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# Tissue Localization of Lymphocytes Bearing a Membrane Receptor for Antigen–Antibody–Complement Complexes

**Peter Dukor, Celso Bianco, and Victor Nussenzweig**

NEW YORK UNIVERSITY SCHOOL OF MEDICINE, DEPARTMENT OF PATHOLOGY, NEW YORK, N. Y. 10016

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**Abstract.** To determine the tissue localization of lymphocytes provisionally termed "complement-receptor lymphocytes," which are characterized by having a membrane receptor for antigen–antibody–complement complexes, we investigated the adherence of sensitized and nonsensitized sheep red cells to frozen sections of mouse lymphoid organs. Nonsensitized erythrocytes became bound exclusively to sinus-lining cells of spleen and lymph nodes, whereas erythrocytes sensitized with antibody and complement adhered to lymphocytes in the follicular areas and the marginal zone of the spleen and in the true cortex of lymph nodes. However, the doubly sensitized erythrocytes failed to bind to the "thymus-dependent" areas of peripheral lymphoid organs or to the thymus itself. We suggest that complement-receptor lymphocytes are of extrathymic origin and that they contribute substantially to follicular antigen localization, which appears to be complement-dependent.

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Certain immune responses seem to depend on the cooperation of thymus-derived and non-thymus-derived lymphocytes.[1–4] In studies of the interaction between these cell lines it may be desirable to make use of morphologic or functional markers unique to either one of the two subpopulations.

In lymphocyte suspensions from different mammalian species, including man, a proportion of cells were regularly found[5,6] to react with sheep erythrocytes (E) which had been sensitized with antibody (A) and complement (C). The adherence of these sensitized erythrocytes (EAC) to such lymphocytes—provisionally termed "complement-receptor lymphocytes" (CRL)—results in the formation of characteristic rosettes (Fig. 1). The complement component required for this interaction was tentatively identified as C3.[7] While similar rosettes were also formed with other leukocytes,[5,6,8] adherence of EAC to macrophages and polymorphs was shown to require divalent cations and to be blocked by EDTA.[5,6,9] In contrast, EAC-reactivity of CRL could not be abolished by EDTA.

As compared with lymphocytes that lack the ability to bind EAC (non-CRL), CRL from mouse lymphoid tissues showed an increased adherence to nylon wool and accumulated in the lighter bands of an albumin density gradient.[7] Moreover, most of CRL were found to bear immunoglobulin determinants on their surface.[7]

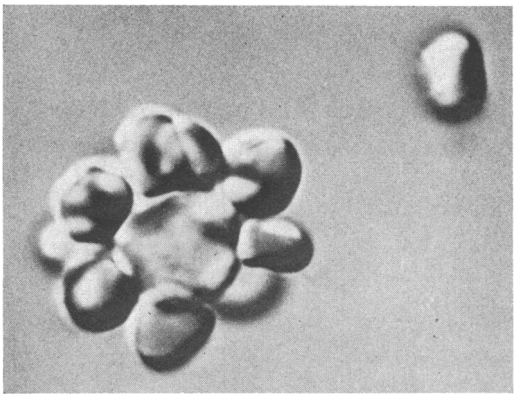

FIG. 1. "Rosette" with EAC on a mouse lymphocyte (differential interference, ×2500).

Cell suspensions from different lymphoid organs contained strikingly different proportions of EAC-reactive cells. CRL were conspicuously absent from thymus, but present in low numbers in bone marrow (5–8%), lymph nodes (10–25%), and thoracic duct (10–20%); there were more (20–40%) in spleen.[7] The tissue distribution of CRL is thus almost exactly the reverse of the one described for lymphocytes carrying the $\theta$-isoantigen,[10,11] which appear to be thymus-derived[12,13]

Hence the question arises whether CRL are non-thymus-derived lymphocytes, originating perhaps directly from bone marrow. If so, CRL would be expected to show a characteristic localization within organized lymphoid tissues: to occupy the "thymus-independent" follicular areas of peripheral lymphoid organs and to be absent from the "thymus-dependent"[14] regions, such as the periarteriolar lymphocyte sheaths of the spleen and the paracortical areas of the lymph nodes. The results presented here are in agreement with this prediction.

