX 22 h; and γ-irr).

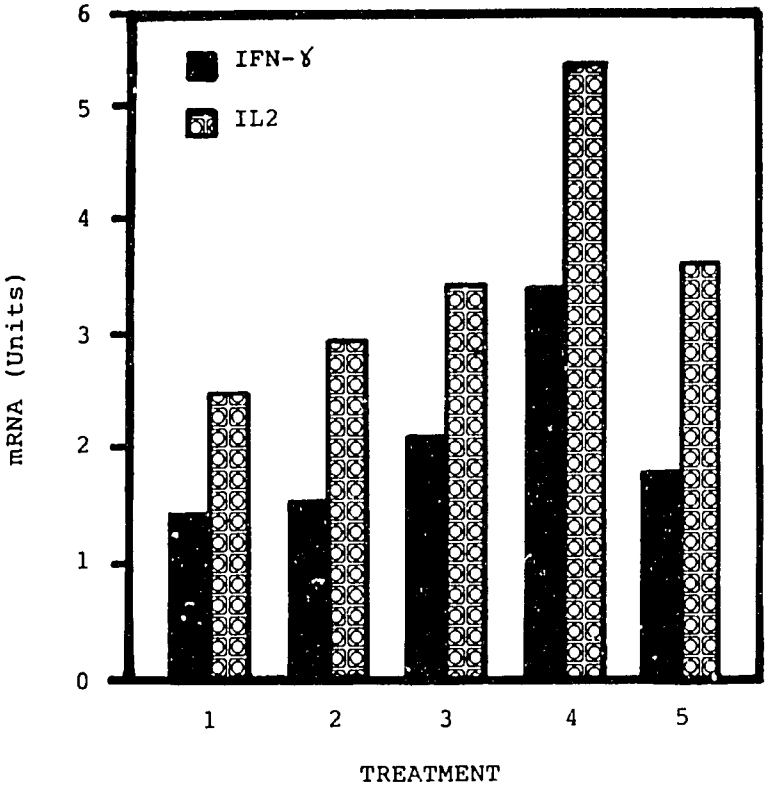

Figure 16. Average level of IL2 mRNA and IFN-γ mRNA from 8 normal subjects' PBML cultured at various conditions (1=control; 2=PHA 18h; 3=PHA 22h; 4=CHX 18-22h; and 5= γ-irr 22h).

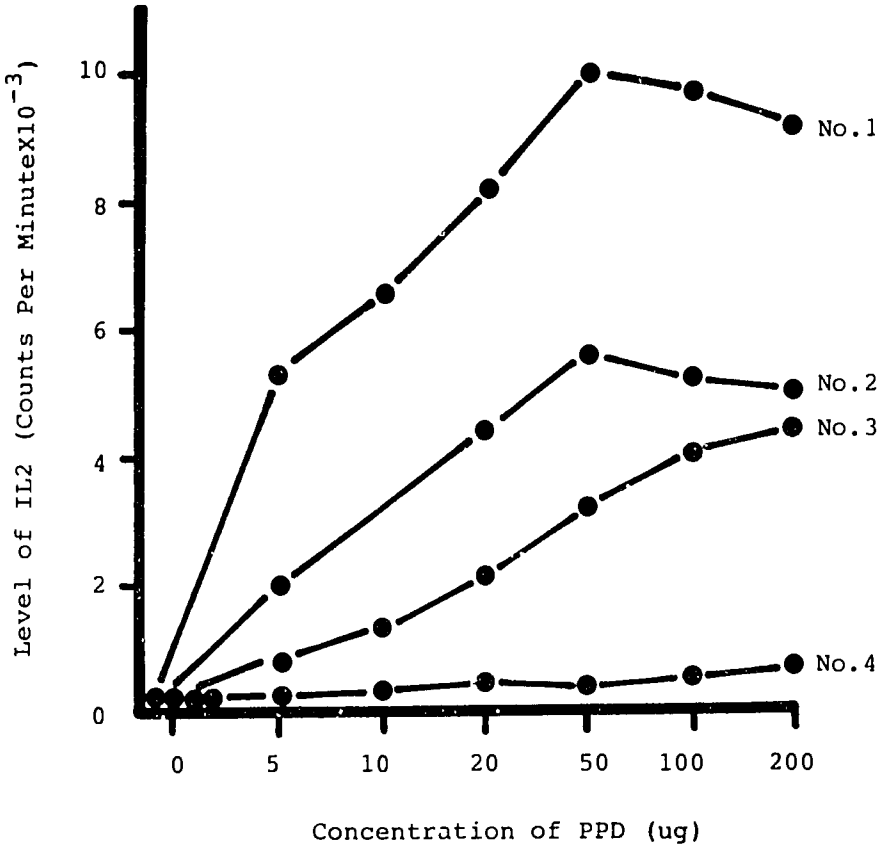

Figure 17. Determination of optimal [illegible] ration of PPD for stimulation of IL[illegible] [illegible]tion from PBML (Subject number 1, [illegible] skin test positive for PPD while 4 [illegible]

US-ISRAEL CDR PROJECT C8-196

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

INTERIM SCIENTIFIC REPORT

June 30, 1989

Prepared by

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Department of Molecular Virology

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91010 Jerusalem, Israel

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Objectives

The aims of this project are:

1. To train, at The Hebrew University of Jerusalem, personnel from the team of Dr. Sanit Makonkawkeyoon at the University of Chiang Mai, Thailand, in techniques of molecular biology to permit performance of those steps in 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 that must be done in Chiang Mai.

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.

It is clear from the description of these goals that training must precede analysis of leprous patient material.

Abstract

1. Training of a Thai scientist in Jerusalem has been completed successfully.

2. Analysis of patient material is about to start in Chiang Mai at this point in time. The Jerusalem end of this process is firmly in place and ready to receive material.

3. In Jerusalem, a study of Down syndrome, a model of immunodeficiency in man, has been completed, using a combination of newly developed molecular and analytical methods. The results show that in this case, deep insight into the nature of the lesion can indeed be obtained. This study may serve as a valuable basis for, and *allow rapid progress in, the work on leprosy that is now about to begin.*

Introduction

Interleukin-2 (IL-2), an inducible cytokine produced upon antigenic or mitogenic stimulation by helper T lymphocytes, is the essential growth factor for all types of T cells. IL-2 activates natural killer cells and is able to induce cytotoxic T cell activity. The strength of an immune response is determined to a large extent by the amount of IL-2 elicited by a stimulus. Interferon-Y (IFN-γ) is also an inducible cytokine possessing a wide range of immmunoregulatory properties, including antiviral activity, activation of macrophages as well as natural killer cells and potent anti-tumor activity in synergy with the tumor necrosis factors. Murine IL-2 and IFN-γ genes are expressed in the same subset of helper T cells and in man, this may also be the case.

Induction of human IL-2 and IFN-γ gene expression by mitogen is dependent upon *de novo* transcription and results in the appearance of a wave of mRNA. The amplitude of the IL-2 mRNA wave can be superinduced up to 50-fold in the presence of

inhibitors of translation, yet the shape of the wave remains unchanged. The amplitude of the IFN-γ mRNA wave is likewise superinducible. Commensurate superinduction of IL-2 and IFN-γ protein is observed upon the removal of the translation inhibitor, cycloheximide (CHX). These observations support the concept that expression of IL-2 and IFN-γ genes is down-regulated strongly through a mechanism involving a labile protein component. This mechanism acts post-transcriptionally.

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 largely 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. Indeed, expression of IL-2 and IFN-γ genes can be superinduced by low doses of Y-irradiation, a treatment thought to prevent the activation of suppressor T cells. The effect of Y-irradiation is also to increase the amplitudes of the induced waves of IL-2 and IFN-γ mRNA.

Down syndrome serves as a model of immunodeficiency in man and as such may be relevant to understanding the immune failure observed in leprous patients. Despite intensive study, the mechanism of this cell-mediated immunodeficiency has remained uncertain. Individuals with Down syndrome (trisomy 21) are known to suffer an increased incidence of severe infections. They display thymic abnormalities. Functional studies of PBMC from such subjects yielded variable results in terms of either a normal or reduced *in vitro* proliferative response to mitogens or antigens. Total T cell number, furthermore, was reported in different studies to be either normal or depressed; a decrease in helper T cell number and CD4/CD8 T cell subset ratio has also been observed. To obtain more precise insight into the molecular basis underlying defective immune cell function in Down syndrome, we have examined, in a project unlinked yet relevant to the present study on leprosy, the dynamics of IL-2 and IFN-γ gene expression and its control.

