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ROSETTE FORMATION BY MOUSE LYMPHOCYTES

III. RECEPTORS FOR IMMUNOGLOBULIN ON NORMAL AND ACTIVATED T CELLS

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SUMMARY

By the use of an indirect immunofluorescent method for the identification of mouse T lymphocytes forming rosettes with antibody-coated erythrocytes, it was found that about 10% of the T cells in normal mouse spleen have receptors for antibody. Inhibition experiments with purified aggregated immunoglobulins showed that IgG2 is the major class involved. The same receptor was found on T cells activated against H-2 isoantigens. The importance of using the appropriate red cells and antiserum for demonstrating receptors on T cells is stressed.

INTRODUCTION

Immunoglobulin (Ig) is associated with the surface of many types of cell, but only in the case of the lymphocytes and plasma cells of the antibody-forming (B) cell series is the Ig clearly endogenous (Raff, Feldmann & DePetris, 1973). Macrophages (Lay & Nussenzweig, 1969; Liew, 1971; Rhodes, 1973), mast cells and basophils (Wilson *et al.*, 1972), monocytes (Hay, Torrigiani & Roitt, 1972), cytotoxic myeloid cells (Greenberg, Shen & Roitt, 1973), and B cells themselves (Basten *et al.*, 1972a), can acquire surface Ig by uptake from the serum, each cell type showing a predilection for the Fc region of particular Ig classes or subclasses. In the case of the T cell, opinions differ as to whether the surface Ig is endogenous (Marchalonis, Cone & Atwell, 1972) or passively acquired (Hudson *et al.*, submitted for publication). Support for the latter possibility was strengthened by the experiments of Yoshida & Andersson (1972) suggesting binding of aggregated or antigen-complexed Ig to T cell-rich spleen cells. The availability of a simple method for staining T-cell rosettes (Gyöngyössy & Playfair, 1974) prompted us to investigate whether these Ig-binding cells were in fact T cells. We have found that at least 10% of spleen T cells can bind antigen-complexed Ig of the IgG2 class.

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MATERIALS AND METHODS

Mice

(C57Bl × BALB/c)F_1 and NZB mice were bred in our laboratory as previously described. Non-inbred nu/nu (Nude) mice were obtained from the Laboratory Animals Centre, Carshalton, Surrey.

Cells

Spleen, thymus, and lymph node cells (axillary, inguinal and mesenteric) were teased from the organs into Hanks's buffered salt solution (HBSS), washed three times at 4°C, at 150 *g*, counted in a haemocytometer, and made up to 2×10^6 per ml in HBSS containing 0·1% gelatin (Yoshida & Andersson, 1972).

'Cells responding to oxazolone' were from the inguinal lymph nodes of mice whose shaved abdomen had been painted 4 days earlier with 0·1 ml of 3% oxazolone in ethanol. 'Activated T cells' were from the spleen and lymph nodes of (C57Bl × BALB/c)F_1 mice irradiated with 850 rad of 250 KVP X-rays, injected with 10^8 parental (BALB/c) thymus cells intravenously, and killed 6 days later.

Sheep red blood cells (SRBC) were obtained from Burroughs Wellcome, ox RBC by courtesy of the Royal Veterinary College, and chicken RBC from our own birds. RBC were washed three times in HBSS and made up to 2×10^8 per ml for sensitization.

Antisera

Mice were injected with 2×10^8 sheep or ox RBC and bled 5, 9 or 14 days later ('primary sera'). Some mice were given sheep or chicken RBC in Freund's complete adjuvant, boosted 10 days later, and bled after another 10 days ('hyperimmune sera'). All sera were inactivated at 56°C for 30 min.

Rabbit anti-mouse brain-associated theta antigen (BAθ) serum was made and absorbed as described previously (Gyöngyössy & Playfair, 1973, 1974).

Sensitization of RBC

Red blood cells were suspended in HBSS containing various dilutions of the appropriate antibody for 15 min at 37°C, followed by two washes in HBSS and final restoration to 2×10^8 per ml in HBSS with gelatin. Subagglutinating doses were chosen for rosette formation as follows: anti-SRBC 5-day serum, 1/200; 9-day serum, 1/400; 14-day serum, 1/400; hyperimmune serum, 1/800; anti-chicken serum, 1/800; anti-ox serum, 1/2–1/100; (ox RBC do not agglutinate).

Rosette formation

The method was adapted from that of Yoshida & Andersson (1972). 0·1 ml of the nucleated cell suspension and 0·1 ml of the sensitized RBC (EA) were mixed in small flat-bottomed tubes and allowed to stand for 1–2 hr at room temperature. Control tubes contained nucleated cells with unsensitized RBC (E), or sensitized RBC alone. The mixture was resuspended on a slow vertical rotor (8 rev/min) and a sample placed in a haemacytometer for rosette counting. One drop of 0·01% Acridine Orange allowed visualization under u.v. light of the central rosette-forming cell (RFC); four or more RBC surrounding a nucleated cell were scored as a rosette. All counts were made in quadruplicate by two observers, and expressed as the percentage of RFC per nucleated cells.

