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Separation of Human Suppressor and Helper T Cells by Concanavalin A-Coated Sheep Erythrocytes

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SUMMARY Normal human peripheral blood mononuclear leukocytes (PBML) were activated by concanavalin A (Con A). Con A-activated and non-activated T cells were separated by E (AET) rosettes (2-aminoethylisothiouronium hydrobromide treated sheep erythrocyte rosettes). Purified T cells were rosetted with Con A-coated sheep red blood cells (Con A-SRBC) at 37° C resulting in Con A-SRBC rosetted and non-rosetted T cells. The Con A-SRBC rosetted T lymphocytes in the T lymphocytes from Con A-activated and non-activated PBML were 44.4 ± 5.4 percent and 16.0 ± 7.5 percent (Mean ± S.D.) while the Con A-SRBC non-rosetted T lymphocytes were 55.6 ± 5.4 percent and 84.0 ± 7.5 percent respectively. The Con A-SRBC rosetted and non-rosetted T cells were separated by Ficoll-Hypaque gradient centrifugation.

Functional studies of Con A-SRBC rosetted and non-rosetted T cells were performed by *in vitro* tests using pre-amplified reverse hemolytic plaque assay for measuring numbers of immunoglobulin G (IgG) secreting cells and ELISA quantitation of IgG concentration. Both techniques were used to assess the suppressor and helper functions of the Con A-SRBC rosetted and non-rosetted T cells. The Con A-SRBC rosetted cells obtained from T cells of Con A-activated PBML showed strong suppressor activities to normal PBML in both pre-amplified reverse hemolytic plaque assay and sandwidh ELISA of IgG concentration, while the Con A-SRBC non-rosetted T cells demonstrated strong helper activities to normal PBML in both assay systems. The amount of Con A-SRBC rosetted and non-rosetted T cells added to normal PBML to show the suppressing or enhancing activities is impressively small in number and in dose response. When this separation technique is evaluated using PBML from lepromatous leprosy, similar results are obtained as compared to those obtained by conventional techniques. The Leu-3a/Leu-2b or CD4/CD8 (helper or inducer T cells/cytotoxic or suppressor T cells) ratios of T cells is significantly higher than the Con A-SRBC rosetted T cell subpopulations ($p < 0.05$). The Leu-3a/Leu-2b (CD4/CD8) ratios of Con A-SRBC non-rosetted T cells is also significantly higher than that of the Con A-SRBC rosetted T cells ($p < 0.05$).

Several reports have shown evidence of the existence of T lymphocyte subpopulations in man. These studies employed differential Fc-receptor binding, hetero-and auto-immune antisera, and monoclonal antibodies to separate the subpopulations of human T cells. An autologous erythrocyte rosette technique has also been used to separate Con A-induced suppressor and helper T cells. [1]

For the Fc-receptor binding, it was demonstrated that two human T cell subsets, T cells bearing receptors for the Fc portion of IgG ($T\gamma$) and T cells bearing receptors for the Fc portion of IgM ($T\mu$), could be isolated via surface receptors capable of binding the Fc portion of IgG or IgM respectively. [2] Functionally, $T\mu$ and $T\gamma$ populations were distinct with respect to phytohemagglutinin (PHA) responsiveness and capacity to effect help or suppression of B cell-immunoglobulin-production in a pokeweed-mitogen-driven system. [2]

Auto- and hetero-immune antisera and monoclonal antibodies have provided an alternative method for human T cell identification. [3-5] It has been reported that approximately 20 percent of peripheral blood T cells are reactive with OKT5 and OKT8 monoclonal antibodies both of which define the suppressor cytotoxic population. [4] Another monoclonal antibody, OKT4, reacted with 60 percent of the total T cell population

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and was restricted in its reactivity to the TH_2^- ($OKT5^-$) T cell subpopulation.[6] This population was the helper population, because it was required for the optimal development of $OKT5^+$ cytotoxic cells in cell-mediated lympholysis, induction of B cell differentiation, proliferation and immunoglobulin synthesis in a pokeweed-mitogen driven system. Therefore, the $OKT4^+$ and $OKT5^+$ cells belonged to reciprocal subsets, and defined the human helper and suppressor T cell subpopulations respectively.