Using a convenient, sensitive and quantitative assay for specific mRNA developed in this laboratory, suitable for quantitating mRNA levels expressed in cells from as little as 1 ml of peripheral blood, we have analyzed the regulated expression of IL-2 and IFN-γ mRNA in PBMC derived from 29 noninstitutionalized Down syndrome individuals and compared it to that of 32 normal donors. Culture conditions were chosen that measure the transient, PHA-induced expression of IL-2 and IFN-γ mRNA, as well as the intactness of post-transcriptional and suppressor T cell-dependent mechanisms that control this expression. The latter was achieved by analyzing, respectively, the superinduction of IL-2 and IFN-γ mRNA occurring upon culture with cycloheximide or after low-dose Y-irradiation. A convenient, sensitive and quantitative assay for specific mRNA was devised, suitable for measuring mRNA levels expressed in cells from 1 ml of peripheral blood. Analysis of individuals with Down syndrome revealed a striking decrease in inducibility of the IL-2 gene. By contrast, induction of IFN-γ mRNA was as vigorous as that observed for normal donors. In cells from trisomic subjects, superinduction of IFN-γ mRNA by cycloheximide was at least as pronounced as for normal donors, while in the case of IL-2 mRNA, it was weaker. These abnormal patterns of IL-2 gene expression were seen irrespective of age. Our findings indicate a selective impairment of IL-2 gene expression in Down syndrome, rather than a general deficiency in helper T cell function (1).

Methods

Plasmids and hybridization probes. Human IL-2 cDNA (p3-16 provided by T. Taniguchi) and IFN-γ cDNA (pBR327 10 provided by R. Devos and W. Fiers) inserts were excised and placed under the phage T7 promoter in pGEM-3 (Promega Biotec). These constructs were used to generate anti-sense RNA transcripts labeled with [α-^{32}P]ATP (Amersham; specific activity, 400 Ci/mmole). Sense RNA transcripts, generated from the SP6 promoter with [α-^{32}P]UTP, yielded no detectable signal upon hybridization. Upon agarose gel electrophoresis of total RNA isolated from peripheral blood cells after induction with mitogen and blotting in 0.1 M NaOH, a

condition that allows transfer of high-molecular weight RNA species, these IL-2 and IFN-γ antisense RNA transcripts each hybridize to a single band migrating, as expected for mRNA, at 1,000 and 1,300 nt, respectively, that comprises at least 85% of the total hybridization response; the remaining response is associated with bands that migrate more slowly than rRNA and are, presumably, precursor RNA forms for IL-2 and IFN-γ mRNA.

Cell culture and induction of gene expression. A volume of 10 ml of peripheral blood is taken from each donor, except for one donor aged 2 wks (7 ml). After a 2-fold dilution with PBS, 7-ml aliquots of blood are layered onto 5 ml of Ficoll Paque (Pharmacia) and centrifuged for 30 min at 190 x g. PBMC are collected, washed once with 50 ml of RPMI 1640 medium, and once more with 25 ml of this medium. The cells are resuspended at a density of 0.5 - 4 x 10^6 cells/ml in 5 ml of culture medium (RPMI 1640 containing 2% FCS, 2 mM glutamine, 10 mM MEM nonspecific amino acids, 100 mM Na-pyruvate, 10 mM Hepes pH 7.2, 5 x 10^{-5} M 2-mercaptoethanol, 100 u/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml nystatin). Specific conditions of induction are detailed in the next section. PHA-P (Difco) is added to 0.4% (v/v). CHX is added to 20 µg/ml. γ-irradiation to a dose of 1,500 rad is performed before the addition of PHA, using a ^{60}Co source. For each individual, five 1-ml cultures, each in a 10-ml round-bottom tissue culture tube, are incubated (for 18 or 22 hr as specified) at 37°C in a water-saturated atmosphere of 5% CO_2 in air.

Strategy for Analysis of Regulated IL-2 and IFN-γ Gene Expression. To allow convenient analysis of regulated expression of IL-2 and IFN-γ genes in PBMC derived from individual donors, we defined, first, a set of conditions that measure not only the induced expression of these genes, but also the intactness of mechanisms that control their expression. Second, we devised a method for detecting gene expression, to allow sensitive and quantitative measurement of specific RNA encoded by these genes, expressed in microcultures containing PBMC derived from 1 ml of peripheral blood. 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.

Five different conditions of induction were chosen, based on our earlier studies on the regulation of IL-2 and IFN-γ gene expression in mononuclear cells from tonsils or peripheral blood. These are: (A) culture of cells for 18 hr in the absence of inducer; (B) culture of cells for 18 hr in the presence of the mitogen, PHA; (C) culture of cells for 22 hr in the presence of PHA; (D) culture of cells with PHA for 22 hr, but in the presence of CHX between 18 and 22 hr; (E) γ-irradiation of the cells, followed by induction with PHA for 22 hr. A wave of IL-2 and IFN-γ mRNA is induced by exposure to PHA reaching a maximum for both genes in the 18-22 hr time range. For both genes, the amplitude of the wave of mRNA can be superinduced in the presence of inhibitors of translation, for example, CHX. Expression of IL-2[2] and IFN-γ genes, both at the level of mRNA and of active protein, can also be superinduced by exposing the cells to low doses of γ-irradiation (1500 rad) before mitogenic stimulation. The effect of γ-irradiation is to prevent the activation of suppressor T cells that are induced concomitantly with IL-2 and IFN-γ genes, and act to down-regulate their expression severely. Such doses of γ-irradiation do not inhibit IL-2 and IFN-γ gene expression. These five conditions of induction are designed to determine if the 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.

Quantitation of specific mRNA in microcultures. Each cell culture is transferred to a sterile 1.5-ml polypropylene microtube and centrifuged for 5 min at 2,000 x *g*. The supernatant is removed and the cell pellet is agitated briefly on a vortex mixer before the addition of 0.4 ml of 7.5 M guanidinium-HCl. The tube is agitated on a vortex mixer for 1 min. To ensure efficient cell lysis, it is important that NaOAc be added only after lysis has been completed. The cell lysate is made 0.1 M in NaOAc, pH 5.1, and 0.25 ml of absolute ethanol is added. RNA is precipitated overnight at -20°C and is recovered by centrifugation at 4°C for 30 min at 16,000 x *g*. After thorough draining of the supernatant on tissue paper, the pellet is dissolved into 100 µl of 37% formaldehyde by agitation on a vortex mixer.

The RNA solution is incubated for 15 min at 60°C and 110 µl of 20 x SSC is added. One aliquot of 100 µl is transferred to a microtiter plate where 8 serial 2-fold dilutions are made in 10 x SSC. A volume of 80 µl of each dilution is transferred to a nitrocellulose sheet (pore size, 0.45 µm), using a 96-well dot blot apparatus. Each well is washed with 100 µl of 10 x SSC. The procedure is repeated for each of 5 cell cultures from a given donor. A duplicate sheet of nitrocellulose is prepared, using a second aliquot of 100 µl of each RNA solution. After baking for 2 hr at 80°C in a vacuum oven, the sheets are hybridized separately with ^{32}P-labeled RNA probes for IL-2 and IFN-γ, respectively.

To generate an RNA standard for hybridization analysis, a 300-ml culture of human tonsil cells at a density of 4 x 10^6 cells/ml is induced with PHA for 24 hr and total RNA is extracted by the guanidinium thiocyanate procedure. Starting with an amount of 0.25 µg of RNA/dot, serial dilutions are applied repeatedly to nitrocellulose sheets using the dot blot procedure described above. Strips of nitrocellulose containing single dilution series are excised from the sheets and included with each hybridization.

