PN-ABC-895

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: February 13, 1989

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. PR No. 1 CS-196
"Regulation of Lymphokine Gene Expression And In Vitro Immunotherapeutic Effect of Interleukin 1 and 2 on Monocytes and Lymphocytes in Leprosy"

Attachment

PN-ABC-895

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PROGRESS REPORT No.1

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

A RESEARCH PROJECT

CDR RESEARCH PROPOSAL C8-196

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

SUBMITTED BY

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CHIANG MAI, THAILAND

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

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BACKGROUND/INTRODUCTIONS

Leprosy is a chronic infectious disease caused by a bacterium, *Mycobacterium leprae*. This disease has a wide distribution throughout the world, and it is most prevalent in the tropics and subtropics. It is estimated that there are approximately 15 million leprosy cases in this world. The vast majority of these patients are found in the "medical tropics" and outlying foci. The Indian subcontinent probably has the greatest number, four million, while the countries of tropical Africa have the highest prevalence rates, often 20-50 per thousand. Most of the countries of South-East Asia (Burma, Thailand, Indonesia, Malaysia, etc.) have considerable numbers, and many countries in South America have leprosy problem (Browne, 1984).

In Thailand there are approximate 200,000 leprosy patients among 50 million population, with about 500 new cases annually. Statistical data from the Division of Health Statistics, Office of the Permanent Secretary, Ministry of Public Health, shows that new cases of leprosy were 340 cases in 1979, 447 in 1980, 591 in 1981, 515 in 1982, 583 in 1983, 433 in 1984, and 279 in 1985.

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

tuberculoid (BT), borderline (BB), borderline lepromatous (BL) and lepromatous (LL), which is subdivided into subpolar lepromatous (LLs) and polar lepromatous (LLp).

It is generally accepted that the different clinical forms of leprosy are clearly associated with varying host defense and cellular immunity to *M.leprae* (Jopling, 1984). However, the immunologic defect(s) leading to the reduction or absence of cellular immunity to *M.leprae* in lepromatous leprosy are not yet fully understood. Early studies of patients with lepromatous leprosy suggested that there is a generalized anergy or unresponsiveness for cell-mediated immunity to various antigens. Other investigators suggested that in the early stages of lepromatous leprosy, while unresponsive to *M.leprae* antigens, appear quite capable of manifesting normal response to other skin test antigens such as coccidioidin, histoplasmin, and PPD (Turk and Bryceson, 1971; Myrvang et al., 1973; Mendes et al., 1974).

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

deficiency was restored by the addition of purified human IL2 to the *in vitro* lymphocyte cultures (Nogueira et al., 1983).

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

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

OBJECTIVES

OVERALL AIM AND SPECIFIC OBJECTIVES.

1. Overall Aim. The aim of this research proposal is to elucidate the abnormality in cell-mediated immunity (CMI) in human leprosy by study of the quantity and functional intactness of T lymphocytes producing IL2 and monocytes producing IL1, by

examination of the function of T helper and T suppressor cells, and particularly their role in the regulation of IL2 and IFN-γ gene expression in this disease.

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

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

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

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

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

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

MATERIALS AND METHODS

PRODUCTION AND DETERMINATION OF IL1 LEVEL

1. Separation and Purification of Monocytes.

Heparinized blood, 10-20 ml, from normal controls and leprosy patients were diluted 1:2 with sterile 0.01 M. phosphate

buffer saline (PBS) pH 7.2 and centrifuged on Ficoll-Hypaque gradient (Boyum, 1968). Peripheral blood mononuclear leukocytes (PBML) was collected, washed, and adjusted to $1x10^6$ cells/ml with 5% FBS-CRPMI medium.

PBML, $2.0x10^6$ cells in 2.0 ml medium were added onto a 35x10 mm plastic plate, incubated at 37 ^oC 5% CO_2 for 2-4 hours. Nonadherent cells were washed off 3 times with 5% FBS-CRPMI medium. Nonadherent and adherent cells were stained with nonspecific esterase stain (NSE). The exact number of monocytes adhering on the plate could be calculated from NSE positive cells on the plate and the number of nonadherent cells washing out from the plate. The desired concentration of monocytes on the plate could be achieved by adding a certain amount of 5% FBS-CRPMI medium.

2. Production of IL1.

The adhered monocytes on plastic plate were adjusted to $2x10^5$ cells/ml by adding various amounts of 5% FBS-CRPMI medium. Lipopolysaccharide (LPS from E.coli, 055:B5, Difco Laboratories, Michigan, USA), 30 ug/ml, was added, incubated at 37 ^oC, 5% CO_2 for 24 hours. Cell suspension was collected and centrifuged, supernatants assayed for IL1 levels.

3. Quantitation of IL1 Level.

Thymocytes from 8 weeks old Balb/c inbred strain were killed by cervical dislocation and the thymus gland was removed under sterile conditions. Thymocyte suspension was prepared by gently homogenizing the thymus gland in a sterile mesh with a sterile

rubber plunger. The thymocytes were washed twice, counted, and then adjusted to $3x10^7$ cells/ml.

Samples were diluted 1:2, 1:4, 1:8 and 1:16 with a final volume of 500 ul for each dilution. Thymocyte suspension, 100 ul, were added to each dilution, mixed, then 30 ul of PHA-P (20 ug/ml) added, mixed. All sample dilutions were assayed in triplicate using 96 wells tissue culture plate. Thymocyte and PHA-P mixture were pipetted, 200 ul, into each well, incubated at 37^oC 5% CO_2 for 72 hours. Tritiated thymidine (8 uCi/ml) was added, 25 ul, into each well, mixed, then incubated 2 hours. The culture was harvested onto glass microfiber filter by a cell harvester, then counted in a liquid scintillation counter (LS 3801, Beckman).

PRODUCTION AND DETERMINATION OF IL2 LEVEL

1. Production of IL2.

PBML, $1x10^6$ cells/ml were stimulated with 2 ug/ml PHA-P or 10 ug/ml PPD, incubated at 37^oC 5% CO_2 18 hours for mitogen and 48 hours for antigen. Cell cultures were harvested and centrifuged, supernatant added with 20 mg/ml of α-methyl-D-mannoside, filtered sterile and then stored at -70^oC for IL2 assay.

2. Assay of IL2 Activity.

Samples were diluted in serial dilutions 1:2, 1:4, 1:8, 1:16 to 1:256 in 100 ul in each well of a 96 wells tissue culture plate. CTLL-2 or 3-day Con A blasts cell suspension containing $8x10^3$ or $8x10^4$ cells/100 ul respectively, were added into each well, mixed, and incubated 24 hours. ^{3}H-TdR containing 0.2 uCi/50

ul were added into each well and incubated for 18 hours. Cultures were harvested onto glass microfiber filters and ^{3}H-TdR incorporation was determined by a liquid scintillation counter (LS 3801 Beckman).

DETERMINATION OF THE NUMBER OF IL2-PRODUCING CELLS.

1. Preparation of stimulated PBML.

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

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

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

3. Preparation and Seeding of Culture Plates.

Soft agar gel technique was used for the determination of IL2-producing cells. Each plastic petri dish (35x10 mm) contains lower agar layer consisting of 1 ml 0.5% agarose in 10% FBS-CRPMI medium, and an upper layer consisting of 0.7 ml of 0.4% agarose in 10% FBS-CRPMI medium, 0.2 ml of responder cells (IL2-dependent

cells) and 0.1 ml of effector cells. In controls, the upper layer contains only effector or responder cells. All cultures in the plastic petri dishes were incubated in 5% CO_2 at 37 ^{o}C for 5 days. The number of IL2-producing cells surrounded by IL2 responsive cells was enumerated under a dissecting microscope.

SEPARATION OF HELPER AND SUPPRESSOR T LYMPHOCYTES AND FUNCTIONAL EVALUATION

Isolation and Separation of Helper and Suppressor T lymphocytes by Con A-SRBC Rosetting Technique.

This simple technic was developed from our laboratory for the separation of suppressor T lymphocytes from helper T lymphocytes. The principle of this technic is coating Con A onto SRBC. The SRBC-Con A will rosette with suppressor T lymphocytes leaving nonrosetted T lymphocytes as helper T lymphocytes. This technic can be performed as follows:

1. Induction of Suppressor T Lymphocytes.

PBML, 4×10^{6} cells in 4 ml medium were stimulated with 50 ug/50 ul Con A, mixed and incubated at 37 ^{o}C 5% CO_2 for 18 hours. After twice washing with RPMI medium, the cell suspension was incubated for another 42 hours, washed twice with RPMI medium, and the cell concentration adjusted to 1×10^{6} cells/ml in 10% FBS-CRPMI medium.

2. Separation of Suppressor and Helper T Cells.

The Con A-treated PBML was separated for T cells by E rosetting technique. T cells at 1×10^6 /ml were mixed with 0.1 ml AET of 1% SRBC-Con A, centrifuged at 600xG for 2 min and incubated at 37 °C 5% CO_2 for 1 hour. The cell pellet was resuspended gently by aspiration with a pasteur pipette. Con A-SRBC rosetted T lymphocytes and Con A-SRBC nonrosetted T lymphocytes were separated by Ficoll-Hypaque gradient centrifugation. Con A-SRBC in the rosetted layer was lysed by hypotonic ammonium chloride solution. The Con A-SRBC rosetted T cells and Con A-SRBC nonrosetted T cells were studied for the immunoregulatory role in HMI by reverse hemolytic plaque assay and production of IgG level by ELISA.

RESULTS

PRODUCTION AND DETERMINATION OF IL2 LEVEL

Figure 1 shows the titration of optimal concentration of 3-day Con A blasts for maximum proliferative response in IL2 levels assay. The optimal concentration of 3-day Con A blasts for IL2 levels assay is 2×10^4 cells/well. Figure 2 depicts effect of IL2 levels assay without adding α-methyl-mannoside into the tested supernatant fluid. It is neccessary to add α-methyl mannoside into the tested supernatant fluid to abolish the inhibitory effect of Con A. Figure 3 illustrates the optimal concentration of 3-day Con A blasts for IL2 levels assay = 2×10^4 cells/well. Figure 4 demonstrates the optimal incubation time for IL2 levels

assay = 24 hours while the optimal pulsing time is 18 hours as shown in figure 5.

