Journal of the Indian Institute of Science

Vol. 64 DECEMBER 1983 No. 12

Section C: Biological Sciences

J. Indian Inst. Sci., 64 (C), Dec. 1983, Pp. 157–168

Dichotomy of lymphocyte population and cell-mediated immune responses in a fruit bat, *Pteropus giganteus*

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Received on June 11, 1983 ; Revised on November 19, 1983.

Abstract

Dichotomy of lymphocyte population in the line of T and B cells in a fruit bat, *Pteropus giganteus* has been shown by differential cytotoxic killing with anti-lymphocyte serum and the serum after absorption with brain cell homogenate. The rationale and necessity of the experiment have been discussed.

Then T-cell mediated response in the bat was measured by delayed-type skin sensitivity test and mixed lymphocyte culture assay ; these reactions were delayed and of lesser degree. Modes of these responses in the bat are interesting from several points of view including the role of bats as reservoir of dreaded bacteria and viruses. Present investigation for the first time looked into details of cell-mediated immune response (CMI) in the bat.

Key words: Bat, lymphocyte dichotomy (B and T), delayed-type hypersensitivity, MLC.

1. Introduction

During the last decade, attempts to identify the different types of lymphocytes have been extended horizontally among the different species of the class Aves and Mammalia and vertically in other classes of the vertebrates. Identification of the different types of lymphocytes in an animal is necessary for understanding the immune mechanisms operative in a species. This becomes imperative in the case of the order Chiroptera. This group of animals has been least studied so far from immunological point of view and is known to be reservoir of dreaded disease-causing viruses and bacteria[1-3].

We have reported elsewhere the antibody-mediated response in a common Indian fruit bat, *Pteropus giganteus*,[4,5] and in this study we intend to explore the cell-mediated immune response (CMI) in this animal. In other groups of mammals, T cells are considered mainly responsible for CMI response. Although it is expected, there is no unequivocal proof for the existence of T cells or at least heterogeneity in the lymphoid cell population in the bat.

In the present investigation, heterogeneity of lymphocytes in the bat has been shown by differential cytotoxic indices with anti-lymphocyte serum and the serum after absorption with bone marrow cells or brain homogenate. Brain cells in mouse have been found to carry the antigen like Thy–1 expressed on the thymus cells[6]. The CMI response has been measured here by delayed-type skin sensitivity test and mixed leucocyte culture.

2. Materials and methods

2.1. *Animals*

Collection, feeding and maintenance of bats were carried out as previously described[4]. The adult animals were fairly large, length from snout to tip of the tail in average was 24 cm and across the patagium or wing was 97 cm. The average weight of an animal was 480 g.

2.2. *Collection of different types of cells*

Spleen and lymph node cells : Spleen and lymph node cells were obtained following the usual procedure of dissociation with a stainless steel grid of wire mesh. RBCs and debris were separated from the suspensions by Ficoll–Hypaque gradient centrifugation as indicated elsewhere[7].

Bone marrow cells : Bone marrow was flushed out of long bones. A uniform cell suspension was made by repeated passage through the 27 gauge needle fitted on a syringe. Debris was separated from suspension by flotation on Ficoll–Hypaque gradient.

Peritoneal exudate cells : 2 ml of Freund's Incomplete Adjuvant was injected intraperitonially one day before sacrificing the bat. After sacrificing, peritonial cavity was flushed with 15–20 ml of PBS and cell suspension was drawn out following the technique of Landahl[8].

Periferal blood lymphocyte (PBL) : Blood was collected by heart puncture in heparinized solution and the tubes were kept in room temperature for 2–3 hr. Upper buffy layer containing WBCs was collected in a separate tube. Lymphocytes were purified by Ficoll–Hypaque gradient centrifugation.

