INTERACTION OF TARGET CELL-BOUND C3bi AND C3d WITH HUMAN LYMPHOCYTE RECEPTORS
Enhancement of Antibody-mediated Cellular Cytotoxicity*

BY HEDVIG PERLMANN, PETER PERLMANN, ROBERT D. SCHREIBER, AND HANS J. MULLER-EBERHARD

From the Department of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037; and the Department of Immunology, University of Stockholm, S-10691 Stockholm, Sweden

In antibody-dependent, lymphocyte-mediated cytotoxicity (ADCC), the interaction between IgG antibodies on the target cells and Fc IgG receptors (FcγR) on the effector cells affords close contact between the cells resulting in lysis of the targets. Present evidence suggests that the Fc-FcγR interaction triggers the cytolytic reaction. (1) It has been shown earlier that lymphoid effector cells with C3 receptors can bind C3-fragment-bearing erythrocytes as assessed by rosette formation. However, this interaction did not result in lysis of the target cells in absence of IgG antibody (2-4). In the presence of antibody to the target cells, target cell-bound C3 fragments strongly enhanced ADCC (1, 5). It was concluded that target cell-associated C3 amplifies ADCC by improving effector cell-target cell contact.

Cell-bound C3 may exist in several molecular forms, designated C3b, C3bi, and C3d (reviewed in [6]). C3 is deposited on particles as C3b which subsequently is cleaved by factor I to form C3bi without reduction of molecular weight. C3bi is then converted to C3d by enzymatic removal of the C3c fragment. The relative proportions of the three fragments on the surface of a target are determined by exposure to controlling enzymes.

The question of the role of these different C3 fragments in regulating cellular effector functions such as ADCC or phagocytosis is largely unresolved. Although

* Supported by grants AI 07007, CA 27489, HL 07195, and HL 16411 from the U. S. Public Health Service, grant 2032-105 from the Swedish Natural Science Research Council, and grant B81-16X-00148 from the Swedish Medical Research Council. Publication 2370 from the Research Institute of Scripps Clinic, La Jolla, Calif.

‡ Department of Immunology, University of Stockholm, Stockholm, Sweden.
§ Department of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, Calif.
¶ Cecil H. and Ida M. Green Investigator in Medical Research, Research Institute of Scripps Clinic, La Jolla, California.

** Abbreviations used in this paper: ADCC, antibody-dependent, lymphocyte-mediated cytotoxicity; complete TCM, Hepes (10 mM)-buffered RPMI-1640 containing 0.4% HSA and supplemented with 100 U penicillin, 100 μg Fungizone, and 2 mM L-glutamine; Es, Ee, Ex, bovine, chicken, or sheep erythrocytes, respectively; EbC3b, EbC3bi, EbC3d, C3b-fragment-, C3bi-fragment-, and C3d-fragment-bearing Eb, respectively; FcγR, Fc IgG receptor; FIP, Ficoll-Isopaque; GBV**, veronal-buffered physiological saline, pH 7.4, containing 0.15 mM CaCl2; HSA, human serum albumin; NF, nephritic factor; PFC, plaque-forming cells; PMN, polymorphonuclear leukocytes; TH, Hanks' balanced salt solution containing 15 mM Tris, pH 7.2; UV, ultraviolet.
previous studies suggested that target cell-bound C3b and C3d might enhance ADCC, no conclusions as to their relative importance could be drawn (3). Moreover, C3bi was not clearly defined at that time. This is the first description of a role for C3bi in lymphocyte function. The present study shows that cell-bound C3bi can directly interact with certain lymphocytes and that in the case of ADCC, this interaction greatly enhances lysis of target cells. The enhancing effect of C3bi is greater than that of C3d, and considerably greater than that of C3b.

Materials and Methods

Buffers. Buffers used were as follows: GVB++: veronal-buffered physiological saline, pH 7.4, containing 0.15 mM CaCl₂, 0.5 mM MgCl₂, and 0.1% gelatin; and TH: Hanks' balanced salt solution containing 15 mM Tris, pH 7.2.

Purified Proteins. The proteins C3 (7), factor B (8), C3b inactivator (9), and β1H (9) were prepared as described previously. Partially purified nephritic factor (NF) was prepared as described previously (10). 1 ml of NF was absorbed three times for 20 min at 0°C with 10⁹ erythrocytes and one time with 10⁹ erythrocytes bearing C3 fragments (EC3b). Bovine conglutinin was prepared by the method of Lachmann (11). Trypsin (TPCK) was purchased from Worthington Biochemical Corp. (Freehold, N. J.)

Erythrocytes. Bovine erythrocytes (Eb) and sheep erythrocytes (Es) were purchased from Colorado Serum Co., Denver, Colo. and used within 2 wk. Chicken erythrocytes (Ec) were prepared from heparinized blood of 10-20-wk-old white Leghorns as previously described (12).

