PN-AAZ-753

Asian Pacific Journal of Allergy and Immunology (1987) 5 : 129-136

# Enumeration of Interleukin 2-Producing Cells from Rat Spleen

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**SUMMARY** A method for the enumeration of IL2 – producing cells from rat spleen has been developed. Rat spleen cells were stimulated with concanavalin A (Con A), washed, then mixed with IL2 – dependent cells (3 day Con A blasts) and plated in soft agar. Clusters of IL2 – dependent cells formed around IL2 – producing cells, giving colonies which were easy to count under a dissecting microscope. All experimental factors influencing development of colonies of IL2 – producing cells surrounded by IL2 – dependent cells were evaluated and set up. Optimum number of effector cells was 2.5 × $10^5$ cells/culture, while optimum number of IL2 – dependent cells was 6 × $10^6$ cells/culture. Optimum concentration of Con A for IL2 stimulation was 40 μg/ml with an optimal stimulation time of 10 hours. Optimum incubation time for development of IL2 – producing cell colonies was 5 days. The number of IL2 – producing cells by this technique had a good correlation with the level of IL2 in the cell culture fluid (r = 0.885). When colonies were aspirated from agar and stained by Wright stain, a big purple stained cell at the center was surrounded by small cells in almost all colonies examined. All cells from colonies were fluoresed with anti-mouse Thy 1.2 – fluorescein conjugate. However, they were negative with anti – mouse Ig – fluorescein conjugate. The number of IL2 – producing cells was 816 – 2080 cells/$10^6$ of rat spleen cells with mean ± S.E.M. = 1404 ± 154/$10^6$ cells.

When stimulated by recall antigens, lectins or bacterial products, monocytes and lymphocytes release biologically highly active soluble mediators termed monokines and lymphokines respectively. One of the monokines is a non-antigen specific peptide, originally designated lymphocyte activating factor (LAF) and now called interleukin 1 (IL1). One of the most important lymphokines previously designated T-cell growth factor (TCGF), is now called interleukin 2 (IL2).[1] The monokine IL1 seems to exert its effects on immune responses by inducing putative IL2-producing cell to differentiate to the point where subsequent ligand activation triggers the production and release of IL2. Such a scheme truly separates the effect of the monokine IL1 from that of the lymphokine IL2.[2,3]

After the discovery of IL2, this lymphokine has attracted considerable scientific attention because of its ability to stimulate continuous growth of T lymphocytes. IL2 is not only a useful reagent for the maintenance of large numbers of effector T lymphocytes, but also a potentially potent immunoregulatory molecule. It could be of therapeutic usefulness in a number of clinical conditions. Many investigators have successfully worked on the production, isolation, and purification of human IL2, rat IL2 and murine IL2.[1-3] The molecular, biological and biochemical characteristics of IL2 from these three species have also been investigated.[4]

Although the production of IL2 from human peripheral blood mononuclear leukocytes (PBML),[5] murine or rat spleen cells[6] and murine or human tumor cell lines[7] has been studied intensively in many laboratories, a quantitative assay of IL2 in the tissue culture fluid from a cell source has only measured the amount of IL2 per unit of cells, and has not determined the number of cells producing IL2. Accurate enumeration of the number of IL2-producing cells is essential to the understanding of immune

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responses both in humoral-mediated immunity (HMI) and cell-mediated immunity (CMI). We have developed a simple technique which can quantitate the number of IL2-producing cells from spleen cells of rats. When IL2-producing cells are cultured with IL2-dependent cells in semisolid agar medium, colonies of IL2-dependent cells form around IL2-producing cells. All colonies formed in the semisolid agar can be enumerated directly under a dissecting microscope.