Figure 6 shows the concentration of $1x10^6$ PBML/ml is the optimal concentration for maximum IL2 production. The optimal PHA-P concentration for maximum stimulation of IL2 production is 2 ug/ml as shown in figure 7. Figure 8 depicts 18 hours being the optimal stimulation time of 2 ug/ml PHA-P for maximum IL2 production from $1x10^6$ PBML/ml.

From this study, the optimal conditions for IL2 levels assay and production are investigated. For optimal IL2 levels assay, $2x10^4$ cells/well of 3-day Con A blasts is incubated with tested sample for 24 hours then pulses with ^{3}H-thymidine for 18 hours. For optimal IL2 production, PBML $1x10^6$ /ml is stimulated with 2 ug/ml PHA-P for 18 hours then supernatant fluid harvested for IL2 levels assay.

DETERMINATION OF THE NUMBER OF IL2-PRODUCING CELLS

Figure 9 demonstrates the optimal concentration of 3-day Con A blasts (responder cells) for IL2-producing cells assay, the optimal concentration is $6x10^6$ cells/plate. Figure 10 shows $2.5x10^5$ PBML/plate is the optimal concentration of effector cells for IL2-producing cells assay. Figure 11 illustrates the optimal incubation time after mixing effector and responder cells for IL2-producing cells assay is 6 days. Figure 12 depicts the optimal stimulation time of PBML by 2 ug/ml PHA-P for IL2-producing cells assay is 4 hours.

SEPARATION OF HELPER AND SUPPRESSOR T LYMPHOCYTES AND FUNCTIONAL EVALUATION

Table 1 shows the percentage of Con A-SRBC rosetting T lymphocytes from Con A-stimulated and nonstimulated PBML of 13 subjects. The $\bar{X}\pm$S.D. of percent T cells in PBML stimulated with Con A = 70.2±5.4 while percent T cells in $E_{(AET)}$ rosette T cells = 86.0±3.2. The percentage of Con A-SRBC rosette and nonrosette T cells of $E_{(AET)}$ T cells = 44.4±5.4 and 55.6±5.4 respectively. The percentage of T cells in nonstimulated PBML = 66.2±4.1 while the percent T cells in $E_{(AET)}$ T cell = 82.7±2.4. The percentage of Con A-SRBC rosette and nonrosette T cells of $E_{(AET)}$ T cells from nonstimulated PBML = 16.0±7.5 and 84.0±7.5 respectively.

Table 2 shows the suppressor activities of Con A-SRBC rosette T cells by plaque forming cells (PFC) assay using reverse hemolytic plaque assay technique in 18 normal subjects. It is noted that the suppressive activities of Con A-SRBC rosette T cells is dose dependent. Table 3 shows the helper activities of Con A-SRBC nonrosette T cells by PFC assay in 8 normal subjects. It is noted that the enhancing activities of the Con A-SRBC nonrosette T cells is also dose dependent.

Figure 13 illustrates the enhancement and suppressive activities of Con A-SRBC nonrosette and rosette T cells by PFC assay. It can be seen that both enhancement and suppressive activities of the Con-SRBC nonrosette and rosette T cells respectively are highly impressive.

Table 4 shows the suppressive activities of Con A-SRBC rosette T cells by determination of secreted IgG in culture

supernatant fluid in 11 normal subjects. The suppressive activities of Con A-SRBC rosette T cells is dose dependent. Table 5 lists the helper activities of Con A-SRBC nonrosette T cells in 4 normal subjects by determination of secreted IgG.

Figure 14 depicts the enhancement and suppressive activities of Con A-SRBC nonrosette and rosette T cells by ELISA determination of IgG concentration in culture supernatant. The enhancing and suppressive activities of both T cell subpopulations are also very impressive.

Table 6 shows the suppressor and helper activities of Con A-stimulated T cells in 9 normal subjects by PFC assay. Table 7 also shows the suppressive and helper activities of non-Con A-stimulated T cells in 6 normal subjects by PFC assay.

Figure 15 illustrates the suppressor and helper activities of Con A-stimulated and non-Con A-stimulated T cells by PFC assay.

DISCUSSION AND CONCLUSION

Optimal conditions for IL2 production and IL2 levels assay have been established. Optimal conditions for IL2 production was $1x10^6$ PBML/ml stimulated with 2 ug/ml PHA-P for 18 hours. Optimal conditions for IL2 levels assay was $2x10^4$ cells/ml of 3-day Con A blasts incubated with tested sample for 24 hours then pulsed with ^{3}H-thymidine for 18 hours. We carefully investigated each step of IL2 levels determination to obtain the precise IL2 quantitation.

This report also shows that all optimal conditions for quantitation of IL2-producing cells have been investigated. PBML $5x10^6$ cells/ml are stimulated with 2 ug/ml PHA-P (Wellcome) for 4 hours, washed twice with RPMI 1640 medium, then adjusted to $2.5x10^6$ cells/ml by 10% FBS-CRPMI medium. For quantitation of IL2-producing cells, $2.5x10^5$ cells/100 ul of effector cells was mixed with $6x10^6$ cells/200 ul of responder cells and 0.7 ml of 0.4% agarose in RPMI medium. After incubation at 37 ^oC 5% CO_2 for 6 days, the number of IL2-producing cells were quantitated by a dissecting microscope.

During this research period we have set up optimal conditions for Con A-stimulation of suppressor T cells in PBML, separation of Con A-SRBC rosette and nonrosette T cells. We have also evaluated function of each isolated T cell subpopulation by measuring PFC and IgG concentration by reverse hemolytic plaque assay and a sandwich ELISA technique respectively. From this study, Con A-SRBC rosette T cells showed impressively strong suppressor activities while Con A-SRBC nonrosette T cells showed very strong helper activities.

WORKPLAN FOR THE NEXT PERIOD

The relationship of IL2 levels and numbers of IL2-producing cells in various types of leprosy patients and normal subjects will be evaluated. Quantitation of IL1-producing monocyte is on the way of setting up. We are also setting up the procedure for separation of suppressor T cells from helper T cells by specific

monoclonal antibodies and complement lysis system. We will try to investigate the *in vitro* effect of recombinant IL1 and/or IL2 on the defective IL1-producing monocytes and the defective IL2-producing T lymphocytes of leprosy patients.

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Table 1. Percentage of Con A-SRBC rosetted and non-rosetted T lymphocytes from Con A-stimulated and non-stimulated PBML.

Subject	Con A-Stimulated				Non-Con A-Stimulated			
Number	%T cells in PBML	%T in E rosette T	%Con A-SRBC rosette T	%Con A-SRBC non-rosette T	%T cells in PBML	%T in E rosette T	%Con A-SRBC rosette T	%Con A-SRBC non-rosette T
1	77.5	91.5	49.0	51.0	68.5	85.0	11.5	88.5
2	75.5	86.0	47.0	53.0	64.0	85.0	14.0	86.0
3	74.0	85.5	54.0	46.0	70.0	80.0	24.0	76.0
4	71.5	81.0	37.0	63.0	68.0	82.0	33.0	67.0
5	73.0	85.0	49.0	51.0	68.5	80.5	13.0	87.0
6	68.0	88.0	45.0	55.0	66.5	82.0	19.5	80.5
7	73.5	84.0	43.0	57.0	72.5	87.5	18.5	81.5
8	69.0	88.0	39.0	61.0	68.0	82.5	11.0	89.0
9	73.0	82.0	37.0	63.0	67.0	81.0	13.0	87.0
10	66.5	93.0	49.0	51.0	59.0	81.5	9.0	91.0
11	69.0	83.0	40.0	60.0	61.0	ND	8.0	92.0
12	65.0	89.0	40.0	60.0	60.0	ND	9.0	91.0
13	57.0	ND	48.0	52.0	68.0	ND	25.0	75.0
$\bar{X}\pm$S.D.	70.2±5.4	86.0±3.2	44.4±5.4	55.6±5.4	66.2±4.1	82.7±2.4	16.0±7.5	84.0±7.5

Table 2. Suppressor and Helper Activities of Con A-SRBC Rosetting T cells by Plaque Forming Cells.

Subject Number	Per Cent of Con A-SRBC Rosetting T Cell Added					
	0	5	10	20	40	80
1	7660*	58.2**	ND	ND	ND	ND
2	7660	53.0	ND	ND	ND	ND
3	9300	21.5	57.0	71.0	ND	ND
4	9600	22.4	38.0	44.3	60.9	ND
5	8550	ND	85.0	94.2	99.7	ND
6	8550	ND	88.3	92.7	96.5	ND
7	8550	ND	84.2	89.2	98.5	ND
8	8550	ND	64.9	91.2	93.9	ND
9	9200	ND	49.5	53.3	72.3	77.7
10	9200	ND	11.0	14.7	41.8	83.7
11	4300	69.8	79.3	97.0	98.4	99.5
12	4300	11.6	77.0	84.9	99.1	100
13	4200	75.0	79.9	95.9	96.4	99.5
14	4200	23.3	71.9	81.7	97.1	100
15	5520	5.8	11.6	58.0	74.6	81.9
16	5520	16.7	42.0	58.0	74.6	85.5
17	3870	10.1	20.7	65.1	77.5	93.8
18	3870	34.4	43.1	66.4	82.7	97.2
$\bar{X}\pm$S.D.	0	33.5±24.3	56.5±26.5	72.4±23	84.3±17.2	91.9±8.8

* Number of PFC/1 x 10^6 PBML ** Percent suppression.

Table 3. Suppressor and Helper Activities of Con A-SRBC Non-rosetting T Cells by Plaque Forming Cells.

