Erythrocyte Rosettes – A Marker for Bovine T Cells

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ABSTRACT

Many species of erythrocytes were investigated for their ability to form spontaneous rosette with bovine peripheral blood leukocytes and fetal thymocytes. Only sheep and chicken red blood cells gave rosettes. Using conditions shown optimum for the demonstration of human rosette forming cells, only low numbers of bovine rosettes were demonstrable. By changing culture conditions to include 100% fetal calf serum, neuraminidase treated erythrocytes and/or lymphocytes and optimizing the incubation times and temperature, up to 38% of peripheral blood leukocytes and 52% of thymocytes formed rosettes. A thymic origin of rosetting cells was ascribed to T cells for the following reasons: 1) thymocytes gave higher numbers than did peripheral blood leukocytes, 2) rosette forming cell numbers were increased in peripheral blood leukocyte subpopulations enriched in T cells by nylon column separation and 3) only very few rosette forming cells had surface immunoglobulin, a marker of B lymphocytes. The reasons why all T cells were not detected by the technique were discussed.

RÉSUMÉ

Les auteurs ont étudié les hématies de plusieurs espèces, relativement à leur capacité de former des rosettes spontanées avec les leucocytes du sang circulant de bovins et les thymocytes de foetus bovins. Seules les hématies des moutons et des poulets formèrent de ces rosettes. Dans des conditions jugées optimales pour la démonstration des cellules humaines formant des rosettes, ils ne réussirent à démontrer que peu de rosettes bovines. En changeant les conditions de culture de façon à inclure 100% de sérum foetal bovin et des hématies et/ou des lymphocytes traités à la neuraminidase, ainsi qu'en rendant optimales les périodes et la température d'incubation, jusqu'à 38% des leucocytes du sang circulant et 52% des thymocytes formèrent des rosettes.

Les auteurs attribuèrent aux cellules T l'origine thymique des cellules formant des rosettes, pour les raisons suivantes: 1) les thymocytes en donnèrent un plus grand nombre que les leucocytes du sang circulant, 2) le nombre de cellules formant des rosettes se trouva augmenté dans les subpopulations de leucocytes du sang circulant enrichies en cellules T, au moyen de la séparation par une colonne de nylon et 3) seulement quelques cellules formant des rosettes possédaient des immunoglobulines superficielles, un marqueur des lymphocytes B. Les auteurs commentent aussi les raisons pour lesquelles cette technique ne réussit pas à déceler toutes les cellules T.

INTRODUCTION

In man and several other mammals, thymocytes and thymus derived lymphocytes can be distinguished from other lymphocytes and mononuclear cells by their ability to bind heterologous erythrocytes (E rosettes) (1, 5, 10, 11, 12, 16). This rosetting phenomenon is nonimmunological, but as a convenient marker of T cells has led to an increased understanding of the part played by T cells in health and disease (1, 9, 24). Working with the ox, we have recently demonstrated antiviral effects *in vitro* that we attributed to T cells (18). However, because we lacked a positive cell surface marker for bovine T cells these results remain to be confirmed. There appears to be no report in the literature of a marker for bovine T cells and our experience with E

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rosettes, using conditions shown to be optimum in man, gave very variable results. The present investigation was undertaken to determine if E rosettes could be used as a marker of T cells in the ox and if so to optimise the *in vitro* conditions for their demonstration.

MATERIALS AND METHODS

Preparation and Source of Erythrocytes (RBC)

Heparinized venous blood collected from healthy man, monkey, dog, cat, horse, sheep, goat, pig, guinea pig, mouse, rat, rabbit and chicken was centrifuged at 650 x g at 40°C for 30 min. After removal of the buffy coat with a Pasteur pipette the RBC were either used immediately or stored in Alsever's solution at 4°C and used within one week. Prior to use, RBC were washed in PBS (0.15M NaCl, 0.01M phosphate pH 7.2) at least three times and made up in a chosen medium to the desired concentration.

Preparation of Peripheral Blood Leukocytes (PBL)

These were prepared as described previously (19). Briefly heparinized venous blood was centrifuged at 650 x g at 40°C for 30 min to collect buffy coat cells. The latter were separated on ficoll-hypaque (density at 25°C 1.077 gm per ml) by the method of Boyum (6). Contaminating erythrocytes were lysed with 0.83% ammonium chloride solution at 37°C for five min. The PBL were washed three times in Pucks solution (PS) to remove debris and platelets. The cells were 98% viable as determined by trypan blue exclusion. The mononuclear cells so prepared consisted of 80 to 90% lymphocytes with 10 to 20% monocytes.

