ULTRASTRUCTURAL CHARACTERISTICS OF THE NON-IMMUNE ROSETTE-FORMING CELL

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SUMMARY

Non-immune rosette formation, where a non-sensitized lymphocyte is surrounded by three or more sheep red blood cells, is an in vitro technique which is thought to be specific in the identification of thymus-dependent (T) lymphocytes. Using the electron microscope and tissue culture techniques, we have studied the ultrastructure of the rosette-forming lymphocyte which has been stimulated by various cellular mitogens. Non-sensitized but stimulated lymphocytes, which form rosettes, have a morphology similar to that of previously identified T cells, adding further credence to the concept that non-sensitized rosette-forming cells represent a population of T cells. Changes in the binding pattern of the sheep red cell membrane to the lymphocyte have been identified which may represent an early phase of cell 'activation.' This marker of activation offers a potential method of studying membrane responses in attempts to localize cellular defects involving the T lymphocyte. In addition, preliminary studies using fluorescein-labelled sheep red blood cell fragments suggest specific attachment of labelled membrane to the T lymphocyte, allowing rapid identification of T cells in smears or tissue preparations.

INTRODUCTION

Lymphocyte function, as determined by in vitro methods, is generally assessed through the detection of biologically active molecules liberated by stimulated lymphocytes (Daguillard, Heiner, Richter & Rose, 1969; Rocklin, Rosen & David, 1970); certain stimulants (mitogens) [e.g. phytohaemagglutinin (PHA), concanavalin A (Con A), pokeweed (PWM)] result in the release of migration inhibition factors, cytotoxic factors, blastogenic factors, etc., while other mitogens have an effect on transformation and the release of immunoglobulins (e.g. PWM).

Non-immune rosette formation, using sheep red blood cells (srbc) and unsensitized human lymphocytes, is considered a reliable index of the presence of thymus-dependent ('T') lymphocytes (Stites, Wybran, Carr & Fudenberg, 1972; Jondal, Holm & Wigzel, 1972; Brain, Gordon & Willets, 1970; Papamichail, Holborrov, Keith & Currey, 1972). The nature of spontaneous srbc attachment and the relationship to 'T' cell function is presently unclear.

We have studied the morphologic character of rosette formation by both stimulated and non-stimulated human lymphocytes in order to determine the ultrastructural characteristics of the rosette-forming cell (RFC). Since different subpopulations of lymphocytes with different functional characteristics have been tentatively assigned unique ultrastructural markers (Matter, Lisowske-Bernstein, Ryser, Lamelin & Vassalli, 1972), we have similarly categorized the lymphocytes involved in non-immune
rosette formation. The results of these observations suggest a method for detecting early lymphocyte activation.

MATERIALS AND METHODS

Lymphocytes (Parker, Schreinemachers & Hilaire, 1972; Harris & Ukaejiofo, 1970)

Fifty millilitres of venous blood were drawn from healthy young volunteers into a 50-ml plastic syringe containing 200 u. heparin (Fellows Medicine Manufacturing Co., without preservative). The syringe was then centrifuged at 400 g for 10 min at room temperature. After centrifugation, the plasma, buffy coat and some red cells were expressed into a plastic centrifuge tube, 50 ml conical (Falcon Plastics, Oxnard, CA), followed by dilution with unfortified RPMI-1640 (Roswell Park Memorial Institute, GIBCO, Grand Island, N.Y.). The cell-rich medium was then passed over 20 ml of 2-mm glass beads to remove fibril strands and subsequently gently layered over a Ficoll-Hypaque gradient in a 250-ml round-bottom glass centrifuge bottle (Ficoll-Hypaque: 24 parts 9% Ficoll, Pharmacia Co.; 10 parts 34% Hypaque, Sodium diatrizoate, Winthrop). The cells and gradient were then centrifuged for 30 min at 400g, measured at the gradient-media interface. Following centrifugation, approximately two thirds of the supernatant was gently aspirated. The slightly turbid, leukocyte-rich interface was carefully aspirated with a Pasteur pipette and the cells were then diluted in RPMI to a volume of 40 ml and centrifuged at 200g for 10 min. This resulted in a small button, which, after aspiration of the supernatant, was resuspended in 40 ml of medium and again centrifuged. At this time the cells were resuspended in 5 ml of medium and counted on a haemocytometer; a trypan blue staining for viability was performed at the same time (90-95% lymphocytes, 95-100% viable). The cells were diluted in medium for culture or in Hanks' Balanced Salt Solution (BSS), pH 7.4 (Microbiological Associates, Inc., Bethesda, MD), for rosette formation.

