CYTOLYTIC LYMPHOCYTIC CELLS WITH COMPLEMENT RECEPTOR IN HUMAN BLOOD

Induction of Cytolysis by IgG Antibody but not by Target Cell-Bound C3*

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We wish to present evidence that antibody-dependent cytolytic lymphoid cells from human blood have receptors for activated C3. Using target cells bearing activated C3 no cytolysis was induced by these effector cells in the absence of IgG antibody. These results point to the importance of the interaction between the cellular Fc receptor and the Fc part of the inducing antibody as the triggering event for the antibody-dependent cytolytic reaction.

Purified lymphocytes from the blood of normal donors are cytolytic for various target cells in the presence of antitarget cell antibodies. In a model system consisting of ^{51}Cr-labeled chicken erythrocytes (E_c)[1] and IgG anti-E_c from hyperimmune rabbits, we have previously shown that T cells have no effector function in this system (1). Similar findings have been reported for animal models (2, 3). It has also been reported that mature B cells with high concentration of surface-bound immunoglobulin seem to be inactive in this cytolytic system (4, 5). It is not known if the effector cells are true lymphocytes or are cells of lymphocytic appearance but belonging to a nonlymphocytic lineage. The cytolytic reaction does not require participation of complement (C) factors C5-C9 (6, 7).

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[1] *Abbreviations used in this paper:* A, rabbit antibody to boiled sheep erythrocyte stromata, IgM fraction; a-E_c, antiserum from rabbits, hyperimmunized to chicken erythrocytes; C(hu), human complement; C(ra), rabbit complement; C3b INA, C3b inactivator; CRL, complement receptor lymphocytes; E_c, chicken erythrocytes; E_s, sheep erythrocytes; ER, lymphocytes forming rosettes with E_s; FCS, fetal calf serum; HGG, human gamma globulin; HSA, human serum albumin; NRS, normal rabbit serum; TH, Tris-buffered Hank's balanced salt solution; VBS, veronal-buffered saline.

Materials and Methods

Solutions. Tris-buffered Hank's balanced salt solution (TH) (pH 7.4) and veronal-buffered saline (VBS) were prepared as described (8, 9). VBS-Ca,Mg contained 0.15 mM Ca^{2+} and 0.54 mM Mg^{2+}. Tissue culture medium RPMI 1640 (Bio-Cult, Paisley, Scotland) was supplemented with 2 mM glutamine, 100 IU penicillin, 100 μg streptomycin/ml and, when indicated, with HEPES-buffer. Mixtures of Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and Isopaque (Nyegaard, Oslo, Norway) (sp gr 1.070–1.078) were prepared according to the method used by Böyum (10). Human serum albumin (HSA) (Kabi, Stockholm, Sweden) was used as 8% stock solution in TH. Gelatin (Edible Compounds Ltd., Stone Ferry, Hall, Yorkshire, England) was prepared as described earlier (8).

Sera. Antiserum from rabbits hyperimmunized to E_c (a-E_c) and normal rabbit serum (NRS) were prepared as previously described (8). The IgM fraction of rabbit antiserum against boiled sheep erythrocyte stromata (A) was obtained by gel exclusion chromatography through Sephadex G-200 (Pharmacia Fine Chemicals, Inc.). Fetal calf serum (FCS) was from Flow Laboratories, Inc., Irvine Scotland. All sera were heat inactivated at 56°C for 45 min.

Complement (C). Purified Human C Components. C1 (11), $C1\bar{s}$ (12), C4 (13), C2 (14), and C3 (15) were prepared as described. Serum from C6-deficient rabbits (Rancho de Conejo, Vista, Calif.) served as the source of rabbit C [C(ra)]. When necessary, the rabbit serum was freed from natural antibodies to sheep erythrocytes (E_s) or E_c by absorption in the presence of 0.005 M EDTA.