2.3. *Raising of anti-lymphocyte serum (ALS)*

Spleen and lymph node cell suspensions were made following the above-mentioned procedure. RBCs were disrupted with 0·85% NH_4Cl. 1 ml cell suspension containing 10^8 cells was mixed well with 1 ml of Freund's Complete Adjuvant. The mixture was injected subcutaneously in the thigh region of a rabbit. Repeated sensitization was done for seven to eight times at ten days interval. After seven days of last immunization, blood was collected and allowed to clot at room temperature. Serum aliquots were collected and kept in a deep freeze (− 70° C) until use.

2.4. *Lytic reaction with ALS*

Heat-inactivated ALS was diluted from 1 : 20 to 1 : 80. To 0·4 ml of diluted serum in a small glass tube, 0·1 ml suspension of a particular cell type at the concentration of 10^7 cells/ml was added. 20 μl fresh pre-absorbed guineapig serum as a source of complement was added to each tube, mixed well and kept for 1 hr at 37° C.

Pre-absorption of guineapig serum was done by 1 ml packed spleen and erythrocyte cells of bat for 10 ml of the serum. The mixture was allowed to stand for 30 min at 4° C and then centrifuged. This pre-absorption step was followed to remove any non-specific cytotoxic factor from the guineapig complement.

Control tubes were maintained for each dilution with heat-inactivated normal rabbit serum. The reaction was stopped by keeping them on ice bath. Living cells were counted by dye exclusion principle. Lytic index for the particular dilution of the serum was calculated as follows :

$$\text{Lytic index (\%)} = \frac{\text{Total no. of living cells with NRS} - \text{Total no. of living cells with ALS}}{\text{Total no. of living cells added in each tube}} \times 100$$

where NRS indicates normal rabbit serum. (Percentage of dead cells with NRS treatment varied in different experiments from 3 to 8%.)

2.5. *Lytic reaction after absorption of ALS with different types of cells*

Spleen, lymph node and bone marrow cells were obtained following the procedure described previously. Brain was homogenized in cold PBS with the help of tissue homogenizer. Cell suspensions of each kind and brain homogenate were washed two times with PBS. Absorption of different aliquots of ALS was done at the ratio of 0·1 ml packed cells and 1 ml ALS at 4° C for 30 min. Absorbed serum was separated by centrifugation. Then cytotoxicity experiments with absorbed serum were carried out in the same fashion as described earlier.

2.6. *Delayed-type hypersensitivity test with 2-4 Dinitrofluorobenzene (DNFB)*

Bats were first immunized by application of 0·1 ml of 2% DNFB in acetone on shaved surface below the armpit region of the left side. On the seventh day after first application they were re-sensitized with 0·1 ml of 0·02% DNFB on the same region of the right side. Size of resensitized spot was measured by slide calipers for six days from the 8th to the 13th day at 24 hr interval.

2.7. *One way mixed lymphocyte culture (MLC)*

Spleen and lymph node cell suspensions were made aseptically in 10 ml of minimum essential medium (MEM) supplemented with 5% autologous serum, nystatin (50 U/ml) and penicillin–streptomycin (50 U/ml). Lymphocytes were purified by Ficoll-hypaque gradient centrifugation, washed with media and cell number was adjusted at three different concentrations, 10^6, 5×10^5 and 10^5 cells per ml.

Stimulator cells were collected from spleen and lymph nodes of another normal bat and treated with Mitomycin C (Mito-C) (Biochem. Pharmaceutical Industries, Bombay, 25 μg/ml) at the concentration of 2×10^7 cells per ml for 25 min at 37° C. Then cells were washed twice with media and adjusted to 10^6 cells/ml. Triplicate cultures for each concentration of responder cells were maintained. Each tube contained 1 ml of responder and 1 ml of stimulator cells with 5% autologous serum of responder cell type. For control experiments, isologous cells were used as stimulator and treated with Mito-C as usual and added to the control cultures containing three different concentrations of effector cells. The cell cultures were incubated at 37° C in a humidified atmosphere containing 5% CO_2 in air. In one set of experiments, the MLC was continued for four days without changing the culture medium. In the second set of experiments, after three days of incubation, all the tubes were centrifuged, supernatant discarded and 2 ml of fresh medium, supplemented with antibiotics and serum, was added to each tube. These tubes were incubated for another four days. ^{3}H-Thymidine (specific activity 15·8 Ci/mM ; Bhabha Atomic Research Centre, Bombay) was added at the concentration of 1 μCi/ml to each tube 16 hr prior to the termination of the culture. Cells were harvested on the small discs of Whatman filter-paper No. 3 ; the filter-paper discs were dried and total radioactivity counted in a scintillation counter as per method described elsewhere[9].

