In vitro properties of heterologous anti-lizard thymocyte serum*

RM. Pitchappan and VR. Muthukkaruppan

Department of Biological Sciences, Madurai University, Madurai 625021

Abstract

A potent and specific antiserum was raised in rabbits by injecting a single cell suspension of thymocytes of the lizard, *Calotes versicolor*. Rabbit anti-*Calotes* thymocyte serum (ATS) was characterized *in vitro* by using cytotoxicity assay and quantitative absorption analysis. The cytolytic activity of ATS was always higher towards thymocytes than towards lymphoid cells of spleen, bone-marrow and peripheral blood. Various absorption experiments indicate the existence of two antigens among thymocytes—one distinct for thymocytes and thymus derived cells and another, common to lymphocytes. Further, thymus-brain antigenic correlation has been demonstrated in this reptilian species. The *Calotes* complement is less efficient than that of guinea pig in mediating the cytolysis of target cells by ATS. Further, ATS did not have cytotoxic effect on antibody producing cells. The significance of these findings has been discussed with reference to the dichotomy of lymphoid systems at this phylogenetic level.

1. Introduction

Heterologous anti-thymocyte serum (ATS) and anti-lymphocyte serum (ALS) have been widely used in mammals and birds as powerful tools for immunosuppression, for understanding the function of a specific population of lymphocytes and for tracing the origin and differentiation of the particular type of lymphocytes.[1-8] Hitherto, no attempt has been made in lower vertebrates to use heterologous ALS as a tool to investigate such immunological problems. It is felt that reptilian system would form a more appro-

priate model because of its greater potential in providing information concerning the origin of cellular and functional dichotomy of vertebrate immune systems.[9,10] Such a comprehensive approach has already been initiated in a species of squamate reptile the garden lizard, *Calotes versicolor* in our laboratory.[11-23] In order to delineate the role of thymus in immune functions and to trace the origin and distribution of lymphocytes of thymic lineage, it was proposed to raise a potent antiserum in rabbits against thymocytes of the lizard *C. versicolor*. The present paper deals with the *in vitro* characterization of rabbit anti-lizard thymocyte serum (ATS) using cytotoxicity assay and quantitative absorption analysis.

2. MATERIALS AND METHODS

EXPERIMENTAL ANIMAL

The garden lizard, *C.versicolor*, a squamate reptile belonging to the family Agamidae, found in common among hedges and vegetations was obtained from local animal suppliers and maintained in the laboratory as described earlier.[12] Lizards weighing 20–30 g were used in the present experiments. The thymus in such animals consists of a well defined cortex and medulla and contains about 2–4 million thymocytes.[19]

ANTI-THYMOCYTE SERUM

The method of Gozzo *et al*[24] with minor modifications was followed to raise the antiserum. Thymuses were removed from ten lizards and a single cell suspension was prepared in phosphate buffered saline (PBS). Thymocytes were washed thrice in ice cold PBS (4°C) by centrifugation. Ten to thirty million thymocytes, suspended in 0·2 ml of saline were mixed with an equal volume of complete Freund adjuvent (Difco, Detroit, Michigan). The mixture was emulsified and injected subcutaneously at two sites in nuchal region of a rabbit, weighing 1·5 kg. A month later the rabbit was given a booster series intravenously. The same number of thymocytes was injected on each day for three consecutive days and after a week, the rabbit was bled by cardiac puncture. The anti-serum was separated by centrifugation, distributed in aliquotes of 3 ml and stored at —20° C until used. Normal rabbit serum (NRS) was obtained from healthy rabbits in a similar fashion.

Antisera and NRS were heat inactivated at 57°C for 30 min and absorbed with *Calotes* red blood cells (CRBC) until no further agglutination was evident.

CYTOTOXICITY ASSAY

Saijo's[25] method with suitable modifications was adopted for cytotoxicity assay. Target cell suspension was prepared from adult lizard thymus and spleen in ice cold Tris-HCl buffer (pH 7·2) and after washing, white cell concentration was adjusted to five million per ml. For bone-marrow cells the humerus and femur were flushed through by the buffer using a hypodermic needle. Blood leucocytes were obtained by differential spinning of the buffy coat to remove CRBC.

