

CLASSIFICATION OF THYMUS-DERIVED AND MARROW-DERIVED  
LYMPHOCYTES BY DEMONSTRATION OF THEIR  
ANTIGEN-BINDING CHARACTERISTICS\*

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It is now established that both thymus-derived (T)<sup>1</sup> and bone marrow-derived (B) immunocompetent cells bear specific antigen-binding receptors on their surface (1, 2), although there may be differences between receptors on T and B cells (3, 4). It is estimated that B cells bind considerably more antigen than T cells (4) and quantitation of cellular antigen binding might therefore distinguish T and B cells. Rosette formation provides a simple and rapid test for the antigen-binding capability of a lymphoid cell (5), and it seems that for sheep erythrocytes (SRBC) specific antigen-reactive cells (ARC) of both T and B type can bind antigen in this way (3, 10). The present study illustrates the use of rosette formation as a tool for characterizing a heterogeneous population of lymphoid cells with specificity for SRBC.

To assay rosettes,  $1 \times 10^6$  nucleated cells plus  $10 \times 10^6$  SRBC were centrifuged to a pellet in pH 7.4 tris(hydroxymethyl)aminomethane-buffered Eagle's minimal essential medium. The pellets were left for 60 min at 4°C and then gently resuspended by pipetting. Within 10 sec glutaraldehyde was added to achieve an over-all concentration of 0.6%. The rosettes were left for 20 min at 4°C to fix fully, then diluted with distilled water, centrifuged, and resuspended. Suspensions were adjusted to appropriate concentrations, 25- $\mu$ l drops were placed on microscope slides, allowed to dry, rinsed in methanol, and stained with methyl green pyronin. Numbers of rosette-forming cells (RFC) and of SRBC per RFC were counted under oil immersion. In order to obtain the profiles reported a minimum of  $10^5$  nucleated cells were scanned. Frequently, as in the case of bone marrow and normal thymus, this number exceeded  $10^6$  cells. Rosettes binding 25 or more SRBC were all placed in the category of 25, as higher counts were not accurately assessable. Adult thymectomy was performed as previously described (6). Mice were thymectomized at 6 wk of age and assayed 3 wk later. Serum for the anti- $\Theta$  experiments was kindly provided by Dr. M. Greaves; it was AKR anti-C3H derived from ascitic fluid, used at a 1 in 6 dilution, and showed maximum cytotoxicity at 1 in 4. Anti- $\Theta$  inhibition was performed in the usual manner (7); the guinea pig serum employed was absorbed with mouse thymocytes and then had no detectable cytotoxicity for mouse spleen

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<sup>1</sup> Abbreviations used in this paper: ARC, antigen-reactive cells; B, bone marrow; PFC, plaque-forming cells; RFC, rosette-forming cells; SRBC, sheep erythrocytes; T, thymus.

cells; rosettes were prepared after washing to remove guinea pig serum. Low dose immunization consisted of  $1 \times 10^8$  SRBC injected into the vein 5 days before assay; high dose immunization was similar except that  $1.5 \times 10^8$  SRBC were given. "Educated" thymus cells