The separation of suppressor from helper T cells by autologous erythrocyte rosette technique was reported by Sakane *et al.*[1] They fractionated the Con A-activated T cells with autologous erythrocytes into auto-rosetted and non-rosetted cells. The auto-rosetted population showed suppressor activity, whereas the non-rosetted population showed helper functions.

We have found a simple and reliable technique to separate T suppressor cells from the T helper subpopulation. The suppressor and helper activities of each cell population have been evaluated by *in vitro* tests for both the numbers of IgG-secreting cells and concentrations of secreted IgG using pre-amplified reverse hemolytic plaque assay and sandwich ELISA respectively.

MATERIALS AND METHODS

Cell separation.

Leprosy patients were seen at the McKean Rehabilitation Institute in Chiang Mai, Thailand. All leprosy patients were diagnosed by the Ridley Jopling classification based on clinical and histopathological diagnosis.[7] Normal control donors were laboratory personnel and student volunteers. Heparinized venous blood was obtained from normal adult volunteers and leprosy patients. Peripheral blood mononuclear leukocytes (PBML) were prepared by the standard Ficoll-Hypaque density centrifugation method.[8] The PBML were washed three times with RPMI 1640 (Gibco, Grand Island, N.Y.) and adjusted to a final concentration of 1×10^6 cells/ml in RPMI supplemented with 10 percent fetal calf serum (FCS, Gibco). The RPMI medium always contained 100 units of penicillin and 100 μg streptomycin per milliliter.

Con A activation of PBML.

Four milliliters of PBML suspension containing 4×10^6 cells were placed in a sterile plastic tube (Falcon, Cockeysville, M.D.). Fifty micrograms of Con A (Sigma) in 50 μl RPMI medium were added to the cell suspension and incubated at 37° C, 5 percent CO_2 for 16-18 hours. The Con A-activated and non-activated PBML were washed once with RPMI medium, and 4 ml of fresh 10 percent FCS in RPMI medium was added to each tube. Cell suspensions were cultivated for a total period of 60 hours, then washed twice more with 0.3 M α-methyl-D mannoside in a RPMI medium.

Separation of T cells.

T cells from Con A-activated and non-activated PBML were separated by adding 0.1 ml of 1 percent AET-SRBC (2-aminoethylisothiouronium-hydrobromide treated SRBC)[9] to 1×10^6 PBML, incubated at 37° C for 10 minutes, then centrifuged at 600 × G for 2 minutes, and kept at 4-8° C overnight. The rosette forming cells in the pellet were resuspended and further purified by Ficoll-Hypaque gradient centrifugation. The AET-SRBC were lysed with an ammonium chloride buffer solution.

Separation of Con A-SRBC rosetted T cells.

A one percent Con A-SRBC suspension was prepared by mixing equal volumes of 2 percent SRBC in PBS (pH 7.4) with 1:800 of Con A solution [1:800 Con A = 1 μg Con A/μl diluted with PBS (pH 7.4) to 1:800]. The mixture was incubated for 1 hour at 37° C with frequent mixing, then washed twice with PBS. The Con A-SRBC was diluted to 1 percent with RPMI medium.

Purified T cells, 1×10^6 cells/ml, were mixed with 0.1 ml. of 1 percent Con A-SRBC. The mixture was incubated at 37° C, 5 percent CO_2 for 30 min, centrifuged at 600 × G for 2 min, then incubated at 37° C for another 30 min. The Con A-SRBC rosetted T cells were separated from the non-rosetted T cells by Ficoll-Hypaque gradient centrifugation. Con A-SRBC were lysed with an ammonium chloride buffer solution. The Con A-SRBC rosetted and non-rosetted T cells were washed once with RPMI medium, then adjusted to a concentration of 1×10^6 cells/ml.

Culture conditions.