**Methods.** To study the tissue localization of CRL, we examined the pattern of adherence of sensitized and nonsensitized sheep erythrocytes to frozen sections of mouse lymphoid tissue. Erythrocytes (E), erythrocytes sensitized to antibody (EA), and EAC were prepared as described before.[7] Briefly, equal volumes of E (5% washed suspension in tissue culture medium 199) and A (rabbit anti-E boiled stroma, 1:500 in saline) were incubated at 37°C for 30 min. The resulting EA were washed, resuspended (5%) in Veronal-buffered saline pH 7.4 (VBS), and incubated with an equal volume of C (CF1 mouse serum, 1:10 in VBS) for a further 30 min at 37°C. Hemolysis was negligible. After three washings in VBS, EAC were resuspended (0.5%) in medium RPMI 1640 or medium 199 for final use. Suspensions of E and EA were prepared in a similar fashion. In order to prevent adherence of erythrocytes to macrophages or polymorphs,[5,6,9] one part of 0.1 M $Na_2H_2$ EDTA (pH 7.6) was added to nine parts of erythrocyte suspension in most experiments.

Lymphoid organs were obtained from 7- to 9-week old male (C57B1/6 × DBA/2)$F_1$ mice. Thymus, spleen, Peyer's patches, and mesenteric, inguinal, and axillary lymph nodes were embedded in gelatine blocks and rapidly frozen in petroleum ether at −72°C. Cryostat sections (6–8 $\mu$m) were mounted on slides, thawed in cold phosphate-buffered saline (PBS), overlayered with E, EA, or EAC, and incubated in a moist chamber at 37°C or at room temperature for 30 min. (Adherence of erythrocytes to tissue sections was greatly diminished by incubation at 4°C, though incubation at room temperature had no distinct disadvantage. Thawing of the frozen sections in alcohol, formaldehyde, or acetone prevented shrinkage and disruption in some of the sections but greatly interfered with the subsequent binding of erythrocytes to both sinuses and follicles.) The

FIGS. 2–5. Adherence of E and EAC to cryostat sections of mouse lymphoid organs. The overlayered red cells (appearing as dark dots) are not in the same plane with the tissue section.

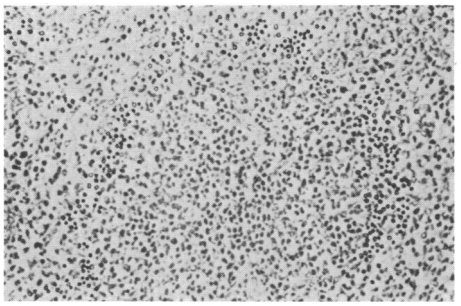

FIG. 2. Nonsensitized erythrocytes (E) adhering to a marginal sinus of the spleen (phase, ×166).

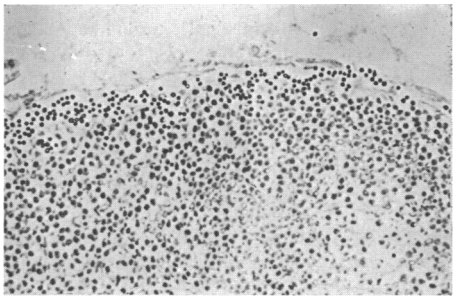

FIG. 3. Nonsensitized erythrocytes (E) adhering to the subcapsular sinus of an inguinal lymph node (phase, ×166).

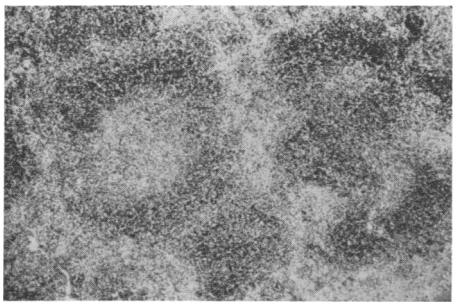

FIG. 4*a*.