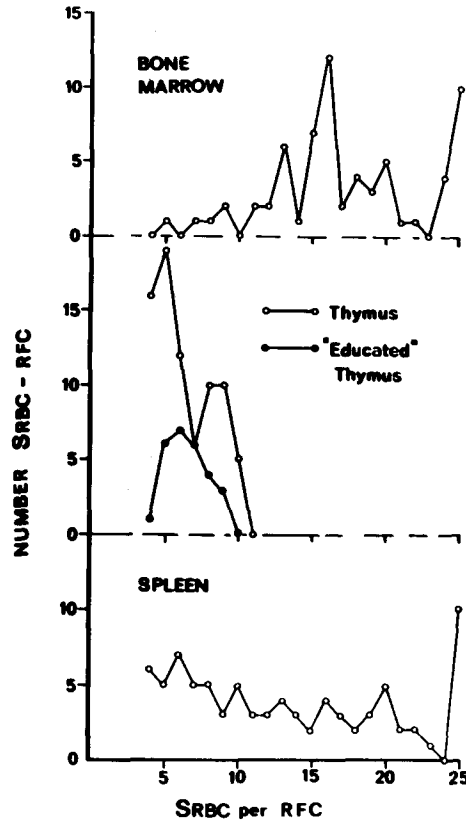


FIG. 1

FIG. 1. Quantitation of the number of sheep erythrocytes bound per lymphoid rosette-forming cell (RFC). Distribution curves were determined on normal marrow and thymus and on thymus cells educated with SRBC in irradiated recipients. All animals were 8-wk old CBA mice.

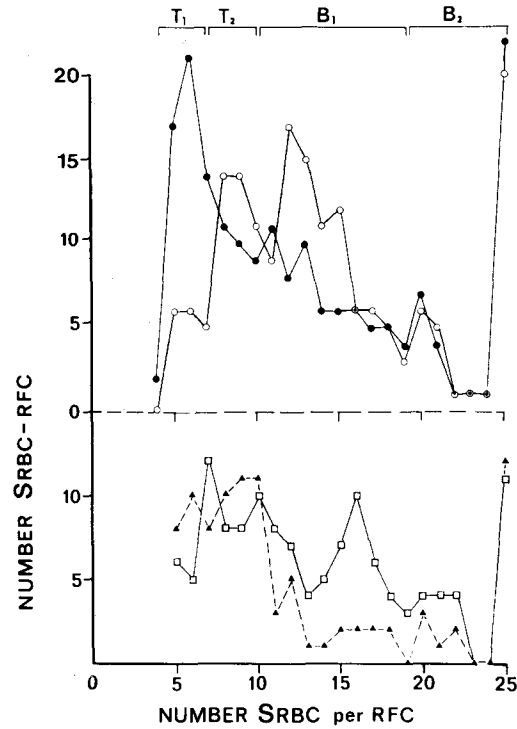


FIG. 2

FIG. 2. Influence of thymectomy and anti- $\theta$  sera on the sheep erythrocyte binding distribution curves in low dose immune spleens. Both CBA and DBA/2 strains of mice were used for these experiments, with no significant differences. GPS, spleen cells treated with guinea pig serum alone; anti- $\theta$  + GPS, spleen cells treated with anti- $\theta$  serum followed by guinea pig serum. ●—●, sham thymectomized; ○—○, thymectomized; □—□, GPS; ▲—▲, anti- $\theta$  + GPS.

were obtained in the spleen by intravenous injection of  $5 \times 10^6$  thymocytes with  $2 \times 10^8$  SRBC into 900 R irradiated mice; the SRBC dose was repeated 4 days later and spleens were harvested after a further 3 days.

The uppermost portion of Fig. 1 shows the antigen-binding distribution of

RFC in bone marrow of normal adult CBA mice. At least two features are apparent: almost all the RFC present bind 10 or more SRBC, and the distribution suggests the existence of several distinct peaks. The midportion of Fig. 1 shows the distribution found with normal and educated thymic lymphocytes. These RFC were morphologically only of small and medium lymphocyte types. The RFC population in normal thymus is seen to lack lymphocytes which bind more than 10 SRBC. The T RFC population is also seen to fall into two main groups: one with an SRBC binding capacity of around 5, and the other with a binding capacity of around 9. Educated thymocytes are shown as still displaying the narrow low antigen-binding range found with normal thymic lymphocytes.

The lowermost portion of Fig. 1 shows the antigen-binding distribution on RFC in normal spleen. By comparison with the previous data there is a strong suggestion that the splenic distribution results from a composite of T and B populations.