$EC4^{oxy}23(hu)$. Intermediates of either E_c or E_s were prepared by fluid-phase activation without antibody (16). 800 μg active C4 and 280 μg $C1\bar{s}$ were mixed and added to 8 ml 5% E (8×10^9 E_s or 3×10^9 E_c), washed three times with VBS-Ca,Mg. After 15 min incubation at 37°C and two washes in VBS, 500 μg $C2^{oxy}$ (17) were added. After 5 min at 37°C,280 μg $C1\bar{s}$, and after additional 10 min 1.4 mg C3 were added. The cells were further incubated for 30 min at 37°C. They were then washed twice in VBS-Ca,Mg and resuspended in 40 ml to give 1% suspensions. The activity of cell-bound C3b was assessed by immune adherence (18). The number of cell-bound C3 molecules was also measured directly by building up intermediates with ^{125}I-labeled C3. The sp act of two different preparations of C3 were 30,000 and 50,000 cpm/μg, respectively.

EAC(ra). To form EA, 1 ml 5% washed E in TH (10^9 E_s or 3.7×10^8 E_c) were incubated for 30 min at 37°C with 1 ml of A. This IgM fraction which reacted with both E_s and E_c was used at a subagglutinating dilution in TH (1:200). EAC(ra) were prepared by mixing 0.25 ml EA with 0.25 ml C6-deficient rabbit serum, diluted 1:4 (TH). After 30 min at 37°C, the cells were washed twice with 10 ml cold TH. For control, portions of EA were mixed with heat-inactivated serum, or with TH only. EAC(ra) were lysed when incubated with NRS in microtitration plates (end point for lysis 1:64–1:125).

Isolation of Human Lymphocytes from defibrinated blood was performed by gelatin sedimentation, iron uptake, and Ficoll isopaque centrifugation (1, 8, 19). To remove remaining monocytic cells, the suspensions were incubated in 10% FCS over night in Falcon tissue culture flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). This procedure gives mononuclear cell suspensions consisting to ~99% of viable lymphocytes.

Fractionation of Lymphocytes on $EC4^{oxy}23(hu)$ columns. Acid-treated glass beads (superbrite 3M, type 100–500; 3M Co., St. Paul, Minn.) were treated with an equal volume of poly-L-lysine (hydrobromide, lot ly-151, mol wt 25,000–50,000; Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill.), 20 μg/ml in sterile NaCl (20). After incubation at 4°C overnight the beads were washed once with saline and poured into glass columns, 15 × 300 mm, 25- to 30-ml beads/column. The columns were charged with 10 ml of a 10% suspension of $E_sC4^{oxy}23$(hu), (10^{10} E/column) and washed with saline until the eluates were free of cells. Control columns were charged with untreated E_s. The erythrocytes on the columns were lysed with 20 ml of distilled water and washed with saline until completely free of hemoglobin. 10 ml of 2% E_s were added at this stage and passaged through the column with ~20 ml saline. Saturation was reflected by passage of the erythrocytes as a narrow red band. The column was filled with RPMI 1640 containing 0.4% HSA, immediately followed by addition of lymphocytes (~20×10^6 lymphocytes/column) which were passaged through the column at a rate of 2–3 ml/min with 40–50 ml HSA-containing medium.

Receptor Lymphocytes (CRL) (21). The fraction of lymphocytes forming rosettes with E_sAC(ra) or $E_sC4^{oxy}23$(hu) was determined according to the method used by Jondal et al. (22). 0.25 ml of

lymphocytes (1×10^6) in HEPES-RPMI were incubated with 0.25 ml of 0.5% suspensions of the C-treated erythrocytes (2.5×10^7). Lymphocytes binding four or more erythrocytes were scored as CRL.

CRL Depletion. Rosettes formed by lymphocyte-E_sAC(ra) mixtures and control mixtures were suspended in 5 ml 8% HSA plus 1.7 ml 3% gelatin in TH, and centrifuged for a few minutes at ~350 *g*. The supernate containing lymphocytes depleted of CRL was withdrawn.