Inhibition of rosettes

Purified myeloma proteins were kindly provided by Dr F. Hay as follows: IgG1, RPC 23; IgG2a, 5563; IgG2b, MOPC 141; IgM, MOPC 104 E; IgA 47 A. 0·05 ml of suitable dilutions were added to the rosette mixture before incubation to give final concentrations of 100, 10, 1 or 0·1 μg per ml. Before use the myeloma proteins were aggregated by heating at 63°C for 20 min.

Immunofluorescent staining of rosettes

This procedure has been described in another paper (Gyöngyössy & Playfair, 1974). Briefly, the rosettes were fixed in 0·6% glutaraldehyde, washed, incubated with the anti-BAθ serum, washed, incubated with an FITC-conjugated goat anti-rabbit Ig antiserum, washed, and mounted for immunofluorescent viewing by incident u.v. light in a Leitz Ortholux microscope. Under these conditions few if any rosettes are lost, and T cells appear strongly ring-stained, even when viewed through surrounding RBC. The number of RFC that contained a T cell was estimated on coded slides by two observers, and expressed as a percentage of all RFC.

RESULTS

Formation of EA rosettes

In preliminary experiments various combinations of indicator RBC and antiserum were tried. Spleen and lymph node cells gave approximately similar numbers of rosettes with sheep RBC sensitized with subagglutinating doses of all the primary antisera used, with chicken RBC sensitized with hyperimmune serum, and with ox RBC sensitized with high concentrations of primary antibody. Sheep and chicken RBC also gave rosettes with thymus cells, but ox RBC did not. The hyperimmune anti-sheep RBC serum gave higher numbers of rosettes with spleen (30%) and lymph node cells (20%), but the same levels with thymus (3–4%) as the primary sera. Since we were interested in the possibility of T-RFC, we adopted a standard preparation of sheep RBC sensitized with 1/400 14-day primary serum, and Table 1 shows the numbers of rosettes obtained from various cell types; these values were not altered by one further dilution of the antiserum, but stronger antiserum led to agglutination, so we cannot be certain that they are plateau values. Under these conditions, rosettes formed with unsensitized RBC never exceeded 0·5%.

Replacement of the HBSS by phosphate-buffered saline did not cause any change in the number of rosettes obtained. However, the addition of 0·01 M EDTA did cause some reduction, the nature of which is presently being investigated.

'Activated T-cell' preparations

The local lymph nodes at the height of a response to oxazolone showed a relative and absolute increase of EA RFC (Table 1). At this time it is said that predominantly T cells are activated (Davies *et al.*, 1969). In the irradiated 'activated T cell' spleens and lymph nodes, there was a slight relative increase in EA RFC compared to irradiated uninjected controls, more marked in lymph nodes (Table 1). When the much larger size of the organs in the mice injected with thymus cells is taken into account, it can be seen that there is a three-fold increase in the absolute numbers of EA rosettes in the spleen and a thirty-fold increase in the lymph nodes.

Staining by anti-T-cell serum

In normal spleen, about 40% of the EA RFC were stained by the anti-BAθ serum, and 26% in the lymph nodes. The calculated percentage of normal splenic T cells which make EA rosettes is about five times higher than in lymph nodes (Table 1). In the 'activated T cell'

TABLE 1. EA rosettes and their staining by anti-T serum in various organs of (C57Bl × BALB/c) F_1 mice

Mice	Cells	EA rosettes*		T-RFC†			
		Percentage of cells	Per organ	Percentage of RFC	Percentage of cells	Percentage of T cells	Per organ
Normal	Spleen	10·1‡	15×10^6	40	4	10	6×10^6
Normal	Thymus	3·9	$2{\cdot}7\times10^6$	N.T.			
Normal	Lymph nodes (pooled)	3·4	$2{\cdot}4\times10^6$	26	0·9	1·8	6×10^5
Normal	Inguinal lymph nodes	3·4	$5{\cdot}8\times10^5$	N.T.			
4 days after oxazolone	Inguinal lymph nodes	6·4	$1{\cdot}7\times10^6$	N.T.			
850 rad	Spleen§	10·2	$5{\cdot}9\times10^5$	56	5·7		$3{\cdot}3\times10^5$
850 rad	Lymph node	4·9	$3{\cdot}2\times10^4$	N.T.			
850 rad + syngeneic thymus	Spleen	11·4	$6{\cdot}8\times10^5$	40	4·6		$2{\cdot}7\times10^5$
850 rad + syngeneic thymus	Lymph node	7·2	$1{\cdot}6\times10^5$	N.T.			
850 rad + parental thymus	Spleen	12·2	$1{\cdot}9\times10^6$	57	7·0		$1{\cdot}1\times10^6$
850 rad + parental thymus	Lymph node	11·3	$1{\cdot}0\times10^6$	70	8·0		7×10^5

N.T. = not tested.
* SRBC sensitized with 14-day primary serum at 1/400 dilution.
† EA rosettes stained by anti-BAθ serum.
‡ All values for normal and experimental mice are based on five to ten animals.
§ Six days after irradiation.

lymph nodes, 70% of the RFC were T cells. Unfortunately we found it difficult to estimate the percentage of T-RFC in the irradiated control lymph nodes, which were practically acellular, but even in the unlikely event that they were all T cells, the injection of parental thymus cells must have led to at least a twenty-fold increase in T-RFC numbers. One experiment where syngeneic thymus cells were injected gave results close to the irradiated uninjected controls, suggesting that histoincompatibility constituted a stimulus for the production of EA RFC. Although the point was not studied systematically, our impression was that the RFC were made by the larger T cells, and that they also bound fewer RBC than non-T-RFC.

