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ROSETTE FORMATION BY MOUSE LYMPHOCYTES

I. DEMONSTRATION BY INDIRECT IMMUNOFLUORESCENCE OF T CELLS BINDING SHEEP ERYTHROCYTES

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SUMMARY

A method is described for indirect immunofluorescent staining of mouse T cells forming rosettes, involving fixation of rosettes by glutaraldehyde and staining with a rabbit anti-mouse brain serum absorbed so as to be specific for T cells. Using this technique, it was shown that about 44% of spleen cells forming rosettes with sheep erythrocytes both in non-immunized and immunized mice, are T cells. The number of T rosette-forming cells increased about ten-fold after immunization with sheep erythrocytes, but only when serum antibody was detectable.

INTRODUCTION

Divergent opinions as to how many, if any, antigen-binding cells in mice are thymus-dependent lymphocytes (T cells) have been put forward by many workers (Bach, 1973; Hunter, Munro & McConnell, 1972; Wilson & Miller, 1971). Most attempts to identify antigen-binding cells as T cells have been indirect, resting largely on the demonstration of antigen-binding cells in the thymus (Modabber, Morikawa & Coons, 1970; Bach, Muller and Dardenne, 1970), or on showing that a proportion of rosette-forming cells (RFC) can be depleted either by treatment with anti-θC3H serum and complement (Bach *et al.*, 1970; Greaves & Moller, 1970; Greaves, Moller & Moller, 1970; Schlesinger, 1970; Roelants, 1972), or by adult thymectomy (Haskill *et al.*, 1972). Recently, a direct demonstration of anti θAKR-sensitive (T) RFC in AKR mice has been published (Ashman & Raff, 1973).

In a previous paper, we described a simple method of indirect immunofluorescence of mouse T cells by means of a hetero-antiserum raised against mouse brain (Gyöngyössy & Playfair, 1973). This specific anti-'brain-associated θ' (BAθ) serum seemed applicable to a study of RFC by immunofluorescence in strains of mice carrying the θC3H as well as the θAKR marker.

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MATERIALS AND METHODS

Mice

(C56Bl × BALB/c) F_1 mice were bred in our laboratory from inbred parental strains originally obtained from the Laboratory Animals Centre, Carshalton (Playfair, 1968).

Nude (nu/nu) mice, non-inbred, were also obtained from Carshalton.

Antisera

Anti-BAθ serum was raised in rabbits and absorbed with irradiated, bone marrow repopulated spleen cells, as previously described (Gyöngyössy & Playfair, 1973), and used at dilution of 1/8. Further absorptions of anti-BAθ were carried out at 1/4 dilution at 4°C, overnight, using equal volumes of washed normal mouse brain homogenate, normal thymocytes or nude spleen cells.

Normal rabbit serum (NRS) used in control experiments was always obtained before immunization from the same rabbit.

Anti-θC3H serum was prepared by injecting CBA thymocytes into AKR mice (Raff, 1969).

Normal mouse serum (NMS) was obtained from AKR mice.

Rosette and staining technique

Fresh sheep red blood cells (SRBC) obtained from Burrough's Wellcome, were washed three times in Hanks's balanced salt solution (HBSS) and adjusted to a concentration of 4×10^8 per ml.

Spleen cells were teased into HBSS, then passed through a 25-gauge needle to break up clumps. The cells were then washed three times (150 *g*) in HBSS, at 4°C for 10 min. The pellet was resuspended, nucleated cells counted in a haemocytometer and the cell concentration adjusted to 4×10^7 per ml.

Duplicate samples were set up in round-bottomed tubes by mixing a 0·5-ml aliquot of SRBC with 0·5 ml of the lymphocyte suspension. The mixture was centrifuged at 50 *g* at 4°C, for 15 min, then allowed to incubate at 4°C for a further 45 min. After incubation, the duplicate tubes were resuspended on a vertical rotor at 8 rev/min. One sample was diluted 1 : 1 with HBSS, then fixed by the addition of glutaraldehyde at a final concentration of 0·6% for 30 min at 4°C (Haskill *et al.*, 1972). The fixed rosettes were then transferred into 8 ml of HBSS, and centrifuged at 150 *g* at 4°C for 10 min, the supernatant was removed and the pellet stained with anti-BAθ followed by FITC-labelled goat anti-rabbit serum, as previously described (Gyöngyössy & Playfair, 1973). Care was taken to resuspend the pellet by gentle tapping. After indirect immunofluorescent staining, the washed pellet was resuspended in one drop of HBSS, and one drop of mounting medium, a drop was placed on a clean glass slide, covered by a coverslip and sealed with nail varnish. Rosettes were examined under a Leitz Ortholux microscope using a × 54 objective under oil immersion, first with phase contrast illumination, then with incident ultraviolet light and a yellow barrier filter ($\lambda > 480$) to determine fluorescence of the central cell. A minimum of 100 rosettes was examined per sample.

