INHIBITION OF THE Fc RECEPTOR OF HUMAN LYMPHOID CELLS BY ANTISERA-RECOGNIZING DETERMINANTS OF THE HLA SYSTEM*

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B lymphocytes are equipped with a receptor for the recognition of the Fc part of IgG (1, 2) which is not identical to the surface Ig receptor (3). Dickler and Sachs (4) found that anti-Ia antibodies inhibit the binding of aggregated IgG to this receptor, whereas anti-H-2D or K antibodies did not have the same effect. On the other hand, Schirrmacher et al. (5) found that also antibodies against other B-lymphocyte surface constituents than Ia could interfere with the Fc receptor. However, the Ia antigens are probably not part of the Fc receptor molecule (6), and the observed inhibition may best be explained by a close association between Fc receptors and the Ia antigens in the B-cell membrane. Since Ia antibodies do not inhibit the Fc receptors of macrophages or fetal liver cells (7), the association between Ia and Fc on these cells may be less pronounced.

We have investigated the relationship between HLA antigens and Fc receptors on human lymphoid cells. The inhibitory activity of different HLA antisera on cells forming rosettes with antibody-coated erythrocytes (FcRFC) and K-cell cytotoxicity was studied with antisera recognizing determinants coded for by different regions of the HLA chromosomal complex. The HLA-A, -B, and -C regions are the human analogues of the mouse H-2D and H-2K regions, while the HLA-D region codes for mixed lymphocyte culture (MLC)-activating determinants which may be analogues to some of the mouse Ia antigens.

Materials and Methods

Antisera. Serum EA from a multiparous woman is specific for HLA-B7 in lymphocytotoxicity tests and does not contain detectable antibodies specific for HLA-Dw2-associated B-cell antigens. Rabbit antihuman β-2-microglobulin (β-2-m) (16-U72; Dakopatts A/S, DK-2000 Copenhagen F, Denmark) was used, since it is part of the HLA-A, -B and -C, but not the HLA-D-associated molecules in the cell membrane (8, 9). Antisera reactive with determinants of the HLA-D region were sought by immunization in HLA-A, -B (and -C?) compatible, but HLA-D-incompatible MLC-positive unrelated combinations. Serum producer S6W is HLA-1A,3; B7,8; Dw3,Ld-oh, and TH is A2,3; B7,12; Dw1,2; their immunizing donors are A1,3; B7,8; Dw2,3, and A2,3; B7,12; Dw6,x, respectively. Details of these sera are presented elsewhere (8). They are reactive with human B lymphocytes and monocytes in cytotoxicity and immunofluorescence tests, but not with normal T lymphocytes. Serum S6W reacts with a determinant which, in population studies, is very closely associated to HLA-Dw2, while serum TH may be directed against a more public determinant including HLA-Dw3, -Dw6, and others.

Rabbit antihuman IgG (10-090) and antihuman IgG-Fc fragment-specific (10-00G) and swine antirabbit IgG (21-090) were all delivered as isolated immunoglobulins by Dakopatts A/S. They

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were excessively absorbed with sheep red blood cells (SRBC) to remove heteroantibodies.

**F(ab')2 Fragments.** F(ab')2 fragments of serum SSW, TH, and EA were prepared by pepsin (2 × crystallized and lyophilized; activity 2,500–3,200 U/mg protein, Sigma Chemical Co., St. Louis, Mo.) digestion of IgG fractions at 37°C pH 4.2 in 0.1 N acetate buffer for 24 h at enzyme/protein ratio of 2:100. IgG was isolated from antiserum by salting out and batch absorption with DEAE Sephadex A50 equilibrated with 0.1 M Tris buffer pH 8.0. By double diffusion in 1% agarose, no precipitin lines were detectable with Fc-specific anti-IgG, while the reaction with anti-IgG was unaltered. Fab' fragments were prepared by reduction and alkylation of the F(ab')2 fragments by dialysis at 4°C against 0.2 M 2-mercaptoethanol followed by 0.001 M iodoacetamide (10). F(ab')2 fragments of rabbit antihuman IgG, rabbit antihuman ～2-m and swine antirabbit IgG were prepared by digesting the immunoglobulin preparations under conditions identical to those applied to human IgG. The precipitating activity in agarose gel was identical for F(ab')2-anti-β-2-m and original anti-β-2-m when tested against β-2-m. 10 ml of serum resulted in the production of 2.5 ml F(ab')2 fragments, while no significant volume changes occurred when IgG preparations were digested. All sera were ultracentrifuged immediately before use, for elimination of IgG aggregates.

