Serological Crossreactivity Between H-Y (Male) Antigens of Mouse and Man
(histocompatibility-Y antigen/cytotoxicity tests/mammals)

STEPHEN S. WACHTEL, GLORIA C. KOO, EVELYN E. ZUCKERMAN, ULRICH HAMMERLING,
MARGRIT P. SCHEID, AND EDWARD A. BOYSE

Memorial Sloan-Kettering Cancer Center, New York, N.Y. 10021

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ABSTRACT Antisera to H-Y (male-specific) antigen were prepared by immunizing female mice with sperm cells from males of the same inbred strain. These antisera were used in mixed hemadsorption and cytotoxicity tests with cells of rats, guinea pigs, rabbits, and humans. The results showed that the H-Y components of all four species are antigenically related to H-Y of the mouse.

The H-Y (histocompatibility-Y) antigen characteristic of male cells was discovered with the observation that female mice often reject skin grafts from males of the same inbred strain (1, 2). It is now established that female mice grafted with male skin produce antibody (H-Y antibody) recognizable in cytotoxicity tests with sperm (3) or with dissociated epidermal cells (4) from male mice. [H-Y antigen occurs on most if not all other male cells as well, but evidently not in a manner rendering them sensitive to lysis by H-Y antisera; its presence on these cells is demonstrable by their capacity to absorb H-Y antibody and thereby abolish the cytotoxicity of H-Y antisera for sperm (3).]

At present the only indication of interspecies H-Y cross-reaction is the observation that injections of male lymphoid cells from some strains of rat sensitize C57BL/6 (B6) female mice against B6 male skin grafts (5). The cytotoxicity test on sperm, and a new serological assay, the "mixed hemadsorption hybrid antibody" (MHA.HA) test that we describe in this report, make detailed analysis of H-Y systems practicable. We have now used these techniques to study the antigenic relations of H-Y components on cells from males of different mammalian species.

MATERIALS AND METHODS

Animals. B6 and BALB/c (BALB) mice, and inbred W/Fu rats, were obtained from colonies maintained by one of us (E.A.B.). Strain-13 inbred guinea pigs were kindly provided by Dr. H. Oettgen of Memorial Sloan-Kettering Cancer Center. NZW rabbits were obtained from Hare Rabbits for Research, Inc., Hewitt, N.J.

Cell Suspensions. Epididymal sperm suspensions were prepared from mice according to Goldberg et al. (3): The epididymis was removed and cut into several pieces in phosphate-buffered saline (pH 7.0) containing 0.5% fructose and 5% fetal-bovine serum (56°C/30 min). After several minutes, the sperm were pipetted off and placed in the phosphate-buffered saline. The suspension was maintained at room temperature until use.

Solutions of leukocytes used for immunizations, absorptions, and serological tests were prepared from the spleen and mesenteric lymph nodes of mice, rats, guinea pigs, and rabbits by mincing the tissues in Earle's balanced salt solution (EBSS). The supernate, containing free cells in suspension, was removed and centrifuged at 100–120 X g for 10 min. The cells were washed twice, counted, and resuspended in a convenient volume of EBSS. Human leukocytes and erythrocytes were separated as follows: Whole blood (12–15 ml) was drawn into heparinized syringes and placed in tubes containing 2 ml of a 1.7% sodium citrate solution and 2 ml of a 5% dextran solution (average molecular weight, 252,000; Sigma Chemical Co., St. Louis, Mo.). Erythrocytes were allowed to settle for 30–45 min, after which the leukocyte-rich supernate was removed. The erythrocyte fraction was resuspended in an equal volume of EBSS containing citrate and dextran and allowed to sediment a second time to increase the yield of leukocytes. Leukocytes were then pooled, centrifuged at 100 X g for 10 min, washed twice, and resuspended in EBSS. Erythrocytes were centrifuged at 900 X g for 10 min, washed twice, and resuspended in EBSS.

