

C3-MEDIATED CYTOADHERENCE

Formation of C3 Receptor Aggregates as Prerequisite for Cell Attachment*

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It is widely accepted that the antibody-triggering mechanism of thymus-dependent antigens relies on cooperation among thymus-derived lymphocytes (T cells), bone marrow-derived lymphocytes (B cells), and macrophages (1-4). These cells maintain close contact during their cooperation (4-6). When C3, the third component of complement, was hypothesized to be another participant in the antibody-triggering mechanism, besides acting directly on B lymphocytes (7,8), C3 was suggested to facilitate cell cooperation (9), e.g., C3-carrying macrophages could adhere to B lymphocytes via their characteristic C3b receptors. Therefore we undertook to clarify what properties are required for such a C3-dependent cell to cell adherence. As test model we used adherence of C3b-carrying sheep erythrocytes (EAC142 3b cells) to human lymphoid cells. When we assayed this interaction in the rosette formation test, a certain density of C3b receptor sites on the receptor carrying cells was critical. This was achieved only by locally concentrating the C3b receptors in small aggregates.

Materials and Methods

Reagents and Buffers. Alpha-iodoacetamide, diisopropylfluoro phosphate (DFP), phenyl-methylsulfonyl fluoride, (PMSF), and colchicine were purchased from Calbiochem, La Jolla, Calif.; bovine serum albumin (BSA) from Miles Laboratories, Kankakee, Ill.; glutaraldehyde (50% in H₂O), sodium azide from Matheson, Coleman and Bell, Norwood, Ohio; and EDTA (sodium salt of ethylenedinitrilo tetraacetic acid) from Mallinckrodt, St. Louis, Mo. Fluorescein isothiocyanate (FITC) anti-C3 serum was a gift from Dr. V. A. Bokisch (Scripps Clinic and Research Foundation, La Jolla, Calif.). The buffers used were phosphate-buffered saline (PBS), Hanks' balanced salt solution (HBSS) and Eagle's minimum essential medium (MEM, Autopow Flow Laboratories, Rockville, Md.).

Cells. EAC142 3b cells were prepared (10) from sensitized sheep erythrocytes (EA) and purified human complement components (a gift from Dr. N. R. Cooper, Scripps Clinic and Research Foundation, La Jolla) so that each cell carried about 10⁵ C3b sites. For some experiments EAC142 3b cells were prepared with varying amounts of C3b per cell. Normal human peripheral blood lymphocytes and cultured human lymphoid cells (Raji) were obtained as described (10).

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Rosette Formation Test. 100 μ l of a lymphocyte suspension (1×10^6 cells/ml HBSS) + 100 μ l of HBSS containing 6×10^{-2} M EDTA + 100 μ l EAC142 3b cells (5×10^7 cells/ml PBS) were left standing 30 min at 37°C and shaken by hand every 10 min. In some experiments the temperature was 23°C and in others 3°C. Then the percentage of rosetted lymphocytes was determined by counting under a light microscope.

Glutaraldehyde Treatment. Samples of 1 ml of a Raji cell suspension (2×10^6 cells/ml HBSS) were incubated with 1 ml of glutaraldehyde (2×10^{-5} to 2×10^{-1} % in PBS) for 10 min at 37°C. Then the cells were washed once in 1% BSA and three times in HBSS and were tested in the rosette formation test.

Immunofluorescent Staining. 5×10^5 Raji cells (treated with and without glutaraldehyde) were suspended in 50 μ l C3 (0.8 mg/ml), incubated for 30 min at 37°C and washed three times in PBS containing 0.1% BSA. The cells were pelleted and 50 μ l of FITC-anti-C3 serum was added. After an incubation of 30 min at 4°C, the cells were washed three times in MEM and for a wet preparation a drop of the cell suspension was sealed with paramount under a cover slip. A Zeiss interference filter system (Carl Zeiss, Inc., New York) utilizing transmitted light from an HBO 200 watt mercury light source was used to observe the results. In some experiments Raji cells were incubated with C3 at 3°C and at 37°C, washed, treated with 0.01% glutaraldehyde, washed again, and then processed for staining with FITC-anti-C3 serum.

Results and Discussion

By reacting EAC142 3b cells with lymphocytes from patients with chronic lymphatic leukemia in the rosette formation test, it was shown that the percentage of rosettes depends on the input of C3 (11). In our experiments 1×10^4 C3 sites per EAC142 3b cell and 3.5×10^4 C3 sites per EAC142 3b cell, respectively, were required to allow 50% of the maximally possible rosette formation with human peripheral blood lymphocytes or Raji cells (Fig. 1). The smallest average distance between the C3 sites is about 450 Å in the case of 3.5×10^4 per EAC142 3b cell, assuming a diameter of 5 μ m for sheep erythrocytes and homogeneous distribution of the C3b sites over the surface of the EAC142 3b cells. Since the C3b sites on EAC142 3b cells were shown to be grouped in clusters (12), the actual distance between the C3b sites must be much smaller.