Normal PBML were used as indicated cells for measuring the augmentation or suppression of the isolated T lymphocytes and its subpopulations. The PBML 1×10^6 and 5×10^5 cells/ml in a 10 × 75 mm plastic tube (Falcon) were mixed with different concentrations of T cell subpopulations (such as Con A-SRBC rosetted or non-rosetted T cells). A final dilution of 1:200 of Pokeweed mitogen (PWM; Gibco) was also mixed in the cell culture. All cultures were incubated at 37° C in 5 percent CO_2 in air at 100 percent humidity on a rocker platform (Belco) set at 7 rpm.[10] Cultures were kept in the CO_2 incubator for 6 days.

Pre-amplified reverse hemolytic plaque assay.[11]

1) Coupling of Staphylococcal protein A to SRBC. SRBC were coated with Staphylococcal protein A (SPA) with chromic chloride ($CrCl_3$) as the coupling agent. Briefly, one milliliter of thrice washed packed SRBC was added into 0.9 ml of 0.85 percent sodium chloride, blended with 0.1 ml of SPA (5 mg/ml SPA, Pharmacia, Piscataway, N.J.), and

mixed well. Ten milliliters of a 0.1 mg/ml solution of chromic chloride (Baker Co., Phillipsburg, N.J.) in normal saline was added to the SPA and SRBC mixture in 0.85 percent sodium chloride solution. The solution was mixed gently, incubated at 30° C, immersed 40 min in a water bath with frequent mixing, and then centrifuged at 1000 × G for 5 min. The SPA-SRBC were washed once with 0.85 percent sodium chloride, then the cell button was brought up with 3 ml of RPMI medium to give a 25 percent suspension.

2) Pre-amplification of SPA-SRBC. The SPA-SRBC were preamplified with human IgG (Cappel Labs., Dowington, P.A.), according to the method of Makonkawkeyoon and Vithayasai.[11] The 25 percent SPA-SRBC in RPMI was incubated with the appropriate dilution of human IgG in equal volumes, and the mixture was placed at 37° C for 30 min. The pre-amplified IgG-SPA-SRBC was washed once with RPMI and resuspended in RPMI at a concentration of 25 percent.

3) Hemolytic plaque assay. Plastic petri dishes 60 × 15 mm. (Falcon) precoated with 4 ml of 0.7 percent agarose (Accurate Chemical, Hicksville, N.Y.) in RPMI medium were prepared and stored at 4° C not more than 1 week. Lymphocytes from various cell mixing cultures were harvested by washing once with RPMI, and 0.1 ml were mixed with 0.85 ml of 0.7 percent agarose and 0.06 ml of 25 percent IgG-SPA-SRBC which had been kept in a 50° C water bath. The mixture was vortexed and the contents of the entire tube poured onto the agarose-precoated petri dish, swirled, cooled at room temperature, and then kept in a 37° C 5 percent CO_2 incubator for 2 hours. One milliliter of 1:50 dilution of IgG fraction of rabbit anti-human IgG (gamma chain specific) (Cappel) or 1 ml medium (as control) was then layered onto the plates and incubated for an additional 2 hours. The developing antiserum or medium was removed and 1 ml of 1:40 diluted, fresh guinea pig complement in buffer was added. The plates were incubated for another 1 hour at 37° C, the diluted complement removed and plates kept at 4-8° C overnight before counting the number of plaque forming cells (PFC) under a dissecting microscope. Data were expressed as PFC per 10^6 PBML.

***In vitro* immunoglobulin secretion.**

A total of 1.0×10^6 PBML in 1 ml RPMI 1640 medium were added to 0, 5, 10, 20, 40 and 80 percent concentrations of Con A-SRBC rosetted and non-rosetted T lymphocytes. All cultures were incubated with PWM in a final dilution of 1:200 at 37° C in air with 5 percent CO_2 at 100 percent humidity for 9 days. At the end of the period, culture supernatants were harvested, and the IgG concentrations determined by sandwich ELISA.

Sandwich ELISA for quantitation of IgG.

