

SCANNING ELECTRON MICROSCOPY OF HUMAN LYMPHOCYTE-SHEEP ERYTHROCYTE ROSETTES*

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Bone marrow or bursal-derived (B) cells and thymus-processed (T) cells can be distinguished by their surface receptors and membrane properties (1-5). Human T lymphocytes form spontaneous rosettes with sheep erythrocytes (E-R)¹ (6, 7) whereas some B cells bear a complement receptor which enables them to form rosettes with erythrocytes coated with antibody and complement (EAC-R) (8). However, despite these functional differences B and T lymphocytes have not been distinguished morphologically by either standard light or electron microscopic techniques (9, 10) even when rosetting and nonrosetting cells are compared (7, 11).

Recently, scanning electron microscopy (SEM) has been applied to the study of human B and T lymphocytes and a spectrum of lymphocyte surface morphology was described (12, 13). In many instances it was possible to distinguish between the smoother surfaced T cells and the more villous B cells; however, a proportion of cells with an intermediate-type of surface morphology could not be accurately classified by SEM.

The purposes of the present study were to: (a) compare E-rosettes and EAC-

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¹Abbreviations used in this paper: EAC, sheep erythrocytes coated with antibody and complement; E-R, spontaneous sheep erythrocyte rosettes; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; PBL, peripheral blood lymphocytes; R, rosettes; SEM, scanning electron microscopy.

rosettes by SEM, (b) establish the surface architecture of the rosetting B and T lymphocytes, (c) describe the nature of the contact between the different rosetting lymphocytes and sheep erythrocytes, and (d) document whether the generally smooth surface of the T cell altered during the process of rosette formation.

Materials and Methods

The following samples were examined by SEM: different suspensions of sheep erythrocytes (SRBC); peripheral blood lymphocytes (PBL) from normal individuals, thymic cells from two children; the MOLT-4 cultured T-cell line and the 8866 cultured B-cell line; EAC-rosettes with PBL and cultured B-cells and E-R with PBL, human thymocytes and cultured T cells.

PBL and Thymic Cells.—The Ficoll-Hypaque density gradient method (14) was used to separate monocytes and lymphocytes from other leukocytes in normal blood samples. The number of contaminating granulocytes still present in these samples was usually less than 6%. The cells were washed three times and resuspended in Hanks' Balanced Salt Solution (HBSS). Fresh human thymus was obtained from two young patients with Fallot's tetralogy who were undergoing cardiac surgery. The tissue was minced, passed through a wire mesh, and the cells were processed as for PBL.

Cultured Cells.—Suspension cultures of the MOLT-4 cell line (15) (generously provided by Dr. J. Minowada, Roswell Park Memorial Institute, Buffalo, N. Y.) were maintained in RPMI-1640 medium with 20% fetal calf serum (FCS). Nine different samples of the MOLT-4 cells were examined by SEM in order to study the effect on surface architecture of the following factors: concentration of FCS (10% or 20%); frequency of changing the culture medium (1, 2, or 3 days); exposure to 4°C temperature; addition of AB serum and phase of *in vitro* growth (logarithmic or stationary phase). Four different samples of the 8866 cell line (B cells) (16) (a gift of Dr. Gary Hoffman, The Rockefeller University, New York) maintained in RPMI-1640 with 20% FCS were also studied under different *in vitro* conditions. Cell suspensions were centrifuged twice at 1,000 rpm for 5 min and the pellet resuspended in fresh culture medium without fetal calf serum.

SRBC.—SRBC were obtained under sterile conditions in Alsevers solution, or heparin, from a number of sheep maintained at The Rockefeller University animal quarters. The cells were generally stored at 4°C for up to 14 days and washed two or three times in HBSS before use. In view of the changes in SRBC surface architecture seen during rosette formation, a number of freshly withdrawn SRBC samples were examined in order to assess the effects of storage, of cold, washing in HBSS, and rosetting procedures on the surface of SRBC.

Preparation of E-R.—Rosettes were prepared according to the method described earlier (7). 0.1 ml of a SRBC suspension in HBSS, 0.1 ml lymphocyte suspension (5×10^6 cells/ml) and 0.02 ml of heat-inactivated pooled human AB serum (Grand Island Biological Co., Grand Island, N. Y.) which had previously been absorbed with an equal volume of packed SRBC or HBSS, were mixed thoroughly and incubated at 37°C for 5 min. The mixture was then centrifuged at 50 g for 5 min at room temperature and incubated at 4°C for 60 min. A minimum of 200 lymphocytes were counted in a haemocytometer and rosettes were defined by the adherence of any number of SRBC. When rosettes were separated from nonrosetting cells the following procedure was used: 0.4 ml of SRBC-R containing suspension was layered on 1.5 ml of the Ficoll-Hypaque mixture and centrifuged at room temperature for 20 min at 400 g. In addition, E-R were prepared according to the method described by Minowada et al. (15). Heparinized SRBC were mixed with the lymphocyte suspension within half an hour of their withdrawal from the sheep, washed three times with phosphate-buffered saline and allowed to stand for 2 h at room temperature. The number of rosette-forming cells was determined in the usual manner.