Preparation of EC3b. EC3b were prepared by a modification of the procedure published elsewhere (13). 6 × 10⁹ washed erythrocytes were mixed with 800 µg C3 and 1.5 µg ¹²⁵I-C3 in a total vol of 500 µl GVB++. Deposition of C3b from the fluid phase onto the cell surface was effected by addition of 24 µg trypsin and incubation for 2.5 min at 23°C. Cells were washed 3 times with 10 ml GVB++ and resuspended in 500 µl GVB++. Formation of cell-bound, alternative C3 convertase was accomplished by addition of 500 µg factor B, 300 ng factor D, 30 µg NF, and sufficient MgSO₄ to give a final concentration of 1.2 mM and incubation for 15 min at 37°C. Cells were washed two times in GVB++ and suspended in 0.5 ml GVB++ containing 800 µg C3 and 1.5 µg ¹²⁵I-C3. C3 deposition by the cell-bound C3 convertase was effected by incubation for 2.5 h at 37°C or 1 h at 37°C and 16 h at 4°C. Cells were washed three times into the appropriate buffer and standardized to 10⁹/ml. The number of C3b bound per cell was determined by assessing the radioactivity associated with a 50-µl sample of cells. Erythrocytes bearing C3bi fragments (EC3bi) were produced by incubation for 45 min at 37°C of EC3b with purified C3b inactivator (8.0 µg/ml) and trypsin (40.0 µg/ml). Erythrocytes bearing C3d fragments (EC3d) were produced from EC3bi by incubation with 10 µg/ml trypsin.

Testing of Cellular Intermediates. The functional state of C3 fragments on erythrocytes was ascertained using a series of assays. C3/C5 convertase formation was determined by the ability of erythrocytes to be lysed by incubation with factors B and D and guinea pig C3-9 as described (14). Immune adherence reactivity was determined in microtiter plates using human erythrocytes as published previously (15). Conglutinin-dependent hemagglutination was performed in microtiter plates containing 2.5 × 10⁶ erythrocytes, serial-dilutions of bovine conglutinin, and GVB++ to a total vol of 75 µl.

Antisera. Antisera to Eb or Ec were prepared from rabbits by six intravenous injections during a period of 10 d. IgG fractions of the antisera were prepared by gel-exclusion chromatography on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) (16). Anti-Eb reacted with Eb but not with Ec as determined by hemagglutination (titer: 21 µg/ml) and ADCC-induction, whereas the reverse was true for anti-Ec. Both antibody preparations reacted with Ec.

Lymphocytes. Human peripheral blood lymphocytes were isolated and purified as previously described (12). In brief, after defibrination of the blood with glass beads, a leukocyte-enriched fraction was prepared by gelatin sedimentation. Phagocytes and adhering cells were removed by treatment with carbonyl iron powder, followed by centrifugation through Ficoll-Isopaque (FIP; Pharmacia Fine Chemicals). The lymphocytes, collected at the interface, were kept at 4°C overnight in Hepes (10 mM)-buffered RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) containing 0.4% human serum albumin (HSA; Cutler Laboratories, Berkeley,
Calif.) and supplemented with 100 U penicillin, 100 μg/ml Fungizone (Grand Island Biological Co.), and 2 mM L-glutamine (complete TCM). They were used the next day after centrifugation and suspension in fresh complete TCM. HSA was used as protein supplement throughout these experiments.

**FcyR Modulation of Lymphocytes.** 1.6 × 10⁸ lymphocytes and 4 × 10⁶ Eb or Ec were mixed in a 50-ml Falcon tube (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) in a total vol of 12 ml complete TCM in the presence of anti-Eb or anti-Ec (IgG fractions). The antibody concentrations were chosen to give 80-90% lysis of ⁵¹Cr-labeled erythrocytes when mixed with aliquots of the lymphocytes at the same ratios under ADCC conditions. After 20 h (or, in one experiment, 40 h) of incubation at 37°C, the cells were washed once in TH, and were then suspended in complete TCM at a concentration of 4 × 10⁸ lymphocytes/ml. Control lymphocytes were mixed with erythrocytes and processed similarly but in the absence of antibody. When lymphocytes, modulated by exposure to antibody-coated erythrocytes, were mixed with fresh ⁵¹Cr-labeled target erythrocytes (Es) under ADCC conditions but without addition of fresh antibody, no lysis ensued (cf. Table II), indicating that no antibody had been transferred with the lymphocytes in these experiments (17).

**Fractionation of Lymphocytes by Adsorption to Monolayers of C3-fragment-bearing Eb.** Tissue culture dishes (Falcon 3002; 60 × 15 mm) were treated for 1 h with 2 ml TH containing 50 μg/ml poly-L-lysine (70,000 mol wt; Sigma Chemical Co., St. Louis, Mo.). After washing, 2 ml TH containing 4 × 10⁸ Eb bearing either C3b, C3bi, or C3d, were added to the dishes. The erythrocytes were allowed to settle at room temperature. After 40 min of incubation, nonadsorbed Eb were washed off with TH. 8 × 10⁶ lymphocytes in 3 ml of complete TCM were added to the monolayers and incubated for 15 min at 37°C. The dishes were then centrifuged for 5 min at 100 g. After 15 min at 4°C, the nonadhering lymphocytes were carefully decanted with a Pasteur pipette and pooled with three additional washes (2 ml of TH wash per dish). The lymphocytes were then washed in TH, and finally suspended in complete TCM at a concentration of 4 × 10⁸ living cells/ml. Recovery of lymphocytes from C3-free Eb control monolayers was ~100%.

