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PROGRESS REPORT No.4

"EVALUATION IN VITRO OF INTERLEUKIN 1 AND  
INTERLEUKIN 2 AS POSSIBLE IMMUNOTHERAPEUTIC  
AGENTS IN LEPROSY"

A RESEARCH PROJECT  
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## 1. BACKGROUND/INTRODUCTIONS:

Leprosy is a chronic infectious disease characterized by a unique spectrum of human immune responses to a single causative agent, M. leprae. Lepromatous patients, at one extreme, are almost totally but specifically non-responsive to M. leprae in vivo and in vitro; strong delayed hypersensitivity and cell-mediated immunity (CMI) mark tuberculoid patients at the other extreme, and most patients exhibit various intermediate (borderline) CMI responsiveness. Various defects in CMI have been demonstrated both in vivo and in vitro, especially in lepromatous patients, but their underlying cause and pathogenesis are unknown.

Recent developments in immunology and molecular biology now make it plausible to hypothesize that the range of aberrant CMI in leprosy is due to inadequate or inappropriate production of IL1 and/or IL2 by host mononuclear cells, resulting in a range of immunoregulatory disturbances as is observed in the immunopathologic spectrum of leprosy. If this is true, host immunity to M. leprae might be modified by immunotherapy to "normalize" IL1 and/or IL2 production or responsiveness; if false, it would exclude a number of presently known immunoregulatory mechanisms and re-direct work toward other immune cells and interactions. This hypothesis will be tested as follows:

## 1. Evaluation of IL1 and IL2 Production In Vitro :

Peripheral blood mononuclear leukocytes (PBML) will be obtained from healthy volunteers and various types of leprosy patients, classified clinically and pathologically. The PBML will be stimulated with concanavalin A, phytohemagglutinin, or *M. leprae* antigens to produce IL2, which will be assayed using an IL2-dependant cultured T cell line (CTLL-2). Adherent monocytes from patients' PBML will be stimulated with lipopolysaccharide (LPS) to produce IL1, which will be assayed using a mouse thymocyte bioassay.

## 2. Evaluation of Patient Lymphocyte Responsiveness to Exogenous IL1 and IL2 as Possible Immunomodulators In Vitro.

IL1 will be obtained from supernatants of LPS-stimulated cultures of normal human monocytes and from commercial sources. IL2 purified from products of a recombinant DNA bacterial strain is available from another laboratory. PBML of different types of leprosy patients, with or without these lymphokines, will be assayed for their ability to divide in response to mitogens, purified *M. leprae*-derived and/or synthesized antigens, and control antigens as a measure of T cell responsiveness. B-cell response to pokeweed mitogen will be measured using a modified reverse hemolytic plaque assay (PFC) and the secretion of IgG and IgM will be measured in culture supernatants using ELISA techniques.

### 3. Determination of Cell Types Required for IL2 Production.

If defects in IL2 production are demonstrated in the above studies, PBML from healthy persons and leprosy patients will be separated into monocyte, B-lymphocyte, T-helper and T-suppressor lymphocytes population by monoclonal antibody rosetting techniques. The different fractions will be assayed in different combinations to determine a) the cell types necessary for optimal IL2 production in response to mitogens and M. leprae antigens, and b) whether any observed defects in IL2 production by leprosy patients' lymphocytes are due to a consistent dysfunction of particular subset(s) or combinations. If such a defect is observed, relevant cell subsets from HLA-D-matched patients and healthy individuals will be co-cultured to see if such defects can be reversed.

### 2. OBJECTIVES:

The overall objectives of this research project are :

- (1) To determine the capability of monocytes and lymphocytes from leprosy patients and healthy subjects to produce IL1 and IL2 respectively.
- (2) To assess the effect, *in vitro*, of exogenous IL1 and IL2 on lymphocytes from leprosy patients.
- (3) Determination of cell types required for IL2 production.

## 3. MATERIALS AND METHODS:

### PART I. PRODUCTION AND DETERMINATION OF IL1.

#### 1. Separation and Purification of Human Monocytes.

Ten to twenty milliliters of heparinized blood is obtained from subjects. The blood is diluted 1:2 with sterile 0.01 M phosphate buffer saline (PBS) pH 7.2 and centrifuged on Ficoll-Hypaque. The peripheral blood mononuclear leukocytes (PBML) is collected, washed 3 times with RPMI 1640 media, cell concentrations adjusted with RPMI 1640 containing 100 units/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamine and 10% heat inactivated fetal calf serum (FCS).

PBML  $2 \times 10^6$  cells in 2.0 ml media are added onto a 35x10 mm. plastic plates, incubated at 37°C 5% CO<sub>2</sub> for 2-4 hrs. Non-adherent cells are washed off 3 times with 10% FCS-RPMI 1640. PBML, non-adherent and adherent cells are stained with nonspecific esterase stain (NSE). The exact number of monocytes adhering on the plate is calculated and the concentration of monocytes can be achieved by adding a certain amount of 10% FCS-RPMI 1640 media.

#### 2. Production and Quantitation of IL1.

##### 2.1 Production of IL1.

The adhered monocyte of normal or leprosy patients on plastic plates are adjusted to  $2 \times 10^5$  cells/ml by adding various amounts of 10% FCS-RPMI 1640 media. Twenty ug/ml of lipopolysaccharide (LPS) are added and incubated at 37°C, 5% CO<sub>2</sub> for 24 hours. Cell suspension is collected and centrifuged

and supernatants assayed for IL1.

## 2.2 Quantitation of IL1.

Thymocytes from 8 week old BALB/C inbred strain mice are used as the indicator cells in IL1 assay. Animals are killed by cervical dislocation and the thymus gland removed under sterile conditions. Thymocyte suspension is prepared by gently homogenizing the thymus gland in a sterile mesh with a sterile rubber plunger. The thymocytes are washed twice with IL1 media, counted, and then adjusted to  $3 \times 10^7$  cells/ml.

Samples for IL1 assay are diluted 1:2, 1:4, 1:8 and 1:16 by IL1 media with a final volume of 600 ul for each dilution. One hundred microliters of thymocyte suspension are added to each tube, and thoroughly mixed. Thirty microliters of PHA-P (conc. 20 ug/ml) are added to each tube and mixed.

All IL1 sample dilutions are assayed in triplicate using 96 well tissue culture plates. Two hundred microliters of the above mentioned mixture are pipetted into each well. The microtiter tissue culture plates are incubated at  $37^\circ\text{C}$  5%  $\text{CO}_2$  for 72 hours. Thymidine incorporation into DNA is measured by adding 25 ul of tritiated thymidine (8 uCi/ml) into each well, mixed, and incubated 2 hrs.

The culture is harvested onto glass microfiber filters using a cell harvester. The glass microfiber filters are dried at room temperature overnight and transferred into scintillation counting vials containing 4.0 ml liquid scintillation fluid. All samples are counted in a liquid scintillation counter (LS 3801 Beckman

Liquid Scintillation Counter, Beckman Scientific Instruments, Inc., Fullerton, CA 92634 U.S.A.).

## PART II. PRODUCTION AND DETERMINATION OF IL2.

### 1. Production of IL2.

PBML,  $1 \times 10^6$  cells/ml are stimulated with 2 ug/ml PHA-P (Wellcome), or 40 ug/ml Con A (Sigma) or 10 ug/ml PPD (Connaught), incubated at 37 C 5% CO<sub>2</sub> 18 hours for PHA-P and Con A and 48 hours for PPD. Cell cultures are harvested and centrifuged, supernatant added with 10 mg/ml of  $\alpha$ -methyl-D-mannoside, filtered sterile and stored at -70 C for IL2 assay.

### 2. Assay of IL2 Activity.

Tested samples are diluted in serial dilution 1:2, 1:4, 1:8, 1:16 to 1:256 with ILL media in 100 ul volume in each well of a 96 well tissue culture plates. One hundred microliters of twice washed CTLL-2 containing  $8 \times 10^3$  cells/100 ul are added into each well, mixed, and incubated 24 hours. Fifty microliters of <sup>3</sup>H-TdR containing 0.2 uCi/50 ul are added into each well and incubated for 18 hours. Cultures are harvested onto glass fiber filters and <sup>3</sup>H-TdR incorporation is determined by a liquid scintillation counter (Beckman LS 3801).

## PART III. PRODUCTION AND DETERMINATION OF INTERFERON.

### 1. Production of Interferon.

PBML,  $1 \times 10^6$  cells/ml, stimulated with PHA-P 2 ug/ml or Con A 40 ug/ml or PPD 10 ug/ml at 37 C, 5% CO<sub>2</sub> for 18 hr for mitogens

and 48 hr for antigen. Supernatants are collected and kept at -20 C.

## 2. Assay of Interferon Activity.

Tested samples are diluted in serial two fold dilutions from 1:2, 1:4, to 1:1024 in 75 ul/well in a 96 well tissue culture plate. Well numbers 11 and 12 are virus and cell controls respectively. FL 5-1 cells are added, 75 ul/well ( $1.5 \times 10^5$  cells/ml). After 24 hours of incubation, 50 ul of Vesicular Stomatitis Virus (VSV) is inoculated into each well containing  $2 \times 10^3$  plaque forming units (pfu), except the cell control wells which are filled with VSV free-culture media. After 24 hours of incubation, complete or 100% CPE is observed in virus control wells, while in cell control wells FL 5-1 is completely intact.

Cells in each well is fixed with 10% formalin for 10 minutes and then stained with 0.4% crystal violet in 70% methanol for 5 minutes. Antiviral activity is expressed in interferon titer units. It is defined as the reciprocal of the highest dilution of the sample reduced by 50% of viral plaque. In our IFN assay, referent laboratory standard IFN were produced by PHA-P (2 ug/ml) stimulation of  $2 \times 10^6$  cells/ml human PBML for 48 hours.

## PART IV. DETERMINATION OF CELL TYPES REQUIRED FOR IL1 PRODUCTION

1. PBML. Heparinized blood was underlaid with Ficoll-Hypaque, centrifuged at 400xG for 30 min. Generally we get PBML with more than 95% purity.

2. Monocytes. Two milliliters of  $1 \times 10^6$  /ml PBML were added onto a 15x80 mm plastic plate, incubated at 37 C 5% CO<sub>2</sub> for 2 hours. Non-adherent cells were washed out twice with warm RPMI medium. Adherent cells on the plastic plate were positive for nonspecific esterase staining more than 90%.

3. T cells. Ten milliliters of  $2 \times 10^6$  /ml PBML were added into a Nylon wool column, incubated at 37 C 5% CO<sub>2</sub> for 1 hour. Non-adherent cells were then eluted with warm RPMI medium. The resulting non-adherent cells were rosetted with AET-SRBC overnight at 4 C. The rosetted T cells were separated from non-rosetted cells by Ficoll-Hypaque gradient centrifugation. AET-SRBC were lysed by hypotonic ammonium chloride solution. When this AET-SRBC rosetting cells were mixed with AET-SRBC, more than 95% give rosette formation.

4. IL2 production. Two milliliters of  $5 \times 10^5$  /ml T cells were added onto plastic plate containing autologous or heterologous monocytes. The cell mixtures were then stimulated with 2 ug of PHA-P for 18 hours. Supernatants from cell culture were assayed for IL2 activity by CTL-2 proliferation.

#### PART V. DEVELOPMENT OF NEW TECHNIQUE.

At the present time we can only detect the level of IL2 in the culture fluid. However if we have a technique which will be able to quantitate the numbers of cells producing IL2, we will open up new possibilities in the study of the immunoregulatory role of IL2 and IL2 production

We have developed a new technique which can quantitate the number of IL2-producing cells from rat spleen. We have also now finished adapting this technique for the human system. Our research article entitled, "Enumeration of Interleukin 2 - Producing Cells from Rat Spleen", has been published in "Asian Pacific Journal of Allergy and Immunology 5:129, 1987." The other research article entitled "Agar Plating Technique for Enumeration of IL2-Producing Cells in Human Peripheral Blood Mononuclear Leukocytes" has been submitted to the "Journal of Immunological Methods" for publication.

#### 4. RESULTS

##### 1. PRODUCTION AND DETERMINATION OF IL2.

Approximately 30 LL/BL, 4 BB, 23 BT/TT and 20 normal subjects were studied for IL2 production in this research period. IL2 production from PBML of LL/BL, BB, BT/TT and normal subjects is not significantly different when stimulated with Con A or PHA-P (figures 1 and 2). However, when stimulated with PPD, IL2 production from LL/BL, BB and BT/TT patients are significantly lower than normal controls (figure 3).