One tenth ml of cell suspension was incubated with one twentieth-ml of ATS dilutions at 37°C. Controls were maintained by replacing ATS with NRS and 15 min later, the reaction mixture was supplemented with 0·05 ml of either fresh guinea pig or *Calotes* complement. After incubating for a further 15 min, the tubes were transferred to an ice cold water bath and 0·05 ml of 1% trypan blue was added. After 2 min, the percentage of dead cells was determined by counting a minimum of two hundred cells. Non-specific staining of lymphocytes was avoided by keeping the treated cells at 4°C.[26]

ABSORPTION ANALYSIS

For absorption of ATS with thymus, blood, spleen and bone-marrow cells, 0·5 ml of antisera diluted 1 : 100 was incubated with different numbers of cells for 30 min at 25°C with occasional shaking. For absorption with brain, liver and kidney, homogenized tissues of known wet weight were incubated with aliquotes of antiserum as described above. The absorbed antisera were recovered by centrifugation and tested for their residual cytotoxicity on lizard thymocytes.

SUSCEPTIBILITY OF HAEMOLYTIC PLAQUE FORMING CELLS TO ATS

Lizards were injected intraperitoneally with 0·1 ml of 25% sheep red blood cells (SRBC).[16] Spleen cells of immunized lizards were treated with various dilutions of ATS and the number of plaque forming cell (PFC) was determined following the techniques of Cunningham and Szenberg[27] as modified for lizards.[16]

3. RESULTS

Each experiment was repeated at least thrice with different batches of ATS. The trends of the graphs were consistently similar, except the variations in the titer value for different batches of antisera. Hence, only results of the representative experiments are depicted here. Heat inactivated NRS was found to have no cytotoxic activity with any of the lymphoid cells tested.

CYTOTOXIC ACTIVITY OF ATS ON LYMPHOID CELLS

The cytotoxic activity of ATS was higher with respect to thymus cells than to cells of other lymphoid organs tested (figure 1). ATS killed 95–100% thymocytes up to a dilution of 1:100 and thereafter the cytotoxic activity declined gradually. The cytotoxic activity of ATS against spleen cells was comparatively low killing as much as 92% at higher concentrations (1:10) followed by a very drastic decline at higher dilutions. The activity was more on blood cells than on bone-marrow cells at different dilutons of ATS tested. Thus among different lymphoid populations marrow cells were least sensitive to the cytotoxic activity of ATS.

At 1:2 dilution of ATS 100% of thymocytes, 90% of spleen and blood cells and 70% of bone-marrow cells were killed. At a dilution of 1:100, where ATS killed still 100% of thymocytes, only about 62% of spleen cells
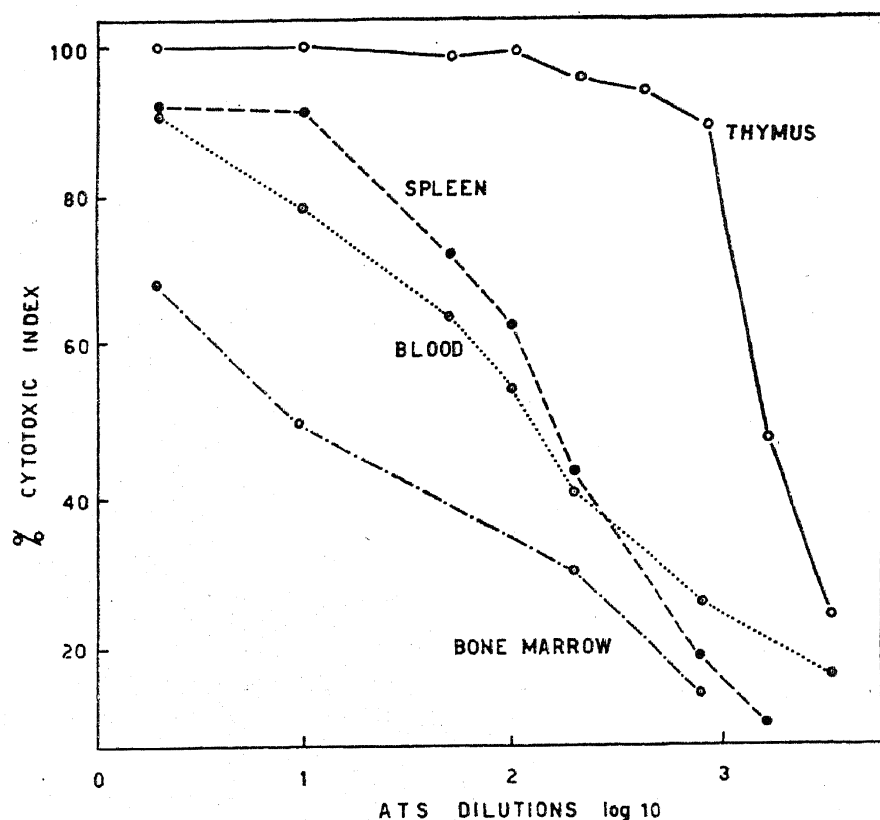