Cytotoxicity assay

Cytotoxic tests using anti-θC3H serum and complement were carried out as previously

described (Gyöngyössy & Playfair, 1973). Viability was assayed by Trypan Blue dye exclusion test.

Gluteraldehyde-fixed SRBC for immunization

Fixed SRBC were prepared by the same method as glutaraldehyde-fixed rosettes.

Haemagglutination

Serum haemagglutinins were assayed in doubling dilutions in microplates with 'V' shaped wells.

RESULTS

Demonstration of T rosette-forming cells

Lymphocytes binding SRBC were first examined under phase contrast illumination. In some cases, the central cell could be clearly discerned, while in others only a 'morula' of

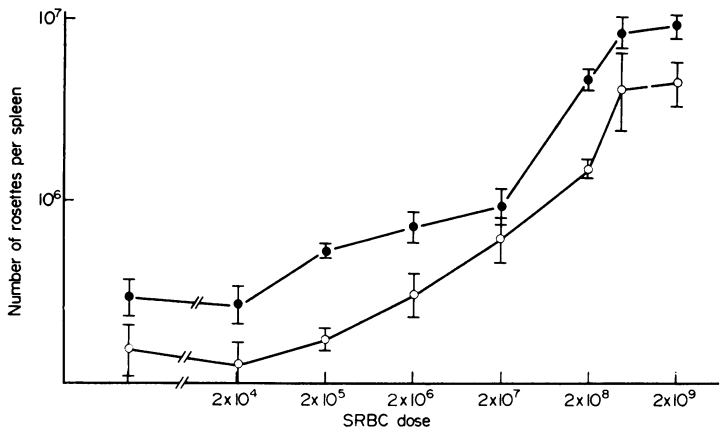

FIG. 1. Total RFC (●) and T-RFC response (○) 5 days after the injection of various numbers of SRBC.

SRBC was visible. When viewed under u.v. RFC positive for BAθ showed a typical ring fluorescence even in those rosettes where the central lymphocyte was completely obscured by SRBC if viewed under phase contrast. RFC showing no fluorescence, or a very pale 'all-over' fluorescence which were also seen in the NRS and conjugate controls were scored as BAθ-negative. In practice there was no difficulty in classifying cells as positive or negative.

Fixation does not alter the numbers or pattern of stained cells; when comparing fixed and non-fixed whole (non-rosetted) spleen cells, no significant difference ($2P<0{\cdot}7$) between the two groups was found. It was also shown by calculation of the absolute numbers of RFC, that essentially no rosettes were lost during fixation.

Dose response

The dose response to SRBC was measured 5 days after immunization. In Fig. 1, RFC are expressed as numbers per spleen. Since i.p. and i.v. injections of SRBC gave closely similar

results, each point on the curve is a pool of a minimum of three i.p. and three i.v. injected mice.

The total number of RFC and the number of RFC labelled with anti-BAθ (TRFC) increased in a parallel fashion, so that the percentage of TRFC remained as a constant 44% of the total.

Background rosettes in non-immunized mice also showed 44% RFC labelled with anti-BAθ.

The smallest dose of SRBC tested, 2×10^4, did not give a significant increase of RFC or TRFC over background levels. A dose of 2×10^5 SRBC was the lowest to elicit a significant increase in the number of rosettes, while 1×10^9 and 2×10^9 both gave a maximal response. Serum haemagglutinins were also measured 5 days after immunization. Both 2×10^4 and 2×10^5 SRBC gave very low haemagglutinating titres (1/4), while mice injected with 1×10^9 SRBC showed a titre of 1/32. Intra-peritoneal injection of 2×10^8 glutaraldehyde-fixed