**HLA Typing.** Mononuclear cells were prepared from fresh defibrinated blood by Ficoll-isopaque flotation (11). HLA-A, -B, and -C typing was performed with 54 highly selected sera by the microcytotoxicity test (12). HLA-D typing was performed by the use of HLA-D homozygous stimulating cells (13). Typing with serum S6W and TH was performed by microlymphocytotoxicity tests using as target cells lymphocytes enriched for B cells (8).

**Production of IgG-Coated SRBC.** SRBC, washed three times with phosphate-buffered saline, were made up to a 1% suspension. The IgG fraction of rabbit anti-SRBC, produced by Sephadex G-200 column chromatography, was diluted to the subagglutinating concentration 1/320 and mixed with the red cells and incubated at room temperature for 60 min. Thereafter, red cells were washed and resuspended in 1% suspension in balanced salt solution (BSS).

**Inhibition of FcRFC by Antisera.** 2 × 10⁶ peripheral blood mononuclear cells were mixed with antisera, diluted 1/2, 1/100 (as indicated) in a total vol of 0.2 ml and incubated for 30 min at +4°C. Thereafter, the cells were washed once in ice-cold buffer and resuspended in 0.5 ml BSS. In instances where two different antisera were used in consecutive steps for inhibition, both incubations were carried out at +4°C for 30 min with cell washes in between. Rosettes were allowed to form by the addition of 0.1 ml of antibody-coated 1% SRBC to 0.5 ml BSS containing 2 × 10⁶ cells. The cell mixture was spun in the cold at 800 rpm for 10 min and thereafter put on ice for another 30 min before examination for the number of rosettes in a light microscope at 400 × magnification. All cells binding five or more red cells were considered positive. At least 200 lymphocytes were scored in each cell suspension.

**Inhibition of K-Cell Cytotoxicity.** Antibody-dependent cell-mediated cytotoxicity (ADCC) was performed as a micro-method (14) with an HLA-B27 antiserum (serum FJH) as sensitizing antibody and human peripheral blood lymphocytes as target cells. Peripheral blood mononuclear cells which did not carry HLA-B27 and which were depleted of adherent cells by incubation in a 3024 Falcon plastic flask (Falcon Plastics, Oxnard, Calif.) for 60 min at 37°C were used as effector cells.

Effector cells (10⁵ in triplicates) were incubated at 4°C for 30 min with 0.15 ml antibody diluted 1/3, 1/100, as indicated on the tables, in medium (RPMI 1640 added 10% fetal calf serum). Thereafter the supernate was pipetted off, the cells washed once with ice-cold medium, 0.1 ml medium with sensitizing antibody was added, and then 5 × 10⁶⁵²⁵Cr-labeled target cells were jetted into the wells in a vol of 0.05 ml. Samples were then incubated at 37°C for 16 h in humidified CO₂ atmosphere, harvested with a semiautomatic harvesting machine (Skatron A/S, Lierbyen, Norway), and chromium release measured in a well type Packard Auto-Gamma Scintillation Spectrometer (Model S78). The percent chromium release was calculated by the formula (ER-SR)/(MR-SR) × 100, where ER is experimental release; SR is spontaneous release in the presence of effector cells without antiserum; MR is maximum release obtained by adding Cetavlon (Imperial Chemical Industries Ltd., Cheshire, England) to a final concentration of 5% to the target cells.

**Results**

**Inhibition of FcRFC.** Mononuclear cells were pretreated with various sera as described above, washed, and thereafter mixed with IgG-coated SRBC. The
results of one experiment are shown in Table I. Pretreatment with anti-HLA-B7 serum did not interfere with Fc rosette formation, neither by FcRFC carrying HLA-B7, nor by those lacking this antigen. However, serum SöW inhibited rosette formation only by FcRFC carrying Dw2, whereas serum TH did not affect the HLA-Dw2 carrying FcRFC, but inhibited the FcRFC from the other donor. Inhibition was found in serum dilution up to 1:10 (cytotoxic titer: 1/2). Altogether, FcRFC from six different individuals were tested with serum SöW. Inhibition of FcRFC was caused by serum SöW on all the four target cells carrying Dw2, but not on other cells.

Inhibition of FcRFC by Antibody Fragments. F(ab’)2 fragments from serum SöW were tested. Table II shows that F(ab’)2 from serum SöW caused a weak but significant inhibition of the two Dw2-positive donors, but had no effect on the Dw2-negative FcRFC. Fab’ fragments were not inhibitory. However, the addition of F(ab’)2 antihuman IgG as a second treatment of the cells increased the inhibitory capacity of dimeric antibody fragments and also caused inhibition by monomeric antibody fragments. The additive effect was most pronounced on monoclonal antibody fragments. F(ab’)2 anti-IgG antibody alone did not interfere with Fc rosette formation.

