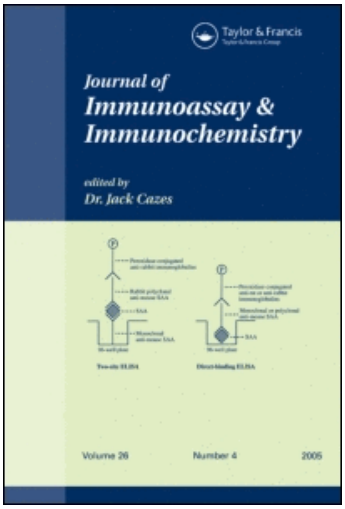

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DETECTION OF SOLUBLE T CELL RECEPTOR -RELEASING CELLS BY ELISPOT ASSAY

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ABSTRACT

A specific and sensitive enzyme-linked immunospot (ELISPOT) assay has been developed for the detection and enumeration of soluble T cell receptor (TCR)-releasing cells. Using this method,we readily detected at the single cell level the release of soluble TCR by living T lymphoma cells (MT-2 and HSB-2) but not by human B lymphoma cells (DAKIKI), mouse hepatoma cells (MH134) and dead MT-2. Furthermore, distinct spots in MT-2 cell culture were not visualized using several monoclonal antibodies against antigens unrelated to TCRs as a primary antibody. The specific and quantitative detection of soluble TCR-releasing cells using ELISPOT assay will certainly provide a valuable tool to better characterize soluble TCRs and their relationship to immune regulation and a number of diseases.

(KEY WORDS: soluble TCR; ELISPOT; T lymphoma; cycloheximide)

INTRODUCTION

Natural soluble membrane receptors such as epidermal growth factor receptors(1), interleukin-2 (IL-2) receptors(2), Fc γ receptors(3), IL-4 receptors (4), IL-6 receptors(5) and tumor necrosis factor receptors(6) are well known to exist in serum or urine. In addition, it has recently become apparent that a T cell suppressor factor (TsF) produced by suppressor T hybridomas shares some epitopes with TCR (7,8). The suppressive effect on lymphocytes attributed to TsF was confirmed by blocking its activity with a monoclonal antibody specific for the TCR α-chain, but not for the TCR β-chain (9,10). A study by Behlke and Loh(11) suggests that a soluble form of the α / β receptor is synthesized via alternative RNA splicing in the constant region of the TCR. Based on these findings, Takata et al.(10) have interpreted that the TsF is not a shed form of the membrane TCR.

Other investigators have demonstrated that the TsF shares serologically common determinants with both α - and β-chains of TCR(7,12-14). It has previously been shown that helper T cells also produce antigen-specific helper factors bearing TCR V β_8 determinants (15). These findings raise the interesting possibility

that T cells secrete soluble factors that are antigenically related to TCRs. This possibility has prompted us to evaluate the applicability of the ELISPOT assay (16,17) for detecting soluble TCRs-releasing cells, using T lymphoma cells as model system.

MATERIALS AND METHODS

Cell lines

MT-2 (human T lymphoma), HSB-2 (human T lymphoblastoid cells), DAKIKI(IgA secreting human B-cell lymphoblastoid cells) and MH134(murine hepatoma cells) were maintained in RPMI1640 medium supplemented with 1 % fetal bovine serum(FBS), 60 mg/L kanamycin and 2 mM glutamine. To detect soluble TCRs-releasing cells, each cell line (10^4 cells/0.5 ml per well) was incubated in RPMI1640 medium containing 1 % FBS and 2 mM glutamine for 6 h at 37 °C in humidified atmosphere of 5 % CO_2 in air. The cell viability was always more than 95 % using the method of Stewart and Ingram (18). Dead MT-2 cells were obtained by freeze-thawing of HT-2 cells ($2x10^4$ cells/ml) in distilled water.

