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FINAL REPORT

Covering Period : June 1988-December 1992

Submitted to the Office of the Science Advisor
U.S. Agency for International Development

"Regulation of Lymphocyte Gene Expression and
In vitro Immunotherapeutic Effect of
Interleukin 1 and 2 on Monocytes and Lymphocytes in Leprosy"

Principal Investigator : Sanit Makonkawkeyoon, Ph.D.

Grantee Institution : Chiang Mai University

Collaborator : Raymond Kaempfer, Ph.D.

Institution : The Hebrew University of Jerusalem, Israel

Project Number : C8-196

Grant Number : 936-5544-G-00-8048-00

AID Grant Project Officer : L.A. Twentyman

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3. Executive Summary

The purpose of this project is to elucidate the abnormality in cell-mediated immunity (CMI) in human leprosy in which it is still ambiguous whether immunologic unresponsiveness are related to lymphokine production or the expression of genes responsible to those lymphokines. The project objectives are divided into 2 parts; a) to investigate the level and numbers of IL1- and 1L2- producing cells in leprosy patients comparing to normal subjects, and b) to investigate IL-2 and IFN- gamma gene regulation in leprosy of different types and normal subjects and the relation between suppressor T cells and expression of IL2 and IFN-gamma genes.

Problem in obtaining PPD at appropriately high concentration for our study has been solved by our own preparation. Our PPD satisfactorily stimulated PBML to produce high level of IL2, and it will be very useful to other scientists who require PPD since the activities of our PPD are equivalent to commercial PPD.

Techniques for enumeration of IL1-producing cells and levels of IL1 produced were set up in our laboratory in Thailand. From this we published a research article entitled 'Enumeration of IL1-producing monocytes from human peripheral blood mononuclear leukocytes by agar plating technique' in the Journal of Immunological Methods, 140:173-180, 1991. We then determined the numbers of IL1- producing cells in PBML of leprosy and normal subjects. There was no statistical difference in both numbers of IL1-producing cells and the level of IL1 in leprosy and normal subjects. This suggests that unrespensiveness in

leprosy patients unlikely to involve exclusively on the production of IL1 by monocytes.

Optimal conditions for IL2 production and assay for IL2 level have been carefully established. All conditions for quantitation of IL2-producing cells have also been investigated. There was no difference numbers of IL2-producing cells whether the PBML were unstimulated or with PHA as stimulant. In addition numbers of IL2-producing cell in PBML of leprosy stimulated with PPD produced IL2 at the same levels as those in normal subjects. PPD-stimulated PBML from LL patients produced IL2 significantly lower level than other typed of leprosy (BL,BB,BT and TT) and normal subjects.

We have set up conditions for Con A-stimulation of suppressor T cells in PBML separation of Con A-SRBC rosette and nonrosette T cell subpopulations by measuring plaque forming cells and IgG concentration by reverse hemolytic plaque assay and a sandwich ELISA technique, respectively. We found that Con A-SRBC rosette T cells showed helper activities. The effect of Con A-rosetting and non-rosetting T cells on the response of PBML to PPD and lepromin has been further studied. It was found that, at the same percentage of T cells added to PBML-antigen cultures, Con A-rosetting T cells had strongly effect on the response of PBML to PPD than non-rosetting T cells. The results add more information to our previous study in that the Con A-rosetting T cells not only have effect on the production of immunoglobulin of the HMI system but also the production of IL-2 of CMI system. We showed that M.leprae was able to inhibit IL2- production by PBML from normal subjects, TT,BT and BL leprosy patients. We further

elucidated the inhibitory effect of indomethacin on the suppression induced by M.leprae. There was significant enhancement of IL2 production in normal and BT/TT subjects but no such effect in BL/LL patients. Indomethacin alone had no direct effect on PBML. Our results should be useful for the study on suppression or unresponsiveness in leprosy patients especially of LL/BL types. However, more information is needed before application for its uses.

From studies on IL2 and IFN-gamma genes, our data show that the two genes remain inducible throughout the development of leprosy but significantly below normal in the TT,BB and LL. It is thus a discernible defect in the capacity of PBML from these stages of the disease to respond to a general stimulus, mitogen, as judged by accurate quantitation of IL2 and IFN- gamma mRNA at the time of maximal expression. This finding is new. Moreover, in LL, there is a striking absence of superinduction responses for the IL2 gene, accompanied by greatly reduced superinduction responses for the IFN-gamma gene. This implies post-transcription and suppressor T cell dependent downregulation are not operating normally in leprosy patients. Our molecular approach is able to reveal aberrant patterns at various degree of IL2 and IFN-gamma gene expression in different types of leprosy, most pronounced in lepromatous leprosy, but not in the normal controls from the same area.

4. Research Objectives

It is generally accepted that different clinical forms of leprosy are associated with varying host defense particularly to cellular immunity (CMI) to M. leprae (Jopling 1984). However, the immunological defect(s) leading to the reduction or absence of CMI to M. leprae in lepromatous leprosy (LL) are not yet fully understood. Early studies in LL patients suggested that there is a generalized anergy or unresponsiveness to various antigens. Others suggested that LL patients at early stages are capable of responding to other skin test antigens such as coccidioidin, histoplasmin and PPD, while unresponsive to M.leprae antigen (Turk and Bryceson 1971, Myrvang et al 1973; Mandez et al 1974).

Evidence for suppression of the immune response by suppressor T lymphocytes or monocytes in LL patients were suggested (Mehra et al, 1979,1980,1984). However, other attempts to find disease-related suppression in leprosy have yielded conflicting results (Bjune 1979, Nath and Singh 1980, Bullock et al. 1982, Stoner et al. 1982). Leprosy-specific in vitro unresponsiveness 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 LL patients failed to produce IFN-gamma upon exposure to M.leprae and defect was restored by the addition of purified human IL2 to the lymphocyte cultures (Nogueira et al. 1983). In our study, the immunoregulatory mechanisms in leprosy will be evaluated at the cellular level by measuring levels of IL1 produced from monocytes and IL2 from T lymphocytes. Numbers of IL1-producing monocytes and IL2-producing T

lymphocytes will be also studied. Molecular analysis on the genes controlling IL2 and IFN-gamma production will be investigated from the same groups of leprosy patients and normal subjects. Active collaboration between cellular immunology laboratory in Chiang Mai University, Thailand, and a molecular biology laboratory in Jerusalem, Israel, provides a bridge between cellular and molecular analysis of this ancient disease. Results of this project are expected to provided novel insight into immunoregulatory mechanisms in leprosy. Our overall aim and specific objectives are as follow.

Overall Aim : The aim of this project is to elucidate the abnormality in CMI in human leprosy by study on the quantity and functional intactness of T lymphocytes producing IL2 and monocytes producing IL1 and particularly their role in the regulation of IL2 and IFN-gamma gene expression in the disease. The specific objectives of this project are as follow :

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

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

3. To assess the <u>in vitro</u> effect of exogenous IL1 and/or IL2 on defective IL1-producing monocytes and/or defective IL2-producing T lymphocytes of various types of leprosy and normal subjects.

4. To evaluate the function of helper and suppressor

T lymphocytes in different types of leprosy and normal subjects.

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

5. Methods and Results

5.1 Preparation of PPD

M tuberculosis H37Rv was grown in Long's synthetic medium for 6 weeks. The bacteria were killed by heating in water bath at 100°C for 3 h. Culture supernatant was collected and passed twice through sterile gauze. The cell free culture supernatant was added with 4% trichloroacetic acid (TCA) and precipitate was 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 3000 rpm for 2 h. Supernatant was collected and filtered through 0.45 u millipore filter. Protein in supernatant was precipitated with saturated ammonium sulphate solution, pH 7.2. The precipitate was washed 5 times with 1% TCA and then dissolved in M/30 phosphate buffer. This purified protein derivative (PPD) was filtered through millipore filter and protein content was determined by Biuret and Lowry method. Three lots of PPD were prepared and evaluated for in vitro stimulation of IL2 production from PBMC of PPD-positive normal subjects. All of them showed good stimulation of IL2 production of all normal subjects. An example of the stimulation effect of our PPD is shown in Figure 1.

5.2 Separation and Purification of Monocytes

PBML ($2x10^6$ cells/ml) was added onto a 10x35 mm plastic plates and incubated for 2h at 37°C in 5% CO_2. Non-adherent cells were washed out three times with warm RPMI medium. The adherent cells on plastic plates were stained with non-specific esterase stain and positive for more than 90%.

5.3 Production of IL1

The adherent monocytes ($2x10^5$ cells/ml) were incubated with LPS(30 ug/ml) at 37°C, 5% CO_2 for 24 h. Supernatant was collected and assayed for IL1 level.

5.4 Quantitation of IL1 level

The method was set up based on the principle that when monocytes are stimulated with LPS, they synthesize and secrete IL1. Mixing the cells or its supernatant with mouse thymocytes, the later cells proliferate due to the stimulatory effect of IL1 resulting in clusters of thymocytes around the IL1-producing cells or proliferation of thymocytes. Thymocytes were harvested from 6-8 week old BALB/c mice and cell suspension was adjusted to $3x10^7$ cells/ml. Unknown samples were serially diluted and thymocyte suspension were mixed with PHA-P (20 ug/ml), incubated at 37°C 5% CO_2 for 72 h. Tritiated thymidine was added and incubated for 2h before the culture was harvested onto glass fiber filter and counted in a scintillation counter.

5.5 Determination of IL1-producing monocytes and IL1 level in leprosy and normal subjects

Number of IL1-producing cells and IL1 level were determined in PBML of leprosy patients and normal subjects. The mean ± SEM of the normal subjects for IL1-producing cells was 2938±540

cells/$2x10^5$ monocytes, and 42.45±9.32 units/ml for IL1 level. There was no significant difference in both the numbers and the level in LL/BL (3534±1264 and 57.95±21.28), BB (4053±3340 and 81.21±81.17), and TT/BT (3971±1065 and 58.42±18.01) (Table 1).

Specificity of the IL1-producing cell assay is shown in Table 2. Anti-IL1 at low concentration was able to partially inhibited the development of IL1-induced colonies. When the concentration of anti-IL1 was increased, the numbers of IL1-induced colonies was gradually decreased until completely inhibited. However, anti-IL6 and anti-TNF-alpha had very low effect on the numbers of IL1-induced colonies. The correlation ofthe numbers of IL1-producing cells and the levels of IL1 produced in normal subjects is shown in Figure 2 with r = 0.981.

5.6 Production of IL2

PBML ($1x10^6$) were stimulated with PHA (2ug/ml) or PPD (10 ug/ml) at 37°C 5% CO_2 for 18 and 48 h, respectively. The cultures were centrifuged and supernatant was added with alpha-methyl-D-mannoside (20 mg/ml) prior to storage at -70°C for IL2 assay.

5.7 Quantitation of IL2 activity

Unknown samples were serially diluted in tissue culture microtiter plates and added with 3-day Con A blast spleen cell suspension ($8x10^4$/100 ul) for 24 h. Tritiated thymidine (0.2 uCi/50 ul) was added to each well and incubated for 18 h. The cultures were harvested and counted for radioactivity by a liquid scintillation counter.

5.8 Preparation of 3-day Con A blast spleen cells

Spleen cell suspension ($1x10^9$/ml) was prepared from BALB/c mice of 6-8 weeks old and stimulated with Con A (5 ug/ml) for 3

days. The cells were washed and readjusted to $3x10^7$ cells/ml.

5.9 Determination of IL2-producing cells and IL2 levels produced by PBML of leprosy and normal

For the determination of IL2-producing cell numbers, soft agar gel technique was used. Each plastic petridish (35x10 mm) contains 1 ml of lower agar layer (0.5% agarose in 10% FCS/RPMI) and 0.7 ml of upper agar layer (0.4% agarose in 10% FCS/RPMI) mixed with 0.2 ml of responder cells (IL2-dependent 3-day Con A blasts) and 0.1 ml of effector cells. In controls, the upper layer contains only effector or responder cells. All cultures were incubated in 5% CO_2 37°C for 5 days. Numbers of IL2-producing cells surrounded by IL2-responder cells were enumerated under a dissecting stereomicroscope. It was found that PBML of either unstimulated, stimulated with PHA or PPD in leprosy of all types contain IL2-producing cells that are not statistically different from normal controls (Table 3). However, the IL2 level produced by PBML strimulated with PPD in LL/BL leprosy was significantly higher than other types of leprosy and normal controls ($p<0.05$) (Table 4).

5.10 Induction and separation of suppressor and helper T cells

PBML ($4X10^6$/4 ml) were stimulated with Con A (50 ug/50 ul) at 37°C 5% CO_2 for 18 h. The cells were washed and further incubated for 42 h. After washing the cells were adjusted to $1x10^6$ cells/ml in 10% FCS in RPMI. The Con A-stimulated PBML were separated for T cells by E-rosetting technique. The T cells ($1x10^6$/ml) were mixed with 1% Con A-coated SRBC, centrifuged at 600xg for 2 min and incubated at 37°C 5% CO_2 for 1 h. The cell pellet was resuspended gently with a pasteur pipette. Con

A-SRBC rosetted and non-rosetted T cells were separated by Ficoll-Hypaque centrifugation. ConA rosetted T cells at the bottom were lysed by hypotonic ammonium chloride solution. The Con A-SRBC rosetted and non- rosetted T cells were studied for their regulatory function in HMI, by reversed hemolytic plaque assay and production of IgG, and in CMI by IL2 production of PBML.

5.11 Study on the effect of Indomethacin on suppression of M. leprae on IL2 production.

Study of immunoregulatory mechanism in leprosy was carried out by incorporating indomethacin into culture of PBML stimulated with M. leprae. There was very low or no IL2 production in the PBML of normal (skin test +ve for PPD) or TT/BT or LL/BL patients stimulated with M. leprae. However, when indomethacin was added into the cultures, there was significant increase of IL2 production in PBML from normal and TT/BT subjects. In contrast, there was no increase from PBML of LL/BL cultures. Indomethacin alone did not have direct effect on PBML (Figure 3, 4, 5, 6)

When PBML of normal(skin test +ve for PPD), TT/BT or LL/BL subjects were cultured with PPD, there was high in normal but very low IL2 production in TT/BT and LL/BL subjects. Nevertheless, indomethacin incorporated into the cultures significantly enhanced the production of IL2 in all groups of subjects tested. (Figure 7, 8, 9)

Our study suggests a difference in immune response to M. leprae and PPD in LL/BL patients, and this difference was not found in the TT/BT and normal subjects. A precised abnormal mechanism of this finding needs further elucidation for more

information to understand immunoregulatory mechanism in leprosy.