Preparation of Fetal Thymus Cells

Fetal thymus tissues were collected immediately following slaughter. These tissues were placed in an excess of ice cold PS, trimmed of extraneous connective tissue, cut into small pieces and single cell suspensions made by passing through a 80 gauge stainless steel mesh. After passage through three layers of cotton gauze pad, cells were washed three times with PS. Cell viability varied but ranged from 40-70%. The fetuses used in studies were 12″ to 24″ in size (four months to six months of age).

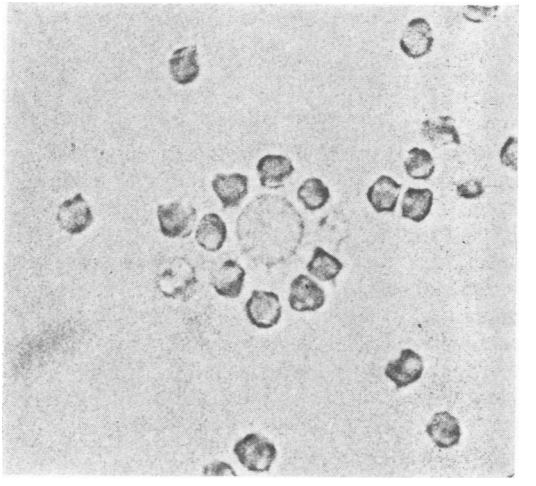

Fig. 1. Lymphocyte from PBL showing rosette formation with neuraminidase treated sheep RBC.

E Rosette Technique

For initial experiments, optimum conditions for the demonstration of human rosettes were used (1). Briefly, PBL or thymocytes at 3 x 10^6/ml in 50 µl of PBS with 0.2% BSA were mixed with an equal volume of 1% sheep RBC in the same medium. After mixing, cells were incubated at 37°C for ten min, then centrifuged at 200 g for five min at 22°C. Cells were then incubated for 24 hours at 4°C. For enumeration of rosette forming cells (RFC), the supernatent fluids were removed and the cells were gently dispersed in 0.1% trypan blue using a short wide bore Pasteur pipette. The preparations were examined after one min and before ten min had elapsed by light microscopy. At least 200 viable cells were recorded (dead cells were excluded from consideration). A cell with three or more RBC adhering to its surface was considered as a rosette (Fig. 1). In some cases cells were fixed in 0.6% glutaraldehyde and stained by Wrights-Giemsa stain. The technique permitted morphological examination of RFC. The great majority of RFC were lymphocytes. Modifications of the rosetting method are described under results.

Media and Sera Used

Various media used for making cell suspensions were PBS (0.15M NaCl. 0.01M phosphate, pH, 7.2), Puck's solution, RPMI-1640 and Eagle's MEM. Other media tried were BSA (10%, 5%, 1%, 0.5% solutions in PBS) and Ficoll (36%, 18%, 9% and 4.5% in PBS). Sera from different sources used were horse serum[1], calf serum[2], freshly prepared autologous bovine serum and plasma, autologous sheep serum and fetal calf serum[3]. All sera used in the studies were heat inactivated at 56°C for 30 min. Most sera were absorbed with sheep RBC prior to use.

Serum Absorption with SRBC and Bovine PBL

For absorption of sera with SRBC, 1 vol of serum was reacted with 0.1 vol of packed, washed fresh SRBC at room temp for four hr and second absorption at 40°C overnight. For absorption of sera with bovine PBL, 50 x 10^6 PBL cells were reacted with 3.0 ml of serum at 40°C for 24 hr.

Detection of Cell Surface Immunoglobulins by Immunofluorescence

Washed cells at 10 x 10^6/ml were reacted in 200 µl volume with fluorescein-labelled rabbit antibovine IgG[4] (Heavy & light chains) for 45 min at 4°C with frequent gentle agitation. Cells were washed three times in ice cold PS, suspended in cold 10% glycerol saline and immediately examined for membrane fluorescence by phase fluorescence using a Leitz, Orthoplan, orthomat-W microscope.