Preparation of Sheep Erythrocyte Antibody-Complement Rosettes (EAC-R).—To preparations containing 6×10^6 lymphocytes in medium TC-199 were added 2×10^8 sheep erythrocytes coated with varying dilutions of Forssman antibody and subhemolytic amounts of complement (EAC). The cells were incubated at 37°C for 2 h. The number of rosettes (a lymphocyte surrounded by at least three erythrocytes was considered positive) per 200 lymphocytes was counted.

Preparation of Specimens for SEM.—Cells were collected and harvested by aspiration on silver membranes (Selas Flotronic, Spring House, Pa.) of 0.45 μm porosity, as described in earlier studies (17). Approximately $6-8 \times 10^6$ cells were required to give a monolayer-like distribution of cells on a 25-mm diameter silver membrane. The silver membranes layered with cells were fixed initially at room temperature and then for at least a day at 4°C, with 1% glutaraldehyde (pH 7.3, 310–320 mOsm), rinsed twice with buffer, dehydrated in a graded series of alcohol, and then in amyl acetate or Freon 113. The soaking specimen was quickly transferred to a previously cooled high pressure chamber (Cepede-Seven critical point apparatus, courtesy of Dr. V. Bystricky, New York) and critical point dried in carbon dioxide or Freon 13 using the method described by Anderson (18).

Portions of the membrane were attached to stubs using double-sided sticky tape and coated with a thin layer of carbon and gold on a rotatory stage (DV-502 Denton vacuum apparatus, Denton Vacuum Inc., Cherry Hill, N. J.) at an angle of 15°. Using standardized conditions the gold deposition averaged 250 Å as calculated by nomograms and the carbon film 120 Å as checked by direct densitometry to standards measured by a crystal quartz thin film monitor. The specimens were then stored under vacuum until examination.

Examination of Specimens by SEM.—A Cambridge S4 scanning electron microscope (Kent Cambridge Scientific Co., Inc., Morton Grove, Ill.) was used at an accelerating voltage of 20 kV, and with 100 or 200 μm diameter illuminating apertures. Micrographs were recorded on Polaroid type 55 P/N films at direct magnification from 1,000–14,000 \times . Generally, a large number of cells were first scanned on the SEM screen in order to evaluate cell size and surface morphology of a large population of cells before recording micrographs. A few random low magnification micrographs were recorded from each specimen for initial counting purposes. For more detailed observation of surface architecture micrographs were taken at direct magnification between 5,000 and 14,000 \times .

RESULTS

PBL Before Rosetting.—As in other studies (12) lymphocytes were classified on the basis of their surface architecture and relative numbers of microvilli. From 5–30% of PBL had many microvilli (average cell diameter of 5.4 μm) covering their surface, while 55–90% of PBL (average cell diameter 4.5 μm) had smoother surfaces, most having no or only a small number of microvilli. The remaining number of cells, up to 28% in some samples, had an intermediate type of surface morphology with a moderate number of microvilli, and were difficult to classify accurately.