## MATERIALS AND METHODS

### Preparation and stimulation of rat spleen cells

Lewis strain of albino rats from our own animal colony at the age of 6-8 weeks were killed by diethylether vapor in a closed jar. Spleen was removed aseptically, cut into small pieces, crushed, and passed through a 40-gauge stainless steel mesh screen in RPMI 1640 medium. The contaminating rat erythrocytes in the spleen cell suspension were lysed by 0.155 M, pH 7.2 hypotonic ammonium chloride solution. The isolated spleen cells were washed twice and adjusted to $1 \times 10^6$/ml with RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS, GIBCO), 10 mM HEPES, 100 units/ml penicillin G, 100 $\mu$g/ml streptomycin, 1 mM sodium pyruvate and $5 \times 10^{-5}$M 2-mercaptoethanol (2-ME). Ten milliliters of rat spleen cell suspension containing 40 $\mu$g/ml concanavalin A (Con A, Sigma) was added to a 60-mm diameter tissue culture plate (Falcon). All culture plates were incubated at 37°C, 5% $CO_2$ in air with humidified atmosphere for 10 hours. Cultured cells were harvested from plates and washed twice with RPMI 1640 medium by centrifuging at 600×g 10 min at room temperature. Spleen cells were adjusted to $2.5 \times 10^6$/ml with supplemented RPMI 1640 medium.

### Three-day Con A blasts

Three-day Con A blasts were prepared from inbred strains of BALB/c mice with some modification of the method of Granelli-Piperno *et al.*[8] Briefly, 6-8 weeks old animals were killed by cervical dislocation; spleens were removed aseptically and crushed through a stainless steel mesh into RPMI 1640 medium. After erythrocytes were lysed and the mouse spleen cells washed twice, the cell suspension was adjusted to $1 \times 10^6$/ml with supplemented RPMI 1640 medium. Twenty milliliters of spleen cell suspension, containing 5 $\mu$g/ml Con A, was cultured in a 100-mm diameter tissue culture plate (Falcon). After 3 days, the nonadherent cells were resuspended by gentle pipetting, collected, and washed twice with RPMI 1640 medium. Cell suspension was adjusted to $3 \times 10^7$/ml with supplemented RPMI 1640 medium.

### Preparation and seeding of culture plates

The culture method used was the soft agar gel system for culturing bone marrow cells[9] with some modification using 35 × 10-mm tissue culture plates (Falcon). The lower agar layer of each culture plate contained 1.0 ml of a mixture composed of 0.5 ml of double strength RPMI 1640 with supplements and 0.5 ml of 1% agarose in distilled water (Agarose Indubiose A45, Accurate Chemical, Hicksville, NY). The upper layer, with a volume of 1 ml, was composed of 0.28 ml of 1% agarose in distilled water, 0.28 ml of double strength RPMI medium, 0.14 ml of RPMI medium, 0.2 ml of responder cell suspension (3-day Con A blasts), and 0.1 ml effector cell suspension (Con A-stimulated rat spleen cells). The mixture was permitted to gel at room temperature for 15 minutes. For controls, cultures were set up in which the upper agar layer consisted of only effector cells or only responder cells. All culture plates were incubated in a $CO_2$ incubator at 37°C 5% $CO_2$ for 5 days. All colonies were enumerated under a dissecting microscope with ×30 magnification.

### Experimental factors influencing development of colonies of IL2-producing cells

*1. Effect of number of responder cells.* Various cell numbers of 3-day Con A blasts, 0, $3.0 \times 10^6$, $4.5 \times 10^6$, $6.0 \times 10^6$ $7.5 \times 10^6$, $9.0 \times 10^6$, $10.5 \times 10^6$ and $12.0 \times 10^6$ cells were plated with $2.5 \times 10^5$ of effector cells (Con A-stimulated rat spleen cells). Colonies developed in the agar plates were counted after 5 days of incubation.

*2. Effect of number of effector cells.* Various cell numbers of Con A stimulated rat spleen cells, *i.e.*, $1.25 \times 10^5$, $2.5 \times 10^5$, $5.0 \times 10^5$, $7.5 \times 10^5$ and $1.0 \times 10^6$ cells were plated with $6.0 \times 10^6$ responder cells (3-day Con A blasts). Colonies in the agar plates were counted after 5 days of incubation.

*3. Effect of Con A concentrations for stimulation of rat spleen cells.* Rat spleen cell suspension, $1 \times 10^6$/ml was stimulated with 0, 10, 20, 40 and 80 $\mu$g/ml Con A for 10 hours. The cell cultures were washed twice and resuspended in RPMI 1640 with supplements. The effector cells, $2.5 \times 10^5$ cells in 100 $\mu$l, were mixed with $6.0 \times 10^6$ cells in 200 $\mu$l of responder cells. Colonies in the agar plates were counted after 5 days of incubation.