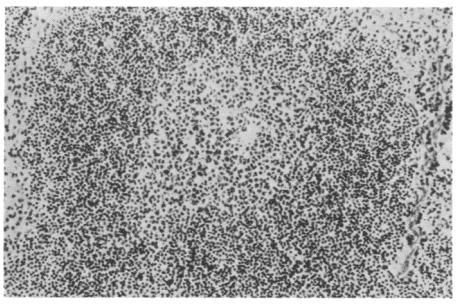

FIG. 4*b*.

FIG. 4. EAC adhering to follicular areas of the spleen (*a* ×50; *b* phase, ×104); note absence of EAC from periarteriolar lymphocyte sheaths.

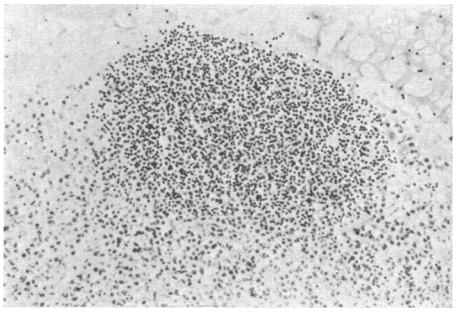

FIG. 5. EAC adhering to cortical areas of the lymph node (phase, ×105); there are no red cells bound to the adjacent paracortical region.

slides were then placed in racks, and repeatedly immersed in PBS until no more erythrocytes were floating off. The washed slides were again placed horizontally and gently flooded with cold glutaraldehyde (3% in phosphate buffer, pH 7.2), fixed for 1 hr, washed in PBS, and post-fixed in 2% $OsO_4$ for 30 min. Finally, sections were stained with hematoxylin and eosin and examined under the light microscope.

TABLE 1. *Adherence of erythrocytes to frozen sections of mouse lymphoid tissues.**

| Type of red cell | Spleen | | | Lymph nodes | | | Thymus |
|---|---|---|---|---|---|---|---|
| | Marginal sinus | Marginal zone and follicles | Peri-arteriolar lymphocyte sheaths | Marginal and medullary sinuses | Cortex | Para-cortical area | |
| E (sheep) | 10/11 | 0/11 | 0/11 | 6/7 | 0/7 | 0/7 | 0/14 |
| E (mouse) | 4/4 | 0/4 | 0/4 | — | — | — | — |
| E + EDTA | 8/8 | 0/8 | 0/8 | 7/8 | 0/8 | 0/8 | — |
| EA | 11/13 | 0/13 | 0/13 | 8/10 | 0/10 | 0/10 | 0/4 |
| EAC | (−)† | 11/11 | 1/11 | 9/9 | 9/9 | 0/9 | 0/4 |
| EAC + EDTA | (−)† | 15/15 | 0/15 | 7/7 | 7/7 | 0/7 | 0/5 |

* The figures denote numbers of positive sections over numbers of sections examined.
† Marginal sinuses were masked by the massive adherence of EAC to adjacent areas.

**Results.** The results are summarized in Table 1. Nonsensitized erythrocytes (E) were found to adhere only to the marginal sinuses of the *spleen* (Fig. 2) and to the subcapsular and medullary sinuses of *lymph nodes* (Fig. 3). In order to ascertain the exact localization of E on spleen sections, titanium dioxide was injected intravenously into a group of mice 15–30 min before they were killed. Indeed, in such preparations the distribution of E coincided with that of titanium particles which had been engulfed by the sinus-lining macrophages. No erythrocytes were ever found to adhere to the *thymus*.

So far, these results confirm similar observations made in rats by Stejskal and Fitch.[15] However, in contrast to what was reported by these authors, sinus localization in mice was not inhibited by antibody. Tissue adherence of EA followed exactly the same pattern as that observed with E. Moreover, sinus localization of both E and EA was not decreased by the presence of EDTA or of 30% normal mouse serum. Also, homologous erythrocytes from $CF_1$ mice showed the same type of tissue adherence. The underlying cause of this phenomenon is not clear.