The amount of secreted IgG in culture supernatants were determined by sandwich ELISA.[12] Briefly, 200 μl of rabbit anti-human IgG (Cappel; dilution 1:2000) was coated onto each well of a polystyrene microtiter plate (Nunc, Kamstrup, Denmark) at 37° C for 3 hours, and washed 4 times with PBS-Tween. Two hundred microliters of diluted test culture supernatants were added, incubated 1 hour at 37° C, and washed 4 times with PBS-Tween. Two hundred microliters of rabbit anti-human IgG peroxidase conjugated (Dakopatts, Glostrup, Denmark; 1:8000) were added, incubated 1 hour at 37° C, and washed 4 times. Two hundred microliters of substrate were added, and the reaction stopped by 50 μl 8 N H_2SO_4 after 30 min. The reacting microtiter plate was read at 492 nm in a spectrophotometer (Microplate Autoreader EL 309, Bio-Tek Instruments, Winooski, VT. U.S.A.). The concentrations of IgG were calculated from a standard curve obtained using known concentrations of standard human IgG.

Surface markers on T cell subpopulations.

Con A-activated T cell and its subpopulations obtained by Con A-SRBC rosette technique were analyzed by a series of monoclonal antibodies reactive to peripheral T cells ($CD3^+$ cells, Leu-4), helper/inducer T cells ($CD4^+$ T cells, Leu-3a), and cytotoxic/suppressor T cells ($CD8^+$ T cells, Leu-2b) (Becton Dickinson, Mountain View, CA). $CD3^+$, $CD4^+$, and $CD8^+$ T cells were determined by indirect immunofluorescence using fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Becton Dickinson) and a fluorescent microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Statistical Analysis.

The student's *t*-test was used.

RESULTS

Table 1 shows the percentage of Con A-SRBC rosetted and non-rosetted T lymphocytes from Con A-stimulated and non-stimulated PBML. The average values ($\bar{x} \pm$ S.D.) of Con A-SRBC rosetted and non-rosetted T lymphocytes from the T cells population were 44.4 ± 5.4 percent and 55.6 ± 5.4 percent in 13 con A-stimulated human PBML, and 16.0 ± 7.5 percent and 84.0 ± 7.5 percent in 13 non-Con A-stimulated human PBML respectively. Figure 1 depicts the enhancement activity of Con A-SRBC non-rosetted T lymphocytes and the suppressive activity of Con A-SRBC rosetted T lymphocytes from 18 Con A-stimulated human PBML by measuring the number of IgG-secreting cells using pre-amplified reverse hemolytic plaque assay. Figure 2 shows the enhancement activity of Con A-SRBC non-rosetted T lymphocytes and the suppressive activity of Con

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

Normal Subject Number	Con A–stimulated				Non–Con A–stimulated			
	%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

A-SRBC rosetted T lymphocytes from 10 Con A-stimulated human PBML by measuring concentrations of IgG from cell cultures using sandwich ELISA technique. The enhancement or suppressive activity patterns from both techniques are in good correlation. Figure 3 illustrates the suppressor or helper activities of 9 Con A-stimulated and 6 non-Con A-stimulated T lymphocytes by measuring the numbers of IgG-secreting cells using pre-amplified reverse hemolytic plaque assay. When concentrations of T lymphocytes are added, the enhancing and suppressive activities of both Con A-stimulated T cells and non-Con A-stimulated T cells are not statistically different ($P > 0.05$). Our separating technique was further evaluated for reliability by testing PBML from lepromatous leprosy patients. The Con A-SRBC rosetted T lymphocytes from Con A-activated PBML of lepromatous leprosy patients show significantly less suppressive activity than Con A-SRBC rosetted T lymphocytes from Con A-activated PBML of normal controls (figure 4). Table 2 shows the cell surface markers of T cell and its subpopulations in five normal subjects. The ratios of Leu-3a/Leu-2b or CD4/CD8 are calculated from the ratios of 5 normal subjects.