Subject Number	Per Cent of Con A-SRBC Non-rosetting T Cell Added			
	0	5	10	20
1	9300*	104.3**	123.7	ND
2	9300	66.7	92.5	128.0
3	1600	37.5	68.8	181.3
4	1600	8.1	75.0	200.0
5	2300	12.2	21.8	147.8
6	2300	ND	57.8	156.5
7	3500	2.6	ND	ND
8	3500	11.4	ND	20.0
$\bar{X} \pm$ S.D.	0	34.7±37.9	73.3±34.1	138.8±63.5

* Number of PFC/1x10^6 PBML **Percent enhancement

Table 4. Suppressor and Helper Activities of Con A-SRBC Rosetting T Cells by Detection of Secreted IgG.

Subject	Per Cent of Con A-SRBC Rosetting T Cell Adaed					
Number	0	5	10	20	40	80
1	100*	54.73**	72.15	87.02	85.18	88.34
2	100	48.03	64.61	ND	72.83	71.49
3	100	35.07	ND	61.19	57.38	81.45
4	100	42.07	65.38	73.58	69.60	76.80
5	100	51.58	ND	62.55	54.67	71.38
6	100	58.06	ND	64.45	58.56	75.22
7	100	40.09	53.55	54.67	67.34	74.48
8	100	24.86	45.41	60.24	78.63	82.69
9	100	ND	35.44	90.56	90.10	92.73
10	100	59.62	ND	77.73	93.16	93.16
11	100	47.86	45.36	64.33	69.36	84.96
$\bar{X}\pm$S.D.	0	46.0±3.8	54.6±5.0	69.6±3.9	72.4±4.0	81.2±2.4

* Concentration of secreted IgG at the base line was 100%

** Percentage of suppression

Table 5. Suppressor and Helper Activities of Con A-SRBC Non-rosetting T Cells by Detection of Secreted IgG.

Subject	Per Cent of Con A-SRBC Non-rosetting T Cell Added			
Number	0	5	10	20
1	100*	1.62**	71.90	24.87
2	100	ND	35.26	60.13
3	100	59.94	70.72	53.52
4	100	3.09	12.16	15.90
$\bar{X}\pm$S.E	0	20.5±19.8	47.5±14.5	38.6±10.8

* Concentration of secreted IgG at the base line was 100%

** Percentage of enhancement

Table 6. Suppressor and Helper Activities of Con A-Stimulated T Cells.

Subject Number	Per Cent Con A-Stimulated T Cells Added				
	0	5	10	15	20
1	18500*	-1.6**	-1.1	3.2	10.3
2	18500	3.2	9.2	15.7	18.9
3	8000	-16.3	10.0	22.5	48.7
4	8000	0	16.2	28.7	45.0
5	6200	-8.1	8.1	8.1	9.7
6	8900	11.2	22.4	42.7	46.1
7	8900	5.6	4.5	12.4	13.5
8	3870	7.2	8.3	ND	36.9
9	3870	-3.4	-0.8	ND	29.5
$\bar{X} \pm$ S.D.	0	-0.24 ± 8.4	8.5 ± 7.5	19.0 ± 13.0	24.3 ± 16.0

* Number of PFC/1 x 10^6 PBML ** Percent suppression

Table 7 Suppressor and Helper Activities of Non-Con A-Stimulated T Cells

Subject Number	Per Cent Non-Con A-Stimulated T Cells Added				
	0	5	10	15	20
1	18500*	-5.4**	-1.1	3.8	3.2
2	8000	6.2	17.5	18.7	31.2
3	8000	-21.3	2.5	22.5	37.5
4	6200	-9.7	-4.8	-14.5	11.3
5	6200	-11.3	11.3	4.8	19.3
6	8900	-25.8	-7.9	11.2	23.6
$\bar{X} \pm$ S.D.	0	-11.2±11.4	-0.85±10	7.75±13	21±12.7

* Number of PFC/1 x 10^6 PBML

** Percent suppression

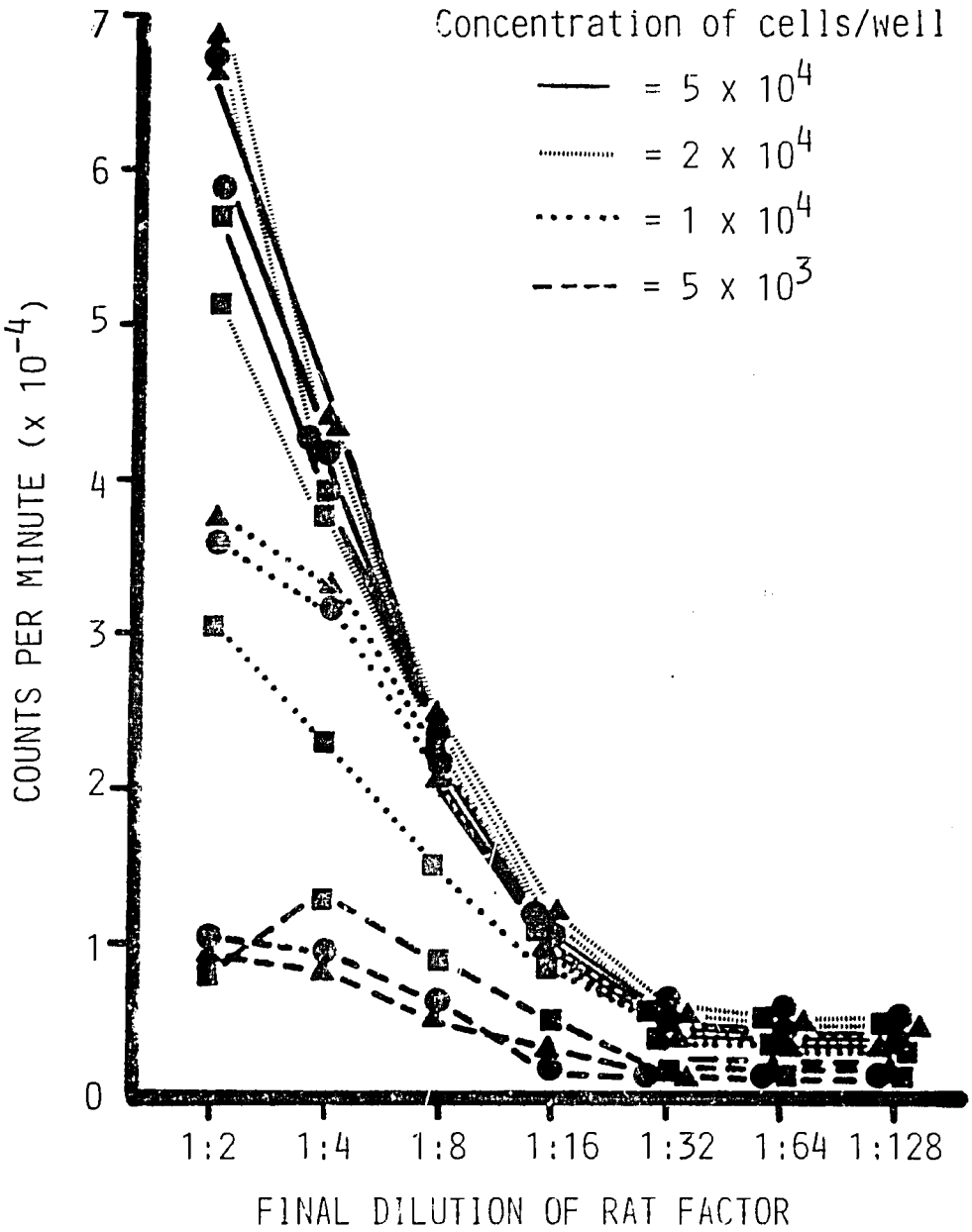

Figure 1. Titration of optimal concentration of 3-day Con A blasts for maximum proliferative response in IL2 levels assay (incubation and pulsing time = 24 and 18 hours respectively; 3 experiments).

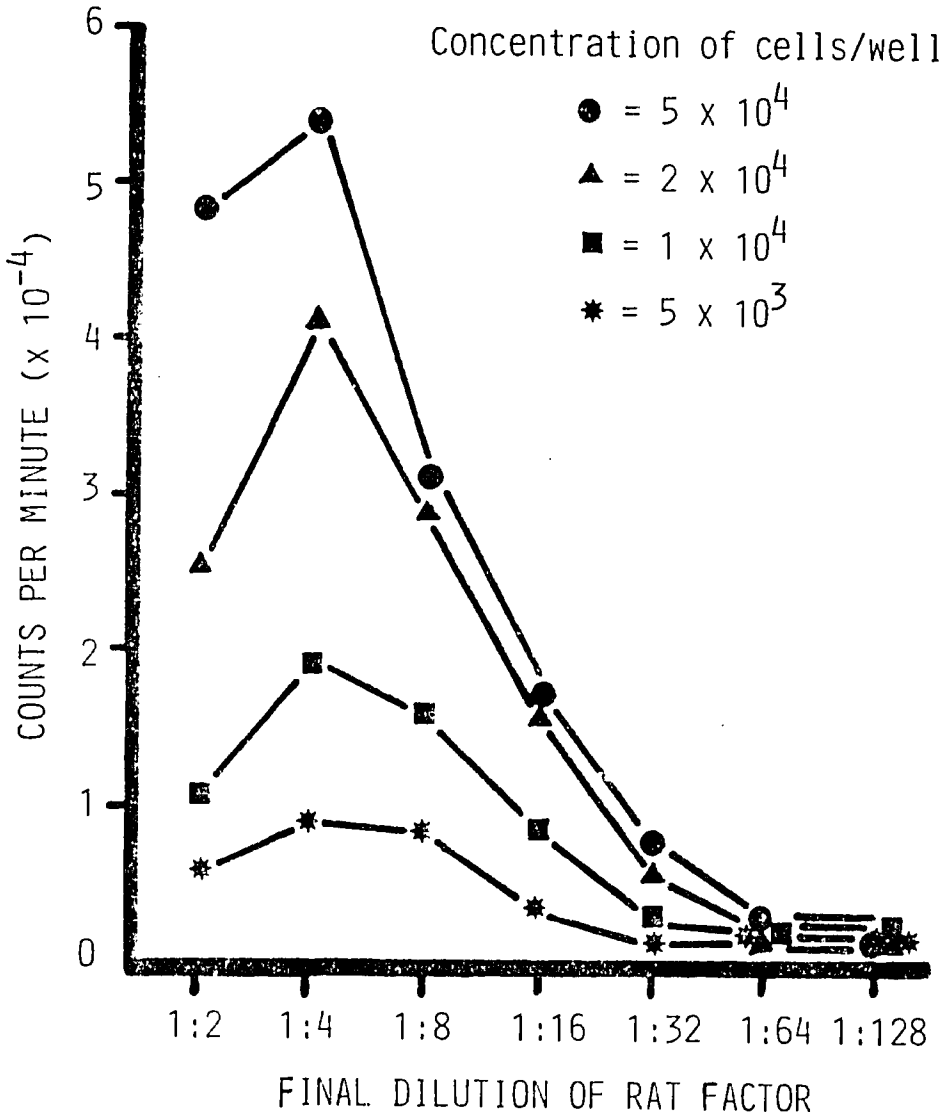

Figure 2. Effect to IL2 levels assay without adding α - methyl-mannoside into the tested supernatant fluid (incubation and pulsing time = 24 and 18 hours respectively).