**Fractionation of Lymphocytes by Rosetting with C3-fragment-bearing Eb.** 1.6 × 10⁸ lymphocytes, suspended in 1 ml complete TCM, were mixed in conical 15-ml tubes (Falcon 2098) with 1 ml TH containing 4 × 10⁴ Eb bearing either C3b, C3bi, or C3d (EbC3b, EbC3bi, and EbC3d). After 5 min of incubation at 37°C, the tubes were centrifuged for 5 min at 100 g. After 1 h additional incubation at 4°C, the cell mixtures were gently suspended, diluted with 3 ml complete TCM, and layered over 1.5 ml FIP. After centrifugation for 20 min at 1,200 g, the nonrosetting lymphocytes were collected at the interface washed in TH and suspended in complete TCM at a concentration of 3 × 10⁶ living cells/ml.

**ADCC.** ADCC of erythrocytes (Eb, Ec, or Ec, respectively) was assayed as described (12). 4 × 10⁶ lymphocytes were mixed with 10⁶ ⁵¹Cr-labeled erythrocytes, either untreated or bearing C3 fragments, in the presence or absence of anti-erythrocyte antibodies (IgG fractions). The total vol of the incubation mixture was 0.3 ml. All mixtures were prepared in duplicate and incubated for 18 h at 37°C. After centrifugation for 10 min at 800 g, the release of ⁵¹Cr was determined.

**Rosette Formation with C3-Fragment-bearing Eb.** 0.1 ml lymphocytes in complete TCM (4 × 10⁸ lymphocytes/ml) were thoroughly mixed with 0.1 ml Eb, EbC3b, EbC3bi, or EbC3d (10⁶/ml). In total the mixtures were incubated for 15 min at 37°C, centrifuged for 5 min at 100 g and held at 4°C overnight. Raji and Daudi cells were washed three times with TH and suspended in complete TCM at a concentration of 2 × 10⁶ cells/ml. For rosetting, 0.1 ml of the cells was mixed with 0.1 ml C3-fragment-bearing Es as described above. Drops of the cell mixtures were transferred to slides and covered with cover slips, coated with 0.001% ethanol solution of acridine orange for nuclear staining of the lymphocytes (18). The percentage of rosetting cells was determined by counting 600-800 cells (two slides per sample) in the ultraviolet (UV) microscope under visible and UV light.

**Results**

Identification of Target Cell-bound C3 Fragments. Table I defines the characteristics of C3 fragments present on target cells. Cell-bound C3b was capable of producing
TABLE I

Identification of C3 Fragments on Cellular Intermediate Complexes

| Property                    | Intermediate complex |       |       |       |
|-----------------------------|----------------------|-------|-------|-------|
|                             | EC3b | EC3bi | EC3d |
| C3-associated $^{125}$I*    | 100  | 100   | 10   |
| C3/C5 convertase‡           | 100  | 0     | 0    |
| Immune adherence§           | 100  | 0     | 0    |
| Conglutination§             | 0    | 100   | 0    |

* Data are represented as percentages of C3b radioactivity.
‡ Data are represented as percentages of lysis.
§ Data are represented as percentages of activity.

Fig. 1. Enhancement of ADCC by target cell-bound C3 fragments. $^{51}$Cr-labeled E$_b$ lacking C3 fragments ($\bullet$) or bearing 45,000 C3b ($\Delta$), C3bi ($\bigcirc$), or C3d ($\bigtriangleup$) molecules per cell were incubated with lymphocytes and anti-E$_b$ antibody (13 mg IgG/ml stock solution) for 18 h. at an E:T ratio of 4:1 as described in Materials and Methods. Spontaneous $^{51}$Cr release was 8% for E$_b$ and E$_b$C3b, and 13% for E$_b$C3bi and E$_b$C3d. The release in the antibody-free lymphocyte controls was the same as the spontaneous release ($\pm$ 1%).

positive immune adherence reactivity with human erythrocytes and of forming the alternative pathway C3/C5 convertase with factors B and D and magnesium ions. Cells bearing C3bi were lacking the above functions, but exhibited the unique ability to be agglutinated by purified bovine conglutinin in the presence of calcium ions. Cells bearing C3d lacked all the above functions, but retained 10% of the radioactivity previously associated with C3b. A minimum of 100 C3b molecules or 500 C3bi molecules could be detected per cell by the functional tests.

