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Inhibition of T Lymphocyte Rosetting by HL-A Alloantisera and Complement[1] (39201)

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(Introduced by R. A. Reisfeld)

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In the fluid mosaic model of the mammalian cell membrane (1) HL-A antigens, which are protein in nature, are mobile structures embedded in lipid bilayers. In fact, after human lymphoid cells were reacted with specific alloantibodies, HL-A antigens moved from a homogeneous distribution to form discrete aggregates or caps on the cell's surface (2). In such experiments, HL-A antigens were not associated with immunoglobulins (3) but were closely linked to β_2-microglobulins on the cell surface (4). Therefore, the spatial and molecular relationships of HL-A determinants to other cell surface structures are under active investigation to define the architecture of cell membranes and the role of HL-A in it. In the present study we have coated peripheral human lymphocytes with HL-A antibodies to investigate their influence on rosette formation with sheep red blood cells (SRBC).

Materials and methods. Human peripheral lymphocytes. Heparinized peripheral blood was taken from healthy subjects with known HL-A phenotypes and lymphocytes were isolated by differential centrifugation on Ficoll-Isopaque gradient (5).

Cultured human lymphoid cells. Cell lines RPMI 1788 and RPMI 4098 were perpetuated in medium RPMI 1640 containing 10% fetal calf serum. The culture flasks were rotated continuously on a platform shaker. Cell viability was assessed by the uptake of trypan blue. Before use, cells were washed 3 × with Hanks' balanced salt solution (HBSS).

Antisera. 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. All of the sera had been frozen and thawed several times. A goat anti-human γ-globulin serum was a gift from Dr. H. Spiegelberg, Scripps Clinic and Research Foundation. IgG were obtained from an anti-HL-A5 alloantiserum ("VICTOR") by 15% Na_2SO_4 precipitation and QAE-Sephadex (A-50) column (6). Fluorescein isothiocyanate (FITC)-conjugated IgG was prepared as described by Goldman (7).

Complement. Rabbit serum deficient in the sixth complement component (C6) and guinea pig serum deficient in the fourth complement component (C4) were obtained from animals with genetic C6 and C4 deficiencies. Rabbit complement (5 vol) was absorbed with 1 vol of packed, washed, cultured human lymphoid cells by incubation at 4° for 60 min with mixing at 10-min intervals. In order to reduce loss of complement activity the absorption of rabbit serum was performed in the absence of free divalent cations (8). Neutralized EDTA (disodium ethylenediamine tetracetate, Mallinckrodt Chemical Works, St. Louis, Mo.) or EGTA (ethyleneglycol-bis β-aminoethyl ether *N*, *N'*-tetracetic acid, Calbiochem, La Jolla, Calif.) were added to complement to a final concentration of 10 m*M* to chelate magnesium and calcium or calcium alone, respectively.

Microcytotoxicity and microabsorption tests were performed as previously described (9, 10).

Rosette formation. This test was per-

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formed as described by Jondal *et al.* (11) and Froland (12) with modifications (5): briefly, 25 μl of a lymphocyte suspension (2 $\times$ 10^6 cells/ml) was mixed with 25 μl of a 1% suspension of SRBC in a Fisher tube, centrifuged at 500*g* for 1 min (Fisher centrifuge), and then incubated at 4° for up to 20 hr. The cell pellets were resuspended by gently tapping the tubes, and a minimum of 200 lymphocytes was counted in a hemocytometer. Lymphocytes were counted as rosette positive if three or more SRBC adhered to them. The percentage of dead lymphocytes was determined by trypan blue exclusion.

When HL-A alloantisera with or without the addition of complement or of anti-human γ-globulin serum were evaluated for inhibition of rosette formation, packed lymphocytes (1×10^5 cells) were incubated with 50 μl of serum for 60 min at room temperature, then washed twice with HBSS and either utilized directly for the rosette formation test or incubated with serum that was the source of complement, with anti-human γ-globulin serum, or with HBSS. For the former procedure, 75 μl of either complement, anti-human γ-globulin serum or HBSS was added to the lymphocytes, and after 30 min of incubation at room temperature, cells were washed twice with HBSS and then mixed with SRBC.