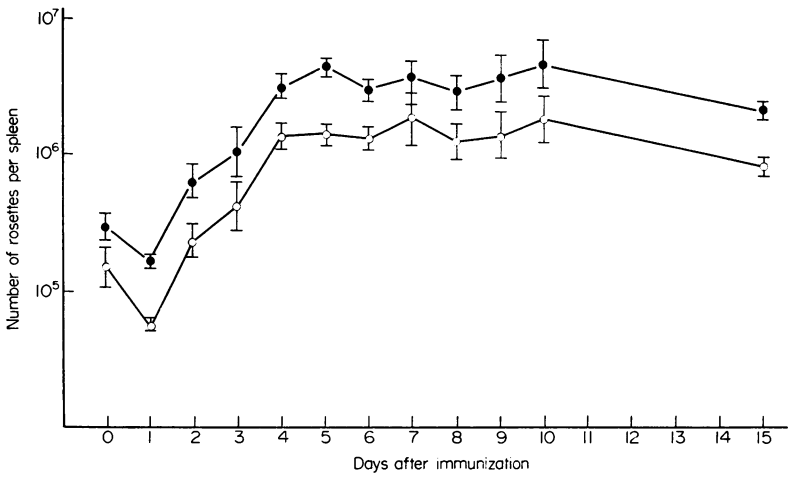

FIG. 2. Time course of the total RFC (●) and T-RFC (○) response after the injection of 2×10^8 SRBC.

SRBC, which are said to 'prime' T cells but not to induce the formation of antibody (Dennert & Tucker, 1972) did not give the same high rosette counts as injection of 2×10^8 fresh SRBC. The rosette response to this dose of fixed SRBC fell in the range expected for 2×10^5 fresh SRBC. In other words, TRFC were not found in the absence of a BRFC or an antibody response.

Time course

The time course of the RFC response after immunization with 2×10^8 SRBC is also expressed as total numbers of rosettes per spleen (Fig. 2).

After a significant drop at day 1 following injection of antigen, both RFC and TRFC reached a maximum at day 5, and remained at about this level until day 15, the last day tested.

Throughout the observed time, 43% of RFC gave fluorescence with anti-BAθ serum.

Specificity of anti-BAθ serum

A standard preparation of spleen cells from (C57Bl × BALB/c)F_1 mice immunized with 2×10^8 SRBC i.p. 5 days previously, was used to establish the specificity of RFC fluorescence (Table 1).

Without pre-treatment, the staining and rosetting properties of the cells fell within the expected range.

After pre-treatment with anti-θC3H serum and complement, using standard cytotoxicity conditions, the numbers of lymphocytes staining with anti-BAθ, and the total numbers of RFC showed a considerable drop. RFC staining with anti-BAθ were completely eliminated. This suggests that the stained RFC were indeed T cells. The fact that some BAθ-positive lymphocytes remained after treatment with anti-θC3H serum and complement can probably be attributed partly to the weakness of our anti-θ serum and partly to the greater sensitivity of the immunofluorescent assay.

Normal AKR serum, and incubation in HBSS alone caused no specific loss of staining, but did cause some decrease in total RFC, probably due to loss of cell surface receptors during the incubation period (Guistino, Hudson & Roitt, in preparation).

A dose of 2×10^8 SRBC given to two athymic nude mice elicited a very small RFC response. Of the observed rosettes, only two were found to be positive for BAθ.

Spleen cells and RFC fluorescing with various antisera

Immunofluorescent staining of spleen cells with anti-BAθ serum shows that around 35% of splenic lymphocytes are T cells, while 44% RFC fluoresce with anti-BAθ serum.

When the anti-BAθ serum is absorbed once with thymocytes or washed brain homogenate, the staining of both lymphocytes and RFC drops to a very low level (2–3%). However, a similar absorption using nude spleen cells did not remove the activity of the antiserum, giving 32% fluorescent lymphocytes and 46% RFC positive for the T marker.

Using anti-mouse light chain serum, 50% of spleen cells and 63% of RFC were shown to carry detectable amounts of light chains on their surface—and were therefore considered to be B cells.

DISCUSSION

From the foregoing results, we conclude that about 44% of both immune and non-immune RFC in mouse spleen are T cells. This agrees fairly well with the results of others (Greaves & Moller, 1970; Wilson & Miller, 1971; Bach, 1973); there is better agreement with respect to the immune than to the background rosette counts, which could be explained purely by strain differences; however, the methods used by other workers, which depend on inhibition of rosette formation, are inevitably less sensitive where small numbers of RFC are concerned —a limitation that the immunofluorescent method does not suffer from.

Specificity of the antiserum

We have already established that the anti-BAθ serum, sutiably absorbed, is a good marker for T cells (Gyöngyössy & Playfair, 1973). However, since RFC, even in immune mice, constitute only about 3% of all cells, we felt it necessary to rule out the possibility that a small number of non-T cells were being stained.