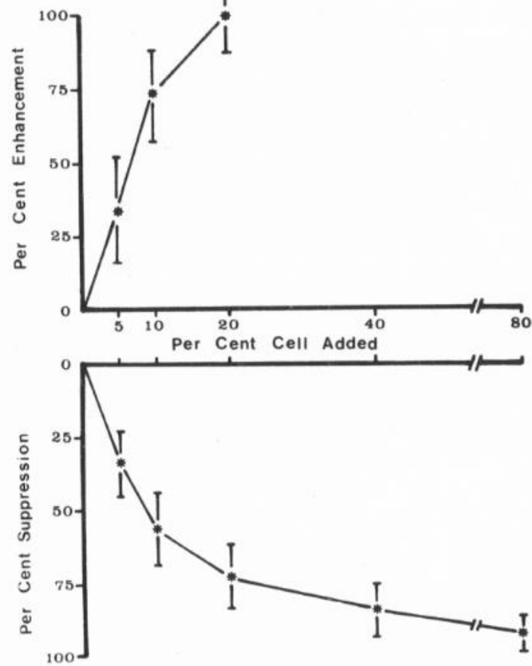

Fig. 1 Enhancement activity of Con A–SRBC non–rosetted T cells and suppression activity of Con A–SRBC rosetted T cells. The numbers of IgG–producing cells are measured by pre–amplified reverse hemolytic plaque assay. Percentage of enhancement or suppression is calculated by comparing numbers of PFC in cells with added concentrations to those in cells (base line control) without added concentrations as follows:

$$\text{Percent Enhancement or Suppression} = 100 - \left(\frac{\text{PFC of indicator cells with added cells}}{\text{PFC of indicator cells without added cells}}\right) \times 100$$

Table 2. Cell surface markers of T cell and its subpopulations.

Cell Type	Leu–4 (CD3)	Leu–3a (CD4)	Leu–2b (CD8)	Leu–3a/Leu–2b** (CD4/CD8)
T cells	95.0 ± 1.4*	52.5 ± 3.5	25.5 ± 3.5	2.07 ± 0.15***
Con A–SRBC rosetted T cells	76.7 ± 11.2	46.8 ± 13.7	47.6 ± 16.4	1.12 ± 0.52
Con A–SRBC non–rosetted T cells	77.3 ± 3.1	54.0 ± 7.0	33.8 ± 8.8	1.75 ± 0.48****

* Mean ± S.D.
** Mean ± S.D. of individual CD4/CD8 ratios.
*** Significantly higher than Con A–SRBC rosetted T cell ($P < 0.05$).
**** Significantly higher than Con A–SRBC rosetted T cell ($P < 0.05$).

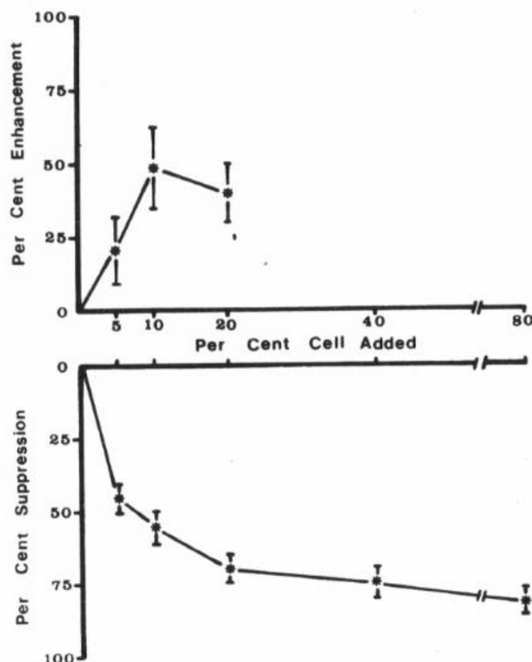

Fig. 2 Enhancement or suppressor activities of Con A–SRBC non–rosetted or rosetted T cells respectively. Concentrations of IgG in cell culture supernatant fluid is measured by sandwich ELISA technique. Percentage of enhancement or suppression is calculated by comparing IgG concentration from cells with added concentrations to those in cells (base line control) without added concentrations as follows:

$$\text{Percent Enhancement or Suppression} = 100 - \left(\frac{\text{IgG of indicator cells with added cells}}{\text{IgG of indicator cells without added cells}}\right) \times 100$$