Figure 1. *In vitro* cytotoxic activity of ATS on lymphoid cells of thymus, spleen, bone-marrow and blood of *Calotes*. Half a million cells were incubated with different dilutions of ATS/NRS at 37° C for 15 min. The mixture was supplemented with guinea pig complement and incubated for another 15 min. Percentage of dead cells was determined by trypan blue dye exclusion and cytotoxic index was calculated using the formula $(A - B)/(100 - B) \times 100$ where A is the percentage of dead cells in ATS and B is the percentage of dead cells in NRS.

were killed. Further, the dilution of ATS capable of effecting 90% lysis of thymus cells was 80–100 times higher than that required to effect similar degree of lysis of spleen cells.

ABSORPTION OF ATS WITH LYMPHOID TISSUES

Among different sources, thymocytes absorbed cytotoxic activity of ATS completely at lower cell concentration (figure 2). After an initial threshold level, an increasing number of thymocytes was found to absorb the cytotoxic activity in a linear fashion. Next to thymocytes, spleen cells were effective in absorbing ATS. However, while the first five million cells decreased the cytotoxic index by 75%, addition of another five million cells reduced it only by 10%. The cytotoxic activity of ATS was also significantly diminished up to about 50% by five million bone-marrow cells and thereafter only less efficient absorption of activity was observed. Blood cells were least capable of absorbing the cytotoxic activity of ATS.
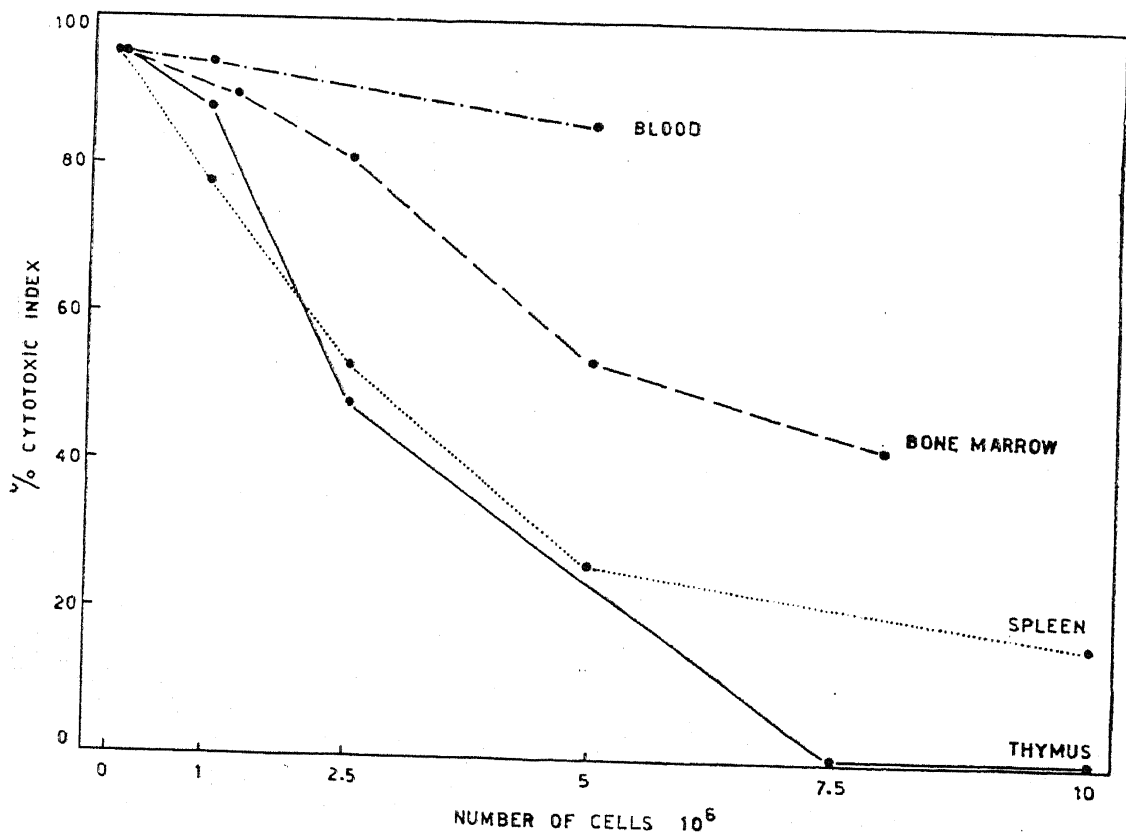
Figure 2. The cytotoxic activity of ATS following absorption with cells of various lymphoid sources. 0·5 ml aliquotes of ATS (diluted hundred-fold) were absorbed with varied numbers of lymphoid cells (abscissa) for 30 min. at 25°C. The residual cytotoxic activity of the absorbed sera was assessed against thymocytes.

