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IMMUNOFLUORESCENCE ANALYSIS OF IMMUNOGLOBULIN BEARING LYMPHOCYTES IN THE INDIAN FRUIT BAT: *PTEROPUS GIGANTEUS*

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ABSTRACT

The immunologic cell types of the Indian fruit bat, P. giganteus, *were characterized on the basis of cell surface Ig markers. Rabbit anti-rat IgM and IgG demonstrated IgM or IgG bearing cells in the nylon wool adherent lymphocyte population, thereby suggesting their equivalence to the B cells of recently evolved mammals including man. Relative proportion of these Ig^+ surface bearing cells was about one-half of the bone marrow lymphocytes, one-third of mesenteric lymph node lymphocytes, and two-thirds of the splenic and peripheral blood lymphocytes. Considering the early origin of Megachiropters (fruit bats) in mammalian evolution, it is suggested that surface characteristics of typical mammalian lymphocytes like Ig markers as such or as genomic messages existed at the time of evolution of Class Mammalia. The high percentage of surface Ig^+ cells in the peripheral circulation of bats suggests a "natural immunodeficiency state", which may be equivalent to immunodeficiency state in humans.*

The immune mechanisms of the Indian fruit bat, *Pteropus giganteus*, have been studied in our laboratory for the past several years (1-9). This mammal belongs to an old evolutionary order, Chiroptera (10), and shows a number of interesting physiological (11) and immunological features. The immunocompetent cell types in the fruit bat have been established earlier (8,9) in reference to their cell surface adhesibility, surface topography and cellular content. In the present investigation, the cell types have been further characterized on the basis of cell surface immunoglobulin (Ig) markers using immunofluorescence techniques. In addition, the distribution of the Ig bearing cells in different lymphoid organs were examined.

MATERIALS AND METHODS

Bats

Healthy, adult Indian fruit bat, *P. giganteus*, from Calcutta of both sexes and weighing from 440 to 520gms were maintained in our Center with food and water *ad libitum.*

Isolation of Bat Ig

Bats were immunized with 25mg bovine serum albumin (BSA) (Sigma, USA) in 1ml phosphate buffered saline (PBS) by intravenous injection. Blood was collected by cardiac puncture from anesthetized bats 14 days after immunization. The serum was passed through a column of Sepherose 6B-100 conjugated with BSA as per standard technique (12). The affinity purified bat Ig thereby isolated was then chromatographed in a 60x2.5cm column of Sephadex G-200