Rosette Formation with E_s (ER). The fraction of lymphocytes forming spontaneous rosettes with E_s in concentrated FCS was determined according to the methods used by Jondal et al. (22) and Yata et al. (23).

ER Depletion. 0.5 ml lymphocytes (8×10^6) and 0.5 ml E_s (1×10^8) in FCS were mixed and incubated in the refrigerator overnight. 2 ml ficoll-isopaque were layered under 2 ml cell suspension (~15×10^6 lymphocytes and 2×10^8 E_s/tube) and the mixtures were centrifuged for 40 min at 800 *g* at room temperature. The ER-depleted lymphocytes were recovered from the interphase.

Lymphocyte-Mediated Cytolysis of Erythrocytes. E_c, or in a few experiments E_s, either untreated or treated with C, were labeled with $Na_2{}^{51}CrO_4$ (0.5–1.0 mCi/ml; 3–10 μg Cr/ml; The Radiochemical Center, Amersham, England) as described (1, 8, 19). When C-treated erythrocytes were used, FCS (2.5%) was substituted with HSA (0.2%) throughout the labeling procedure.

For the cytolysis experiments, all dilutions were made in RPMI 1640 containing 5% FCS or 0.4% HSA. The number of target cells per incubation tube was 2×10^4–8×10^4, while the number of lymphocytes varied from 2×10^5–8×10^5. The final dilution of a-E_c or NRS was 10^{-5} or 3×10^{-6}, total vol of incubation mixture 0.3 ml. In some experiments, the total vol was 1.5 ml, with correspondingly larger numbers of cells. If not otherwise indicated, lysis is given as percent of total isotope released after 15–20 h from cells to medium, corrected by substracting percent spontaneous release in lymphocyte-free control tubes. All tubes were set up in duplicate (SE ≤ 2%) (1, 8, 19).

Results

Experimental procedures. With optimal concentrations of antiserum (final dilutions 10^{-5}–3×10^{-6}) and optimal lymphocyte to E_c ratios (≥10:1) complete lysis is usually seen after about 15–20 h of incubation. At this time, suboptimal ratios, e.g. 5:1, give incomplete but fully significant lysis. 18 h of incubation and different lymphocyte to E_c ratios were therefore chosen to assay lymphocyte activities (1, 19).

Depletion of Antibody-Dependent Effector Cells by Human C3b Columns. $E_sC4^{oxy}23$(hu) intermediates (7,000–10,000 C3 molecules/E_s) were adsorbed to glass bead columns. Their retention on the column was assessed by C3-bound radioactivity. In a typical experiment, the total amount C3b initially retained was approximately 35 μg. The total amount eluted during all subsequent washings and lymphocyte passage was 17%. Almost 10% of the total came off during elution with RPMI 1640 containing 5% FCS. Since FCS contained C3b inactivator (C3b INA) which converts C3b to C3c and C3d (24) and thereby abolished immune adherence activity of $E_sC4^{oxy}23$(hu), it was in all subsequent experiments replaced by HSA. Under these conditions, C3b was not affected and losses were kept to a minimum.

The yield of cells passaged through the $E_sC4^{oxy}23$(hu) column and the E_s control column was ~30%. However, while the loss of lymphocytes on the control column was random, the $E_sC4^{oxy}23$(hu) column gave an eluate in which the T-cell fraction was increased and the fraction of CRL was markedly decreased.

Table I shows the results of a typical experiment in which unfractionated and column-passaged lymphocytes were tested for antibody-dependent cytolysis. In order to increase the sensitivity of the system, effector cell to target cell ratios

TABLE I

Surface Markers and Antibody-Dependent Cytolytic Potential of Lymphocytes Passaged Through Glass Bead Column Charged with E_s Membranes Bearing Human C3b

Lymphocytes	ER	CRL*	% ^{51}Cr release (corrected)‡	
			NRS	a-E_c
	%	%		
Untreated	43	19	1	17
E_s column passaged	36	18	0	13
E_sC4^{oxy}23(hu) column passaged	64	4	0	6

* E_sC4^{oxy}23(hu) rosette-forming cells.