ELISPOT assay for the detection of single cells secreting soluble TCR

MT-2, HSB-2, DAKIKI and MH134 cells (10^4 cells/0.5 ml per well) suspended in RPMI1640 medium containing 1% FBS were added to Millicell (12 mm in diameter; Millipore, Bedford, MA) in flat-bottomed 24-well plates (Corning Laboratory Sciences, Corning, NY). The cultures were placed in a humidified incubator for 6 h in an atmosphere of 5% CO_2 in air at 37 °C. The incubated cells on Millicell membrane were removed with a cell scraper and the membranes were washed three times with Tris-buffered saline pH 7.6 (TBS). Then, the Millicell well was treated for 10 min with TBS-2% H_2O_2 to inhibit endogenous peroxidase activity and subsequently washed five times in TBS containing 1% Tween 20 (TBST). Each well was blocked with 0.5 ml of 3% bovine serum albumin (BSA) in TBS for 1 h at 37 °C, followed by three washes in TBST. The wells were incubated with 0.2 ml of mouse anti-human TCR $\alpha\beta$ monoclonal antibody(mAb)BW242/412 IgG_{2b}(1000^{-1}dilution; T cell Diagnostics, Cambridge, MA), mouse anti-TCR α mAb 3A8 IgG_{2a} (1000^{-1} dilution; T cell Diagnostics), mouse anti-TCR β mAb 8A3 IgG_1 (1000^{-1} dilution; T cell Diagnostics), mouse anti-human epithelial membrane antigen (EMA) mAb IgG_{2a}(predilution; Nichire, Tokyo), mouse anti-hepatitis B surface antigen (HBs) mAb IgG_{2b} (predilution; Nichire) or mouse anti-human

myoglobin mAb IgG_1 (1000^{-1} dilution; ICN Biochemicals, Costa Mesa, CA) in TBS containing 1% BSA overnight at 4 °C, and washed four times with TBST. The wells were subsequently incubated with 0.2 ml of biotin-conjugated goat $F(ab')_2$ fragment anti-mouse IgG (Immunotech, Marseille, France) diluted at 1:200 in TBS containing 1% BSA for 1 h. After four washes in TBST, the wells were exposed to 0.2 ml of a 1:50 dilution of streptavidin conjugated to horseradish peroxidase (Vector Lab. Burlingame, CA) for 30 min, and washed three times with distilled water. The spots were visualized by the addition of substrate solution containing 0.2 mg/ml DAB (3,3'-diaminobenzidine.4HCl; Wako Pure Chemical Industries, Tokyo) and 0.003% H_2O_2 in 50 mM TBS, pH 7.6. The number of spots in the wells was counted with a microscope at 10 x magnification.

Treatment with cycloheximide

MT-2 cells (2×10^4 cells/ml) were pretreated with cycloheximide (5 μg/ml or 50 μg/ml; Sigma, St. Louis, MO) in RPMI1640 medium containing 1% FBS for 1h at 37 °C. After extensive washing, the cells (2×10^4 cells/ml) were incubated once in Millicell in 0.5 ml volumes of RPMI1640 medium containing cycloheximide (5 μg/ml or 50 μg/ml) and 1% FBS for 6 h at 37 °C.

RESULTS

Antigen-specific ELISPOT

MT-2 cells (10^4 cells/well) were incubated in individual wells of Millicell for 6 h at 37 °C. The cells were removed by repeated washes and then blocked by adding BSA. Wells were exposed to either anti-EMA mAb (predilution), anti-HBs mAb (predilution) or anti-human myoglobin mAb (1:200 or 1:1000 dilution) as negative controls or anti-TCR $\alpha\beta$ mAb (1:1000 dilution). After incubation with biotin-conjugated anti-mouse IgG antibodies, the brown spots were detected by adding streptavidin conjugated to horseradish peroxidase and substrate. The spots in MT-2 cultivated wells were detected apparently by anti-TCR$\alpha\beta$ mAb, but neither by the presence of anti-EMA mAb, anti-HBs mAb and anti-human myoglobin mAb nor by the absence of primary antibodies (Fig. 1). The development of spots did not occur in cell-free wells.