ABSORPTION OF ATS WITH NON-LYMPHOID TISSUE

Among the non-lymphoid tissues brain was more efficient than kidney and liver in absorbing the cytotoxic activity (figure 3). This much absorption was achieved by 125 mg of brain tissue, whereas addition of another 375 mg did not significantly remove any more thymocytolytic activity and the curve became asymptotic. On this abscissa, even 1·5 mg (wet weight) of thymocytes (7·5 million cells) were sufficient to completely absorb the cytotoxic activity of ATS.

ABSORPTION OF ATS WITH BRAIN AND BONE-MARROW CELLS

Based on the data presented in figures 2 and 3, further experiments were carried out to elucidate the nature of the remaining cytotoxic activity after absorption with either brain or bone-marrow cells. The same aliquote oj ATS was absorbed in sequence with five million bone-marrow cells and 250 mg of brain tissue and the remaining cytotoxic activity against thymocyte
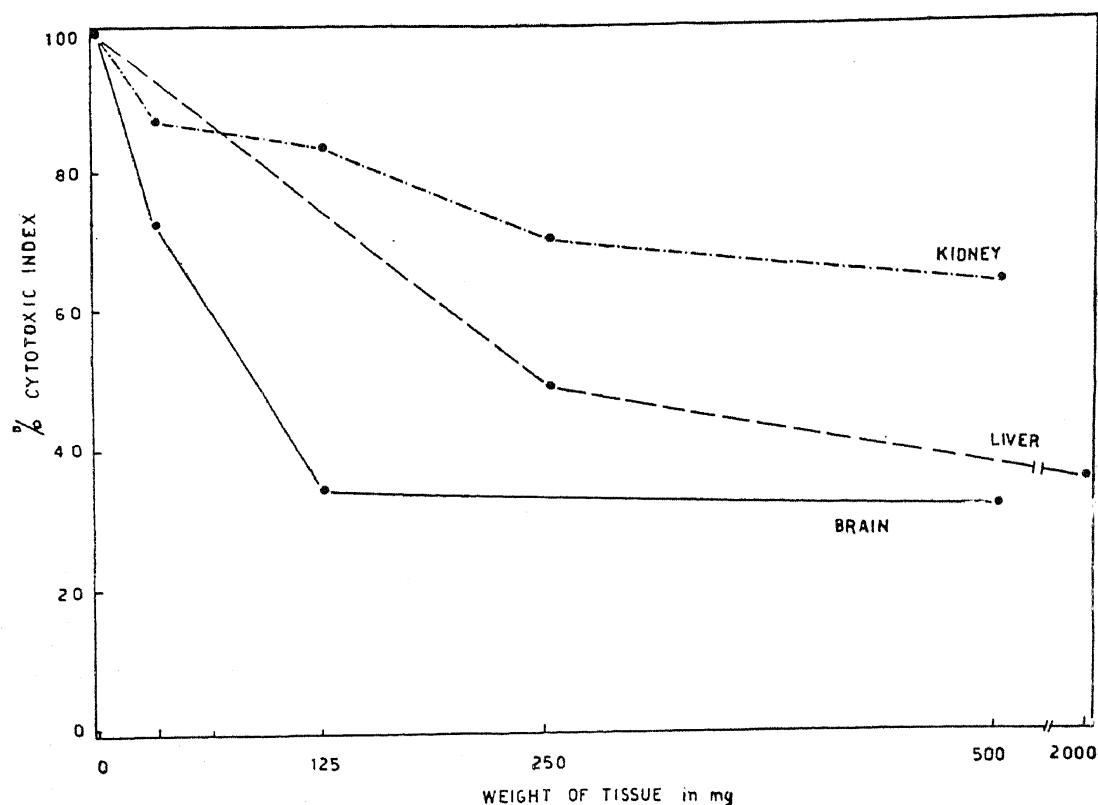

Figure 3. Absorption of ATS with cells of various non-lymphoic tissues. Various amounts of kidney/biain/liver tissues (abscissa) were used to absorb 0·5 ml aliquots of ATS (diluted hundred-fold) for 30 min at 25°C. The residual cytotoxic activity of the absorbed sera was assayed against thymocytes.

was ascertained. As shown in table 1, combined absorption of ATS elimi-
nated the entire cytotoxic activity in contrast to the inability of either of these
tissues.

