# A SCANNING AND TRANSMISSION ELECTRON MICROSCOPE STUDY OF ANTIGEN-BINDING SITES ON ROSETTE-FORMING CELLS*

BY FRED G. GUDAT‡ AND W. VILLIGER

*(From the Institute for Pathology and the Laboratory of Electron Microscopy of the University of Basel, Basel, Switzerland)*

After immunization of mice with sheep red cells (SRC)[1] specific antigen-binding cells can be detected in vitro by their ability to bind target red cells to their surface leading to the formation of a rosette (1, 2). Electron microscopy of such rosettes revealed proliferating lymphoid cells in the center of a rosette (for literature see references 3 and 4). In accordance with the visualization of immunoglobulin on the surface of certain lymphocytes (5, 6), there is evidence for the involvement of specific surface antibody in rosette formation (RF), namely the hemolysis of rosettes by complement (7), and the suppression of RF by antisera directed against immunoglobulins (8–10). Therefore rosette-forming cells (RFC) appeared to be a suitable object for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to study antigen-binding sites on antibody-producing cells.

Both approaches revealed a spotlike binding of the red cell membrane to the surface of the RFC. The direct demonstration of immunoglobulin with ferritin-labeled antibodies in such binding areas and their ultrastructure at high resolution will be discussed in the light of recent findings on the distribution of antigen receptors on the surface of lymphocytes.

## Materials and Methods

Inbred mice were used 5 days after an injection of 0.1 ml of a 20% SRC suspension into both forelegs. Single cell suspension of the regional lymph nodes were incubated for 60 min at 37°C in plastic dishes (Falcon Plastics, Oxnard, Calif.) to eliminate adhering macrophages. RF was then allowed to proceed in 1 ml of Eagle's minimal essential medium containing 10% fetal calf serum, $18 \times 10^6$ cells, and 0.5% SRC at 37°C for 60 min. The rosettes were fixed and processed for TEM as described in detail elsewhere (3), using Epon or Araldit as the embedding medium. Ultrathin sections were viewed in a Siemens Elmiscope I electron micro-

scope (Siemens Corp., Iselin, N. J.) operated at 60 kV and screen magnifications up to × 30,000.

*Immunoferritin Labeling.*—Horse spleen ferritin (Pentex Biochemical, Kankakee, Ill.; twice crystallized) was purified according to Andres et al. (11). A rabbit antimouse immunoglobulin antiserum (Miles-Seravac, Elkhart, Ind.; lot 3784), precipitated twice with 50% ammonium sulfate, was conjugated with the purified ferritin according to Vogt[2] (25 mg of protein, 100 mg of ferritin, 0.8 ml of 0.1% glutaraldehyde in 10 ml 0.1 M phosphate buffer, pH 6.8, at 37°C overnight, followed by dialysis in 0.1% hydroxylammonium-chloride in buffer). The purification of the conjugate was performed in a Spinco ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) by threefold centrifugation at 100,000 g for 4½ h (11). Unfixed rosettes were collected and incubated in a drop of the conjugate at the bottom of a plastic embedding capsule for 30 min at 4°C, followed by several washings in phosphate-buffered saline, double fixation in glutaraldehyde (3%, 30 min) and osmium tetroxide (1%, 30 min), and embedding in Epon. The specificity of the conjugate was confirmed by blocking experiments with unconjugated antibody and incubation of rosettes in free ferritin.

*Scanning Electron Microscopy.*—A total of 50 unfixed rosettes were placed on flying cover glasses with a micropipette and transferred into 1% phosphate-buffered glutaraldehyde (0.15 M, pH 7.4) of varying osmolarities (165, 330, and 375 mosM). After fixation for 30 min at 4°C, the specimens were rinsed extensively in buffer of corresponding osmolarity. After three rinses in distilled water, the adhering rosettes were gradually dehydrated with methanol and finally dried at room temperature. The cover glasses were mounted with silver paint on aluminium specimen holders and coated with carbon (approx. 100 Å) and gold (approx. 150 Å) on a rotating stage in a vacuum evaporator. The preparations were examined with a scanning electron microscope (Stereoscan, model MK II; Cambridge Instrument Co., Ossining, N. Y.).