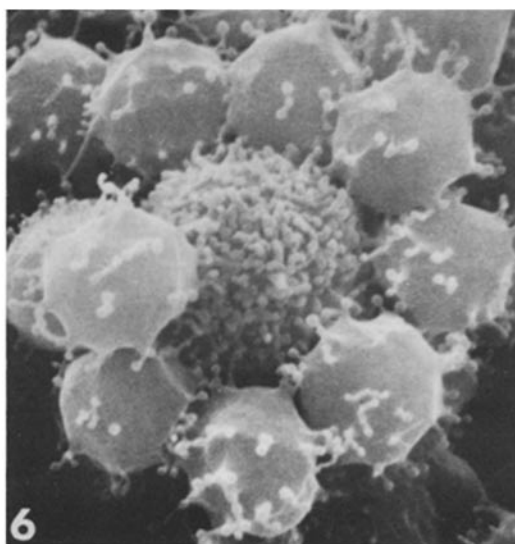
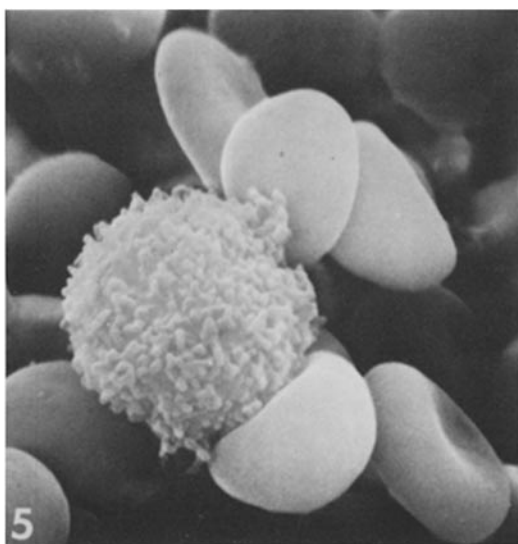
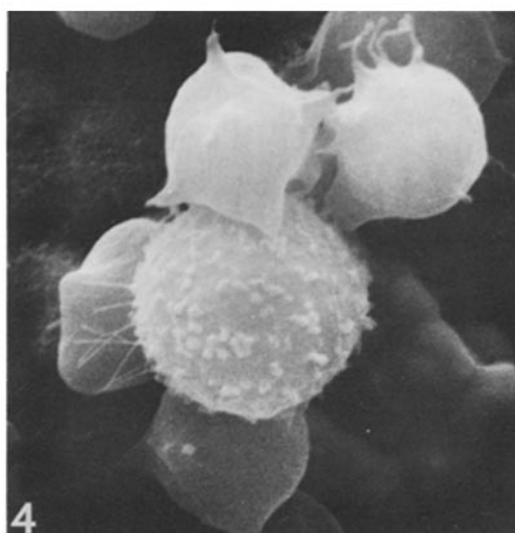
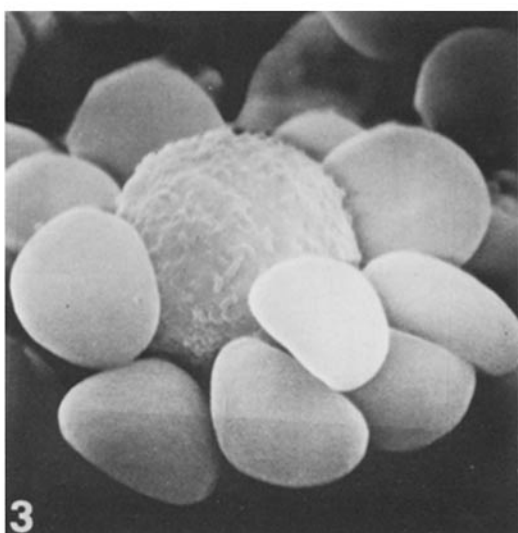
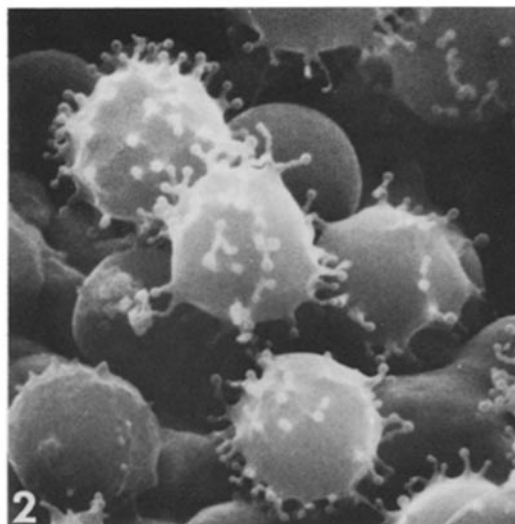
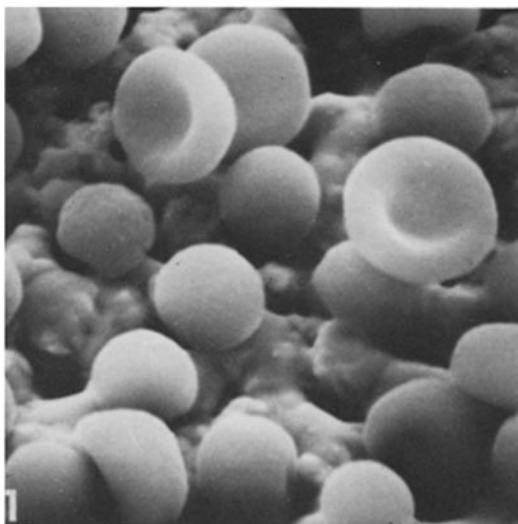
SRBC.—Freshly withdrawn SRBC fixed immediately in 1% glutaraldehyde retained their biconcave shape, but when stored at 4°C or washed in HBSS before fixation, became smaller, and generally spherical in shape (2.0–3.5 μm in diameter) with a varied number of surface projections (average length 640 nm but some up to 1–2 μm). These changes have been defined as spherocytosis transformation and/or microspherulation by Bessis and co-workers (19, 20) and in our study were already recognized when stored SRBC were washed in

HBSS, becoming most prominent, however, after rosetting procedures (Figs. 1, 2). Rosette-forming spheroechinocytes had multiple surface projections (some 1–5 μm in length, 90–160 nm broad) which were frequently branched showing beaded drumstick-like extremities (150–250 nm broad) (Fig. 2). When freshly withdrawn SRBC were used to form rosettes, most SRBC maintained their biconcave shape, although some became spherical and a few showed surface projections.