Lymphocyte cultures

Macrotechnique. Lymphocytes suspended in RPMI-fortified media, concentration of $1 \times 10^6$ cells per ml, were distributed in 0.4-ml aliquots into 12 x 75 mm plastic tubes (Falcon Plastics, Oxnard, CA). (RPMI-1640 complete medium is made up as follows (100 ml): 81 ml RPMI 1640 with glutamine (Gibco catalog no. 187G); 4 ml Hepes buffer pH 8.1; 1 ml 5000 units penicillin (50 units/ml); 5000 µg streptomycin (50 µg/ml); 15 ml heat-inactivated foetal calf serum.) Mitogen was added with a Hamilton syringe to the following concentrations: 20 µg PHA-P (Difco-Bacto-Phytohemagglutin-P), 20 µg PWM (pokeweed mitogen, Grand Island Biological Co.) and 4 µg Con A (concanavalin A, Calbiochem). After appropriate culture periods, the lymphocyte cultures were centrifuged at 400 g for 5 min. The supernatant was aspirated and the cell button resuspended in BSS; the cells were centrifuged again then and resuspended to a final volume of 0.9 ml with BSS. Heat-inactivated foetal calf serum (FCS, GIBCO, Grand Island, N.Y.), 0.05 ml, was added prior to the addition of srbc. (Note: lymphocyte clumping and the prevention of srbc agglutination required thorough washing with BSS.)

Sheep red blood cells (srbc)

All srbc (Mr Earl Fife, Department of Serology, WRAIR) were collected in Alsever's solution, stored at 4 °C and used within 2 weeks of collection. At time of utilization, the cells were washed 3 times in BSS, and centrifuged at 300 g for 10 min. They were finally suspended in BSS at a 1% concentration (approximately 160 x 10^6 cells per ml).

Rosette formation (modified after Jondal) (Jondal, 1972)

To the macro-culture tubes (prepared as above) containing at least 400,000 lymphocytes and 0.05 ml heat-inactivated foetal calf serum, were added $3.2 \times 10^8$ washed srbc (approximately 8:1 ratio, srbc:lymphocytes). The mixture was incubated at 37 °C for 5 min, then centrifuged at 200 g for 5 min and placed at room temperature for 60-90 min. Subsequently, 0.5 ml of the supernatant was aspirated with a Pasteur pipette, the tube gently swirled for partial resuspension,
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and the cells transferred to a counting chamber with a capillary pipette. Duplicates were counted, 400 total lymphocytes, with rosette-forming cells (RFC) defined as those lymphocytes surrounded by three or more srbc (Fig. 1). The result was expressed in percentage of RFC/Total lymphocytes. 

Electron-microscopic technique

All specimens examined by electron microscopy were cell suspensions handled in the same manner as above. The lymphocyte-srbc mixture, as described in the rosette technique, were re-centrifuged at 200 g for 5 min following the room-temperature incubation. The supernatant was aspirated carefully to near dryness with a Pasteur pipette and the cell button was fixed with picric acid-paraformaldehyde (PAF). The pellet was then kept at 22 °C for at least 24 h and processed with osmium tetroxide, dehydrated with graded ethanol and propylene oxide and then impregnated with epoxy resin (EPON 812). Ultrathin sections were cut and stained with lead nitrate and uranyl acetate. Specimens were examined by a Zeiss EM 9S-2 electron microscope.