Ratio of radioactive incorporation by experimental cells to the same of control cells was calculated as stimulation index. The stimulation index at 1·2 or more was considered as positive reaction as shown in graft *versus* host type of cell-mediated reaction in mice by Simonsen[10]. The experiments were repeated several times with different concentrations of the effector cells. The representative experiments where all three concentrations of effector cells had been used at one time are presented in Table III.

Table I

Susceptibility of the lymphocytes from spleen and lymph node to the complement-mediated killing by the anti-lymphotcyte serum (ALS) before and after absorption with lymphocytes from spleen and lymph node

Type of cells	Expt. No.	Lytic index* with different dilutions of the serum					
		ALS			ALS after absorption with lymphocytes from spleen and lymph node		
		1/20	1/40	1/8	1/20	1/40	1/80
Spleen	1.	70	59	45	30	35	32
	2.	69·8	52·6	46·2	17·2	15	11·8
Lymph node	3.	100	88·9	83·3	33·3	22·2	11·1
	4.	87·1	83·8	78·7	35·4	29·0	22·5
	5.	70	66·6	43·3	40·0	35·3	33·3

* Defined in Materials and methods.

3. Results

3.1. *Degree of killing of lymphoid cells from different sources with ALS*

It was found that sensitising spleen and lymph node cells could absorb out a significant level of antibodies in ALS thus causing sharp decrease in the efficacy of ALS (Table I). The residual cytotoxicity after standard absorption of the ALS with spleen or lymph node cells was not negligible. This could be due to allogenic factor ; the different batches of sensitising cells, absorbing and target cells were collected from different bats which were not inbred. Thus some antibody molecules directed against certain determinants other than common antigen on lymphocytes might not have been absorbed out by a particular batch of the absorbing lymphocytes.

Comparatively, ALS-mediated lytic indices with lymph node cells were higher than that of the spleen cells (Table I). This might indicate that lymph nodes harbour more of the lymphocytes. This fact has further been corroborated by the facts demonstrated in fig. 1 where cytotoxic experiment with ALS was performed against cells from different sources. Lytic indices for lymph node cells were followed by spleen cells and peritoneal exudate cells (PEC). There was not much variation in the lytic indices with three different dilutions of ALS in the case of PEC. Lytic indices with bone marrow cells and lymphocytes collected from peripheral circulation did not differ appreciably.

Table II

Lytic index for lymphocytes from spleen and lymph node with antilymphocyte serum (ALS) before and after absorption with bone marrow (BM) cells, brain cell homogenate, BM and brain cell homogenate together

Type of cells	Lytic index with different dilutions of the serum, mean ± S.D. (No. of expts)											
				p value								
	ALS			ALS absorbed with BM cells			ALS absorbed with brain homogenate			BM + Brain homogenate absorbed ALS		
	1/20	1/40	1/80	1/20	1/40	1/80	1/20	1/40	1/80	1/20	1/40	1/80
Spleen	65·44 ±16·23 (8) ...	60·36 ±20·90 (8) ...	45·09 ±20·09 (8) ...	66·93 ±20·62 (6) N.S.	52·33 ±24·20 (6) N.S.	40·64 ±17·16 (6) N.S.	39·06 ±10·76 (6) <·01	33·61 ±12·06 (6) <·02	35·13 ±11·34 (6) N.S.	36·43 ±18·11 (7) <·01	30·09 ±16·97 (7) <·01	31·30 ±11·64 (6) N.S.
Lymph node	85·75 ±12·01 (5) ...	86·81 ±12·63 (5) ...	80·18 ±20·97 (5) ...	75·37 ±10·77 (5) N.S.	62·98 ±16·89 (5) <·05	53·32 ±24·70 (5) N.S.	42·71 ±28·98 (5) <·05	44·78 ±30·05 (3) <·05	12·00 ... (2) ...	35·01 ±20·08 (5) <·01	43·77 ±21·64 (5) <·01	16·42 ±10·48 (3) <·01

* Statistical significance of the difference with the index of corresponding dilution of ALS ; N.S.—Not significant.