Experiments were carried out to establish further that low antigen-binding RFC in spleen are indeed T RFC. These experiments were performed with spleen cells of mice receiving low dose immunization with SRBC, which simplified assays by increasing RFC numbers without fundamentally altering the normal distribution pattern.

First, we looked at the early effects of adult thymectomy on splenic RFC. This procedure is known to deplete swiftly a subpopulation of peripheral T cells as assayed by a variety of approaches including assessment of RFC (8, 9). In the upper part of Fig. 2 may be seen the changes in splenic RFC distribution present 3 wk after adult thymectomy. The data is an aggregate of two experiments. RFC in sham-operated controls resembled those in similarly immunized normal mice (see Table I.) In comparison, adult-thymectomized mice have a marked depletion of T RFC ( $\leq 10$  SRBC/RFC). This depletion appears to have spared the high-binding T region. Concomitantly an increase of low-binding B RFC has occurred, yielding the unchanged RFC totals which have also been noted by others following thymectomy (8, 10) (Fig. 2, Table I).

Second, the effects of preceding rosette formation with exposure to either fresh guinea pig serum or anti- $\Theta$  serum followed by guinea pig serum were compared. The results appear in the lower part of Fig. 2. Guinea pig serum by itself had little effect on RFC. The additional introduction of anti- $\Theta$  serum resulted in a 25% reduction in total numbers of RFC. However, the antigen-binding profile reveals a surprising change. The loss of RFC has occurred exclusively in the B region. In the T region there has, if anything, been an increase of RFC numbers. To clarify this anomaly we first subjected thymus cells to the same treatment with anti- $\Theta$  and guinea pig serum; this completely abolished rosette formation by the thymic lymphocytes. Next, bone marrow cells were identically tested. Here there was little decrease in the total number of RFC after anti- $\Theta$  and guinea pig serum but a profound change in the RFC distribution profile.

The results of this experiment are shown in Table I, which lists the distribution of RFC. These results show that no more than 8% of RFC in normal bone marrow display T characteristics, but after anti- $\Theta$  treatment, T-type RFC have risen to 50%. This occurs at the expense of those B-type RFC of intermediate antigen-binding capacity which we have designated as belonging to the  $B_1$  region (Table I). The noncongenic anti- $\Theta$  used is thus seen to have two effects: it removes T RFC and it reduces the antigen-binding performance of  $B_1$  RFC to the point where they shift into the T region, but not to the point of their elimination as RFC.

TABLE I

*A Summary of the Values of  $T_1$ ,  $T_2$ ,  $B_1$ , and  $B_2$  RFC from a Variety of Sources under Immune and Normal Situations*

	Total rosette-forming cells				Total RFC scored*
	$T_1$ 4-7 SRBC	$T_2$ 7-10 SRBC	$B_1$ 10-18 SRBC	$B_2$ 18 $\rightarrow$ SRBC	
	%	%	%	%	
Thymus	64	36	0	0	78 (6.1)
Educated thymus	63	37	0	0	27 (28)
Bone marrow	2	6	52	40	117 (6.1)
Anti- $\Theta$ bone marrow	43	7	4	46	40 (4.9)
Spleen	33	11	28	28	83 (10.0)
HDI spleen	5	2	33	60	320 (306)
LDI spleen	24	15	39	22	126 (32)
Anti- $\Theta$ LDI spleen	37	25	18	20	94 (24)
STx LDI spleen	25	18	32	25	183 (50)
ATx LDI spleen	8	21	48	23	175 (42)

HDI = high dose SRBC immunized; LDI = low dose SRBC immunized; STx = adult shamthymectomy; ATx = adult thymectomy. Because the defined regions  $T_1$ ,  $T_2$ ,  $B_1$ , and  $B_2$  overlap, numbers of rosettes appearing in the overlapping value for sheep erythrocytes per rosette have been equally divided between the two neighboring regions.

\* Total RFC assessed for the percentage calculations, with RFC/ $10^6$  cells in parentheses. Values for each group represent the sum of from two to four separate experiments, except for educated thymus which is derived from a single experiment.