DISCUSSION

There is much evidence to show that distinct T lymphocyte subsets in man are defined by cell-surface markers. One approach of these studies is to employ differential Fc-receptor binding. Approximately 60-70 percent of T lymphocytes in peripheral blood are Tμ, whereas a smaller portion, representing less than 20 percent, are Tγ cells. Tγ cells serve as suppressors, while Tμ serve as helper T lymphocytes.[2] Hetero- and auto-immune antisera directed at subsets of human T cells have provided another method for T cell subset identification.[3] Twenty percent of peripheral T cells are reactive with TH_2 hetero-antisera (TH_2^+), while the remaining 80 percent of T cells are unreactive with TH_2 sera (TH_2^-). Functionally, the TH_2^+ T cell subsets act as suppressor/cytotoxic populations, while the Th_2^-T cell subsets act as helper/inducer populations.[3] Using murine monoclonal antibodies (mAbs) to human T lymphocytes surface antigens, two major categories of T cells emerge, namely, the T4+ (CD4+) helper/inducer and the T8+ (CD8+) suppressor/cytotoxic subsets.[4,6]

Surface markers of lymphocytes, such as lectin receptors, are of increasing important in immunological regulation studies. Lectin receptors are carbohydrates, residing in the oligosaccharide sequences of membrane glycoproteins or glycolipids. Lectin will specifically combine noncovalently with mono-and oligosaccharides. The gross morphological changes and biochemical events occurring in lectin-stimulated lymphocytes *in vitro* resemble many of the antigen-induced immune reactions that take place *in vivo*. Sakane *et al.*[1] were able to identify and separate Con A-induced suppressor cells from Con A-induced helper cells by autologous erythrocyte rosette technique. Suppressor and helper cells are confined to the auto-rosetted and non-rosetted T cell populations respectively. However, they found that OKT4+ and OKT8+ cell types are equally distributed among both auto-rosetted and non-autorosetted T cell activated by Con A.

Our separation technique for helper and suppressor T lymphocytes stems from the knowledge that Con A can polyclonally activate suppressor

cells *in vitro*.[13] The Con A-activated suppressor cells are then able to inhibit the response of autologous or allogeneic lymphocytes to Con A, PHA, antigens or allogeneic cells in MLC.[13,14] Therefore, we coated SRBC with a maximum concentration of Con A without autoagglutination. The success of Con A coated onto the SRBC was evaluated by rabbit anti-Con A serum for hemagglutination. The Con A-SRBC is used to rosette out Con A activated T lymphocytes, resulting in Con A-SRBC rosetted and Con A-SRBC non-rosetted T cells. The immunoregulatory role of each cell type is determined by mixing the cell type with normal PBML. Their functional role is evaluated by counting IgG-producing cells (using pre-amplified reverse hemolytic plaque assay; PFC)[11] and by measuring the concentration of IgG production (using sandwich ELISA).[12] The helper activity of the Con A-SRBC non-rosetted T lymphocytes is impressively powerful because only small percentages of added cells give very high augmentation of PFC and IgG secretion. Similarly, the suppressor activity of the Con A-SRBC rosetted T lymphocytes is also very powerful because a small percentage of these cells give high suppression. The Con A stimulated T lymphocytes and non-stimulated T lymphocytes have a low suppressive activity.

The reliability of this technique was evaluated using PBML from 16 lepromatous leprosy patients and 16 normal controls. The Con A-SRBC rosetted T lymphocytes from Con A-activated PBML of lepromatous leprosy patients or normal controls was added to normal PBML in different concentrations, then stimulated with PWM. The concentration of IgG in supernatant fluid was determined by sandwich ELISA. One can see that the suppressive activity of Con A-SRBC rosetted T lymphocytes is significantly lower in lepromatous leprosy patients than normal controls in all cell concentrations.