Table III

MLC response of the bat's lymphocytes to the mitomycin-C treated allogenic bat's cells after four days of culture

Expt. No.	Expt./Cont.	Responder cell No.	Responder *a* stimulator*	CPM Mean ± S.E.	Stimulation index** (Expt./Cont.)
1.	Expt.	10^6	A *a* B	4190·0 ± 972·48	
	Cont.	10^6	A *a* A	2870·0 ± 288·77	1·45
	Expt.	5×10^5	A *a* B	2840·33 ± 301·17	
	Cont.	5×10^5	A *a* A	4713·00 ± 1137·30	0·60
	Expt.	10^5	A *a* B	2630·0 ± 368·66	
	Cont.	10^5	A *a* A	4877·66 ± 674·61	0·53
2.	Expt.	10^6	C *a* D	3098·66 ± 392·19	
	Cont.	10^6	C *a* C	2718·66 ± 148·92	1·13
	Expt.	5×10^5	C *a* D	2748·0 ± 268·99	
	Cont.	5×10^5	C *a* C	3226·33 ± 147·45	0·85
	Expt.	10^5	C *a* D	2595·0 ± 263·03	
	Cont.	10^5	C *a* C	2498·0 ± 200·0	1·03
3.	Expt.	10^6	E *a* F	1747·66 ± 225·56	
	Cont.	10^6	E *a* E	1659·0 ± 125·50	1·05
	Expt.	5×10^5	E *a* F	1600·66 ± 111·81	
	Cont.	5×10^5	E *a* E	1565·5 ± 99·50	1·02
	Expt.	10^5	E *a* F	1944·0 ± 464·55	
	Cont.	10^5	E *a* E	1612·33 ± 108·12	1·20

* Stimulator cells were 10^6 in number in each case and always treated with mitomycin-C. *a*-versus.
** Stimulation index of 1·2 or above has been considered as positive.

3.2. *Susceptibility of the lymphocytes from spleen and lymph node to ALS after absorption with bone marrow cells and brain homogenate*

It is apparent from the data in Table II that prior absorption of ALS with bone marrow cells does not significantly reduce the cytolytic ability of ALS whereas absorption of ALS with brain cell homogenate causes significant reduction in lytic activity of ALS