5.12 Study of IL2 and IFN-gamma gene expression in leprosy.

Our data shows that IL-2 and IFN-gamma genes remain inducible throughout the development of leprosy, from TT to LL stages, but significantly below normal in the TT,BB and LL stages. Thus, as measured by induced expression of these two genes, there is a discernible defect in the capacity of PBML from these stages of the disease to respond to a general stimulus, mitogen, as judged by accurate quantitation of IL-2 and IFN-gamma mRNA at the time of maximal expression. Moreover, in LL, there is a striking absence of superinduction responses for the IL-2 gene, accompanied by greatly reduced superinduction responses for the IFN-gamma gene, these responses measure post-transcription and suppressor T cell dependent downregulation. With the decline in inducibility, these results mean that post-transcription and suppressor cell-dependent downregulation are not operating normally in TT subjects. Our molecular approach is able to reveal aberrant patterns of IL-2 and IFN-gamma gene expression in different types of leprosy, most pronounced in lepromatous leprosy, but not in the normal controls from the same area. For more details see attached report from our Israeli counterpart.

6. Impact, Relevance and Technology Transfer

The Israeli Principal Investigator, Dr.R. Kaempfer made 2 visits to Dr. Makonkawkeyoon (SM)'s laboratory in Chiang Mai, Thailand. The first visit, during December 16-21, 1989 included 4 full days of discussion with SM and his team and an extensive guided tour of leprosy patients at the McKean Leprosy Rehabilitation Center in Chiang Mai. Dr.Kaempfer also gave a 2-h seminar, entitted : Regulation of Human Interleukin-2 and Interferon-gamma Gene Expression and Its disturbance in diseases. The second visit was made during December 7-18, 1991, to allow analysis of the results with the Thai counterpart. He also delivered a 2-hr seminar at CMU Medical Center that elicited spirited discussion and transfer of research activities to attendants.

Dr.K. Praputpittaya had been trained in Dr.R.Kaempfer's laboratory at the Hebrew University of Jerusalem, Israel during June 1 - July 4, 1989. The training included transfer of technology on the basic and advanced techniques on gene expression. Such technique had now been set up successfully in the Department of Clinical Immunology, Chiang Mai University. Subsequently Dr.Praputpittaya has transfered those techniques to his colleaques and those working in the same area of molecular studies.

Since our team has been working on leprosy and tuberculosis research for more than ten years, our group, in particular Dr.S. Makonkawkeyoon, is recognised as expert in cellular study in leprosy and tuberculosis. Through support from the World Health Organization, the Royal Thai Ministry

of Public Health regularly sends about 50 health professionals a year to visit our laboratory. In addition, about 150-200 medical doctors from 21 countries in Asia and Australia have visited our laboratory and acquainted with the fruitful research support of USAID for our research in leprosy and tuberculosis. All of these visitors came to visit our laboratory while they were attending a 9-month Diploma Course in Dermatology at the Institute of Dermatology in Bangkok, Thailand.

An extensive financial support from USAID has been used to strengthen our research laboratory and to build it into a modern one. After the completion of our project, our laboratory will be equiped with essential instrument for both cellular and molecular studies. From Dr.Enger's (previous WHO secretary in IMMLEP) personal communication, our laboratory in Thailand has been recognised as expertise in cellular immunological study expecially in leprosy and tuberculosis. Following this we are quite confident to claim that our laboratory could be a centre of cellular study in immunology at least in the ASEAN countries

7. <u>Project Activities and Outputs</u> :

<u>Publication</u>

"Enumeration of interleukin-1 producing monocytes from human peripheral blood mononuclear leukocytes by agar plating technique". Hirunpetcharat C, Maneckarn N, Kasinrerk W, Supajatura V, Praputpittaya K and Makonkawkeyoon S. J Immunological Methods 140(1991): 173-180.

Presented Scientific research

1. Effects of M.tuberculosis on the production of IL2 by peripheral blood mononuclear cells. By Sriburee A, Makonkawkeyoon S, Maneekarn N, Praputpittaya K and Hirunpetcharat C. Presented at the 8th RIHES Annual Meeting, Chiang Mai University, 20 June 1990.

2. Enumeration of Monocytes producing IL1 in normal subjects. By Hirunpetcharat C, Maneekarn N, Kasinrerk W, Supajatara V, Praputpittaya K and Makonkawkeyoon S. Presented at the 8th RIHES Annual Meeting, Chiang Mai, University, 20 June 1990.

3. Effect of Indomethacin on the suppression of IL2-production by M.leprae. Praputpittaya K, Makonkawkeyoon S, Kasinrerk W, Hirunpetcharat C and Sriburee A. Presented at the 8th RIHES Annual Meeting, Chiang Mai University, 20 June 1990.

4. Expression of Interleukin-2 and Interferon-gamma genes in Leprosy. By Praputpittaya K. Present at the Second Meeting of Scientific Working Groups on Leprosy Research in Thailand, held at Research Institute for Health Sciences (RIHES), Chiang Mai University, Thailand.

5. Studies on the immunological defects in leprosy patients. By Kasinrerk W, Sakonwasan C and Praputpittaya K. Presented at the Third Meeting of Scientific Working Group on Leprosy Research in Thailand. Organised by Faculty of Associated Medical Sciences, Faculty of Medicine, Khon Kaen University, Leprosy Division, Department for Communicable Disease Control, Ministry of Public Health, Thailand and Sasakawa Memorial Health Foundation. 12-13 November 1992, Khon Kaen University, Thailand

Training

1. Dr. K.Praputpittaya had been trained in Dr. R. Kaempfer's laboratory at the Hebrew University of Jerusalem, Israel, during June 1, 1989 to July 4, 1989. The training included basic and advanced techniques to start research on gene expression and to practice techniques in *in vitro* culture conditions for measuring both function of the IL2 and IFN-gamma genes in peripheral blood mononuclear cells and to discuss and to plan work for both Thai and Israeli groups throughout the project.

2. Drs. K.Praputpittaya and S. Makonkawkeyoon attended the BOSTID/AID Workshop for PSTC and CDR researchers on Polymerase Chain Reaction Technology; at the Center for Advanced Training in Cell and Molecular Biology (CATCMB), Washington D.C., during 24-28 September 1990.

8. Project Productivity

Our project has accomplished most of the proposed goals. Only that the levels of IL1 and IL2 produced by PBML were measured by bioassay rather by immunoassay. These two techniques are different in that bioassay measures function of the two components whereas the immunoassay measures quantitatively. It is, thus, ideal to measure the two components both by bioassay and immunoassy. Reasons why we firstly did not use the immunoassay was that the technique is too costly. Nevertheless, this could be done later since we have kept supernatant left over from the bioassay.

9. Future Work

Our project could lead to future work in terms of more lymphokines could be studied for their immunoregulatory role in leprosy patients. It is possible that using newly advanced antigens of M.leprae such as recombinant protein, synthetic peptides might be good or even better than native M.leprae antigen like heat-killed bacilli. These antigens are now available from the WHO. This approach might give more clear picture of immunoregulation in leprosy.

10. Reference

1. Jopling WH (1984) Handbook of Leprosy. 3rd ed. William Heinemann Medical Books Ltd. (London).

2. Turk JL and Bryceson ADM (1971) Immunological phenomena in leprosy and related diseases. Adv.Immunol. 13: 209-266.

3. Myrvang B Godal T, Ridley DS, Froland SS and Song YK. (1973). Immune responsiveness to Mycobacterium leprae and other mycobacterial antigens throughout the clinical and histological spectrum of leprosy. Clin. Exp. Immunol. 14. 541-553.

4. Mendes E, Raphael A, Mota NGS, and Mendes NF. (1974) Cell-mediated immunity in leprosy and transfer of delayed hypersensitivity reactions. J. Allerg. Clin. Immunol 53 : 223-229.

5. Mehra V, Brennan PJ, Rada E, Convit J and Bloom BR. (1984). Lymphocyte suppression in leprosy induced by unique M. leprae glycolipid. Nature 308: 194-196.

6. Mehra V, Mason LH, Fields JP and Bloom BR (1979). Lepromin-induced suppresso cells in patients with leprosy. J. Immunol. 123:1813-1817.

7. Mehra V, Mason LH, Rothman W, Reinhertz E, Schlossman SF and Bloom BR (1980). Delineation of a human T cell subset responsible for lepromin-induced suppression in leprosy patients. J. Immunol. 125: 1183-1188.

8. Bjune G (1979). In vitro lymphocyte stimulation in leprosy : simultaneous stimulation with Mycobacterium leprae antigens and phytohemagglutination. Clin. Exp. Immunol. 36:479-487.

9. Nath I and Singh R (1980). The suppressive effect of M.leprae on the in vitro proliferative responses of lymphocytes from patients with leprosy. Clin. Exp. Immunol. 41:406-414.

10. Bullock WE, Watson S, Nelson KE, Schauf V, Makonkawkeyoon S and Jacobson RR (1982). Aberrant immunoregulatory control of B lymphocyte function in lepromatous leprosy. Clin. Exp. Immunol. 49: 105-114.

11. Stoner GL, Mshana RN, Tow J and Belehu A (1982). Studies on the defect in cell-mediated immunity in lepromatous leprosy using HLA-D-identical siblings. Absence of circulating suppressor cells and evidence that the defect is in the T-lymphocyte, rather then the monocyte population. Scan. J. Immunol. 15: 33-48.

12. Haregewoin A, Godal T, Mustafa AS, Belehu A and Yemaneberhan T (1983). T-cell conditioned media reverse T-cell unresponsiveness in lepromatous leprosy. Nature, 303: 342-344.

13. Nongueira N, Kaplan G, Levy E, et al (1985). Defective gamma interferon production in leprosy : Reveral with antigen and interleukin 2. J. Exp. Med. 158 : 2165-2170.

Table 1 Numbers of IL-1 producing cells and IL1 level in leprosy and normal subjects.

Subjects	No. tested	IL1 level (u/ml)	IL1 producing cells (/2x10^5 macrophage)
LL/BL	10	57.95 ± 21.28[a]	3534 ± 1264
BB	3	81.21 ± 81.17	4053 ± 3340
TT/BT	15	58.42 ± 18.01	3971 ± 1065
Normal	14	42.45 ± 9.32	2938 ± 540

a X ± SEM

Table 2 Effect of anti-IL1, anti-IL6 and anti-TNF alpha on the numbers of IL1-producing cells in human PBML

Subject	Antiserum added			
No.	none	1/2500	1/500	1/100
Anti-IL1				
1	708[a]	402 (43.2)[b]	288 (59.3)	31 (95.6)
2	484	388 (19.8)	80 (83.5)	0 (100)
3	698	582 (16.6)	296 (57.6)	0 (100)
4	1121	982 (12.4)	421 (62.4)	ND[c]
Anti-IL6				
4	1121	1150 (-2.6)	925 (17.5)	ND
5	750	722 (3.7)	710 (5.3)	770 (-2.7)
Anti-TNF alpha				
4	1121	1063 (5.2)	978 (12.8)	989 (11.8)
5	750	710 (5.3)	762 (-1.6)	720 (4.0)

a Numbers of IL1-producing monocytes from adhering $1x10^5$ PBML in 2ml medium in plastic culture plate.

b Figures in parentheses are expressed as percent inhibition of IL1-producing cells compared to no antiserum (none).

c Not done

Table 3 Number of IL2-producing cells in PBML of leprosy and normal subjects.

		IL2-producing cells (/10^6 PBML)		
Subjects	No.	unstim.	PHA-stim.	PPD-stim.
LL/BL	5	103 ± 48[a]	666 ± 189	273 ± 47
BB	3	85 ± 34	1025 ± 426	616 ± 362
TT/BT	7	208 ± 53	441 ± 182	427 ± 117
Normal	8	210 ± 57	866 ± 404	572 ± 163

a X±SEM

Table 4 IL2 level produced by PBML of leprosy and normal subjects

Subject	IL2 level (U/ml)		IL2 level (cpm)
	PHA	PPD	*M. leprae*
LL/BL	15.34±1.95[a]	1.84±1.01	1415±277**
	(29)[b]	(29)	(24)
BB	26.84±12.18	2.35±0.84	699±179
	(9)	(9)	(7)
TT/BT	9.56±2.08	2.04±0.63	780±126
	(20)	(22)	(19)
Normal	16.21±2.31	2.05±0.53	823±94
	(31)	(32)	(29)

a X±SEM

b no. of subjects tested

** $p<0.05$ as compared to normal

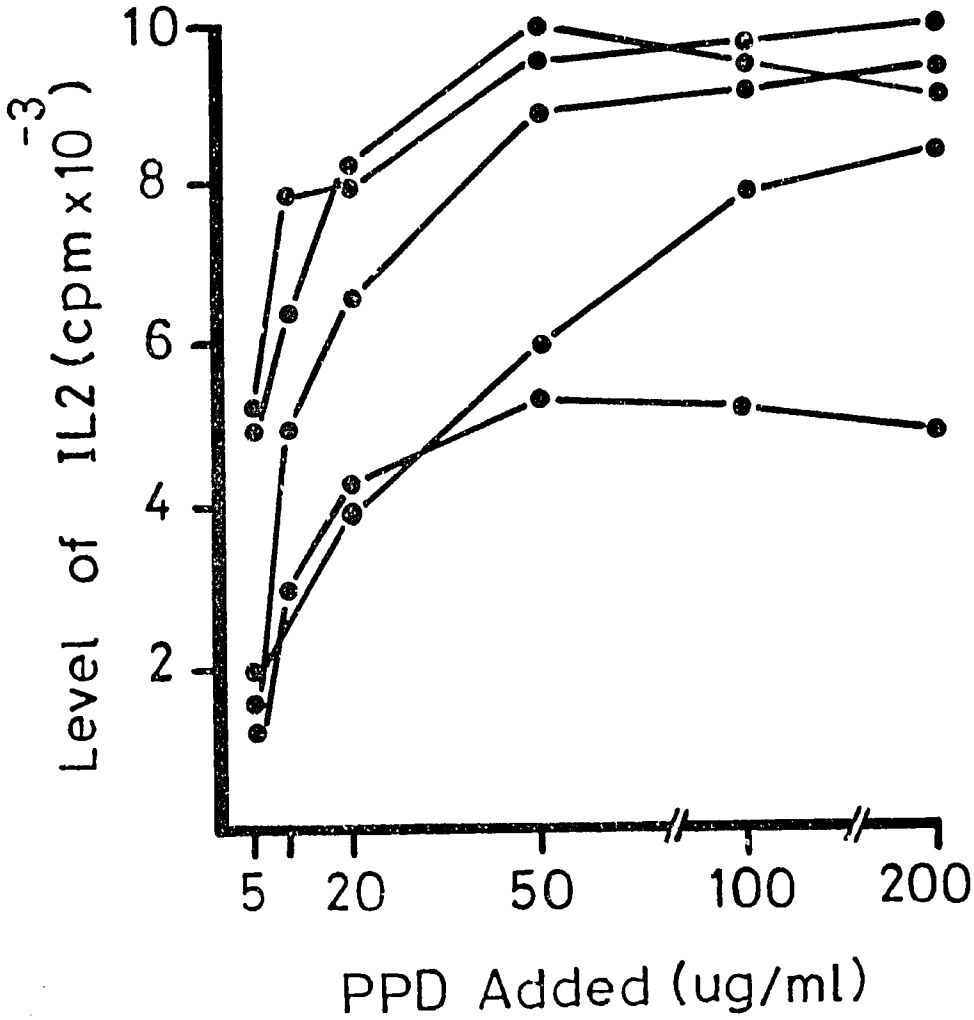

Figure 1. Evaluation of PPD lot 1 for stimulation of IL2 production from PBML of normal subjects (skin test positive to PPD).

