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Activation of Human Complement by Human Lymphoid Cells Sensitized with Histocompatibility Alloantisera*

(membrane/cytotoxic test)

S. FERRONE, N. R. COOPER, M. A. PELLEGRINO, AND R. A. REISFELD

Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037

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ABSTRACT **Cultured human lymphoid cells sensitized with human histocompatibility (HL-A) antibodies were able to activate the human complement system *in vitro*. Some HL-A alloantisera selectively activated the alternate complement pathway while other antisera activated only the classical pathway. A third group of alloantisera was equally able to initiate complement action by way of either pathway. The mechanism of complement activation did not correlate with the HL-A antigen present on the cells or the HL-A specificity of the alloantisera, indicating that the antigenic determinants or distribution on the cell surface play no direct role in selecting the pathway of activation. In this completely homologous system the alternate pathway was found to have the same cytolytic potential as the classical pathway. Thus, an altered or damaged membrane is not a prerequisite for the production of cytolytic damage by the alternate pathway. A complete understanding of the mechanism of interaction of membrane bound antigens and antibodies with the complement system may provide a versatile tool for the investigation of membrane antigen expression.**

Cell membranes play a fundamental role in conditioning host reactions of the cells to the internal and external environment. There is considerable evidence that structural and antigenic characteristics of the cell membrane determine the fate of normal transplanted and tumor cells in a host. Among several antigenic systems of the cell membrane, histocompatibility (HL-A) antigens have been extensively studied primarily for their relevance in clinical transplantation. How they function in cell economy, completely unrelated to the man-made situation of allotransplantation, is still unknown.

Serological characterization of HL-A antigens is currently based on the lysis of lymphoid cells by HL-A-specific alloantisera and complement in the complement-dependent cytotoxic test. Differential cytopathic effects of HL-A alloantisera are a common observation in HL-A typing of cells; variations in the requirement of complement among different HL-A specificities have also been described, and certain combinations of antigen and alloantibody such as HL-A4b are often not cytolytic (1). Further, only certain HL-A cytotoxic antibodies cause a hyperacute rejection of kidney transplants (2).

Two pathways of complement activation have been described (3–5). One of these, the classical pathway, begins with the reaction steps involving the first (C1), fourth (C4), and second (C2) components, while the other, the alternate pathway, involves an unknown number of proteins, of which three, C3 proactivator convertase (C3PAse), hydrazine-sensitive factor (HsFa), and C3 proactivator (C3PA), have been isolated. These pathways converge at the step involving C3, and the remaining steps involving C5, C6, C7, C8, and C9 are common to both pathways. The two pathways differ in requirements for divalent cations; the alternate pathway requires magnesium, in contrast to the classical pathway where calcium is necessary (6).

We have investigated the mechanism of activation of complement by human lymphoid cells sensitized with HL-A alloantisera and determined the relationship of the pathway of complement activation to the cytopathic potential and HL-A specificity of the alloantisera.

MATERIALS AND METHODS

Human Lymphoid Cell Lines. RPMI 1788 (HL-A, 2, 10, 7, 14) and RPMI 8866 (HL-A 2, 3, 7, 12) cell lines were grown in suspension cultures in RPMI 1640 medium containing 10% fetal-calf serum, while WI-L2 cells (HL-A 1, 2, 5, 17) were perpetuated in minimal essential medium containing 10% fetal-calf serum. All cells were washed three times with Hank's balanced salt solution, resuspended in balanced salt solution, and adjusted to the desired concentration before testing.

HL-A Alloantisera. HL-A alloantisera were obtained from the serum bank at the National Institute of Allergy and Infectious Diseases and from the laboratories of Drs. R. Ceppellini, R. Payne, and P. I. Terasaki. These sera are only operationally monospecific as they contain weak, contaminating antibodies. All alloantisera were thawed and frozen several times.

Complement. A pool of fresh sera derived from five healthy human donors with no previous history of pregnancy or blood transfusion served as the source of complement. The complement pool was stored at −70° in small aliquots. Complement components C1, C2, C3, C4, C5, C6, C7, C8, C9, and C3PA, isolated from human serum by published methods (5, 7–11, and Haxby, J. &. Müller-Eberhard, H. J., manuscript in preparation), were used for molecular hemolytic titrations (9, 10, 12, 15) and for binding studies. C2 and C3PA were tested for rheumatoid factor activity and certain allotypic specificities by agglutination of tanned sheep erythrocytes and latex particles coated with human fraction II (FII and Hyland RAf test) and found to be negative at maximal concentrations of 3 and 30 μg, respectively.