All lymphocytes were classified according to the ultrastructural criteria of Matter et al. (1972). ‘B’ lymphocytes were most frequently small-to-medium size cells, with clear cytoplasm, a central nucleus with margined and patchy pyknotic chromatin, and one or more nucleoli. Usually a few mitochondria were seen, with little endoplasmic reticulum and a small Golgi apparatus. ‘B’ blast cells were quite large, with giant nucleoli and abundant ribosomes, but without a microfilament network.

Criteria to differentiate ‘T’ cells were the following: Ti lymphocytes were small-to-medium size lymphocytes with a paucity of organelles and a lack of polyribosomes. T2 lymphocytes contained ‘rosette’-appearing ribosomes, focal areas of cytoplasmic clearing beneath the cell membrane and 0.2-0.4 μm, membrane-bound, electron-dense granules were present. T3 cells had characteristically dark staining with a network of microfilaments, densely packed cytoplasmic monoribosomes and notable areas of clear cytoplasm.

RESULTS

Non-stimulated, uncultured lymphocytes contained a marked predominance of Ti lymphocytes (Fig. 2). These cells closely resembled ‘B’ lymphocytes but had fewer cytoplasmic organelles and no polyribosomes.

In the non-stimulated, uncultured rosette preparation, the rosette-forming cells were 95-100 % Ti lymphocytes (Fig. 3). The sheep red blood cells were bound to these lymphocytes by a narrow area of cell membrane usually as a thin pseudopodial extension. The red cells were similarly distorted at the binding site. The distance between membranes at the binding site (binding gap) was a constant 7.5-10 nm (Fig. 4). There appeared to be only a single binding site between individual srbc and the lymphocyte in each plane of sectioning.

When non-stimulated rosettes were subjected to hypotonic lysis, with phosphate buffer saline, pH 7.4, 24 mosmol, there was little dissociation of the ruptured srbc membrane from the lymphocyte. The binding gap remained constant at 7.5-10 nm and there appeared to be layering of the srbc against the lymphocyte as though the srbc fragment was more easily able to conform to the lymphocyte surface (Fig. 5).

When PHA was added to the lymphocyte-rich media, either 15 min prior to adding the srbc or 15 min into the lymphocyte-srbc incubation period, there was a change in the lymphocyte population acting as rosette formers. Sixty-seven and 33 % of the RFC were T2 and Ti cells, respectively. No T3 lymphocytes were noted. In addition to the
change in RFC population from T1 to predominantly T2 lymphocytes, there was also an increase in the numbers and length of srbc-lymphocyte binding sites in each ultrathin section (Figs. 6, 7). The separation between membranes, the binding gap, remained 7.5-10 nm.

Lymphocyte cultures maintained for 48 h showed increased numbers of T2 lymphocytes. These cells were manifested by the development of numerous rosette-appearing polyribosomes and submembrane clearing. When lymphocytes cultured for 48 h with or without mitogen were subjected to rosette formation, the number of binding sites between individual srbc and the RFC increased and underwent marked continuous elongation (Fig. 8). The width of the binding gaps remained unchanged.

There were no demonstrable differences in binding morphology of RFC in cultures with PWM as compared to PHA or Con A. However, cultures containing PHA and Con A, incubated for 48 h, showed a new population of lymphocytes; more than 50% of the RFC were T3 lymphocytes. These T3-RFC demonstrated broad areas of srbc attachment with multiple sites per srbc, but the binding gap, as before, was a constant 7.5-10 nm (Figs. 9, 10).

DISCUSSION

Spontaneous rosette formation probably represents a non-immunologic T-cell marker (Lay, Mendes, Blanco & Nussenzaeig, 1971). The nature of the binding is not clear and the wide range of reported 'normal' percentages of rosette-forming cells among circulating lymphocytes indicates the differences in experimental techniques. The effect of temperature, time, pH and cation concentration (Lay et al. 1971) and the inhibition of rosette formation by various anti-immunoglobulins, anti-lymphocyte sera and cellular toxins has been well documented (Wybran & Fudenberg, 1971; Bach, Dorment, Dardenne & Balmer, 1969).