In some assays after preparation of membrane fluorescence the cell populations were processed for rosetting cells. This was done by reacting cells at 3 x 10^6/ml in 100% FCS with 1% SRBC in 100% FCS for ten min at 22°C. After centrifugation at 200 x g for five min at 22°C cells were incubated overnight at 4°C then examined for fluorescing and rosetting cells by phase fluorescence. These extra steps did not diminish the percentage of membrane fluorescing cells from the cell populations.

Nylon Wool Column Separation of Lymphocytes

The method was described in detail elsewhere (18).

Enzyme Pretreatment of Cells

For treatment of RBC with neuraminidase the method of Baxley *et al* (2) was used. One vol of packed washed RBC was mixed with five vol of neuraminidase[5] (One vial reconstituted in 5 ml of PBS). The mixture was incubated at 37°C for one hr, washed three times with PBS and resuspended in the incubation medium to be used. For treatment of PBL with neuraminidase, 10 x 10^6 cells in 200 µl of neuraminidase enzyme (concentration as above) were incubated at 37°C for 30 min, washed three times with PS and resuspended in 100% FCS. An aliquot of neuraminidase treated lymphocytes was processed for surface membrane fluorescence by immunofluorescence as described previously. Erythrocytes were pretreated with papain by the method of Wilson *et al* (23).

One vol of washed 20% RBC in PBS was added to one vol of papain mixture — one part of 0.25% papain[6] in PBS plus one part 3.6% $Na_2HPO_4.12H_2O$ in distilled water plus two parts 0.2% L-cysteine-HCL. The mixture was incubated at 37°C for 15 min, washed three times in PBS and resuspended in 100% FCS.

Trypsin treatment of RBC was achieved by mixing a 5% RBC suspension in PBS with an equal vol of varying concentrations (1%, 0.5%, 0.25%) of trypsin[7] at 37°C for one hr. The reaction was terminated by adding FCS. Cells were washed three times with PBS and resuspended in 100% FCS.

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[1]Grand Island Biologicals, N.Y. Lot 91650.

[2]Grand Island Biologicals, N.Y. Lot 234404.

[3]Grand Island Biologicals, N.Y. Lots: C552307, A251507, R347317, C65217.

[4]Cappel Labs. Inc. Downington, U.S.A.

[5]Vibrio cholera filterate, Sigma Chem. Comp.

[6]Grand Island Biological Comp. Lot. 70550, N.Y.

[7]Fisher Scientific, Comp., U.S.A.

TABLE I. Effect of Culture Conditions on Number of E Rosettes in Bovine PBL and Fetal Thymocytes

Incubation Medium	Absorbed with	No. of RFC[a] PBL Mean	PBL Range	PBL # of Expts	Fetal Thymocytes Mean	Fetal Thymocytes Range	Fetal Thymocytes # of Expts
PBS	—	0	0	5	5.7	4— 7	3
PBS+5%FCS-1[b]	—	2	1— 3	5	9.6	8—11	3
10%FCS-1	—	4.6	3— 6	5	16.0	14—18	3
20%FCS-1	—	6.6	5— 8	5	18.0	16—20	3
40%FCS-1	—	10.0	8—12	5	25.0	24—28	3
60%FCS-1	—	11.0	9—14	5	30.0	29—32	3
80%FCS-1	—	15.0	13—17	5	34.0	31—36	3
100%FCS-1	—	16.0	14—19	12	33.0	32—35	3
100%FCS-1	sheep RBC	15.0	14—18	2	34.0	30—36	3
100%FCS-1	bovine PBL	16.5	15—18	2	ND[c]		
100%FCS-2	sheep RBC	8.0	7—9	2	ND		
100%FCS-2	bovine PBL	8.5	7—10	2	ND		
100%FCS-3	sheep RBC	10.0	9—11	2	ND		
100%FCS-3	bovine PBL	9.0	9—10	2	ND		
100%FCS-4	sheep RBC	7.5	7— 8	2	ND		
100%FCS-4	bovine PBL	7.0	6— 8	2	ND		

[a]50 μl of 3 x 10^6/ml suspension of PBL or thymocytes in a given medium mixed with 50 μl of 1% sheep RBC suspended in the corresponding medium in 10 x 75 mm glass tubes in duplicate. Mixtures were incubated at 22°C for 10 min, centrifuged at 200 x g for 5 min at room temp and then incubated at 4°C for 20-24 hr. These preparations were examined as described in materials and methods
[b]FCS-1, FCS-2, FCS-3 and FCS-4 refer to different batch numbers of FCS
Not done