Abbreviations: HL-A, human histocompatibility antibodies; C1, C2, ..., complement components; C3PAse, C3 proactivator convertase; C3PA, C3 proactivator; HsFa, hydrazine-sensitive factor.

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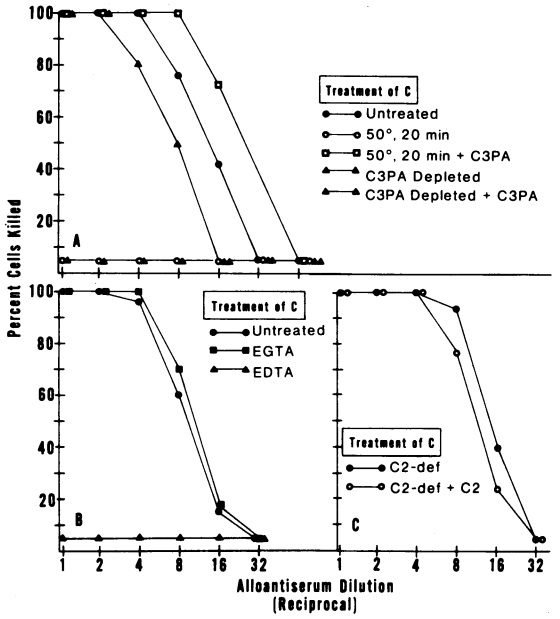

FIG. 1. Cytolytic activity of some HL-A alloantisera against human lymphoid cells (WI-L2 or RPMI 8866) in conjunction with human complement inhibited in the classical and/or alternate pathway. Alloantisera: (*A*) Stockenberg; (*B*) Pinquette; (*C*) Jackson. *def*, deficient.

C3PA antiserum was produced in goats as described (5). Preparations of C3, C5, C8, C9, and C3PA were trace labeled with ^{125}I or ^{131}I by the chloramine T method (14) without loss of activity and used to quantitate cell binding. The specific radioactivity was, respectively, 0.35×10^5, 6.6×10^5, 1.9×10^5, and 7.8×10^4 cpm/μg of C3, C5, C8, and C9, values which correspond to 0.035, 0.66, 0.19, and 0.78 μCi/μg.

Human complement deficient in C2 was obtained from a patient with inherited C2 deficiency (15). This serum had no detectable hemolytic activity or deficiency of other components of the complement system (15). Full lytic activity was restored on addition of purified C2 to this serum. Human complement was depleted of C3PA by heating of the serum at 50° for 20 min or through the use of an immunoabsorbent composed of unsolubilized antiserum to C3PA. Quantitative immunodiffusion studies showed that 60–70% of the C3PA protein was destroyed by 20 min of heating at 50°; no C3PA was detectable in serum treated with an immunoabsorbent to C3PA. No C3 destruction was observed on addition of inulin (10 mg/ml) to sera depleted of C3PA, documenting the inability of such sera to sustain alternate pathway activity. The levels of C3, C4, and C8 in the complement source after incubation with lymphoid cells sensitized with HL-A alloantisera were quantitated by hemolytic measurements (9, 10, 12, 13). The results were expressed as the percent of the number of effective molecules remaining in comparable samples prepared with normal human serum in place of HL-A alloantisera.

Human complement treated with 0.01 M EGTA [ethylene glycol-bis(betaamino ethyl ether)-*N*,*N*'-tetraacetic acid, Calbiochem, La Jolla, Calif.] demonstrated no hemolytic activity for sensitized sheep cells. C3 cleavage was observed on addition of inulin (10 mg/ml) to such sera, documenting the integrity of the alternate pathway in the sera. Sera treated with 0.01 M EDTA (disodium ethylenediaminetetraacetate; Mallinckrodt Chemical Works, St. Louis, Mo.) were unable to lyse sensitized cells or to mediate C3 destruction after addition of inulin.