Time course of soluble TCR $\alpha\beta$ secretion

The number of TCR $\alpha\beta$ spots produced by MT-2 (10^4 cells/well) augmented rapidly in a time-dependent fashion. The maximal number was reached between 3 and 6 h, whereas TCR $\alpha\beta$ spots in dead MT-2 cells failed to be detected (Fig. 2). Based on these observations,

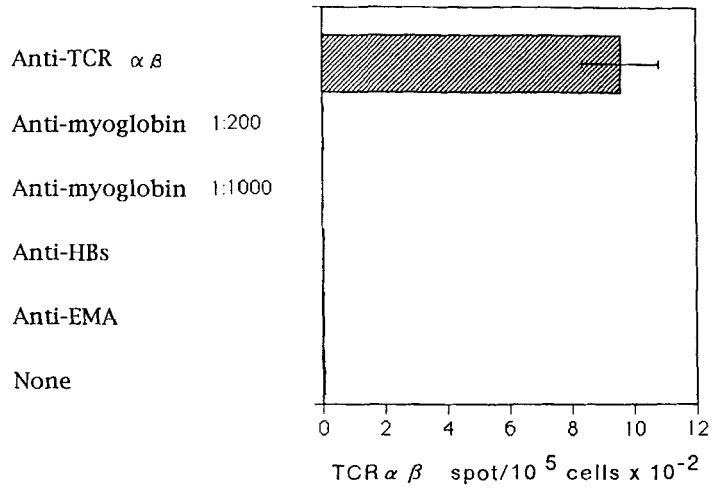

FIGURE1 Detection of primary antibodies-specific spots in MT-2 cells using ELISPOT assay. MT-2 cells(10^4 cells per well) were incubated in Millicell for 6 h. After washing, anti-human TCR mAb(1000^{-1} dilution), anti-human EMA mAb(predilution), anti-HBs mAb(predilution) or anti-human myoglobin mAb(200^{-1} and 1000^{-1} dilution) were added to each well, followed by biotin- labelled anti- mouse IgG, streptavidin conjugated to horse radish perioxidase and the substrate. Each bar is the means of five separate experiments performed in duplicates. Brackets represent S.E. of the means.

an incubation time of 6 h was chosen for all subsequent experiments.

Concentrations of primary antibodies

After incubation of MT-2 cells (10^4 cells/well) for 6 h, primary antibodies against TCR $\alpha\beta$, TCR α and TCR β of dilutions ranging from 1:250 to 1:4000

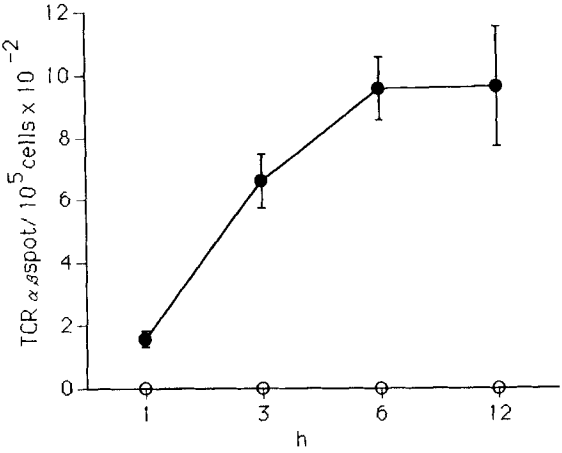

FIGURE2 Kinetics of TCR $\alpha\beta$ spots formed by living and dead MT-2 cells. Living(●) and dead(○) MT-2 cells(10^4 cells/well) were incubated for 12 h. The number of TCR $\alpha\beta$ spots was estimated on the indicated hours. Each point represents the means of triplicated experiments. Each verical bar represents S.E. of the means.

were added. By using anti-TCR $\alpha\beta$, anti-TCR α or anti-TCR β mAb at dilutions between 1:250 and 1:1000, we were able to detect efficiently TCR-releasing cells. The addition of anti-TCR antibodies diluted to less than 1:1000 led to a progressive decline of the number of TCR spots (Table 1).

TCR spot formation by several different tumor cells

When TCR releasing cells in MT-2 and HSB-2 as a T lymphoma cell line were measured by ELISPOT assays,

TABLE 1

Dilution Analysis of Anti-TCR mAb in ELISPOT Assay *

Dilution	TCR $\alpha\beta$ spots/10^5 cells	TCR α spots/10^5 cells	TCR β spots/10^5 cells
1:250	936 ± 157	912 ± 163	528 ± 54
1:500	990 ± 85	925 ± 140	525 ± 97
1:1000	964 ± 218	942 ± 155	569 ± 105
1:2000	225 ± 37	203 ± 61	153 ± 42
1:4000	41 ± 19	48 ± 27	23 ± 7