RESULTS

Two sources of cells were routinely used for the demonstration of E rosettes, peripheral blood leukocytes (PBL) isolated by Ficoll-hypaque floatation and fetal thymocytes collected from recently slaughtered animals. In initial experiments, erythrocytes from different species of animals were tested using conditions shown optimum for demonstration of E rosettes in man (see materials and methods). Human, monkey, horse, sheep, goat, pig, dog, cat, guinea pig, rat, rabbit, mouse and chicken RBC were tested and of these only sheep and chicken RBC gave rosettes and these were of low number (PBL gave 4-6% with sheep RBC and 2-4.8% with chicken RBC). Corresponding percentage of fetal thymocytes were 8-22% for sheep RBC and 6-10% with chicken RBC. None of the other species of RBC tested formed rosettes either with PBL or fetal thymocytes.

As shown in Table I, the number of E rosettes could be elevated by increasing the concentration of fetal calf serum (FCS) in the incubation medium. In fact, 100% FCS gave the maximum percentage of rosettes with both PBL (mean 16%) and fetal thymocytes (mean 33%). Other batches of FCS were tested and found to give few rosettes. These sera also caused

TABLE II. Effect of the Concentration Bovine PBL and Sheep RBC on the Number of RFC[a]

PBL (x 10^6/ml)	Mean Number of RFC — SRBC[c] Concentration 2.0%	1.0%	0.5%	0.25%
10	ND	24	18	8
8	ND	22	17	10
6	27[b]	23	19	12
4	25[b]	28	20	15
3	24[b]	30	23	16
2	ND	30	25	18
1	ND	31	24	20
0.5	ND	31	25	19

[a]Method as described in Table I except that medium used for PBL and SRBC suspension was 100% FCS-1
[b]Difficult to read due to overcrowding by RBC
[c]Neuraminidase treated SRBC
ND = Not done

lymphocyte clumping. The use of other tissue culture media, RPMI 1640 or Eagles MEM instead of PBS, made no difference in the numbers of rosettes produced (data not shown). Other additants were also investigated including Ficoll (36%, 18%, 9% and 4.5%), BSA (10%, 5%, and 1%), calf serum (10%, 40%, 80%, 100%), autologous bovine serum and plasma (10%, 40%, 80%, 100%), autologous sheep serum (100%) and horse serum (100%) but these gave lower and more variable numbers of ro-

TABLE III. Effect of Enzyme Pretreatment of RBC and Lymphocytes on Number of Bovine RFC

Treatment of RBC	Percent RFC[a]		
	PBL		
	Untreated	Neuraminidase Treated	Thymocytes Untreated
Neuraminidase	24(18 — 32)[c]	32(27 — 38)[d]	45(42 — 52)[d]
Control[b]	16(10 — 20)[c]	26(20 — 35)[d]	34(32 — 38)[d]
Papain	20(13 — 26)[d]	ND[e]	ND
Control[b]	15(9 — 18)[d]	ND	ND
Trypsin — 1%	0	ND	ND
— 0.5%	3(1 — 5)	ND	ND
— 0.25%	8(6 — 9)	ND	ND
Control[b]	16(11 — 18)	ND	ND

[a]Method as described in Table I, except that medium for suspending cells (RBC, PBL, thymocytes) was 100% FCS
[b]Control RBC were incubated under same conditions as enzyme treated cells except that enzymes were omitted
[c]Values indicate means plus the range of values obtained in six experiments
[d]Values indicate means plus the range of values obtained in four experiments
[e]Not done

settes than obtained with FCS. Also shown in Table I are results indicating that FCS absorbed with sheep RBC gave approximately the same number of rosettes as did unabsorbed FCS. Furthermore, absorption of sera with PBL did not alter the numbers of RFC. The effect of varying the concentration of lymphocytes (0.5×10^6 to 10×10^6 ml) and of erythrocytes (0.25, 0.5, 1.0, 2.0% was investigated. The highest percentage of rosettes was obtained with a lymphocyte concentration of 3×10^6/ml and a SRBC concentration of 1% (Table II). At lymphocyte numbers of higher than 3×10^6/ml a tendency for cell clumping made enumeration difficult.