*4. Optimal stimulation period of Con A for effector cells.* Rat spleen cell suspension of $1 \times 10^6$/ml was stimulated with 40 $\mu$g/ml Con A for a period of 5, 10, 18 and 24 hours respectively. After each stimulation period, cultured cells were washed twice and resuspended in RPMI 1640 medium with supplements,

and 2.5 × $10^5$ cells of Con A stimulated rat spleen cells were agar plated with 6.0 × $10^6$ of responder cells. Colonies were counted after 5 days of incubation.

*5. Optimum incubation period for development of colonies.* After stimulation with 40 μg/ml Con A for 10 hours, the rat spleen suspension of 1 × $10^6$/ml was washed twice and 2.5 × $10^5$ cells/100 μl were mixed with 6.0 × $10^6$/200 μl of responder cells. Colonies developed in agar plates were counted every day for 7 days.

**Correlation between number of IL2-producing cells and level of IL2**

Various numbers of Con A-stimulated rat spleen cells, 0.625 × $10^5$, 1.25 × $10^5$, and 2.5 × $10^5$/ml, were mixed with 6.0 × $10^6$ responder cells and agarose. Colonies of IL2-producing cells were enumerated after an incubation period of 5 days. The level of IL2 in each supernatant fluid was determined by microassay using CTLL-2 as the indicator cells[10] (CTLL-2 was kindly provided by Professor Ward E. Bullock, Division of Infectious Diseases, Department of Internal Medicine, University of Cincinnati, Ohio, USA). The level of IL 2 production and the number of IL2-producing cells were plotted to see the correlation.

**Morphological and cell surface markers studies**

Colonies in each culture were punched out using a capillary pipette and the contents were blown into 1.0 ml RPMI medium (37°C). In each experiment, 10 colonies from one plate of culture were pooled. The morphology of cells was examined by phase contrast microscopy and by staining with Wright's stain. Cells from colonies were also stained with goat anti-mouse Ig-fluorescein conjugate and anti-mouse Thy 1.2 fluorescein conjugate (Becton Dickinson, Mountain View, CA.) for detection of mouse B and T lymphocytes respectively. Individual colonies were also stained and examined by light microscope.

## RESULTS

**Experimental factors influencing development of colonies of IL2-producing cells**

Figure 1 shows the influence of the number of responder cells on the development of colonies of IL2-secreting cells in 4 rat spleens. The optimum cell concentration of responder cells was 6 × $10^6$ cells/culture. The appropriate concentration of effector cells from 5 rats was 2.5 × $10^5$ cells/culture as shown in Figure 2. Figure 3 shows the optimal concentration of Con A for stimulation of IL2-producing cells was 40 μg/ml in 3 rats, while Figure 4 shows that the optimal stimulation time was 10 hrs using 40 μg/ml con A (5 rats). Figure 5 shows optimum incubation time for IL2 colony development in 2 rats was 5 days. The relationship between the number of IL2-producing cells and the level of IL2 in 4 rats is shown in Figure 6. There is a significant correlation between the number of IL2-producing cells and the level of IL2 in the culture supernatant fluid (r = 0.885)

Figure 7 shows the IL2 dependent properties of 3-day Con A blasts and CTLL-2. After rat factor (containing IL2) was adsorbed with 3-day Con A blasts or CTLL-2, the adsorbed rat factor was assayed for IL2 with 3-day Con A blasts (Fig. 7A) or with CTLL-2 (Fig. 7B). All IL2 was adsorbed out by 3-day Con A blasts or CTLL-2. Figure 8 shows the morphology of an IL2-producing colony [A, × 30] and IL2-producing and non-IL2-producing cells [B, × 200].