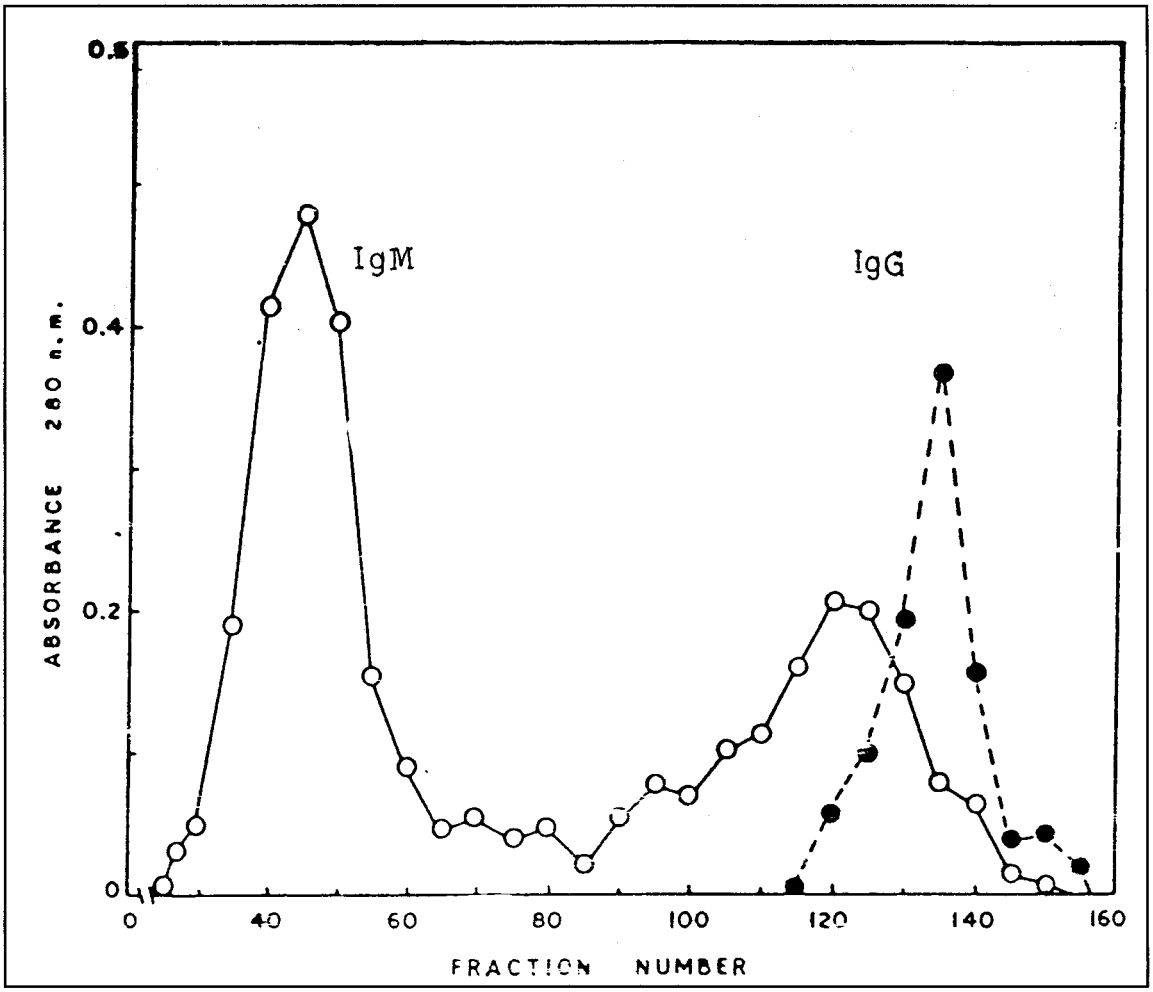

Fig. 1. Sephadex G-200 elution profile of bat immunoglobulins showing separation of two classes of Ig, one eluting in the void volume, and the other eluting later, in a similar location as purified human IgG run separately under similar conditions. o —— o; ●- - -● purified human IgG.

equilibrated with 0.1M Tris-HCl (pH 7.2) in accordance with standard methodology (13). The absorbance at 280nm of the elute collected in 3ml fractions at a flow rate of 5ml/hr were read in a Shimadzu UV-160 spectrophotometer and plotted. Purified human IgG (Sigma, USA) as standard marker protein was also run in the same column under similar conditions. Fractions corresponding to each peak were pooled separately, lyophilized and stored at -20°C until used.

Polyacrylamide Gel Electrophoresis

The characteristic electrophoretic mobility of the affinity purified bat IgM and IgG was studied in 10% polyacrylamide gel following the method of Davis (3). BSA and purified human IgG were used as standard marker proteins.

Anti-bat Ig Antibodies

Rabbits from which normal serum had already been collected were immunized with 2mg of either bat IgM or IgG emulsified in Freund's Complete Adjuvant, and injected