TABLE 1. Distribution of T cells and T-RFC in the spleens of SRBC-injected (C57Bl × BALB/c) F_1 and nude mice. The results of pre-treatment with anti-θ serum are shown

Spleen cells	Pre-treatment	Percentage killed	Percentage stained with anti-BAθ serum	Total number of RFC per spleen	RFC as percentage of total spleen cells	Percentage of RFC stained with anti-BAθ serum	Total number of TRFC per spleen	TRFC as percentage of total spleen cells
2×10^8 SRBC i.p., 5 day immune	—	—	25·3	$9\cdot0\times10^6$	4·5	42·9	$3\cdot9\times10^6$	1·93
	Anti-θ serum $\frac{1}{2}$ + complement	33·2	8·5	$1\cdot25\times10^6$	1·1	0	0	0
	NAKR serum $\frac{1}{2}$ + complement	7·3	26·3	$3\cdot2\times10^6$	1·6	37·0	$1\cdot2\times10^6$	0·39
	HBSS 1 hr at 37°C	0	31·1	$4\cdot5\times10^6$	2·3	44·0	$1\cdot9\times10^6$	0·99
2×10^8 GA-fixed SRBC i.p., 5 day immune	—	—	37·9	$1\cdot1\times10^5$	0·08	25·5	$3\cdot3\times10^4$	0·02
Nu/nu 2×10^8 SRBC i.p., 5 day immune	—	—	5·4	$1\cdot0\times10^5$	0·5	(2/13)*	—	—

Except where otherwise indicated, each point represents a minimum of three separate experiments.

* Only two mice tested.

The specificity of the anti-BAθ serum was first assessed on nude mouse spleen cells, less than 5% of which showed positive staining. This agrees with the data of Raff (1973) who finds a small percentage of θ-positive cells in nude mice. In a second experiment, treatment of normal spleen cells with anti-θC3H serum and complement before rosette formation specifically elimated the BAθ-positive RFC (Table 1). Similarly, a single absorption of the anti-BAθ serum with washed brain homogenate or normal thymocytes removed the ability of the serum to stain RFC, whilst absorption with nude mouse spleen cells did not (Table 2). These controls indicate that the BAθ-positive RFC are indeed T cells.

Fixation of rosettes

Fixation and subsequent immunofluorescent staining of RFC appeated to be the best method for demonstrating T rosettes for several reasons. In the first place, preliminary experiments showed that anti-BAθ serum itself blocks T rosette formation; thus direct or

TABLE 2. Percentage of lymphocytes and of RFC fluorescing with various antisera, in spleen cells from (C57Bl × BALB/c) F_1 mice given 2×10^8 SRBC i.p. 5 days earlier

Antiserum*	Percentage of lymphocytes fluorescing	Percentage of RFC fluorescing
Anti-BAθ 1/8	34·7	44
Anti-BAθ absorbed with thymocytes 1/8	2	3
Anti-BAθ absorbed with mouse brain 1/8	0	3
Anti-BAθ absorbed with nude mouse spleen 1/8	32·0	46
Anti-mouse light chain 1/8	50·0	63

Except where otherwise indicated, each point represents a minimum of three separate experiments.

* Indirect immunofluorescence using FITC-labelled goat-anti-rabbit serum.

indirect staining followed by rosette formation would not show TRFC. By allowing the labelled BAθ antigens to cap at 37°C, new SRBC receptors can be exposed for rosette formation, however the same incubation will also cause a loss of receptors (Guistino *et al.*, in preparation). Staining after rosette formation also entails a loss of rosettes during the necessary washes, unless the rosettes are fixed with glutaraldehyde (Haskill *et al.*, 1972).

Significance of TRFC

We have observed that TRFC remain as a constant fraction—about 44% of spleen RFC. Also, about 60% of RFC appear positive when labelled by anti-light chain serum (Table 2). Although we have not carried out double labelling experiments, our presumption is that most or all of the non-TRFC are probably BRFC.

Low immunizing doses of SRBC or doses of G-SRBC, both of which have been shown to prime T cells but not detectably to raise antibody levels (Dennert & Tucker, 1972) did not

cause any increase in the number of rosettes (Table 1). This failure of both B and TRFC numbers to increase when little or no antibody is being made suggests that rosette formation by T cells might be B cell-mediated (Webb & Cooper, 1973). Since mice have low levels of 'natural' anti-SRBC antibody, it is conceivable that even the background TRFC are of this type. The rapid loss of rosette-forming ability by T cells at 37°C also suggests that this may be so (Guistino *et al.*, in preparation). The implications of the idea that T-rosette formation may be due to antibody not made by the T cell, are reviewed elsewhere (Playfair, 1974).

In this paper, we have described an immunofluorescent method using anti-BAθ to demonstrate that some SRBC-RFC are T cells. This method is clearly applicable to the study of other rosetting systems, such as the Fc and complement receptor rosette tests, and these are reported in the following papers.

ACKNOWLEDGMENTS

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