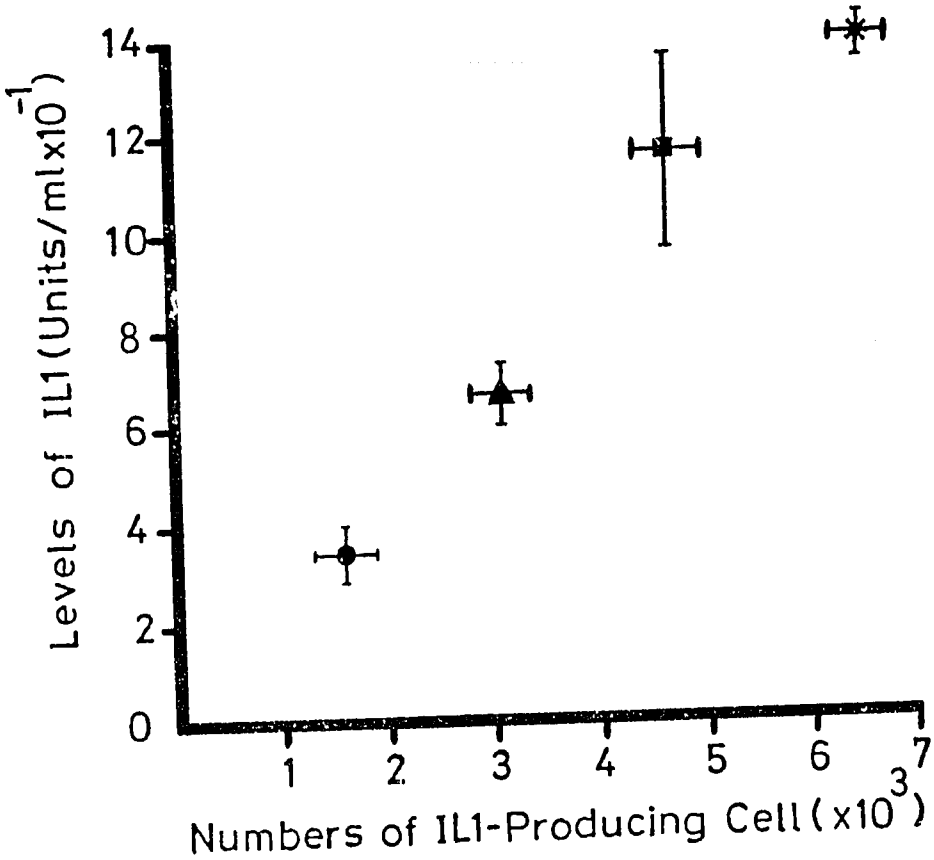

Figure 2. Correlation of IL1 levels and numbers of IL1-producing monocytes in 4 different concentrations of monocytes, each concentration consisted of 3 normal subjects (= mean±S.D. of 2.5x10^{5} PBML; = mean±S.D. of 5x10^{5} PBML; = mean±S.D. 1x10^{6} PBML; = mean±S.D. of 2x10^{6} PBML).

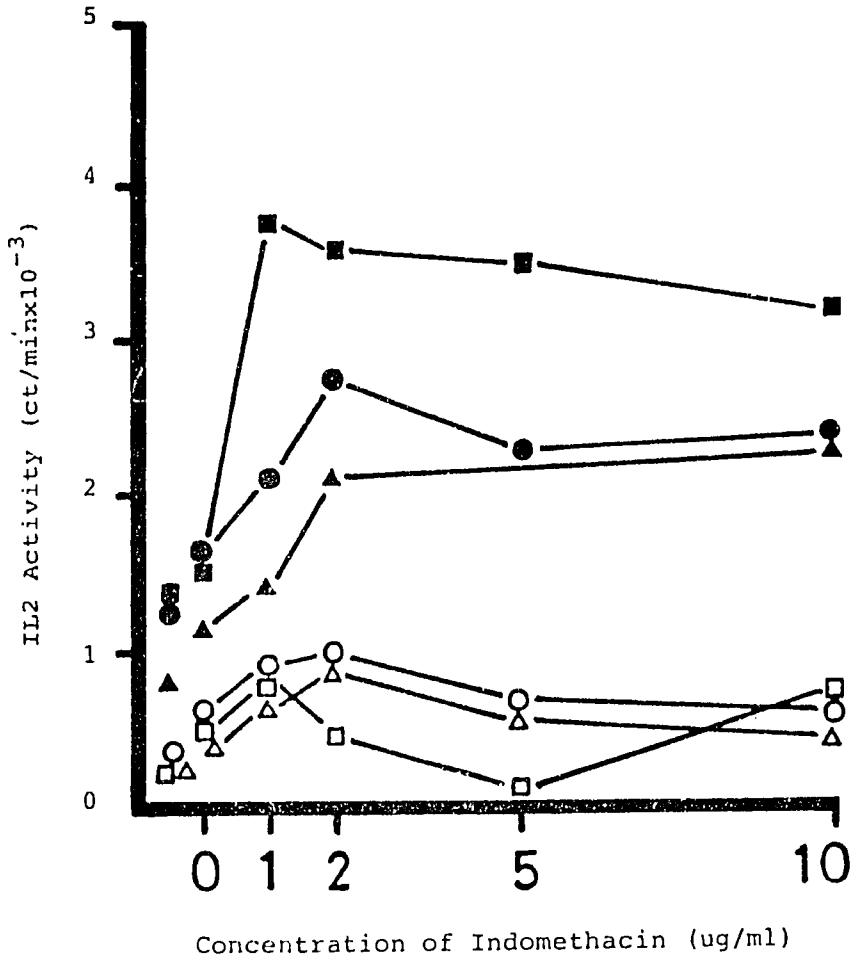

Figure 3. Effect of indomethacin on the suppressive activity of M.leprae on IL2 production of PBML from normal subjects (M.leprae 50 ug/culture)

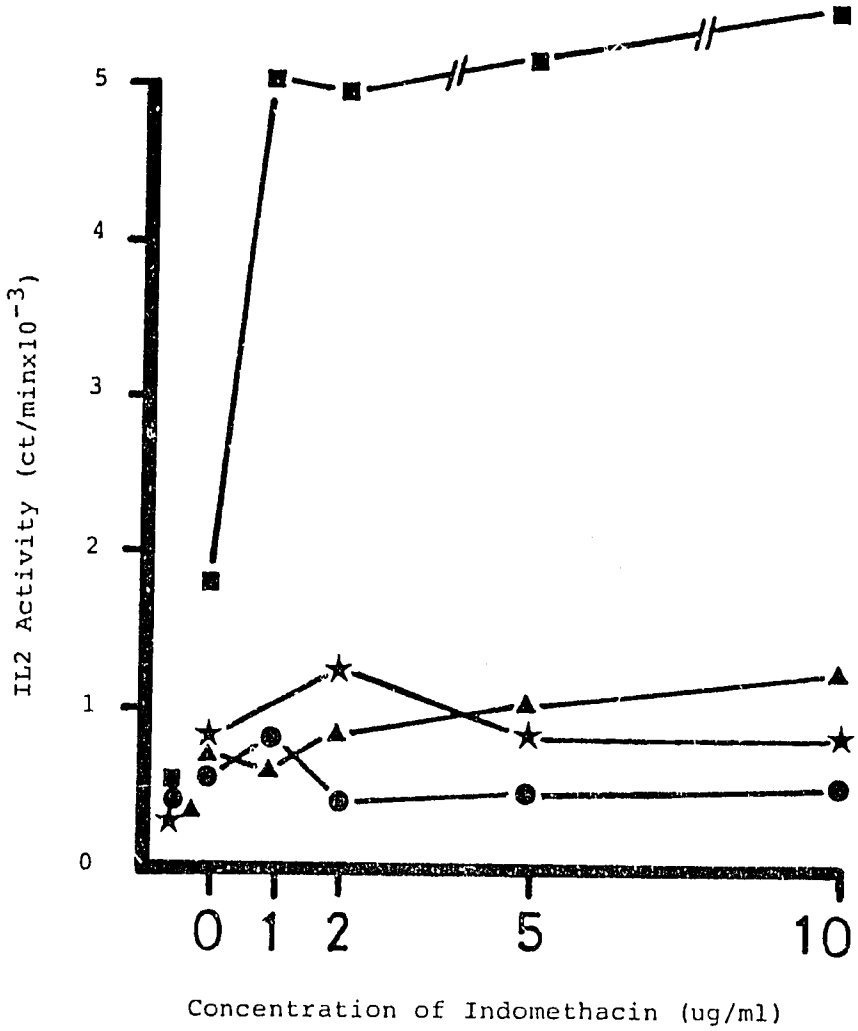

Figure 4 . Effect of indomethacin on the suppressive activity of M.leprae on IL2 production of PBML from BT/TT leprosy patients (M.leprae 50 ug/culture).

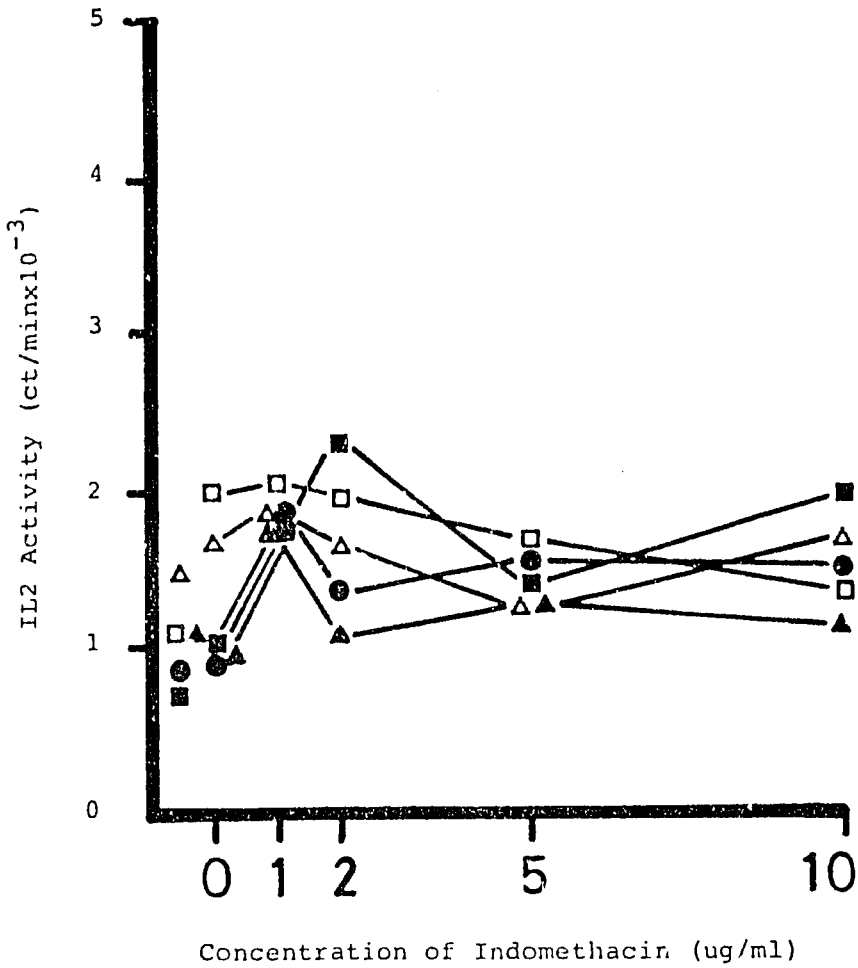

Figure 5. Effect of indomethacin on the suppressive activity of M.leprae on IL2 production of PBML from BL/LL leprosy patients (M.leprae 50 ug/culture).

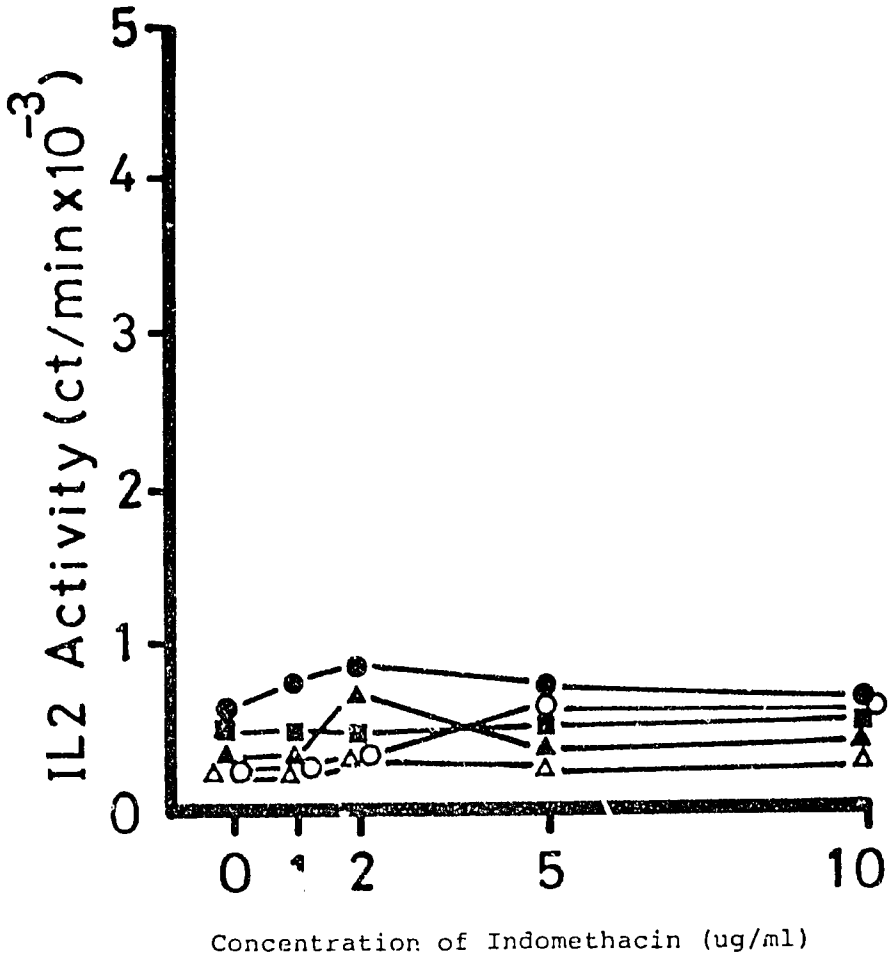

Figure 6. Effect of indomethacin on the IL2 production from PBML of normal subjects.