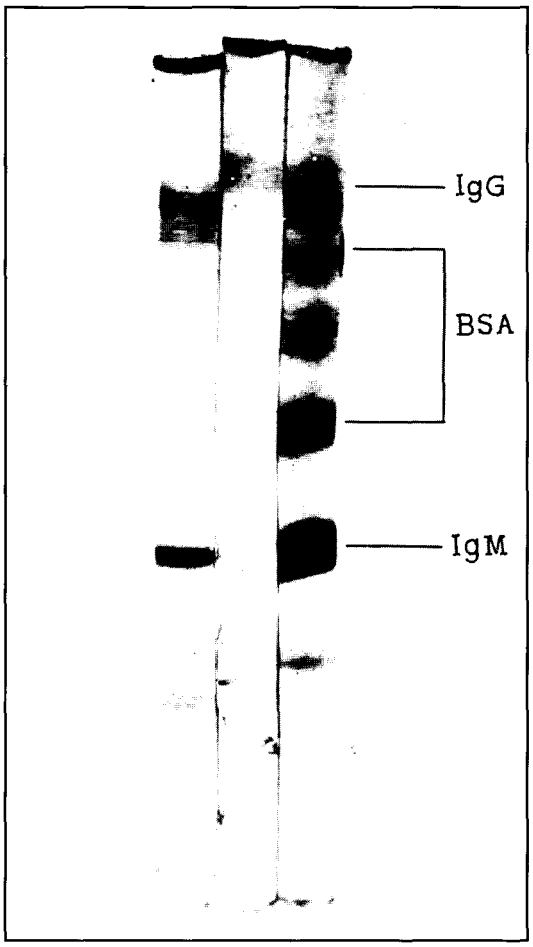

Fig. 2. Separation of two major classes of bat Ig in polyacrylamide gel. Lane at the left contains affinity purified bat Ig; Lane 2 (middle) contains human IgG; and Lane 3 at the right contains a mixture of bat Ig, human IgG and BSA.

subcutaneously in the thigh region of the hindleg. After 6 weekly injections, the respective rabbit antisera against bat IgM or IgG were collected. Ig fraction of all sera were precipitated by 33% $(NH_4)_2SO_4$, desalted through Sephadex G-25 gel filtration, concentrated by vacuum dialysis and stored at -20°C until used.

Fluorochrome Conjugate

Fluorescein isothiocyanate (FITC, Sigma, USA) was conjugated with goat anti-rabbit Ig (Sigma, USA) at a ratio of 1:10 in the dark (15). Unreacted dye was removed by Sephadex G-25 gel filtration of the FITC-protein mixture. Fluorescein/protein (F/P) ratio of the conjugate was about 1:87 which we considered satisfactory.

Separation of Bat Immunocompetent Cells

The plastic adherent (PA), nylon wool adherent (NA), and nylon wool non-adherent (NNA) cells of the bat were separated as described earlier (8). Briefly, leukocytes from the spleen and lymph nodes were suspended in Earle's Balanced Salt Solution (EBSS) containing 10% decomplemented goat serum and antibiotics at a concentration of 10^7 cells/ml and incubated in plastic petri dishes at 37°C for 45 min. The PA cells were separated by scraping them off with a rubber device. The plastic non-adherent cells were then fractionated by passage through a nylon wool fiber column into the NA and NNA populations. Cell viability in the three cell populations was more than 90% as judged by trypan blue dye exclusion technique.

Immunofluorescence Microscopy

100µl aliquots containing $2x10^6$ cells from isolated PA, NA or NNA populations or mononuclear cells from bone marrow, spleen, mesenteric lymph node and peripheral blood were incubated with 100µl of rabbit anti-bat IgM or IgG antibody at 1:10 dilution for 30 min at 4°C. Control cells were incubated with normal rabbit Ig. Cells were then washed twice with chilled PBS containing 0.1% NaN_3 and further incubated with 100µl of fluoresceinated goat anti-rabbit Ig at 1:10 dilution for 30 min at 4°C in the dark. Cells were then washed twice with chilled PBS and resuspended in PBS-Glycerol (1:1) as per standard protocols (14,15). Cells were examined on a hemocytometer slide under a Zeiss FLUOVAL microscope equipped with epi-illumination from HBO-202 lamp, D-224 excitation and BG-47 barrier filters. Photographs were taken using 400 ASA KODAK

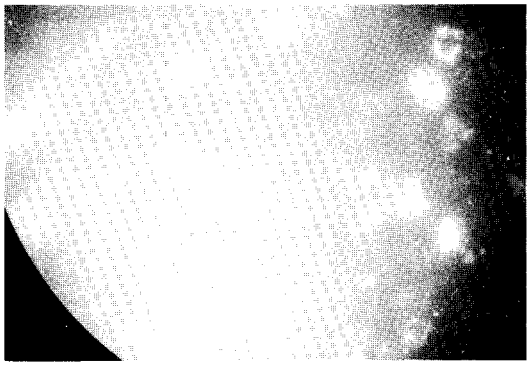

Fig. 3. Photomicrograph of nylon wool adherent lymphocytes of the fruit bat treated with rabbit anti-bat IgM and then goat anti-rabbit IgG coupled with FITC showing immunofluorescent staining of membrane Ig in the form of rings and patches.

color film exposed for 4-6 min and boosted to 800 ASA during development.