Results. Human peripheral lymphocytes sensitized with HL-A alloantisera were perfectly capable of forming rosettes with SRBC even after incubation with anti-human γ-globulin serum (Table I). In contrast, the addition of C6 deficient rabbit serum to HPL sensitized with HL-A alloantisera inhibited this reaction (Table I). C6 deficient rabbit serum was chosen as a source of complement because it does not lyse HL-A antibody coated lymphocytes, since only viable human T lymphoid cells can form rosettes with SRBC. Previous studies have shown that immune lysis of nucleated cells like that of red blood cells requires activation of complement components C1-C9 (8). That the inhibition of rosette formation was caused by HL-A alloantibodies is shown by the fact that only HL-A alloantisera directed against determinants present on T human lymphocytes used in a given assay could inhibit the rosette formation in conjunction with C6 deficient rabbit serum (Table II).

TABLE I. BLOCKING BY C6 DEFICIENT RABBIT SERUM (C6RS) OF ROSETTE FORMATION BETWEEN SRBC[a] AND HUMAN PERIPHERAL LYMPHOCYTES (M.P.:HL-A 2,5,12) SENSITIZED WITH HL-A ALLOANTISERA.

Alloantiserum	First incubation with: HL-A Specificity	First incubation with: Rosettes (%)	Second incubation with: HBSS[b] (% Rosettes)	Second incubation with: aHuIg[c] (% Rosettes)	Second incubation with: C6RS (% Rosettes)
–	–	69	71	68	71
Eriksson	2	75	77	76	0
Victor	5	69	69	73	0
Jackson	7	70	70	65	70
Daines	12	69	70	65	2

[a] Sheep red blood cells.
[b] Hanks' balanced salt solution.
[c] No capping was observed under these experimental conditions, when a FITC-aHuIg was used.

Furthermore, when HL-A alloantibodies were specifically absorbed with cultured human lymphoid cells bearing the corresponding HL-A specificities, they lost their ability to block rosette formation in conjunction with C6 deficient rabbit serum (Table III).

The titer of inhibitor activity of HL-A alloantisera was significantly lower than the titer of cytotoxic activity with rabbit complement in the lymphocytotoxicity test (Fig. 1) as already observed with anti-human lymphocyte sera (13). As a matter of fact, HL-A alloantisera of low cytotoxic titer did not inhibit rosette formation at all. The necessity for complement in the inhibition of rosette formation is indicated by the loss of this effect when C6 deficient rabbit serum is treated to inactivate the complement system, such as by heating at 56° for 30 min or by adding EDTA (Table IV). The natural antibodies present in rabbit serum (8) and directed to a polymorphic antigenic system represented on human lymphoid cells (14) do not play any role in the inhibition of the rosette formation since absorption of such antibodies from C6 deficient rabbit serum did not effect the results (Table IV).

To determine whether the classical or the alternate pathway of the complement system is involved in the inhibition of rosette formation, C6 deficient rabbit serum was

TABLE II. CORRELATION BETWEEN CYTOTOXIC ACTIVITY AND BLOCKING OF ROSETTE FORMATION BY HL-A ALLOANTISERA

Alloantiserum	HL-A specificity	Test	Cells			
			M.P. (2, 5, 12)	A.C. (1, 8, 7)	A.P. (2, 9, 5)	C.S. (1, 3)
Gaulier	1	C[a]	–[b]	+[b]	–	+
		B[c]	–	+	–	+
Eriksson	2	C	+	–	+	–
		B	+	–	+	–
Storm	3	C	–	–	–	+
		B	–	–	–	+
Grubisch	9	C	–	–	+	–
		B	–	–	+	–
Victor	5	C	+	–	+	–
		B	+	–	+	–
Jackson	7	C	–	+	–	–
		B	–	+	–	–
Willet	8	C	–	+	–	–
		B	–	+	–	–
Daines	12	C	+	–	–	–
		B	+	–	–	–

[a] Cells incubated with alloantisera and normal rabbit serum.
[b] – and + symbols indicate positive or negative cytotoxic reaction (Test C) and blocking or not of the rosette formation (Test B).
[c] Cells incubated with alloantisera and C6 deficient rabbit serum.

TABLE III. ROSETTE FORMATION BETWEEN SRBC[a] AND HUMAN PERIPHERAL LYMPHOCYTES (A.P.:HL-A 2, 9, 5) SENSITIZED WITH ANTI-HL-A2 SERUM ABSORBED WITH HL-A2 (+) OR HL-A2 (–) CELLS.