Effect of Enzyme Treatment of Erythrocytes and Lymphocytes

Treatment of sheep RBC and/or lymphocytes with the enzymes neuraminidase (22) and papain (8) were shown to increase the number of E rosettes in human and other species. In the bovine, enzyme pretreatment of both RBC and lymphocytes led to increased numbers of E rosettes (Table III). Neuraminidase pretreatment of RBC increased rosettes from a mean of 16% to 24% and papain pretreatment from 15% to 20%. Following enzyme pretreatment of lymphocytes alone, E rosette numbers were also increased from a mean of 16% to a mean of 26%. Highest numbers of E rosettes were obtained if both lymphocytes and RBC were pretreated with neuraminidase (mean of 32%). Whereas neuraminidase and papain pretreatment of RBC led to increased numbers of E rosettes, trypsin treatment diminished the numbers (Table III).

Using the optimum culture media (100% FCS), cell concentration (3×10^6/ml) and neuraminidase pretreatment of SRBC the effect of time and temperature of incubation on the rosette response was investigated. As can be seen from the results shown in Table IV, the optimum time was ten min at 22°C followed by overnight incubation at 4°C. Not only was there an increase in numbers of rosettes after overnight incubation but the rosettes produced were more stable (less tendency to disrupt during handling). In comparison to PBL, thymocytes showed a higher percentage of rosettes which occurred earlier and were also more stable.

Evidence that E Rosettes are T Cells

Morphological studies on Wright's-Giemsa stained cytospin preparations revealed that the majority of RFC were medium (50-65%) and small (20-35%) lymphocytes. Between 5-10% of RFC were large lymphocytes and about 2% of RFC had monocyte morphology. Evidence for the assumption that the majority of E rosettes detected were T cells is presented in Table V. The nylon wool technique of Julius *et al* (13) has been shown in several species including the ox (18) to be a convenient method of obtaining enriched populations of T cells, since T cells adhere weakly to nylon. Using this technique, nonadherent

TABLE IV. Effect of Time and Temperature of Incubation on Number of RFC in Bovine PBL and Fetal Thymocytes

Constant Condition	Temp of Incubation	Time of Incubation Prior to Centrifugation in Min 5	10	15	30	45	60
Postcentrifugation[a] overnight incubation at 4°C	37°C	23.7[b] (23 - 26)	25.5 (23 - 28)	24.5 (21 - 26)	20.7 (18 - 23)	17 (14 - 19)	12 (11 - 14)
	22°C	25 (22 - 28)	17.5 (25 - 30)	24.5 (22 - 27)	22 (20 - 25)	16 (14 - 20)	15 (13 - 16)

Precentrifugation incubation at 22°C for 10 min	Cells	Postcentrifugation Time of Incubation in Hrs 2	4	8	20 - 24
	PBL	6 (4 - 7)	10 (7 - 11)	13 (10 - 15)	25 (21 - 27)
	Thymocytes	22 (19 - 24)	23.5 (21 - 25)	30 (28 - 32)	48 (42 - 50)

[a]Centrifugation at 200 x g for 5 min at 22°C
[b]Mean values — figures in brackets represent range found in four experiments

TABLE V. Rosetting Characteristics and Immunoglobulin Containing Cells in Bovine PBL and PBL Subfractions

Cell Type	%RFC	%MF[a]	Mean % Age of Cells with +MF +RFC	+MF −RFC	−MF +RFC	−MF −RFC
PBL	21[b] (19 -24)	32 (24 - 38)	0.2	33.2	23.2	43.0
Effluent	31 (27 - 34)	4.4 (1.6 - 7)	ND[c]	ND	ND	ND
Adherent	11 (8 - 16)	50 (46 - 58)	ND	ND	ND	ND
Neuraminidase treated PBL	32 (27 - 38)	34 (26 - 37)	0.1	30.4	30.0	39.5