‡ Means of duplicate incubations, ranges <2%. 0, release equal or less than in lymphocyte-free controls (≤2%). Lymphocyte/E_c, 5:1.

were kept at a suboptimal level (5:1). All column-passaged samples had reduced lytic activity. However, while reduction of cytolysis by the control lymphocytes was slight (~20%), that of the E_sC4^{oxy}23(hu) column-passaged cells was significantly stronger (~65%), corresponding to a ~75% reduction in CRL.

Depletion of Antibody-Dependent Effector Cells by Centrifugation of $E_sAC(ra)$ rosette-forming cells (CRL). Centrifugation methods to deplete mouse lymphocytes with receptors for mouse C have been described (21, 25). The mean percentage of CRL found here with the lymphocytes from 10 normal donors was 19.1 ± 1.2 (SE). After centrifugation through HSA-gelatin, the fraction of CRL recovered from the interphase was 2.4 ± 1.3 (SE).

Table II shows that depletion of cells with receptor for C(ra) strongly reduced cytolytic activity. As was to be expected (4), cell preparations depleted of T cells had a strong antibody-dependent cytolytic potential (exps. 5 and 6, Table II).

C on Target Erythrocytes does not Trigger Lymphocyte-Mediated Cytolysis. E_cC4^{osy}23(hu) (~30,000 molecules C3b/erythrocyte) were employed as target cells. They were strongly active in immune adherence. The results of a typical cytolysis experiment in which the medium was supplemented with either 5% FCS or 0.4% HSA are shown in Table III. E_cC4^{oxy}23(hu) were not lysed in either case in spite of the high lymphocyte to target cell ratio applied in this experiment. This suggests that neither C3b nor C3d formed in the presence of FCS, were able to trigger a cytolytic response. When a-E_c (IgG) at high dilution was added to the lymphocyte/E_c mixture, complete lysis was obtained with both media.

Purified lymphocytes were first passaged through columns charged with human gamma globulin (HGG)/rabbit anti-HGG, previously shown to retain antibody-dependent effector cells (1, 4). Unfractionated and column-passaged lymphocytes were incubated with three types of target cells (Table IV). No cytolysis was induced in the absence of anti-E_c in any case. However, in the presence of anti-E_c, cytolysis of E_cC4^{oxy}23(hu) was significantly elevated over that of E_c. This was particularly pronounced with effector cells passaged through the HGG/anti-HGG column.

TABLE II

Cytolytic Potential of CRL- or ER-Depleted Lymphocytes

Exp.	Lymphocytes	CRL*	% ^{51}Cr release (corrected)‡	
			NRS	a-E_c
		%		
1	Untreated	20	0	93
	CRL depleted	2.5	0	1
2	Untreated	17	0	72
	CRL depleted	1	0	0
3	Untreated	23	1	16
	CRL depleted	2	0	1
4	Untreated	26	0	55
	CRL depleted	6	0	2
5	ER depleted	40	0	60
	CRL depleted	1	0	2
6	ER depleted	49	0	54
	CRL depleted	2	0	8

* E_sAC(ra) rosette-forming cells.

‡ Lymphocyte/E_c, 10:1. 0, release equal or less than in lymphocyte-free controls ($\leq$2.5%).

TABLE III

Effect of Lymphocytes on E_c Bearing Human C3b

Medium	% ^{51}Cr release (total)*		
	E_c	E_c + a-E_c	E_cC4^{oxy} 23(hu)
RPMI 1640 + 0.4% HSA	1	94	5
RPMI 1640 + 5% FCS	2	91	3

* Total (uncorrected) release. Lymphocyte/E_c, 40:1. Spontaneous release in lymphocyte-free controls, 9–12%.