Sheets and standard strips are prehybridized for 4 hr at 60°C in 37.5% (v/v) formamide, 10% dextrose sulfate, 1% sodium dodecyl sulfate, 50 mM Tris-HCl pH 7.8, 0.1% pyrophosphate, 0.1 mg/ml salmon sperm DNA, 0.1 mg/ml *Escherichia coli* MRE600 tRNA and 0.6 M NaCl. The hybridization mixture is brought to a final concentration of 2.5 x Denhardt solution, 0.1 mg/ml tRNA and 5 x 10^6 cpm/ml of [^{32}P]RNA probe. After hybridization for 16-18 hr at 60°C, sheets are washed twice for 15 min at room temperature with 2 x SSC, twice for 30 min at 68°C with 2 x SSC containing 1% sodium dodecyl sulfate, and once for 30 min at 68°C with 0.2 x SSC containing 0.2% sodium dodecyl sulfate. After exposure to X-ray film, autoradiograms are scanned at 630 nm in a microelisa autoreader.

Data processing. For each individual dilution series, A_{630} values of hybridization intensity between 0 and 2 are corrected for film background and used

for linear regression analysis. The slope obtained is a measure of mRNA concentration for the gene being probed. To convert this value into standard units of mRNA/cell, it is divided by the slope obtained with standard RNA and by the cell density during culture. We have written a specific program to allow computerized data analysis and presentation of results in a series of graphic displays as chosen by the operator.

Statistical analysis. The significance of the difference of means is determined using the paired, two-tailed *t* test.

Training and Exchange of Scientists

With the start of this project in the fall of 1988, the Israeli PI turned to his Thai co-PI in order to initiate the training that forms an indispensible first step in this research [see *Appendix I* (letter)]. Unfortunately, some delay was caused in Chiang Mai. In December, 1988, Dr. Kriangsak Praputpittaya joined the project in Chiang Mai, and we organized a training session of 5 weeks in our laboratory that took place, again after some delay, during the period June 1 - July 4, 1989. A detailed report on this training is attached as *Appendix II*. There is no doubt that Dr. Praputpittaya has mastered all those steps in the technique for analysis of gene expression that must precede the point where further work can be done in Jerusalem (see *Appendix II* for details). It is hoped that a minimum of time will pass before needed chemicals are available on the Thai side, and the flow of samples to Jerusalem can begin. The essential dot blot apparatus was purchased in Jerusalem and taken along to Chiang Mai. Given Dr. Praputpittaya's very high experimental proficiency and the fact that he obtained excellent results in our laboratory, we are optimistic that the pace of progress will increase steeply in the immediate future.

The Israeli PI did not travel to Chiang Mai as yet, because it made no sense to do so before the training in Jerusalem had taken place. Since it will take a few

months before experimental work in molecular biology in Chiang Mai has reached momentum, the PI plans to visit Chiang Mai in December, 1989, now seen as an optimal time for fruitful consultations, data interpretation and formulation of research emphases. Communication between our laboratory and that in Chiang Mai has been excellent throughout.

Discussion

Results with Down syndrome, obtained in our laboratory using the same experimental approach as in the present project, serve as a demonstration that the type of analysis anticipated for leprosy can not only be completed successfully, but may indeed serve as a powerful and original tool to obtain insight into the mechanism of the defective immune response in that disease. It is thus relevant to analyze here our results with Down syndrome. Our major finding (1) is that regulated expression of the IL-2 gene is severely impaired in Down syndrome, yet expression of the IFN-γ gene is essentially normal. Aberrant expression of the IL-2 gene is independent of age. The approach used in these experiments was to analyze the intactness of the mechanisms regulating expression of both genes in the same cell population. RNA obtained from individual cell cultures was hybridized in parallel to IL-2 and IFN-γ RNA probes. Since the impairment in expression of the IL-2 gene observed in Down syndrome is accompanied by near normal expression of the IFN-γ gene, the latter gene provides an internal standard. The mitogen-induced expression of IL-2 and IFN-γ genes is thought to occur largely in the same subset of $CD4^+$ helper T cells. Therefore, our results point to a specific defect in the function of the IL-2 gene, rather than a general deficiency in helper T cell function, as a molecular basis for the decreased immune response in Down syndrome.

The human IL-2 and IFN-γ genes are located on chromosomes 4 and 12, respectively. Expression of each of these genes is controlled at the post-transcriptional level by a mechanism involving a labile protein component whose neutralization, in the presence of inhibitors of translation such as CHX, leads to

extensive superinduction of the transient wave of mRNA that is expressed by these genes upon mitogenic stimulation. In the presence of CHX, a significant superinduction of both IL-2 and IFN-γ mRNA is observed within 4 hr in cultures of cells from normal donors. In subjects with Down syndrome, both genes also respond with a significant superinduction to CHX, although the response of the IL-2 gene is weaker than for normal donors. This weaker response to CHX is in line with the decline in inducibility of the IL-2 gene. The essential observation is, however, that the defect in IL-2 gene expression is not restored to normal values when CHX is present. Excessive repression of the IL-2 gene by this post-transcriptional mechanism thus cannot account for the aberrant expression that is observed.

Expression of IL-2 and IFN-γ genes is also controlled by the action of suppressor T cells. Concomitant with the induction of IL-2 and IFN-γ gene expression, mitogenic stimulation induces a transient activation of T cells able to effectively suppress the expression of these genes. This regulation is manifested, among other evidence, by a superinduction of IL-2 mRNA by up to 10-fold in human tonsil cells exposed to low doses of γ-irradiation. A decrease in the CD4/CD8 cell subset ratio was reported for trisomic subjects (as for leprous subjects) in some but not all previous studies. The CD8 subset comprises cytotoxic as well as suppressor T cells. Although counting of T cell subsets does not report on their functional activity, it is possible that in Down syndrome, excessive suppressor T cell activity may cause the observed deficiency in IL-2 gene expression.

In PBMC derived from the group of normal donors, expression of IFN-γ mRNA is superinduced to a significant extent by exposure of the cells to Y-irradiation prior to induction with PHA. By contrast, the IL-2 gene in these donors is not responsive to superinduction by Y-irradiation. The most likely interpretation of this finding is that the IFN-γ gene is considerably more sensitive than the IL-2 gene to down-regulation by suppressor T cells.

If enhanced suppressor T cell activity were to occur in Down syndrome, one

would have expected, therefore, to observe a selective inhibition of IFN-γ gene expression. This is not the case. Instead, our results show that in Down syndrome, expression of the IFN-γ gene is essentially normal, although the extent of superinduction of IFN-γ mRNA after γ-irradiation is slightly reduced relative to that seen for normal donors. By contrast, it is the IL-2 gene whose expression is selectively impaired and cannot be restored, even in part, by γ-irradiation. Since, as we have just seen, this gene is actually less sensitive to the action of suppressor T cells than the IFN-γ gene, the failure of IL-2 gene expression in Down syndrome is inconsistent with an explanation based on the concept of enhanced suppressor T cell activity.

In cells derived from normal donors, the index of induction of the IFN-γ gene consistently exceeds that of the IL-2 gene, by about twofold. This observation demonstrates that the IFN-γ gene responds more vigorously than the IL-2 gene to a mitogenic stimulus. The induction of IL-2 and IFN-γ mRNA by mitogen is dependent upon *de novo* transcription. Hence, our finding that in Down syndrome, induction of the IFN-γ gene is as vigorous as that observed for normal donors, while induction of the IL-2 gene is severely reduced, is consistent with an explanation based on reduced transcriptional activity of the IL-2 gene. Nuclear runon analysis requires the availability of a 50-fold greater number of cells than was used in these experiments, a limitation that prevented the quantitation of the primary transcription rate of IL-2 and IFN-γ genes for statistically significant groups of trisomic and normal subjects. Yet, our findings indicate that the selective impairment of IL-2 gene expression in Down syndrome is not caused by the CHX-sensitive, post-transcriptional mechanism, nor by suppressor T cell action, but most likely occurs at transcription.

Since the IL-2 and IFN-γ genes are expressed transiently upon induction, aberrations in gene expression are most readily detected early in the induction process. Several previous studies did not reveal a defective response to mitogens in PBMC populations in Down syndrome, as judged by proliferation or

production of IL-2, although decreased responsiveness to specific antigens was observed. These biological responses are, however, the cumulative result of a sequence of events. Determination of mRNA, on the other hand, gives dynamic information about the responsiveness of the genes at a time of maximal expression and is more direct and specific. Levels of mRNA can be measured over a great range and can illuminate function of regulatory mechanisms. We have subjected separate aliquots of cells to a plurality of induction conditions, capable of providing measurement not only of the induced expression of these cytokine genes but also of the functional intactness of regulatory mechanisms that control their expression. By comparing the relative performance of the IL-2 and IFN-γ genes, as we have done, any departure from normal behavior is detected with greater sensitivity.