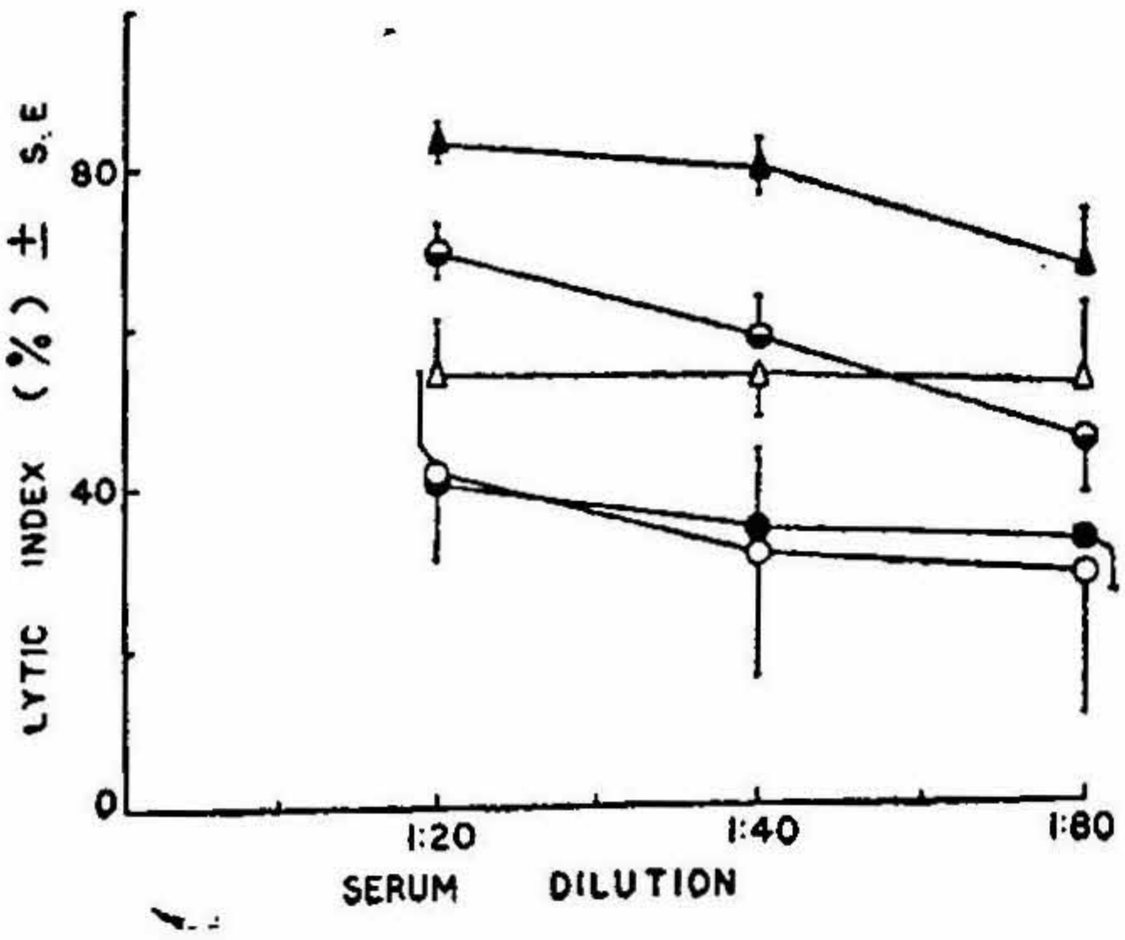

FIG. 1. Lytic index indicating the degree of susceptibility of the lymphoid cells from different sources to the treatment of anti-lymphocyte serum. Lymph node cells (▲), spleen cells (◒), peritoneal exudate cells (△), peripheral blood cells (●) and bone marrow cells (○). Each point in the figure represents the mean of 3–9 separate experiments and vertical bars represent standard error.