SUSCEPTIBILITY OF ANTIBODY PRODUCING CELL TO ATS

The splenic population containing antibody producers was treated with
ATS and complement and then the number of plaque forming cells (PFC)
were determined (figure 4). In NRS treated spleen cells, there was no change
in the proportion of PFC. On the other hand, there was an increase in the
number of PFC per million viable cells after ATS treatment. There was
a two fold enrichment of PFC at 1 : 100 dilution of ATS and at this dilution
only about 50% of the total number of cells were alive (figure 4). Whereas
at 1 : 10 dilution, PFC number shot up to 170 per million viable cells and
at this point only about 12% of the original number of cells were alive, thus
indicating that PFC was not affected by ATS.

EFFICIENCY OF Calotes COMPLEMENT

Since the very purpose of raising ATS was to use it in experiments for
inducing lymphoid depletion, and since avian complement was unable to
mediate cytolysis by mammalian antibody[28] it was necessary to verify whether
Calotes complement was efficient enough to mediate cytolysis of target cells
by the antibody of mammalian origin. As shown in figure 5, guinea pig
complement mediated 85% lysis of the target cells at the ATS dilution of
1 : 1000, whereas Calotes complement required an antiserum dilution of 1 : 10
to effect the same degree of lysis. Thus, lizard complement, although less
efficient mediated definite lysis of thymocytes in vitro by the antibody of
mammalian origin.

Table 1. Residual cytotoxic activity of ATS after absorption with brain and bone-marrow cells.

Absorption of ATS[1] (1 : 100)	% cytotoxic index[2]
Nil	97%
Brain 250 mg	58%
Bone-marrow cells (5 × 10⁶)	44%
Brain 250 mg + bone-marrow cells (5 × 10⁶)	5%

[1] absorbed with Calotes RBC.

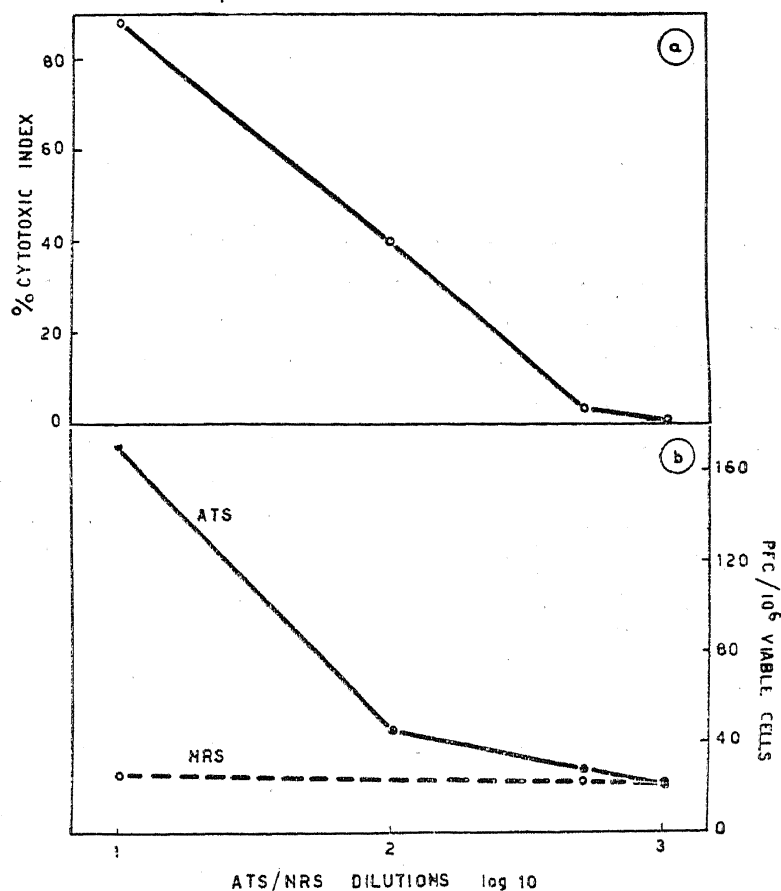[2] 4% of thymocytes were dead in NRS contro tube.