RESULTS

Isolation of Two Classes of Ig From Bat

The affinity purified bat immunoglobulins when fractionated by Sephadex G-200 gel filtration revealed two peaks *(Fig. 1)*. Ig corresponding to the first peak was eluted in the void volume and the other was eluted in the fractions corresponding to that of purified human IgG chromatographed under similar conditions.

Polyacrylamide gel electrophoresis of the affinity purified bat Ig also showed two major bands *(Fig. 2)*, one with a higher relative electrophoretic mobility like IgM and the other with a mobility close to that of human IgG.

Enumeration of Surface IgM and IgG Bearing Cells

Bat IgM and IgG, as characterized earlier in the study, were used to generate antisera for the immunofluorescent detection of Ig bearing lymphocytes of the bat. The positively stained cells showed bright immunofluorescence in the form of patches and rings around the cell periphery *(Fig. 3)*, whereas a dull and diffuse fluorescence over the entire cell body indicated dead cells. In control experiments, normal rabbit Ig did not produce positive staining, thus suggesting specificity of the antisera to bat IgM and IgG.

The NA cell population was represented by the prominent positively stained cells (sIg^+), the percentage of which approximated 90% *(Fig. 4)*. Of these, about 54% were surface IgM positive ($sIgM^+$ cells) and the rest were surface IgG ($sIgG^+$ cells). In contrast, the NNA and the PA populations showed only about 20% and 15% SiG^+ cells, respectively.

Distribution of sIg^+ Cells in Lymphoid Organs and Peripheral Blood

The number of sIg^+ cells varied in different lymphoid organs and peripheral blood as shown in *Fig. 5*. About one-third of the mononuclear cells from a mesenteric lymph node were sIg^+ and were slightly higher (38%) in the bone marrow. In spleen, the mean number of sIg^+ cells was closer to two-thirds (69%) of the mononuclear cells. In contrast, most cells (~82%) in the peripheral circulation were sIg^+. The ratio of $sIgM^+$ and $sIgG^+$ cells were approximately 3:2 for bone marrow, spleen and peripheral blood and was slightly higher for a mesenteric lymph node (4:2) *(Table 1)*.

DISCUSSION

Two distinct classes of immunoglobulins, IgM and IgG in bat serum have been characterized by their electrophoretic mobility and gel filtration elution profiles, expanding on earlier studies using ß-mercaptoethanol sensitivity of the Igs (2). Subsequently, the surface Ig^+ immunocompetent cells in different lymphoid populations of the bat were identified by antisera generated against purified bat IgM and IgG. The sIg^+ cells were maximum in nylon wool adherent (NA) cell population in comparison to the nylon wool non-adherent (NNA) and plastic adherent

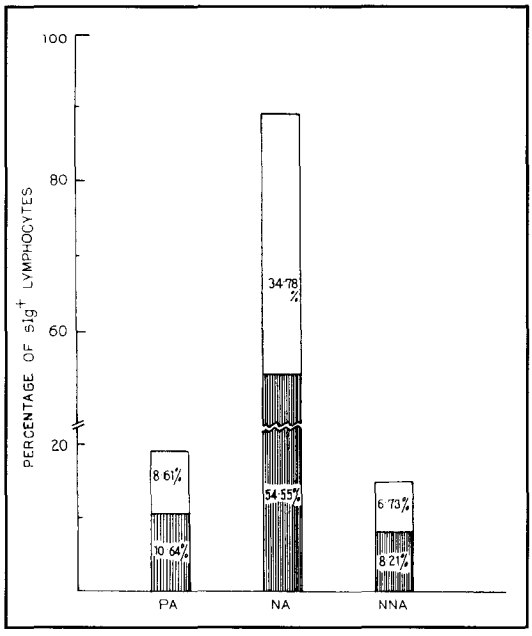

Fig. 4. Percentage of surface Ig bearing (sIg⁺) lymphocytes in the plastic adherent (PA), nylon wool adherent (NA) and nylon wool non-adherent (NNA) cell populations of the fruit bat. ❑ surface IgM bearing cells; ❑ surface IgG bearing cells.