## RESULTS

*Scanning Electron Microscopy.*—The general appearance of rosettes as seen in the scanning microscope after isotonic fixation is shown in Figs. 1 and 2. The fixation in hypotonic solution of 165 mosM (Fig. 3) was employed in order to cause swelling of the red cells, thus exerting a tension on the cell contacts and exposing the binding sites more clearly. At first glance, the binding of the SRC appeared to involve mostly broad, evenly attached areas, even at higher magnification (Figs. 2 and 7). In hypotonically fixed rosettes these broad contacts of the white cells became particularly prominent as cytoplasmatic caps covering considerable parts of the swollen red cells (Fig. 3). There were, however, several indications of circumscribed spots of adhesion. The most frequent in this respect were folds and extensions of the cytoplasm which rose from the body of the white cell and formed a spotlike contact with the SRC (Fig. 5). Occasionally these contacts were seen as irregularly outlined patches on the red cells (Fig. 4). Another conspicuous observation were thin extensions of the red cell surface which formed pointlike contacts with the white cell. These extensions were also identified in detached erythrocytes (Fig. 2).

The RFC adhered to the glass with footlike processes (Fig. 6) which were more developed in the larger cells. The surface which was not covered by red

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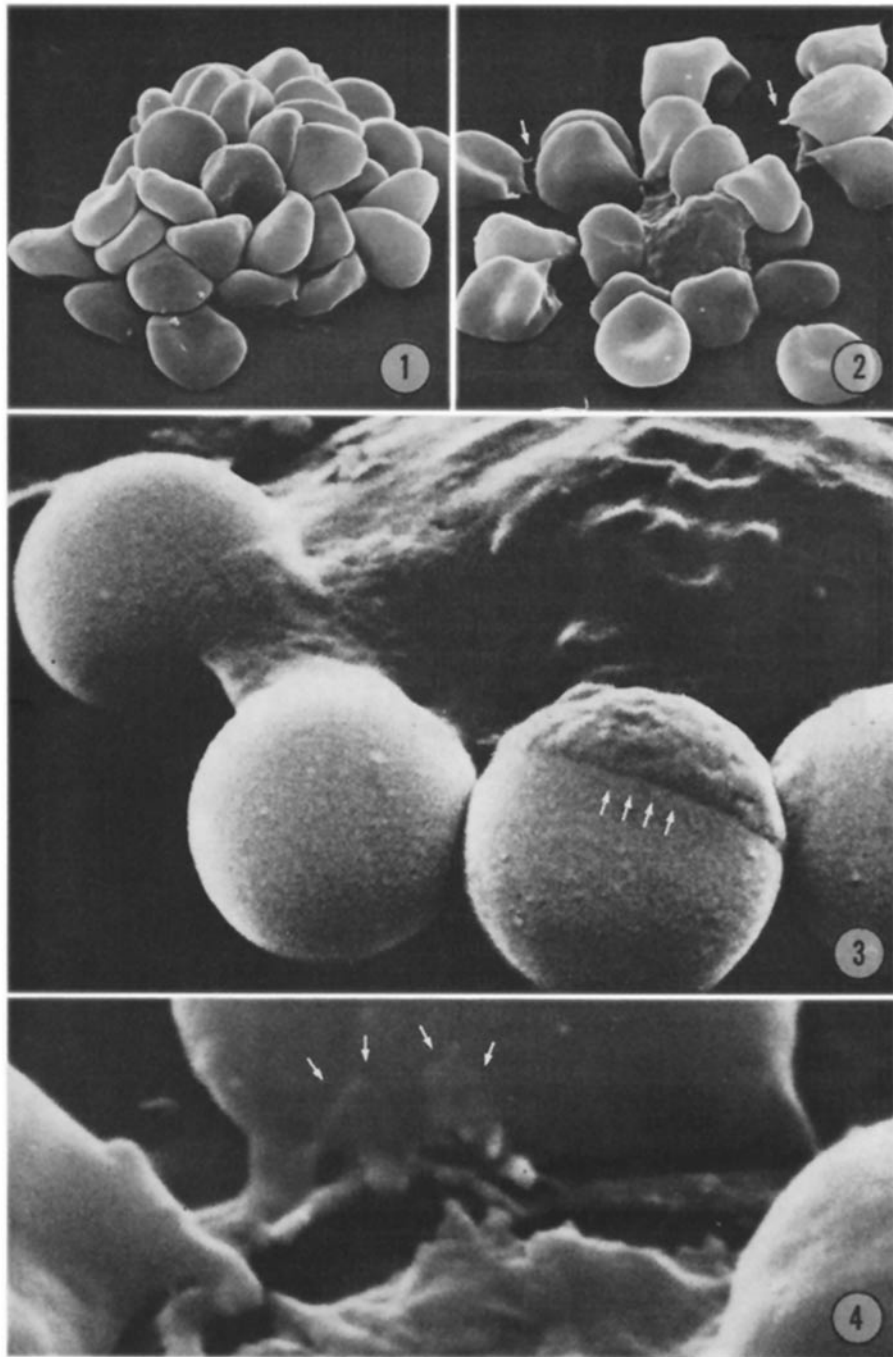[2] Vogt, A. 1970. Hygiene Institut, University of Freiburg, Freiburg i.Br., Germany. Personal communication.