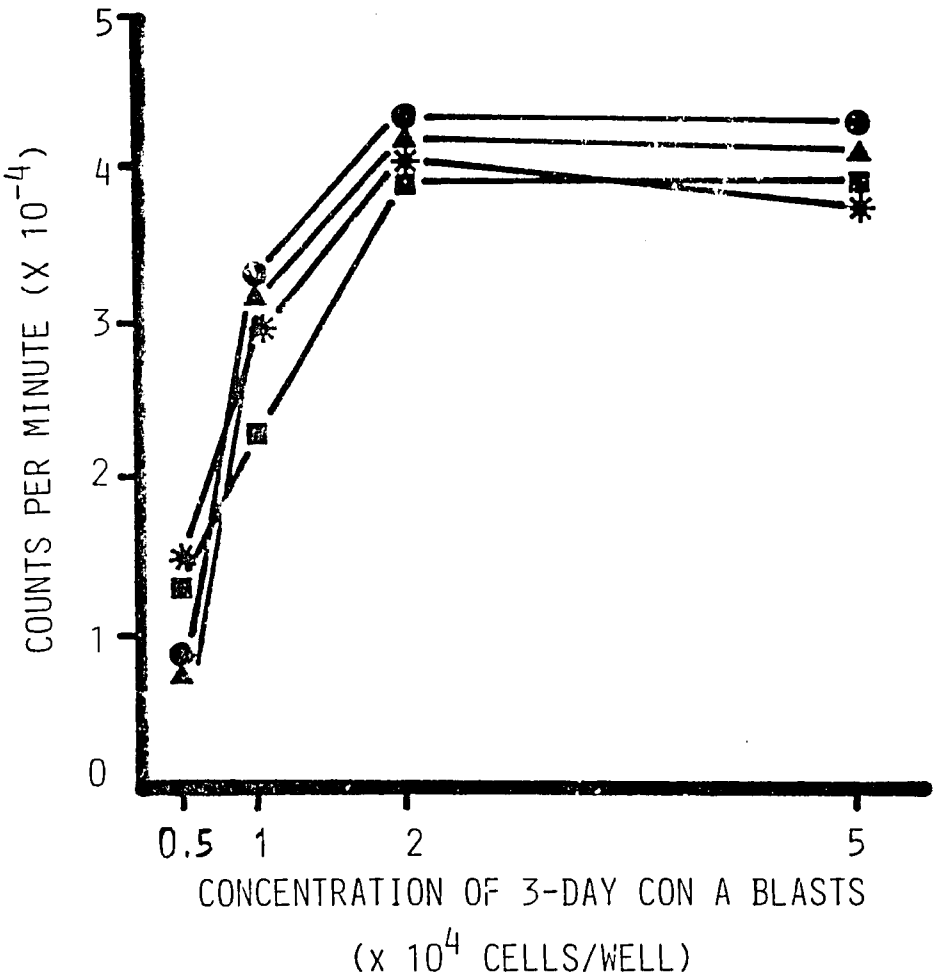

Figure 3. Comparison of 3-day Con A blasts concentrations for maximum proliferative response against rat factor in I'2 levels assay (incubation and pulsing time = 24 and 18 hours respectively; 4 experiments).

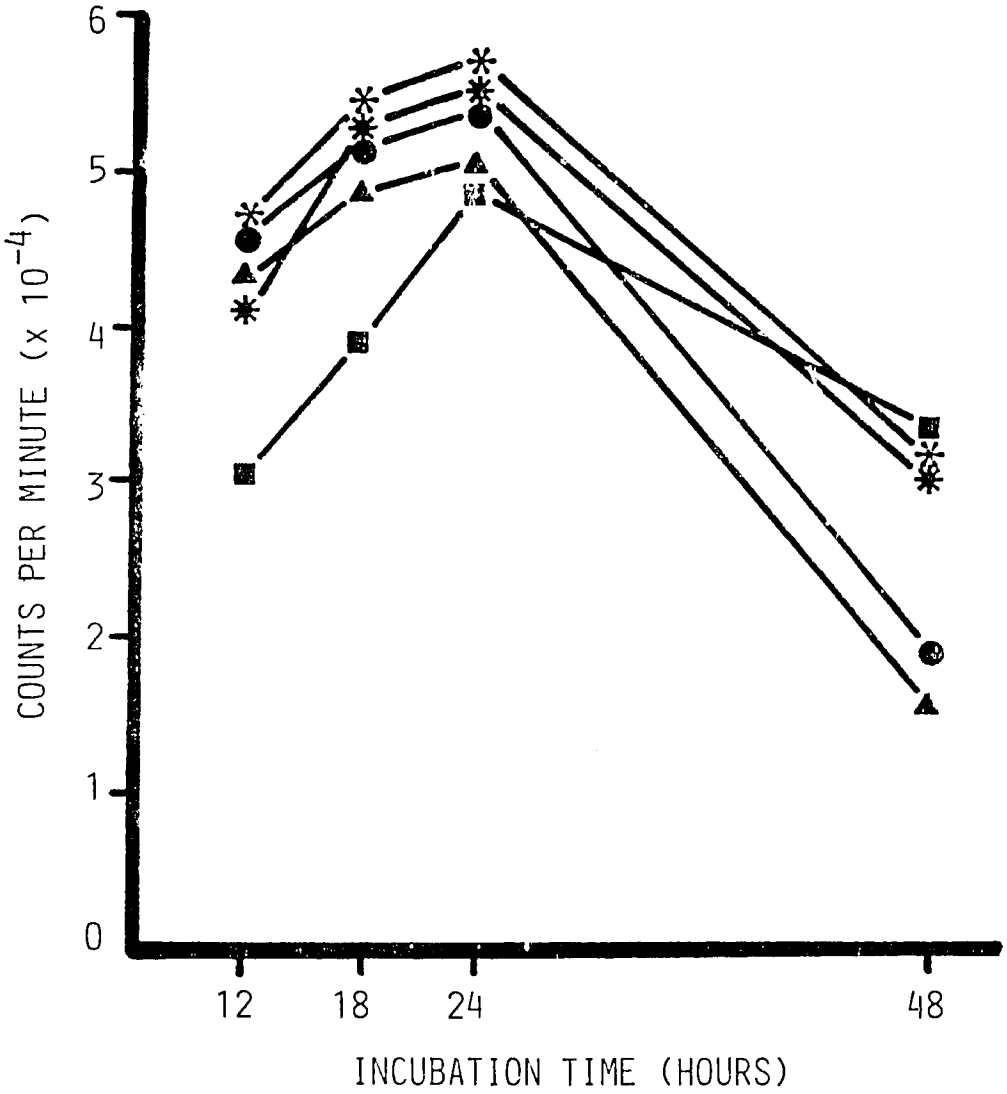

Figure 4. Determination of optimal incubation time for IL2 levels assay (concentration of 3-day Con A blasts = 2 x 10^4 cells/well; pulsing time = 18 hours; 5 experiments).

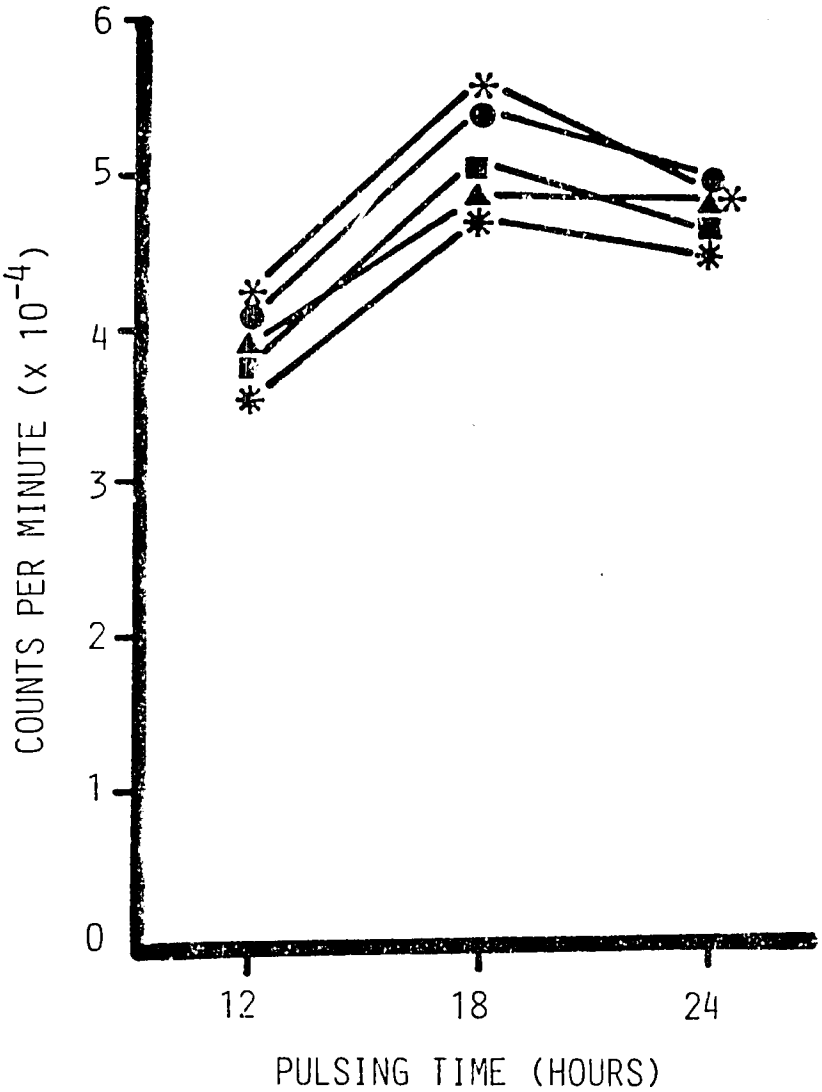

Figure 5. Determination of optimal pulsing time for IL2 levels assay (concentration of 3-day Con A blasts = 2×10^4 cells/well, incubation time 24 hours; 5 experiments).