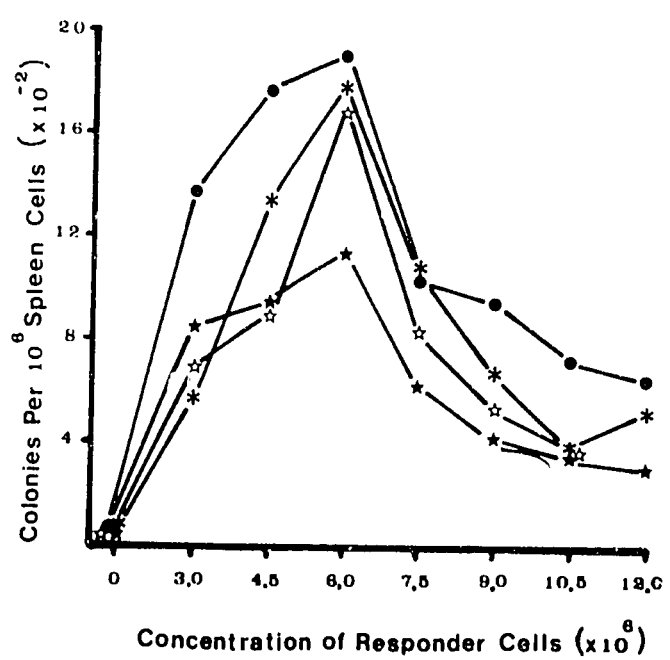


Fig. 1 Optimal concentration of responder cell (3 – day Con A blasts) for development of colonies of IL2 – producing cells (4 experiments).

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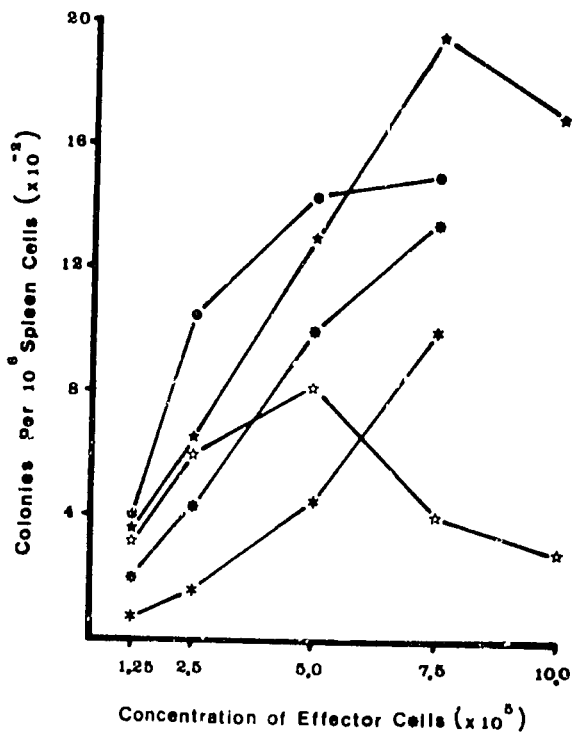


Fig. 2 Titration of effector cells (Con A – stimulated rat spleen cells) for development of colonies of IL2 – producing cells (5 experiments).

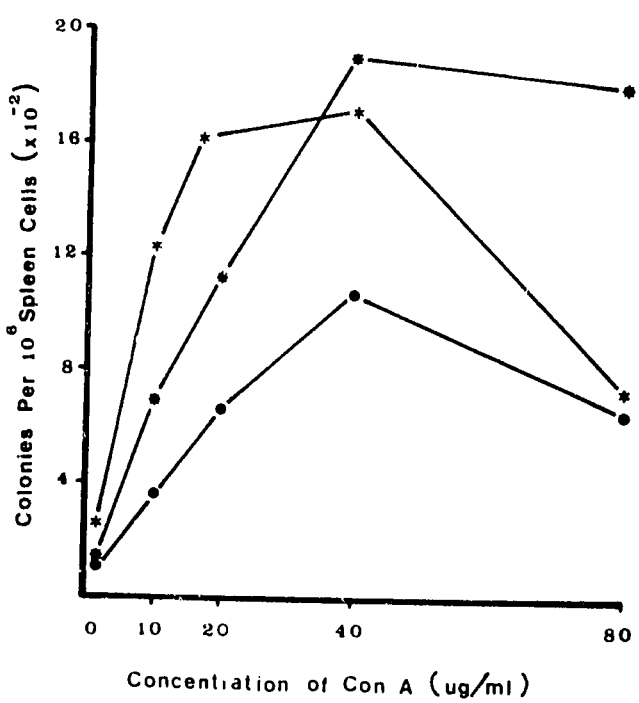


Fig. 3 Titration for the optimal concentration of Con A for stimulation of IL2 – producing colonies (3 experiments).