The procedure we devised for quantitating mRNA is especially suitable for studies of regulated expression of genes encoding bioregulatory proteins in PBMC obtained from small volumes of peripheral blood, making it a method of choice for studies of patient material. The mRNA species encoded by such genes are normally expressed to low levels of abundance, and in a minority of the cells in the population. Quantitation of gene expression thus requires relatively large numbers of cells and a greater sensitivity of detection than is the case for more abundantly expressed genes or for cell lines.

Procedures known to be effective for abundantly expressed genes in small numbers of cells in other systems, we found, are inappropriate for study of IL-2 and IFN-γ gene expression in PBMC. Thus, direct blotting of guanidinium thiocyanate cell lysates is limited to no more than 10^5 cells, too few for the detection of cytokine gene expression, as viscosity of lysates from more cells prevents its filtration through nitrocellulose. Lysis in guanidinium thiocyanate and precipitation of RNA with LiCl yields pure RNA, but involves too many steps to be convenient. Detergent lysis yields partly degraded RNA contaminated with protein that acts to mask RNA sequences from IL-2 and IFN-γ hybridization probes.

Our procedure offers the advantages of being simple, sensitive and quantitative. One-step lysis and processing in a single tube prevent loss of material. The method is sensitive: a hybridization signal of IL-2 or IFN-γ mRNA can be detected within 4-48 hr of film exposure in as little as 5×10^5 cells. A 10-ml sample of blood usually yields 10^7 PBMC, permitting multiple analyses of gene expression. The response is linear with increasing RNA input as well as cell number over a wide range of intensities, allowing reliable analysis of gene expression in small and variable numbers of cells from peripheral blood of individual subjects. Film blackening is quantitated in an Elisa reader, generating numerical data. By including a reference RNA standard for hybridization, specific mRNA content for each cell culture can be expressed in terms of standard units per cell, to permit direct comparison of results obtained with individual subjects in different experiments. Finally, the method is exceptionally suitable for this collaboration with Chiang Mai, because the experimental phases that must be carried out there, including cell preparation, culturing, harvesting and extraction of RNA, and dot blotting of RNA samples on nitrocellulose sheets to be shipped to Israel for further analysis with radioactive hybridization probes, requires only light equipment and simple chemicals, but not enzymes or radioactive agents.

References

For brevity's sake, references cited in the Research Proposal are not repeated in this Report.

1. L. Gerez, L. Madar, G. Arad, T. Sharav, A. Reshef, M. Ketzinel, D. Sayar, C. Silberberg and R. Kaempfer. Aberrant Regulation of Interleukin-2 But Not of Interferon-γ Gene Expression in Down Syndrome (Trisomy 21). *J. Clin. Invest.*, submitted for publication, July, 1989.

APPENDIX I

THE HEBREW UNIVERSITY OF JERUSALEM

Faculty of Medicine

RAYMOND KAEMPFER, Ph.D.

Dr. Philip M. Marcus Professor of Moleculnr Biology and Cancer Research

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November 23, 1988

Dr. Sanit Makonkawkeyoon
Chairman, Department of Clinical Immunology
Faculty of Associated Medical Sciences
Chiang Mai University
Chiang Mai 50002, THAILAND

Dear Sanit,

Now is the time to activate our collaboration, project number 936-5544-G-00-8048-00, and I propose as follows:

Stage 1: You yourself or one of your seniors visits our laboratory for a period of about four - five weeks in order to learn preparation of peripheral blood cells, their induction in culture under various conditions, and extraction of RNA. The aim is to reach the point where these procedures can be applied reproducibly back in your laboratory. Thorough discussion of the patient-groups available in Chiang Mai and desirable objects for study. Appreciation of the procedures outlined in Stage 2. Given the need to think together, it would, of course, be best if you came yourself. We need ample advance notice to allow us to find a room in town with an easy reach of the laboratory.

Stage 2: Preparation of RNA samples in your laboratory to the point where ethanol precipitates in Eppendorf tubes can be air-mailed to us for dot-blotting, hybridization with riboprobes, exposure, optical scanning, data-processing, and graphic display. The person here during Stage 1 will be able to observe all these steps, and in the future, dot-blotting may be conducted in your laboratory as well.

Stage 3: Analysis of the first stage of results. Our data will show, if expression of IL-2 and IFN-Y genes is normal or abnormal in terms of: basal level, inducibility, superinducibility by CHX, superinducibility by Y-irradiation. See our proposal for the meaning of these measurements.

Stage 4: Integration of the laboratory and clinical data on patients chosen by you for analysis and our findings. Here,

integration with results with IL-1 and IL-2 in your laboratory on cells from leprosy patients, as well as other indicators of immune function or disease available, is the objective.

Stage 5: Sharper definition of the patients to be chosen and assays to be done, on the basis of previous data.

Note: We find that our procedures allow handling of cells from four to five patients simultaneously. For starters, we should aim at pilot-sized patient numbers. Strategy should be discussed during Stage 1.

I enclose a letter received today from your dean. Please inform him of my correct name and address (see my letterhead).

Cordially,

Raymond Campbell

Enc.

APPENDIX II

Report on training of Dr. Kriangsak Praputpittaya in Israel

Trainee: Dr. Kriangsak Praputpittaya, Ph.D. K. Praputpittaya

Address: Department of Clinical Immunology
Faculty of Associated Medical Sciences
Chiang Mai University
Chiang Mai 50002

Israeli supervisor: Professor Raymond Kaempfer, Ph.D. R. Kaempfer

Place of training: Department of Molecular Virology
The Hebrew University of Jerusalem
91010, Jerusalem, Israel

Duration of training: June 1, 1989 - July 4, 1989.

Purposes of training:

- to learn basic and advanced techniques to start research on gene expression.
- to learn and practice techniques involved in *in vitro* culture conditions for measuring both function of the IL-2 and IFN-γ genes in peripheral blood mononuclear cells (PBMC), and also the activation of suppressor T cell circuits that may regulate the expression of the two genes in leprosy.
- to discuss and to plan steps of research to be carried out by both Thai and Israeli groups concerning type, number and other criteria on sample collection of leprosy and normal subjects.

TRAINING PERFORMED:

Week 1 - (June 4-8, 1989)

-I have learned some basic techniques related to the study on gene expression of IL-2 and IFN-γ in lymphocytes. The techniques, demonstrated by Ms. Lisya Gerez, Dr. Gila Arad and others of the Kaempfer team, were as follows:

-*In vitro* cell culture of PBMC
-Harvest of cell culture
-Cell lysis with guanidine HCL
-RNA extraction and solution
-Dot blotting and dilution
-*In vitro* transcription of RNA hybridization probe
-Prehybridization and hybridization
-Autoradiography
-Scanning of films
-Data processing and graphic analysis on the computer

-I have also checked a list of equipment, chemicals and supplies, both available and not yet available on the Thai side. Information has been sent out to Dr. Sanit in Chiang Mai, to expedite acquisition of key items of equipment. A dot blot apparatus will accompany me back to Thailand.

Weeks 2-4 - (June 11-30, 1989)

I have performed the following techniques:

-cell culture, harvesting of the cells for mRNA extraction and dot blotting, of 12 blood samples from normal blood donors.
-measuring film density of dot blots upon autoradiography and entering of data into a computer for data analysis.
-Performance of data analysis.

I have also discussed with Dr. Kaempfer and his colleagues research to be performed on leprosy.

RESULTS:

Human peripheral blood mononuclear cells (PBMC) of 12 normal blood donors were tested for their IL-2 and IFN-γ gene expression in response to PHA-P, in untreated cells and in cells treated with cycloheximide or γ-irradiation. Results are expressed as absorbance of dot blots at
630 nm.

Figures 1 and 2 show IL-2 mRNA levels of PBMC cultured at various conditions as indicated in attached procedures. It was found that in seven out of twelve (#1,2,3,4,5,6 and 7; Figures 1 and 2) samples the IL-2 mRNA could be induced by PHA since their IL-2 mRNA levels of PHA stimulated PBMC were significantly higher than those of control cells without any treatment. The effect of PHA was comparable whether the PBMC were stimulated with PHA for 18 or 22 hours. Superinduction which is normally induced by treatment with cycloheximide or γ-irradiation was also observed. Cycloheximide mediated a superinduction of IL-2 in

7 subjects (#2,5,6,8,9,10 and 12; Figure 1). However, γ-irradiation was not effective in any subject tested, confirming results of the Israeli team.