[a]Membrane fluorescence detectable after reaction with labelled rabbit antibovine IgG serum
[b]Numbers are mean values with ranges of four experiments shown in brackets
[c]Not done

cells had higher numbers of rosetting cells (27-34%, mean 31% $P < 0.05$) and cells mechanically removed from the column were depleted of rosette forming cells (8-16%, mean 11% $P < 0.05$). Also shown in Table V are the results of experiments in which membrane fluorescence with fluorescein-labelled antiimmunoglobulin serum was used as a marker of B lymphocytes. Membrane fluorescing cells were depleted from the effluent cell population (1.6-7%, mean 4.4%) as compared to whole PBL (24-38%, mean 32%) and enriched in the nylon wool adhering cell population (46-58%, mean 50%). In addition, when the membrane fluorescence technique was performed prior to the development of rosettes only a very few cells with membrane fluorescence also formed rosettes (mean < 0.2% of total RFC). Around 40-45% of cells formed neither rosettes nor showed membrane fluorescence. The identity of these cells remains to be determined but up to 20% of the cells could be monocytes. Monocyte identification by specific means is needed to enhance the precision of our experiments.

Also shown in Table V are the results that neuraminidase treatment of PBL did not change the pattern of responsiveness.

DISCUSSION

The present work permits us to add the ox to the list of species in which E rosettes can be used as a marker of T cells. Of 13 species of erythrocytes listed, only two (sheep and chicken RBC) gave rosettes using conditions shown to be optimum for the demonstration of human E rosettes. Moreover the percentage of cells forming rosettes under optimized conditions was very low, far lower than the percentage of T cells present. Thus the fetal thymus cells examined, which presumably consist of

greater than 90% T cells, only gave a mean of 45% rosette forming cells (RFC). Human fetal thymocytes usually give up to 100% RFC (15). We have found (Grewal, A. S. and Rouse, B. T. Unpublished observations.) that <0.2% bovine fetal thymocytes have immunoglobulin bearing cells, a marker of B lymphocytes (17, 18). By changing the experimental conditions, up to 38% of PBL and 52% of fetal thymocytes gave E rosettes. These conditions included the use of 100% FCS, neuraminidase treatment of both erythrocytes and lymphocytes, a ten min incubation at 22°C, centrifugation at 200 g for five min at 22°C followed by overnight incubation at 4°C. Neuraminidase (22) as well as papain (8) pretreatment of RBC has been shown in other species to increase E rosette formation. Some confusion exists as to whether neuraminidase pretreatment of lymphocytes increases the number of T cells detected or if non T cells account for the increase of rosettes (3). Our data discussed below would tend to support the idea of an increase in the detection of T cells.

Although 100% FCS was found optimum, considerable variation amongst batches of FCS was noted. Less supportive batches of FCS also caused lymphocyte clumping, leading us to suspect that perhaps they contained antilymphocyte antibodies. These antibodies have been shown to cause a decrease in E rosette formation in other species (7, 14). However, absorption with lymphocytes (and SRBC) neither precluded clumps nor increased the percentage of E rosettes detected. Consequently, the cause of batch variation among FCS remains undetermined. However, the variation among batches of FCS is a common phenomenon within immunological techniques and the cause is usually not known (20, 21).

Even under the optimum conditions reported herein, not all T cells formed rosettes. In most species, the percentage of T cells in peripheral blood is 70-80% (4) and in the ox we have, from the negative approach of subtracting the number of cells showing surface immunoglobulin (B cells) from total lymphocyte numbers, assumed that T cells account for around 65% of circulating lymphocytes. Thus in the ox, like other species, T cells appear to predominate over B cells. However, in the ox, presumably only a subset of T cells form E rosettes although it remains probable that even further refinement of the experimental conditions or the use of other species of erythrocytes may detect all T cells. Our evidence that the E rosettes detected were indeed T cells was based on the results of several experimental approaches. Thus thymocytes consistently had higher numbers of E rosettes than did PBL. Secondly, nylon wool fractionation, a technique that yields a nonadherent cell population enriched in T cells (18), gave higher percentages of RFC than did the unseparated cells or cells mechanically removed from the nylon. Finally, when employing a combination of membrane fluorescence to detect immunoglobulin bearing cells together with rosetting, less than 0.2% of RFC were found to fluoresce, evidence that the RFC were essentially not B lymphocytes. To further prove that RFC are T cells will require functional tests on separated cells. One way would be to separate RFC from nonrosetting cells and examine the response of such cells both to mitogens and their ability to provide helper function *in vitro* antibody production. These approaches are under investigation in our laboratory. Should these procedures be successful then the solution to the question of the role of T cells in antiviral defense mechanisms may be more easily approached.

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