Morphologically, the srbc-lymphocyte attachment in spontaneous rosette formation noted in this study appears to be a firm apposition of cell membranes without fusion. The attachment is strong enough to persist despite hypotonic lysis of the red cells and there is a constant distance between bound membranes measuring 7.5-10 nm. The maintenance of this attachment would support the findings of Silveira, Mendes & Tolnai (1972), where, using fixed tissue preparations, he was able to demonstrate srbc binding to lymphocytes in patterns consistent with known 'T' and 'B' cell distribution. The binding gap and general appearance of apposition between membranes are similar to those documented by Gudat & Villiger (1973). In his study, however, he was using sensitized lymphocytes and ferritin-labelled anti-immunoglobulin; he was unable to detect immunoglobulin within the binding sites and showed a relatively consistent non-specific electron-dense pattern appearing like intercellular bridges. Such structures were inconstantly seen in our studies and the exact nature of these bridges is unknown; however, Zucker-Franklin & Bernley (1972), using ferritin-labelled srbc and ferritin antisera with immune RFC documented the presence of similar intercellular bridges but the exact membrane-to-membrane boundaries were not apparent.

The increased length of membrane binding in mitogen-stimulated lymphocytes has
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not previously been reported. Storb, Bauer, Storb, Fliedner & Weiser (1969) documented 2 distinct patterns of adherence in immune (previously sensitized) RFC; macrophage rosettes have a broad zone of adherence to the red cell, a majority of white lymphocytes show a 'point adherence' with a narrow zone of attachment. The distance between membranes was not determined.

This increased length of membrane attachment, along with increased binding sites per srbc, possibly represents lymphocyte 'activation.' No such lengthening or multiple binding sites were seen in unstimulated, uncultured RFC. The nearly immediate development of such changes with short PHA, Con A and PWM incubation, as well as the presence of similar changes in non-mitogen containing cultures at 48 h, is intriguing. Further, all cells, at 48 h, showed such binding changes and most of them showed intracellular structural changes compatible with the 'activated' (T2) or 'differentiated' (T3) lymphocyte of Matter et al. (1972). Other studies have shown that the percentages of RFC increase in cultures stimulated by Con A and PHA, immediately after addition of the mitogen, while PWM-RFC levels remain the same. However, [¹H]thymidine uptake by cultured cells increases in cultures stimulated by PHA, Con A and PWM, although to a notably lesser extent when PWM is the stimulant (unpublished data).

The development of T3 cells (Matter's differentiated lymphocyte) in all mitogen-containing cultures does not necessarily reflect the change in srbc-binding morphology. This is shown by the absence of T3 cells in the non-stimulated cultures at 48 h, yet the srbc-lymphocyte attachment was not distinguishable from the RFC formed in the presence of mitogen.

It is possible that the alterations of srbc-binding characteristics described here represent a means of recognizing alterations which 'virgin' T cells undergo during the process of cell activation. Certainly the earliest consistent changes documented in these studies of activated cells were the alterations in srbc-binding patterns, i.e. an increased number of binding sites per srbc and marked lengthening of each individual attachment. Although the results presented cannot substantiate a definite correlation between altered srbc binding and T cell activation, they suggest that further evaluation of the concept would be worthwhile.

The absence of B cells in rosette formation based on this classification further supports the specificity of spontaneous rosette formation as a T cell marker.