The effect of anti-β-2-m was also investigated. No effect on FcRFC could be detected. Furthermore, F(ab’)2 fragments against rabbit IgG were used in an attempt to enhance a possible inhibitory effect of anti-β-2-m, but without success.

Inhibition of K-Cell Cytotoxicity. Effector lymphocytes selected for the presence or absence of HLA-B7, Dw2, and Dw3, were used. Table III summarizes the results. Serum SöW and TH induced some HLA-D-correlated inhibition of the effector cells, but a stronger inhibition was observed with the anti-HLA-B7 antibodies on the HLA-B7-positive effector cells. Anti-β-2-m gave marked inhibition of all tested effector cells.

Experiments were also performed with F(ab’)2 fragments. None of the F(ab’)2 fragments demonstrated any inhibitory activity although they were reactive with the relevant antigens when tested by immunofluorescence. Thus, the inhibition of K-cell cytotoxicity seems to depend upon an intact Fc part in the blocking antibody.

Discussion

Striking differences in the ability of HLA antisera to inhibit Fc rosette-forming cells and K-cell cytotoxicity were demonstrated. Antisera recognizing HLA-A, -B, and -C antigens had no inhibitory effect on FcRFC, while two antisera, which may recognize determinants identical to or associated with those of the HLA-D series, specifically inhibited FcRFC. Inhibition, though somewhat decreased, was also expressed by the F(ab’)2 fragments, while Fab’ alone was inactive. Subsequent treatment of the Fab’-incubated cells with F(ab’)2 anti-IgG produced specific inhibition.

The Fc part of the shed AgAb complexes appears not to be the main inhibitory component. The experiments were performed under conditions where the cell membrane was rigid (4°C), and only antigens with very weak attachment to the membrane could possibly be shed. Capping/stripping experiments have indicated a firm attachment of
TABLE I

Inhibition of FcRFC by Serum S6W and TH but not by Anti-HLA-B7 Antibodies

| Serum          | Dilution | FcRFC + with cells from donor with HLA phenotype |
|----------------|----------|--------------------------------------------------|
| None           |          | A3;B7,Dw2                                       |
|                |          | A3,w19; B12,w15,Dw4,x                           |
| Normal AB serum| 1/2      | 35                                               |
|                |          | 35                                               |
| Anti-HLA-B7 (EA)| 1/2      | 36                                               |
|                |          | 24                                               |
|                | 1/10     | 33                                               |
|                |          | 36                                               |
| Serum S6W      | 1/2      | 9                                                |
|                |          | 35                                               |
|                | 1/10     | 11                                               |
|                |          | 35                                               |
|                | 1/100    | 32                                               |
|                |          | NT                                               |
| Serum TH       | 1/2      | 33                                               |
|                |          | 18                                               |
|                | 1/10     | 20                                               |
|                |          | 17                                               |
|                | 1/100    | NT                                               |
|                |          | 29                                               |

NT, not treated.

TABLE II

Inhibition of FcRFC by Antibody Fragments of Serum S6W

| First treatment | Second treatment | FcRFC + with cells with phenotypes |
|-----------------|------------------|-----------------------------------|
|                 |                  | A3;B7;Dw2                         |
|                 |                  | A3,w19; B12,w15,Dw4,x             |
| Normal serum    | Normal serum     | 37                                |
|                 | F(ab')1, antihuman IgG | 29 | 33 |
| S6W F(ab')1     | Normal serum     | 37                                |
|                 | F(ab')1, antihuman IgG | 19 | 26 |
| S6W Fab'        | Normal serum     | 35                                |
|                 | F(ab')1, antihuman IgG | 15 | 24 |
| NT, not treated. |                  |                                   |

HLA-A, -B, -C, and probably -D associated antigens to lymphocyte membranes (8, 15). The reason that F(ab')2 fragments are less efficient, and that Fab' are inefficient may be due to the fact that the serum S6W is low in titer, and that the antibodies have low avidity (8). Thus a much decreased binding may be expected by the Fab' fragments. Inhibition after "coating" with F(ab')2 anti-IgG can be due either to decreased dissociation from the cells, increased steric hindrance, or both. Schirrmacher et al. (5) in the mouse system also found that F(ab')2 fragments were less effective.