Table 1 shows numbers of colonies when effector cells were mixed with responder cells. The resulting number of IL2-producing cells is 816-2080 cell/$10^6$ spleen cells with a mean $\pm$ S.E.M. = 1404 $\pm$ 154/$10^6$ cells. When responder or effector cells were cultured alone in agar, the numbers, of spontaneous colonies were 0-79 colonies/6 $\times$ $10^6$ (Mean $\pm$ S.E.M. = 21 $\pm$ 10 colonies) or 0 colony/2.5 $\times$ $10^5$, respectively.

## DISCUSSION

T cells and their secretory products, lymphokines, are the major regulators of the immune system. IL2, one of lymphokines, has been shown to act in a variety of immune responses. Purified IL2 has shown, *in vitro*, a wide variety of activities such as to trigger the proliferative expansion of activated T cell clones, enhance thymocyte mitogenesis, provide T cell help for the generation of nude spleen-cell plaque-forming cell responses to heterologous erythrocytes, and induce alloantigen-specific thymocyte and nude spleen cytolytic T cell reactivity. The *in vivo* administration of purified IL2 results in the augmentation of two distinct cytolytic responses, alloantigen-specific CTL activity and resident NK-cell responses.[4] These findings confirm that IL2 may be an important regulator of immune function and, as such, warrants examination as an active therapeutic agent against clinically deficient, aberrant immune responses. Therefore, IL2 has attracted considerable scientific attention, not only as a useful reagent for the maintenance of large numbers of effector T lymphocytes, but also as a potentially potent immunoregulatory molecule, which could be of therapeutic usefulness in a number of clinical conditions.

There are many methods used to detect the IL2 level in cell culture

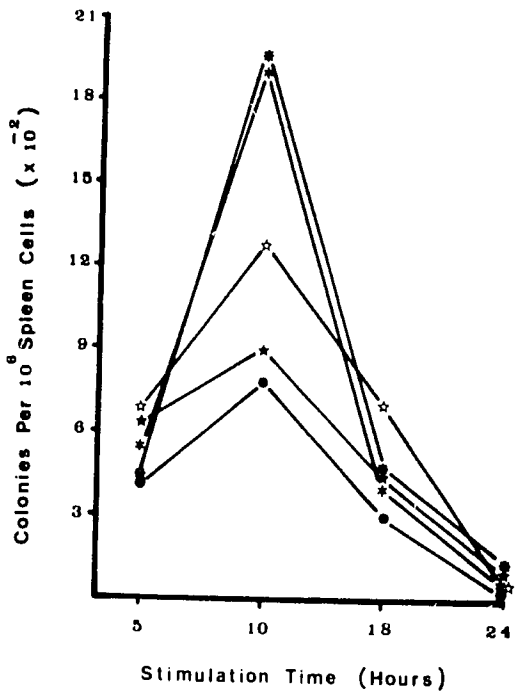


Fig. 4 Optimal stimulation time of 40 μg/ml Con A for development of colonies of IL2 – producing cells (5 experiments).