H-Y Antisera. H-Y antisera were prepared in adult B6 and BALB female mice by not less than 3 weekly intraperitoneal injections of from 50 to 100 X 10^6 spleen cells from B6 and BALB males, respectively. The mice were bled between 7 and 14 days after inoculation, and the serum was separated and stored immediately before thawing, if not used again within 24 hr, were discarded.

H-Y antisera, particularly those prepared by skin-grafting, frequently contain autoantibody reactive with epidermal cells from males or females and with sperm (ref. 6; and see ref. 9), and lower levels of such antibody are found in the serum of immununized mice. We have found that H-Y antisera prepared against male spleen cells contain far less of this autoantibody than those prepared by skin-grafting, in accord with the unpublished observations of Scheid and Flaherty that sperm and epidermal cells share an autoantigen that is absent from spleen cells (see ref. 7). Thus in the case of H-Y antisera prepared against spleen cells exclusively, titration in the cytotoxicity test with sperm (see Results) is a generally useful and valid way of screening sera for H-Y activity. With regard to use of the cytotoxicity test for H-Y typing, of different species

Abbreviations: MHA.HA, mixed hemadsorption hybrid antibody; B6, C57BL/6; BALB, BALB/c; EBSS, Earle's balanced salt solution; Ig, immunoglobulin.
Fig. 1. Density gradient containing reagents for the MHA.HA test. Gradients of the immune reagents, interspersed with wash solutions (EBSS), are established in tubes made from 1-mL disposable pipettes (internal diameter, 2.5 mm), siliconized with 1% dimethyldichloro silane in benzene (Bio-Rad Laboratories, Richmond, Calif.). One end of the tube is heat-sealed. Heat-inactivated fetal-bovine serum (FBS) (immunoprecipitin-tested, free of IgG; Grant Island Biological Co., Grand Island, N.Y.) is included in each layer in a concentration that prevents intermixing of layers during subsequent centrifugations of the cells. The wash layers are visually distinguished by the addition of phenol red. One-tenth milliliter of (1 × 10⁸) test cells are presensitized in H-Y antiserum diluted 1/2 (0.1 mL) for 20 min on ice. Test cells are then placed in the gradient tube which is centrifuged at 40 × g for 10 min and then at 100 × g for 10 min.

(Results), absorption of sperm autoantibody (if present) from the H-Y antiserum was unnecessary because positive scoring in the test depended entirely on the contrasting results of absorption with male cells and female cells.

The Mixed Hemadsorption Hybrid Antibody (MHA.HA) Test. In this test, 1 × 10⁸ lymph-node cells (from rats, guinea pigs or rabbits), or 1 × 10⁹ leukocytes (from humans), prepared as above, were reacted sequentially with: (a) 0.1 mL of H-Y antiserum diluted 1/4, followed by (b) the hybrid antibody: antibody against mouse immunoglobulin (IgG) prepared in rabbit hybridized with antibody against sheep erythrocytes prepared in rabbit, followed by (c) sheep erythrocytes (2 × 10⁸/mL). Test cells carrying H-Y antigens bind sheep erythrocytes, forming “rosettes.” To ensure that all major classes of mouse antibody would be detectable in this assay, the specificities of the anti-Ig arm of the hybrid reagent we used included at least anti-h, anti-κ, and anti-μ.

The reaction was carried out by centrifuging the cells through a discontinuous density gradient containing the reagents and wash layers in sequence (7); see Fig. 1. Gradients were prepared and maintained at 4°C, and used within 1–2 hr after preparation. After centrifugation, the tubes were placed on ice for at least 30 min. Each tube was cut several centimeters above the pellet. For reading (at 1 hr and again at 16 hr after centrifugation), the pellet was gently resuspended and the number of rosettes and free leukocytes were counted in a hemacytometer. Any leukocyte to which five or more sheep erythrocytes had adsorbed was scored as a rosette; many rosettes contained more than five sheep erythrocytes and more than one leukocyte. Toluidine blue (1%) was used to identify free test cells.