We are trying to see the effect of suboptimal doses of PHA-P and Con A on IL2 production of leprosy patients and normal subjects. When suboptimal doses of PHA-P used at 1, 0.5 and 0.25 ug/ml (optimal dose of PHA-P = 2 ug/ml), there is no significant difference in IL2 production by PBML in LL/BL patients and normal controls at any PHA-P concentrations (Figure

4). When suboptimal doses of Con A used at 20, 10 and 5 ug/ml (optimal dose of Con A = 40 ug/ml), there is also no significant difference in IL2 production between LL/BL patients and normal controls (figure 5).

## 2. EFFECTS OF INTERLEUKIN 2 ON PRODUCTION OF INTERLEUKIN 1 BY MONOCYTES.

Various concentrations of interleukin 2 produced by human PBML stimulated with PHA-P in our laboratory are incubated with monocytes for 2 or 5 hours, washed, then stimulated with LPS. Level of interleukin 1 is assayed by mouse thymocytes proliferation. Figures 6-10 show effect of different doses of IL2 on IL1 production by monocytes of LL patients. Figures 6, 7 and 8 show 6 LL patients' monocytes preincubated with 200, 100, 50 or 25 units/ml of Lab IL2 for 2 hours, washed, then stimulated with LPS. Production of IL1 from IL2-pretreated monocytes are not significantly different from the untreated monocytes. Figures 9 and 10 show the same result in 3LL although IL2 preincubation period is extended to 5 hours. Figure 11 shows thymidine uptake in count per minute (cpm) of external standard (Ultrapure IL1, Human, Genzyme) and internal standard I and II (Human IL1 produced in our laboratory). Our laboratory produced IL1 (lot I and II) have comparable activities to the external standard IL1.

### 3. Effect of Exogenous IL1, IL2 and IL1 + IL2 on Lymphocyte Transformation Test and IL2 Production.

IL1 or IL2 or IL1 + IL2 are mixed with leprosy patients' or normals' PBML with or without lepromin or PPD. Lymphocyte transformation was determined by <sup>3</sup>H-thymidine uptake while IL2 level was determined by proliferation of IL2- dependent 3-day Con A blasts.

Figures 12 and 13 show the effects of laboratory produced human IL1 (Lab. IL1) on lymphocyte transformation of 2 normal and 5 leprosy patients' PBML with and without lepromin or PPD stimulation. IL1 does not have any enhancing or suppressing effect on lymphocyte transformation of normal or leprosy patients' PBML.

Figures 14 and 15 show effect of laboratory produced human IL2 (Lab. IL2) on lymphocyte transformation test of 2 normal and 6 leprosy patients' PBML with and without lepromin or PPD stimulation. Figure 14 shows lymphocyte transformation in 2 normal subjects. Both respond nicely to 50-100 Units/ml of IL2 (Figure 14A), while lepromin and PPD have strong stimulating effect until effect of IL2 is masked by these antigens (Figure 14B and C). These 2 normal controls are PPD skin test positive. Figure 15A shows the ability of PBML of leprosy patients' response to Lab. IL2. When lepromin or PPD is added to the cultures, lymphocyte transformation dose not significantly increase compared to cultures containing only IL2 (see figure 15A, 15B and 15C).

Figure 16 shows the effect of exogenous recombinant IL2 on lymphocyte transformation in 2 normal controls. There are very low response in these 2 normals' PBML to r IL2 (figure 16A). However, when lepromin or PPD is added a nice response in lymphocyte transformation occurs in both normal subjects without any effect from exogenous r IL2 (figure 16B and C). Figure 17A shows 2 LL respond nicely to exogenous r IL2. Adding lepromin does not increase the response. However, adding PPD increases the response (figure 17B and C respectively). Figure 18A illustrates one LL patient and one BT patient having no response to exogenous r IL2. When lepromin is added, only the BT patient shows a good response, while the LL patient has no response to both lepromin and PPD (figure 18B and C). Figure 19A also shows no response to exogenous r IL2 in one LL patient and one BT patient. Again, when lepromin is added only the BT patient responds, while the LL patient does not respond (figure 19B). However, both LL and BT patients respond nicely to PPD (figure 19C).

Figures 20 and 21 show effect of IL1 + IL2 on lymphocyte transformation of leprosy patients and normal PBML. PBML is incubated with 0.5 Unit of IL1 and various different doses of Lab IL2 with or without lepromin or PPD. Figure 20 shows one normal PBML responded nicely to IL1 + IL2 alone or lepromin + IL1 + IL2 or PPD + IL1 + IL2. However, PBML in one LL and one BB patients respond poorly to IL1 + IL2 alone and also poorly to IL1 + IL2 + lepromin or IL1 + IL2 + PPD (figure 21A, B and C).

Figure 22 shows effect of exogenous Lab IL1 on the production of IL2 from PBML of one LL patient, one BT and 2 TT patients. All leprosy patients do not respond to exogenous Lab IL1 or exogenous Lab IL1 + PPD.

Figure 23 depicts effect of exogenous Lab IL2 on the production of IL2 from PBML of one LL, one EL, one BT and 2TT patients. All patients respond nicely to exogenous Lab IL2. The addition of PPD into the culture does not increase IL2 production.

Figure 24 shows levels of IFN production from LL/BL, TT/BT and normal controls. The levels of IFN production in LL/BL, TT/BT and normal subjects are not significantly different when stimulated with PHA-P or Con A (figure 24A and B). However, when stimulated with PPD, the IFN level in normal subjects is significantly higher than in TT/BT and LL/BL (figure 24C).

## 5. DISCUSSION AND CONCLUSION

At this stage of our research, we have completed all of our research goals except one problem we are working on now, namely, what cell types are abnormal for IL2 production in leprosy patients?. We expect to finish in March 1988. In this research period, we have determined IL2 levels in approximately 30 LL/BL, 4 BB, 23 BT/TT and 20 normal subjects. The level of IL2 from LL/BL, BB, BT/TT are not statistically different from normal subjects when PBML is stimulated with mitogens, PHA-P or Con A. However, when PPD is used as the stimulating agent, there is significant difference between LL/BL vs normal, BB vs normal and

TT vs normal.

We have also studied levels of IL2 when PBML is stimulated with suboptimal doses of PHA-P or Con A in 24 LL/BL and 8 normal controls. IL2 level is not significantly different between LL/BL and normal subjects when suboptimal doses of PHA-P or Con A is added.

Effects of exogenous IL2 to IL1 production in leprosy patients are carried out in 9 LL patients. Exogenous IL2 has no effect on IL1 production in LL patients.

We tried to study the effects of exogenous IL1, IL2 or IL1 + IL2 on IL2 production in leprosy patients. However, we found that at least 3 lots of our lepromin were not able to stimulate IL2 production even at a very high concentrations (100  $\mu\text{g}/\text{ml}$ ). Therefore, we have to use lymphocyte transformation test in this study. Our study shows that IL1 does not have any enhancing or suppressing effect on lymphocyte transformation of normal controls or leprosy patients.

From our study, most of PBML from normal subjects are able to respond nicely to exogenous IL2. However, some LL patients respond nicely to exogenous IL2 while some LL patients respond poorly. When exogenous IL1 + IL2 is added into PBML, lymphocyte transformation respond nicely in normal controls, while one LL and one BB responded poorly.

We tried to see the effect of exogenous IL1 or IL2 on the production of IL2. Exogenous IL1 has no effect on IL2 production in leprosy patients. However, exogenous IL2 has enhancing effect on IL2 production in leprosy patients.

Interferon production in normal subjects and leprosy patients has been studied by stimulation of PBML with PHA-P or Con A or IPD. IFN production from LL/BL, BT/TT and normal controls is not significantly different when stimulated with PHA-P, or Con A. However, when PBML is stimulated with PPD, the level of IFN production is significantly different between LL/BL vs normal; TT/BT vs normal; and LL/BL vs TT/BT.

#### 6. WORKPLAN FOR THE NEXT PERIOD

We will try to finish the evaluation of PBML, monocytes, T cells, T helper and T suppressor cells from normal and leprosy patients in the regulation of IL2 production. A final report of all of our research will be prepared for submission to USAID/PSTC program.



Figure 1. Interleukin 2 Production by PBMC of Leprosy and Normal Subjects Stimulated with Con A (40 ug/ml) (  $\bar{x}$  = Mean  $\pm$  S.E.).

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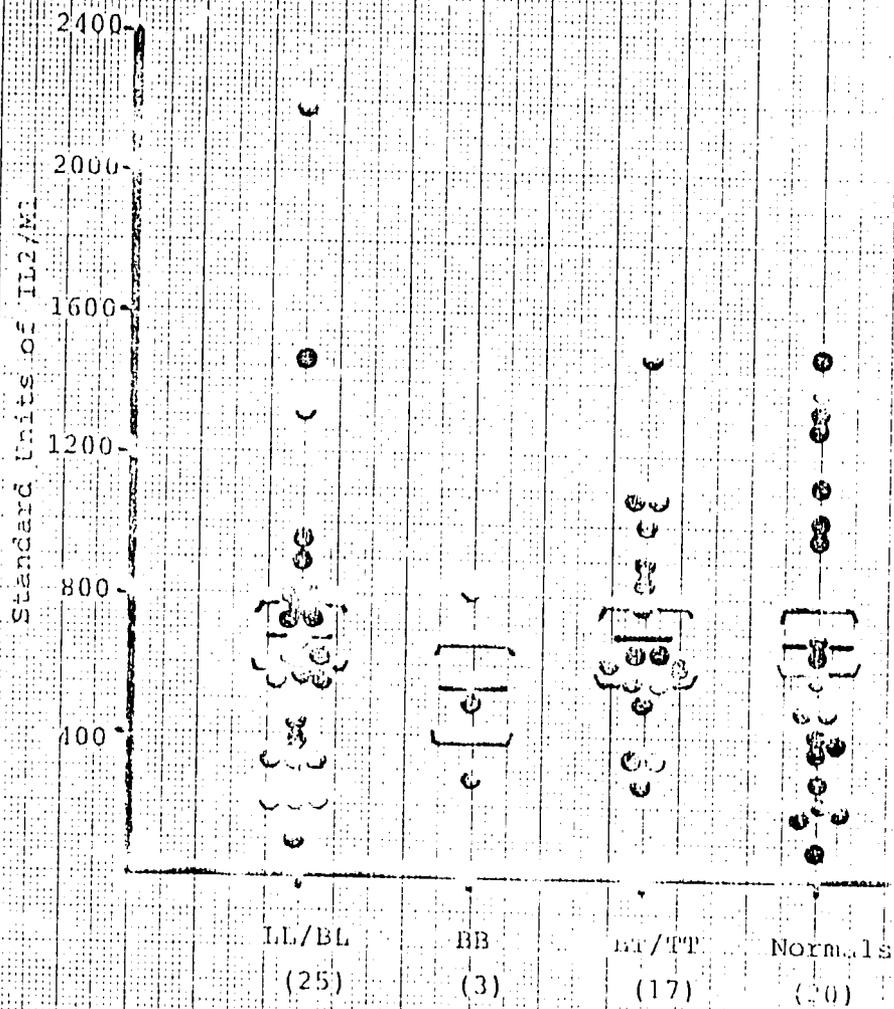


Figure 2. Interleukin 2 Production by PBML of Leprosy and Normal Subjects Stimulated with PHA-P (2 ug/ml) (  $\square$  = Mean  $\pm$  S.E.).