Enhancement of ADCC by Target Cell-bound C3bi and C3d. To establish the effect of target cell-bound C3 fragments on cell-mediated lysis, E$_b$C3b, E$_b$C3bi, or E$_b$C3d were incubated with highly purified lymphocytes either in the absence or in the presence of rabbit anti-E$_b$ (IgG fraction). Fig. 1 shows specific $^{51}$Cr release from the target cells after 18 h of incubation. The spontaneous $^{51}$Cr release from C3-fragment-bearing erythrocytes was comparable to that observed with erythrocytes lacking C3 fragments. Target cells with C3b, C3bi, or C3d were not lysed by the lymphocytes in absence of antibody. However, all three fragments enhanced ADCC. Enhancement was between 5- and 15-fold at suboptimal antibody concentration. The number of C3 molecules per target cell varied from 15,000 to 100,000, and very similar results were obtained in replicate experiments. The enhancing effects of the C3 fragments ranked in the
order of C3bi > C3d >> C3b. The enhancing effect of C3b was very weak. The magnitude of enhancement by the fragments varied with different lymphocyte preparations.

To study the effect of enhancement by C3 fragments on modulated lymphocytes, lymphocytes were incubated at 37°C with antibody-coated Eb or Ec before they were used as effector cells. This treatment is known to remove FcyR from the lymphocyte surface (19, 20) and, hence, to strongly reduce their ADCC potential. However, their natural cytotoxicity is unchanged or even increased as determined by lysis of nucleated target cells in the absence of antibody (21, 22). Table II shows the results of three experiments in which modulated lymphocytes were used with Eb with or without C3 fragments in absence or in presence of antibody. In experiments 2 and 3, modulation markedly reduced ADCC. The presence of C3 fragments on the target, however, markedly enhanced the cells' cytotoxicity, which was dependent on antibody dose. In experiment 3, lysis of C3-fragment-bearing cells could be detected even in the absence of antibody.

**Demonstration of C3bi Receptors on Lymphocytes in Addition to Receptors for C3b and C3d.** The interaction of lymphocytes with EbC3b, EbC3bi, or EbC3d was studied using a rosetting assay. Table III shows the results obtained with lymphocytes from six different donors. Three different preparations of C3 were used. As control, the lymphoblastoid cell lines Raji and Daudi were included. Peripheral blood lymphocytes formed rosettes with all three types of indicator cells. There was no difference in the size of the rosettes, regardless of the type of fragments used. However, the percent of rosettes formed with different indicator cells varied significantly. The percent rosettes formed with EbC3b was almost twice that with EbC3bi and 5- to 10-fold higher than with EbC3d. Both Raji and Daudi cells formed very strong rosettes with

| Table II |
|-------------------|
| **Effect of Target Cell-bound C3 Fragments on Cytotoxicity of FcyR-modulated Lymphocytes** |

| Exp.* | Target cells | Spontaneous release‡ | &superscript;Cr release at different concentrations of anti-Eb§ |
|-------|--------------|----------------------|-------------------------------------------------------------|
|       |              |                      | Control lymphocytes (µg IgG/ml)                              | Modulated lymphocytes (µg IgG/ml) |
|       |              |                      | 0      | 0.13 | 0.4 | 1.3 | 4.0 | 13 |
| 1     | Eb           | 5                    | 9      | 23   | 40  | 51  | 13  | 18 |
|       | EbC3b        | 10                   | 12     | 47   | 62  | 71  | 16  | 53 |
| 2     | Eb           | 20                   | 12     | 28   | 11  | 17  | 14  | 43 |
|       | EbC3b        | 23                   | 11     | 39   | 14  | 43  | 15  | 53 |
|       | EbC3bi       | 18                   | 16     | 56   | 15  | 53  | 14  | 17 |
| 3     | Eb           | 13                   | 12     | 44   | 14  | 17  | 14  | 17 |
|       | EbC3b        | 20                   | 26     | 58   | 33  | 64  | 35  | 71 |
|       | EbC3bi       | 22                   | 30     | 77   | 35  | 71  | 24  | 57 |
|       | EbC3d        | 24                   | 27     | 72   | 24  | 57  |

* Experiment 1: ~50,000 C3b-Eb; lymphocytes modulated with Eb-anti-Eb for 20 h. Experiment 2: ~20,000 C3b/Eb; lymphocytes modulated with Eb-anti-Eb for 40 h. Experiment 3: ~90,000 C3b/Eb; lymphocytes modulated with Ec-anti-Ec for 20 h.

‡ Percent &superscript;Cr-release from Eb in lymphocyte-free controls.

§ Percent total &superscript;Cr-release (uncorrected) after 18 h, lymphocyte:target cell ratios of 4:1.
Table III

| Exp. | EbC3b | EbC3bi | EbC3d |
|------|-------|--------|-------|
|      | Lymphocytes | Raji | Daudi | Lymphocytes | Raji | Daudi | Lymphocytes | Raji | Daudi |
| 1    | 13.0  | 64    | <1    | 8.8   | >95  | >95  | 1.4   | >95  | >95  |
| 2    | 10.1  | 20    | 0     | 5.2   | >95  | >95  | 5.2   | >95  | >95  |
| 3    | 10.5  | <5    | 0     | 7.1   | >95  | >95  | 1.7   | >95  | ~60  |
| 4    | 10.4  | ~90   | ND    | 3.0   | >95  | ND   | 0.5   | >95  | ND   |
| 5    | 11.3  | 42    | 0     | 4.3   | >95  | ND   | 1.0   | >95  | ~60  |
| 6    | 10.2  | ND    | ND    | 5.5   | ND   | ND   | 1.0   | ND   | ND   |

10.9 ± 1.1§ | ~44 | 0 | 5.7 ± 2.1 | ~95 | ~95 | 1.8 ± 1.7 | ~95 | ~70 |

* C3-fragment-bearing Eb made up freshly for each experiment. No rosettes were formed in Eb controls included in every experiment.
‡ From six different donors.
§ Means ± SD.
∥ Not done.