E-Rosettes.—Using the E-R (at 4°C) as a T-cell marker, 70–80% of normal PBL were identified as T cells under the light microscope. When E-R were prepared at room temperature and without AB serum only 28–40% of PBL formed rosettes. With the SEM, a varying number of SRBC were seen clustered around a lymphocyte, the surfaces of which remained in most cases readily visible. Occasionally, however, large clumps of SRBC covered the surface of some lymphocytes entirely making their identification impossible. Other rosettes were apparently labile, the smooth lymphocytes completely losing their constellation of SRBC during the preparatory procedures. Over a hundred rosettes were examined and counted directly with the SEM and from micrographs enlarged to twice their original magnification. The majority of rosetting T lymphocytes (average diameter 4.6 μm) had smooth, slightly irregular surfaces, while some however, had a small number of microvilli (Fig. 3). Of the remaining rosetting lymphocytes 15–25% had an intermediate-type of surface morphology with moderate numbers of microvilli (Fig. 4) and 10–20% had a more complex surface architecture similar to that of villous B cells (illustrated in Fig. 5). The stublike microvilli projecting from their surfaces were generally 100–250 nm broad and similar in their dimensions to the beadlike extremities of the spheroechinocyte projections (Fig. 6). Contact between spheroechinocytes and T lymphocytes was invariably of point-type (100–250 nm broad) via microvilli projecting from the SRBC (Fig. 7). Broader zones of attachment, illustrated in Fig. 8, were less frequently observed. Point-contact was less frequently seen when SRBC maintained their biconcave shape.

EAC-R.—About 15% of PBL formed EAC-R and most of these lymphocytes carrying complement receptor had villous surfaces as seen by SEM (average diameter 5.5 μm). The majority of rosetting cells had multiple microvilli (Fig. 5), 15–30% had an intermediate-type of surface morphology with a moderate number of microvilli (Fig. 8), and less than 10% were almost entirely smooth and resembled lymphocytes illustrated in Fig. 3. The rosetting SRBC were mostly spheroechinocytic or ovoid with a moderate number of surface projections. Contact between B-PBL and SRBC was more frequently over a broader zone of surface (generally 0.8–2 μm) (Fig. 8). Point contact was less frequent. Monocytes rosetting with EAC were recognized by their surface architecture (presence of ridgelike profiles and ruffled membranes with lack of fingerlike microvilli) and evidence of SRBC phagocytosis (Fig. 9).

Cultured T-Cell Line (MOLT-4).—



Before rosetting: The MOLT-4 cells have been shown to be of T-origin (15). 65–90% of them form E-rosettes. Most of these large cells had the T-cell type of surface architecture. After examination of more than a thousand cells from nine different samples, it appeared that the majority of the cells were almost entirely smooth (Fig. 10), although a spectrum of surface morphology was seen, 5–27% (average 20%) having a relatively small to moderate number of microvilli. 5–26% (average 16%) of cells had a larger number of microvilli, but in contrast to most cultured B cells, areas of exposed surface were often visible between the microvilli (Fig. 11 compared with B cell in Fig. 12). Changes in the in vitro culture conditions did not significantly alter the spectrum of surface morphology of the cells although the number of smoother cells was slightly increased after exposure to cold (21). Although synchronized cells were not examined there appeared to be no significant surface differences between cells examined at logarithmic or stationary phases of the cell growth. Examination of one sample of very rapidly proliferating cells, however, revealed a larger percentage of villous cells which may be related to in vitro growth conditions or cell cycle variations (22).

After rosetting: After E-rosette formation with a particular sample containing 90% smooth cells, only 10% of the rosette-forming T cells remained smooth and 90% had a moderately large number of microvilli. After examination of four other rosette suspensions, it was found that only 30% of the rosette-forming cells were almost entirely smooth, the remaining cells having an intermediate number of microvilli or being villous (Fig. 13). Almost all stored SRBC used for rosette formation showed spherocytosis and alterations in contacts with the cultured T cells were invariably point to point as in T-PBL rosettes. When freshly withdrawn SRBC were used, their biconcave shape was maintained but contacts with lymphocytes were still generally over a limited area (250–600 nm broad) either via a tapered portion of SRBC which had attached to the surface of the T cell or through lymphocyte microvilli (Fig. 14–16). Occasional MOLT-4 cells (8–15%) were found to rosette with EAC. In

FIG. 1. SRBC washed with HBSS showing spherocytosis. Two SRBC have maintained their biconcave shape. $\times 3,600$.