The effects of anti- $\Theta$  on RFC in both spleen and marrow (Fig. 2, Table I) show a further distinction between  $B_1$  RFC and  $B_2$  RFC. Whereas anti- $\Theta$  considerably reduced the antigen-binding performance of  $B_1$  RFC there was little, if any, effect on the multilayered  $B_2$  RFC.

Multilayered rosettes clearly suggest themselves as the products of receptor-secreting cells. In corroboration, we found an association between such RFC and plaque-forming cells (PFC) in two separate types of experiment. In the first type of experiment rosette-containing spleen cell suspensions from low dose immunized mice were fractionated by velocity separation under unit gravity (13). Individual fractions were then assayed for both RFC and PFC; PFC and

multilayered RFC were found to concentrate in identical fractions. In the second type of experiment the splenic RFC distribution was determined in high dose immunized mice, in which there were numerous PFC; at this time a distinct shift of RFC to the multilayered ( $B_2$ ) region was also present (Table I).

T RFC were seen to segregate under two peaks, one at a binding capacity of around 5 SRBC, and the other at around 9 SRBC. Adult thymectomy depleted the low-binding T RFC while sparing the high-binding ones. In contrast we have recently found that it is the high-binding T RFC which are preferentially reduced by *in vivo* administration of anti-lymphocyte globulin. We designate these low- and high-binding T RFC as  $T_1$  and  $T_2$ , respectively, and note that their response to adult thymectomy is similar to that of the  $T_1$  and  $T_2$  populations of Raff and Cantor (9).

Noncongenic anti- $\Theta$  serum abolished T RFC but also, as suggested previously (11, 12), affected B RFC; more particularly it reduced the antigen-binding performance of  $B_1$  RFC while having little, if any, effect on the multilayered  $B_2$  RFC. The effect on  $B_1$  RFC might well be due to non- $\Theta$  specificities and if so would not be demonstrable if congenic anti- $\Theta$  were used.  $B_2$  RFC are probably actively releasing antibody. The anti- $\Theta$  results suggest that  $B_2$  RFC might also differ antigenically from  $B_1$  RFC. The  $B_1$  RFC group is seen as one in which antibody-forming cell precursors are found. This view obtains some support from our finding that the sedimentation velocity of  $B_1$  RFC (of 8–12 mm/hr) fits with the known post-rosette formation sedimentation velocity of antibody precursor cells for SRBC in normal mice (13).  $B_1$  RFC should prove PC 1 negative in the system of Takahashi et al. (11) and this would accord with failures of PC antisera to depress immunocompetence.

In our experiments, ARC of the T type were clearly distinguishable from ARC of the B type. With glutaraldehyde fixation of freshly formed rosettes, dissociation was avoided; such dissociation probably underlies some of the controversy concerning T RFC (11, 12, 14). In the absence of fixation we have found T RFC to be extremely unstable; even with the most careful handling they display, after resuspension, a half-life of just a few minutes at 4°C. Staining rosettes increased the accuracy with which true rosettes could be identified microscopically. The experiments reported have been limited to the model of reactivity to SRBC in mice. However, the same experimental protocol is in principle equally applicable to studies in other species and of other antigens.

#### SUMMARY

Antigen-binding cells of T and B origin can readily be determined by quantitating the number of sheep erythrocytes per rosette after glutaraldehyde fixation. The  $T_1$  and  $T_2$  populations have low antigen-binding properties and are very unstable without fixation. The  $B_1$  and  $B_2$  populations are stable and correlate with precursor and secretory cells. Fixation of rosettes permits a sensitive test for studying differentiation of T and B cells.

*Note Added in Proof.*—Recent evidence indicates that the instability of T rosettes may be related to cap formation of the surface receptors. We have been able to stabilize T rosettes by the addition of sodium azide and by concanavalin A, both of which are agents known to inhibit cap formation.

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