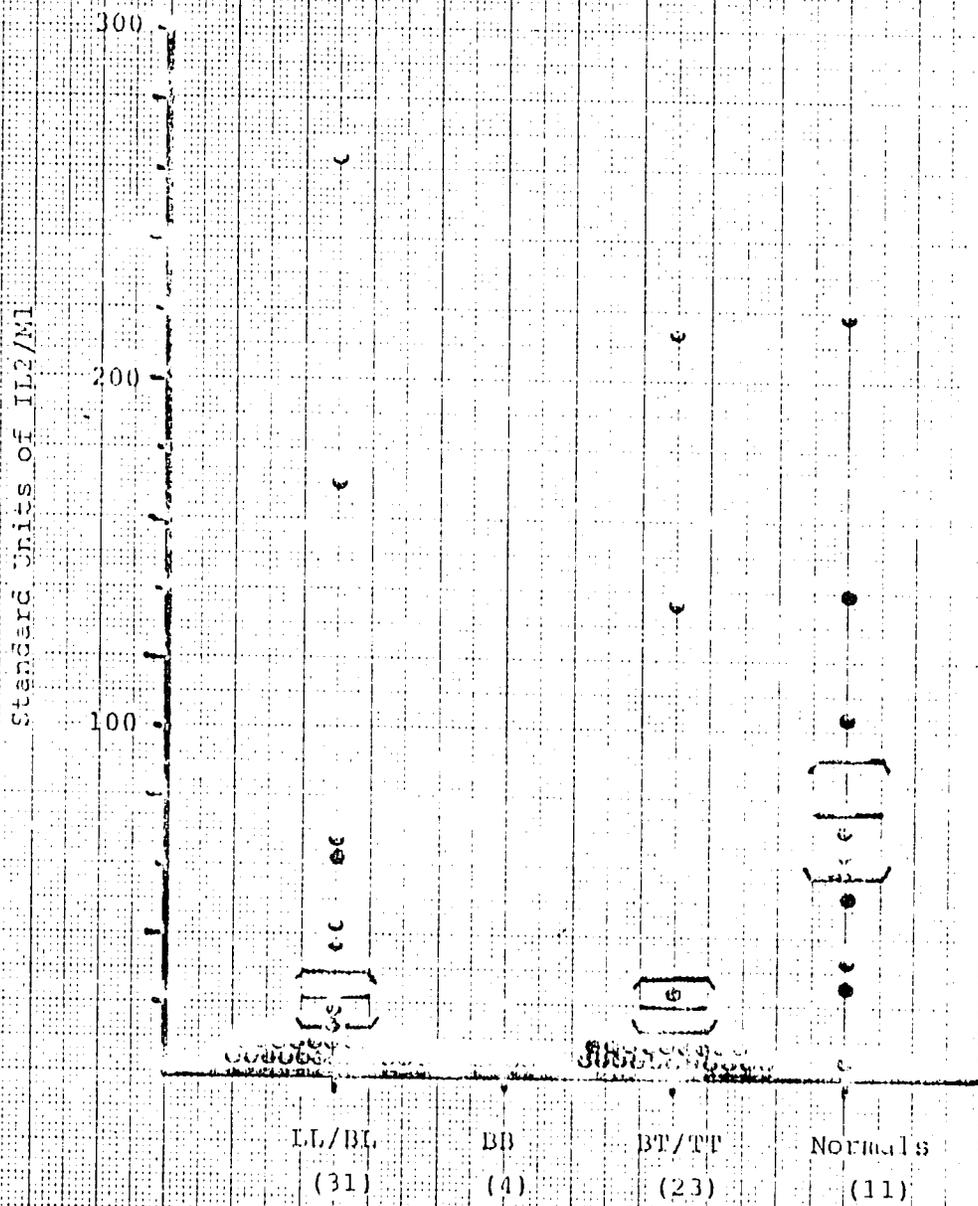


Figure 3. Interleukin 2 Production by PBML of Leprosy Patients and Normal Subjects Stimulated with PPD (10 ug/ml) (  $\square$  = Mean  $\pm$  S.E.).

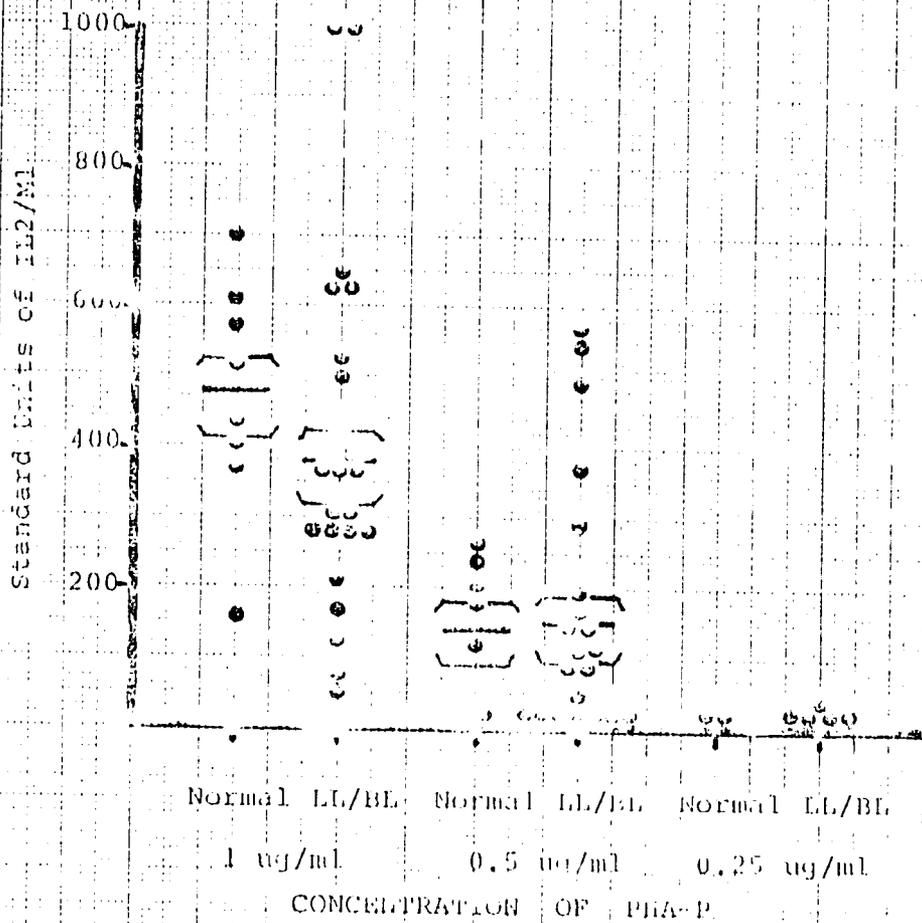


Figure 4. Interleukin 2 Production by PBML of Leprosy Patients and Normal Subjects Stimulated with Suboptimal Doses of PHA-P (  $\bar{x}$  = Mean  $\pm$  S.E.).

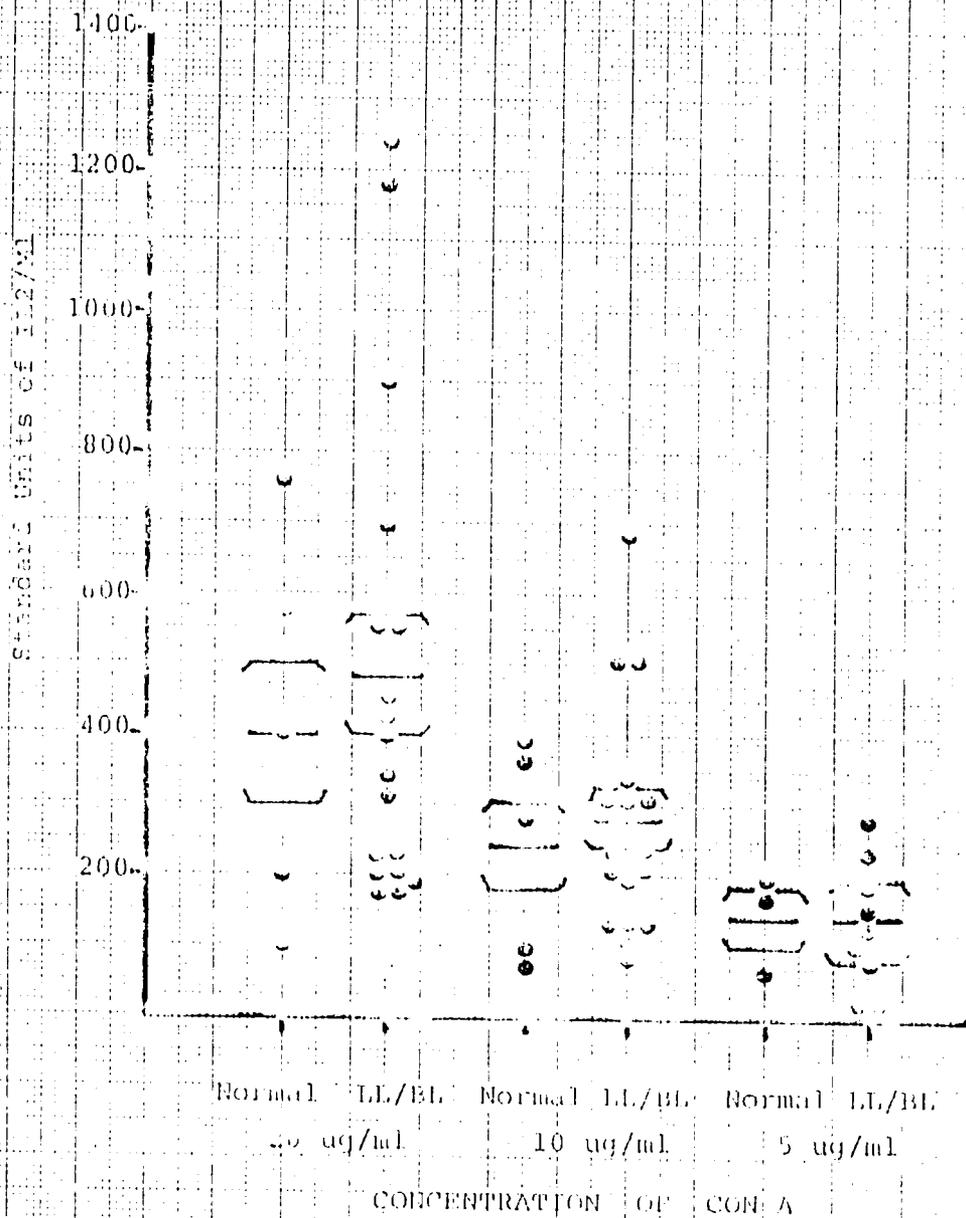


Figure 5. Interleukin 2 Production by PBMC of Leprosy Patients and Normal Subjects Stimulated with Suboptimal Doses of con A (  $\bar{x}$  = Mean  $\pm$  S.E.).

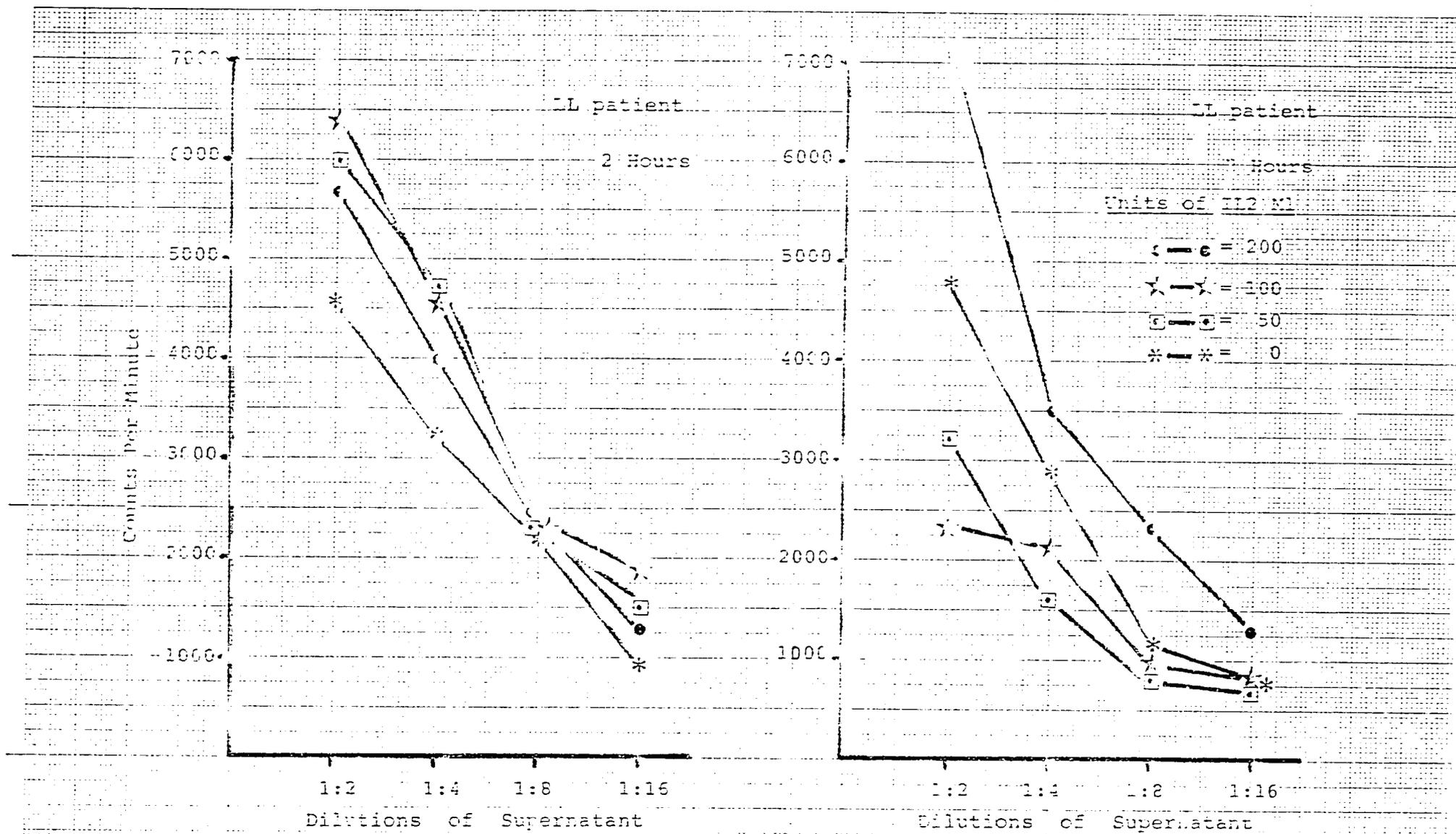


Figure 6. Effect of Interleukin 2 on Interleukin 1 Production by Monocytes of Leprosy Patients.