Inhibition of EA rosette formation by Ig

When EA rosettes were formed with the standard 14-day anti-SRBC serum, the addition of heat-aggregated IgG2a or IgG2b at concentrations of 10 μg/ml or above completely

inhibited rosette formation from both normal spleen cells and 'activated T cell' lymph node cells. There was some inhibition at higher concentrations of IgG1, but none with IgM or IgA (Fig. 1). Similar results were obtained with the 5-day antiserum and the hyperimmune serum, so it is probably not an artefact due to the Ig class distribution in the antisera. However, it is quite possible that none of our sera contained enough IgG1 antibody to bind the SRBC to IgG1 receptors.

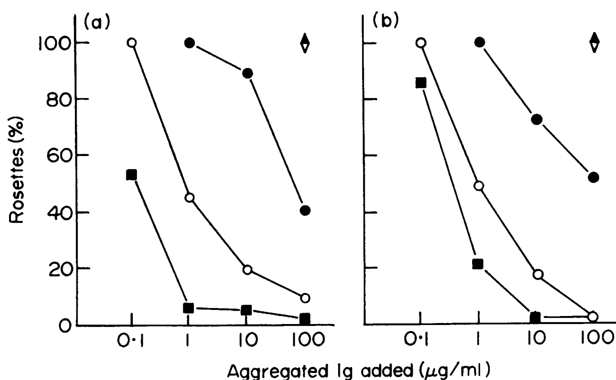

FIG. 1. Inhibition by aggregated Ig classes and subclasses of EA rosette formation by (a) normal spleen cells and (b) 'activated T cell' lymph node cells. (●) IgG1. (○) IgG2b. (■) IgG2a. (▲) IgM. (△) IgA.

DISCUSSION

Our findings confirm those of Yoshida & Andersson (1972) that some T cells, particularly when activated to major histocompatibility antigens, can bind SRBC–IgG antibody complexes. The evidence from the inhibition study (Fig. 1) also agrees with the finding of Lee & Paraskevas (1972) that the immune complexes binding to T cells were of antigen with IgG2a, and not IgG1, antibody. Interestingly enough, mouse macrophages and cytotoxic myeloid cells also bind the IgG2 subclasses preferentially (Greenberg *et al.*, 1973), whilst B cells appear to bind predominantly IgG1 (Basten, Warner and Mandel, 1972b). Thus our evidence suggests that from the point of view of their Ig Fc receptor, T lymphocytes have more in common with the macrophage–monocyte cell series than with the B lymphocytes. One implication of this is that IgG found on T cells, especially IgG2, may well be cytophilic. However, in view of our failure to inhibit EA RFC with IgM, even when the SRBC must have carried considerable amounts of IgM, we cannot exclude the possibility that T cell-associated IgM might be endogenous. We propose to investigate the rosette-forming and inhibiting properties of monomeric IgM (Rhodes, 1973).

We have, of course, no proof that all cells with Ig Fc receptors are detected by the method used here. Indeed it is evident that most of the B cells in spleen and lymph node are not binding EA, whereas they label with radioactive soluble complexes (Basten *et al.*, 1972a). Conversely, thymus cells appear to bind EA better than soluble complexes. The failure of antibody-coated ox RBC to make rosettes with thymus cells suggests that they may be unsuitable for demonstrating T cells with Fc receptors, though this point has not been checked with immunofluorescence. Since chicken RBC gave results identical to sheep RBC, it seems likely that the ox, rather than the sheep, RBC are atypical.

A curious finding in the 'activated T cell' experiments was that while, as time proceeded, there was an increase of EA RFC (most of which were T cells), there was a simultaneous decrease to zero of complement (EAC) rosettes (Arnaiz-Villena, Gyöngyössy & Playfair, unpublished). This is our only evidence as yet that EA rosette-forming and EAC rosette-forming T cells are not necessarily identical cells. One interpretation might be that 'activation' of T cells is associated with expression of the Fc receptor and loss of the C3 receptor. However there is the alternative possibility that the C3 receptors are blocked by split complement products (Eden *et al.*, 1973) resulting from irradiation or the graft-versus-host reaction. In preliminary experiments with the autoimmune NZB strain of mice, we have again found high T-RFC with EA and low EAC RFC, which may signify that NZB T cells are in a state of chronic activation.

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