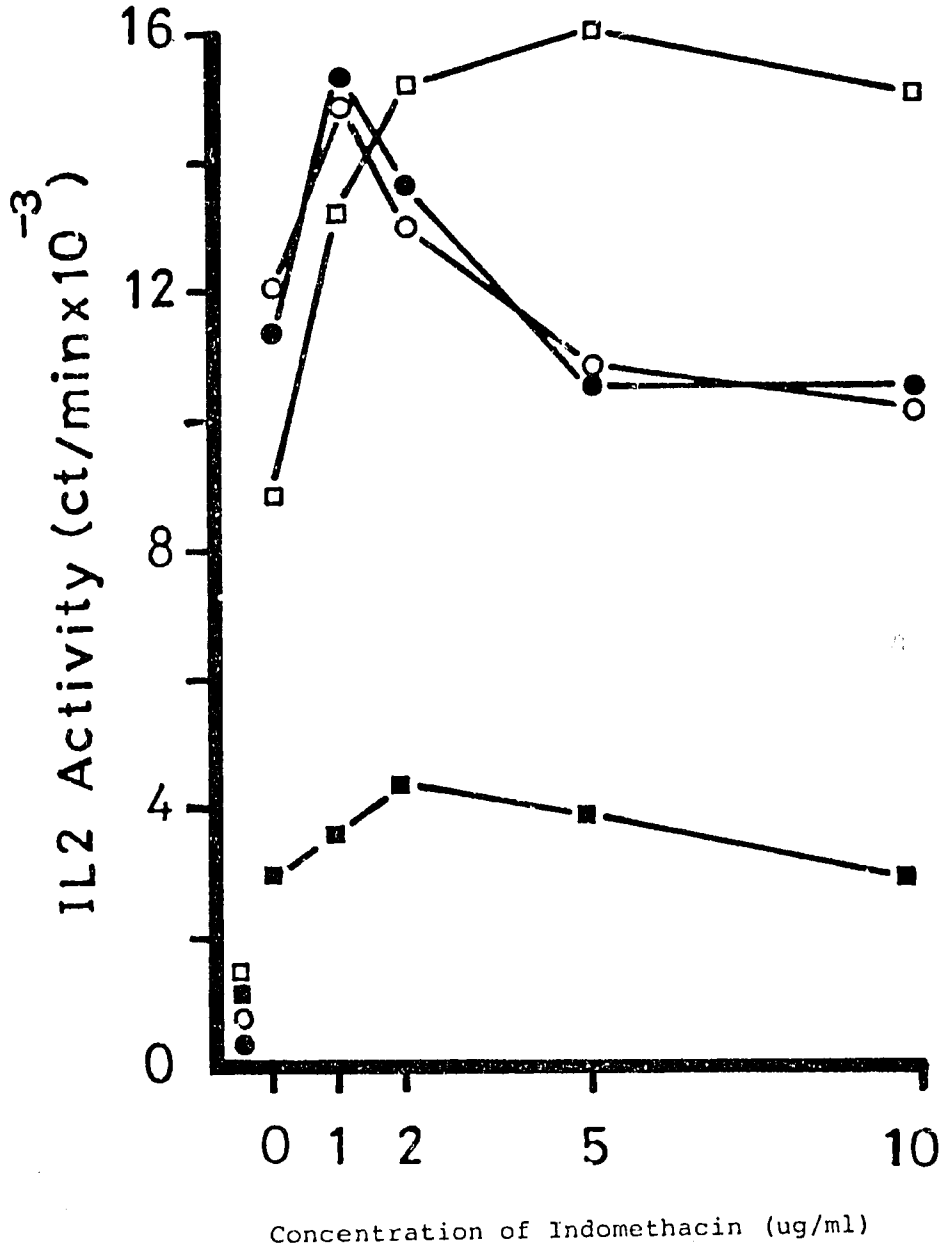

Figure 7. Effect of indomethacin on the IL2 production from PBML of normal subjects when stimulated with PPD.

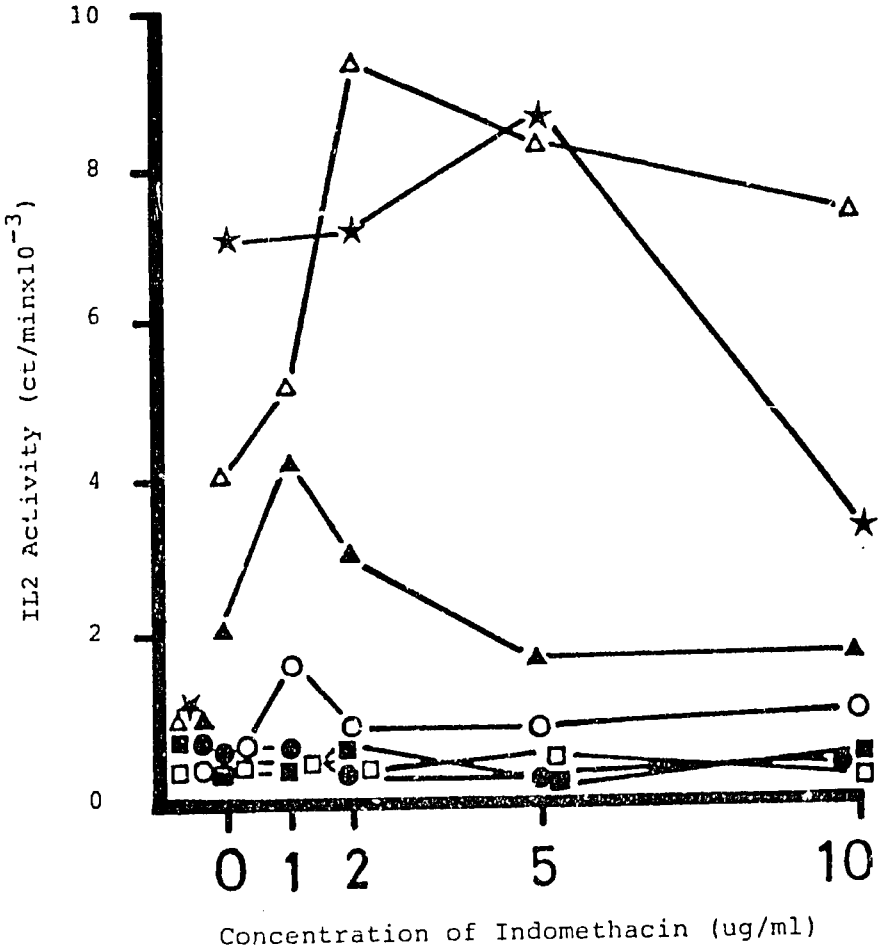

Figure 8. Effect of indomethacin on the IL2 production from PBML of BT/TT leprosy patients when stimulated with PPD.

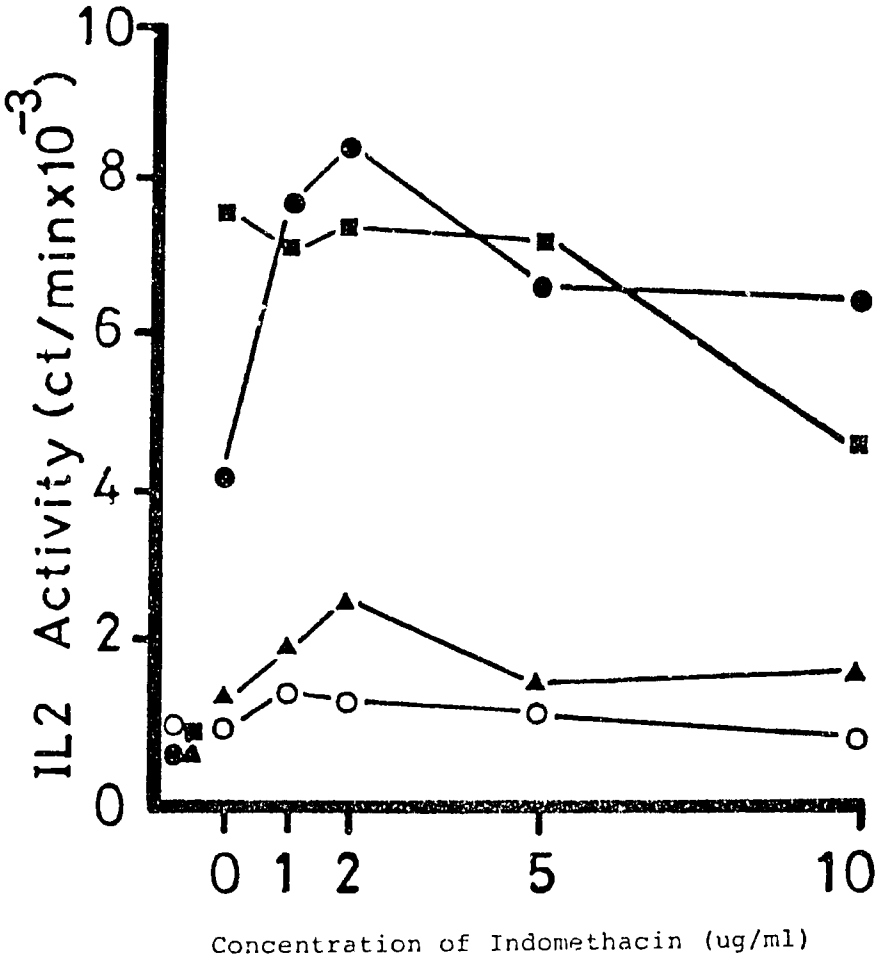

Figure 9. Effect of indomethacin on the IL2 production from PBML of BL/LL leprosy patients when stimulated with PPD.

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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

December 31, 1991

Prepared by

Dr. Raymond Kaempfer

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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 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-gamma) 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-gamma 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-gamma genes in cell populations from patients with various forms of leprosy.

REPORT

1. Site Visit of Israeli PI to Chiang Mai

The Israeli Principal Investigator visited Chiang Mai December 7-18, 1991, to allow analysis of the results in hand so far with both Dr. Kriangsak Praputpittaya in the department of Dr. Sanit Makowkawkeyoon and meet with the clinicians at McKean Leprosy Rehabilitation Center responsible for the patients analyzed in this study. He also delivered a 2-hr seminar at CMU Medical Center (see attached announcement) that elicited spirited discussion.

An unexpected bonus was the coincidental visit to CMU Medical Center of a leprosy researcher from Hanoi, Vu Tan Trao of the National Institute of Hygiene and Epidemiology. Results of her team show that cellular immune response function, judged by PHA response, is impaired in leprosy, fluctuating with the clinical status of patients and their bacterial antigen load, rather than with stage of the disease. She attended the above-mentioned seminar; in addition, we had a full discussion of her data, which fit remarkably well with the findings presented in this report and strengthen its conclusions. This is particularly important because our findings tend to demand revision of current views on the defect in the immune response in leprosy.

2. Dynamics of Regulated IL-2 and IFN-Gamma Gene Expression

In the Report dated June 30, 1991, we presented results on 61 leprosy subjects and 25 normal donors. In the interim period,

there was a problem to collect PBMC from additional patients in Chiang Mai based on a temporary suspension of AID funding due to the unstable political situation in Thailand, now reversed, as well as to a prolonged breakdown of the gamma-irradiation facility at Chiang Mai University Medical Center that is expected to be overcome only by mid-December, 1991. At that time, active collection of blood samples will resume; nitrocellulose filters containing their RNA will again be mailed to Jerusalem for moleculnr analysis, with healthy controls included in each set of donors.

We presented the results of analysis of 81 donors, distributed as follows:

Table 1. Distribution of Leprosy Subjects

CONDITION	DESIGNATION	NUMBER OF CASES
Normal donors	NORMAL	24
Tuberculoid	TT	7
Borderline tuberculoid	BT	18
Borderline	BB	5
Borderline lepromatous	BL	11
Lepromatous	LL	16
TOTAL		81

To allow convenient analysis of regulated expression of IL-2 and IFN-gamma genes in PBMC derived from subjects, 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, based on earlier studies of the regulation of IL-2 and IFN-gamma gene expression in mononuclear cells from tonsils or peripheral blood. A wave of IL-2 and of IFN-gamma mRNA is induced by exposure to PHA, reaching a maximum for both genes in the 18-22 hr time range. The amplitude of the waves of mRNA can be superinduced in the presence of inhibitors of translation, for example, cycloheximide (CHX). Expression of IL-2 and IFN-gamma genes into mRNA and active protein can also be superinduced by exposing cells, before mitogenic stimulation, to low doses of gamma-irradiation that prevent activation of suppressor T cells. These conditions of induction are designed to determine if IL-2 and IFN-gamma 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, are functioning normally.

PBMC isolated from each donor in Chiang Mai were cultured in 5 conditions. 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) during 18-22 hr;

(E) for 22 hr with PHA, but after gamma-irradiation of the cells with a dose of 1,500 rad.

Cells from each culture were collected and lysed in 7.5 M guanidinium-HCl. RNA, precipitated overnight in ethanol at -20°C, was dissolved into formaldehyde and incubated for 15 min at 60°C. Eight serial 2-fold dilutions, made in 10x saline sodium citrate, were applied in duplicate to nitrocellulose sheets, using a 96-well dot blot apparatus. The filters were mailed from Chiang Mai to Jerusalem for the next steps. After baking in a vacuum oven, sheets were hybridized separately with ^{32}P-labeled RNA probes for IL-2 and IFN-gamma, respectively. Each hybridization included a strip of nitrocellulose containing serially diluted standard RNA, purified from human tonsil cells induced with PHA for 24 hr. Exposed autoradiograms were scanned at 630 nm in an ELISA reader. For each dilution series, hybridization intensities were subjected to linear regression analysis. The slope is proportional to the mRNA concentration for the gene being probed. This value was corrected for the total number of PBMC cultured/ml and is expressed

in our data processing program as standard units of mRNA/cell. An expandable data base was compiled for these donors, enabling graphic display of IL-2 and/or IFN-gamma gene expression patterns by individual, group of subjects, or index of induction.

3. Analysis of IL-2 and IFN-Gamma mRNA Expression Patterns

Figure 1 presents, in 12 panels, the levels of IL-2 mRNA (left) and IFN-gamma mRNA (right) for each of the 6 groups of donors. In each panel, mRNA level, expressed in standard units of mRNA/cell, is shown as the group average value, with standard error of the mean (SEM), in 5 bars denoting, from left to right, each of the 5 conditions of induction A-E defined above. Analysis of Fig. 1 was presented in the previous report.