Counts Per Minute

5000

4000

3000

2000

1000

IL patient

2 Hours

1:2 1:4 1:8 1:16

Dilutions of Supernatant

5000

4000

3000

2000

1000

IL patient

2 Hours

IL2 Units/Ml

● = 200

★ = 100

□ = 50

\* = 0

1:2 1:4 1:8 1:16

Dilutions of Supernatant

Figure 7. Effect of Interleukin 2 on Interleukin 1 Production by Monocytes of Leprosy Patients.

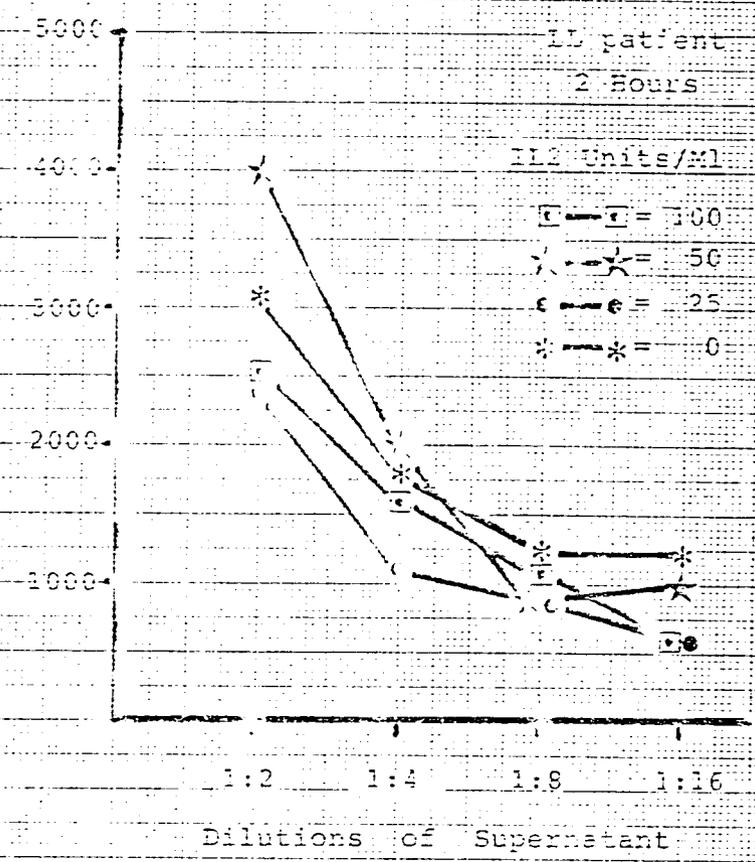
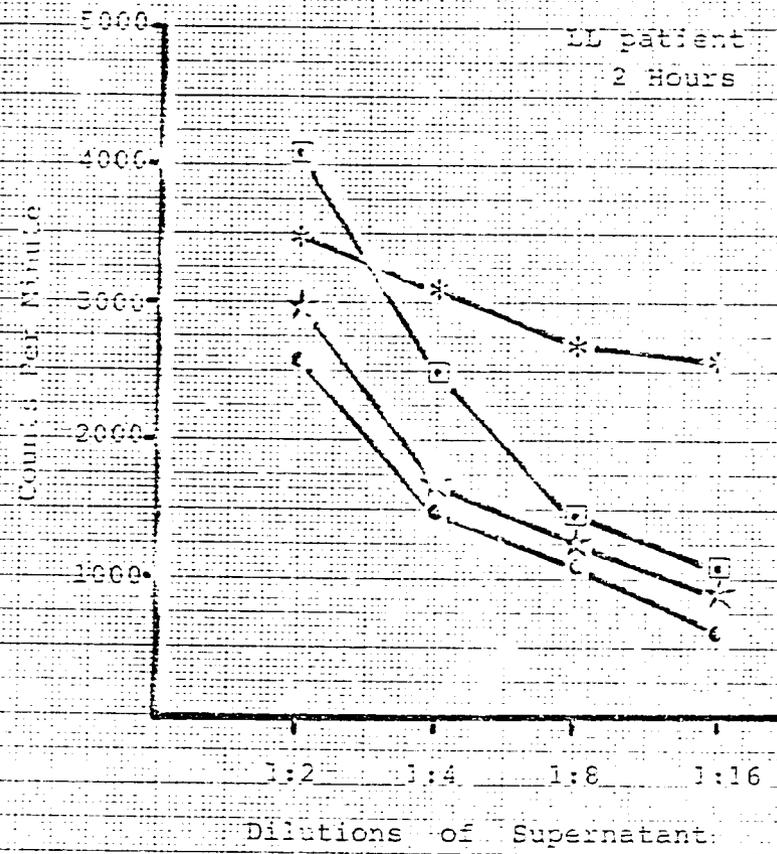


Figure 8. Effect of Interleukin 2 on Interleukin 1 Production by Monocytes of Leprosy Patients.

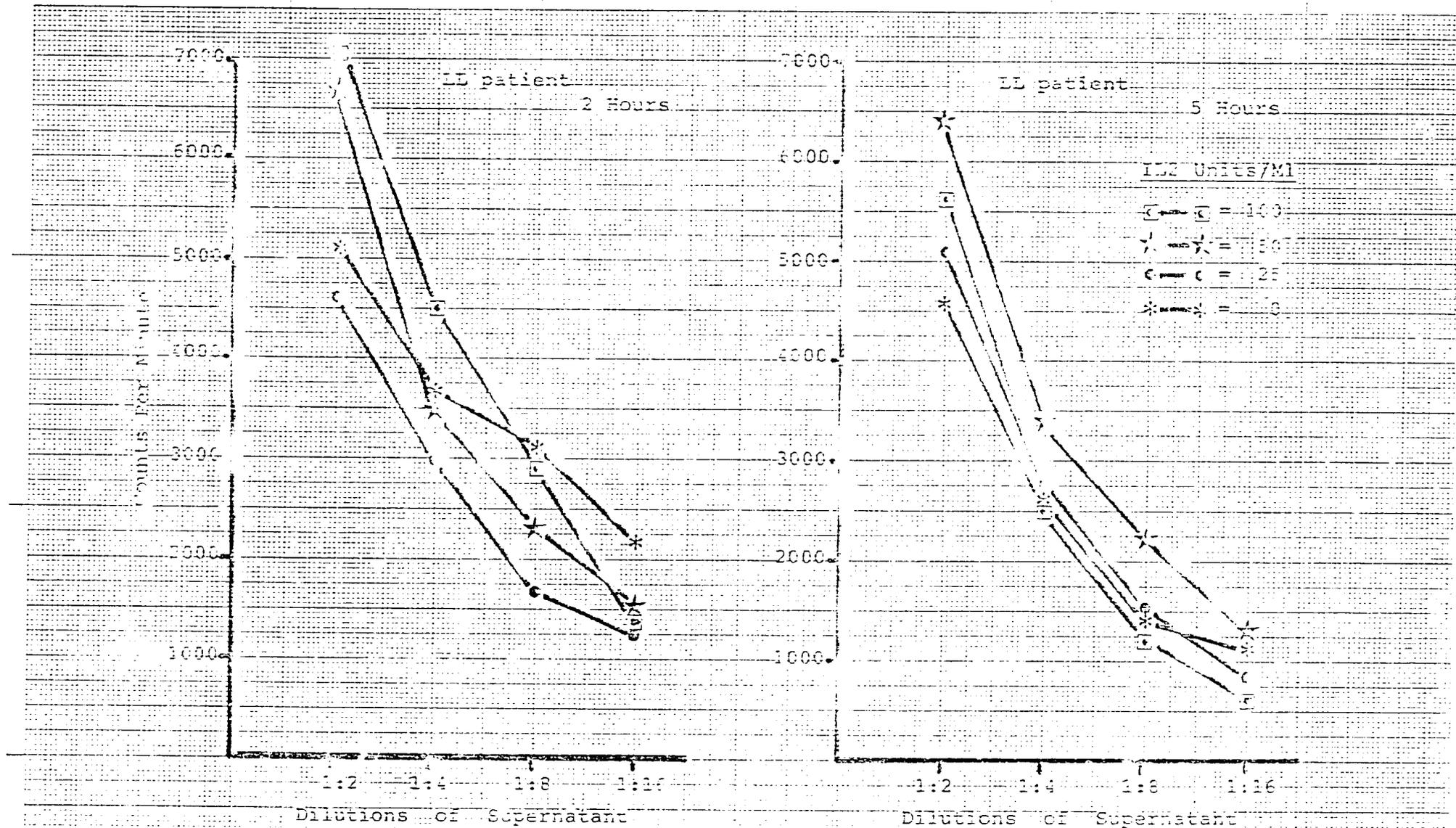


Figure 9. Effect of Interleukin 2 on Interleukin 1 Production by Monocytes of Leprosy Patients.

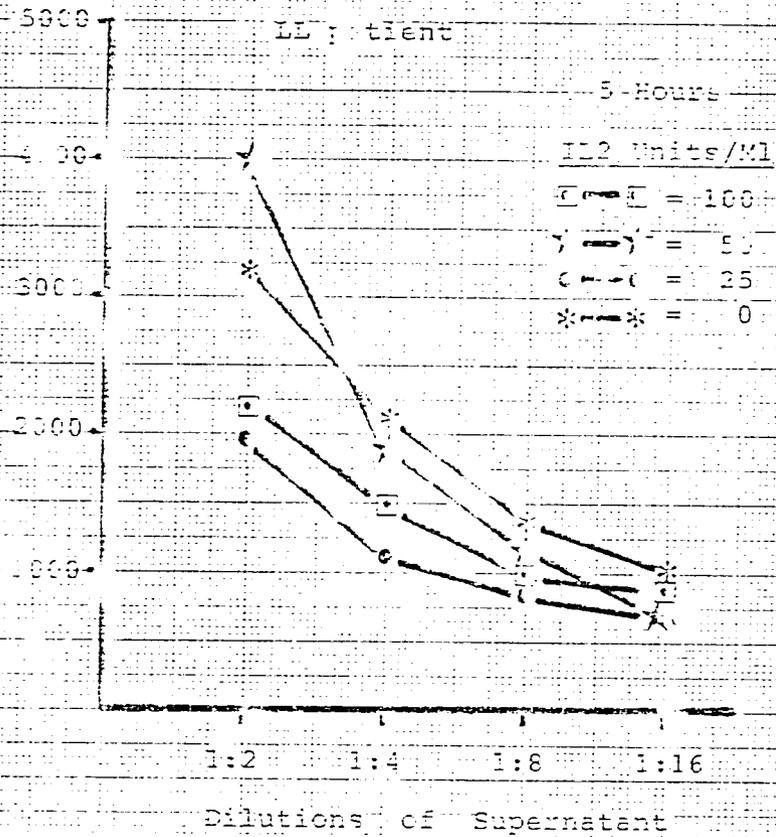


Figure 10: Effect of Interleukin 2 on Interleukin 1 Production by Monocytes of Leprosy Patients.