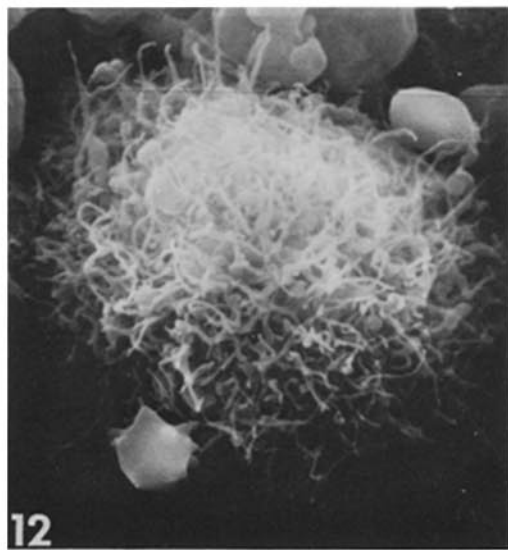
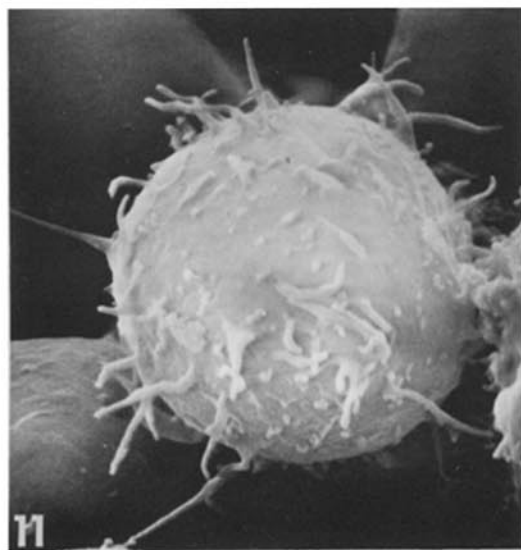
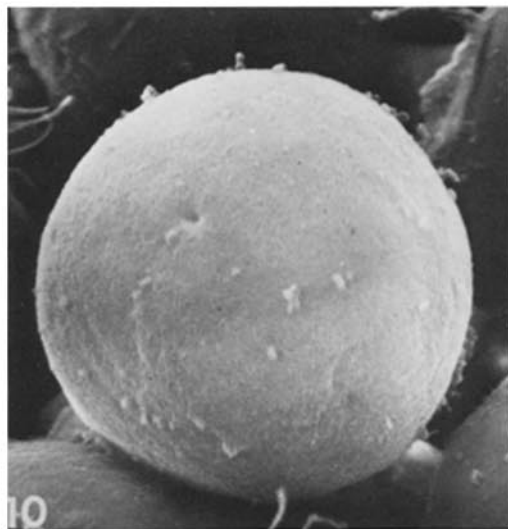
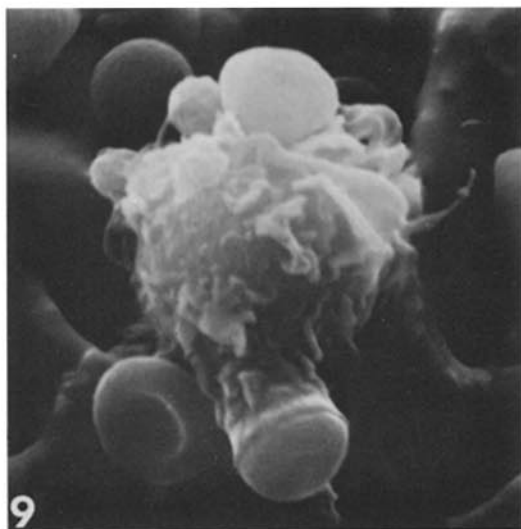
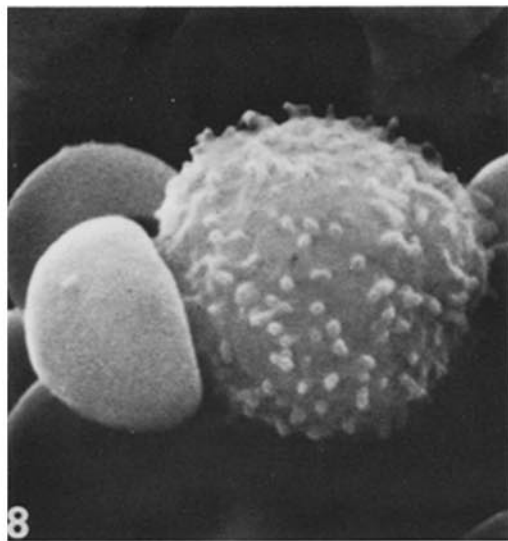
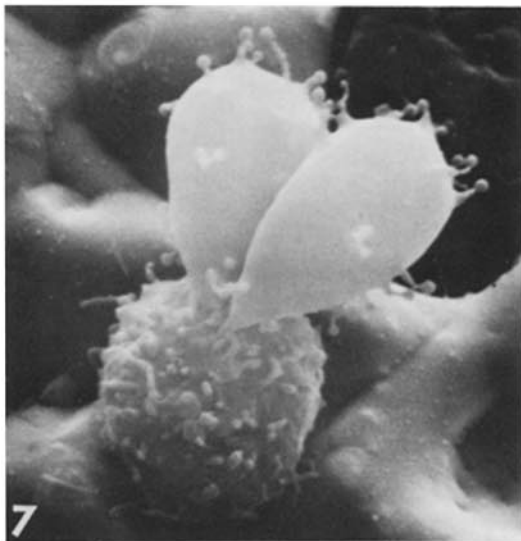
FIG. 2. SRBC showing microspherulation, with multiple microprojections with beaded ends. $\times 6,900$.

FIG. 3. E-rosette showing a smooth surfaced T lymphocyte surrounded by SRBC; the majority of T cells and less than 10% of rosetting B cells had this type of surface. $\times 5,700$.

FIG. 4. E-rosette showing intermediate type of T lymphocyte. Up to 30% of rosetting B and T cells had this type of surface. $\times 6,000$.

FIG. 5. EAC-rosette, showing villous B cell. The majority of B cells and about 15% of T cells showed this surface morphology. $\times 5,700$.

FIG. 6. Rosetting T lymphocyte with a villous surface, surrounded by spherocytotic SRBC which have multiple projections with beaded extremities of similar dimensions to lymphocyte microvilli. This type of picture suggested that deposition of portions of SRBC projections to the lymphocyte surface may occur. $\times 6,600$.



most of these cases, however, the contact between SRBC and cultured T cell was point attachment, suggesting that these rosettes were randomly formed spontaneous E-R.