An altogether different pattern of localization was observed with EAC. In the *spleen*, these adhered to the follicular areas of the white pulp and to the surrounding marginal zone, but spared both the hemopoietic regions of the red pulp and the periarteriolar lymphocyte sheaths (Fig. 4). Similarly, in *lymph nodes* EAC became bound exclusively to the cortical region and the sinuses, but were conspicuously absent from the paracortical area (Fig. 5). Again, on sections of *Peyer's patches*, EAC localized over the central follicles, but did not adhere to the more loosely packed lymphocytes of the interfollicular zone. Localization of EAC over germinal centers of spleen, lymph nodes, or Peyer's patches was variable. In general, large, active centers containing abundant blast cells were always covered with sensitized erythrocytes, whereas small, possibly inactive centers failed to fix EAC. No binding of EAC was ever observed with sections of *thymus*.

It appears, therefore, that follicular localization of sensitized sheep erythrocytes on tissue sections is mediated by complement. Moreover, tissue adherence of EAC is restricted to the "thymus-independent" regions of peripheral lymphoid organs.

Although the predominant cells in the areas that bind EAC are lymphocytes,

the possibility must be entertained that sensitized erythrocytes interact with other components prevalent in follicles. Indeed, antibody-mediated follicular localization of antigen *in vivo*[16,17] has been mainly attributed to trapping by dendritic reticular cells and their lacy processes.[18-20] Nevertheless, the following reasons strongly support the contention that most EAC adhering to frozen sections were actually bound to lymphocytes: (*a*) the extent of erythrocyte adherence to frozen sections from different lymphoid organs parallels the proportions of CRL found in lymphocyte suspensions of the same tissues, and (*b*) contacts between sedimented EAC and lymphocytes in spleen slices were observed under the electron microscope.

In the last-mentioned experiments, freshly excised mouse spleens were cut with a razor blade into thin slices, pinned onto waxed Petri dishes, repeatedly washed with medium 199, and overlayered with E or EAC for 30 min in the presence of EDTA. Slices were then extensively rinsed with medium 199, flooded with cold buffered glutaraldehyde (4%), fixed for 2 hr at 4°C, postfixed with buffered $OsO_4$ (2%) for 30 min, and rinsed again. Subsequently the Petri dishes were filled with 4% buffered agar (40°C). After solidification, agar blocks containing embedded tissue slices were cut vertically to the erythrocyte-exposed spleen surface, dehydrated, embedded in Epon 812, and sectioned. Grids were stained with uranyl acetate and lead citrate, and examined with a Zeiss model 9 electron microscope.

Very few nonsensitized erythrocytes could ever be detected on the surface of the spleen slices. In contrast, a relatively large number of EAC was found to bind predominantly to small and medium lymphocytes (Fig. 6). In some in-