Figure 4. Susceptibility of antibody producing cells (PFC) to ATS treatment *in vitro* Spleen cells were obtained from SRBC immunized lizards and were treated with various dilutions of ATS/NRS and guinea pig complement. Percentage cytotoxic index was determined in one-third of the treated cells utilizing trypan blue dye exclusion method (*a*). The reaction in the remaining cell was stopped by abrupt dilution and the number of plaque forming cells were determined and expressed as per million viable cells (*b*).

4. DISCUSSION

From the *in vitro* cytotoxicity assay it is evident that the ATS under investigation was specific towards thymocytes. At any given dilution of ATS, the cytotoxic activity was more towards thymocytes than towards cells of other lymphoid tissues (figure 1). This observation is well correlated with the ability of thymocytes to absorb the cytotoxic activity of ATS exhaustively (figure 2). Similar findings on the specificity of isologous or heterologous ATS have already been reported for higher vertebrates.[28-32]

THYMOCYTE ANTIGENS

Based on the present observations, we postulate that the thymus in the lizard, *C. versicolor* consists of a heterogenous population of lymphocytes
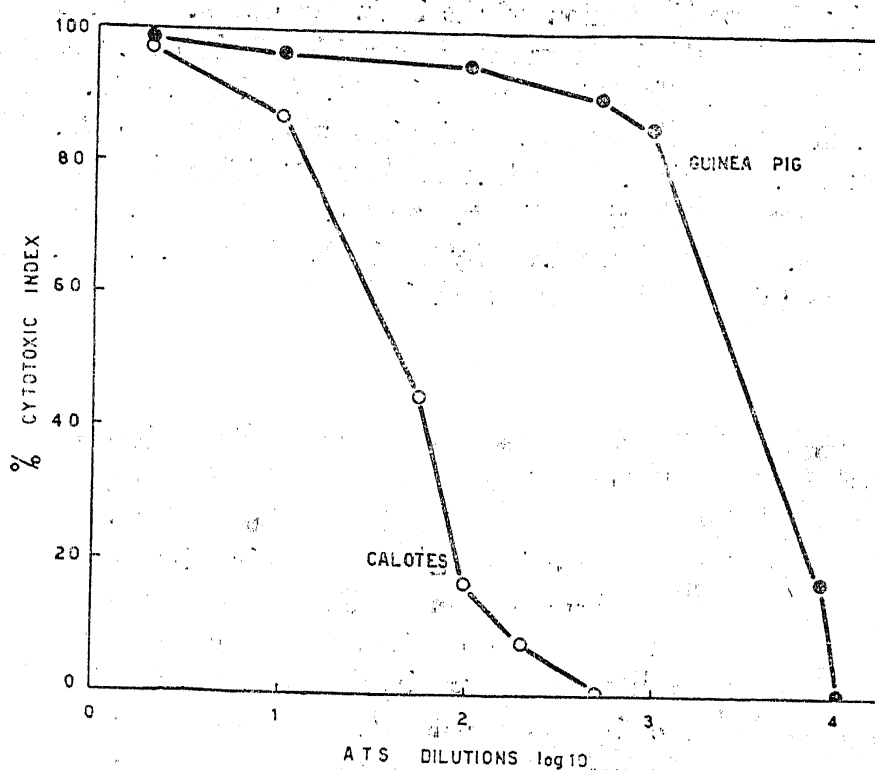
Figure 5. Efficiency of *Calotes* and guinea pig complements in mediating cytolysis of lizard thymocytes by ATS. In the cytotoxicity assay, either guinea pig complement or *Calotes* complement was added and their efficiency was correlated.

carrying two antigenic determinants : one distinct for thymocytes and thymus derived lymphocytes (comparable to T-cells of birds and mammals) and the other, common to all lymphocytes. We can tentatively call the first one as T-antigen and the second, L-antigen.

T-ANTIGEN

The present analysis on ATS absorption using the various tissues such as brain, liver, and kidney shows that the brain matter is more efficient in absorbing the cytotoxic activity of ATS (figure 3), thereby suggesting a greater antigenic correlation (T-antigen) between brain and thymus. In the light of the findings in mammalian system[29,30,33-35] which described the presence of thymic theta antigen on nervous, brain and epidermal tissues, it may be anticipated that thymus-brain antigenic relationship exists even at this lower phylogenetic level.