* MT-2 cells(10^4 cells/well) were incubated for 6 h. After washing and blocking, serial dilutions of anti-TCR$\alpha\beta$ mAb, anti-TCR α mAb or anti-TCR β mAb were added to each well, followed by biotin-labelled anti-mouse IgG, streptavidin conjugated to horse radish perioxidase and the substrate. Results are expressed as the means ± SEM of three separate experiments performed in triplicates.

both cell lines formed considerably TCR $\alpha\beta$ -, TCR α - and TCR β -specific spots. In contrast, no TCRs-specific spots could be demonstrated in DAKIKI (human B-cell lymphoblastoid cells) and MH134 (murine hepatoma cells)(Table 2). Spot formation by tumor cells did not occur when primary anti-TCRs mAb had been omitted (Table 2).

Treatment of MT-2 cells with cycloheximide

To investigate whether protein synthesis was required for TCR-specific spot formation, MT-2 cells were pretreated and subsequently incubated in the presence of cycloheximide. The number of TCR $\alpha\beta$ -specific spots was reduced remarkedly by treatment of MT-2 cells with cycloheximide (Fig. 3).

DISCUSSION

The ELISPOT assay for detecting single cells producing various cytokines is now well established (19-24). In this report we have made an attempt to better detect soluble TCR-releasing cells using ELISPOT assay. The membrane in Millicell was coated with anti-TCR α or anti-TCR β mAb in our initial experiments. The wells were reacted with primary anti-TCR β or TCR α mAb and secondary biotin-conjugated

TABLE 2

TCR-Specific Spot Formation in Several Different Tumor Cells *

Cell line	mAb(-)	TCR $\alpha\beta$ spots/10^5 cells	TCR α spots/10^5 cells	TCR β spots/10^5 cells
MT-2	0	947 ± 15	961 ± 74	510 ± 102
HSB-2	0	415 ± 97	654 ± 217	527 ± 96
DAKIKI	0	0	0	0
MH134	0	0	0	0

* MT-2, HSB-2, DAKIKI and MH134 cells at 10^4 cells per well were incubated for 6 h. The number of TCR-specific spots was estimated by the ELISPOT assay. Results are the means ± SEM of five separate experiments performed in duplicates.

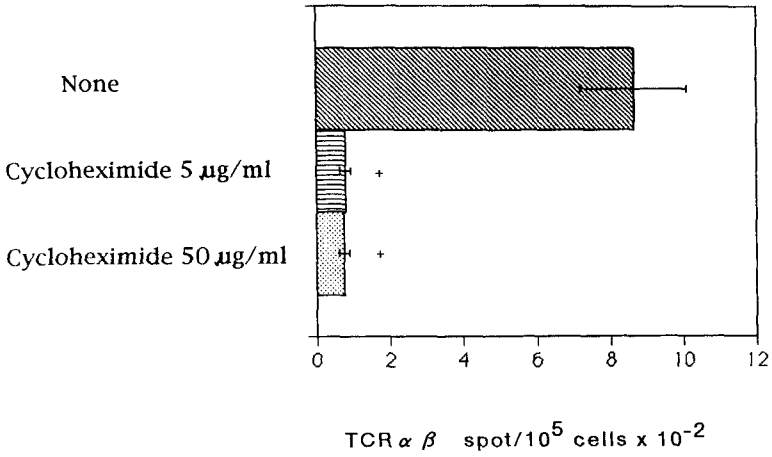

FIGURE3 Effects of cycloheximide on TCR $\alpha\beta$ spot formation in MT-2 cells. MT-2 cells(2 x 10^4 cells/ml) were pretreated for 1 h and incubated for 6 h in the presence of cycloheximide(5 μg/ml or 50 μg/ml). Each bar is the means in three separate experiments. Brackets represent S.E. of the means. A comparison of cycloheximide treatment with controls; +, $p<0.001$

anti-mouse IgG antibodies. Soluble TCR$\alpha\beta$ complex-releasing cells could be detected by this ELISPOT assay. Since the single TCR α - or TCR β -chain releasing cells failed to be observed by this assay, the cells were incubated directly on the uncoated membrane of Millicell wells. For the detection of soluble TCR-releasing cells, cells were suspended in RPMI1640 medium containing 1% FBS and added to the uncoating wells. When the cells were incubated in medium with 1% FBS or in protein-free PFHF-II medium