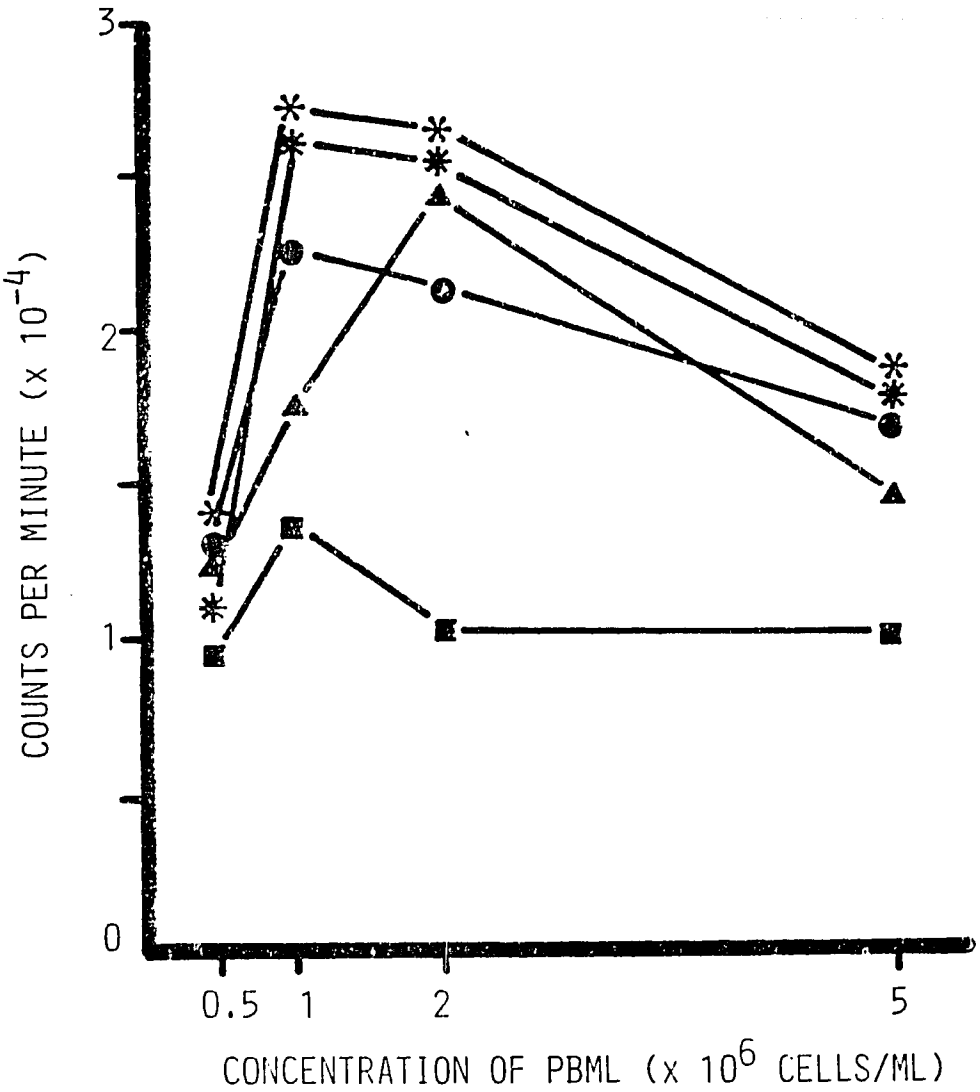

Figure 6. Determination of optimal PBML concentration for maximum IL2 production (PHA-P 2 ug/ml and stimulation time 18 hours; IL2 assay: 3-day Con A blasts 2 x 10^4 cells/well, incubation and pulsing time 24 and 18 hours respectively; 5 Subjects).

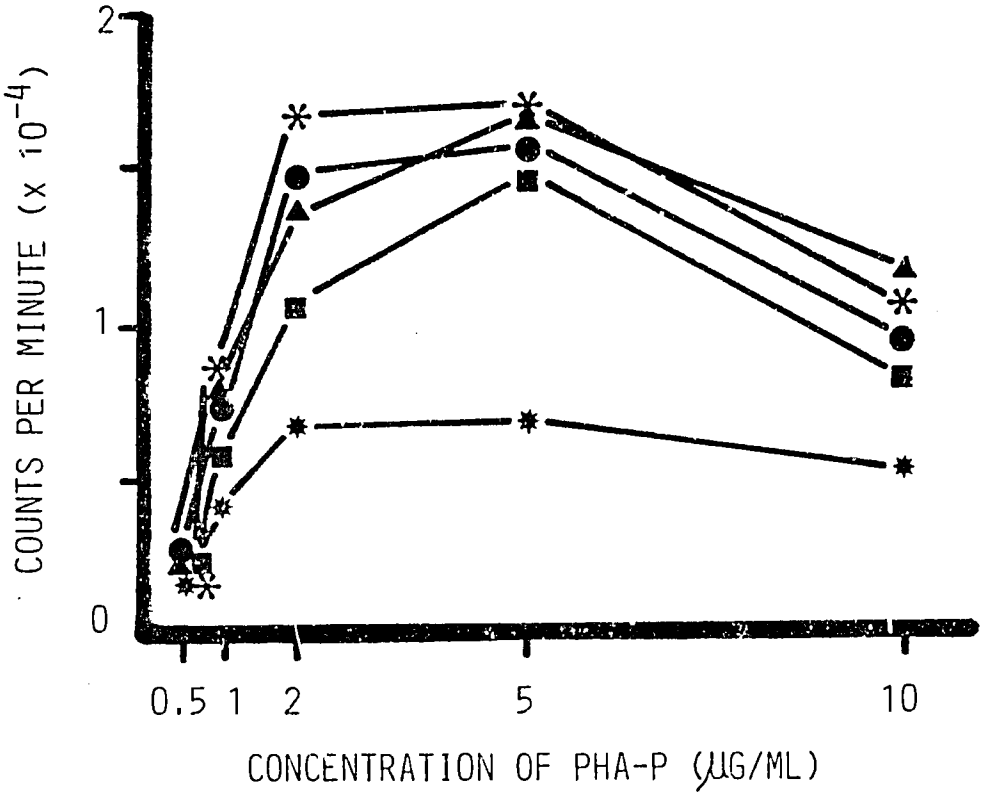

Figure 7. Titration of optimal PHA-P concentration for maximum IL2 production (concentration of PBML 1 x 10^6 cells/ml, stimulation time 18 hours; IL2 assay: 3-day Con A blasts 2 x 10^4 cells/well, incubation and pulsing time 24 and 18 hours respectively; 5 Subjects).