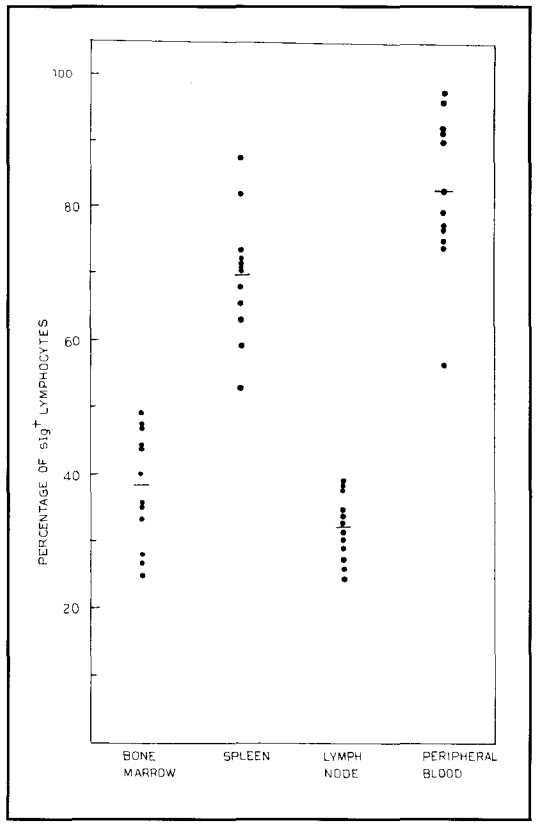

Fig. 5. Percentage of sIg⁺ lymphocytes in different lymphoid organs and peripheral blood of the fruit bat. Each point represents an individual reading and the bar denotes the mean.

(PA) cell populations *(Fig. 4)*. Thus, by convention of adherence to nylon wool and having surface Ig, NA cells can be properly equated with the B lymphocytes of other mammals (16-20), birds (21), and reptiles (22). Similarly, the nylon wool NNA cells being negative for sIg, their sensitivity to anti-brain serum (9) and their cell surface morphology (8), may be categorized at T lymphocytes. The PA cells, by virtue of adhesiveness and lack of sIg, may be classified as macrophages. Moreover, most B cells in the bat bear sIgM molecules *(Table 1)* which is likely in monomeric form as in other mammals.

Presence of sIgG on certain B cells of the bat favors the mammalian feature of the order which originates at an early point of mammalian evolution (10), and specifically distinguishes the bat from sIgY bearing reptilian or avian B cells (21,22). If the expression of surface Ig at the origin point of mammals is not taken for granted, however, the genomic reserve of the message for such expression at that stage needs to be considered.

The percentage of B cells in bone marrow and the B and T cell ratio in spleen and lymph node of the bat *(Fig. 5)* resemble mouse or other recently evolved mammals (23,24). However, the percentage of B cells in the peripheral circulation is much higher (about 82%) *(Fig. 5)* when compared with 15-30% in normal mouse or man (23-25). Such high B cell percentages are encountered in sick patients only with selective deficiencies of T cells, where 80-90% of circulating lymphocytes may be B cells (25-28). The B cell percentage in women is also high (about 70%), between the 7th and 14th week of pregnancy (29). Perhaps for survival of the fetus, the

TABLE 1
Surface IgM and IgG Bearing Lymphocytes (%) From Lymphoid Organs and Peripheral Blood of Bats