The persistent attachment of the srbc fragments in the lysed preparation initiated an additional study utilizing srbc membrane ghost fragments. Staining of the lymphocyte cultures, stimulated and non-stimulated, with the fluorescein-conjugated srbc membrane fragments confirm the initial electron-microscopic finding that the binding of srbc to T lymphocytes does not require an intact srbc (Fig. 11). Follow-up work to determine the specificity (although no cross-reactivity was seen with labelled anti-immunoglobulins) and the usefulness in tissue sections will be documented.
CONCLUSION

The pattern of srbc-RFC attachment is possibly a method of detecting early lymphocyte activation. By using a previously described ultrastructural lymphocyte classification along with the pattern of RFC-srbc membrane attachment, we have separated T lymphocytes into 3 distinct groups:

T1-RFC (‘resting’ lymphocytes) generally formed by the uncultured lymphocytes without mitogen.

T2-RFC (‘activated’ lymphocytes), which show ultrastructural characteristics similar to Matter’s T1 or T2 lymphocyte but which have a distinct pattern to the srbc-lymphocyte membrane attachment. These cells were seen in all RFC when treated with mitogen and in cells not exposed to mitogen when cultured for 48 h.

T3-RFC (‘differentiated’ lymphocytes) which have characteristic intracellular changes. These cells were seen only in the mitogen-containing cultures and they maintained the membrane-attachment pattern of the ‘activated’ cells.

As a part of this study, preliminary work is presented using fluorescein-labelled srbc-membrane fragments as a rapid method of detecting ‘T’ lymphocytes.

REFERENCES


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Fig. 1. Lymphocyte surrounded by sheep red blood cells = 'rosette' formation. × 450.
Fig. 2. High-power electron micrograph of the characteristic rosette-forming lymphocyte in the resting or 'virgin' state (T↓ lymphocyte). × 33 250.
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Fig. 3. Low-power electron micrograph of non-stimulated rosette-forming (T1) lymphocyte. Binding sites are by attenuated pseudopodial cytoplasmic extensions from both the lymphocyte and srbc. × 7500.

Fig. 4. High-power electron micrograph of the binding site between a non-stimulated rosette-forming (T1) lymphocyte and a sheep red blood cell showing a 7.5–10 nm separation at the binding site. × 57000.
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Fig. 5. High-power electron micrograph of a non-stimulated uncultured rosette-forming lymphocyte with sheep red blood cells which have been subjected to phosphate buffer (24 mosmol) lysis. There are numerous breaks in the red cell membrane and loss of most of the stroma. The remaining membrane fragment is bound to the T1 lymphocyte by a constant 7.5–10 nm electron-lucent gap. x 27000.

Fig. 6. Low-power electron micrograph of a T1 lymphocyte which has been incubated with PHA following rosette formation. The individual sheep red blood cells show multiple points of binding with the lymphocyte. x 10000.
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Fig. 7. Low-power electron micrograph of a T2 lymphocyte with 0.2–0.4 μm membrane-bound, dark round granules within the cytoplasm, rosette-like polyribosomes and increased cytoplasmic organelles. The preparation was incubated with PHA for 15 min prior to rosette formation and shows an increase in the number of binding sites and an increased length of the individual membrane attachments. (The large dark round bodies primarily on the erythrocytes are precipitated lead artifact.) \( \times 10000 \).

Fig. 8. High-power electron micrograph of the binding site of a 48-h incubated T2 lymphocyte and a sheep red blood cell. There is marked elongation of the binding site on the lymphocyte surface; however, the binding gap remains constant at 7.5–10 nm. \( \times 60000 \).
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Fig. 9. Low-power electron micrograph of a rosette-forming lymphocyte stimulated for 48 h with Con A. The lymphocyte is a T3 cell and forms broad areas of binding to the sheep red blood cells. × 7500.

Fig. 10. High-power electron micrograph of the binding site of the sheep red blood cell to a T3 lymphocyte which has been incubated for 48 h with Con A. The binding gap is 7.5–10 nm and extends over a broad area of the lymphocyte surface. × 55000.
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Fig. 11. Normal human lymphocytes stimulated in culture for 96 h with Con A and stained by the direct fluorescein-conjugated sheep red blood cell fragments. $\times 450$. 