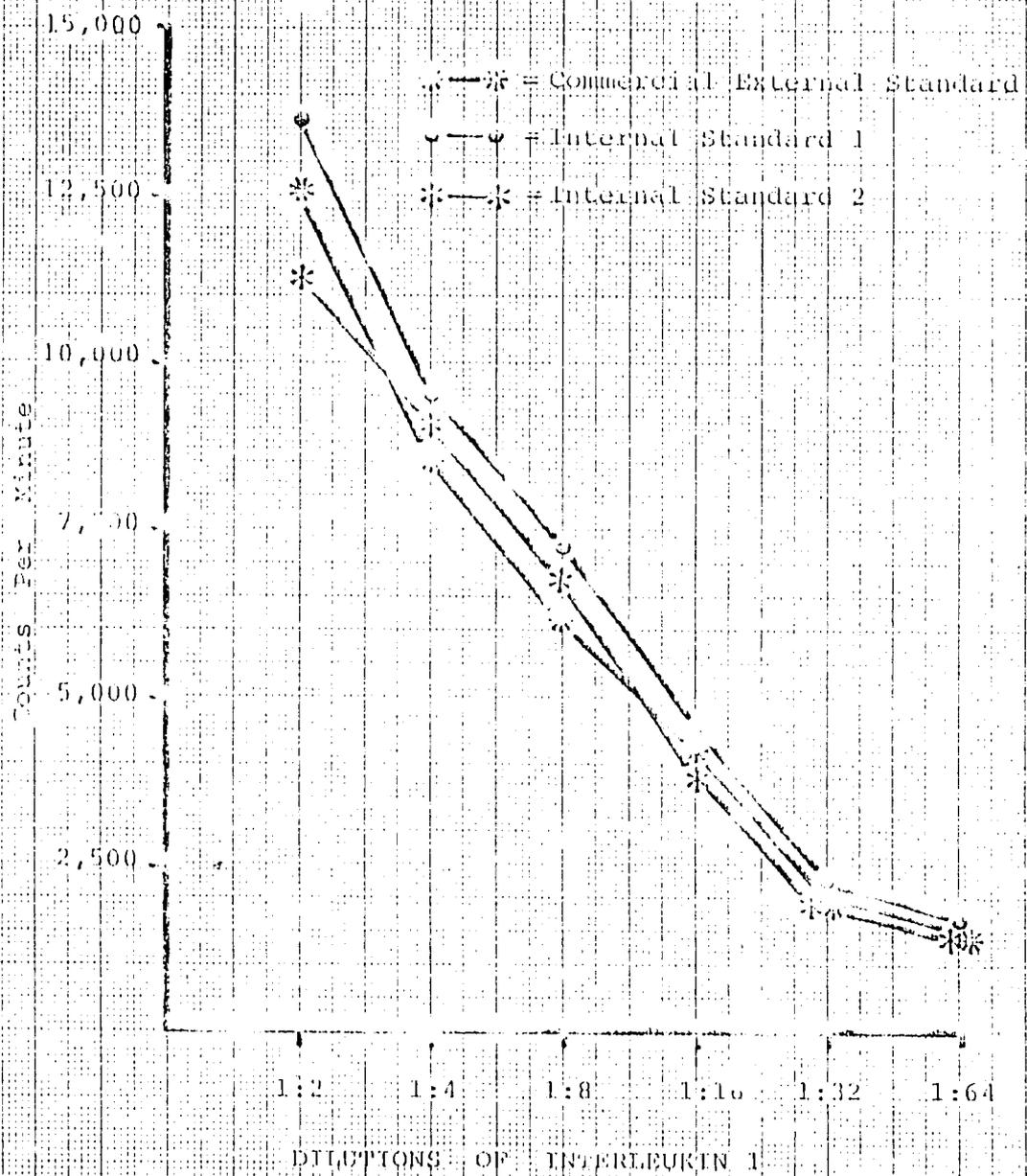


Figure 11. External and Internal Standard III for Control of III Assay.

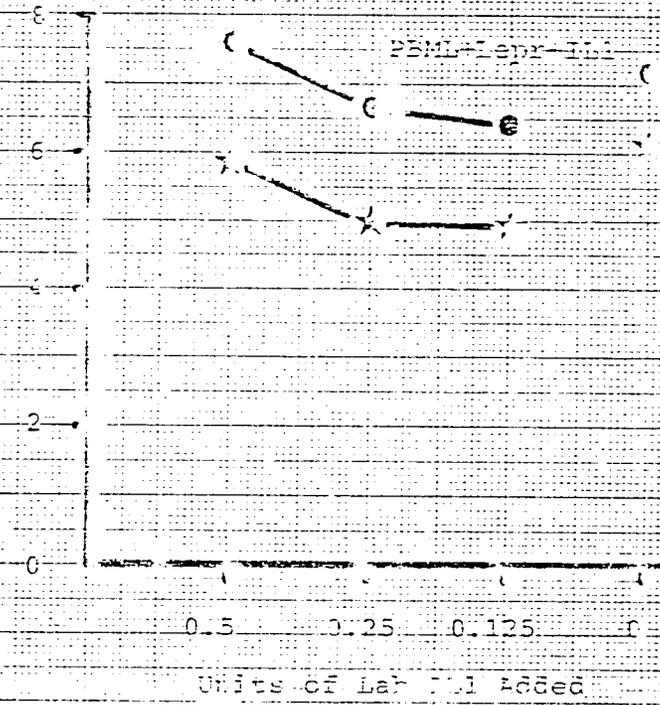
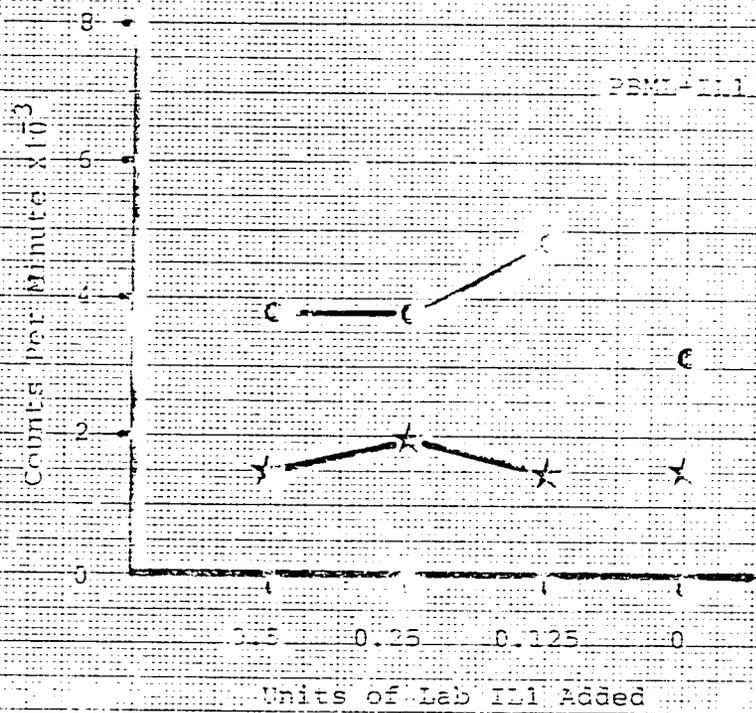


Figure 12. Effect of Exogenous Laboratory Produced IL1 on Lymphocyte Transformation of PBML from Normal Controls.

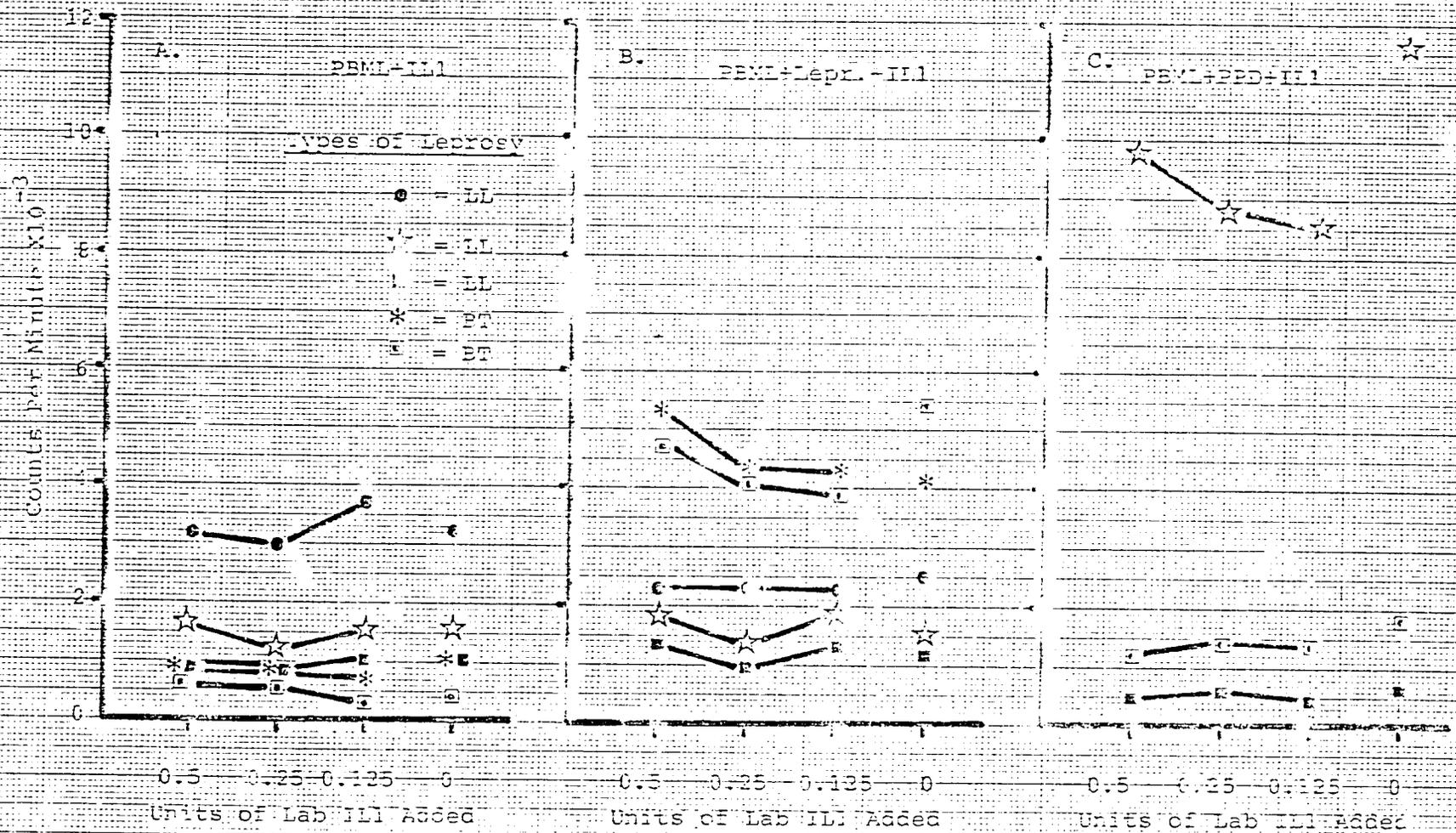


Figure 13. Effect of Exogenous Laboratory Producing III on Lymphocyte Transformation of PBML from Leprosy Patients.

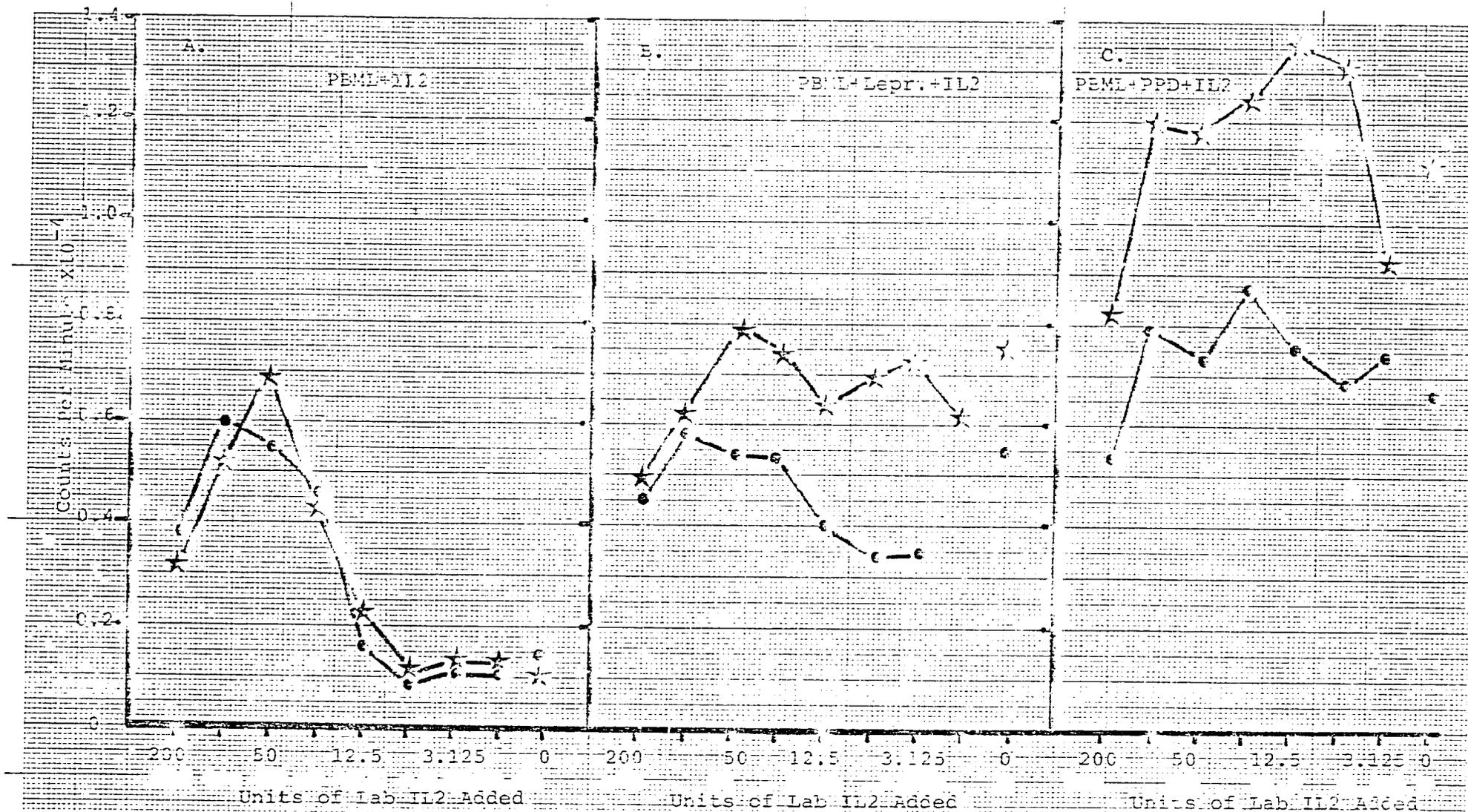


Figure 14. Effect of Exogenous Laboratory Producing IL2 on Lymphocyte Transformation of PEML from Normal Subjects.