Cell Source	Expt. #	$sIgM^+$ cells	Mean (± SE)	$sIgG^+$ cells	Mean (±SE)	$sIgM^+ : sIgG^+$ (mean ratio)
BM	1(a)	24.00		23.53		
		20.69	20.16	7.41	13.34	
		15.79	(2.38)	9.09	(5.12)	
	(b)	25.00		10.71		
		22.73	22.36	10.53	9.64	
		19.35	(1.64)	7.69	(0.98)	
						3.14 : 2.00
	2(a)	20.69		28.57		
		25.81	24.47	18.52	20.04	
		26.92	(1.92)	13.04	(4.55)	
	(b)	24.24		22.73		
		19.05	23.40	16.67	18.93	
		26.92	(2.31)	17.39	(1.91)	
S	1(a)	44.44		27.27		
		41.67	42.99	28.57	26.75	
		42.86	(0.80)	25.00	(1.04)	
	(b)	45.45		27.78		
		30.77	41.19	23.57	24.05	
		47.37	(5.24)	15.79	(4.13)	
						3.00 : 2.00
	2(a)	45.83		36.00		
		48.00	41.99	23.08	30.80	
		32.14	(4.96)	33.33	(3.94)	
	(b)	40.91		30.77		
		34.48	40.41	18.52	30.32	
		45.83	(3.29)	41.67	(6.67)	
MLN	1(a)	18.18		9.52		
		21.43	19.38	11.76	9.66	
	(b)	30.77		4.17		
		15.79	21.27	22.22	13.24	
		17.24	(4.77)	13.33	(5.21)	
						3.84 : 2.00
	2(a)	17.39		7.41		
		25.93	22.77	13.16	11.20	
		25.00	(2.70)	13.04		
	(b)	19.23		12.50		
		24.14	20.81	8.00	10.28	
		19.05	(1.67)	10.34	(1.29)	
PB	1(a)	46.67		10.00		
		36.36	44.34	37.50	24.92	
		50.00	(4.10)	27.25	(8.02)	
	(b)	33.33		44.44		
		66.67	51.85	25.00	34.74	
		55.56	(9.79)	34.78	(5.61)	
						3.08 : 2.00
	2(a)	43.75		38.89		
		46.15	49.58	33.33	36.57	
		58.82	(4.68)	37.50	(1.67)	
	(b)	57.14		35.29		
		40.00	53.59	35.00	34.93	
		63.64	(7.05)	34.50		

BM=Bone marrow; S=spleen; MLN=mesenteric lymph node; PB=peripheral blood

percentage of peripheral circulating T cells is especially low at this stage and "graft rejection" is suppressed. A high percentage of peripheral circulating B cells tends to corroborate the relative immunodeficient status of the bat as suggested by earlier delay in immune response (2,5), a ten-fold lesser density of ligand receptors on bat T cells (6) and a slower energy turnover (7). Whereas the increased number of B cells may compensate for the T cell deficiency by greater antibody production, alternatively, the antibodies generated may act as "blockers" and may impede immunoresponsiveness. The relative immunodepression in bats may allow proliferation of pathogens in this species (30) and render them as carriers for human disease. Perhaps, a similar situation exists in other vertebrates known to be carriers of pathogens.