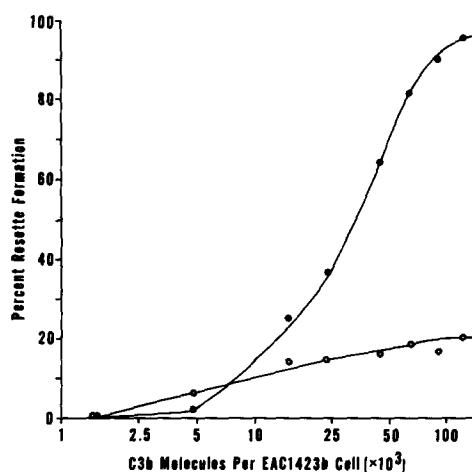


FIG. 1. Dependence of rosette formation on the amount of C3b molecules per EAC142 3b cell. Closed circles: reaction with Raji cells; open circles: reaction with human peripheral blood lymphocytes.

Requirements on the part of C3b receptor carrying lymphocytes for C3b-dependent cytoadherence are more difficult to assess. We questioned whether lymphocytes in their native state would allow C3-dependent cytoadherence or if the lymphocytes' surfaces would have to undergo certain changes, such as redistribution of surface proteins, to be ready for cytoadherence. It is known that rosette formation is temperature-dependent (13). In line with this finding we did not obtain any rosettes at 3°C but at 23°C the yield was 48% rosettes and at 37°C, 89%. This result has two possible explanations: involvement of a temperature-dependent enzymatic process or involvement of a rearrangement process, like patch and cap formation, whose temperature dependence is well documented. The first possibility was checked by exposing Raji cells for 20 min at 37°C to varying concentrations of several enzyme inhibitors, then adding EAC142 3b cells (37°C, 10 min) and assessing the inhibitors' influence on the Raji cells' capacity to form rosettes. None of the inhibitors interfered with the receptors' activity. The highest concentration at which the inhibitors were applied was: 1.3×10^{-2} M sodium azide, 1×10^{-4} M DFP, 5×10^{-4} M PMSF, 1×10^{-2} M alpha-iodoacetamide, 1×10^{-2} M EDTA. Also colchicine (3×10^{-3} M) was without effect.

To challenge the second possibility we partially or completely fixed the surfaces of Raji cells in their native state by pretreating the cells with increasing amounts of glutaraldehyde (up to $5 \times 10^{-2}\%$). As seen in Fig. 2, with increasing glutaraldehyde concentrations, the Raji cells lost their capacity to bind EAC142 3b cells, indicating a role of free receptor movement for C3-dependent cytoadherence. The same was true for EAC142 3d cells (unpublished data).

To rule out the chance that this loss of binding capacity was from inactivation of the C3 receptors themselves rather than from reduction of free movement by membrane constituents, the following experiment was done. Raji cells, which

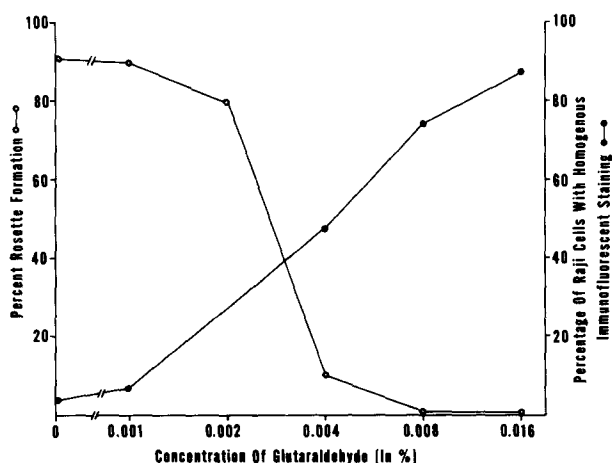


FIG. 2. Effect of glutaraldehyde fixation on Raji cells. Open circles: effect on rosette formation between Raji cells and EAC142 3b cells. Closed circles: effect on C3 modulated C3 receptor distribution as demonstrated by exposing the Raji cells to C3, and consecutively to FITC anti-C3 serum.

had been treated with PBS or with different concentrations of glutaraldehyde (10 min, 37°C) and then washed, were incubated for 30 min at 37°C with and without soluble C3, washed in PBS and then stained with FITC anti-C3 serum. All cells were stained, except those which had not encountered C3. Raji cells which had been treated with glutaraldehyde before exposure to C3 showed a homogeneous pattern of the stain (Fig. 3 A); whereas, if glutaraldehyde was omitted, C3