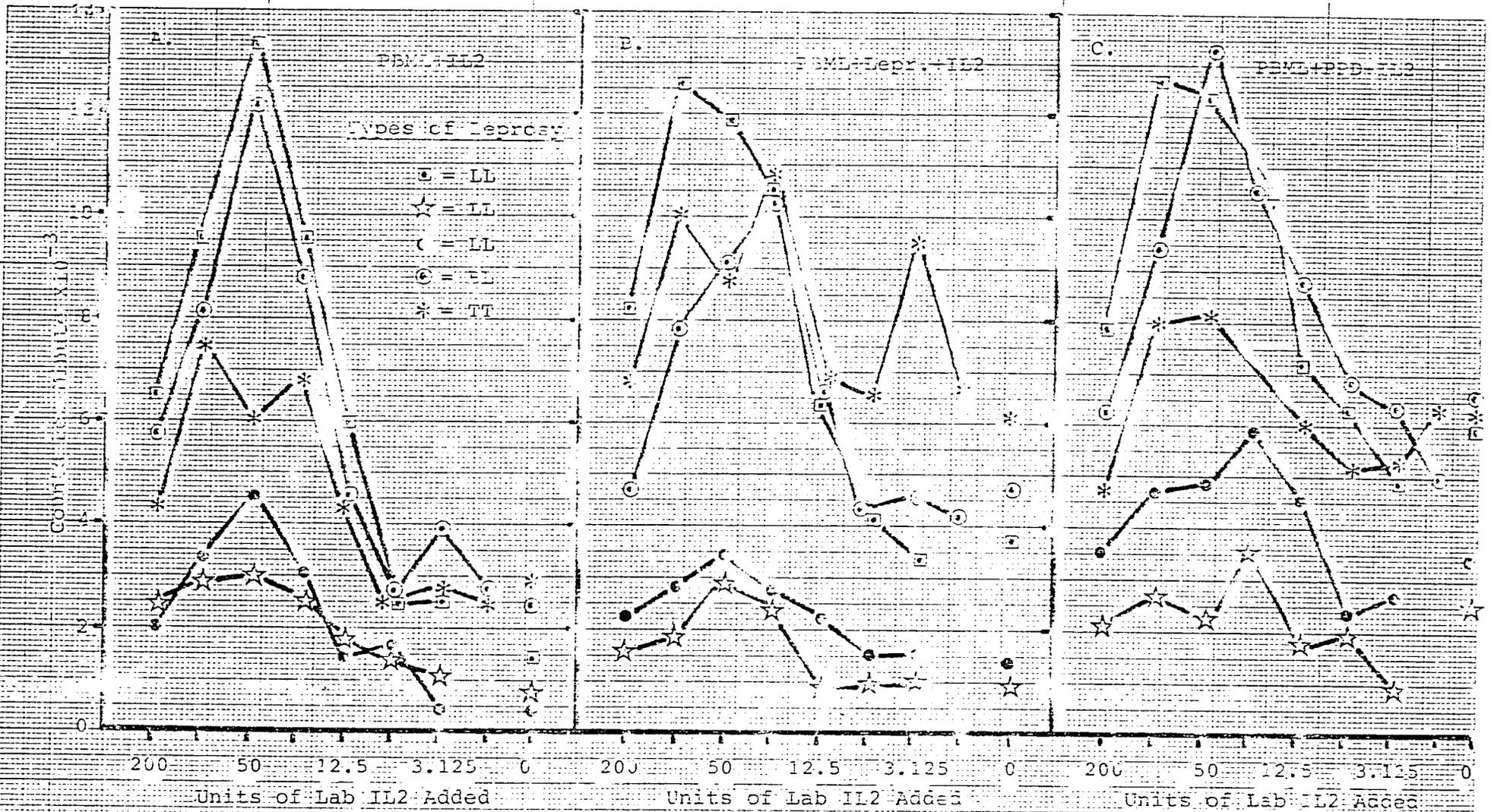


Figure 15. Effect of Exogenous Laboratory Producing IL2 on Lymphocyte Transformation of PBML from Leprosy Patients.

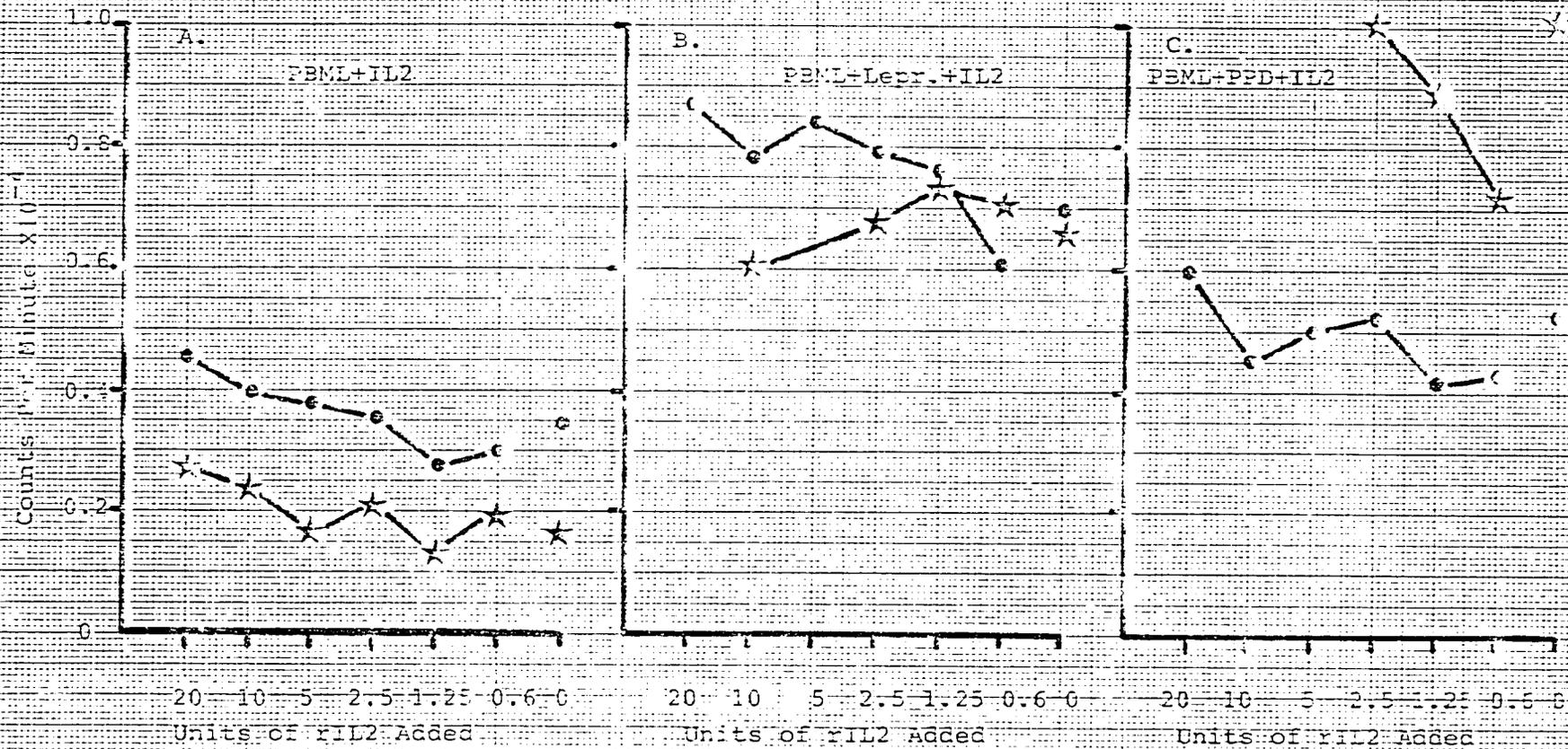


Figure 16. Effect of Exogenous Recombinant IL2 on Lymphocyte Transformation of Normal Controls.

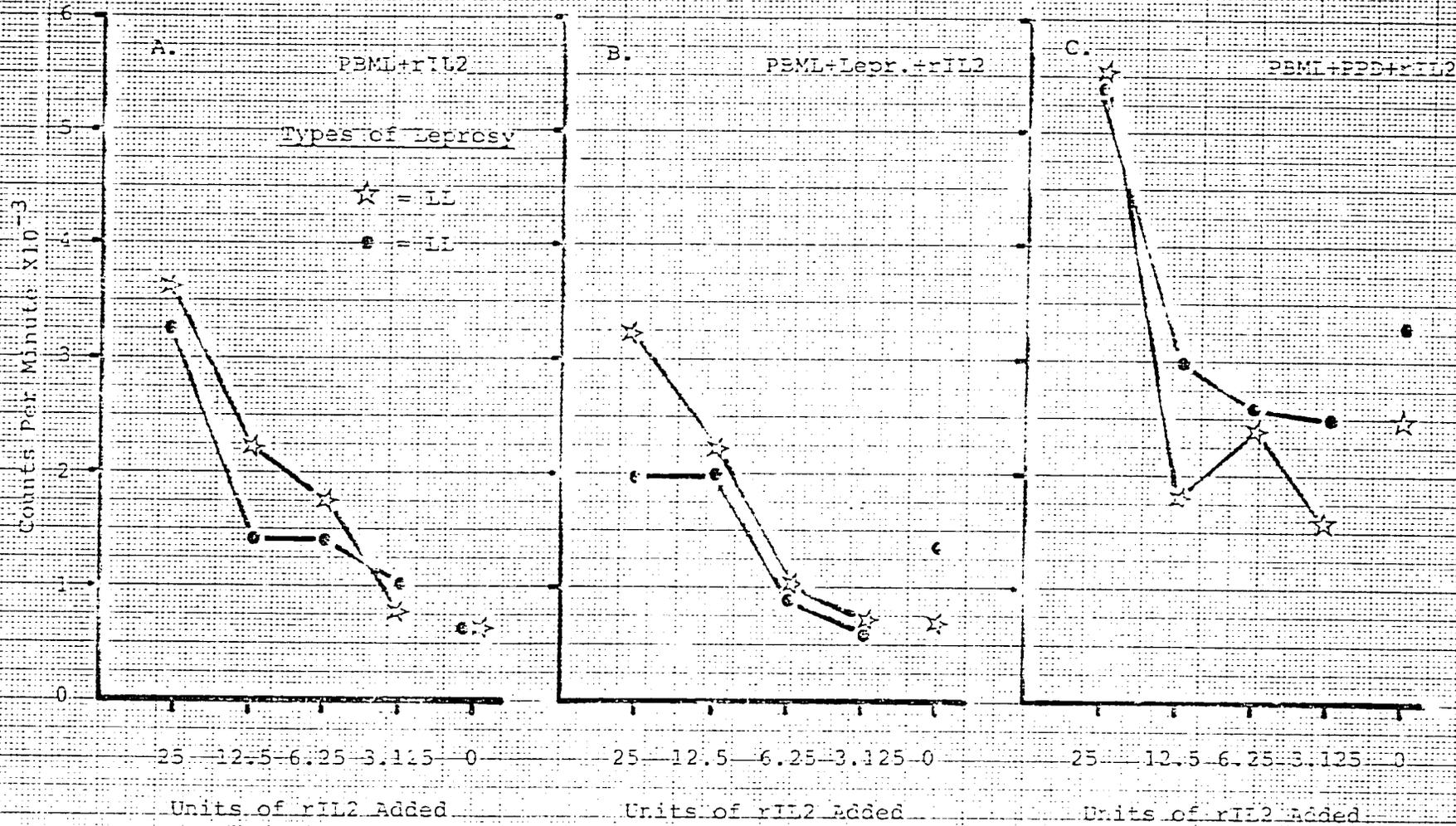


Figure 17. Effect of Exogenous Recombinant IL2 on Lymphocyte Transformation of Leprosy Patients.