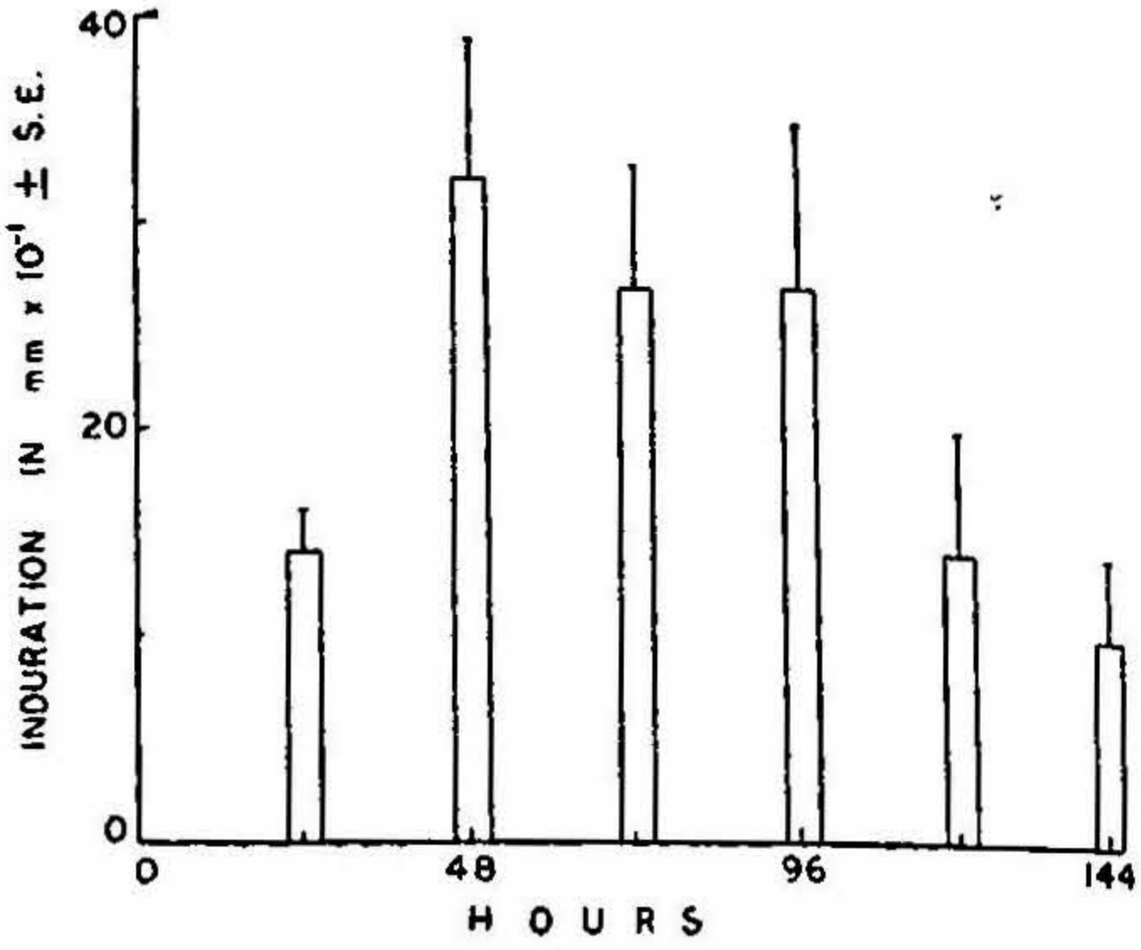

FIG. 2. Degree of erythema at different hr after resensitization on the seventh day of the initial sensitization with 2, 4 DNFB.

to both spleen and lymph node cells. The reduction of lytic activity of ALS after absorption with brain cells was approximately up to 40% in the case of spleen cells. Absorption of ALS with bone marrow and brain homogenate at a time causes the reduction comparable to the results obtained with ALS absorbed with brain homogenate only.

3.3. *Cell-mediated immune response*

a. *Skin sensitivity test to* 2, 4 *DNFB* : Skin sensitivity of 12 bats to 2,4 DNFB was studied. Only three bats showed proper skin reaction where maximum induration was observed at 48 hr of second application of DNFB, accompanied by maximum erythema also (fig. 2). Then gradually the size of the reaction spot decreased to normal. Rest of the bats manifested slight response accompanied by small induration which was not practically measurable.

b. *Mixed lymphocyte culture* (*MLC*) *reaction* : Three different concentrations of effector cells were mixed with a fixed number of stimulator cells and the cultures were incubated for four and seven days in two separate experiments (Tables III and IV). Ratio of incorporation of ^{3}H-TdR by experimental and control cells was taken into consideration for calculating the stimulation index and enumerating the positive reactions (1·2 or above) as defined in Materials and methods.