(Gibco BRL, Gaithersburg, MD), the results were similar. Therefore, we have chosen to use RPMI1640 medium containing 1% FBS in this report. The treatment with H_2O_2 after cell incubation, which inactivates endogenous peroxidase derived from adherent cells, significantly reduced the background spots. Further cleaning of the solid phase with a cell scraper to remove the cells and their debris from the membrane also helped to reduce both the background and false spots. TCR spots by MT-2 cells were not detected by primary anti-EMA mAb IgG_{2a}, anti-HBs mAb IgG_{2b} and anti-human myoglobin mAb IgG_1 which have the same IgG isotype as anti-TCRs mAb used in this study (Fig. 1). When we have tested the specificity of six different secondary antibodies in ELISPOT assay, biotin-conjugated goat $F(ab')_2$ anti-mouse IgG employed in this ELISPOT assay failed to make the background spots among other tested secondary antibodies. TCR-specific spots were produced by MT-2 and HSB-2 cells as a human T lymphoma cell line but not by DAKIKI (human B-lymphoblastoid cells) and MH134 (murine hepatoma cells) (Table 2). Furthermore, soluble TCR secreting cells in human peripheral blood mononuclear cells have been detected by ELISPOT assay (submitted).

On the basis of these findings, it strongly suggests that T cells spontaneously release soluble TCR.

Since the number of TCR $\alpha\beta$-specific spots in MT-2 cells was greatly reduced by cycloheximide, an inhibitor of protein synthesis (Fig. 3), TCR spot formation appears to depend on de novo protein synthesis by MT-2 cells. In addition, TCR spot formation by dead MT-2 cells did not occur (Fig. 1). These results indicate that spot formation resulted from secretion of rather than from shedding form of the membrane TCR.

Soluble TCRs may play an important role in regulating various types of immune responses. Some reports (7,10,12,14,25) demonstrate that the TsF shares an α chain and / or a β-chain epitope with TCR. Therefore, the soluble TCR appears to cause the suppressive activity in the immune responses. In addition, the soluble TCR may possess the capacity to trigger helper or effector functions, because soluble helper T cell factors also share antigenic determinants with TCRs (15). The ELISPOT assay for the detection of soluble TCR-releasing cells increases the potential usefulness of this technique for studying the immunoregulation and the understanding of disease processes.

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REFERENCES

1. Weber, W., Gill, G.N. and Spiess, J. Production of an epidermal growth factor receptor-related protein. Science 1984; 224: 294-7.

2. Rubin, L.A., Kurman, C.C., Fritz, M.E. et al. Soluble interleukin 2 receptors are released from activated human lymphoid cells in vitro. J. Immunol. 1985; 135: 3172-7.

3. Blank, U., Fridman, W.H., Daeron, M., Galinha, A., Moncuit, J. and Neauport-Sautes, C. Size and charge heterogeneity of murine IgG-binding factor(IgG-BF). J. Immunol. 1986; 136: 2975-82.

4. Mosley, B., Beckmann, M.P., March, C.J. et al. The murine interleukin-4 receptor: Molecular cloning and characterization of secreted and membrane bound forms. Cell 1989; 59: 335-48.

5. Novick, D., Engelmann, H., Wallach, D. and Rubinstein, M. Soluble cytokine receptors are present in normal human urine. J. Exp. Med. 1989; 170: 1409-14.

6. Gatanaga, T., Hwang, C.D., Kohr, W., et al. Purification and characterization of an inhibitor(soluble tumor necrosis factor receptor) for tumor necrosis factor and lymphotoxin obtained from the serum ultrafiltrates of human cancer patients. Proc. Natl. Acad. Sci. USA 1990; 87: 8781-4.

7. Fairchild, R.T., Kubo, R.T. and Moorhead, J.W. Soluble factors intolerance and contact sensitivity to 2,4- dinitrofluorobenzene in mice. IX. A monoclonal T cell suppressor molecule is structurally and serologically related to the alpha/beta T cell receptor. J Immunol. 1988; 141: 3342-8.