Figure 3 shows levels of IFN-γ mRNA for 12 normal subjects, the same group described above. The IFN-γ gene could be induced in all subjects, except #8 and 11, with at least a 2-5 fold increase of IFN-γ mRNA after treatment with PHA for either 18 or 22 hours. IFN-γ gene superinduction by cycloheximide was found to be to moderate in 3 subjects (#5,8 and 10; Figure 3), but strong in 3 subjects (#6, 9 and 12; Figure 3). Other subjects showed no significant superinduction response to cycloheximide.. In contrast to the IL-2 gene, the IFN-γ gene was superinducible by γ-irradiation in 3 subjects (#3, 4 and 6; Figure 3). It is interesting that IFN-γ gene of some subjects (#8 and 12; Figure 3) could be superinduced only by cycloheximide, and other subjects only by γ-irradiation (#3 and 4; Figure 3). This discrepancy could be explained by different modes of action for cycloheximide and for γ-irradiation. These mechanisms are under study by Dr. Kaempfer and his group.

Figure 4 and 5 depict examples of autoradiography of IL-2 (Figure 4) and IFN-γ (Figure 5) mRNA dot blots which were subsequently measured for absorbance at 630 nm with an ELISA reader.

Figure 6 shows a summary of IL-2 and IFN-γ mRNA of PBMC of 8 normal subjects. The levels of mRNA are averaged and expressed as units after being compared to standard IL-2 and IFN-τ RNA. It is clearly demonstrated that IL-2 and IFN-γ genes are induced by PHA, and superinduced by the treatment of cycloheximide. γ-irradiation could induce superinduction only of IFN-γ gene expression, but not of IL-2 gene expression.

PLAN OF WORK IN THAILAND CONCERNING IL-2 AND IFN-γ GENE EXFRESSION IN LEPROSY:

- To order essential equipment and supplies required for the gene expression study, such as a vacuum oven, dot blot apparatus and a microcentrifuge.
- When all essential equipment, chemicals and supplies are available, blood samples of normal Thai subjects will be collected and processed to the point that harvested mRNA is dot-blotted onto nitrocellulose membranes. These nitrocellulose membranes will then be packed and mailed to Israel for hybridization analysis with radioactive RNA probes for IL-2 and IFN-γ . This action will enable us to evaluate cell culture conditions and the mRNA harvesting system in Thailand. Analysis in Israel will guide the cell culture work in Thailand. Graphic representations of gene expression patterns generated in Jerusalem will be sent to Chiang Mai.

- Subsequently, blood samples of leprosy patients will be collected, processed, and sent to Israel. In addition to leprosy samples, normal blood samples will be jointly processed

in order to create a data base for normal donors and minimize experimental variation. The following information will be gathered for each blood sample:

Date collected	Serial number
Family name	Number of cells (x 10^6/ml)
First name	Treatment (drug)
Birth date	Duration of treatment
Identification number	Complications
Type of disease	Treatment of cells in vitro (with recombinant IL-1 etc.)

PROCEDURES:

Preparation of mRNA from cell culture:

The procedure described below is for a one-patient sample.

WARNING: USE GLOVES AT ALL TIMES FOR SAFETY AND TO PREVENT CONTAMINATION OF SAMPLE BY RIBONUCLEASE ON HANDS!

Day 1 - Culture of lymphocytes

1. Collect peripheral blood from subject in heparinized tubes. Ten to twenty ml of whole blood is sufficient. Blood samples should be processed as soon as possible on the day of collection and should be kept at room temperature to ensure optimal results.
2. Dilute blood with PBS at 1:1 (v/v) in a 50 ml centrifuge tube. Mix the sample evenly.
3. Prepare 15 ml centrifuge tubes each containing 5 ml Ficoll-Hypaque. Mark the tubes with patient identification.
4. Carefully, using a 10 ml pipette, layer 8-10 ml from the diluted blood over a bed of 5 ml Ficoll-Hypaque in each tube. Do not mix the F-H and the blood sample.
5. Centrifuge at 200 g (1000 rpm) in a cell centrifuge for 30 min at 18-20°C without using the brake.
6. Collect the white interface layer containing the lymphocytes with a pasteur pipette and transfer to a clean 50 ml centrifuge tube. It is essential to remove all the material in a minimal volume.
7. Wash cells with at least 3 volumes of RPMI 1640 warmed to room temperature. Centrifuge at 200 g for 15 min at 18-20°C. Do second wash with 25 ml RPMI 1640.
8. Discard supernatant. Resuspend the cells in 5 ml of complete medium warmed to 37°C.
9. Count cells.
10. Adjust cell density to be at 4 x 10^6 cells/ml. Although this cell density is optimal, the assay will give satisfactory results with as few as 0.5 x 10^6 cells/ml. Thus, if lower cell density is obtained, keep total volume at 5 ml.
11. Dispense 1 ml of suspension into each of five culture 10-ml tubes and treat them accordingly as follows:

Induction of mRNA:

Tube 1: Control - not induced - harvest at 18 h.
Tube 2: Add 4 μl of PHA-P at 0 time and harvest cells at 18 h.
Tube 3: Add 4 μl of PHA-P at 0 time and harvest cells at 22 h.
Tube 4: Add 4 μl of PHA-P at 0 time, superinduction with 2 μl cycloheximide (10 mg/ml) at 18 h, and harvest cells at 22 h.
Tube 5: Gamma-irradiation the cells with 1500 rad with a Co^{60} gamma cell 220 (Atomic energy of Canada Ltd. or equivalent) at 0 time, then add 4 μl PHA-P. Harvest cells at 22 h.

10. Incubate cultures at 37°C in 5% CO_2 atmosphere for 18 h to 22 h.

Day 2 - Cell harvesting and mRNA extraction

1. Thaw 7.5 M guanidine HCl solution kept at -20°C.
Harvest cells at the time indicated in step 11 of Day 1. Take out cultures from the incubator. Mix the cells in the tubes by tapping the bottom and by pipetting the suspension in and out of a sterile pasteur pipette or 1 ml pipette tip.
3. Transfer the cell suspension into a sterile Eppendorf tube.
4. Centrifuge at 5000 rpm (Eppendorf centrifuge) at room temperature for 5 min.
5. Discard the supernatant using a sterile tip or vacuum. Vortex the pellet to disperse the cells.
6. Add to each tube 0.4 ml of 7.5 M guanidine HCl solution.
7. Dissolve pellet by vortexing the solution for at least 20 sec. or until no aggregates are found in the solution.
8.Add to each tube 20 μl of 2 M Potassium acetate pH 5.1.
9. Add to each tube 250 μl of absolute ethanol kept at -20°C.
10. Mix and leave at -20°C overnight. The preparation is stable at this point and can be stored at -20°C for longer periods if necessary.

Note: It is suggested that samples be stored at this point of the procedure until material from 2-4 subjects is available for further processing.

Day 3

1. Centrifuge the Eppendorf tubes at 12000-14000 rpm for 30 min at 4°C in an Eppendorf centrifuge.
2. Prepare a 60°C water bath.
3. Thaw 37% formaldehyde solution.
4. Discard the supernatant carefully with a sterile tip. Mix on vortex until no clumps are found.
5. Dissolve pellet in 100 μl of 37% formaldehyde, as the RNA is to be tested against 2 probes (IL-2 and IFN-γ). Take care to vortex until RNA pellet dissolves completely.
6. Add 110 μl of 20x SSC, and vortex.
7. Place the tubes on polystyrene foam in 60°C water bath for 15 min for RNA denaturation.
8. Prepare 100 ml of 10x SSC by diluting the 20x SSC solution 1:1 with sterile double distilled water.
9. Wet the 9 x 13 cm nitrocellulose paper and the GB003 blotting sheet with sterile double distilled water. The nitrocellulose sheet should be wet gradually. Change the water to 10x SSC solution. Soak for at least 10 min.
10. After RNA denaturation, dilute each sample by eight 2-fold serial dilutions with 10x SSc: [Add to each well of sterile microplate 100 μl of 10x SSC. Pipette 100 μl of the sample to first well and then serially dilute by pipetting to adjacent wells]. For each patient one will finally have 40 wells and one blot will be loaded with 80 wells (2 patients). The unused wells will serve as machine blank. In a case where 2 probes are tested, there are 210 μl in each sample.
11. Prepare the Biodot apparatus. First lay the blotting sheet, then the nitrocellulose on top. Close tightly the apparatus and connect it to the vacuum trap fully opened.
12. Transfer 80 μl from each well of the microplate to the corresponding well of the Biodot. Apply the samples to the middle of the well to ensure that no RNA adheres to well walls.
13. Once dry, wash each well with 100 μl of 10x SSC and let it dry for 1-2 min.
14. Withdraw the nitrocellulose sheet and air dry between a folded Whatman #3 sheet. Bake at 80°C in a vacuum oven for 2 hrs.
15. Repeat steps 10-14 to prepare a second RNA blot.
16. Wrap the nitrocellulose in Whatman #3 sheet and pack sheets together to be sent to Israel between cardboard supports by registered airmail. [NB: This material is not radioactive].
17. The following steps will be carried out by the Israeli group:

- Pre-hybridization of the nitrocellulose membranes
- Hybridization
- Autoradiography
- Scanning for density of dot blots
- Analysis of results in the computer
- Statistical analysis

Preparations of reagents and chemicals

1. Culture medium
   -RPMI 1640 containing:
   * 2 mM glutamine
   * 10 mM MEM non-essential amino acids
   *100 mM MEM sodium pyruvate
   *10 mM HEPES pH 7.2
   *100 u/ml penicillin
   *100 μg/ml streptomycin
   *40 μg/ml gentamycin
   *5 x 10^{-5} M 2-mercaptoethanol
   *2% fetal calf serum, heat inactivated
   *5 μg/ml nystatin
2. 7.5 M guanidine HCl (MW 95.53) Ultrapure grade (IBI)
   guanidine HCl 500g
   Sterile distilled water up to 700 ml
3. 2M Potassium acetate (KOAc) pH 5.1-5.2
   KOAc (Sigma, M&B) 9.82 g
   Sterile water
   adjust pH with acetic acid, with final volume of 50 ml.
4. Deionization of formaldehyde
   Formaldehyde (37%) (Merck) mixed with AG 501-x8(D) resin (Bio-rad) at a ratio of 1 gm per 10 ml formaldehyde solution. The mixture is stirred on a magnetic stirrer for 30 min at room temperature. The mixture is then filtered with Whatman #3 filter paper, aliquoted and kept at -20°C.
5. 20x SSC (0.015M Sodium citrate, 0.15M Sodium chloride solution)
   Na citrate 88.2 g
   NaCl 175.3 g
   Sterile Double distilled water 1 liter
6. PHA-P (Difco)
   Reconstitute PHA-P (in lyophilized form) with 5 ml sterile double distilled water per bottle. The reconstituted solution is then kept at 4°C, ready for use.
7. Cycloheximide(10 mg/ml) (Sigma)
   Cycloheximide 10 mg in 1 ml sterile distilled water, then use at 2 μl per 1 ml cell culture to obtain 20 μg/ml final concentration.
8. PBS