In a second series of experiments E_cAC(ra) or E_sAC(ra) were used as target cells. The IgM fraction of the anti-E_s stroma antibody (A), employed for C activation did not induce lymphocyte-mediated cytolysis. Table V presents the results of six independent experiments, performed with the lymphocytes from six different donors. No lysis was seen when E_cAC(ra) or E_sAC(ra) were used as target cells although they carried enough C for optimal rosette formation. Addition of IgG anti-E_c induced cell-mediated lysis in all cases.

TABLE IV

Effect of Fractionated Lymphocytes on E_c Bearing Human C4b or C3b

Lymphocytes	ER	CRL*	% ^{51}Cr release (corrected)‡				
			E_c		E_cC4(hu)	$E_cC4^{oxy}23$ (hu)	
			NRS	a-E_c	NRS	NRS	a-E_c
	%	%					
Untreated	54	15	0	35	0	0	42
FCS column passaged§	57	23	0	26	1	1	44
HGG/anti-HGG column passaged§	77	4	0	1	1	0	11

* $E_sC4^{oxy}23$(hu) rosette-forming cells.

‡ Lymphocyte/E_c, 12.5:1. RPMI 1640 + 0.4% HSA. NRS or a-E_c final dilutions 10^{-5}. 0, release equal or less than in lymphocyte-free controls [E_c, 1%; E_cC4(hu), 4%; and $E_cC4^{oxy}23$(hu), 12%].

§ Glass beads treated with FCS or HGG/rabbit anti-HGG (1, 4, 20).

TABLE V

Effect of Lymphocytes on E_c or E_s Bearing IgM Antibody and C(ra)

Exp.	CRL*	Target cells	Lymphocyte/E	% ^{51}Cr release (corrected)‡			
				EA		EAC(ra)	
				NRS	a-E_c	NRS	a-E_c
	%						
1	ND	E_c	50:1	5	ND	6	80
2	ND	E_c	25:1	4	ND	4	80
3	ND	E_c	5:1	8	55	6	59
4	15	E_s	100:1	8	35	10	ND
5	12	E_s	50:1	4	34	3	ND
6	12	E_s	10:1	5	33	3	39

* Lymphocytes forming rosettes with portions of E_sAC(ra) used as target cells in exp. 4–6.

‡ RPMI 1640 + 0.4% HSA. NRS or a-E_c final dilutions, 3×10^{-6}. Release in lymphocyte-free controls: exp. 1, 10–13%; 2, 6–8%; 3, 4–6%; 4, 10–12%; 5, 9–11%; and 6, 12–17%.

Discussion

Depletion of cells with C receptors was achieved by two independent methods and with C of two different sources. Columns charged with membranes of E_sAC (mouse) have recently been used by Jondal et al. (26) to remove CRL from human blood. In our experiments, the glass beads were coated with cell membranes bearing human C3b, produced by fluid-phase activation of C4 without application of antibody. This procedure excludes removal of effector cells with Fc receptors. In order to avoid breakdown of cell-bound C3b to C3d by C3b

INA (24), HSA was used as the only protein supplement in the medium. These experiments therefore suggest that antibody-dependent effector cells have receptors for human C3b. Under the conditions used, no reaction with E_sC4 was seen in rosette experiments. Lack of expression of bound C4b activity in these experiments is due to the relatively small number of C4 molecules per cell employed. C4b-dependent immune adherence (18) or rosette formation (27, 28) requires 3,000–5,000 C4b molecules/cell.

The experiments in which cells with receptors for C(ra) were removed by centrifugation of E_sAC(ra) rosettes confirmed the results of the column fractionation experiments with $E_sC4^{oxy}23$(hu). Since the C6-deficient rabbit sera contained C3b INA activity, both C3b and C3d are likely to be present on the C-bearing E_s. Preliminary experiments (unpublished results) suggest that antibody-dependent cytolytic effector cells also have receptors for C3d, as has been reported for CRL (29).