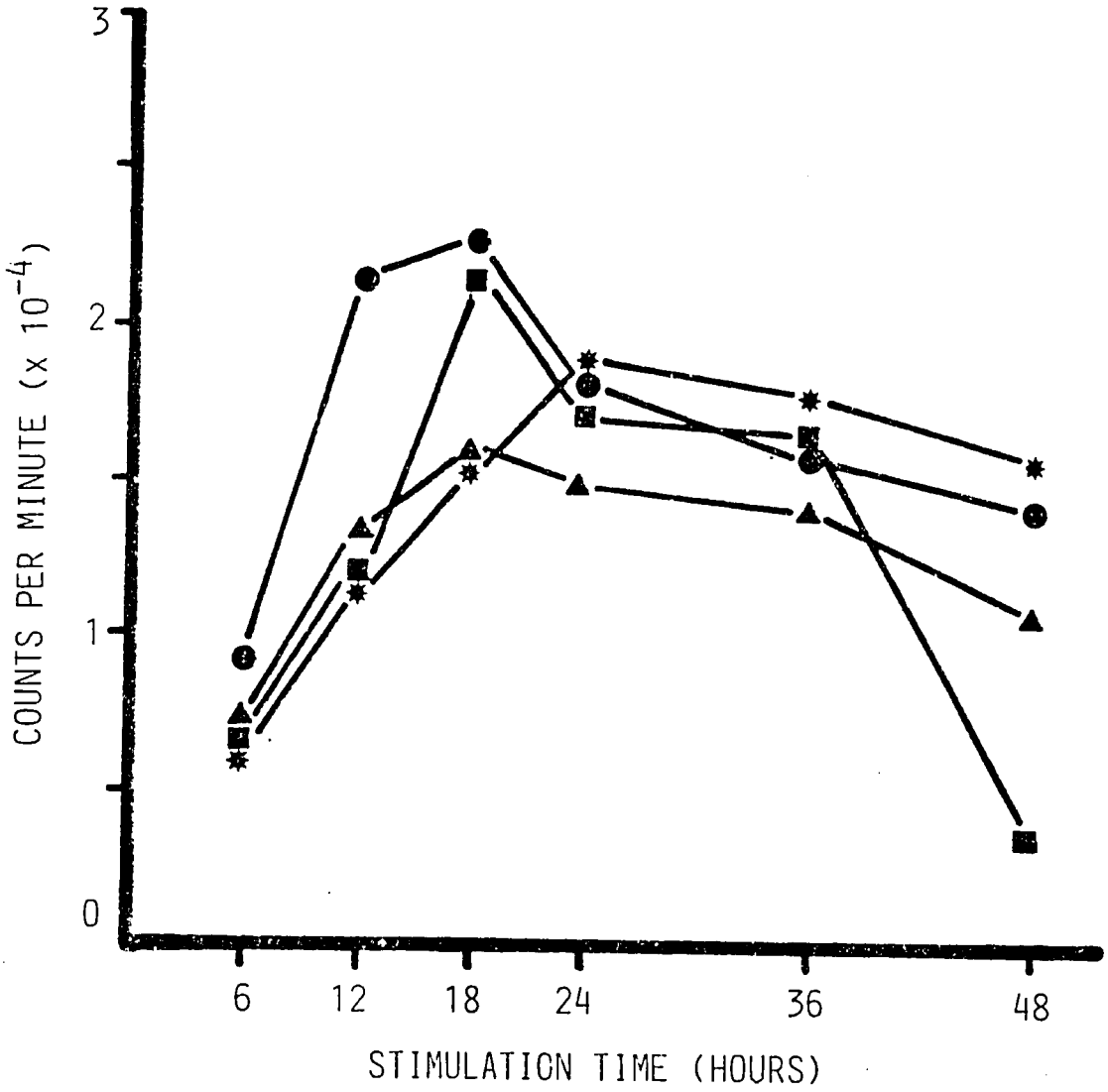

Figure 8. Titration of optimal stimulation time of 2 μg/ml PHA-P for maximum IL2 production from 1 x 10^6 PBML/ml (IL2 assay; 3 day Con A blasts 2 x 10^4 cell/well, incubation and pulsing time 24 and 18 hours respectively; 4 Subjects).

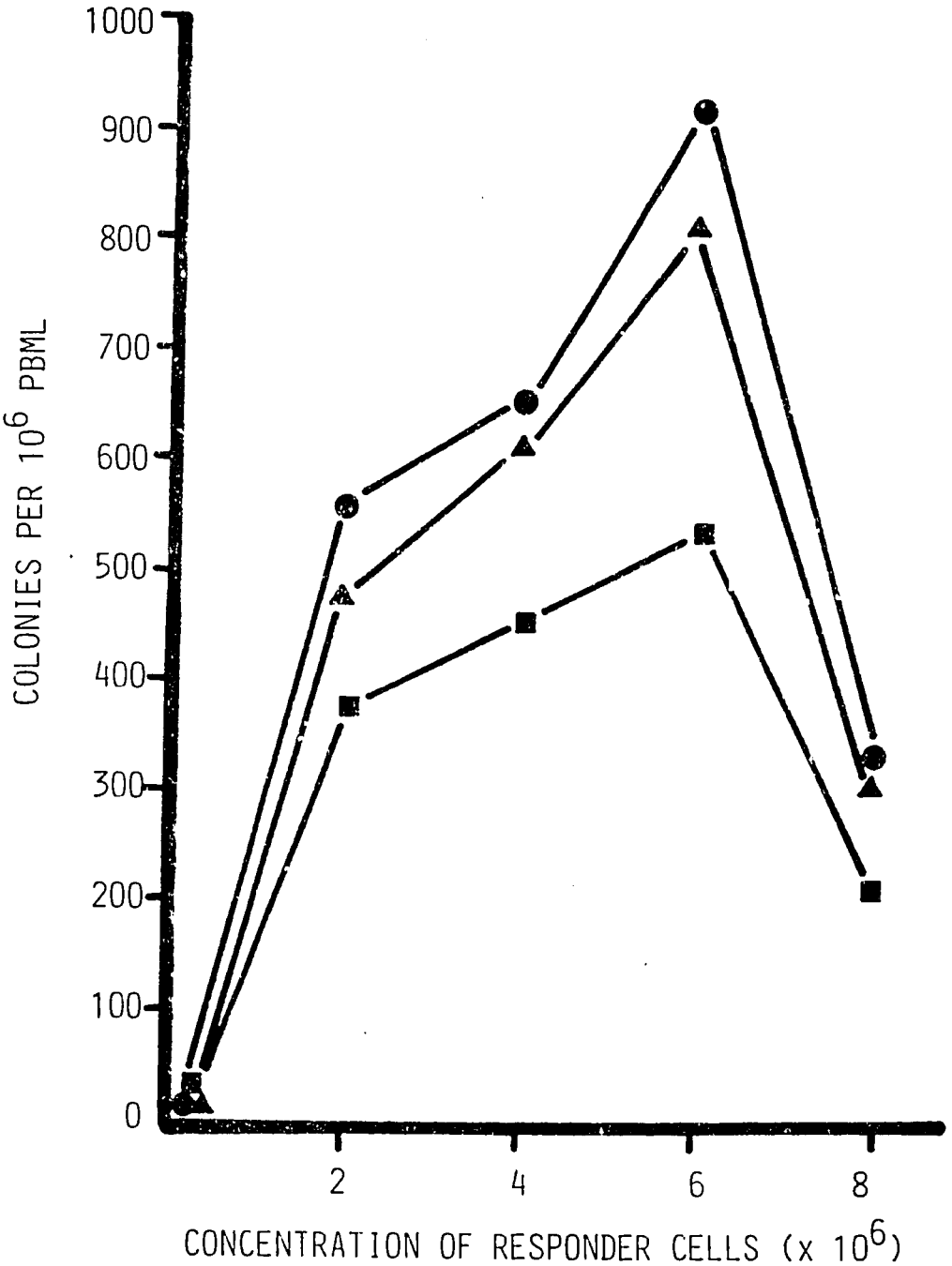

Figure 9. Determination of optimal concentration of 3-day Con A blasts for IL2 producing cells assay (effector 2.5 x 10^5 cells/plate, PHA-P 2 μg/ml for 4 hours and incubation time 5 days; 3 Subjects).

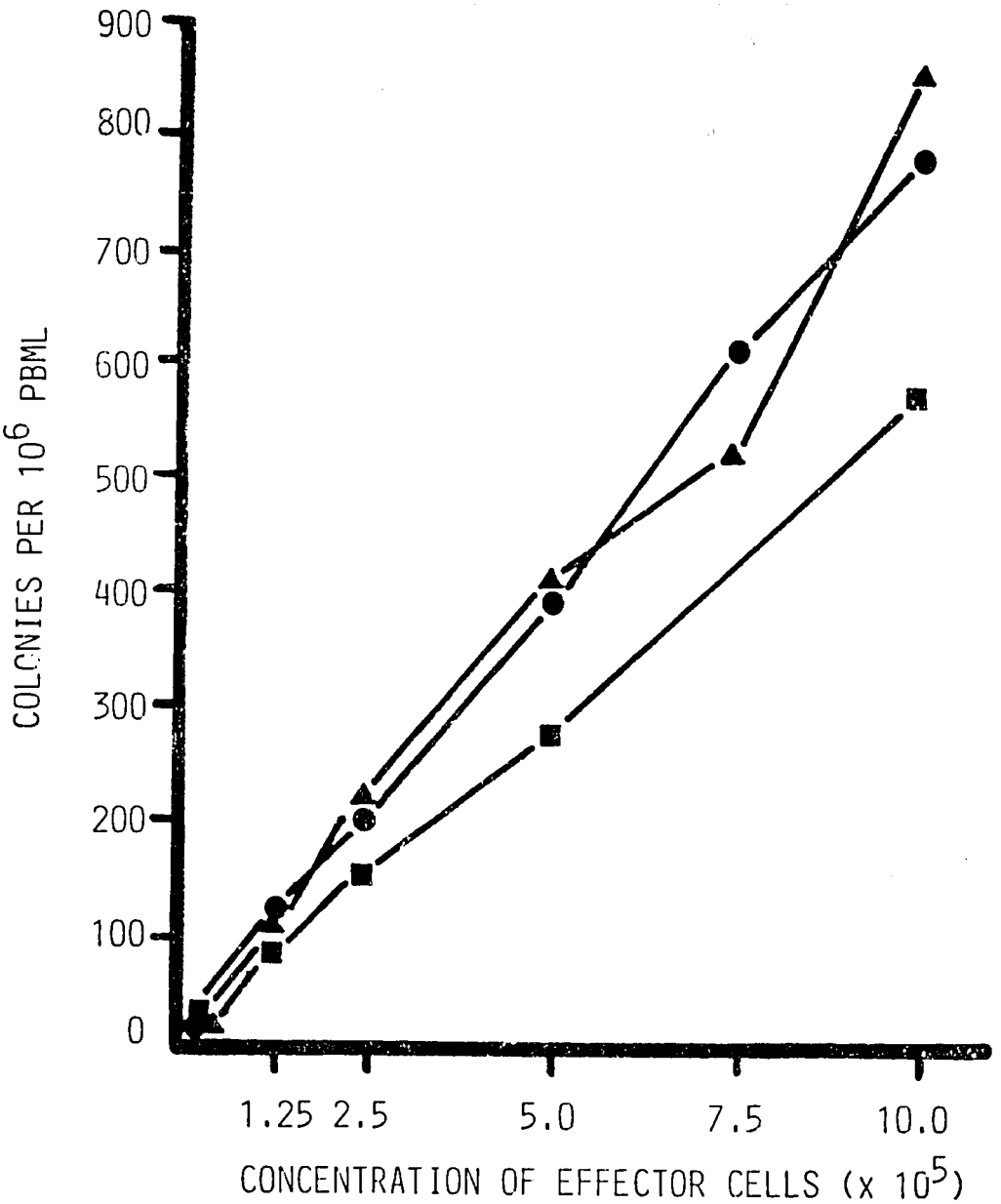

Figure 10. Titration of optimal concentration of effector cells for IL2 producing cells assay (responder 6 x 10^6 cells/plate, PHA-P 2 μg/ml for 4 hours and incubation time 5 days; 3 Subjects).