Cytotoxic Assay. The eosin exclusion microcytotoxic test with cultured lymphoid cells was performed as described (16). Human complement was treated so as to inactivate one or the other complement pathway as described below.

Complement Studies. Packed washed cultured human lymphoid cells (5×10^5) in the logarithmic stage of growth were incubated at 37° for 10 min with 20 μl of HL-A alloantisera previously heated at 56° for 30 min. Then 40 μl of human complement was added, and incubation was continued for 60 min. Subsequently the cells were sedimented and the supernatant serum was frozen in aliquots for subsequent C3, C4, and C8 titrations.

In order to determine binding of complement components, ^{125}I- and ^{131}I-labeled C3, C5, C8, or C9 were incorporated into the human complement source. In a typical experiment 0.53 or 0.22 μg of ^{125}I-labeled C3 or C9 and 0.31 or 0.44 μg of ^{131}I-labeled C5 or C8 were added to the approximately 54.0, 3.0, 3.4, and 6 μg of C3, C5, C8, and C9 (Calcott, M. A. & Müller-Eberhard, H. J., manuscript in preparation), respectively, contained in the human complement source. The studies were performed as described above except that the cells were washed three times after the 60-min incubation period and analyzed for radioactivity in a well-type scintillation counter. Nonspecific binding of complement components was determined from reaction mixtures, which included heat-inactivated normal human serum in place of specific antisera. Nonspecific cellular binding of C3, C5, C8, and C9 averaged 1% of the total radioactivity offered to the cells. Molecular weights of 185,000, 200,000, 150,000, and 79,000 were used to calculate the number of bound C3, C5, C8, and C9 molecules, respectively. In calculating these values, only the bound

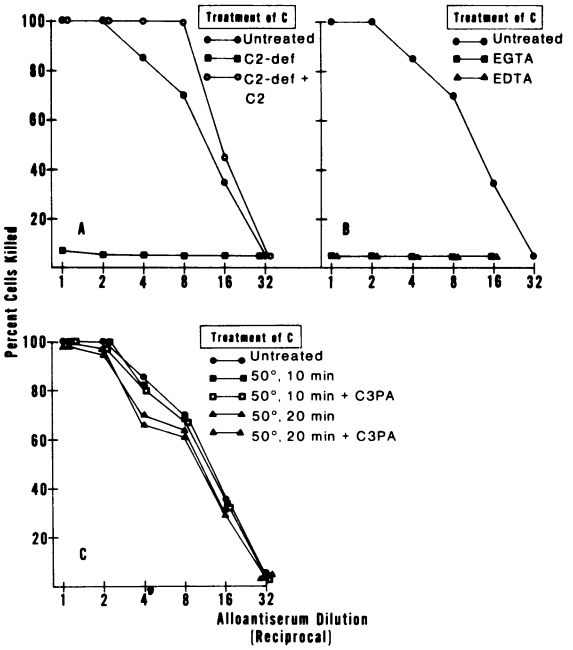

FIG. 2. Cytolytic activity of HL-A alloantisera (De Sande) against human cultured lymphoid cells (WI-L2) in conjunction with human complement inhibited in the classical and/or alternate pathway.

radiolabeled molecules were considered, since the number of unlabeled active C3, C5, C8, and C9 molecules in the alloantisera and complement sources and bound to the cells was unknown.

RESULTS

The following approaches have been used to determine the mechanism of activation of the complement system by lymphoid cells sensitized with HL-A alloantisera. First, the ability of HL-A alloantisera to mediate lysis of cultured lymphoid cells in conjunction with human complement selectively depleted of components of the classical or alternate pathway was measured. Second, the pattern of consumption of C3, C4, and C8 in serum incubated with lymphoid cells sensitized with HL-A antibodies was determined. Third, cellular binding of radiolabeled components in human complement incubated with lymphoid cells sensitized with HL-A antibodies was assessed.

The results indicate that HL-A alloantisera can be divided into three groups: (*i*) those which activate only the alternate pathway of complement, (*ii*) those which activate only the classical pathway, and (*iii*) those which activate both pathways.