4. Analysis of IL-2 and IFN-Gamma mRNA Induction Ratios

Figure 3 depicts the index of induction and superinduction of IL-2 and IFN-gamma mRNA, respectively, in the population of normal donors and leprosy subjects. Index of induction is defined as the ratio of specific mRNA levels obtained after culture in the presence of PHA for 18 hr (P) or 22 hr (Q) and in its absence. Induction occurs only if the index exceeds the value of 1. 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 gamma-irradiation and induced directly. Superinduction occurs only if the index exceeds the value of 1.

Comparison of IL-2 and IFN-gamma gene expression in Figure 3 shows that in NORMAL donors, induction of IFN-gamma mRNA is twofold greater in extent. Though in normal donors, IFN-gamma mRNA is superinduced significantly upon gamma-irradiation (S), that is less the case for IL-2 mRNA. These findings are is also seen for the normal donors studied in Jerusalem (cf. Fig. 2).

Inspection of indices of induction P and Q shows that IL-2 and IFN-gamma genes remain inducible throughout the development of leprosy, from TT to LL stages, as evidenced by values that stay >1, though they are significantly below normal in the TT, BB and LL stages.

This trend is seen far more directly by rearranging the data of Fig. 3 as was done for Fig. 4. It is seen clearly that induction ratio at 18 hr (P in Fig. 3) or 22 hr (Q in Fig. 3) is well below normal controls (N) in the polar types of leprosy, TT and LL, except for the 22 hr value of LL, the significance of which remains to be established, since the IFN-gamma gene shows consistently low inducibility at both times of induction.

A similar trend is evident in the transition states BT, BB and BL that are more unstable and where data are indeed less uniform than in the TT and LL groups. In TT and LL, SEM values are smaller than for transition stages BT, BB and BL, even though group size for some of latter is large (see Table 1).

Inspection, in Fig. 4, of response to superinduction by CHX and gamma-irradiation (indices of superinduction R and S, respectively) shows significantly below normal values in TT and LL, with more

variable results for the transition stages. In both TT and LL, there is a striking absence of CHX and gamma-irradiation superinduction responses for the IL-2 gene (values close to 1), accompanied by greatly reduced superinduction responses for the IFN-gamma gene. Coupled with the decline in inducibility, these results must mean that post-transcriptional and suppressor cell-dependent downregulation are not operating normally in the TT and LL subjects. Since gamma-irradiation prevents activation of suppressor cells, but does not inhibit the action of already activated suppressor cells, there may be a state of high activation in TT and LL subjects that affects the general mitogen response. This is also a new and significant result.

5. General Conclusions

It is clear that our sensitive, molecular approach is able to reveal aberrant patterns of IL-2 and IFN-gamma gene expression in almost all stages of leprosy, most pronounced in tuberculoid and lepromatous stages. These data show that the ability of PBMC from leprous donors to respond to a general stimulus, mitogen, is significantly impaired.

Because we are studying activity of IL-2 and IFN-gamma genes at a time of maximal expression, we could reveal responses that may have been missed in earlier studies employing biological assays that record changes far later in the response. Moreover, quantitation of mRNA levels is linear over a wide range of response, while biological assays reach plateau more quickly and can thus distinguish between partial and full activity only over a narrow range of response.

Given the ongoing uncertainty regarding the question, if unresponsiveness of the immune system in leprosy is antigen-specific, as generally thought, or based on a general impairment of the cellular immune response, our results lead to novel and essential conclusions: they clearly support the latter interpretation.

6. Recommendations for Further Work

At this point in the project, it is important to formulate several ways to improve the data profile towards publication, taking advantage of the remaining period.

(a) The size of each group of patients should be expanded to $\geq$20 each, to allow firm statistical analysis.

(b) Total T cell numbers (CD3), CD4 and CD8 subsets should be quantitated in Chiang Mai for all samples taken from hereon. Information on these numbers, if available for the subjects tested, should be provided. Expression of IL-2 and IFN-gamma genes should be quantitated in Jerusalem not only per total PBMC, but also as standard units of mRNA/T cell. In this manner, it can be shown if T cell function is affected throughout the stages of leprosy.

(c) Individual patterns of gene expression should be matched with drugs or other treatment received, information that should be collected for all donors examined.

(d) ^{3}H-thymidine incorporation response to PHA should be determined for all samples of PBMC collected, as an independent measure of T cell function.

(e) On selected donors, both normal and leprous, a third filter should be prepared for hybridization with a control probe for β-actin. This requires more PBMC and will be done only where that amount is available.

(f) Similarly, on selected donors, both normal and leprous, IL-2 and IFN-gamma production should be assayed in culture medium at 72 hr after stimulation with PHA. This also requires more PBMC for longer incubation. The assays are available in the Chiang Mai laboratory.

Legends

Fig 1 - Regulated expression of IL-2 and IFN-gamma genes in PBMC from normal donors and leprosy subjects in TT, BT, BB, BL, LL stages. IL-2 and IFN-gamma mRNA was quantitated, in standard units/cell, in PBMC from each donor, cultured in 5 different conditions (A-E). See text. Mean ± SEM is depicted for each group.

Fig 2 - Regulated expression of IL-2 and IFN-gamma genes in 32 normal donors. IL-2 and IFN-gamma mRNA were quantitated, in standard units/cell, in PBMC from each donor, cultured in 5 different conditions (A-E), defined in the text. Mean ± SEM is depicted.

Fig 3 - Index of induction (P, Q) and superinduction (R, S) of IL-2 and IFN-gamma mRNA species in PBMC from normal donors and leprosy subjects in TT, BT, BB, BL, LL stages. Ratios P-S (see text) are defined as: P=B/A, Q=C/A, R=D/C AND S=E/C, where A-E are as defined in the text. Specific RNA ratios were calculated first for each individual donor and then averaged for each group. Mean ratio ± SEM is shown for each group.

Fig 4 - Index of induction and superinduction of IL-2 and IFN-gamma mRNA: direct comparison of normal donors and leprosy subjects in TT, BT, BB, BL, LL stages. Specific RNA ratios were calculated first for each individual donor and then averaged for each group. Ratios of induction or superinduction P-S (data from Fig. 3) are compared for the 6 groups. Mean ratio ± SEM is shown for each group.

