INHIBITION OF H-Y CELL-MEDIATED CYTOLYSIS BY H-2D\(^{b}\) ANTISERUM*

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Cell-mediated cytolysis (CMC) of male, H-Y positive cell, by female effector cells, is subject to H-2 restriction (1). Furthermore, skin graft rejection caused by H-Y incompatibility is influenced by the H-2 type of both the male donor and the female recipient (2, 3). These facts led Ohno to surmise that the H-2-\(\beta_2M\) complex might act as an anchorage site for H-Y at the cell surface (4). Both Beutler et al. (5) and Fellous et al. (6) later supported this idea by showing that human male cells of the Daudi line, whose surfaces do not incorporate HLA-\(\beta_2M\), do not express the H-Y+ cell-surface phenotype but are induced to do so when fused with female cells expressing HLA-\(\beta_2M\). Cocapping experiments with antisera to H-Y, \(\beta_2M\) (6), H-2 (7), and HLA (6) have not resolved the question whether H-Y and H-2 are in fact physically associated, and we have sought further evidence of the contiguity of H-Y and H-2 by determining whether H-2 antiserum can inhibit lysis of male target cells by female effector cells in CMC assays (abbreviated as H-Y CMC). We looked especially for possible differences in inhibition by H-2D as compared with H-2K antibody because in mice of the strain we studied, C57BL/6 (B6), efficiency of H-Y CMC depends on compatibility of target and effector cells for D but not for K.

Materials and Methods

**Mice.** C57BL/6 (B6) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. All other mice were obtained from E. A. Boyse at this Institute.

**CMC Assay.** The \(^{51}Cr\)-release CMC assay was performed essentially according to Gordon and Simpson (8).

**Target Cells (T).** B6 male spleen cells were cultured for 5 d in RPMI (Grand Island Biological Co., Grand Island, N.Y.) supplemented with fetal calf serum (15%), glutamine (2 mM), 2-mercaptoethanol (5 \(\times\) 10\(^{-5}\) mM), penicillin, and streptomycin; concanavalin A (4 \(\mu\)g/ml) was added on the 3rd d of culture. The cells were then washed once, labeled with \(^{51}Cr\) and suspended at a concentration of 1 \(\times\) 10\(^5\) cells/ml for the CMC assay (8).

**Effector Cells (E).** B6 females, 2-3 mo old, were inoculated with 1 \(\times\) 10\(^7\) B6 male spleen cells intraperitoneally. Spleen cells were harvested 1-8 wk later, cocultured for 5 d in RPMI (supplemented as above) with irradiated B6 male spleen cells as described (8), and suspended at a concentration of 2-15 \(\times\) 10\(^5\) cells/ml for use in the CMC assay.

**Assay.** Equal volumes (50 \(\mu\)l of target cells and effector cells) were placed in microtiter wells and incubated in 3% \(CO_2\) at 37\(^\circ\)C for 4 h on a rocking platform. The supernates were harvested by the Titertek apparatus (Flow Laboratories, Inc., Rockville, Md.) and tested for \(^{51}Cr\)-release in a gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Each test was set up in triplicate. Results are expressed as: specific \(^{51}Cr\) release percent = \(100 (A-B)/(C-B)\); where A = cpm test sample; B = cpm spontaneous release with no effector cell; and C = cpm maximal release (addition of 50 \(\mu\)l of 1N HCL).

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TABLE I

| Specificity       | Immunization                                      | 1/cytotoxicity titers on B6 LNC* |
|------------------|---------------------------------------------------|----------------------------------|
| α-H-2\(a\)       | B6 anti-BALB ascites sarcoma Meth. A.              | <10                              |
| α-D\(b\)         | (HTI × B6.H-2\(d\))\(F\)\(_1\) anti-B6 leukemia EL4 | 300                              |
| α-K\(b\)         | (BALB.G × B10.A)\(F\)\(_1\) anti-BALB.B spleen and LNC | 600                              |

* LNC, lymph node cells.

Fig. 1. Inhibition of H-Y cell-mediated cytotoxicity, B6\(d\) anti-B6\(d\) (H-2\(d\)), by D-region H-2 antibody. Each point is the mean of readings from seven separate tests (E:T = 15:1).

Corrected lysis percent =

\[
\frac{\text{specific } ^{51} \text{Cr release of test with } \alpha-D^b}{\text{specific } ^{51} \text{Cr release of test with normal mouse serum (NMS)}} \times 100.
\]

Control. Female target cells were substituted for male target cells as a control in every test; in every case 100(A-B)/(C-B) with female cells was <5%.

Antiserum. See Table I.

Results

Blocking of H-Y CMC by Antibody to D-region H-2 Antibody. Fig. 1 shows the effect produced by adding to the H-Y CMC assay the antisera specified in Table I. Addition of antiserum to D-region H-2 antigen (α-D\(b\)) inhibited lysis significantly at an assay dilution of 1:80 and almost completely at 1:20. The addition of K-region H-2 antibody (α-K\(b\)) did not inhibit lysis and titers of these antisera in cytotoxicity assays were similar. Lysis was not inhibited by NMS, nor by the control antiserum, H-2\(b\) anti-H-2\(d\), which could not react specifically with the cells, which were H-2\(b\) (B6). Other antisera, such as α-Thy-1.2 and α-Ly-2.2 at the same dilutions, had no inhibitory effect (data not shown).