Human Thymocytes.—

Before rosetting: The majority of thymic cells (average diameter 4.4 μm) had smooth surfaces with only few microvilli (Fig. 17). A small proportion of the remainder of the cells had moderate numbers of microvilli which were generally stublike and short. Cells with B-type villous surfaces were rarely seen.

After rosetting: By light microscopy, 90–100% of thymic cells formed E-rosettes. By SEM, most of these rosetting cells had smooth surfaces. 10–15% of the rosette forming cells however had a more complex villous surface, some of them developing microvilli, up to 4 μm in length (Fig. 18), suggesting that the surface of some thymic cells may alter during the rosetting procedure.

Cultured 8866 Cell Line (B Cells).—

Before rosetting: Four different samples were examined at different phases of growth (logarithmic and stationary) and under different growth conditions comparable to those described for the MOLT-4 cell line. Most of these cells are known to secrete immunoglobulin (16) and only about 10% of them formed E-R. The majority of the cells were of the very villous type with large numbers of microvilli (Fig. 12).

After rosetting: 35% of cells formed EAC rosettes. Most of the rosetting cells had extremely villous surfaces (Fig. 12). Contact between lymphocyte and SRBC was frequently over a broad zone (up to 2–3 μm) and rarely via narrow point contacts.

DISCUSSION

B- and T-derived lymphocytes can be distinguished in many cases under the SEM although a spectrum of surface morphology was frequently observed. Most circulating B cells had villous surfaces and only a small number were smooth whereas the majority of T cells were relatively smooth. Some lymphocytes had an intermediate number of microvilli and could not be classified

FIG. 7. Rosetting T lymphocyte with intermediate type of surface architecture illustrating the narrow zone of SRBC attachment to T-cell surface. $\times 7,000$.

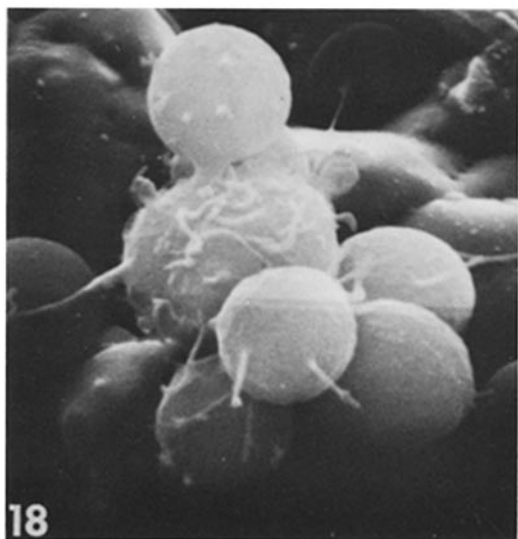
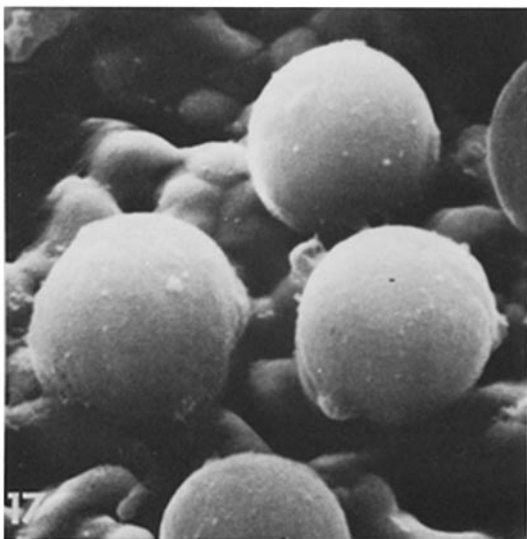
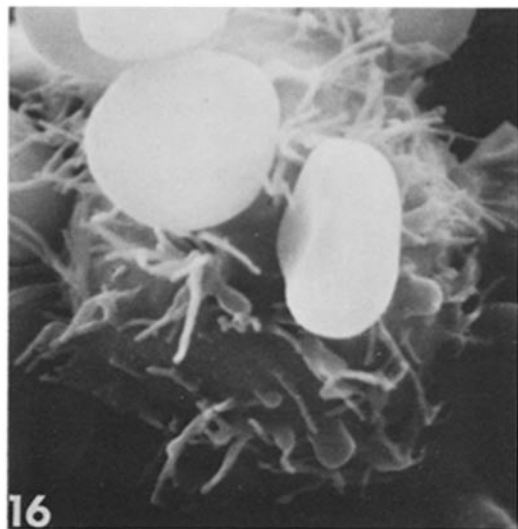
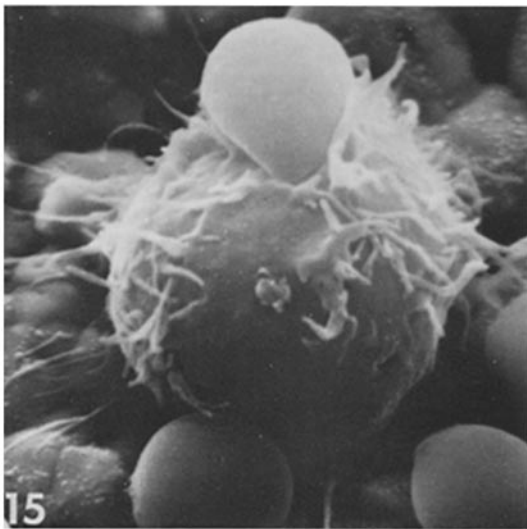
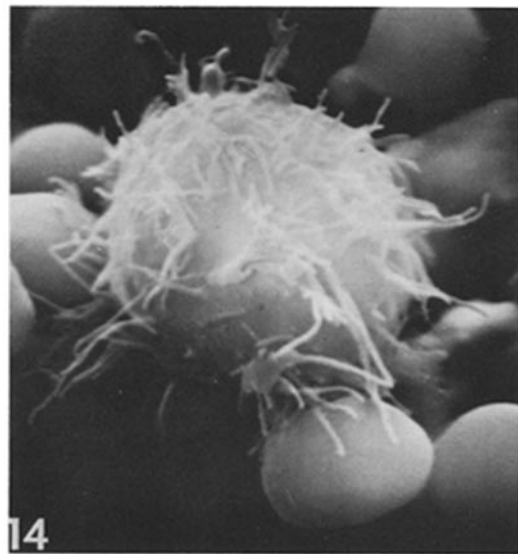
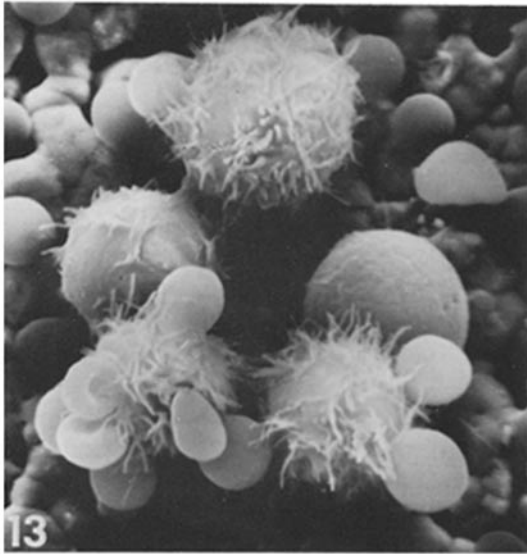
FIG. 8. Rosetting B lymphocyte with intermediate type of surface architecture showing broader zone of contact between SRBC and B-lymphocyte surface. $\times 9,000$.