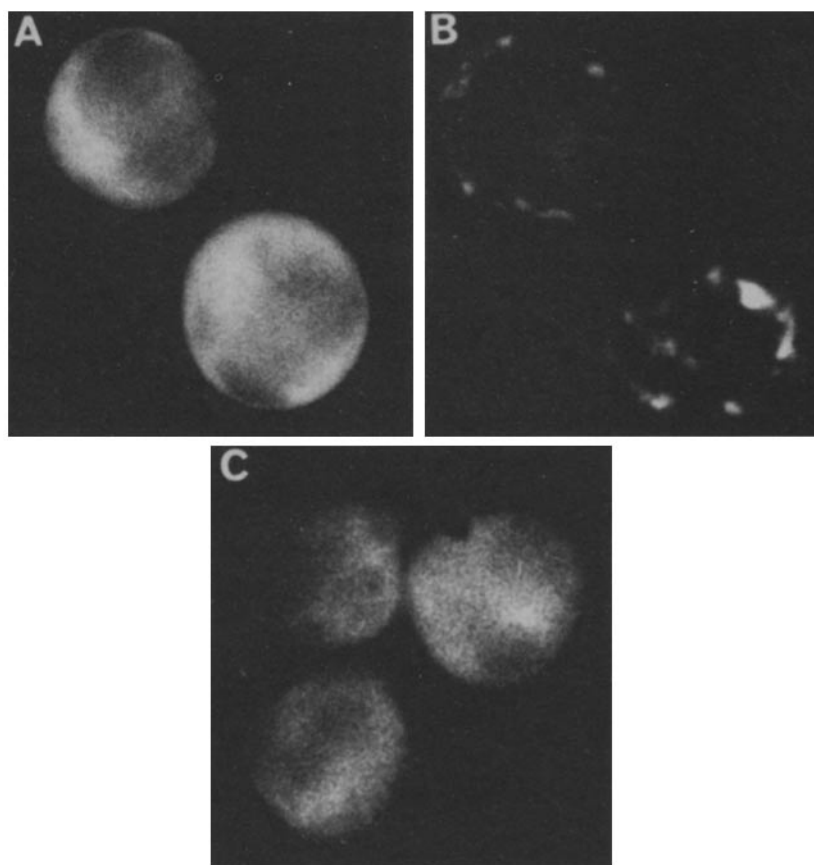


FIG. 3. Immunofluorescent staining (FITC anti-C3 serum) patterns of Raji cells. (A) After incubation in 0.01% glutaraldehyde and exposure to C3 for 30 min at 37°C; (B) after incubation in HBSS and exposure to C3 for 30 min at 37°C; (C) after incubation in HBSS and exposure to C3 for 30 min at 3°C, followed by staining with FITC-anti-C3 serum for 30 min at 4°C.

induced a patchy staining (Fig. 3 B). The latter pattern was achieved also, if after exposure to C3 and before staining with FITC-anti-C3 serum the cells had been fixed with glutaraldehyde, ruling out the need for a cross-linking antibody during C3-induced microaggregation of C3 receptors. Thus, conditions which caused reduction of rosette formation also reduced the C3-mediated, patchy distribution of C3 receptors and increased the percentage of cells with homogeneously distributed C3 receptors (Fig. 2). These findings suggest the importance of

microaggregation of these receptors for C3-dependent cytoadherence. Such a concept is substantiated by the fact that at 3°C, neither rosettes were formed, nor could C3 induce microaggregation, provided also the incubation with FITC-anti-C3 serum was performed at this low temperature (Fig. 3 C). This is in agreement with the temperature dependence of patch formation induced by cross-linking Ig receptors (14-16) or lectin receptors (16,17).

Raji cells reportedly carry on the order to 10^5 C3 receptors per cell (18). This density apparently is not high enough to cause effective adherence of EAC142 3b cells, since local receptor aggregation seems to be a prerequisite for a firm interaction. Since EAC142 3b cells can trigger production of chemotactic factors upon interaction with B lymphocytes (19), it is reasonable to assume that the microaggregation itself or a concomitant alteration of the cell surface is operative in this process.

It will add to our knowledge of cell-cell interactions in general to study if microaggregation of the corresponding receptors is required not only for C3-dependent cell-to-cell adherence but also for cytoadherence mediated by Fc receptors (13, 20) or Ig-like antigen receptors (4).

Summary

Inhibition of free movement of C3 receptors by either applying low temperature (3°C) or fixing the cell surface of lymphocytes with glutaraldehyde (2×10^{-5} to $2 \times 10^{-1\%}$) results in loss of firm attachment of EAC142 3b cells to the lymphocytes as demonstrated here by loss of rosette formation of Raji lymphoid cells. Under the same conditions soluble C3 can still bind but is unable to induce aggregation of C3 receptors into small patches. It is suggested that the local increase of C3 receptor density by aggregation is a prerequisite for C3-dependent cytoadherence. Microaggregation of corresponding receptor sites may be essential also in other recognition systems.

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