Preparation of the Hybrid Antibody Anti-Sheep Erythrocytes/Anti-Mouse Ig. The detailed procedure is described elsewhere (8). The gamma globulin fraction of sheep erythrocytes antiserum prepared in rabbit was digested with pepsin to yield F(ab)₂ fragments. Specific anti-sheep erythrocyte F(ab)₂ fragments were then separated from the normal gamma globulin fraction of the antiserum by adsorption to sheep erythrocyte stroma coupled to APB–cellulose and elution at pH 2. Anti-mouse IgF(ab)₂ fragments were prepared similarly. Two parts of anti-sheep erythrocyte F(ab)₂ were mixed with one part of anti-Ig F(ab)₂, and the mixture was reduced with sodium borohydride and reoxidized. Monovalent fragments that failed to recombine were eliminated by chromatography on Sephadex G-100. Two hybrid antibodies with different anti-Ig specificities were prepared: (a) anti-mouse IgG₁, F(ab)₂/anti-sheep erythrocyte with predominantly anti-κ specificity but also some anti-γ activity; (b) anti-mouse IgM/anti-sheep erythrocyte with anti-μ and anti-λ specificities. (IgM was prepared from MOPC 104E, a mouse plasma-cell tumor producing IgM with μ and λ chains.) Hybrid antibodies were used at 100 µg/mL.

Cytotoxicity Test for H-Y Antiserum. The cytotoxicity test was performed with BALB epithidymal sperm essentially according to Goldberg et al. (3). Equal volumes (0.05 mL) of (a) anti-H-Y serum (serially diluted 1/2 to 1/25), (b) sperm (5 × 10⁶/mL), and (c) selected horse serum (complement source [9]) diluted 1/4, were incubated at 37°C for 50 min. A freshly prepared trypan blue solution (0.1 mL) was added to each suspension during the last 5–7 min of incubation. The tubes were then placed on ice, and dead (stained) and live (unstained) sperm were counted in a hemacytometer.

RESULTS
Experimental procedure
Immediately before each test, H-Y antisera were selected as follows: Sera from individual donor female mice (immunized with male spleen cells) were titrated against BALB sperm for cytotoxic H-Y antibody (3). [There is some strain variability in the proportions of viable sperm (those excluding trypan blue), and in our experience BALB sperm usually give higher viability counts than sperm of several other mouse strains.] Sera with a satisfactory titer (≥ 50% sperm dead at 1/4 dilution of antiserum, ≤ 30% dead in controls) were pooled, diluted 1/5, and divided into three parts. The first part (A) was absorbed with female cells, and the second (B) with male cells, of the species being tested (30 min on ice, 1 or 2 parts packed cells to 1 part serum). The third part (C) was not absorbed. These three serum samples (A, B, and C) were then tested for H-Y antibody by the MHA.HA method and by the cytotoxicity test. In some cases the MHA.HA and cytotoxicity test were performed independently of one another, i.e., on separate days with different serum pools.

Mixed Hemadsorption Hybrid Antibody (MHA.HA) Test. For tests of rat, guinea pig, and rabbit cells, the mouse H-Y antiserum was absorbed once with male (A) or female (B) spleen cells and then reacted with male or female lymph-node cells. For tests with human cells, sera were absorbed twice, first with erythrocytes and then with leukocytes (both from
TABLE 1. Reaction of mouse H-Y antiserum with male antigens of rats, guinea pigs, rabbits, and humans: summary of results with the MHA.HA test