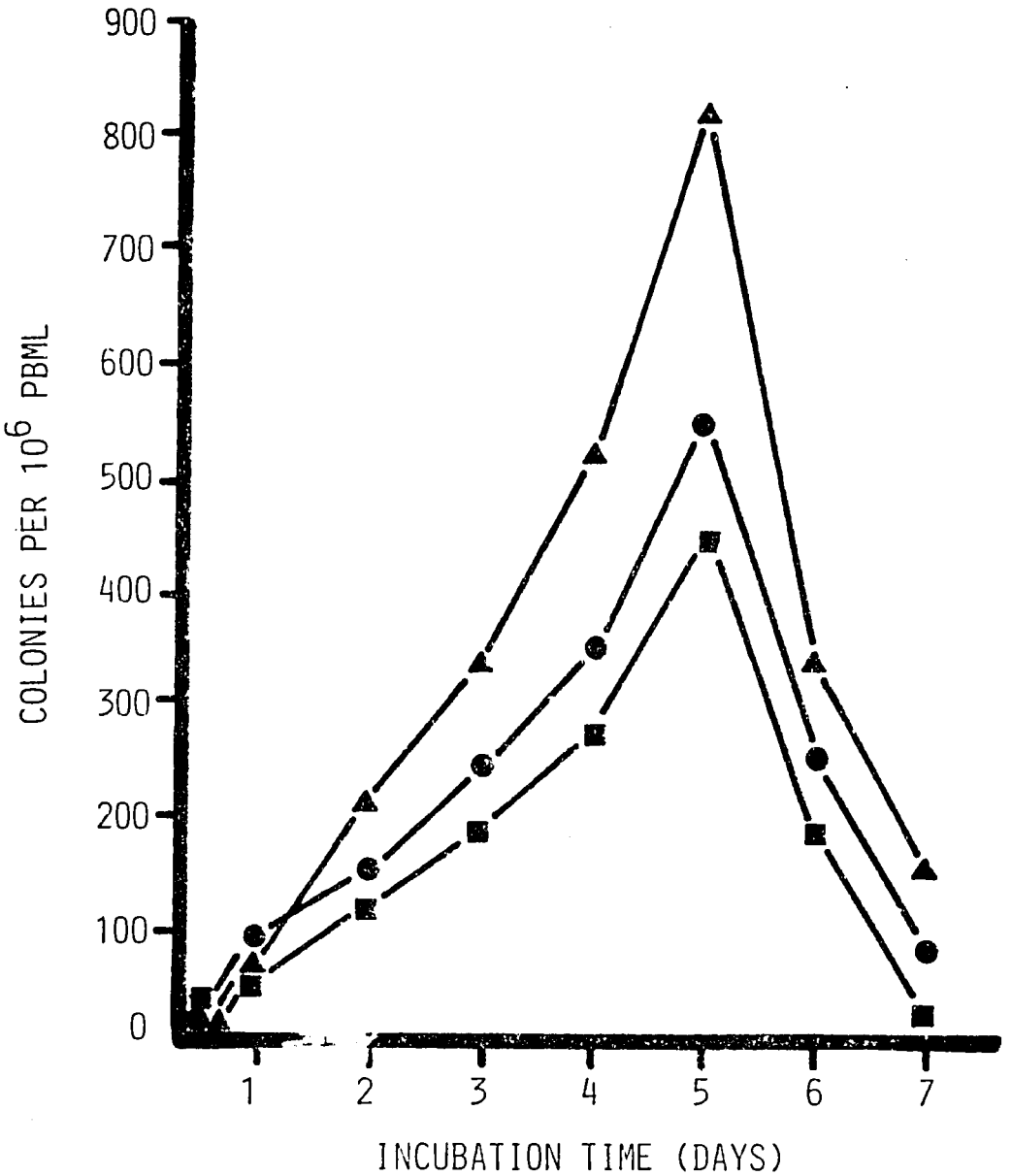

Figure 11. Determination of optimal incubation time after mixing effector and responder cells for IL2 producing cells assay (responder 6×10^6 cells/plate, effector 2.5×10^5 cells/plate and 2 µg/ml PHA-P for 4 hours; 3 Subjects).

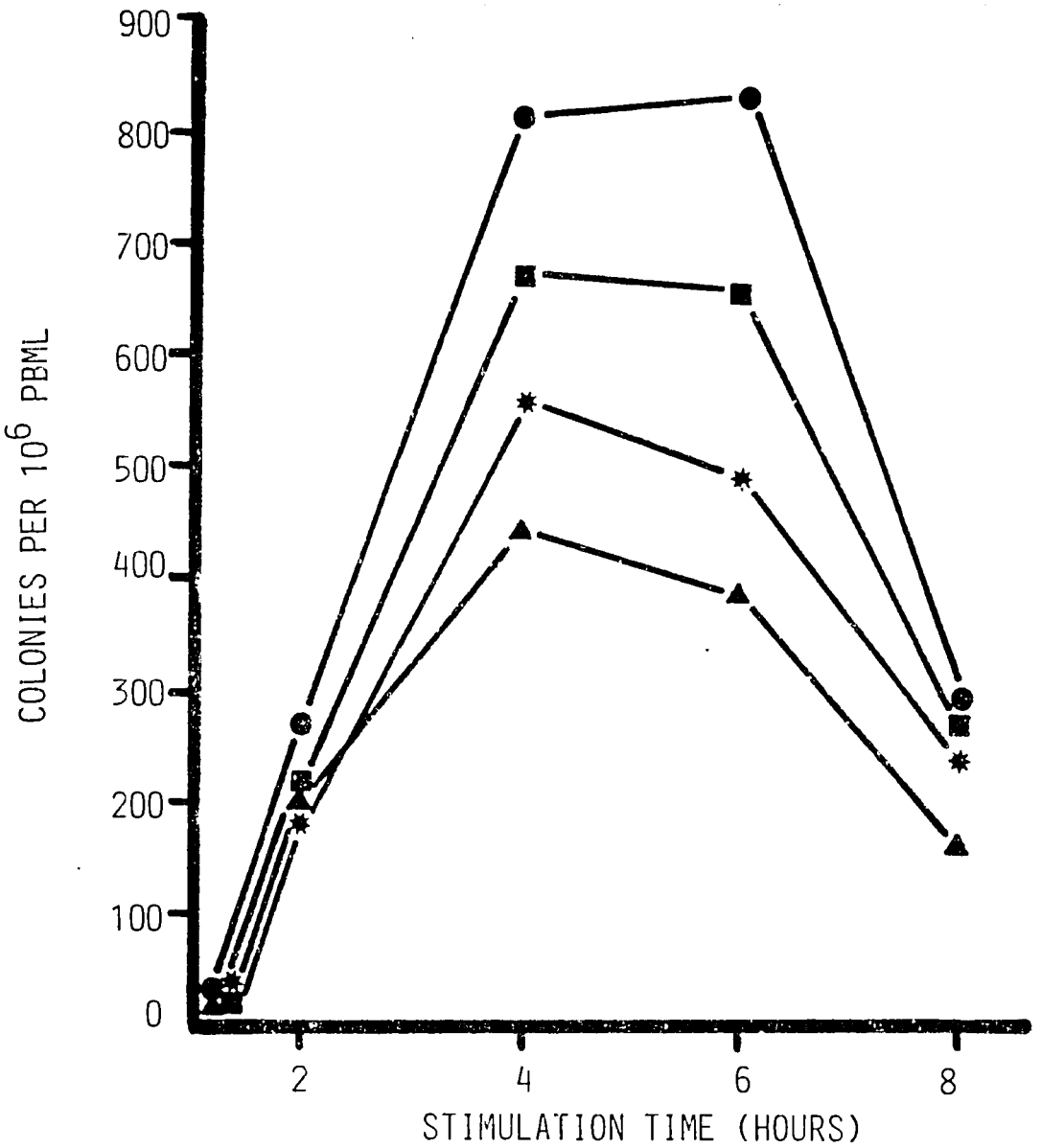

Figure 12. Determination of optimal stimulation time of 2 μg/ml PHA-P for the maximum colonies production (responder 6 x 10^6 cells/plate, effector 2.5 x 10^6 cells/plate and incubation time 5 days; 4 Subjects).