FIG. 9. EAC-monocyte rosette. The rosetting monocyte has ridgelike ruffled membranes and only very few microvilli. An SRBC is in the process of being phagocytosed. $\times 6,000$.

FIG. 10. Smooth cultured T cell (MOLT-4). Most of the cells had surfaces of this type, with very few microvilli. $\times 6,300$.

FIG. 11. Villous cultured T cell (MOLT-4). About 20% of cells had this type of surface but relatively large areas of exposed surface were frequently visible between the microvilli. $\times 6,000$.

FIG. 12. Rosetting cultured B cell (8866). Most of these cells had villous surfaces, both before and after rosetting. $\times 3,100$.



accurately by SEM alone before rosetting, but were thought to represent subpopulations of either B or T cells. A proportion of cultured T cells had a larger number of microvilli but frequently differed from cultured villous B cells by showing larger areas of exposed surface between the microvilli. Further studies with synchronized cells will hopefully reveal how much of the spectrum of the surface morphology of a given cell type might be related to the phases of cell cycle, as suggested by recent studies (22).

Examination and classification of B and T lymphocytes after E and EAC rosette formation provided further evidence for the above mentioned correlation between lymphocyte type and cell surface architecture. Most of the T cells in E-rosettes were of the smooth type, only 20% having an intermediate number of microvilli and 15% being villous. Thymocytes and cultured T cells with more microvilli were evident after rosetting indicating that the surface of some T cells alters and becomes more complex during the process of rosetting. This may account for some of the villous T lymphocytes seen in E-rosettes. Most of the rosetting B cells had moderate to markedly villous surfaces and less than 10% of them were smooth. SEM studies of murine lymphocytes also show that a similar proportion of B cells have smooth surfaces.² It is possible that these smoother cells are precursors or B cells at a different stage of differentiation.² These results with rosetting lymphocytes, which are similar to those described by Lin et al. (13), confirm that most B cells are villous and the majority of T cells are smooth. The findings also indicate that more villous T cells and smoother B cells cannot be distinguished by SEM without immunologic identification. It is possible that some of these cells represent lymphocytes with both B and T markers which have been demonstrated among normal PBL, although in small numbers (7).

In most cases, the type of contact between rosetting B and T lymphocytes

² Polliack, A., U. Hammerling, N. Lampen, and E. de Harven. 1974. Surface morphology of murine B and T lymphocytes: a comparative study by scanning electron microscopy. Submitted for publication.