EbC3bi and EbC3d. In both instances, a large number of indicator cells was bound to lymphoblastoid cells. Raji cells, but not Daudi cells, also bound EbC3b. However, the frequency of these rosettes was low and the rosettes were weak, and contained fewer indicator cells. (Fig. 2)

Heterogeneity of Lymphocytes with Respect to Type of Complement Receptor. C3-fragment-binding lymphocytes were depleted from the effector cell preparations by two different methods: (a) adsorption of lymphocytes to monolayers of C3-fragment-bearing Eb, and (b) formation of rosettes with C3-fragment-bearing Eb, followed by separation from nonrosetting lymphocytes by centrifugation.

Table IV shows the distribution of C3 receptors among cells that did not adsorb to EbC3d monolayers. The cells recovered in the supernate were then layered onto EbC3bi monolayers. Each depletion step removed ~20% of the lymphocytes originally added. There was no selective loss of EbC3b rosetting cells. The yield of EbC3bi-binding cells after C3d depletion was slightly lower than the total yield. C3bi depletion resulted in a very significant, but not complete, reduction of EbC3bi rosetting cells. Similarly, C3d depletion reduced the number of EbC3d rosetting cells. The extent of
Table IV

Fractionation of Lymphocytes by Sequential Adsorptions to C3-fragment-bearing E_b Monolayers

| Lymphocytes                  | Percent recovery after depletion* | EbC3b | EbC3bi | EbC3d |
|------------------------------|----------------------------------|-------|--------|-------|
|                              | Percent rosettes                 | Frequency | Recovery | Frequency | Recovery | Frequency | Recovery |
| Control                      | 100                              | 10.5  | 100    | 7.6    | 100      | 1.9      | 100      |
| EbC3d depleted               | 83                               | 12.3  | ~99    | 6.3    | 74       | <0.5     | <20      |
| EbC3bi depleted after EbC3d depletion | 63                             | 11.0  | 67     | 1.6    | 15       | <0.5     | <20      |

* Recovery of lymphocytes in supernates after monolayer adsorption, given as percent of original input (=100).
‡ Frequencies of rosette-forming lymphocytes in supernates after monolayer adsorption assayed by addition of fresh E_b-intermediates (no rosettes present in supernates before assay).
§ Recoveries of rosette-forming lymphocytes in supernates after adsorption, given as percent of original input (=100).

Table V

Fractionation of Lymphocytes by Rosetting with C3-Fragment-bearing E_b

| Lymphocytes                  | Percent recovery after depletion* | EbC3b | EbC3bi | EbC3d |
|------------------------------|----------------------------------|-------|--------|-------|
|                              | Percent rosettes                 | Frequency | Recovery | Frequency | Recovery | Frequency | Recovery |
| Control                      | 100                              | 10.5  | 100    | 5.5    | 100      | 1.0      |          |
| EbC3d depleted               | 75                               | 10.0  | 71     | 3.5    | 48       | <0.5     | NC       |
| EbC3b + EbC3d depleted       | 66                               | <1    | <6     | 2.0    | 24       | <0.5     | NC       |

* Recovery of lymphocytes in supernates after rosettes depletion, given as percent of original input (=100).
‡ Frequencies of rosette-forming lymphocytes in supernates after depletion, assayed by addition of fresh E_b-intermediates (no rosettes present in supernates before assay).
§ Recoveries of rosette forming lymphocytes in supernates after depletion, given as percent of original input (=100).
|| Not calculated.

this depletion is not certain because of the low frequency of EbC3d rosetting cells in this experiment.

Table V summarizes the results of an experiment in which depletion was performed by rosetting with either EbC3d or with a mixture consisting of equal parts of EbC3b and EbC3d. It will again be noted that C3d depletion did not selectively diminish the number of C3b rosetting cells. Only very few remaining C3d rosettes were seen after this step. The frequency of C3bi rosetting cells was also decreased. Depletion with mixtures of EbC3b and EbC3d removed >90% of the EbC3b rosetting cells and apparently all EbC3d rosetting cells, whereas ~25% of the EbC3bi rosetting cells remained in the supernate.