Fig. 1. General appearance of rosette in SEM with tightly packed SRC of a diameter of 4.1–4.3 nm. Fixed at 330 mosM. × 3,000.

Fig. 2. Rosette with large central cell and several detached red cells. Note thin extensions of red cells (arrow). Fixed at 330 mosM. × 3,000.

Fig. 3. Hypotonically fixed rosette. Arrows indicate a caplike contact of the white cell cytoplasm with the swollen red cell. Fixed at 165 mosM. × 12,000.

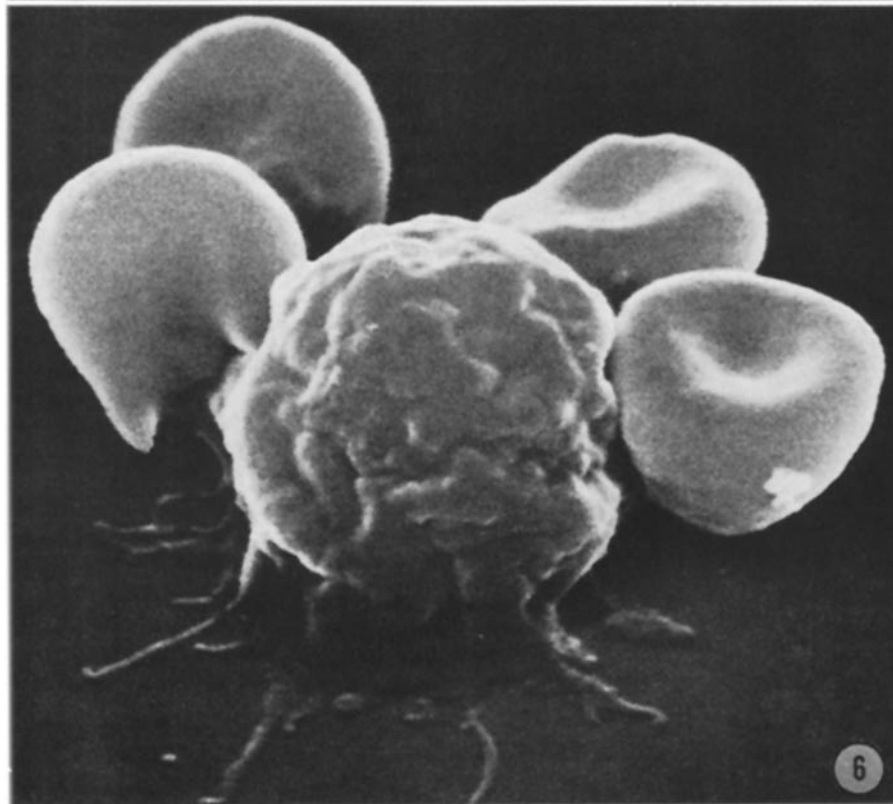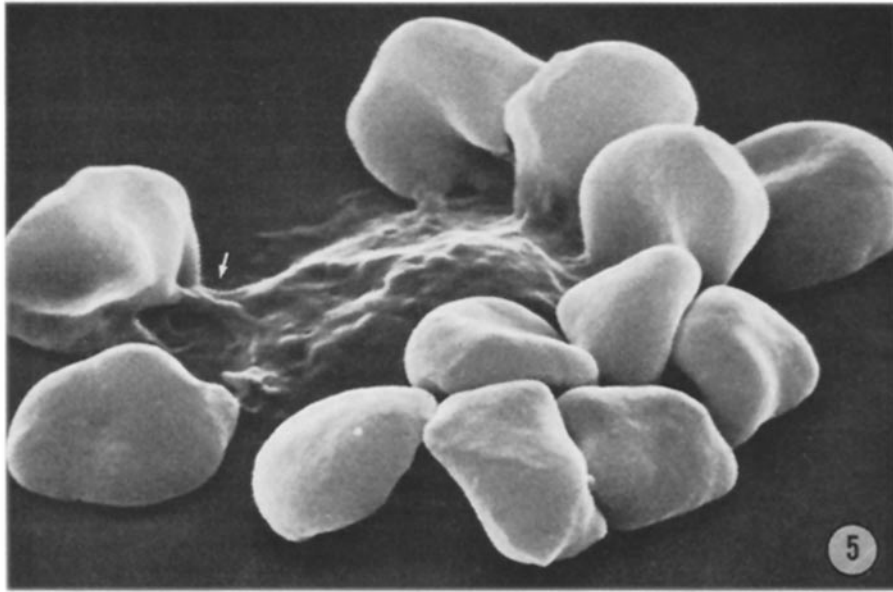Fig. 4. Higher magnification of patchy contact areas on the surface of a red cell (arrows). Fixed at 330 mosM. × 23,000.