Using similar centrifugation techniques for effector cell fractionation, van Boxel et al. recently concluded that the antibody-dependent cytolytic effector cells in mouse spleen belong to a subset of C receptor-bearing bone marrow-derived lymphocytes (25). Cell fractionation in the presence of EDTA suggested that these mouse effector cells had Mg^{2+} independent C receptors. Inhibition of cytolysis by depletion of E_sAC(ra) rosette-forming cells in 0.005 M EDTA also suggest that the cytolytic effector cells studied by us are distinct from mature blood monocytes or polymorphonuclear neutrophiles (unpublished results).

No lysis was obtained when the effector cells were incubated with target cells bearing human C3b in the absence of IgG antibody. Incubation in the presence of FCS, which converted most of the target cell-bound C3b to C3d, was as ineffective in this respect as incubation in HSA which left C3b intact. In these experiments the number of C3 molecules to target cells was ~30,000, which was more than three times the concentration needed for optimal rosette formation or for depletion of effector cells on the C-charged columns, and more than three to five times the concentration of IgG anti-E_c needed for 50% lysis under similar conditions in the absence of C (19). However, while C alone did not induce a cytolytic response of the effector cells, its presence on the target cells significantly potentiated antibody-dependent lysis (Table IV).

Experiments with erythrocytes bearing IgM antibody and C(ra) confirmed the results obtained with C(hu) components. Target cell-bound C concentrations sufficient for rosette formation were not at all capable to induce cell-mediated lysis. That the C receptor on the effector cells has no direct role in the induction of lysis was also apparent from experiments in which 0.2–40 μg purified human C3b[2] were added to incubation mixtures, containing lymphocytes, E_c, and IgG anti-E_c (1.5×10^6–3×10^8 molecules C3b/lymphocyte). Under these conditions, C3b neither inhibited nor enhanced the antibody-dependent cell-mediated cytolysis (unpublished results). These results are in line with those recently reported by van Boxel et al. (7). It thus seems that the interaction between the Fc part of erythrocyte-bound IgG and the Fc receptor of the antibody-dependent

[2] Human C3b was a gift to Dr. V. Bokisch, Scripps Clinic and Research Foundation, La Jolla, Calif. It was prepared by trypsinization of C3 and separated from C3a by Pevikon electrophoresis.

effector cells is of special importance to bring about the surface modulation necessary for triggering the cytolytic events described in this paper. However, the reaction between target cell-bound C and the C receptor of the effector cells seems to amplify the attachment phase which is a prerequisite for antibody-induced cell-mediated lysis. Although antibody-dependent cell-mediated lysis is a nonphagocytic event (8) these results are reminiscent of earlier findings made with mononuclear phagocytes of both human and mouse origin, suggesting that the presence of activated C (C3) on IgM- or IgG-coated erythrocytes enhances attachment to the phagocytes but that only IgG effectively induces ingestion (30, 31).

Summary

Human blood lymphocytes were fractionated on glass bead columns charged with sheep erythrocyte (E_s) membranes-bearing human C3b (7,000–10,000 molecules/E_s). In the passaged cells the proportion of C receptor lymphocytes was strongly reduced, in parallel with the capacity to lyse chicken erythrocytes (E_c) in the presence of IgG-rabbit anti-E_c antibody. In other experiments, lymphocytes forming rosettes with E_s bearing activated rabbit complement [C(ra)] from C6-deficient rabbits were removed by centrifugation through human serum albumin-gelatine mixtures. This procedure also depleted the lymphocyte preparations of antibody-dependent cytolytic effector cells. The results suggest that such effector cells have receptors for human C as well as for C(ra).

Lymphocytes were not able to lyse erythrocytes bearing either human C3b (~30,000 molecules/E_c) or activated C(ra) in the absence if IgG antierythrocyte antibodies. Under the same experimental conditions these target cells were efficiently lysed in the presence of small amounts of IgG antitarget cell antibodies. This suggests that the interaction between the cellular Fc-receptors and the Fc part of the inducing antibodies is of special significance for the triggering of the cell-mediated lytic reaction. However, although target cell-bound C did not trigger cytolysis, it seemed to potentiate antibody-dependent cytolysis, probably by enhancing effector cell-target cell contacts.

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