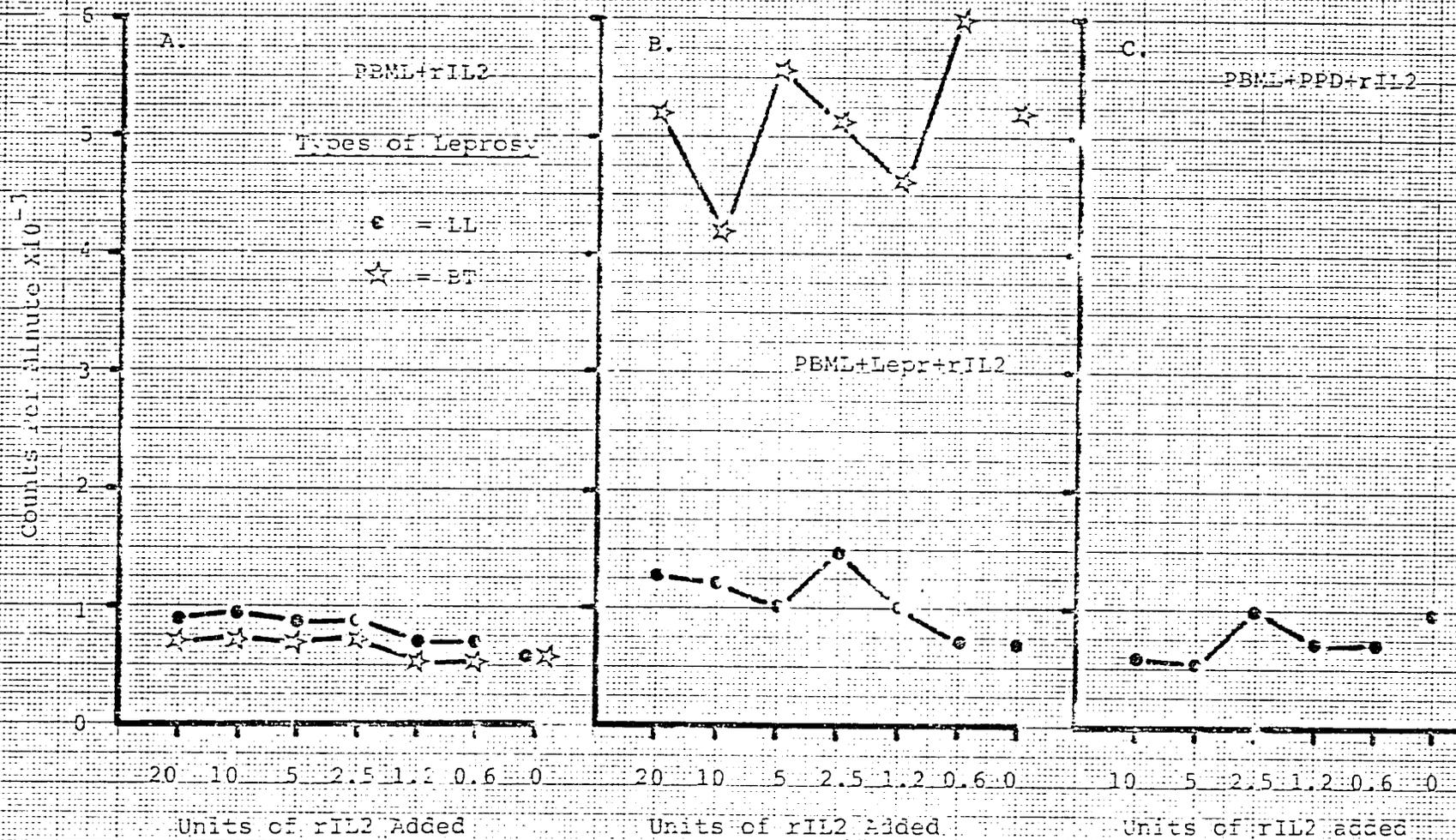


Figure 18. Effect of Exogenous Recombinant IL2 on Lymphocyte Transformation of Leprosy Patients.

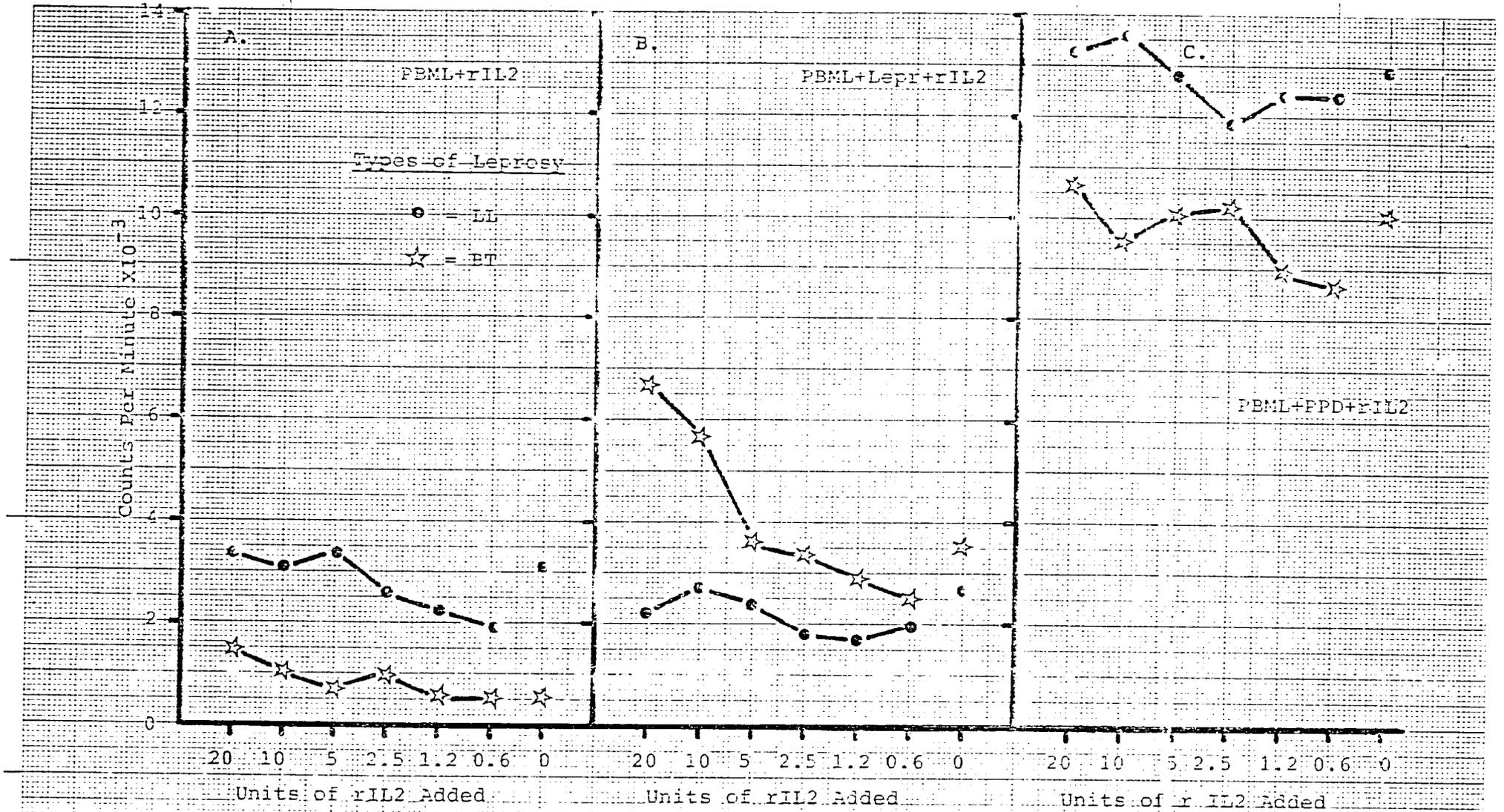


Figure 19. Effect of Exogenous Recombinant IL2 on Lymphocyte Transformation of Leprosy Patients.

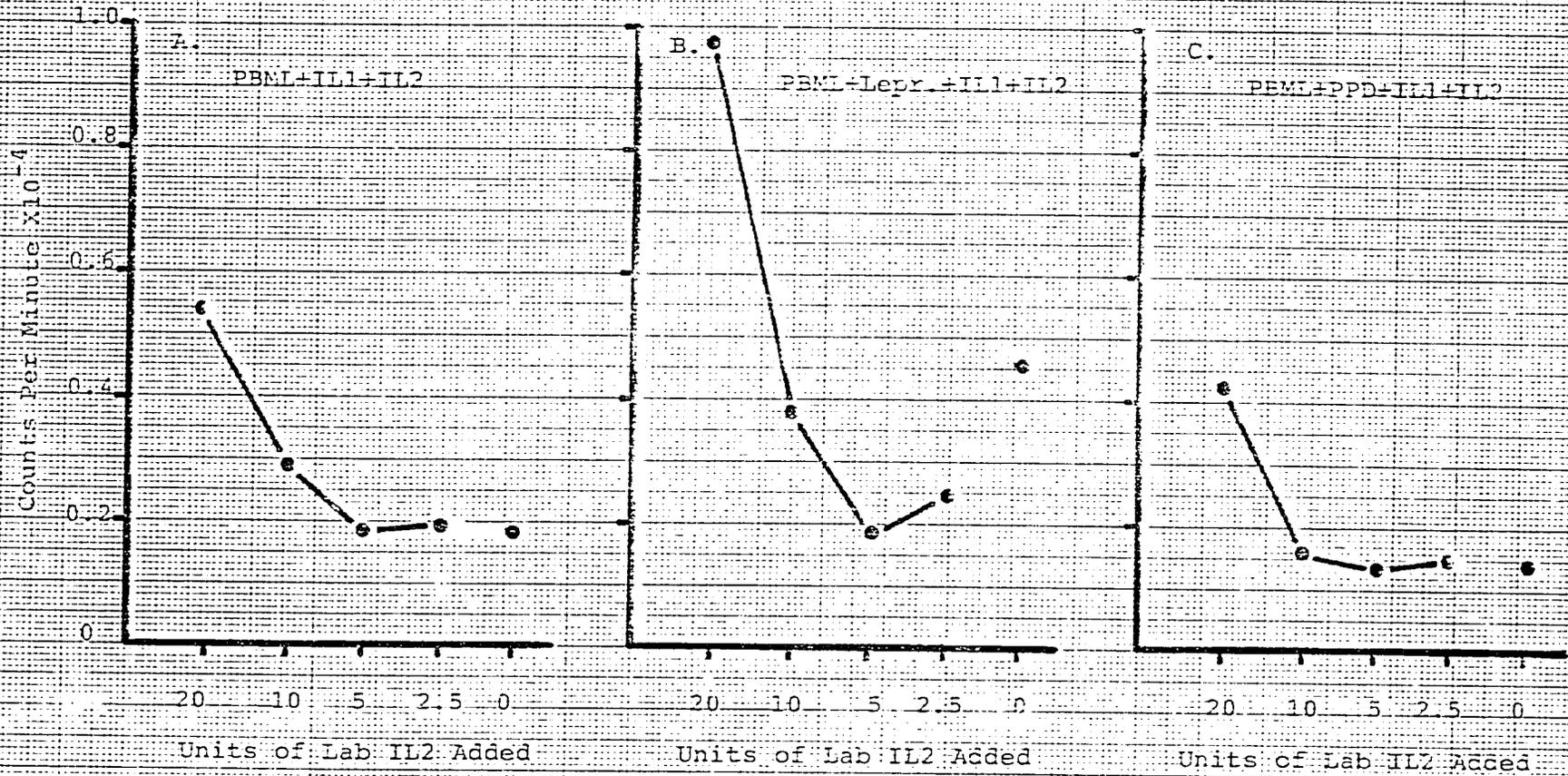


Figure 20. Effect of Exogenous IL1+IL2 on Lymphocyte Transformation of Normal Controls.