Fig. 5. Large RFC with spotlike contacts between cytoplasmic folds and red cells (arrow). Fixed at 330 mosM. × 6,500.

Fig. 6. Small lymphocyte with attached red cells adhering to the glass with small footlike processes. Fixed at 330 mosM. × 11,000.

cells was uneven, but smooth (Figs. 6 and 7). No membranous structures, as characteristic of macrophages (12), were visible on the surface.

*Transmission Electron Microscopy.*—In ultrathin sections the distribution of cell types in rosettes was similar to that described for lymph nodes in an earlier report (3). The majority of the cells belonged to large lymphocytes in various stages of stimulation. There were few plasma cells and exceptionally few macrophages.

Close contacts of the cell membranes basically involved microspikes or short segments on the surface of the lymphocytes, compatible with a spotlike distribution of the binding sites (Fig. 8). Between such close contacts a conformational apposition of the cells with variable distances from each other occurred. On the part of the erythrocytes the contacts involved either segments of the body of the red cells or fine extensions which were attached to the lymphocyte in a small area (Fig. 8), comparable to those seen by SEM. Regardless of their size, the contacts were characterized at high magnification by a gap of 80 Å between the opposing cell membranes with a range of 75–90 Å when measured in those areas in which the contact zone was cut in a fairly perpendicular orientation as judged from the clearly visible bilayered structure of both cell membranes. A common finding in the gap region were irregularly spaced bridges of low electron density between the cell membranes (Figs. 9–11). A less frequent observation was an increase in electron density along the inner aspect of the lymphocyte cell membrane (Figs. 10 and 11).

Ferritin molecules, indicative of mouse immunoglobulin, were found in a spotlike distribution on the free surface of the lymphoid cells. In addition, they were seen either attached to the open end of the contact areas (Fig. 13) or interspersed between the white and red cells (Figs. 12 and 14). In the latter case, however, the distance between the cell membranes was more than 100 Å indicating that such sections were very close to but not inside a contact area. In fact, we were unable to demonstrate ferritin molecules attached to the bridges in the center of a close contact. It is interesting to note that cells with an advanced ergastoplasm showed more ferritin molecules on the surface (Fig. 12) than less differentiated lymphocytes.