Inhibition of ADCC by C3-Fragment-bearing Bystander Cells. To explore whether interaction of lymphocytes with erythrocyte-bound C3 fragments results in the release of a soluble ADCC-enhancing factor, unlabeled E_b, bearing either C3b, C3bi or C3d, were mixed with an equal number of ^51^Cr-labeled erythrocytes not bearing C3
fragments. Different dilutions of antibody to Eb were added to the lymphocyte-Eb mixtures. Fig. 3 indicates that no soluble factor that would enhance lysis of C3-free cells was released under these conditions. On the contrary, the presence of C3-fragment-bearing Eb in the incubation mixtures inhibited lysis of the C3-free targets. Inhibition by C3-fragment-bearing Eb was also observed when Eb was used as target cells in the presence of anti-Ec antibodies which did not cross-react with Eb.

Discussion

The data presented in this paper show that lymphocytes that participate in ADCC may have receptors reacting with target cell-bound C3bi in addition to those reacting with C3b and C3d. All three cell-bound C3 fragments enhanced ADCC, but C3bi and C3d enhanced the reaction much more strongly than C3b. The data also show that in absence of specific antibody, cytotoxicity was not induced in spite of the presence of up to 100,000 C3-fragments/target cell, which is in agreement with our earlier results (2).

Approximately 40% of the lymphocytic effector cells of ADCC have receptors for activated C3 as assessed by rosetting of individual effector cells in the ADCC plaque assay (23, 24). At least two distinct receptors reacting with C3b or C3d have previously been found on human peripheral blood lymphocytes and other cells (25, 26). The C3b receptor (CR1 or immune adherence receptor) is present on erythrocytes, lymphocytes, polymorphonuclear leukocytes (PMN), and monocytes and reacts with the intact C3b-molecule. The C3d receptor (CR2) is present on lymphocytes, but not on PMN or monocytes and reacts with the C3d-fragment (26–28). Recently, the occurrence of an additional receptor on human PMN and monocytes with specificity for C3bi (CR3) was proposed (29). The C3bi receptor has also been found on human glomeruli (24) and on rat peritoneal mast cells (30). In this study, using human peripheral blood lymphocytes, ~11% of the cells bound EC3b, 6% bound EC3bi, and 2% bound EC3d. Raji cells bound EC3b weakly and with different frequencies when different batches of these cells were tested. In contrast, most Raji cells formed large rosettes with either C3bi- or C3d-indicator cells. The latter was also the case with Daudi cells which, however, did not bind EbC3b. This is in accord with previous

![Figure 3](image-url)
findings indicating that Daudi cells lack the C3b receptor, but possess the C3d receptor (31, 32).

Evidence was obtained indicating that the distribution of C3 receptors among human peripheral blood lymphocytes is nonhomogeneous. The results of the depletion experiments indicated that lymphocytes bearing C3b receptors are distinct from those bearing C3bi and/or C3d receptors. No definite conclusion could be reached regarding the differential expression of C3bi and C3d receptors on these lymphocytes. However, in unpublished studies, further insights in these questions have recently been gained by using the ADCC plaque assay (23, 24). C3bi receptor bearing plaque-forming cells (PFC) were more than twice as frequent than C3b receptor PFC. C3d receptor PFC were even fewer than C3b receptor PFC. Further, plaques were formed on monolayers of Eb that bore antibody and either C3b, C3bi, or C3d. Subsequently the PFC were incubated with Eb that bore one of the three fragments but not antibody. Under those conditions, the majority of the PFC rosetted with those cells that carried the same C3 fragments as the target cells. Most of the PFC with receptors for C3d also had receptors for C3bi. The PFC that carried both the C3bi and C3d receptors appeared to be T cells, as judged by surface-marker analysis, whereas the C3b receptor PFC were null cells. A detailed account of these results will be published elsewhere (B. Wählín, H. Perlmann, P. Perlmann, R. Schreiber, and H. Müller-Eberhard, manuscript in preparation).

It has been reported that EAC-rosetting of C3-receptor bearing lymphocytes induces the release of lymphotoxin-like factors, which enhance the natural cytotoxicity of lymphocytes to nucleated target cells (33). The present results do not support a role for soluble factors in this system. The exposure of lymphocytes to C3 fragments before the cytotoxicity assay did not increase their natural cytotoxicity to Eb, nor their ADCC potential. Admixture of C3-fragment bearing target cells to 51Cr-labeled, C3-fragment-free target cells did not enhance ADCC of the labeled cells. Enhancement should have occurred if a soluble enhancing factor had been generated in the presence of C3 fragments.

The target cell-mixing experiments gave additional information. To achieve enhancement of ADCC, antibody and C3 fragments had to reside on the same cell. When cells were introduced into the reaction mixture which carried C3 fragments but no antibody, lysis of antibody-coated cells was inhibited. The inhibitory capacity of the fragments ranked in the same order as their enhancing capacity: C3bi > C3d >> C3b. Presumably, this inhibition of ADCC is a result of competitive binding of the C3-fragment-bearing cells to the effector cells.