NaCl	8 g	KCl 0.2 g
Na_2HPO_4 $7H_2O$	1.93 g	Sterile distilled water 1 liter

9. Ficoll-Hypaque solution (Pharmacia)

Figure 1

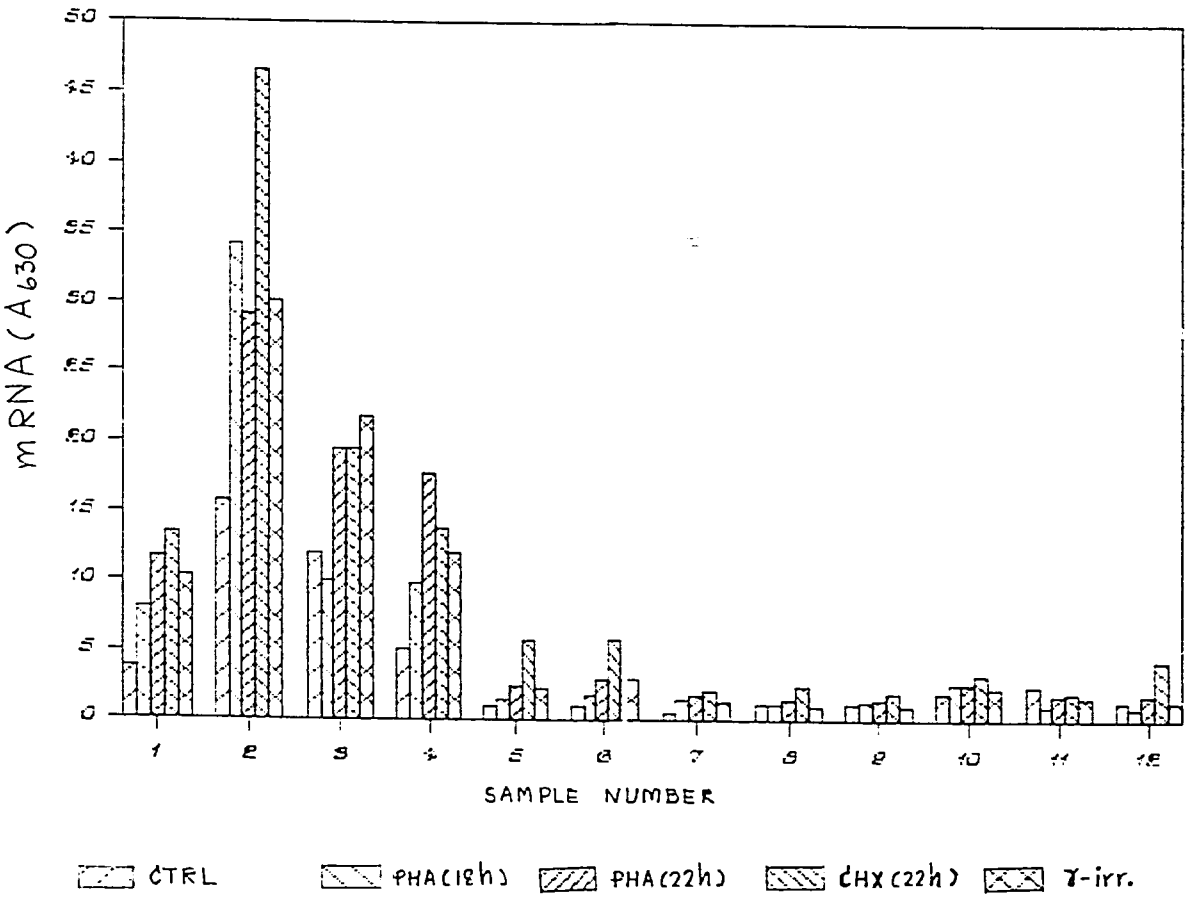


Figure 1

Figure 2

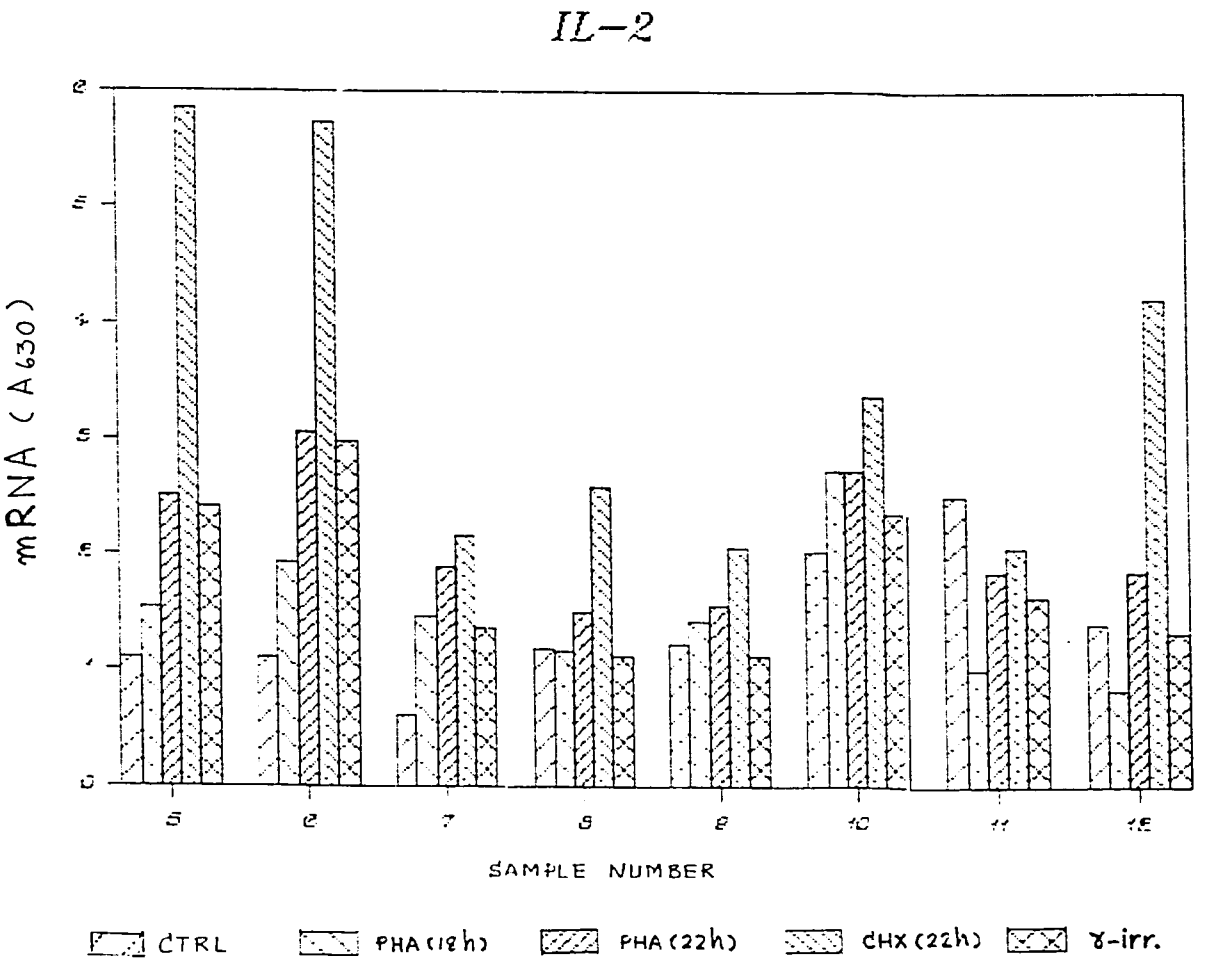


Fig. 2

Figure 3

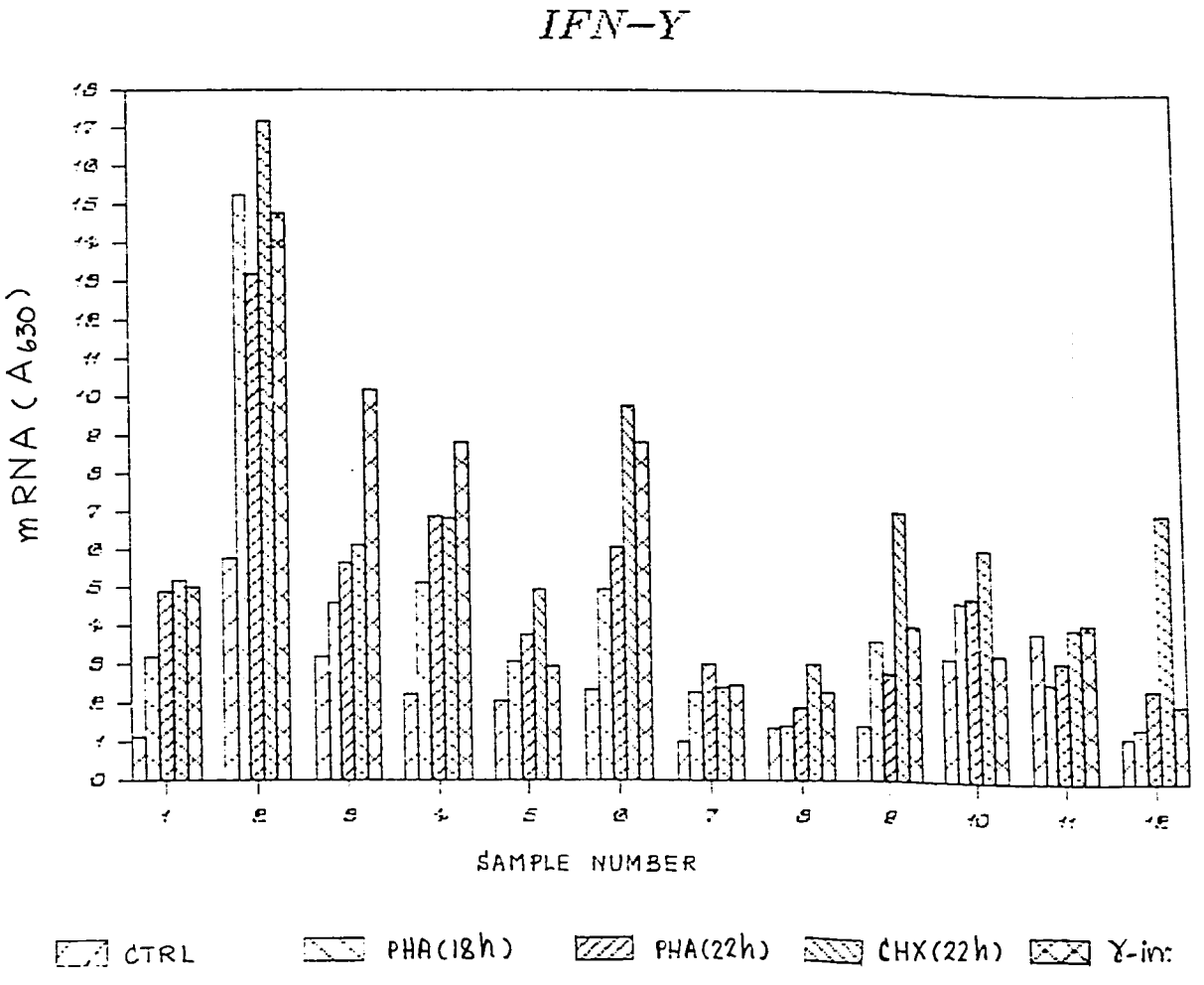


Fig. 3

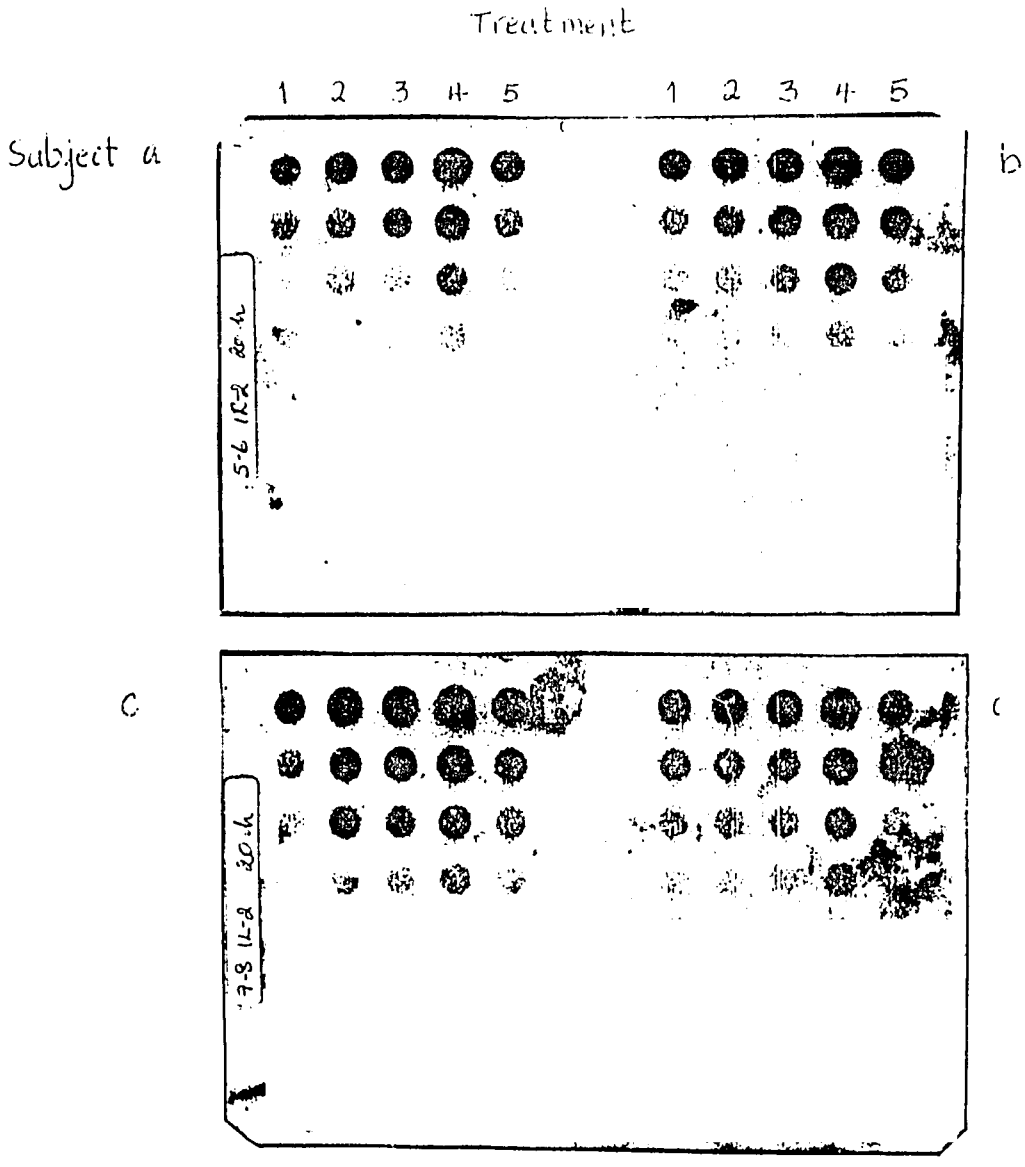


Figure 4. Autoradiograph of IL-2 mRNA dot blots of normal controls [a (#5), b (#6), c (#7) and d (#8)] with different treatment of PBMC (see Figure 6)