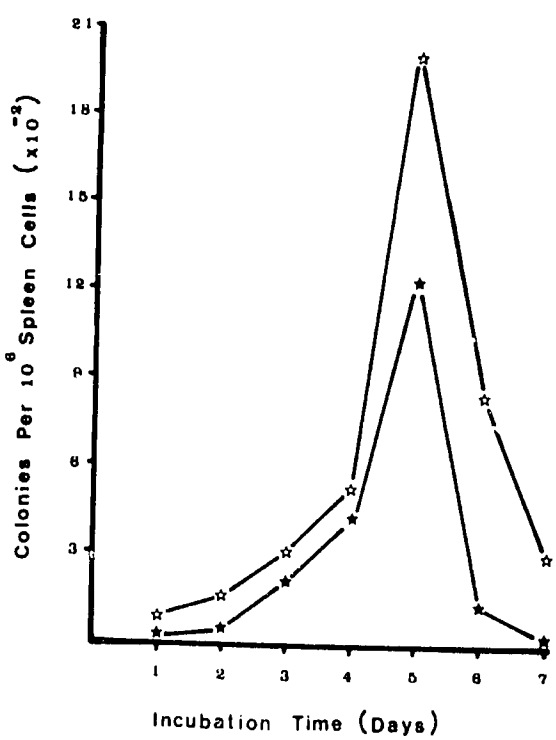


Fig. 5 Determination of optimal incubation time for development of colonies of IL2 – producing cells (2 experiments).

fluid, and the ability of T lymphocytes to produce IL2 in various diseases has been evaluated using these procedures.[11] The unit amount of IL2 per amount of cell culture could be determined by these IL2 assays. However, the number of T lymphocytes that can produce and secrete IL2 cannot be determined by these assays. Therefore, we have developed a simple and reliable technique which can enumerate IL2-secreting cells using a soft agar system.[9]

All experimental factors influencing development of colonies of IL2-secreting cells were evaluated and set up. The optimal number of rat spleen cells (effector cells) which was stimulated to secrete IL2 was $2.5 \times 10^5$ cells/culture, while the optimal number of responder cells (3-days Con A-blasts) was $6 \times 10^6$ cells/culture. The optimal concentration of Con A was 40 μg/ml with the optimal stimulation time 10 hours. When the number of IL2-producing cells was compared with the level of IL2 in the cell culture fluid, there was a significant correlation between the two ($r = 0.885$). The optimal incubation time for development of IL2-producing cell colonies was 5 days. It was noted that after 5 days of incubation, the IL2-producing cell colonies disintegrated and dissolved after 6-7 days. When IL2-producing cell colonies were examined under a light microscope, they showed one big purple stained cell surrounded by smaller IL2-dependent mouse 3-day Con A blasts. When IL2-producing cell colonies were smeared and stained with anti-Thy 1.2-fluorescein conjugate (specific for T cells of mice), all cells were positive like the 3-day Con A blasts. However, Con A-treated rat spleen cells gave negative reaction to the anti-Thy 1.2 antiserum. IL2-producing colonies, 3-day Con A blasts and Con A-stimulated rat spleen cells were negative when stained with anti-mouse Ig-fluorescein conjugate.

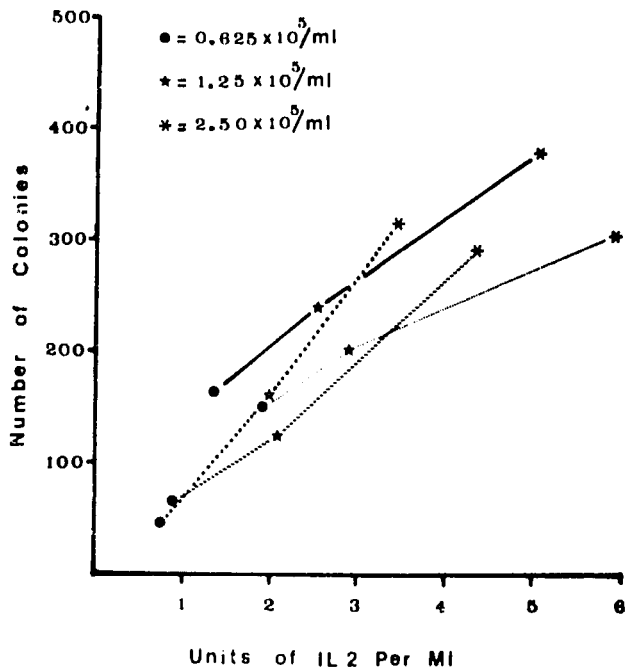


Fig. 6 Relationship between number of IL2 – producing cells and level of IL2 production from rat spleen cells, $0.625 \times 10^5$, $1.25 \times 10^5$ and $2.5 \times 10^5$ per ml (4 experiments).