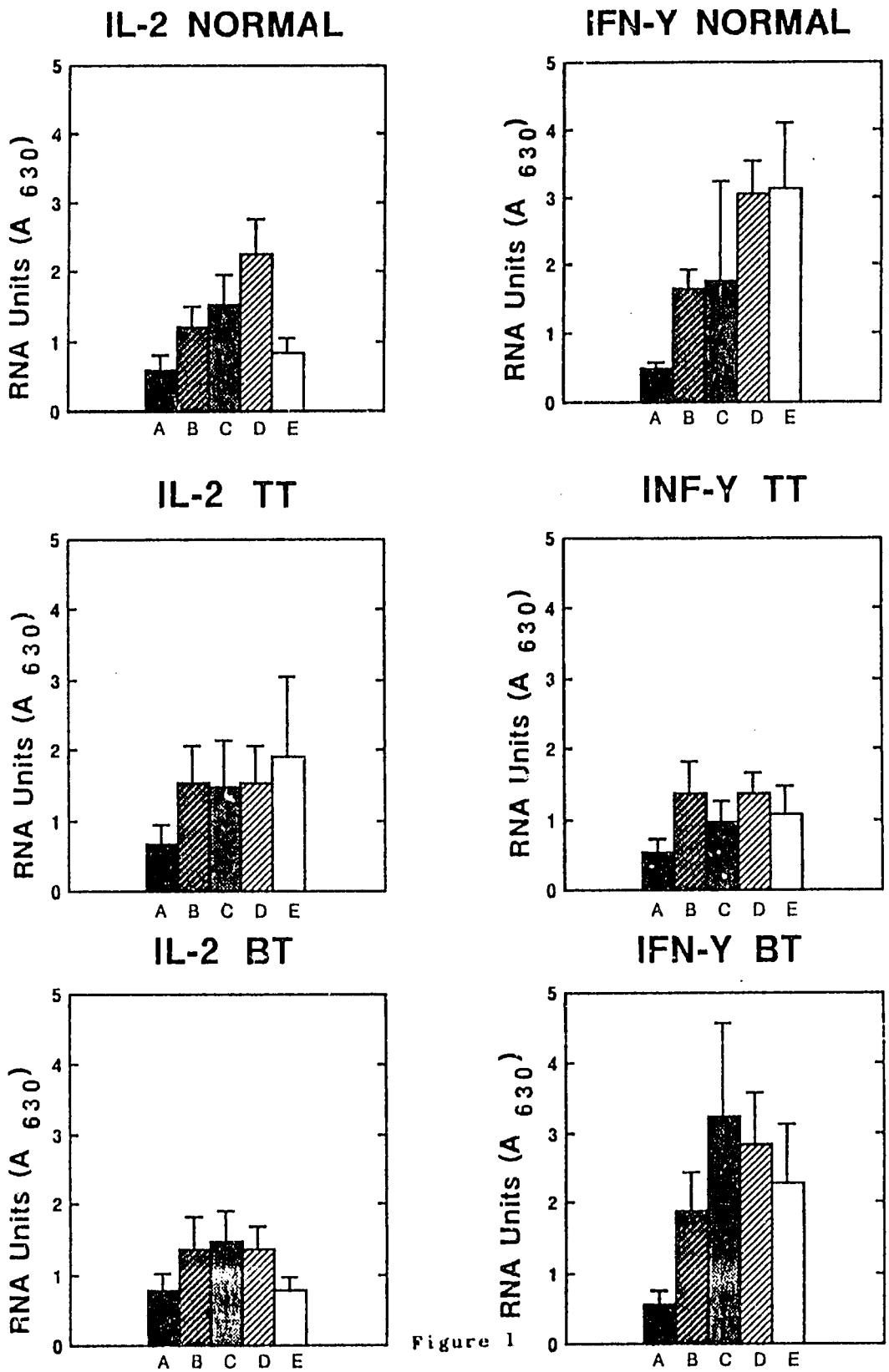

Figure 1

Part I

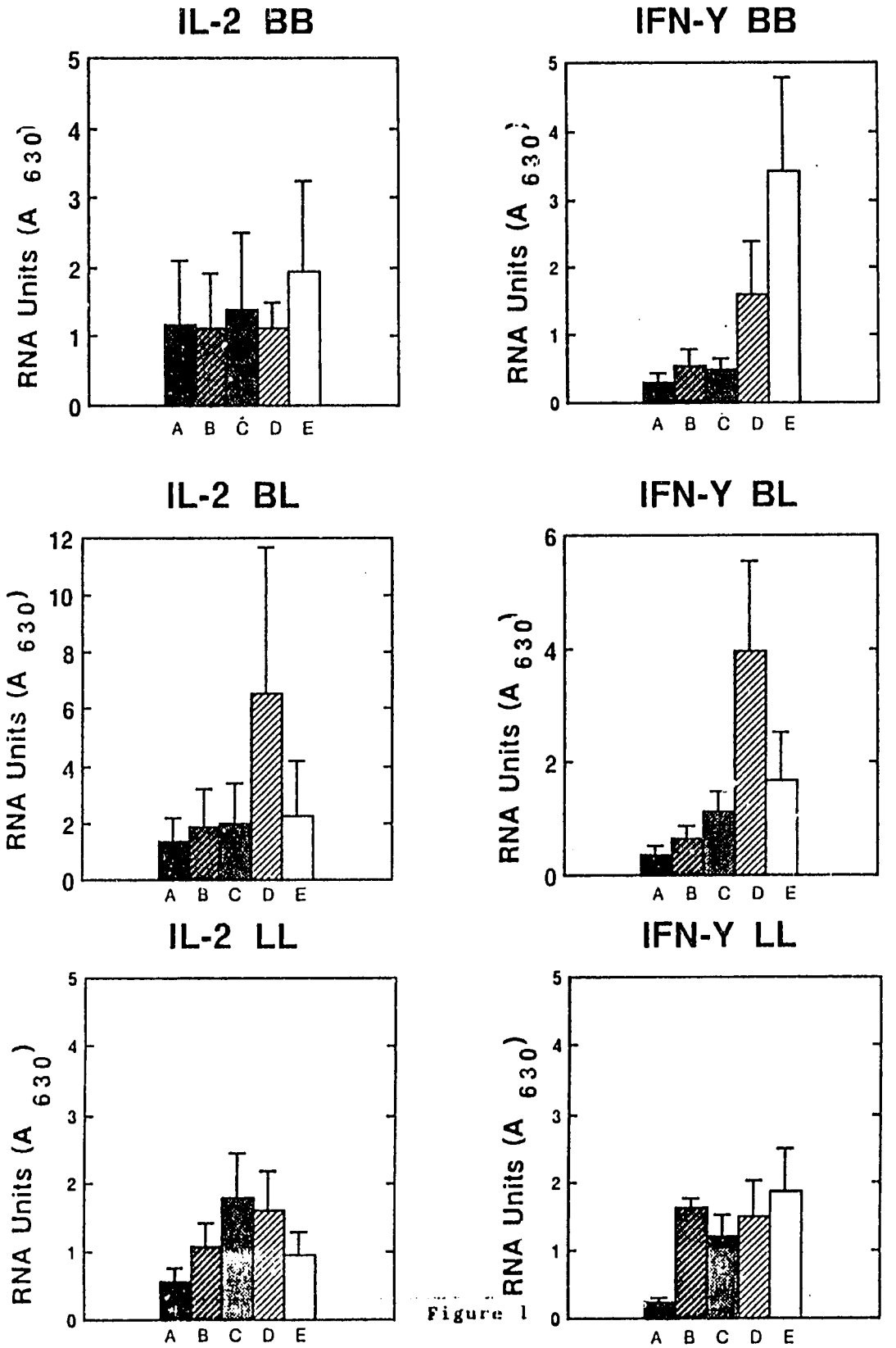

Figure 1

Part II

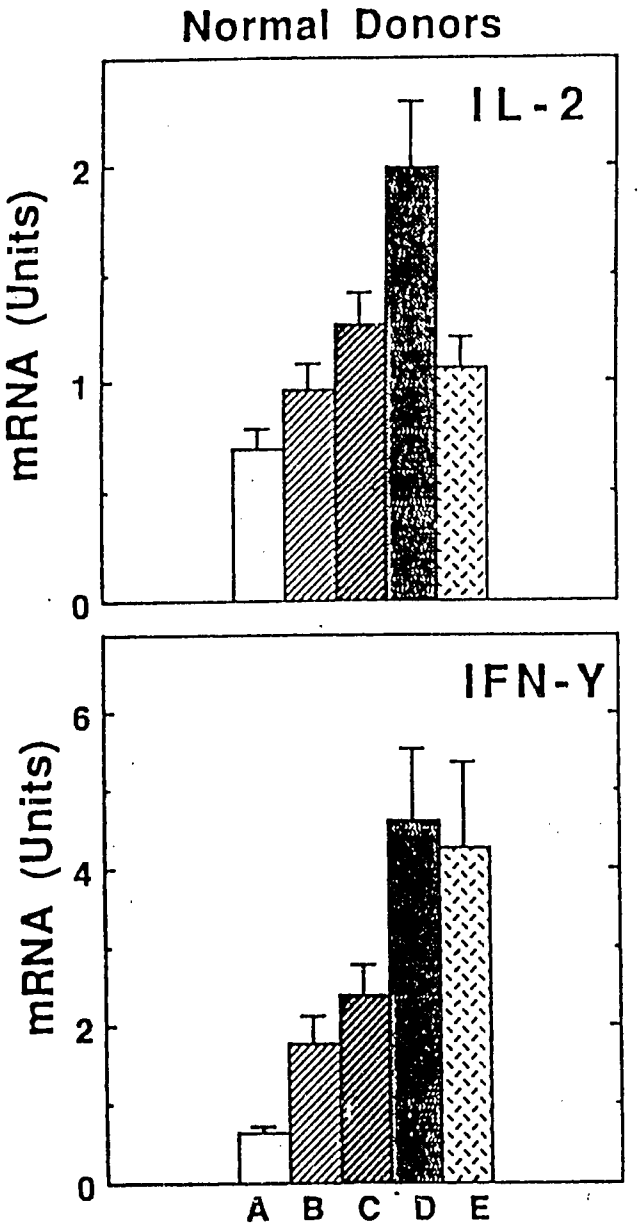

Figure 2

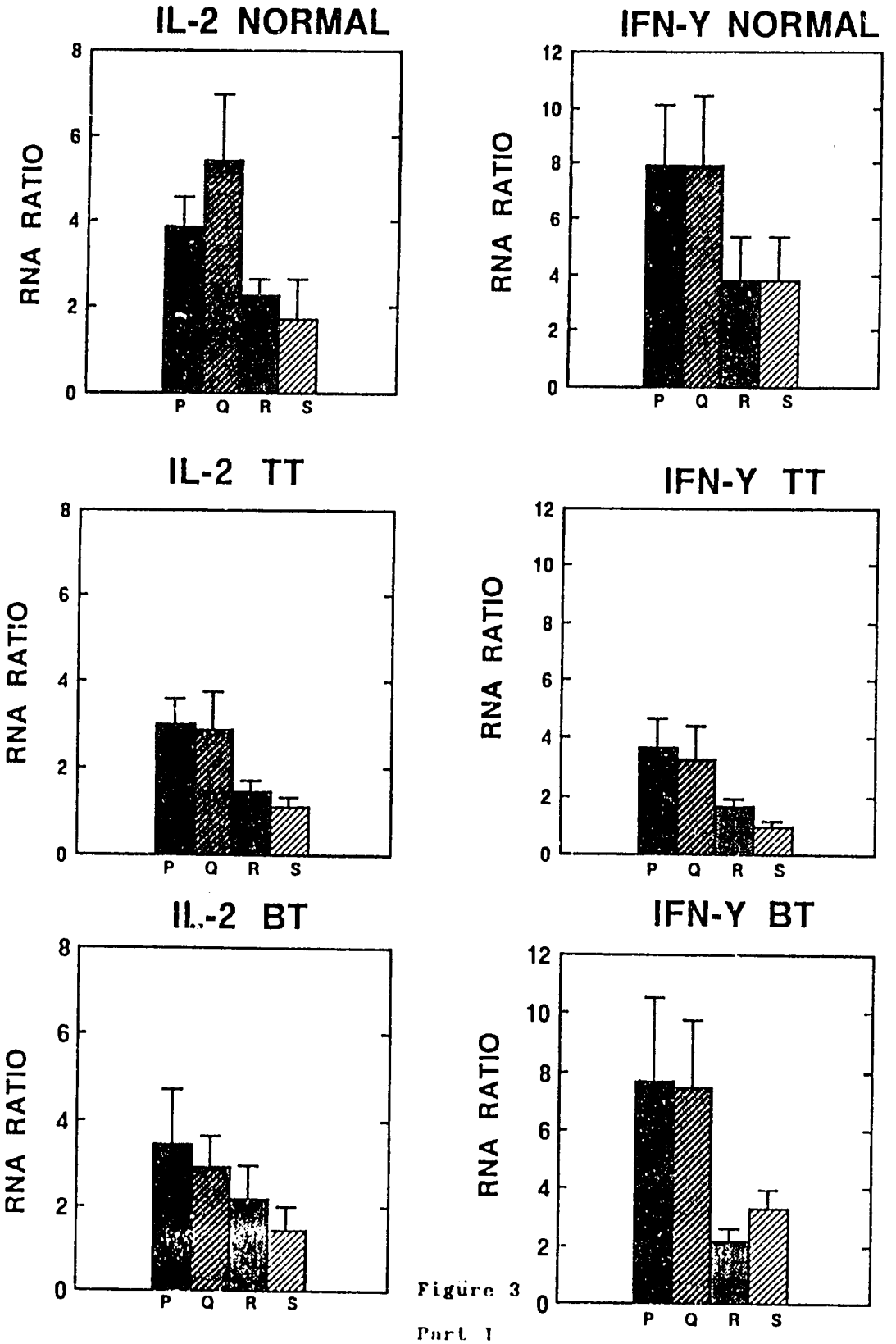

Figure 3

Part 1

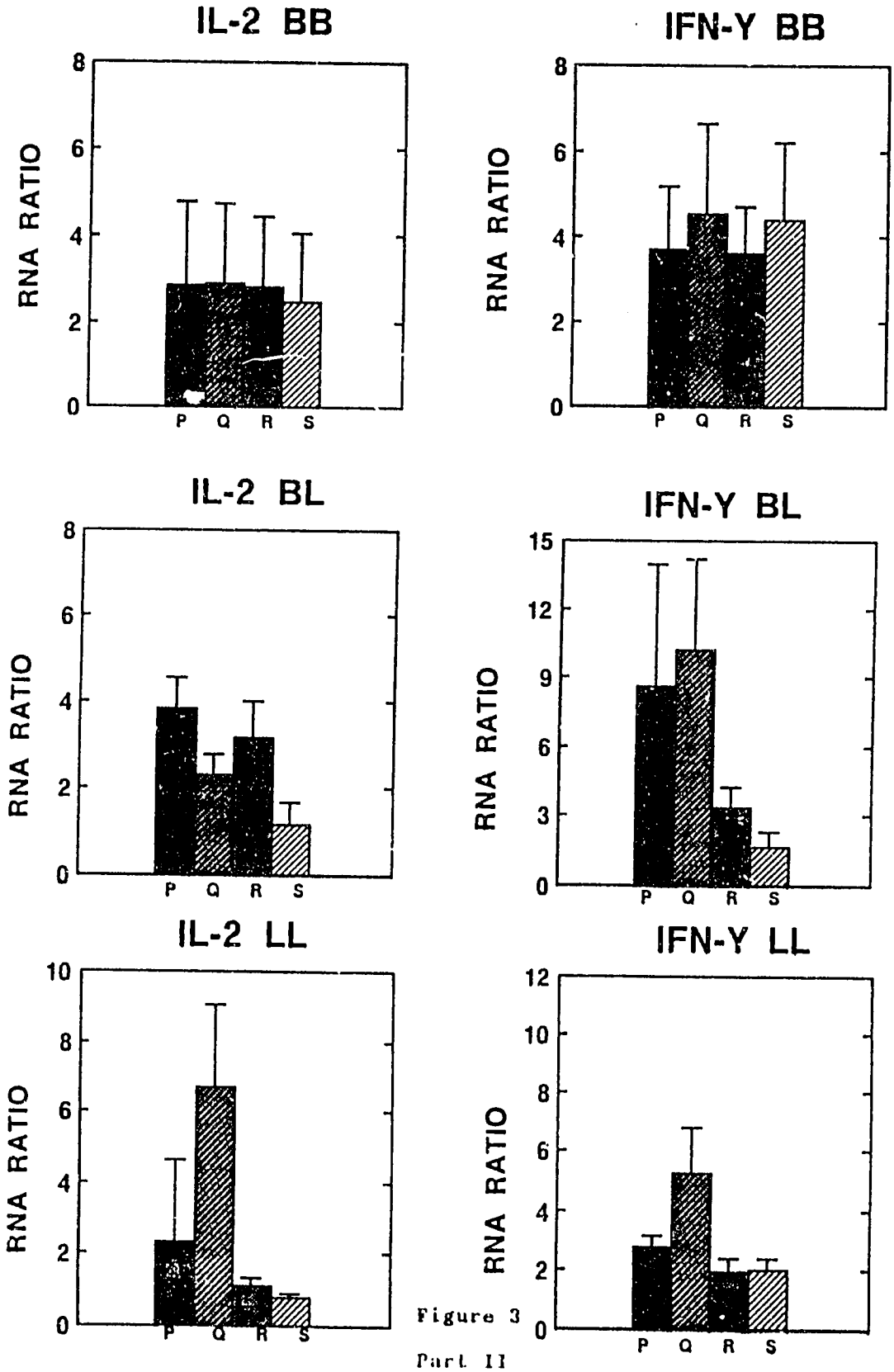

Figure 3

Part II

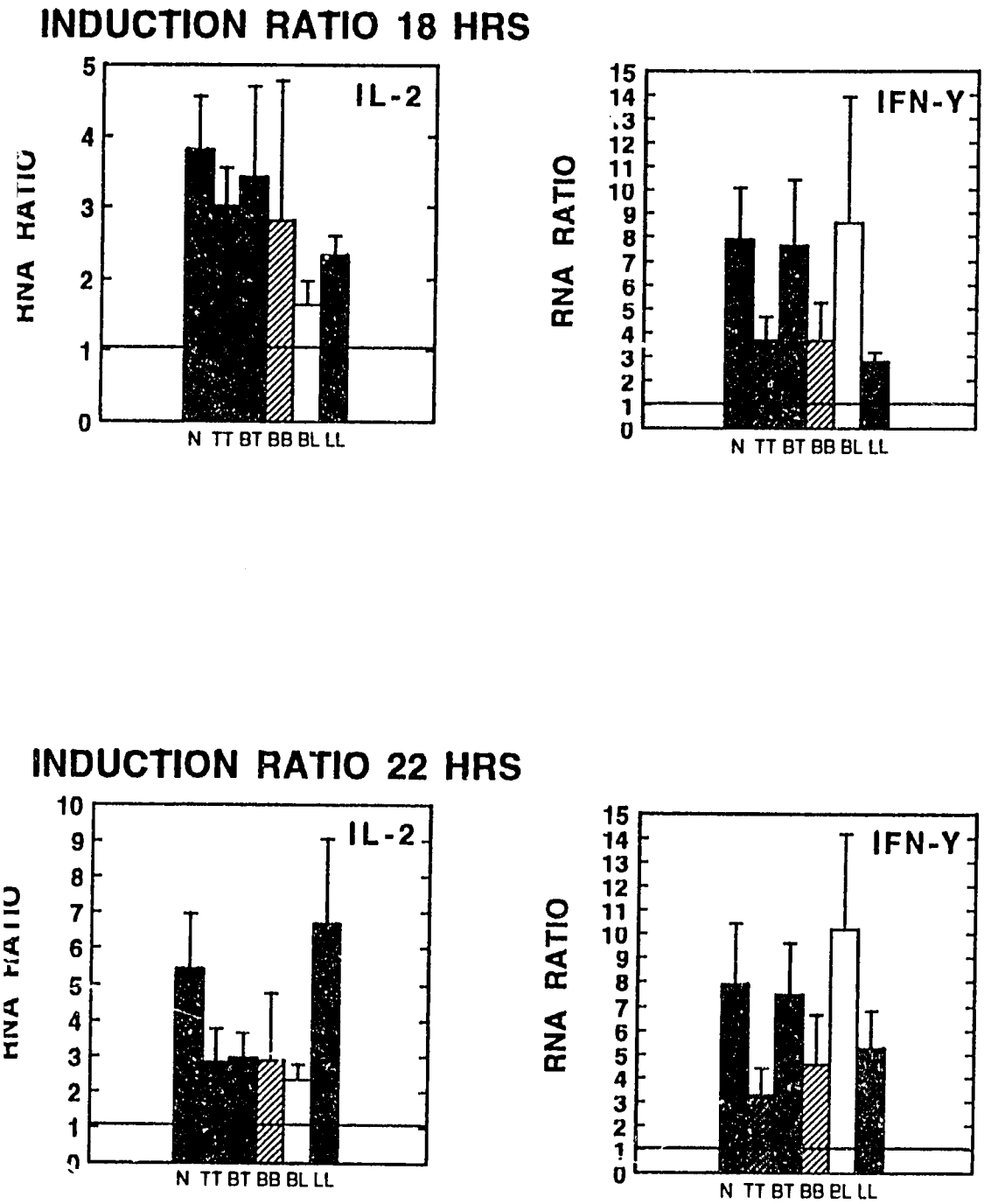

Figure 4

Part I

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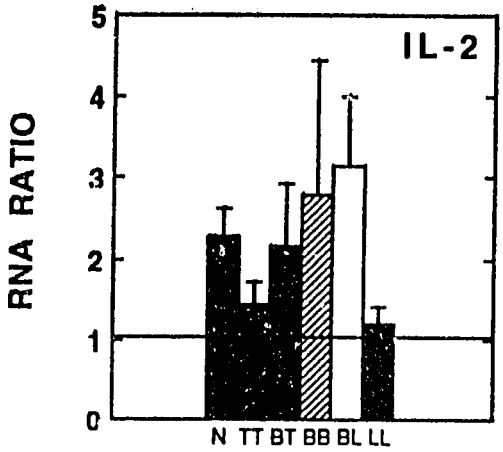
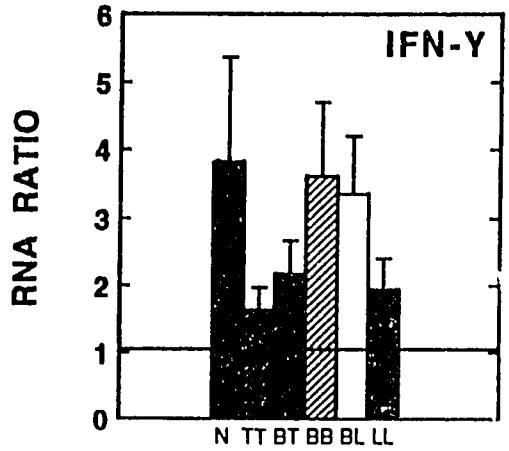
CHX SUPERINDUCTION RATIO





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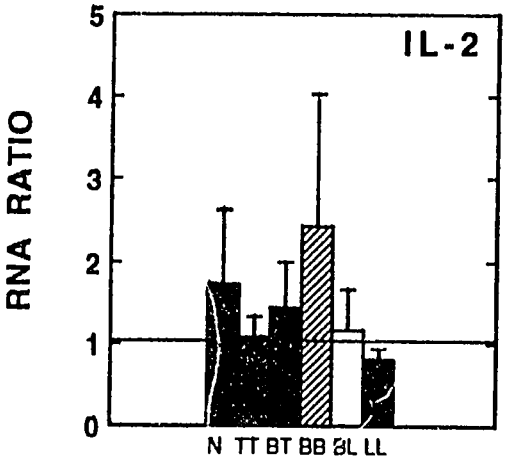

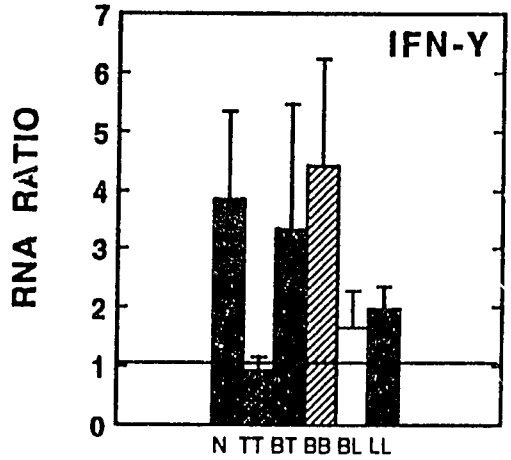

Figure 4

Part II

ภาควิชาภูมิคุ้มกันวิทยาคลินิก

คณะเทคนิคการแพทย์ ม.ช.