## DISCUSSION

To ensure the cellular homogeneity of RFC for SEM, a selected lymphocytic cell population, poor in macrophages, was employed in this study. This was achieved by using plastic-absorbed lymph node cells on the 5th day of primary immunization, all of which is known to favor RF by lymphoid cells (3). Finally, concomitant TEM confirmed that the rosettes, collected in the same way as for SEM, were almost exclusively formed by lymphoid cells. Accordingly, we did not find evidence for phagocytosis of red cells in the scanning microscope. On the other hand, the formation of footlike processes and the adherence to glass is not restricted to macrophages, but does occur in stimulated lymphocytes as
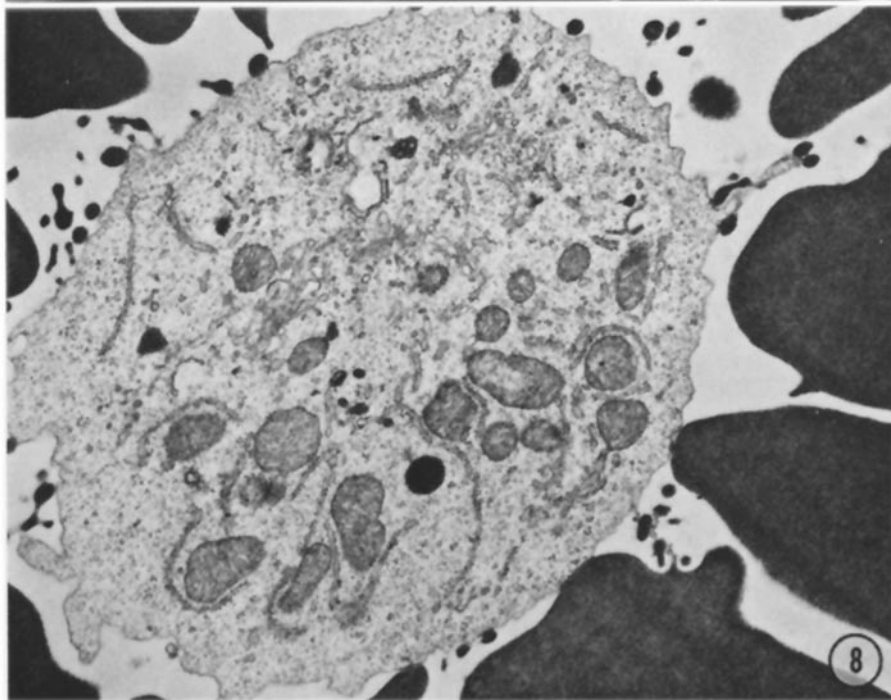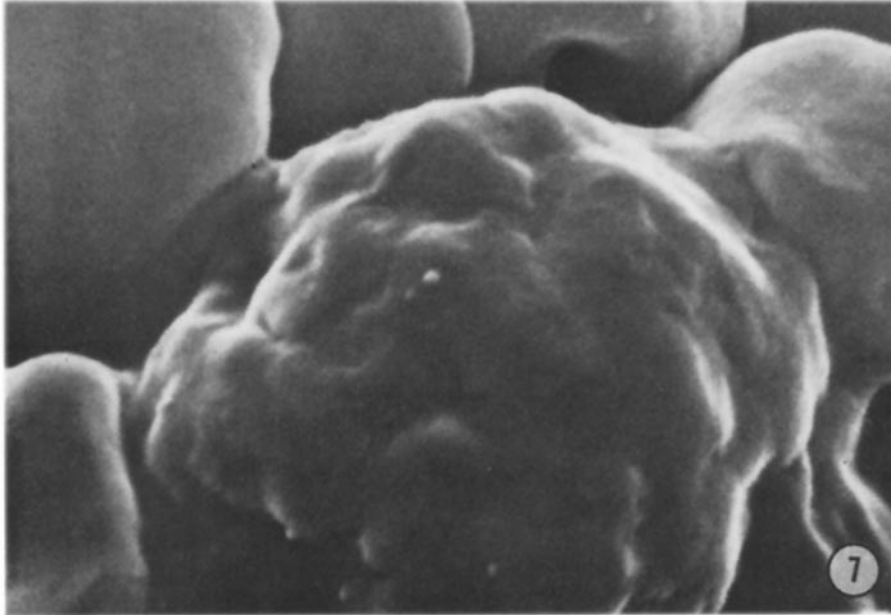
FIG. 7. This surface view of a RFC with broad contacts exemplifies the lack of finer details in such contact areas when viewed through SEM. Fixed at 330 mosM. × 19,000.