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REFERENCES

1. Chakraborty, AK, AK Chakravarty: Dichotomy of lymphocyte population and cell mediated immune responses in a fruit bat, *Pteropus giganteus*. J. Ind. Inst. Sci. 64(c) (1983), 157.
2. Chakraborty, AK, AK Chakravarty: Antibody mediated immune response in the bat, *Pteropus giganteus*. Dev. Comp. Immunol. 8 (1984), 415.
3. Chakravarty, AK, AK Chakraborty: Hisophysiology of the white pulp follicles and the germinal centres in spleen and lymph nodes of a bat, flying fox (*Pteropus giganteus*). Comp. Physiol. Ecol. 9 (1984), 197.
4. Paul, BN, AK Chakravarty: *In vitro* analysis of delayed immune response in a bat, *Pteropus giganteus*: Process of ConA mediated activation. Deve. Comp. Immunol. 10 (1986), 55.
5. Paul, BN, AK Chakravarty: Phytohaemagglutinin mediated activation of bat (*Pteropus giganteus*) lymphocytes. Ind. J. Exptl. Biol. 25 (1987), 1.
6. Paul, BN, AK Chakravarty: Density of ConA receptors on T cells of bat and its implication on delayed activation. J. Ind. Inst. Sci. 68 (1988), 37.
7. Paul, BN, AK Chakravarty: Kinetics of three respiratory enzymes of the lymphocytes from a bat, *Pteropus giganteus* during activation. Ind. Biologist. 23 (1991), 13.
8. Sarkar, SK, AK Chakravarty: Analysis of the immunocompetent cell types in the bat, *Pteropus giganteus*: Scanning electron microscopic characterization of the cells. Dev. Comp. Immunol. 15 (1992), 423.
9. Sarkar, SK, AK Chakravarty: Analysis by surface antigenic marker and anatomical distribution of T cells in the bat, *Pteropus giganteus*. PhD Thesis. Univ. North Bengal, 1991.
10. Young, JZ: *The Life of Vertebrates*. Oxford University Press, New York, 1962.
11. Wimsatt, WA (Ed.): *Biology of Bats*. Academic Press, New York, 1970.
12. Porath, J, R Axen, S Ernback: Chemical coupling of proteins to agarose. Nature 215 (1967), 1491.
13. Nowotny, A: *Basic Exercises in Immunochemistry*. Springer Verlag, Berlin, 1979, 3-8.
14. Davis, BJ: Method and application to human serum protein. Annals N.Y. Acad. Sci. 121 (1964), 404.
15. Goding, JW: Conjugation of antibodies with fluorochromes: Modifications to the standard methods. J. Immunol. Meth. 13 (1976), 215.
16. Loor, F, L Forni, B Pernis: The dynamic state of the lymphocyte membrane. Factors affecting the distribution and turnover of surface immunoglobulin. Eur. J. Immunol. 2 (1972), 203.
17. Raff, MC, M Sternberg, RB Taylor: Immunoglobulin determinants on the surface of mouse lymphoid cells. Nature 225 (1970), 553.
18. Wilson, JD, GJV Nossal: Identification of human T and S lymphocytes in normal peripheral blood and in chronic lymphocytic leukemia. Lancet ii (1972), 788.
19. Reeves, JH, HW Renshaw: Surface membrane markers on bovine peripheral blood lymphocytes. Am. J. Vet. Res. 39 (1978), 917.
20. Lewin, HA, CC Calbert, DVM Bomenico-Bernco: Cross reactivity of a monoclonal antibody with bovine, equine, ovine and porcine peripheral blood B lymphocytes. Am. J. Vet. Res. 46 (1985), 785.
21. Pernis, B, L Forni, L Amante: Immunoglobulins as cell receptors. Ann. N.Y. Acad.

Sci. 190 (1971), 420.
22. Natrajan, K, VR Muthtukkaruppan: Distribution and ontogeny of B cells in the garden lizard, *Calotes versicolor*. Dev. Comp. Immunol. 9 (1985), 301.
23. Rabellino, E, S Colon, HM Grey, et al: Immunoglobulins on the surface of lymphocytes. I. Distribution and quantification. J. Exp. Med. 133 (1971), 156.
24. Roitt, IM: *Essential Immunology*. Blackwell, Oxford, 1988.
25. Cooper, MD, AR Lawton: Circulating B cells in patients with immunodeficiency. Am. J. Pathol. 69 (1972), 513.
26. Preud-homme, JL, M Seligman: Primary immunodeficiency with increased number of circulating B lymphocytes contrasting with hypogammaglobulinemea. Lancet i (1972), 442.
27. Gatti, RA, J Gershanik, AH Levkoff, et al: Immunologic recovery: Spontaneous or induced? Proc. Central Soc. Clin. Res. 44 (1972), 74.
28. Grey, H.M., E. Rabellino, P. Profsky: Immunoglobulins on the surface of lymphocytes. IV. Distribution in hypogammaglobulinemea, cellular immune deficiency and chronic lymphocytic leukemia. J. Clin. Invest. 50 (1971), 2368.
29. Strelkaukas, AJ, BS Wilson, S Dray, et al: Inversion of levels of human T and B cells in early pregnancy. Nature 258 (1975), 331.
30. Sulkin, SE, R Allen: Bats: Carrier of human disease producing agents. In: *About Bats: A Chiropteran Biology Symposium*. Slaughter, BH, W Walton (Eds.), Southern Methodist University Press, Dallas, 1970.

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