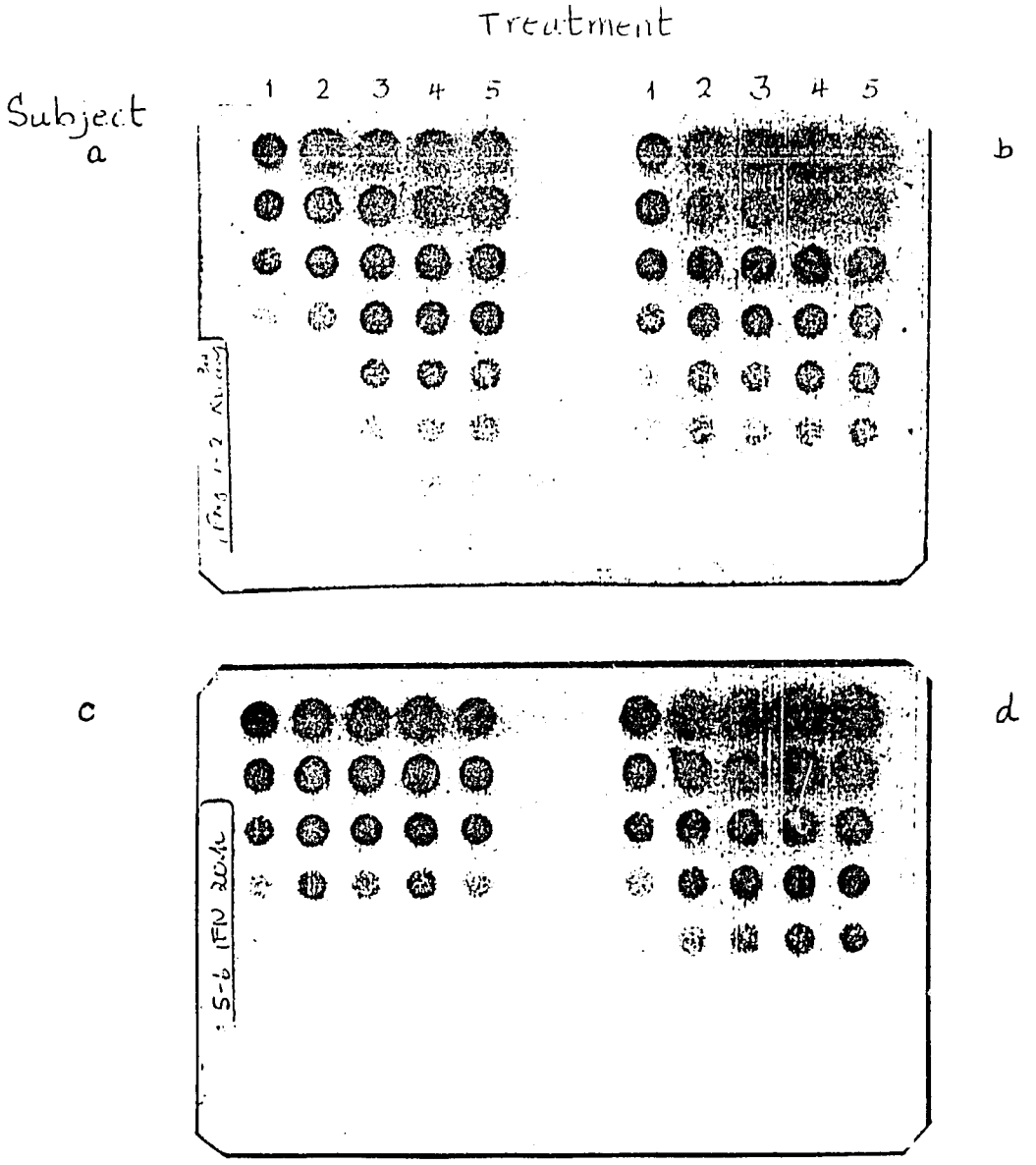
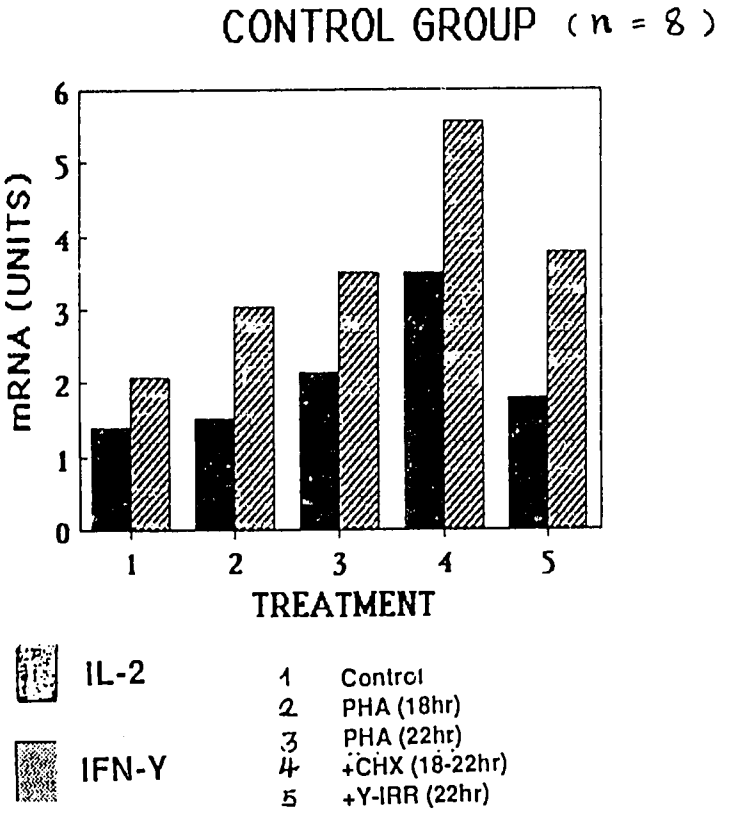


Figure 5. Autoradiograph of IFN-γ mRNA dot blots of normal controls [a (#), b (#), c (#) and d (#)] with different treatment of PBMC (see Figure 6)

Figure 6


CONTROL GROUP (n = 8)
mRNA (UNITS)
0
1
2
3
4
5
6
1
2
3
4
5
TREATMENT
IL-2
IFN-Y
1 Control
2 PHA (18hr)
3 PHA (22hr)
4 +CHX (18-22hr)
5 +Y-IRR (22hr)