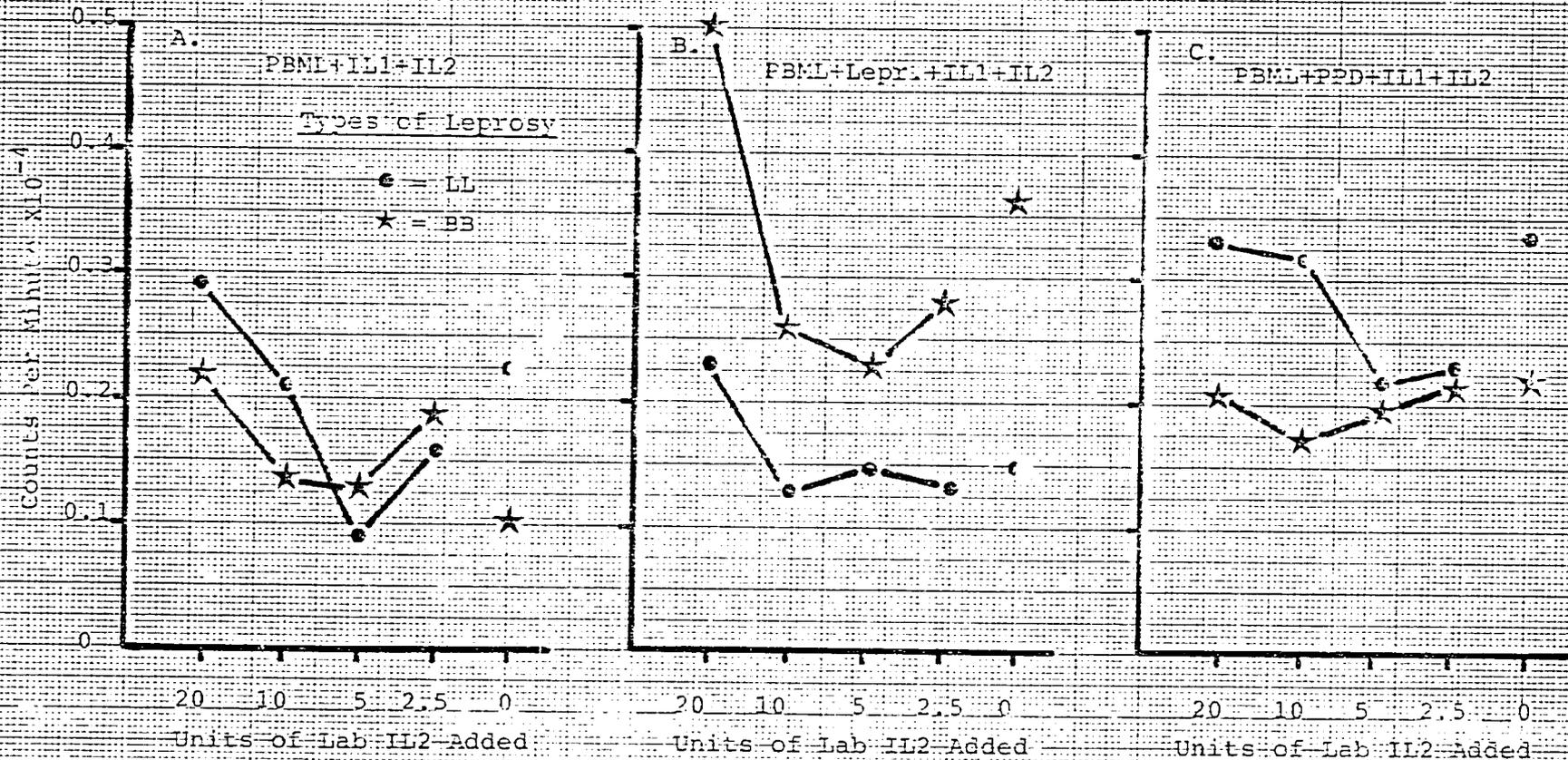


Figure 21. Effect of Exogenous IL1+IL2 on Lymphocyte Transformation of Leprosy Patients.

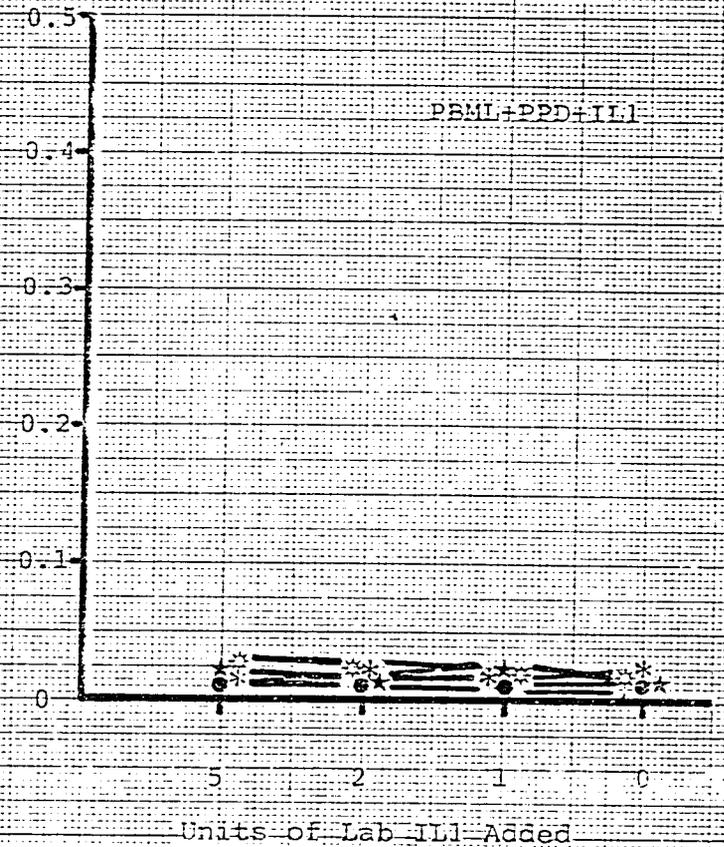
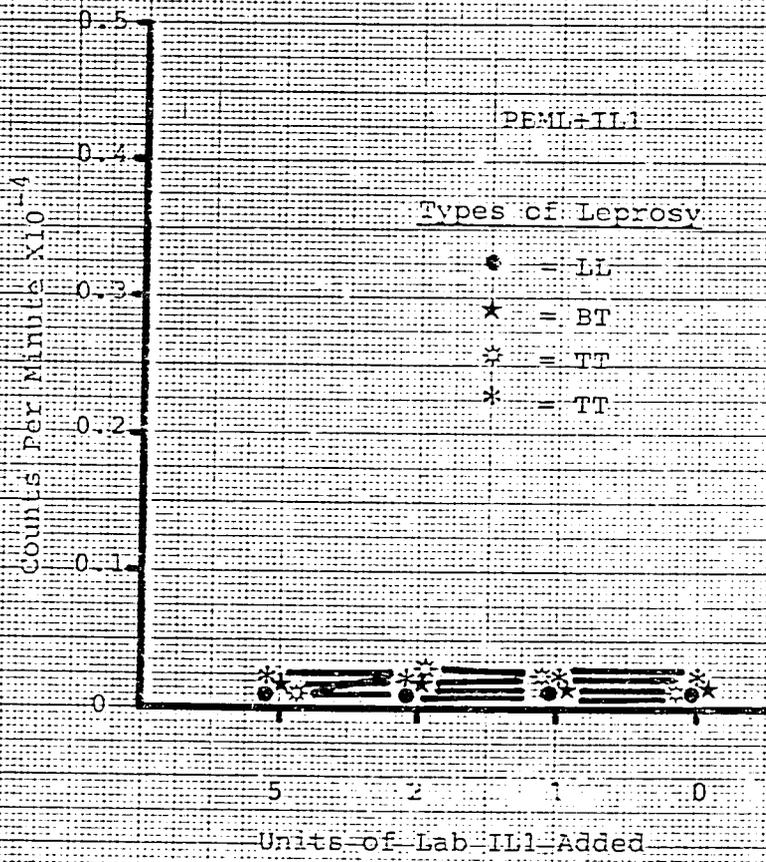


Figure 22. Effect of Exogenous IL1 on IL2 Production from PBML of Leprosy Patients.

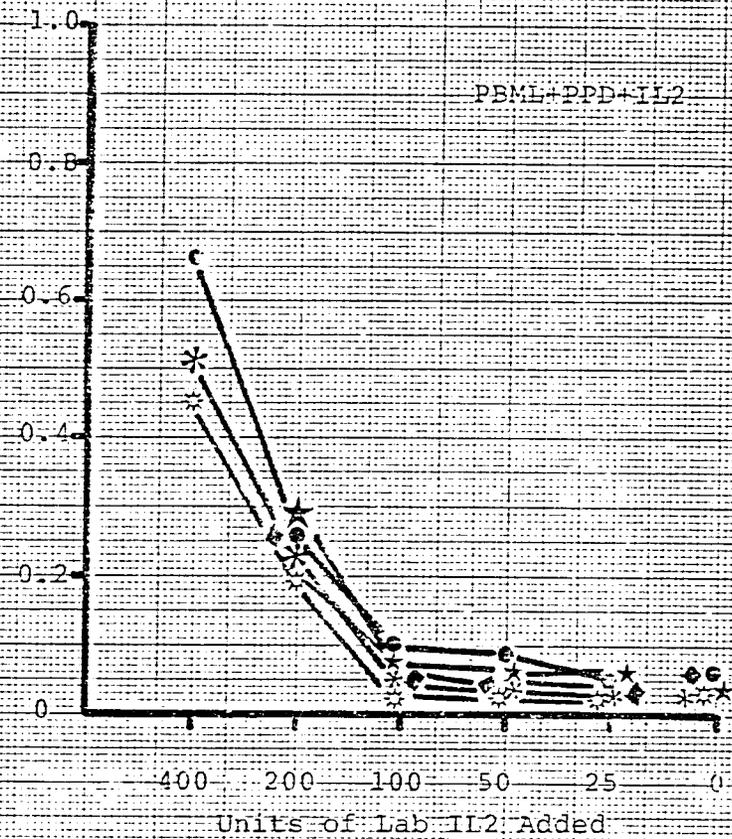
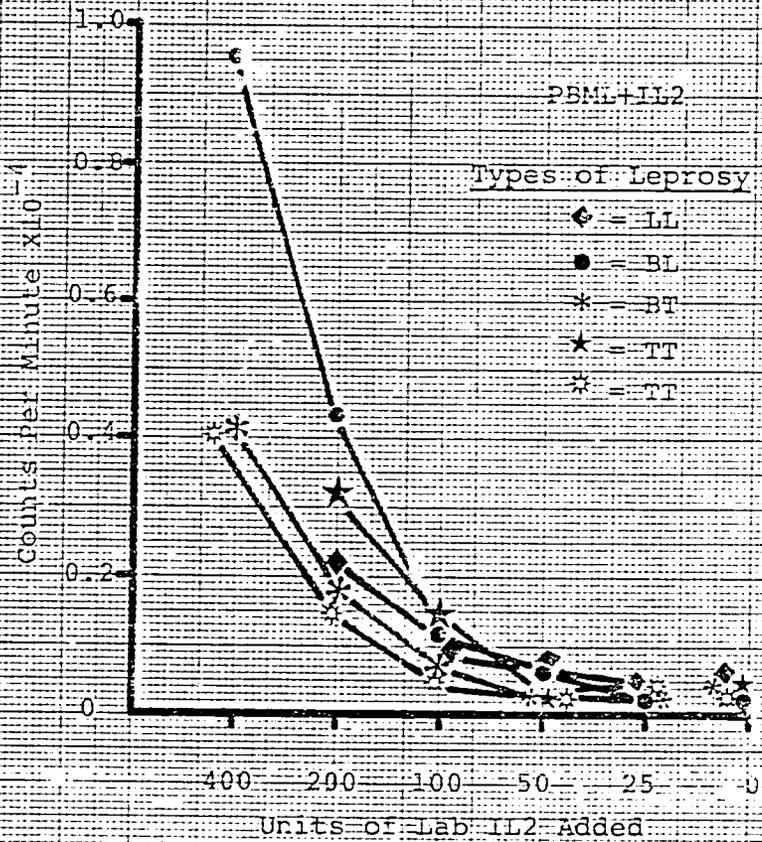


Figure 23. Effect of Exogenous IL2 on IL2 Production from PBML of Leprosy Patients.