Taken together, the results described in this paper show that cell-bound C3 fragments have an important role in regulating ADCC. Because this reaction requires target cell-effector cell contact (1) C3 fragments on the surface of the target cells may enhance cytotoxicity through interaction with C3 receptors on the effector cells. That C3bi and C3d are more efficient in enhancing ADCC than C3b may be biologically significant. Although complement activation via the classical or the alternative pathway leads to deposition of C3b on the target cell surface, the structural and functional integrity of C3b is under rigid temporal constraints. Owing to the cooperative action of β1H and the C3b inactivator, C3b is converted to C3bi, which in turn is degraded to C3d. Because C3b conversion is a rapid process, the predominant forms of cell-bound C3 are C3bi and C3d. Neither of these fragments is capable of sustaining
the cytolytic reaction which would result in the formation of the membrane attack complex and cell death. However, because C3bi and C3d are more efficient in enhancing ADCC than C3b, these fragments may facilitate cellular surveillance in the presence of minute amounts of antibody.

Summary

The occurrence and distribution of distinct receptors for three C3 fragments on purified human blood lymphocytes were studied by rosette formation. Indicator cells were bovine, chicken, or sheep erythrocytes (E) bearing up to 100,000 molecules of human C3b (EC3b) without antibody. EC3b was converted to C3bi-bearing E (EC3bi) with purified C3b inactivator (factor I) and B1H (factor H), and to C3d-bearing E (EC3d) by treatment of EC3bi with trypsin. Using bovine E (Eb) as indicators, ~1% of the lymphocytes bound EbC3b, 6% bound EbC3bi and 2% bound EbC3d. Fractionation of the lymphocytes by adsorption to monolayers of C3-fragment-bearing Eb or by rosetting indicated that most of the cells with receptors for C3b were distinct from those having receptors for C3bi and/or C3d. Cells from two lymphoblastoid cell lines (Raji and Daudi) formed strong rosettes with either EC3bi or EC3d. A fraction of the Raji cells, but not of Daudi cells formed rosettes with EC3b, which were weak. 

A fraction of the Raji cells, but not of Daudi cells formed rosettes with EC3b, which were weak. 


gamma Cr-labeled E was used as a target in antibody-dependent, lymphocyte-mediated cytotoxicity (ADCC). In the absence of antibody, C3-fragment-bearing E was not lysed by the lymphocytes. However, at suboptimal concentrations of IgG anti-E antibody, ADCC of C3-fragment-bearing E was strongly enhanced. The enhancing capacity of the fragments occurred in the order of C3bi > C3d >> C3b. In addition, C3-fragment-bearing cells inhibited the lysis of antibody-coated cells not bearing C3 fragments. Inhibition ranked in the same order as enhancement. It is concluded that target cell bound C3 fragments enhance ADCC by improving contact between target cells and those effector cells which have C3 receptors. Cell-bound C3 fragments inhibit ADCC of C3-free targets by impeding their contact with such effector cells. It is proposed that certain lymphocytes are capable of interacting with C3bi in addition to C3b and C3d and that C3bi and C3d have a greater regulatory effect on their cytolytic function than C3b.

We thank Mrs. Mary Brothers for expert technical assistance, and Dr. Michael Pangburn and Dr. George LaMotte of our Institute for contributing to the supply of proteins.

Received for publication 17 February 1981.

References

1. Perlmann, P., and J. C. Cerottini. 1979. Cytotoxic lymphocytes. In The Antigens. M. Sela, editor. Academic Press, Inc., New York. 5:173.
2. Van Boxel, J. A., W. E. Paul, I. Green, and M. M. Frank. 1974. Antibody dependent lymphoid cell mediated cytotoxicity: role of complement. J. Immunol. 112:398.
3. Perlmann, P., H. Perlmann, and H. J. Müller-Eberhard. 1975. Cytolytic lymphocytic cells with complement receptors in human blood. Induction of cytolysis by IgG antibody but not by target cell bound C3. J. Exp. Med. 141:287.
4. Lustig, H. J., and C. Bianco. 1976. Antibody-mediated cell cytotoxicity in a defined system: regulation by antigen, antibody, and complement. J. Immunol. 116:253.
5. Ghebrehiwet, B., R. G. Medicus, and H. J. Müller-Eberhard. 1979. Potentiation of
antibody-dependent cell-mediated cytotoxicity by target cell-bound C3b. J. Immunol. 123: 1285.

6. Müller-Eberhard, H. J., and R. D. Schreiber. 1980. Molecular biology and chemistry of the alternative pathway of complement. Adv. Immunol. 29:1.

7. Tack, B. F., and J. W. Prah. 1976. Third component of human complement: purification from plasma and physiochemical characterization. Biochemistry. 15:4513.

8. Götz, O., and H. J. Müller-Eberhard. 1971. The C3-activator system: an alternate pathway of complement activation. J. Exp. Med. 135:44.

9. Pangburn, M. K., R. D. Schreiber, and H. J. Müller-Eberhard. 1977. Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein β1H for cleavage of C3b and C4b in solution. J. Exp. Med. 146:257.

10. Schreiber, R. D., R. G. Medicus, O. Götz, and H. J. Müller-Eberhard. 1979. Properdin and nephritic factor dependent C3 convertases: requirement of native C3 for enzyme formation and the function of bound C3 as properdin receptor. J. Exp. Med. 142:760.