8. Iwata, M., Katamura, K., Kubo, R.T., Grey, H.M. and Ishizaka, K. Relationship between T cell receptors and antigen- binding factors. II. Common antigenic determinants and epitope recongnition shared by T cell receptors and antigen-binding factors. J. Immunol. 1989; 143: 3917-24.

9. Collins, M., Kuchroo, V.K., Whitters, M.J., et al. Expression of functional $\alpha\beta$ T cell receptor gene rearrangements in suppressor T cell hybridomas correlates with antigen binding, but not with suppressor cell function. J. Immunol. 1990; 145: 2809-19.

10. Takata, M., Maiti, P.K., Kubo, R.T., et al. Cloned suppressor T cells derived from mice tolerized with conjugates of antigen and monomethoxypolyethlene glycol. Relationship between monoclonal suppressor factor and the T cell receptor. J. Immunol. 1990; 145: 2846-53.

11. Behlke, M.A. and Loh, D.Y. Alternative splicing of murine T-cell receptor β -chain transcripts. Nature 1986; 322: 379-82.

12. Kuchroo, V.K., Steele, J.K., O'Hara, R.H., et al. Relationships between antigen-specific helper and inducer suppressor T cell hybridomas. J. Immunol. 1990; 145: 438-48.

13. Fairchild, R.L., Kubo, R.T. and Moorhead, J.W. DNP-specific / class I MHC-restricted suppressor molecules bear determinants of the T cell receptor α - and β -chains. J. Immunol. 1990; 145: 2001-9.

14. Chen, Y., Takata, M., Maiti, P.K., Mohapatra, S., Mohapata, S.S. and Sehon, A.H. The suppressor factor of T suppressor cells induced by tolerogenic conjugates of ovalbumin and monomethoxypolyethylene glycol is serologically and physiochemically related to the $\alpha\beta$ heterodimer of the T cell receptor. J. Immunol. 1994; 152: 3-11.

15. Guy, R., Ullrich, S.J., Foo-Philips, M., Hathcock, K.S., Appella, E. and Hodes, R.J. Antigen-specific helper function of cell-free T cell products bearing TCR V β_8 determinants. Science 1989;244: 1477-80.

16. Sedgwick, J. D. and Holt, P. G. A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. J. Immunol. Methods 1983; 57: 301-9.

17. Czerkinsky, C., Andersson, G., Ekre, H.-P., Nilsson , L.-Å., Klareskog, L. and Ouchterlony, Ö. Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells. J. Immunol. Methods 1988; 110: 29-36.

18. Stewart, C.C. and Ingram, M. A method for counting phytohemagglutinin stimulated lymphocytes. Blood 1967; 29: 628-39.

19. Versteegen, J.M.T., Logtenberg, T. and Ballieux, R.E. Enumeration of IFN-γ-producing human lymphocytes by spot- ELISA. J. Immunol. Methods 1988; 111: 25-9.

20. Hutchings, P.R., Cambridge, G., Title, J.P., Meager , T. and Cooke, A. The detection and enumeration of cytokine-secreting cells in mice and man and the clinical application of these assays. J. Immunol. Methods 1989; 120: 1-8.

21. Lewis, C.E. Detecting cytokine production at the single-cell level. Cytokine 1991; 3: 184-8.

22. Merville, P., Pouteil-Noble, C., Wijdenes, J., Potaux, L., Touraine, J.L. and Banchereau, J. Detection of single cells secreting IFN-gamma, IL-6 and IL -10 in irreversibly rejected human kidney allografts , and their modulation by IL-2 and IL-4. Transplantation 1993; 55: 639-46.

23. Fujihashi, K., McGhee, J.R., Beagley, K.W., et al. Cytokine-specific ELISPOT assay. Single cell analysis of IL-2, IL-4 and IL-6 producing cells. J. Immunol. Methods 1993; 160: 181-9.

24. King, C.L., Low, C.C. and Nutman, T.B. IgE production in human helminth infection. Reciprocal interrelationship between IL-4 and IFN-gamma. J. Immunol. 1993; 150: 1873-80.

25. Ballinari, D., Castelli, C., Traversari, C., et al. Disulfide-linked surface molecules of monoclonal antigen-specific suppressor T cells: Evidence for T cell receptor structure. Eur. J. Immunol. 1985; 15: 855 -60.