The possible mechanism of IL2-producing cell and subsequent IL2-dependent cell colony occurs when the IL2-producing cell secretes IL2 by the stimulation of Con A. IL2-dependent cells around the IL2-producing cell will respond to the IL2 secretion by dividing and forming a colony around the producing cell. One can hypothesize that all but one cell in each colony will be IL2-dependent (mouse T) cells. Our results, showing that all cells examined from the colony were positive to anti-Thy 1.2-fluorescein conjugate, confirm this possibility. The IL2-dependent cells (3-day Con A blasts) used in our study are truly IL2-dependent cells because when rat factor (containing IL2) is adsorbed with CTLL-2, the 3-day Con A blasts is not able to grow in the adsorbed supernatant fluid.

Some researchers have tried to enumerate IL2-secreting T cells. Miller and Stutman[12] tried to enumerate IL2-secreting T helper

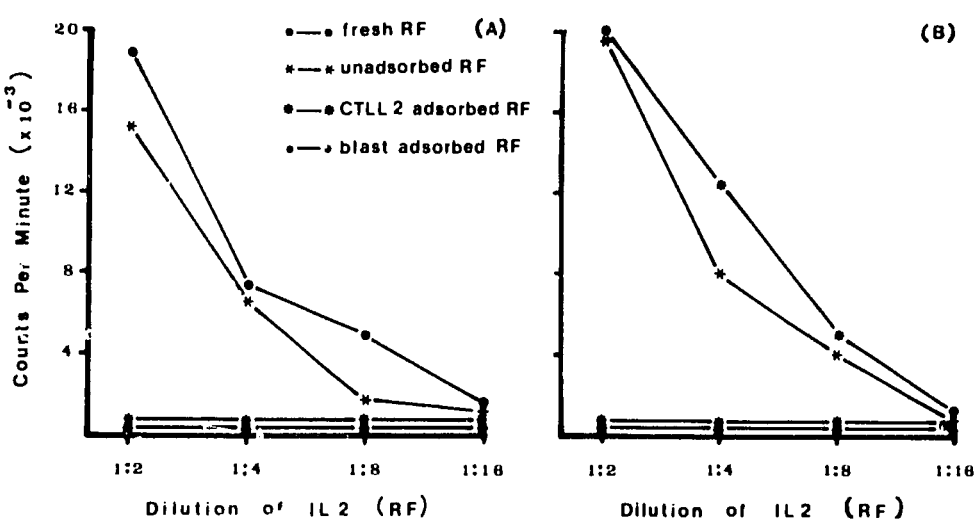


Fig. 7 Adsorption of IL2 (Rat Factor) with CTLL – 2 or 3 – day Con A blasts; supernate assayed for IL2 activity after adsorption by 3 – day Con A blasts (A) or CTLL – 2 (B).