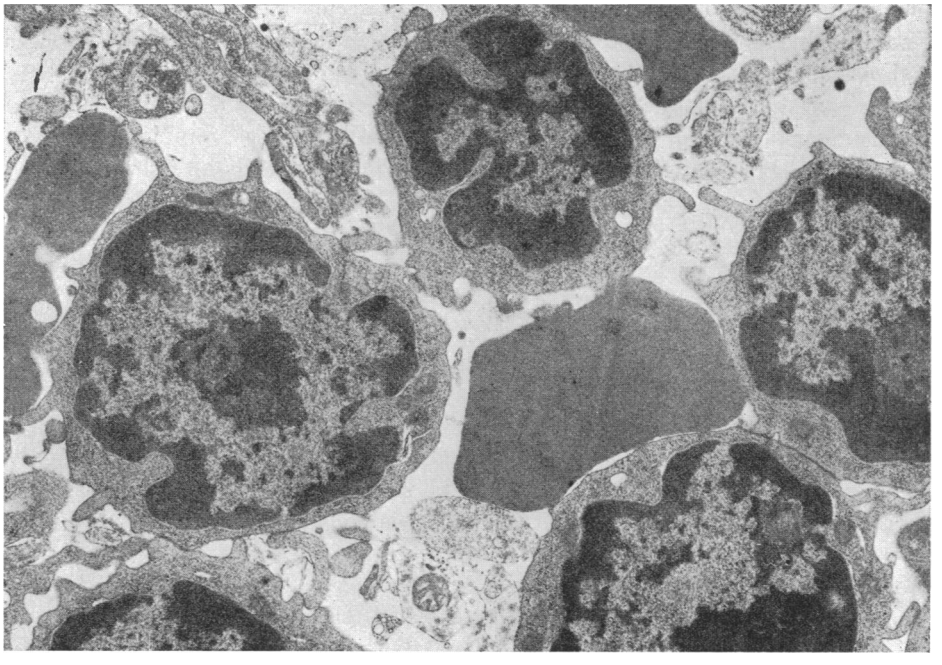

FIG. 6. Electron micrograph of EAC bound to lymphocytes in a section of mouse spleen (×4,760).

stances, single erythrocytes were seen to combine with several lymphocytes. Conversely, single lymphocytes were sometimes surrounded by tightly bound erythrocytes. The actual link between erythrocytes and lymphocytes was frequently limited to a very small region where the plasma membranes of both partners could no longer be resolved and did not apparently involve reticular processes of other elements. (The latter observation should be considered with a note of caution, since the contacts between EAC and CRL were established at 25°C and in the presence of EDTA, which may have interfered with the integrity of the plasma membranes.) Moreover, occasional EAC were also bound to reticular cells. Thus, the electron microscopic findings demonstrate clearly that in the presence of EDTA, most of the actual contacts between EAC and spleen slices were mediated by a complement-dependent membrane receptor of lymphocytes.

**Discussion.** The apparent avidity of follicular lymphocytes for EAC *in vitro* raises important questions as to the nature of follicular localization of antigen *in vivo*. From studies employing electron microscopic autoradiographs it is clear that antigen can bind to the dendritic processes of reticular cells.[18-20] Nevertheless, it was reported that some of the antigen was also found between densely packed lymphocytes where no separating process could be resolved.[19,20] Follicular antigen localization *in vivo* has been shown to be antibody-dependent[20-25] and to involve the participation of the F(c) fragment[20] which is required for complement fixation. From the combined evidence of the *in vivo* and *in vitro* studies, it therefore appears likely that (*a*) specialized lymphocytes participate in follicular antigen trapping and (*b*) antigen localization in follicles is mediated by membrane receptors for complement, common to both lymphoid and reticular cells.

Moreover, the demonstration in the very same lymphoid compartments of a complement-dependent interaction of antigen–antibody complexes (EAC) with lymphocytes on the one hand and with reticular cells on the other may suggest a basic functional relationship between these two cell types. Thus it seems possible that CRL could be specifically bound by antigen–antibody–complement complexes previously deposited on the surface of dendritic reticular cells. Alternatively, CRL carrying such complexes on their membranes may be selectively trapped by the reticular framework of the follicles. In either case, complement would provide the adhesive responsible for the dense follicular accumulations of specialized lymphocytes in peripheral lymphoid tissues.

The data reported in the present paper provide also some clues as to the origin of CRL. The conspicuous absence of such cells from the thymus and from the "thymus-dependent areas" of spleen, lymph nodes, and Peyer's patches strongly suggests an extrathymic origin of CRL. This conclusion is further supported by recent evidence from this laboratory indicating a relative increase in the proportion of CRL in suspensions of lymph node cells from neonatally thymectomized mice and a selective persistence of CRL in tissue sections from such animals.[26] It is probable, therefore, that CRL are directly derived from bone marrow. The membrane receptor for antigen–antibody–complement complexes may represent a useful tool for the identification and separation of these cells.

The actual proof for the extrathymic (marrow?) origin of CRL can be furnished only by cell-transfer experiments. Such studies are in progress.

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Abbreviations: E, sheep erythrocytes; A, antibody; C, complement; EAC, erythrocytes sensitized to both antibody and complement; CRL, complement-receptor lymphocytes.

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