Activation of the Alternate Pathway of Complement. Human complement, which was depleted of an essential component of the alternate pathway by partial destruction of C3PA by heating at 50° for 30 min or by removal of C3PA with an immunoabsorbent, was not capable of mediating lysis of lymphoid cells sensitized with certain HL-A alloantisera (Fig. 1*A*). The loss of lytic activity, which was proportional to the length of heating, was complete after 20 min. The addition of highly purified C3PA fully restored the lymphocytotoxic activity to this previously heated serum, while other complement components such as C1, C3, C8, and C9 were unable to do so. C3PA by itself, with or without human complement, was not cytolytic to human lymphoid cells. Increasing amounts of C3PA progressively restored the lytic efficiency of C3PA-depleted complement in the lymphocytotoxic test; in fact at the highest amount of C3PA added (1.6 μg for 2 μl of serum), the titer of the alloantisera exceeded that obtained with native untreated human complement. The addition of C3PA to untreated human complement also enhanced the lysis of cultured cells sensitized with HL-A alloantisera.

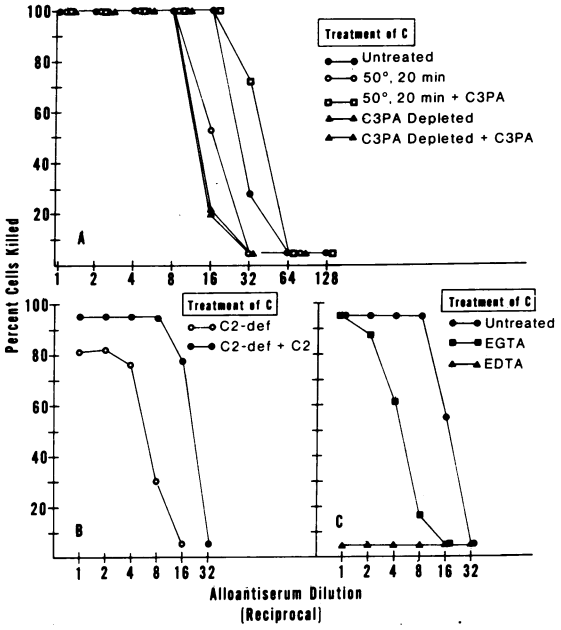

FIG. 3. Cytolytic activity of HL-A alloantisera (Victor) against human cultured lymphoid cells (WI-L2) in conjunction with human complement inhibited in the classical and/or alternate pathway.

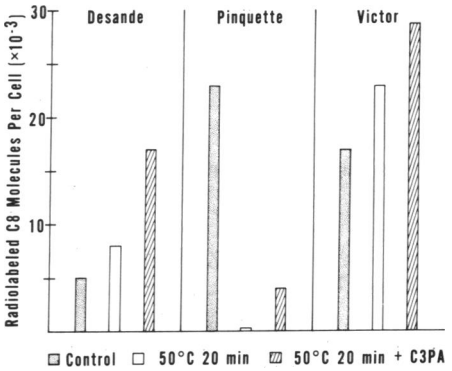

FIG. 4. Binding of radiolabeled C8 by cultured human lymphoid cells WI-L2 sensitized with HL-A alloantisera that activate the classical pathway (*left*), the alternate pathway (*center*), or both pathways (*right*). Comparable results were obtained in identical studies with labeled C3, C5, and C9. The numbers on the *ordinate* have been multiplied by 10^{-3}.

In contrast, these same alloantisera were fully able to initiate lysis of lymphoid cells incubated with complement selectively deficient in a component of the classical pathway. Thus, serum deficient in C2 was able to mediate lysis of sensitized lymphoid cells (Fig. 1*C*). Similarly, serum in which C1 was inactivated by treatment with EGTA (10 mM) was able to initiate lysis (Fig. 1*B*).

Activation of the Classical Pathway of Complement. With other HL-A alloantisera, complement deficient in C2 was not able to mediate cytolysis. Full cytolytic activity was, however, restored on addition of C2 to the deficient complement. The addition of EGTA (final concentration 10 mM) to the complement source to chelate calcium and inhibit the classical pathway completely prevented lysis. In contrast, inactivation of the alternate pathway by gentle heating of the complement source (50°, 30 min) had no essential influence on its cytolytic potential (Fig. 2).