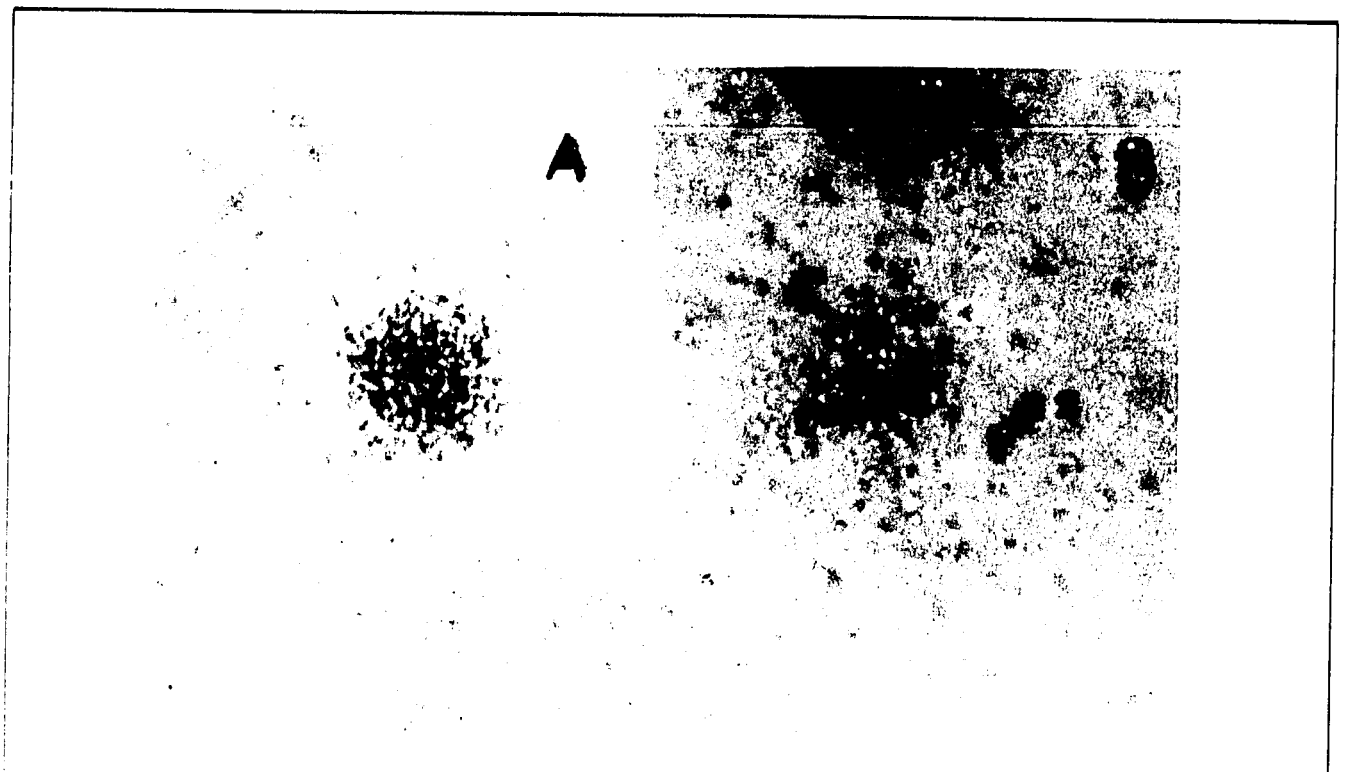


Fig. 8 IL2 – producing cell colony; one whole colony under light dissecting microscope (A, × 30); one big cell at the center of colony surrounded by smaller cells of 3 – day Con A blasts (B arrow 1, × 200) (B arrow 2 Non – IL2 – producing cell).

cells in spleen cell populations of mice by limiting dilution analysis. After alloantigen stimulation, there was about one T cell in every 30 to 300 that could generate detectable levels of IL2. However, their procedure depends on statistical analysis and estimation. Gillis *et al.*[13] tried to identify interleukin 2 producer and responder T lymphocyte subpopulations using monoclonal antibody specific to IL2 and immunoperoxidase staining. They identified a T cell subpopulation in PHA-stimulated human PBML cultures which they believed to be IL2 producer T lymphocytes. Their interpretation was supported by a similar staining pattern of Jurkat FHCRC cell line known to be a human IL2 producer. Their IL2 producer cells also had intense cytoplasmic staining surrounding the IL2 deposit-free nucleus. Vie and Miller[14] presented a culture method for estimating the number of human blood lymphocytes producing IL2 when responding to mitogen. Their method was also based on limiting dilution analysis and estimation. Our procedure, on the other hand, determines the actual number of IL2-producing cells which are surrounded by IL2 dependent cells, providing a simple, accurate and straightforward means to enumerate IL2-producing cells in a variety of immunologic disorders. This technique should be used as a potential procedure to evaluate the abnormality of immunoregulation in various diseases concerning aberrant immune responses.

**Table 1** Number of colonies from different cell cultures

| Cell types | | Number of colonies | |
|---|---|---|---|
| Responder | Effector | Range | Mean±S.E.M. |
| – | + | 0 | 0[a] |
| + | – | 0–79 | 21±10[b] |
| + | + | 816–2080 | 1404±154[c] |

[a]Colonies per 2.5 × $10^5$ of effector cells
[b]Colonies per 6.0 × $10^6$ of responder cells
[c]Colonies per 1.0 × $10^6$ of effector cells

## ACKNOWLEDGEMENTS

Research supported by USAID/PSTC program Grant Number 936-5542-G-00-5056-00 to Chiang Mai University.

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