11. Lachmann, P. J. 1967. Conglutinins and immunoconglutinins. Adv. Immunol. 6:480.

12. Perlmann, H., P. Perlmann, G. R. Pape, and G. Hallden. 1976. Purification, fractionation and assay of antibody dependent lymphocytic effector cells (K-cells) in human blood. Scand. J. Immunol. 5:57.

13. Pangburn, M. K., and H. J. Müller-Eberhard. 1978. Complement C3 convertases: cell surface restriction of β1H control and generation of restriction of neuraminidase treated cells. Proc. Natl. Acad. Sci. U. S. A. 75:2416.

14. Schreiber, R. D., M. K. Pangburn, P. L. Lesavre, and H. J. Müller-Eberhard. 1978. Initiation of the alternative pathway of complement: recognition of activators by bound C3b and assembly of the entire pathway from six isolated proteins. Proc. Natl. Acad. Sci. U. S. A. 75:3948.

15. Cooper, N. R. 1969. Immune adherence by the fourth component of complement. Science (Wash. D. C.). 165:396.

16. Perlmann, H., L. Moretta, and P. Perlmann. Regulation of ADCC in vitro by IgM antibodies. Effector cells and mechanisms. Scand. J. Immunol. In press.

17. Perlmann, P., H. Perlmann, and H. Wigzell. 1972. Lymphocyte mediated cytotoxicity in vitro. Induction and inhibition by humoral antibody and nature of effector cells. Transplant. Res. 13:91.

18. Soulillou, J. P., C. B. Carpenter, A. J. F. d'Apice, and T. B. Strom. 1976. The role of nonclassical, Fc-receptor-associated Ag-B antigens (Ia) in rat allograft enhancement. J. Exp. Med. 143:405.

19. Cordier, G., C. Samarut, and J. P. Revillard. 1977. changes of Fc-receptor related properties induced by interaction of human lymphocytes with insoluble immune complexes. J. Immunol. 119:1943.

20. Moretta, L., M. C. Mingari, and C. A. Romanzi. 1978. Loss of Fc-receptors for IgG from human T-lymphocytes exposed to IgG immune complexes. Nature (Lond.). 272:618.

21. Pape, G. R., L. Moretta, M. Troye, and P. Perlmann. 1979. Natural cytotoxicity of human Fcγ-receptor-positive T-lymphocytes after surface modulation with immune complexes. Scand. J. Immunol. 9:291.

22. Härfast, B., T. Andersson, A. Alsheikly, and P. Perlmann. 1980. Effect of Fc-receptor modulation on mumps virus dependent lymphocyte-mediated cytotoxicity in vitro. Scand. J. Immunol. 11:357.

23. Währin, B., and P. Perlmann. 1976. Detection of K-cells by a plaque assay. In In Vitro Methods in Cell-mediated and Tumor Immunity. B. Bloom and J. R. David, editors. Academic Press, Inc., New York. 523.

24. Währin, B., H. Perlmann, and P. Perlmann. 1976. Analysis by a plaque assay of IgG or IgM dependent cytolytic lymphocytes in human blood. J. Exp. Med. 144:1375.
25. Ross, G. D., M. J. Polley, E. M. Rabellino, and H. M. Grey. 1973. Two different complement receptors on human lymphocytes. One specific for C3b and one specific for C3b inactivator-cleaved C3b. *J. Exp. Med.* 138:798.

26. Ross, G. D., and M. J. Polley. 1975. Specificity of human lymphocyte complement receptors. *J. Exp. Med.* 141:1813.

27. Dierich, M. P., and V. A. Bokisch. 1977. Receptor binding sites on C3 and C3b. *J. Immunol.* 118:2145.

28. Carlo, J. R., S. Ruddy, E. J. Studer, and D. H. Conrad. 1979. Complement receptor binding of C3b-coated cells treated with C3b activator, β1H globulin and trypsin. *J. Immunol.* 123:523.

29. Ross, G. D., and E. M. Rabellino. 1979. Identification of a neutrophil and monocyte complement receptor (CR3) that is distinct from lymphocyte CR1 and CR2 and specific for a site contained within C3bi. *Fed. Proc.* 38:1457.

30. Vranian, G., D. Conrad, and S. Ruddy. 1980. C3 mediates phagocytosis by rat peritoneal mast cells. *J. Immunol.* 124:1544.

31. Dierich, M. P., M. A. Pellegrino, S. Ferrone, and R. A. Reisfeld. 1974. Evaluation of C3-receptors on lymphoid cells with different complement sources. *J. Immunol.* 112:1766.

32. Theoﬁlopoulos, A. N., F. J. Dixon, and V. A. Bokisch. 1974. Binding of soluble immune complexes to human lymphoblastoid cells. I. Characterization of receptors for IgG Fc and complement and description of the binding mechanism. *J. Exp. Med.* 140:877.

33. O'Neill, P., B. F. Mackler, and P. Wyde. 1975. Complement (C3) receptor bearing lymphocyte-mediated cytotoxicity and lymphotoxin responses. *Cell. Immunol.* 20:33.