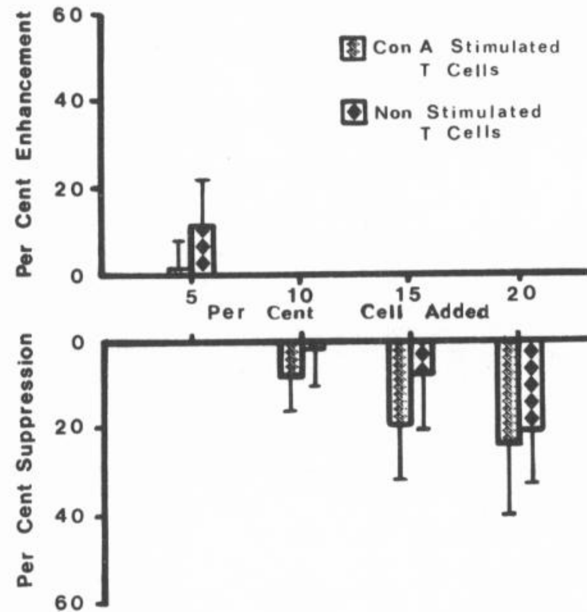

Fig. 3 Suppressor and helper activities of Con A–stimulated and non–stimulated T cells. The numbers of IgG–producing cells are measured by pre–amplified reverse hemolytic plaque assay. Percentage of enhancement or suppression is calculated by comparing numbers of PFC in cells with added concentrations to those in cells (base line control) without added concentrations.

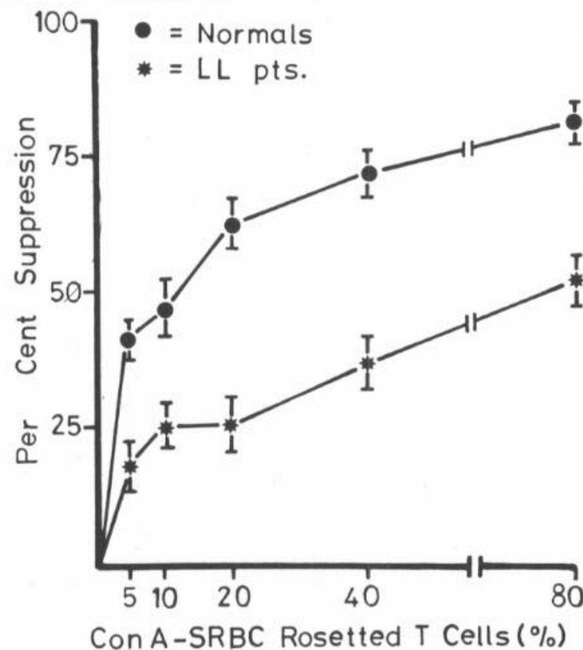

Fig. 4 Suppressive activities of Con A–SRBC rosetted T lymphocytes of normal donors and lepromatous leprosy patients. Mean ± S.D. of 16 normal donors and 16 lepromatous leprosy patients are compared at 5, 10, 20, 40 and 80% of added Con A–SRBC rosetted T cells. The suppressive activities are all significantly different with P values at 5, 10, 20, 40 and 80% = <0.005, <0.010, <0.001, <0.001 and <0.005 respectively.

This finding gives the same result as our previous study in lepromatous leprosy patients when using CD8+ T cells in regulating immunoglobulins-secreting cells. We found that CD8+ T cells in lepromatous leprosy patients had significantly less suppressive activity than normal CD8+ T cells.[15] From phenotypic studies using monoclonal antibodies, we are able to demonstrate that the Leu-3a/Leu-2b or CD4/CD8 (helper or inducer T cells/cytotoxic or suppressor T cells) ratios in Con A-SRBC rosetted T cells is significantly lower than in T cells and in Con A-SRBC non-rosetted T cells. This simple and inexpensive technique should be widely used for separation and isolation of helper and suppressor T lymphocytes for functional studies in various immuno-aberrant conditions and diseases.

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