FIG. 8. Ultrathin section of rosette-forming lymphocyte showing numerous small contacts with red cell extensions and broader contacts directly with red cells. × 14,000.
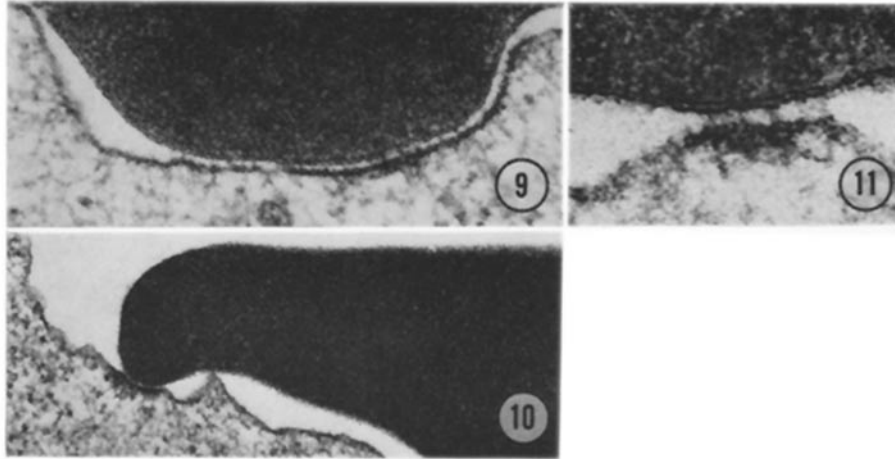
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FIG. 9. This detail of a broad contact shows fine electron-opaque bridges between the cell membranes. × 90,000.

FIG. 10. This pointlike contact shows a circumscribed concentration of electron-opaque material along the inner aspect of the lymphocyte membrane. × 40,000.

FIG. 11. High magnification of a small contact showing electron-opaque bridges between the cell membranes. × 200,000.

well. This was demonstrated after stimulation with phytohemagglutinin and tuberculin (13), and is now shown after stimulation with SRC.

Our results obtained by TEM and SEM indicate that the adherence of the erythrocytes is basically mediated by circumscribed, spotlike binding areas on the antigenic SRC and the lymphocyte surface. The presence of immuno-globulin at these sites was shown by ferritin-labeled antibodies. These findings are in accordance with the ultrastructural demonstration of immunoglobulin and antigen receptors shown in other models (14–18), specifically in direct (19) and indirect rosettes (20). As judged from a large number of ultrathin sections, the binding sites in rosettes vary in size from small pointlike to broader contact areas which correspond to those seen by SEM in connection with cell extensions or cytoplasmic patches on the red cells. Regardless of their overall size, we found as a common feature in TEM an average distance of 80 Å between the white and red cell membranes, and in addition electron-opaque bridges between the membranes of such close contacts. As we could not show ferritin-labeled anti-bodies attached to the bridges of the inner part of the contact areas, but only at their periphery, it remains open to discussion whether these bridges represent immunoglobulin receptors. Since ferritin with a diameter of 120 Å (21) is not apt to penetrate into the 80 Å gap, this lack of direct labeling of the bridges does not exclude the possibility that they are immunoglobulin receptors, indeed. Since single IgG and IgM molecules with average diameters of 150 and 300 Å,
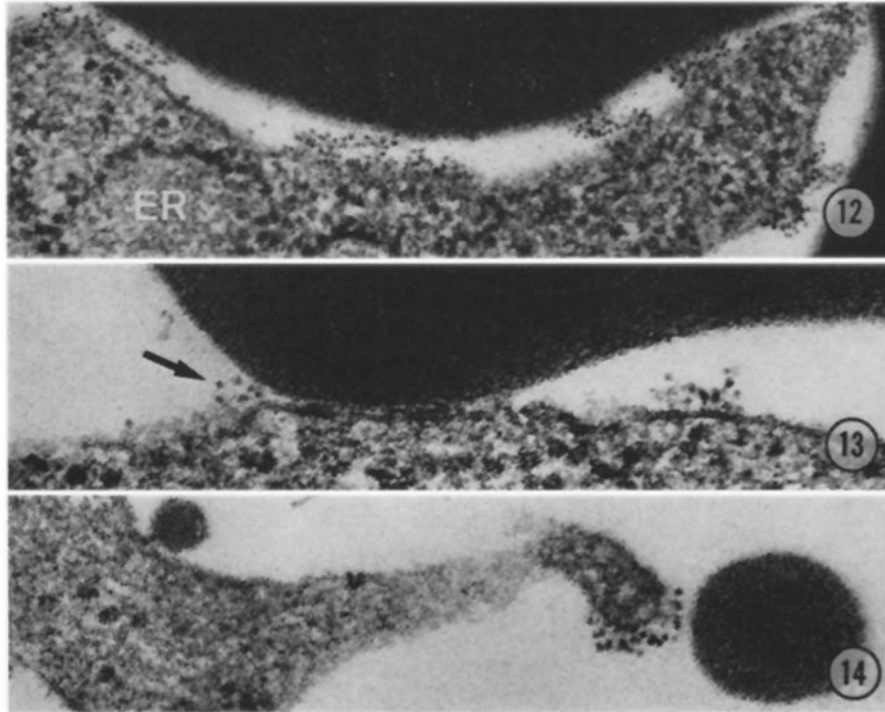
Fig. 12. Plasma cell forming a rosette with several groups of immunoferritin molecules in contact with the white and red cell surface. *ER* denotes an expanded ergastoplasma cisterna. × 70,000.