ขอเชิญฟังการบรรยายพิเศษ

เรื่อง

Dysregulated Expression of IL-2 and IFN-gamma Genes in Down Syndrome, Chronic Renal Failure, Bladder Cancer, Acute Lymphocytic Leukemia and SLE.

โดย

Professor Raymond Kaempfer
Dept. Molecular Virology, Faculty Medicine,
The Hebrew University of Jerusalem, ISRAEL

เวลา 13:30-15:30 น. วันจันทร์ ที่ 9 ธันวาคม 2534

ณ ห้องประชุมภาควิชาภูมิคุ้มกันวิทยาคลินิก คณะเทคนิคการแพทย์

ด่วน

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Enumeration of interleukin-1 producing monocytes from human peripheral blood mononuclear leukocytes by agar plating technique

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An agar plating technique was developed for enumeration of IL-1-producing monocytes based on the principle that when IL-1-producing monocytes were cocultured with mouse thymocytes and PHA in semisolid agar medium in a plate, mouse thymocytes proliferated around IL-1-producing monocytes resulting in the clusters or colonies of cells. The IL-1-produced clusters or colonies of cells can be counted under a dissecting microscope. Optimal conditions were established for induction and development of IL-1-producing monocytes. The numbers of IL-1-producing monocytes ranged from 819 to 1930 cells/10^5 monocytes, with mean ± SEM = 1344 ± 182 cells/10^5 monocytes; the IL-1 activity ranged from 11.7 to 85.9 U/10^5 monocytes/ml, with mean ± SEM = 42.8 ± 11.2 U/10^5 monocytes/ml in seven normal subjects. The IL-1 activity per one monocyte ranged from 12.7 to 86.5 mU, with mean ± SEM = 33.5 ± 9.8 mU. The mean numbers of IL-1-producing monocytes and the mean IL-1 levels produced by monocytes from the same normal subjects were highly correlated (r = 0.981). The numbers of IL-1-produced colonies resulting from IL-1-producing monocytes could be completely abolished by incorporation of rabbit anti-human IL-1 in the semisolid agarose but not by rabbit anti-human IL-6 or anti-human TNF-α.

Key words: Interleukin 1; Monocyte; Agar plating

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Abbreviations: IL-1, interleukin 1; IL-2, interleukin 2; IL-6, interleukin 6; TNF, tumor necrosis factor; PHA, phytohemagglutinin; mRNA, messenger RNA; PBML, peripheral blood mononuclear leukocytes; FBS, fetal bovine serum; NSE, nonspecific esterase stain; LPS, lipopolysaccharide

Introduction

Interleukin-1 (IL-1) is produced by activated mononuclear phagocytes and is essential for supporting both T and B cell responses. Interleukin-2 (IL-2) is produced by activated T cells which supports T cell proliferation in vitro (Aarden et

al., 1979). Interleukin-1 represents a family of polypeptides with a wide range of biological activities, many of which are considered essential for the induction of immune responses, both in vivo and in vitro. The most significant of these activities is the capacity to stimulate the production of IL-2 by T cells. However, IL-1 also causes an increase in body temperature in vivo, which may help in host immune responses to invading microorganisms by enhancing the effects of IL-1 on T cell proliferation. Interleukin-1 is normally produced by activated mononuclear phagocytes derived from peripheral blood or other tissues, or by leukemia monocytes and monocytic tumor cell lines (Lachman, 1983). Human IL-1 plays an important role in the pathogenesis of many diseases and functions as a key mediator of the host response to various infectious, inflammatory, and immunologic challenges (Dinarello, 1989).

It has been shown that there are at least two distinct human IL-1 molecules, termed IL-1α and IL-1β (March et al., 1985). Several laboratories have measured IL-1 in human body fluids for the diagnosis and prognosis of various disease processes and for certain physiologic functions. There is also a need for detecting IL-1 mRNA in certain tissues as an indicator of IL-1 mediated local disease (Giulian and Lachman, 1985). Interleukin-1 can stimulate the proliferation of mouse thymocytes either alone, or in the presence of low concentrations of a mitogenic lectin, such as PHA (Paetkau et al., 1976). Therefore, based upon its ability to directly stimulate the proliferation of mouse thymocytes, human IL-1 is usually assayed by adding IL-1 dilution of preparations onto microplate wells containing thymocytes and PHA. Proliferation is monitored by [^{3}H]thymidine incorporation (Gearing et al., 1985). A more sensitive assay for IL-1 level was developed, in which production of IL-2 is measured by a murine T lymphocyte clone LBRM-33 stimulated with PHA and IL-1 (Gillis and Mizel, 1981; Conlon, 1983). While most studies have focussed on IL-1 level or activity found in monocyte or macrophage culture supernatant, very few studies have been carried out on quantitation of IL-1-producing monocytes or macrophages. In this report, we describe a simple and reliable technique for determining the numbers of human IL-1-producing monocytes. The quantitation is based on the principle that when IL-1-producing monocytes are cocultured with mouse thymocytes and PHA in semisolid agar medium, mouse thymocytes will proliferate around IL-1-producing monocytes. The resulting 'clusters or colonies of cells' can be seen and counted under a dissecting microscope. Each cluster or colony of cells represents an individual IL-1-producing monocyte.

Materials and methods

Isolation of PBML

Informed consent was first obtained after the nature and possible consequences of the studies had been fully explained. Peripheral blood from participating laboratory personnel was drawn into a heparinized test tube (10 U/ml of blood). Peripheral blood mononuclear leukocytes (PBML) were isolated by Ficoll-Hypaque density gradient centrifugation (Böyum, 1968), washed twice with RPMI 1640 medium and resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 10 mM Hepes, 100 U/ml penicillin G, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 5×10^{-5} M 2-mercaptoethanol (complete medium).

Separation of monocytes

PBML, 4×10^6 cells in 2.0 ml complete medium, were introduced into a 35 × 10 mm plastic tissue culture plate (Nunc, Kamstrup, Denmark), and incubated at 37°C, 5% CO_2 for 2–4 h. Non-adherent cells were washed off three times with warm RPMI 1640 media. PBML, non-adherent and adherent cells were stained with non-specific esterase stain (NSE). Adherent cells were NSE-positive for 89.86 ± 2.61% (mean ± SD of ten normal subjects). After calculating the exact number of monocytes adhering on the plate, the necessary volume of 10% FBS-RPMI 1640 medium was added to provide a final concentration of monocytes equal to 2×10^5/ml.

Production and determination of IL-1 level

As explained above, the adhered monocytes on the plastic plate were adjusted to 2×10^5 NSE-positive cells/ml by adding various calculated

amounts of 10% FBS-RPMI 1640 medium. Lipopolysaccharide (LPS, *E. coli* O 127:B8, Sigma), 20 μg/ml, was added to the monocytes and the plates were incubated at 37°C, 5% CO_2 for 24 h. The cell suspension was collected, centrifuged and the supernatants were kept at −70°C for IL-1 assay. The IL-1 level was determined by mouse thymocyte proliferation assay (Paetkau et al., 1976). Levels of IL-1 were expressed in U/ml by comparison to commercial standard human IL-1 (human interleukin-1, ultrapure, Genzyme, Massachusetts, U.S.A.).

Production and quantitation of IL-1-producing monocytes

The method for enumeration of IL-1-producing monocytes was performed by adding 1×10^6 PBML in 2.0 ml supplemented RPMI medium onto 35 × 10 mm tissue culture plates. After 2h of incubation, non-adherent cells were removed by gently expelling culture medium onto cells at the bottom of the plate three times and the total numbers of non-adherent cells were counted. The numbers of cells adhering on the plate were derived by subtracting the total number of PBML with the number of non-adherent cells. The number of NSE-positive cells adhering on the plates were then determined. The concentration of monocytes adhering on plates was adjusted to 1×10^6 cells/ml by adding an appropriate amount of RPMI 1640 medium. Lipopolysaccharide (LPS, 20 μg/ml) was added to the adhering monocytes which were incubated at 37°C, 5% CO_2 for 4 h, washed twice, and then treated with a thymocyte mixture (1.5 ml of 0.3% agarose in medium containing 16×10^6 thymocytes and 1.5 μg PHA-P). After gelling of agarose, the culture plate was incubated at 37°C, 5% CO_2 for 6 days and the number of IL-1-producing cells, appearing as colonies, were counted under a dissecting microscope with × 30 magnification. The amounts of IL-1-producing monocytes in total monocytes was obtained and the numbers of IL-1 producing monocytes/10^6 were then determined.

Determination of optimal conditions for induction of IL-1 producing monocytes

Number of PBML. Various cell concentrations of PBML (0, 0.5, 1, 2 and 4×10^6 cells in 2.0 ml media) were introduced into tissue culture plates. After 2 h of incubation, non-adherent cells were washed off, 20 μg/ml LPS was added and the plates were incubated at 37°C, 5% CO_2 for 4 h. After adherent cells were washed, the thymocyte and PHA-P mixtures in agarose media were added, and the numbers of IL-1-producing monocytes were counted after 6 days of incubation.

Number of thymocytes. Various cell concentrations of mouse thymocytes (0, 4, 8, 12, 16 and 20×10^6 cells in 1.5 ml of 0.3% agarose media containing 1.5 μg PHA-P) were added into LPS stimulated monocytes derived from 1×10^6 PBML adhering on tissue culture plates. After 6 days of incubation, the numbers of IL-1-producing monocytes were quantitated.

Concentration of LPS. Various concentrations of LPS (0, 5, 10, 20, 40 and 60 μg/ml) were incubated with adhering monocytes derived from 1×10^6 PBML for 4 h, washed, and then thymocyte and PHA-P mixtures in agarose media were added. The numbers of IL-1-producing monocytes were determined after 6 days of incubation.

Stimulation period for LPS. Monocytes were stimulated with LPS (20 μg/ml) for periods of 0, 1, 2, 4, 6, and 8 h, respectively. Thymocyte and PHA-P mixtures in agarose media were then added, and the numbers of IL-1-producing monocytes were counted after 6 days of incubation.

Concentration of PHA-P. Various concentrations of PHA-P (Wellcome) (0, 0.25, 0.5, 1, 2 and 4 μg/ml) were added into thymocyte mixtures in agarose media and then the mixtures were added onto LPS-stimulated monocytes. After 6 days of incubation, the numbers of IL-1 producing monocytes were counted.

Incubation period. After the adhering monocytes derived from 1×10^6 PBML were stimulated with LPS (20 μg/ml) they were overlayered with thymocyte and PHA-P mixture in agarose medium. The numbers of IL-1-producing monocytes were counted every day for 7 days.

Correlation of IL-1 level and IL-1-producing cells

Monocytes were separated from PBML by surface adherence on plastic plates as previously described. IL-1 levels and the numbers of IL-1-producing monocytes from normal subjects were

produced and determined as described. Correlation of the IL-1 levels and the numbers of IL-1-producing monocytes was plotted and the value of the correlation coefficient determined.

Effect of anti-IL-1, anti-IL-6 and anti-TNF-α antibodies on the development of IL-1-produced colonies

Various dilutions of rabbit anti-human IL-1, rabbit anti-human IL-6 and rabbit anti-human TNF-α polyclonal antibodies (Genzyme Corporation, Boston, MA, U.S.A.) were incorporated into thymocyte and PHA-P mixtures in agarose medium. After overlayering the thymocytes, PHA-P and various anti-cytokine mixture in agarose medium onto the adhering monocytes, the numbers of IL-1-producing monocytes were determined after 6 days of incubation.

Results

Optimal conditions for development of IL-1-producing cells

The optimal concentration of thymocytes for the maximum development of IL-1-producing cells is 16×10^6 cells/plate while the optimal concentration of PBML is 1×10^5/plate as shown in Figs. 1*A* and 1*B* respectively. Figs. 2*A* and 2*B* show the optimal concentration of LPS (20 μg/ml) stimulation and PHA-P (1.0 μg/plate) co-stimulation for maximum IL-1-producing monocytes respectively. The optimal stimulation time for LPS is 4 h while the optimal incubation period for the development of IL-1-induced colonies is 6 days as depicted in Figs. 3*A* and 3*B* respectively.

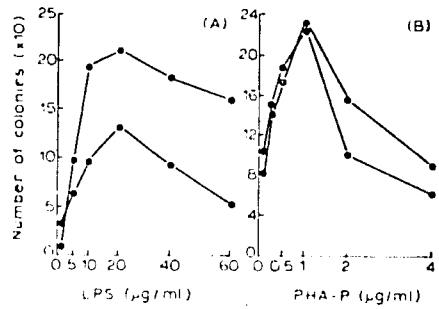

Fig. 2. Determination of optimal LPS (*A*) and PHA-P (*B*) concentrations for the assay of IL-1-producing monocytes.

Correlation of IL-1 levels and IL-1-producing cells

The numbers of IL-1-producing monocytes and the levels of IL-1 activity in culture supernatant fluids of each cell culture were quantitated in parallel in order to determine the correlation as described in the materials and methods section. Fig. 4 shows a very good correlation between the mean numbers of IL-1-producing monocytes and the mean levels of IL-1 in the culture supernatant fluids ($r = 0.981$).