Table IV

MLC response of the bat's lymphocyte to the mitomycin-C treated allogeneic bat's cells after 7 days of culture

Expt. No.	Expt./Cont.	Responder Cell No.	Responder *a* stimulator*	CPM Mean ± S.E.	Stimulation index** (Expt./Cont.)
1.	Expt.	10^6	A *a* B	6913·16 ± 306·78	
	Cont.	10^6	A *a* A	4965·88 ± 346·88	1·39
	Expt.	5×10^5	A *a* B	10339·33 ± 1083·88	
	Cont.	5×10^5	A *a* A	6191·00 ± 693·13	1·67
	Expt.	10^5	A *a* B	5895·00 ± 274·68	
	Cont.	10^5	A *a* A	7723·00 ± 55·02	0·76
2.	Expt.	10^6	C *a* D	6921·50 ± 93·66	
	Cont.	10^6	C *a* C	3631·11 ± 457·36	1·90
	Expt.	5×10^5	C *a* D	3022·33 ± 258·46	
	Cont.	5×10^5	C *a* C	5073·88 ± 452·32	0·59
	Expt.	10^5	C *a* D	4050·77 ± 221·59	
	Cont.	10^5	C *a* C	5009·22 ± 131·82	0·80

* Stimulator cells were 10^6 in number in each case and always treated with mitomycin-C. *a*-versus.
** Stimulation index of 1·2 or above has been considered as positive.

Out of three experiments with three different concentrations of effector cells, only two cases incubated for four days showed positive reaction and one was marginal (Table III). However, assay on the seventh day of culture showed enhanced incorporation of ^{3}H-TdR in the experimental cultures with higher concentration of the effector cells in both the experiments (Table IV).

4. Discussion

Use of anti-thymocyte serum would possibly help to show directly the existence of T-cells in the cell population of secondary lymphoid organs of the bat. Some difficulties such as involuted thymus in adult bats and non-availability of regular supply of neonatal animals restricted us in raising anti-thymocyte serum[7]. Even then it seemed that it is necessary to show, if possible, the existence of dichotomy in lymphocyte population of the bat before measuring CMI response in *P. giganteus*. Thus the readings for differential killing of the lymphocytes by anti-lymphocyte serum before and after absorption with brain homogenate have been taken into account. It was thought that like some

other mammals, brain cells of the bat might share some antigenic determinants with the thymus cells. It was demonstrated in mouse that thymus and brain cells share the θ or Thy-1 antigen[11,12].

It has been observed here that the absorption of anti-lymphocyte serum with brain cell homogenate caused significant decrease in the lytic index of ALS, indicating the possibility that certain antibody molecules, effective against certain lymphocytes, had been absorbed by the brain cell homogenate. From this experiment, it seems that brain cells of the bat possibly share some antigen expressed on its thymocytes. Thus it seems that there is some kind of heterogeneity in the lymphocyte population of the bat in the line of T and B cells which can be determined on the basis of cell surface determinants as in mice[12–16], chicken[17,18] and man[19].

We have also observed that prior absorption of ALS with bone marrow cells did not significantly affect ALS-mediated killing of spleen and lymph node cells. It seems that B type cells in bone marrow are possibly not differentiated enough to possess the cell surface determinants similar to the B cells in the secondary lymphoid organs.

Pteropus giganteus manifests typical delayed-type hypersensitivity reaction, usually characteristic of T-cell-mediated response, resulting in erythematous, maximum induration at 48 hr after secondary exposure to the sensitizing agent like DNFB as in other mammals[20]. The notable difference in the bat is that only three out of twelve bats showed this reaction.

The CMI response in the bat as measured by MLC assay is delayed compared to mice where the peak can be obtained in four or five days[21]. This kind of delay (of 7 days) can be observed in lower vertebrates like marine toad, *Bufo marinus*[22]. In this context, we would like to refer to our earlier work where we observed a delayed antibody-mediated response in *P. giganteus*[4,5]. Furthermore, striking lack of cell-mediated immunity in bats against some mycotic agents was also reported by several workers[23–25]. Having characteristic lymphoid cell types of mammalian immune system why does the bat exhibit lesser degree and delayed antibody mediated and CMI response merits further studies.

Acknowledgements

This study has been carried out with the financial assistance from the University Grants Commission, New Delhi, and the lump grant of the Government of West Bengal to the Centre for Life Sciences.

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