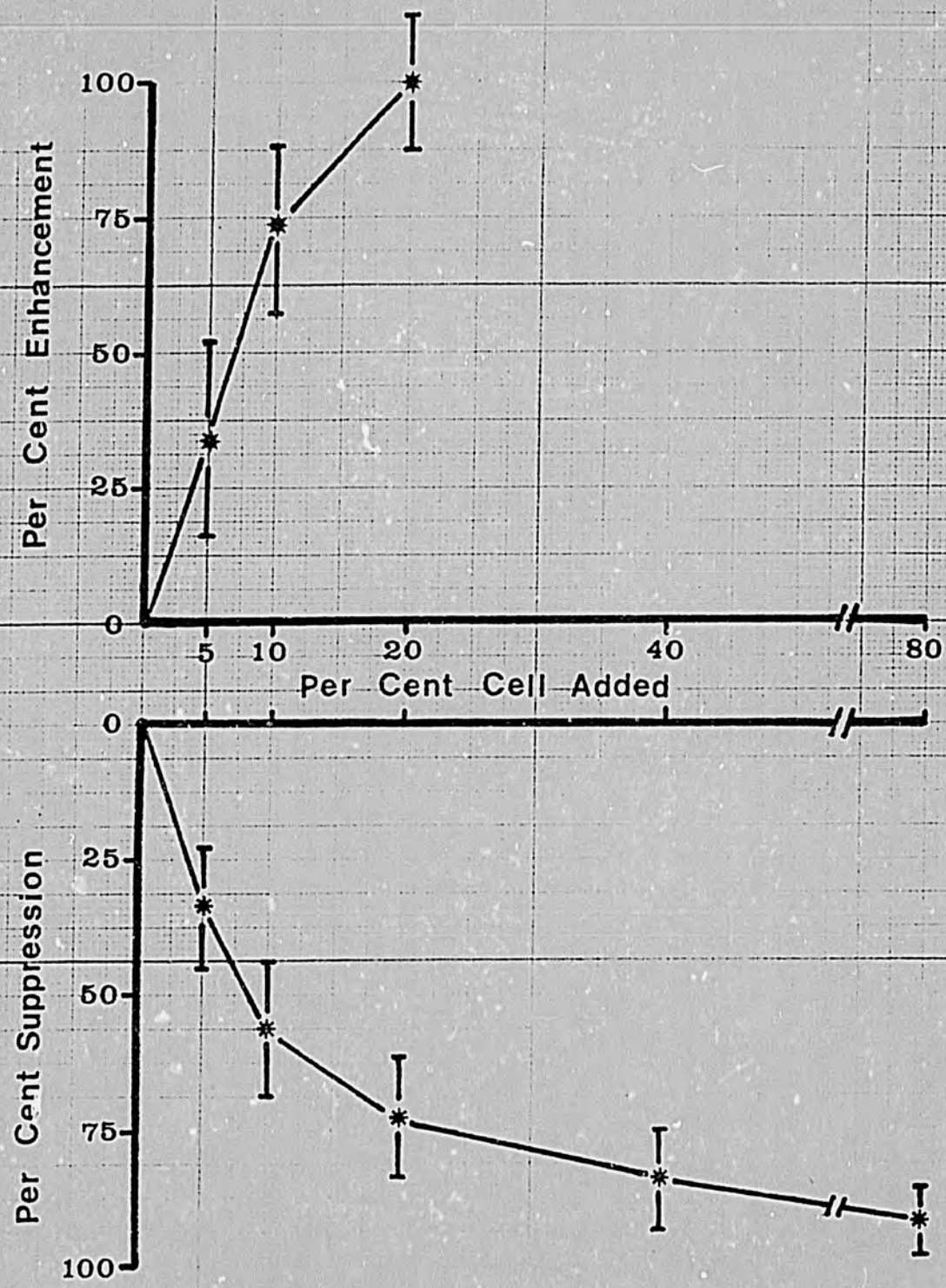

Figure 13. The enhancement activity of Con A-SRBC nonrosette T cells and the suppressive activity of Con A-SRBC rosette T cells (the numbers of PFC were measured by reverse hemolytic plaque assay).

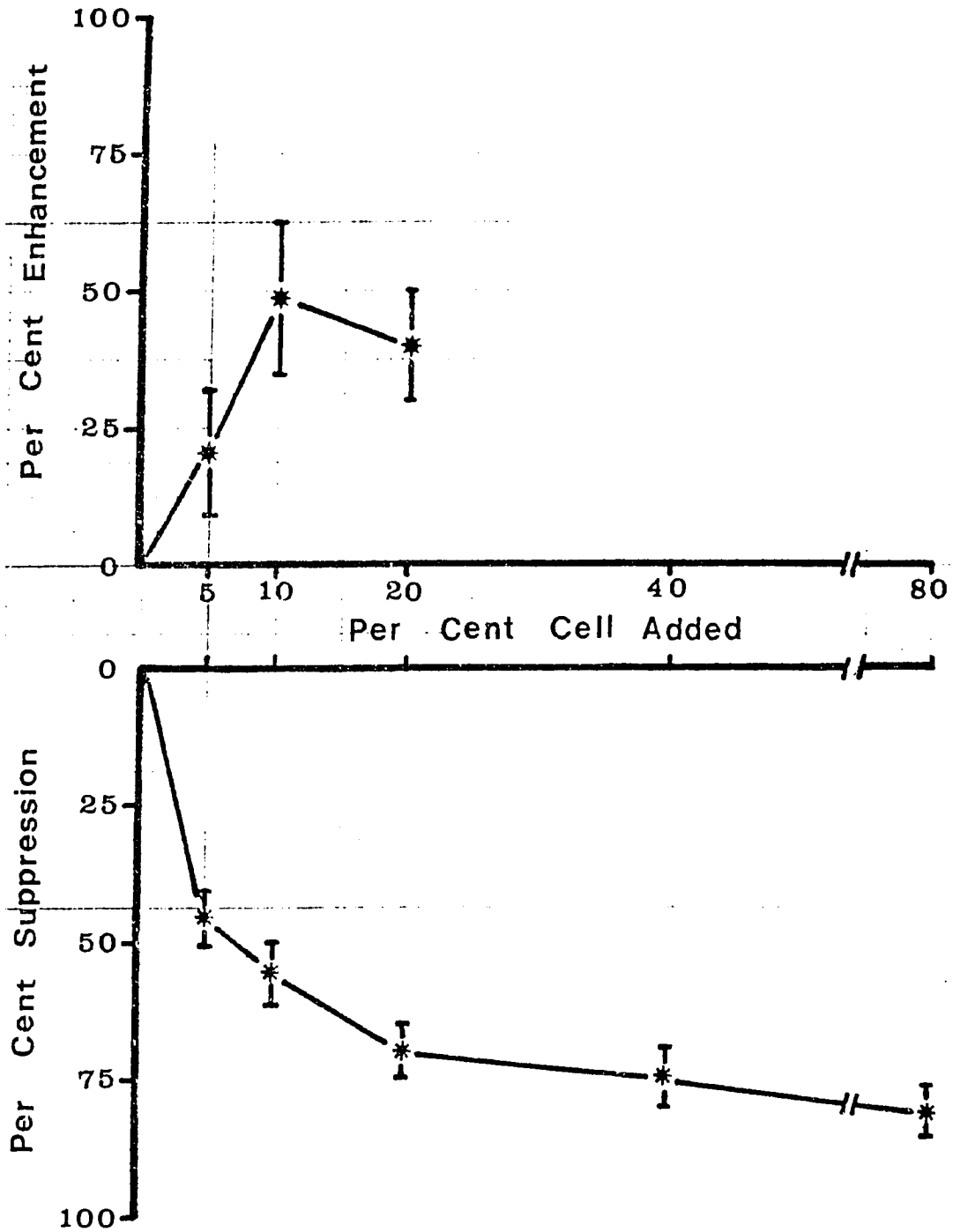

Figure 14. The enhancement activity of Con A-SRBC nonrosette T cells and the suppressive activity of Con A-SRBC rosette T cells (the concentrations of secreted IgG were measured by sandwich ELISA).

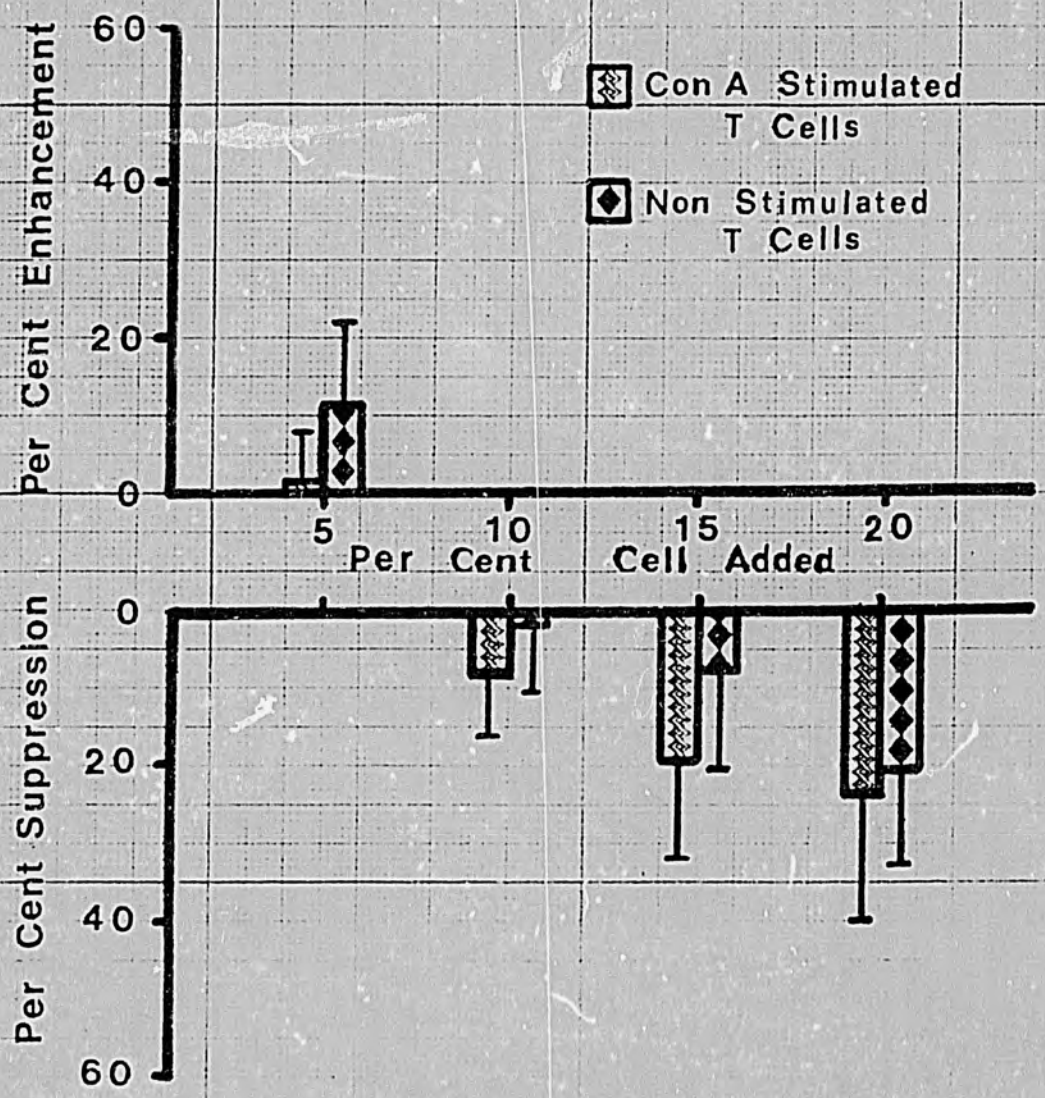

Figure 15. Suppressor and helper activities of Con A stimulated and Con A nonstimulated T cells (the numbers of PFC were measured by reverse hemolytic plaque assay).