While absorption kinetics of brain and thymus were initially parallel causing corresponding reduction of thymocytolytic activity of ATS, even a large amount of brain matter was unable to completely absorb this activity,

as shown by the specific flexion of the brain absorption curve. This observation suggests that the brain matter is capable of absorbing only certain antibodies present in the ATS, presumably anti-T, but not anti-L. These findings in the lizard are identical with absorption curves for anti-Thy-1·2 iso-antiserum and the defined divalent antiserum.[36] Thus there are adequate reasons to suggest that the ATS in question contains antibodies of two specificities: one directed against thymus-brain determinants and another having no complementary structure on brain tissue.

L–ANTIGEN

The cytotoxic index on spleen cells and spleen absorption curve indicate the presence of both T- and L-antigens in this organ (figure 1)· While the first five million spleen cells absorbed 75% of the cytotoxic activity, the remaining activity could not be efficiently absorbed by the addition of another five million cells. Therefore, on the basis of our hypothesis, it may be predicted that the remaining cytotoxic activity at the point of flexion of spleen absorption curve might very well be due to anti-T antibodies. The slow absorption thereafter by spleen cells was due to the lower amount of T-antigen present among spleen cells, as in the case of mouse θ antigens.[37] On the basis of these findings, it may be predicted that the deviating course of brain absorption curve is due to the anti-L antibodies left unabsorbed in ATS and that of bone-marrow absorption curve, anti-T antibodies. This prediction has been subsequently confirmed by the observation that the absorption of ATS with both brain matter and bone-marrow cells eliminated the entire thymocytolytic activity (table 1). Therefore, it could be inferred that in the lizard L-antigen is present on bone-marrow cells as well.

It has been established in higher vertebrates that antibody producing cells were of bone-marrow origin.[38, 39] In birds the incubation of immunized spleen cells in thymus specific antiserum had no significant effect on their plaque-forming capacity; but incubation in anti-bursa serum abolished this capacity.[3] The present finding, that anti-SRBC antibody producers were not affected by ATS (figure 4), suggests that in reptiles too, the antibody producers are not T-cells. It seems that they bear entirely different antigens and belong to the B-cell lineage.

SPECIFICITY OF ATS

The cytotoxic activity could not be absorbed by liver or kidney, and this suggests that the responsible antibodies in ATS were directed against tissue specific rather than species specific or transplantation antigens.[32] It

is quite possible that the absorption of ATS with *Calotes* erythrocytes (nucleated) eliminated all antibodies except those directed against tissue specific antigenic determinants (*cf.* 8). Double diffusion and immuno-electrophoretic analysis of ATS with *Calotes* serum did not show any precipitin bands (unpublished) suggesting the absence of antibody to lizard immunoglobulin in ATS.

ACTION OF ATS

Regarding the mode of action of ATS *in vivo*, a number of mechanisms have been proposed (*cf* 8). Complement dependent immuno-cytolysis was shown not to be mandatory for ALS activity.[40,41] On the other hand, Wick *et al.*[28] attributed the absence of ALS activity in chicken to the inability of mammalian antibody to utilize avain complement. Nevertheless, *Calotes* complement was capable of mediating definite cytolysis with the ATS of mammalian origin, although with less efficiency. Thus, the *in vitro* properties of ATS are well correlated with our *in-vivo* studies, demonstrating the modulation of humoral immuno response to SRBC by ATS treatment in the lizard.[19, 21] However, it remains to be seen whether such a modulation of anti-SRBC response is related to the selective depletion of the population of thymus-derived small lymphocytes. Further, the importance of raising specific antisera for T- or B-cells, especially for the purpose of delineating the ontogeny of lymphoid cells in various lymphoid organs is well indicated.

The heterogeneous nature of thymic cells and their membrane antigens have been demonstrated in rat,[31] rabbit,[32] and mouse.[36] In chicken too, the existence of two kinds of antigens (T and L) among thymocytes has been reported.[28] Our present observation demonstrating the heterogeneous nature of thymic antigens, thymus-brain antigenic relationship and an antibody producer, insensitive to the ATS, suggests that during the phylogeny of immunity, dichotomy of lymphoid system into T- and B-cells has already evolved at the reptelian level.