Our results indicate that the antigenic determinants recognized by our two antisera, S6W and TH, are closely associated with the Fc receptor in the lymphocyte membrane, and that the inhibition therefore may be caused by steric hindrance. Others (16, 17) have also recently reported that the Fc receptors on human lymphocytes and B-lymphoid cell lines are closely associated with determinants recognized with some B-cell alloantisera. In MLC reactions, we have shown that serum S6W (18) and serum TH1 demonstrate a

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strong and specific inhibition of the stimulating cells. Studies by Meo et al. (19) in the mouse have revealed that some of the Ia antigens may be major MLC-activating determinants in this species. Thus, the association between the Fc receptor and the determinants recognized by the SSW and TH antisera is further evidence that these determinants may be human analogues of the mouse Ia antigens.

Both anti-HLA-A, -B, and -C antibodies and serum SSW and TH were able to inhibit K-cell cytotoxicity. No inhibition could be observed with the F(ab')2 fragments of the antibodies, indicating that the Fc part was responsible for the inhibitory activity. The test was performed at 37°C for 16 h, conditions that promote aggregation of the antigens in the cell membrane and shedding. Thus, a steric hindrance of the Fc receptor might be reversed. This makes it difficult to determine the association between Fc receptor on K cells and HLA determinants in the ADCC system. Inhibition by intact IgG antibodies only may be due to the effect of aggregated or shed AgAb complexes with an intact Fc portion.

Our results are parallel to the findings in the mouse by Schirrmacher et al. (5) that the effector cells in an ADCC assay are inhibited by intact anti-H-2D, K, and Ia antibodies, but not by F(ab')2 fragments thereof.

Anti-HLA-B7 and anti-β-2-m were unable to inhibit FcRFC, which agrees well with the observations in mice by Dickler and Sachs (4) and Krammer and Pernis (7), who report that anti-Ia, but not anti-H-2D- or -K-specific antisera, could inhibit FcRFC. Thus, inhibition of Fc rosette formation may be an easy and specific screening method for detection of antibodies recognizing HLA-D-associated (human Ia?) determinants.

**Summary**

The inhibitory effect of HLA antisera on Fe receptors of human lymphoid cells was investigated. The ability of lymphoid cells to form rosettes (FcRFC) with antibody-coated sheep red blood cells and to function as effector cells (K cells) in

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**Table III**

Inhibition of ADCC by Intact IgG Antibodies. Sensitizing Antibody, Anti-HLA-B27; Preincubation of Effector Cells with Anti-HLA-B7, Anti-β-2-m, Serum SSW, or Serum TH

| Preincubation serum | Dilution | Effector cell HLA type | A2; B7, w15; Dw2, x | A1; B8; Dw3 | A1; w29; B8; 15; Dw2, 107 |
|---------------------|---------|------------------------|---------------------|------------|------------------------|
|                     |         |                        | T1* | T2 | T1 | T2 | T3 | T4 |
| Normal AB serum     | 1/3     | 63 | 40 | 53 | 34 | 25 | 46 |
| Serum SSW          | 1/3     | 21 | 25 | 53 | 39 | 29 | 50 |
| Serum TH           | 1/3     | 35 | 19 | 8  | 20 | 11 | 21 |
|                    | 1/25    | NT | NT | NT | NT | 20 | 31 |
| Anti-HLA-B7       | 1/25    | 14 | 8 | 53 | 51 | 35 | 60 |
|                    | 1/100   | 30 | 31 | NT | NT | NT | NT |
| Anti-β-2-m       | 1/25    | -11 | 20 | -3 | 10 | 12 | 13 |
|                    | 1/100   | NT | NT | NT | NT | 17 | 14 |

* Target 1: A9; B13, 27; Dw5, 6 (SR 101, counts per minute, MR 441). Target 2: A9, 11; B27, w35; Cw2, 3; LD 107, x (SR 256, MR 1.146). Target 3: A1, 2; B5, 27; Cw2; Dw6, x (SR 520, MR 2.032). Target 4: A2, w19; B6, 27; Cw2; Dw2, 108 (SR 102, MR 316). SR, spontaneous release; MR, maximal release.

† Percent ⁴Cr release calculated from medians.
antibody-dependent cell-mediated cytotoxicity were used as assay systems. We found that antisera recognizing determinants of the HLA-A, -B, and -C series had no effect on FcRFC, while a specific inhibitory effect was observed of antisera probably reacting with determinants identical to or closely associated with those of the HLA-D series. This inhibitory effect was retained in the F(ab')$_2$ fragments. Specific inhibition of K cells was observed with all HLA antisera, but this effect was lost in the F(ab')$_2$ fragments. We conclude that the Fc receptor of rosette-forming lymphocytes may be closely associated with products of the HLA-D region. This is analogous to the association between the Fc receptor and the Ia antigens on murine rosette-forming B lymphocytes.

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