First incubation		Second incubation	
Alloantiserum	Absorbed with	HBSS	C6RS
–	–	74	70
Eriksson (HL-A2)	–	72	2
Eriksson (HL-A2)	RPMI-4098 (HL-A3, W28)	70	0
Eriksson (HL-A2)	RPMI-1788 (HL-A2, 10, 7, 14)	73	72

[a] Sheep red blood cells.

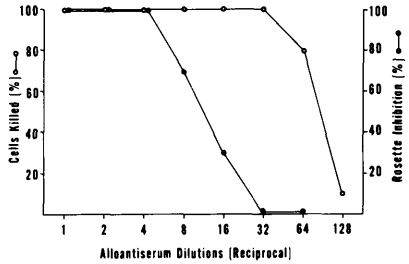

FIG. 1. Comparison between cytolysis (O——O) and rosette inhibition (●——●) of HL-A alloantiserum VICTOR. In the cytotoxicity test, normal human lymphocytes were incubated with alloantiserum and normal rabbit serum. In the rosette inhibition test the cells were incubated with alloantiserum and C6 deficient rabbit serum.

added with EGTA (final concentration 10 m*M*) and Mg^{2+} (final concentration 1×10^{-3} *M*) (15). Unlike EDTA which binds both Ca^{2+} and Mg^{2+}, EGTA chelates primarily CA^{2+} which is not required for alternate complement function (15). When rabbit serum was treated with EGTA, rosette formation was not inhibited, indicating that the classical pathway mediates inhibition (Table IV). This conclusion is reinforced by the inability of C4 deficient guinea pig serum to block the rosette formation in conjunction with HL-A alloantisera (Table IV).

In order to determine whether the inhibition of rosette formation, caused by HL-A alloantisera and complement components, occurs because of capping of HL-A antigens, human lymphocytes sensitized with FITC-HL-A-antibodies and C6 deficient rabbit serum, were examined for capping of HL-A antigens. Under our experimental conditions no capping of HL-A antigens on the cell surface was observed.

Discussion. The present experiments indicated that HL-A alloantibodies and the

TABLE IV. INVOLVEMENT OF THE CLASSICAL PATHWAY OF THE COMPLEMENT SYSTEM IN THE BLOCKING OF ROSETTE FORMATION BETWEEN SRBC[a] AND HUMAN PERIPHERAL LYMPHOCYTES (M.P.: HL-A 2, 5, 12) SENSITIZED WITH HL-A ALLOANTISERA.

First reagent (EDTA 10 mM)	Second reagent						
	HBSS	C6RS[b]	C6RS (EDTA 10 m*M*)	C6RS (EGTA 10 m*M*)	C6RS (56°, 30 min)	C6RS[c] (absorbed)	C4GP[d]
HBSS	72[e]	68	70	61	69	72	67
Eriksson (HL-A2)	78	0	65	68	64	3	69
Victor (HL-A5)	70	4	67	72	65	3	70

[a] Sheep red blood cells.
[b] C6 deficient rabbit serum.
[c] C6RS absorbed with cultured human lymphoid cells in presence of EDTA.
[d] C4 deficient guinea pig serum.
[e] Numbers indicate percentage rosettes.

early components of the complement system inhibited the formation of rosettes with SRBC. This inhibition occurred through the activation of the complement's classical pathway since binding of calcium or deficiency of one of the essential components selectively excluded inhibition. These results agree with our previous finding that HL-A alloantibodies can activate either the classical or alternate pathway of human complement, but only the classical pathway of rabbit and guinea pig complement (16, 17). The involvement of HL-A alloantibodies is clearly indicated by correlation between HL-A phenotype of lymphocytes and HL-A specificity of alloantisera and by lack of blocking activity of these sera once they were absorbed with cultured human lymphoid cells bearing the corresponding HL-A specificities.

Several explanations can be put forward for the inhibitory effects of HL-A antibodies and early complement components on rosette formation: (a) Inhibition might be caused by killing of lymphocytes; however, this seems unlikely because C6 deficient rabbit complement cannot by itself sustain killing of lymphocytes sensitized with HL-A alloantibodies, as shown by trypan blue uptake. (b) HL-A antigens are closely positioned to receptors for SRBC and, thus, the antibodies together with complement components could sterically hinder binding of SRBC. (c) The binding of HL-A alloantibodies and complement components to the lymphocyte surface might cause great configurational changes of its membrane either disturbing the distribution of the receptors or changing the charge of the cell membrane, thus inhibiting rosette formation with SRBC. This latter possibility is favored by our previous finding that antihuman lymphocyte antibodies present in normal sera from different animals species can also inhibit rosette formation when reacted with lymphocytes in conjunction with C6 deficient rabbit serum (6). The role of HL-A antigens, therefore, seems to be only incidental to the inhibition phenomenon, representing simply convenient cell surface molecules required for the activation of complement.