Fig. 13. Contact zone of a lymphocyte and a red cell showing ferritin molecules attached to the free surface of the lymphocyte and to the open end of the contact zone (arrow). × 120,000.

Fig. 14. Ferritin labeling of a contact zone formed by a white and red cell extension. × 120,000.

respectively, (22) would neither give enough contrast in regular TEM nor fit into the contact gap, it would imply that the bridges represent bundles of immunoglobulins either partly immersed into the lymphocyte membrane or changed in conformation so as to fit into this gap. We cannot, however, rule out the interpretation that these densities are condensations, true or artificial, of cell coat material other than immunoglobulin. It seems very unlikely that the bridges are caused by tangential sectioning of the membranes themselves, because they appeared also in contact areas which were cut in a perpendicular orientation. From the molecular dimensions it is also apparent that SEM having a resolution of 200–300 Å for biological material, could not reveal finer details of the contact areas under currently applied techniques. This holds true not only for the intermembranous bridges which theoretically should have been

visible at the periphery of the contacts, but also for the exact three-dimensional differentiation of 80-Å contacts from passive appositions of the cell membranes between such specific bonds.

A final comment on the peculiar red cell extensions which are documented in other publications as well (18, 19) seems justified. Their appearance suggests that they are pulled out of the body of the erythrocyte by some force. This might be due to as yet undefined influences during the preparatory steps, e.g., by centrifugal forces. It is our experience, however, that the appearance of red cell extensions varies from one rosette to the other within the same preparation. Our observation that these extensions are more frequent in rosettes formed by activated lymphocytes, also favors the interpretation that this effect is due to an active biological process. A possible explanation comes from recent demonstrations of the movement of surface immunoglobulin spots which are formed after contact with cross-linking agents like antibodies and antigens (22). Following this conception of freely movable immunoglobulin spots, it is suggested that these red cell extensions are an expression of a movement of the involved contact areas.

### SUMMARY

The ultrastructure of binding sites in rosette-forming cells of mice after immunization with sheep red cells was studied by means of scanning and transmission electron microscopy. It was found that the red cells were bound to the lymphocyte surface in circumscribed, immunoglobulin-containing areas, consistent with a spotlike or patchy distribution of antigen-binding immunoglobulin receptors. In these contact areas the cell membranes formed a gap of 80 Å (range 75–90 Å) which exhibited electron-opaque bridges at high magnification. These results are discussed in the light of the recent recognition of the formation of immunoglobulin spots on the lymphocyte surface after antigen contact. Morphological details suggest that the same mechanism is operating in rosette formation, possibly including the movement of the contact areas on the lymphocyte membrane.

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