REFERENCES

1. Turk, J. L. and Willoughby, D. A., *Lancet* i 249 (1967).

2. Martin, W. J. and Miller, J. F. A. P., *Lancet* ii 1285 (1967).

3. Potworowski, E. F., Richer, G., Borduas, A. and Forget, A., *J. Immunol.* 106 1416 (1971).

4. Kerbel, R. S. and Eidinger ᴐ., *J. Immunol.* 106 917 (1971)

5. Diener, E., Collaghan, F. D. and Kraft, N., *J. Immunol.* 107 1775 (1971).

6. Raff, M. C. and Owen, J. J. T., *Eur. J. Immunol.* 1 27 (1971).

7. Toben, H. R. and Pierre, R. S. St., *Int. Arch. Allergy Appl. Immunol.* 42 454 (1972).

8. Lance, E. M., Medawar, P. B. and Taub, R. N., *Adv. Immunol.* **1** (1973).

9. Cohen, N., *J. Am. Vet. Med. Assoc.* **159** 1662 (1971).

10. Du Pasquier, L., in *Current Topics in Microbiology and Immunology* **61** 38 (1973).

11. Manickavel, V. and Muthukkaruppan, VR., *Transplantation* **8** 307 (1969).

12. Muthukkaruppan, VR., Kanakambika, P., Manickavel, V. and Veeraraghavan, K., *J. Morphol.* **130** 479 (1970).

13. Kanakambika, P., Doctoral thesis submitted to Annamalai University, India (1971).

14. Manickavel, V., Doctoral thesis submitted to Annamalai University, India (1972).

15. Kanakambika, P. and Muthukkaruppan, VR., *Proc. Soc. Exp. Biol. Med.* **140** 21 (1972).

16. Kanakambika, P. and Muthukkaruppan, VR., *J. Immunol.* **109** 415 (1972).

17. Kanakambika, P. and Muthukkaruppan, VR., *Experientia* **28** 1225 (1972).

18. Kanakambika, P. and Muthukkaruppan, VR., *Proc. Indian Acad. Sci.* **B 78** 37 (1973).

19. Pitchappan, RM., Doctoral thesis submitted to Madurai University, India (1975).

20. Jayaraman, S. and Muthukkaruppan, VR., *Experientia* (in press) (1975).

21. Muthukkaruppan, VR. Pitchappan, RM. and Ramila, G., *Amer Zool.* (in press) (1976).

22. Muthukkaruppan, VR. Subramania l illai, P. and Jayaraman, S., In Cleveland clinic symposium (1976).

23. Pitchappan, RM. and Muthukkaruppan. VR., *Proc. Indian Acad. Sci.* **B 84** 42 (1976).

24. Gozzo, J. J., Wood. M. L. and Monaco, A. T. *Transplantation* **14** 352 (1972).

25. Saijo, N. *Immunology* **24** 683 (1973).

26. Zimpel, H., Stark, R. and Thiele, H. G., *J. Immunol. Methods* **3** 411 (1973).

27. Cunningham, A. J. and Szenberg, A., *Immunology* **14** 599 (1968).

28. Wick, G., Albini, B. and Milgrom, F., *Clin. exp. Immunol.* **15** 237 (1973).

29. Reif, A. E, and Allen, J. M. V., *Nature London* **209** 521 (1966).

30. Raff, M. C. and Wortis, H. H., *Immunology* **18** 931 (1970).

31. Colley, D. G., Malkian, A. and Waksman, B. H. *J. Immunol.* **104** 585 (1970).

32. de la Noue, H., Koperstych, S. and Richter, M., *Immunology* **23** 655 (1972).

33. Reif, A. E. and Allen, J. M. V., *Nature London* **209** 523 (1966).

34. Scheid, M., Boyse, E. A., Carswell, E. A. and Old, L. J., *J. Exp. Med.* **135** 938 (1972).

35. Michael, B., Pasternak, G. and Steuden, J., *Nature London New Biol.* **241** 221 (1973).

36. Thiele, H. G., Stark, R., Keeser, D. and Zimpal. H., *Z. Immunol. Forsch.* **146** 346 (1974).

37. Raff, M. C. and Cantor, H., *Progress in Immunology*, ed. B. Amos (Academic Press, New York), p. 83 (1971).

38. Nossal, G. J. V., Cunningham, A., Mitchell, G. F. and Miller, J. F. A. P., *J. Exp. Med.* **128** 839 (1968).

39. Kindred, B., *J. Immunol.* **107** 1291 (1971).

40. Barth, R. F., and Carroll, G. F., *J. Immunol.* **104** 522 (1970).

41. Cinader, B., Jeejeebhoy, H. F., Koh, S. W. and Rabbat, A. G., *J. Exp. Med.* **133** 81 (1971).