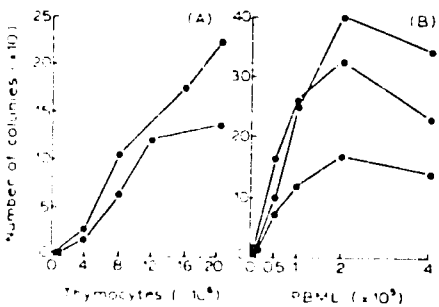

Fig. 1. Determination of optimal thymocytes (*A*) and PBML (*B*) concentrations for the assay of IL-1 producing monocytes. (*Note*: in Figs. 1–3, each line represents the result obtained from one normal subject).

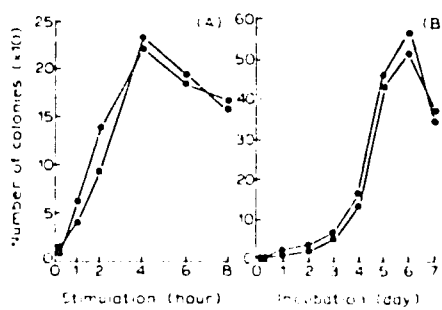

Fig. 3. Determination of optimal stimulation time (*A*) and incubation time (*B*) for the assay of IL-1 producing monocytes.

TABLE I

SEPARATION AND PURIFICATION OF MONOCYTES FROM FRESH NORMAL HUMAN PERIPHERAL BLOOD MONONUCLEAR LEUKOCYTES

Subject number	Total PBML	Non adherent cells	Adherent cells	Adherent cell population	
			% NSE +ve [a]	Total Mϕ [b]
1	90,000	69,000	21,000	99	20,790
2	94,000	83,000	11,000	94	10,340
3	94,000	84,000	10,000	95	9,500
4	100,000	83,000	17,000	97	16,490
5	117,000	100,000	17,000	94	15,980
6	83,800	80,000	3,800	96	3,648
7	63,000	61,000	2,000	97	1,940

[a] NSE, non-specific esterase staining.
[b] Mϕ, total numbers of monocytes in each plate.

Effect of anti-cytokine antibodies on the development of IL-1-produced colonies

Table I shows the yield of monocytes after separation from PBML of seven normal human subjects by surface adherent technique.

Table II shows the effect of anti-IL-1, anti-IL-6 and anti-TNF-α on the development of IL-1-producing monocytes from normal subjects. Anti-IL-1 at low concentration was able to partially inhibit the development of IL-1-induced colonies. When the concentration of anti-IL-1 was increased, the numbers of IL-1-induced colonies were gradually decreased until completely inhibited. However, anti-IL-6 and anti-TNF-α have a very low effect on the number of IL-1-induced colonies.

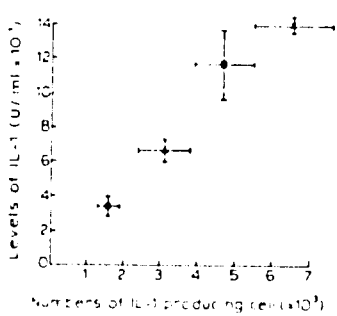

Fig. 4. Correlation of IL-1 levels and numbers of IL-1-producing monocytes in four different concentrations of monocytes. Each concentration consisted of three normal subjects (● = mean ± SD of 2.5 × 10⁵ PBML, ▲ = mean ± SD of 5 × 10⁵ PBML, ■ = mean ± SD of 1 × 10⁶ PBML, + = mean ± SD of 2 × 10⁶ PBML).

Numbers of IL-1-producing monocytes in healthy normal individuals

The numbers of IL-1-producing monocytes per 1.8 × 10⁵ monocytes were determined from seven healthy Thai individuals. The number of IL-1-producing monocytes ranged from 819 to 1930 cells/10⁵ monocytes with an average of 1344 ±

TABLE II

EFFECTS OF ANTI-IL-1, ANTI-IL-6 AND ANTI-TNF-α ON INTERLEUKIN-1-PRODUCING MONOCYTES FROM HUMAN PERIPHERAL BLOOD MONONUCLEAR LEUKOCYTES

Subject number	No anti-serum added	Dilution of antisera added		
		1:2500	1:500	1:100
Anti-IL-1				
1	708 [a]	402 (43.2) [b]	288 (59.3)	34 (95.6)
2	484	388 (19.8)	80 (83.5)	0 (100)
3	698	582 (16.6)	296 (57.6)	0 (100)
4	1,121	982 (12.4)	421 (62.4)	ND [c]
Anti-IL-6				
1	1121	1,150 (-2.6)	925 (17.5)	ND
2	750	722 (3.7)	710 (5.3)	770 (-2.7)
Anti-TNF-α				
1	1,121	1,063 (5.2)	978 (12.8)	989 (11.8)
2	750	710 (5.3)	762 (-1.6)	720 (4.0)

[a] Numbers of IL-1 producing monocytes from adherent 1 × 10⁶ PBML, 2 ml in plastic culture plate.
[b] Figures in parentheses are expressed as percent inhibition of IL-1 producing cells.
[c] ND, not done.

TABLE III

NUMBERS OF IL-1-PRODUCING MONOCYTES, IL-1 ACTIVITY AND UNIT OF IL-1 PER MONOCYTE FROM NORMAL SUBJECTS

Subject number	IL-1-producing monocytes per 10^5 monocytes	IL-1 activity (U/10^5 monocytes/ml)	IL-1 activity (mU/monocyte)
1	1,664	23.0	13.8
2	1,930	35.6	18.4
3	1,927	80.0	41.5
4	924	11.7	12.7
5	993	85.9	86.5
6	1,150	44.8	39.0
7	819	18.6	22.7
$\bar{X}$	1,344	42.8	33.5
SD	483	29.6	26.0
SEM	182	11.2	9.8

Note (1) When 16×10^6 thymocytes were cultured alone in soft agar plate, 75 ± 25 spontaneous colonies appeared.

(2) Monocytes alone derived from 1×10^6 PBML resulting in 0 colony.

(3) The numbers of IL-1-producing monocytes shown in the table are already corrected by subtracting the background.

182 cells/10^5 monocytes (mean ± SEM). The level of IL-1 activity from the same normal subjects ranged from 11.7 to 85.9 U/10^5 monocytes per ml with an average of 42.8 ± 11.2 U. The IL-1 activity per one monocyte/ml ranged from 12.7 to 86.5 mU with an average of 33.5 ± 9.8 mU as shown in Table III. When 16×10^6 thymocytes or monocytes derived from 1×10^6 PBML were cultured alone in the soft agar plates, the average numbers of spontaneous colonies were 75 colonies/16×10^6 thymocytes (mean ± SEM = 75 ± 25 colonies) and 0 colonies/monocytes derived from 1×10^6 PBML respectively.

Discussion

The soluble mediators produced by cells of the lymphoreticular system play major roles in immune responses in the defense mechanism of the host. One of these major mediators, interleukin-1, exerts a wide variety of immunoregulatory effects (Dinarello, 1984). The capacity of IL-1 to augment thymocyte proliferation and, to a lesser extent, T lymphocyte growth, presumably leads to expansion of the lymphocyte population (Dinarello, 1989). IL-1 promotes the specific cytotoxic functions of cytotoxic T lymphocytes (CTL) and natural killer (NK) activity of human peripheral blood mononuclear cells in conjunction with IL-2 and IFN-γ. IL-1 also augments the in vitro proliferation, differentiation, and antibody-producing functions of B lymphocytes. In addition to these immunologic activities, IL-1 promotes the growth or functional activities of a wide variety of non-lymphocytic cell types. These pleiotropic effects of IL-1 can account for a number of manifestations of acute and chronic inflammatory reactions. Both the functional activities and the molecular structures of IL-1 have also been illustrated. At least two monocyte-derived IL-1 species have been identified and sequenced both in man and mouse (March et al., 1985; Oppenheim et al., 1986). These molecules, named IL-1α and IL-1β, although having only 28% amino acid homology, have the same biological activities and bind to the same receptor (Kilian et al., 1986; Rupp et al., 1986).

There are many methods for detecting IL-1 levels in cell culture fluid. The method for IL-1 level assay commonly used is the proliferation of mouse thymocytes stimulated with IL-1 alone or in the presence of IL-1 and low concentrations of mitogenic lectin (Pajetkau et al., 1976). IL-1 activity can be detected when IL-1 is incubated with certain T cell tumor lines. These tumor lines produced IL-2 when stimulated with IL-1, and the IL-2 released can be quantitated by an IL-2-dependent T cell line (Gillis and Mizel, 1981). The leukocyte pyrogen test can also be used to detect IL-1 activity, wherein the increase in body temperature is measured following in vivo administration of the IL-1 preparation (Duff and Durum, 1983). The significant activities of IL-1 on various cell types have prompted studies of the effects of IL-1 in several diseases. Measurement of IL-1 levels in different conditions provide a better understanding of the disease processes. The unit amount of IL-1 per cell culture can be determined by these IL-1 assays. However, the numbers of monocytes that can produce and secrete IL-1 cannot be determined by these assays.

Therefore, we developed a simple and straightforward method for measuring the numbers of IL-1-producing monocytes by overlayering adhering monocytes with thymocytes and PHA-P in semisolid agarose. This resulted in clusters or colonies of thymocytes which responded to IL-1 produced from monocytes.

The optimal conditions for the production and enumeration of IL-1-producing monocytes have been determined in our laboratories. The optimal stimulation time of LPS was 4 h for maximum development of IL-1-producing monocytes. Our data are in good agreement with others that the number of IL-1-producing monocytes are increased tremendously when stimulated with LPS. Bayne et al. (1986) showed that unstimulated monocytes did not contain immunocytochemically detectable levels of IL-1. However, after stimulation with LPS and/or PHA, fluorescent staining of monocytes became apparent after 3.5 h stimulation. The production of IL-1 by normal human peripheral blood mononuclear leukocytes can be detected as early as 30 min intracellularly, and extracellularly within 1 h after stimulation with LPS. Most of the IL-1 activity (97%) was found in the cytosol of LPS-stimulated monocytes (Matsushima et al., 1986).

Using specific monoclonal antibodies to detect IL-1α- and IL-1β-producing cells by an immunofluorescent stain, no staining was seen in unstimulated PBML, but intense, maximal staining of approximately 5% for each type was seen in PHA and PMA stimulated PBML (Barkley et al., 1989). Our study, revealed that the average number of IL-1-producing monocytes was 1344 ± 182 cells/10^5 monocytes. Although our enumeration of IL-1-producing monocytes by semisolid agar resulted in a lower number when compared to the immunofluorescent technique, nevertheless, the principle of each technique was different. The immunofluorescent method will detect IL-1 on the surface of cells, however, the semisolid agar will only detect cells producing and secreting functional IL-1.

The mean numbers of IL-1-producing monocytes and the mean IL-1 levels produced by monocytes from the same normal subjects were highly correlated (r = 0.981). The colonies of thymocytes as detected by our method developed as a result of actual thymocyte proliferation in response to IL-1 produced from monocytes. Evidence for this is the observation that treatment with anti-IL-1 decreased the number of colonies in a dose-dependent manner. The colonies of thymocytes were abolished completely when anti-IL-1 incorporated in agarose was increased to a high concentration. However, anti-IL-6 as well as anti-TNF-α had little or no ability to decrease the number of colonies. This method provides a biological assay for quantitation of IL-1-producing cells by measuring the functional capability of the cell in synthesis and secretion of IL-1. This simple and straightforward technique for measuring the numbers of IL-1-producing cells should therefore be useful for studies seeking to understand the mechanisms of IL-1 production and the cellular immunoregulation in various infections and immunological disorders.

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References

Aarden, L.A., Brunner, T.K., Cerottini, J.C., Dayer, J.M., et al. (1979) Revised nomenclature for antigen nonspecific T cell proliferation and helper factors. J. Immunol. 123, 2928.

Barkley, D., Feldmann, M. and Mann, R.N. (1989) Live detection by immunofluorescence of distinct cell populations producing interleukin-1α and interleukin-1β in activated human peripheral blood. J. Immunol. Methods 120, 277.

Bayne, E.K., Rupp, E.A., Limnico, G., Chin, J. and Schmidt, J.A. (1986) Immunocytochemical detection of interleukin-1 within stimulated human monocytes. J. Exp. Med. 163, 1267.

Boyum, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. 21 (suppl. 97), 77.

Conlon, P.J. (1983) A rapid biologic assay for the detection of interleukin-1. J. Immunol. 131, 1280.

Dinarello, C.A. (1984) Interleukin-1. Rev. Infect. Dis. 6, 51.

Dinarello, C.A. (1989) Interleukin-1 and its biologically related cytokines. Adv. Immunol. 44, 183.

Duff, G.W. and Durum, S.K. (1983) The pyrogenic and mitogenic actions of interleukin-1 are related. Nature 304, 449.

Gearing, A.J.H., Johnstone, A.P. and Thorpe, R. (1985) Production and assay of the interleukins. J. Immunol. Methods 83, 1.

Gillis, S. and Mizel, S.B. (1981) T cell lymphoma model for the analysis of interleukin 1 mediated T-cell activation. Proc. Natl. Acad. Sci. U.S.A. 78, 1133.

Giulian, D. and Lachman, L.B. (1985) Interleukin-1 stimulation of astroglial proliferation after brain injury. Science 228, 497.

Kilian, P.L., Kaffka, K.L., Stern, A.S., Woehle, D., Benjamin, W.R., Dechiara, T.M., Gubler, U., Farrar, J.J., Mizel, S.B. and Lomedico, P.T. (1986) Interleukin 1α and interleukin 1β bind to the same receptor on T cells. J. Immunol. 136, 4509.

Lachman, L.B. (1983) Human interleukin 1: purification and properties. Fed. Proc. 42, 2639.

March, C.J., Mosley, B., Larsen, A., Cerretti, D.P., Braedt, G., Price, V., et al. (1985) Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature 315, 641.

Matsushima, K., Taguchi, M., Kovacs, E.J., Young, H.A. and Oppenheim, J.J. (1986) Intracellular localization of human monocyte associated interleukin 1 (IL1) activity and release of biologically active IL1 from monocytes by trypsin and plasmin. J. Immunol. 136, 2883.

Oppenheim, J.J., Kovacs, E.J., Matsushima, K. and Durum, S.K. (1986) There is more than one interleukin 1. Immunol. Today 7, 45.

Paetkau, V., Mills, G., Gerhard, S. and Monticone, V. (1976) Proliferation of murine thymic lymphocytes in vitro is mediated by the Concanavalin A-induced release of a lymphokine (costimulator). J. Immunol. 117, 1320.

Rupp, E.A., Cameron, P.M., Ranawat, C.S., Schmidt, J.A. and Bayne, E.K. (1986) Specific bioactivities of monocyte-derived interleukin 1α and interleukin-1β are similar to each other on cultured murine thymocytes and on cultured human connective tissue cells. J. Clin. Invest. 78, 836.