Further experimentation will be necessary to establish whether the effect of activated complement on rosette formation is a nonspecific one, i.e., an overloading of cell surface membranes with exogenous reagents, or whether it is linked to more biologically relevant function of the cell surface.

Summary. The relationship between HL-A antigens and rosetting of sheep red blood cells (SRBC) with peripheral human lymphocytes has been investigated by incubating them with HL-A antibodies. Although sensitizing the lymphocytes with HL-A alloantisera had no effect on their ability to form rosettes with SRBC, further sensitization with C6 deficient rabbit serum as a source of early complement components inhibited the formation of rosettes with SRBC. The involvement of HL-A alloantibodies in the inhibition of rosette formation was shown first by correlating the HL-A phenotype of the lymphocytes and the HL-A specificity of the alloantisera and, second, by specifically absorbing the HL-A alloanti-

bodies from the alloantisera. Complement was needed to inhibit rosette formation since this effect was lost when rabbit serum was treated to inactivate complement. The participation of complement's classical pathway in rosette inhibition was shown by chelating the Ca^{2+} ions by EGTA treatment of the C6 deficient rabbit serum. Perhaps, binding of HL-A antibodies and early complement components to the lymphocyte surface disturbs the distribution of the receptors or affects the charge of the cell membrane, thus inhibiting the rosette formation with SRBC.

1. Singer, S. J., and Nicolson, G. L., Science **175,** 720 (1972).
2. Bernoco, D., Mattiuz, P. L., Miggiano, V. C., and Ceppellini, R., G. Bat. Virol. Immunol. **64,** 314 (1971).
3. Preud'Homme, J. L., Neauport-Sautes, C., Piat, S., Silvestre, D., and Kourilsky, F. M., Europ. J. Immunol. **2,** 297 (1972).
4. Poulik, M. D., Bernoco, M., Bernoco, D., and Ceppellini, R., Science **182,** 1352 (1973).
5. Pellegrino, M. A., Ferrone, S., Dierich, M. P., and Reisfeld, R. A., Clin. Immunol. Immunopathol. **3,** 324 (1975).
6. Pellegrino, M. A., Ferrone, S., Dierich, M. P., Pellegrino, A., and Reisfeld, R. A., Clin. Immunol. Immunopathol. **4,** 85 (1975).
7. Goldman, M., "Fluorescent Antibody Methods," Academic Press, New York, (1968).
8. Ferrone, S., Cooper, N. R., Pellegrino, M. A., and Reisfeld, R. A. J. Immunol. **107,** 939 (1971).
9. Pellegrino, M. A., Ferrone, S., and Pellegrino, A., *in* "Transplantation Antigens" (B. D. Kahan and R. A. Reisfeld, eds.), p. 433. Academic Press, New York (1972).
10. Pellegrino, M. A., Ferrone, S., and Pellegrino, A., Proc. Soc. Exp. Biol. Med. **139,** 484 (1972).
11. Jondal, M., Holm, G., and Wigzell, H., J. Exp. Med. **136,** 207 (1972).
12. Froland, S. S., Scand. J. Immunol. **1,** 269 (1972).
13. Bentwich, Z., and Kunkel, H. G., Transplant. Rev. **16,** 29 (1973).
14. Mittal, K. K., Ferrone, S., Mickey, M. R., Pellegrino, M. A., Reisfeld, R. A., and Terasaki, P. I., Tissue Antigens **3,** 88 (1973).
15. Fine, D. P., Marney, Jr., S. R., Colley, D. G., Sergent, J. S., and Des Prez, R. M., J. Immunol. **109,** 807 (1972).
16. Ferrone, S., Cooper, N. R., Pellegrino, M. A., and Reisfeld, R. A., Proc. Nat. Acad. Sci. USA **70,** 3665 (1973).
17. Ferrone, S., Cooper, N. R., Pellegrino, M. A., and Reisfeld, R. A., Transplant. Proc. **6,** 13 (1974).

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