Specificity of Inhibition of H-Y CMC by D-region H-2 Antibody. To further test the antigenic specificity of inhibition of H-Y CMC by D\(b\) antibody, two portions of α-D\(b\) serum were absorbed, one with BALB.G and the other with BALB male spleen and lymph node cells. The H-2 haplotype of BALB.G is H-2\(g\), a recombinant between H-2\(d\) (BALB) and H-2\(b\) (C57BL) (9). Thus, BALB and BALB.G differ in the D-region of H-2 but are similar in the region I to K inclusive. Absorption of α-D\(b\) serum with BALB.G, but not with BALB, abolished its capacity to inhibit H-Y CMC (Fig. 2).
Fig. 2. Specificity of inhibition by D-region H-2 antibody: tests with α-D<sup>b</sup> serum absorbed with BALB.G or BALB male cells. Each point is the mean of readings from two separate tests (E:T = 15:1).

Time Dependency of the Blocking of H-Y CMC by D<sup>b</sup> Antibody. The following tests were conducted with a view to obtaining evidence of the nature of the blocking of H-Y CMC by D<sup>b</sup> antibody. A block developing very rapidly might suggest that binding of D<sup>b</sup> antibody itself is sufficient to impede CMC; a longer time interval might suggest a necessity for subsequent events entailed by the binding of D<sup>b</sup> antibody. Fig. 3A shows a progressive increase in lysis in H-Y CMC assays terminated 1 through 4 h after initiation. Fig 3B shows that blocking was progressively less effective when D<sup>b</sup> antibody was added to the 4-h CMC assays at intervals of 1, 2, and 3 h after initiation. The data shown in Fig. 3C are critical, because they show that pre-exposure of target cells to D<sup>b</sup> antibody, followed by washing before the initiation of the standard 4-h assay, imposed a block to H-Y CMC that was relatively slow in developing. Blocking by pre-exposure of target cells to D<sup>b</sup> antibody was virtually complete, but only after an exposure time approaching 2 h.

Discussion

The finding that H-2D<sup>b</sup> antibody inhibits H-Y-specific CMC by B6 effector cells, whereas H-2K<sup>b</sup> antibody does not, corresponds with the features of H-2 restriction in this system, namely that compatibility for D<sup>b</sup>, but not for K<sup>b</sup>, is required for optimal H-Y specific cytolyis by B6 effector cells (1). This might imply that H-Y and D<sup>b</sup> are so closely situated in the plasma membrane that attachment of D<sup>b</sup> antibody impedes engagement of cytotoxic T cells equipped to recognize a complex H-Y:D<sup>b</sup> target, but certain data conflict with so straightforward an explanation. First, blocking of an H-Y:D<sup>b</sup> complex by D<sup>b</sup> antibody would take only a few minutes, and yet maximal blocking of H-Y-specific CMC by pre-incubation with D<sup>b</sup> antibody required up to
2 h. Furthermore, Flaherty and her colleagues have mapped the relative positions of H-Y and D\textsuperscript{b}, using fixed cells, and find that they are not contiguous, although H-Y antibody applied to unfixed cells initiates a rearrangement that brings H-Y and D\textsuperscript{b} into close proximity. Because approximation of H-Y and D\textsuperscript{b} can also be induced by testosterone,\textsuperscript{1} and because rearrangements of this kind evidently affect particular components of the plasma membrane in an orderly manner (10),\textsuperscript{1} it seems unlikely

\textsuperscript{1} Flaherty, L., D. Zimmerman, and S. S. Wachtel. 1979. H-Y antigen: cell surface mapping and testosterone-induced supramolecular repatterning. \textit{J. Exp. Med.} 150:1020.
that such adjustments in the display of surface molecules are serological artifacts. We propose that H-Y and H-2D\textsuperscript{b} normally are not closely adjacent, but become so in response to activators. The question whether this change in configuration of the target cell surface is an incidental or necessary event in H-Y-specific CMC cannot be answered at the present time.

Summary

Assays of H-Y-specific cell-mediated cytolysis (CMC) in vitro were carried out with B6 female effector cells and B6 male target cells. This lytic system depends on compatibility for H-2D\textsuperscript{b} but not for H-2K\textsuperscript{b}. Antibody to H-2D\textsuperscript{b} but not to H-2K\textsuperscript{b} blocked the CMC assay. However, maximal blocking required exposure of the target cells to H-2D\textsuperscript{b} antibody for up to 2 h. This interval is far longer than would be required for blocking of a pre-existing H-Y:H-2D\textsuperscript{b} complex, and seems more consonant with a membrane reorganization bringing H-Y and H-2D\textsuperscript{b} together, as proposed from mapping studies. The question whether the postulated configurational change affecting H-Y and H-2D\textsuperscript{b} is a necessary feature of CMC awaits further study.

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