Activation of Both Pathways of Complement. Still other HL-A alloantisera were able to activate both pathways of complement. With these sera, lymphocytotoxicity could be mediated by complement depleted of C3PA, deficient in C2, or depleted of calcium. EDTA (final concentration 10 mM), an efficient chelator of calcium and also magnesium which is required for the activation of both pathways, prevented lymphocyto-toxicity on addition to the complement source (Fig. 3).

Consumption of Complement Components. While the extent of depletion of C3 and C8 activity in the complement source did not vary markedly after incubation with lymphoid cells sensitized with the various HL-A alloantisera, C4 consumption was found to be greater with alloantisera that activated

only the classical pathway than with antisera that selectively triggered the alternate pathway. Activation of the classical pathway is known to consume considerably more C4 than the alternate pathway (17).

Effect of C3PA Inactivation on the Binding of Labeled Complement Components. Alloantisera that activated the classical and alternate pathways of complement differed in their ability to bind C8 after destruction of C3PA in the complement source by heating (Fig. 4). Thus, alloantisera that activated the classical pathway exhibited increased C8 binding after heating of the complement source. In contrast, alloantisera that activated complement by way of the alternate pathway mediated decreased C8 binding after heating the complement source. Addition of C3PA to the heated human complement partially restored the uptake of labeled C8. Comparable results were obtained in identical studies with labeled C3, C5, and C9.

Relationship of HL-A Specificity to Complement Activation. The pathway of activation of complement did not appear to be related to the HL-A specificity, since HL-A alloantisera with the same specificity could be shown to activate different pathways. Similarly, no correlation was found between the pathway of activation of complement and the cytolytic activity of the sera. A direct relationship was generally observed between the uptake of labeled C8 and the titer of HL-A alloantisera.

DISCUSSION

Cultured lymphoid cells sensitized with HL-A alloantisera activate the complement system by different pathways. Some bypass C1, C4, and C2 in their attack on C3, as lysis is mediated by C2-deficient serum and is not affected by depletion of calcium from the reaction mixture, an ion required for the activity of C1. No lysis occurs when these alloantisera are tested with complement depleted of C3PA, but lytic activity returns when C3PA is added. Other HL-A alloantisera activate complement through the classical pathway, as deficiency of C2 or inhibition of C1 by chelation of calcium with EGTA completely prevent lysis of cultured lymphoid cells. Still other alloantisera mediate lysis through both pathways, as depletion or inactivation of a factor(s) of one of the pathways does not significantly affect the lysis of target cells.

The choice of pathway does not correlate with the specificity of the HL-A alloantisera or with the target cell, indicating that the antigenic type or distribution on the cell surface play no direct role in activating complement. This property appears to be determined by HL-A alloantibodies. We have not as yet determined if activation of one or another of the pathways correlates with the immunoglobulin class or subclass. If this is the case, this would be analogous to guinea pig immunoglobulin G, where different subclasses activate different pathways of complement (3), and to human immunoglobulins, where different classes activate different pathways (18).

In our work the two pathways of complement were equally able to mediate lysis of human lymphocyte sensitized with human alloantisera. In subsequent work we have found that these same HL-A alloantisera are able to activate only the classical pathway when guinea pig serum is used as the source of complement. The different source of complement and the possible presence of natural antibodies in the reaction mixture (19) probably explain the discrepancy between our results and those of May *et al.* (20). May *et al.* found that murine alloantisera mediated lysis of murine lymphocytes only by way of activation of the classical pathway.

This is the first demonstration of cytotoxic damage by the alternate complement pathway to unaltered cells in a completely homologous system. A recent report by May and Frank (21) indicates that the alternate pathway may also damage sheep erythrocytes heavily sensitized with heterologous antisera. Before this report, numerous studies suggested that the alternate pathway was unable to mediate cytolytic damage unless the cells had undergone membrane damage (20, 22).

The relationship of these findings to the events *in vivo* that occur after allotransplantation cannot be assessed at present. The model system *in vitro* that we have investigated is completely homologous and in all likelihood quite physiologic. In addition to aiding in the selection of compatible donor-recipient combinations in clinical transplantation, it is anticipated that a complete understanding of the mechanism of interaction of membrane-bound antigens and antibody with the complement system may provide a versatile tool for the investigation of membrane antigenic expression.

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