FIGS. 13-16. Villous rosetting cultured T cells. These micrographs illustrate that the rosetting cells frequently had villous surfaces, suggesting that the surface of some T cells alters during rosetting.

FIG. 13. Shows this on lower magnification. $\times 3,000$.

FIG. 14. Shows a villous cell with point contact of lymphocyte microvilli with SRBC. $\times 6,300$.

FIG. 15. Illustrates a smoother type of T cell with point attachment of SRBC to lymphocyte surface and the presence of microvilli in the vicinity of SRBC attachment. $\times 6,700$.

FIG. 16. Shows villous T-cell surface and attachment of two SRBC. $\times 7,400$.

FIG. 17. Human thymocytes with smooth surfaces, before rosetting. $\times 6,500$.

FIG. 18. Thymocyte E-rosette. Central thymic cell has microvilli up to $3\ \mu\text{m}$ in length. $\times 6,800$. 15% of rosetting cells had this type of surface suggesting that the surface of some thymocytes may alter during rosetting.

was different. Contact between T-cell and spheroechinocytic SRBC was invariably through well circumscribed point attachment and broader zones of contact were rare. Point attachment was less frequent in T-PBL rosetted with well preserved SRBC but was commonly encountered with cultured T cells. Similar observations have been made by transmission electron microscopy of E-rosettes (7) where it appeared that contacting microvilli were always separated from each other by 500 Å or more whereas SEM suggests that the cells are in intimate contact. In EAC-B rosettes, point attachment was infrequently observed and broader zones of contact between cells were usually seen, confirming TEM observations on human and murine rosetting cells (7, 11). These differences in the type of contact may be related to basic differences in the nature of the receptor sites and perhaps the microvilli and the more frequent point to point attachment seen in E-R are formed as part of an active biological process which may involve movement of areas of cytoplasm associated with the receptor sites.

Our results indicate that the surface of some T cells becomes more villous during rosetting with SRBC. Initially, we thought that this could result from the deposition of portions of SRBC projections on the lymphocyte surface during rosetting. Many of the SRBC used in E-rosettes showed striking surface changes becoming spherical and developing microprojections. Bessis and co-workers (19, 20) defined this phenomenon as spheroechinocyte transformation and/or microspherulation, noting that it occurs in response to a variety of intrinsic and extrinsic factors. Many of the rosetting SRBC showed up to 30 projections extending from their surface and it was considered feasible that portions of these could be deposited on the surface of the T cell during rosetting as has been suggested by some TEM observations (7, 11). The beadlike terminal portions of the SRBC projections have approximately the same diameter as the stublike lymphocyte microvilli. If these were to adhere to the surface of a proportion of T lymphocytes they would be indistinguishable from microvilli, causing the surface to appear more villous than it really is. Lymphocytes rosetted with freshly withdrawn SRBC, however, were found to have a similar spectrum of T-cell surface morphology and villous T cells were observed; thus, it appears that it is the lymphocyte surface itself which alters during the active process of rosetting and intercellular contact.

Recently Kay et al. (23) have reported much larger numbers of T cells with villous surfaces in E-R, however this report does not take into consideration the fact that smoother T cells alter and become more villous as a result of the rosetting contacts, an observation which has also been reported independently by Lin and Wallach (24). Furthermore, Kay et al. (23) prepare their E-R, which may in fact constitute "active" E-R, differently to those described in the present study and collect them on glass cover slips which is also likely to offer conditions of attachment and sampling different from those in the present study.

SUMMARY

Human lymphocytes of known B or T derivation were examined by scanning electron microscopy (SEM) before and after rosetting with SRBC. After collection of the cells onto silver membranes the samples were prepared for SEM by the critical point drying method. Sheep RBC frequently underwent spherocytosis transformation and multiple projections extended from their surfaces. This was readily noticeable after storage of SRBC in the cold and washing in Hanks, but more prominent after rosetting. These erythrocyte surface alterations were less apparent when freshly withdrawn cells were used. Spontaneous sheep erythrocyte rosettes (E-R), a marker for human T lymphocytes, were prepared with normal peripheral blood lymphocytes (PBL), thymic cells, and cultured T cells. EAC-rosettes (EAC-R), used to identify B lymphocytes with complement receptors, were prepared with normal PBL and cultured B cells. The majority of rosetting T lymphocytes had generally smooth surfaces while about 20% had an intermediate number of microvilli and 15% were more villous and indistinguishable from villous B cells. Studies of rosetting thymocytes and cultured T cells however indicated that the surface of some T cells alters on rosetting, becoming more villous and thus account for the higher numbers of villous T cells seen in E-rosettes. Point to point contact sites between SRBC and T lymphocytes were more frequent than broad zones of attachment. The majority of rosetting B lymphocytes had multiple microvilli, about 25% had a moderate number of microvilli and less than 10% had smooth surfaces similar to those of most T cells. Areas of contact between EAC and B lymphocytes were frequently broad zones of attachment.

The study confirms that in many cases B and T lymphocytes can be distinguished by their surface architecture as seen under the SEM; however, about 20% of rosetting B and T cells have similar surfaces with intermediate numbers of surface microvilli and cannot be distinguished by SEM without parallel immunologic identification.

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