<table>
<thead>
<tr>
<th>Species</th>
<th>Absorbed with spleen cells from</th>
<th>Tested on lymph-node cells from</th>
<th>Average no. of rosettes/100 leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>♂</td>
<td>♂</td>
<td>30.2</td>
</tr>
<tr>
<td>(W/Fu)</td>
<td>♀</td>
<td>♂</td>
<td>2.3</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>♂</td>
<td>♂</td>
<td>1.8</td>
</tr>
<tr>
<td>(strain 13)</td>
<td>♀</td>
<td>♂</td>
<td>15.4</td>
</tr>
<tr>
<td>Rabbit</td>
<td>♂</td>
<td>♀</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>♂</td>
<td>2.7</td>
</tr>
<tr>
<td>Human</td>
<td>♂</td>
<td>♀</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>♂</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Three tests each (rat, guinea pig, and rabbit) and five tests (human) were scored as coded samples by two observers who recorded the counts of rosettes and free leukocytes. The values (percentages) shown in Table 1 were derived from the formula: number of rosettes/number of rosettes + free leukocytes. Each value given is an average of percentages from the several tests scored. They are minimum values, because larger rosettes are formed from the conglomerates of smaller rosettes. A hemacytometer field generally contained between 50 and 200 leukocytes. Controls not shown in Table 1 included substitution of normal mouse serum or H-2 antiserum for the H-Y antiserum. Absorption with either male or female cells in these controls reduces the number of rosettes to background levels.

Male or female donors; the absorbed sera were then reacted in the MHA.HA test with male or female leukocytes. For every test, the erythrocytes and leukocytes used in absorption and as target cells were pools of cells obtained from three to eight blood donors.

Naturally occurring heteroantibody in the mouse H-Y antiserum can react with male or female test cells of other species, causing them to bind the hybrid antibody and produce rosettes. The advantage of the absorption method of demonstrating H-Y antigen, as used here, is that adequate absorption with either male or female cells removes heteroantibody, thus abolishing this source of rosette formation. In the case of human leukocytes, the high count of rosettes in controls (which we suspect is due to especially marked effects by heteroantibody across such a wide phylogenetic gap) necessitated prior absorption with erythrocytes as well as the standard comparative absorptions with male leukocytes (A) and female leukocytes (B) on which the test for H-Y antigen is based. In the case of rat, guinea pig, and rabbit, a single absorption was sufficient to reduce the background counts of rosettes to acceptable levels.

The results of the MHA.HA tests, summarized in Table 1, indicate that H-Y antigen, identical or crossreacting with mouse H-Y, occurs on male cells of all four species tested. This is shown by the fact that in every case absorption with male cells (A) reduced the number of rosettes to background levels whereas absorption with female cells (B) did not. [Human thymus-derived cells bind sheep erythrocytes spontaneously, but this reaction is 95–100% inhibited at temperatures below 10° (10); any contribution this might have made to the formation of rosettes is inconsequential because the scoring of the MHA.HA test depends on the contrast between the number of rosettes formed on male and female cells.]

Cytotoxicity Test. In this procedure, mouse H-Y antiserum is absorbed with male (A) or female (B) cells, as described above, and then titrated for residual cytotoxic activity against BALB sperm. If aliquot A is inactive compared to aliquot B, then H-Y antibody must have been removed specifically by male leukocytes of the species supplying the cells for absorption, and the H-Y antigen of that species must be identical or crossreactive with mouse H-Y.

The results of the cytotoxicity tests are summarized in Fig. 2. In every case absorption with male cells (A) removed or greatly reduced cytotoxic activity against BALB sperm whereas absorption with female cells (B) did not.

**DISCUSSION**

The sharing of H-Y antigens by species as divergent as mouse and man suggests that most or all mammalian species may have H-Y antigen in common. Certainly we can now study serologically the H-Y systems of noninbred species, in which H-Y antisera could be prepared only with difficulty (if at all), using H-Y antisera produced in inbred species such as the mouse, rat, and guinea pig.

The MHA.HA technique is a "direct" test, i.e., the target cells come from the species whose H-Y antigen is being tested for crossreactivity with mouse H-Y; a positive reaction may
therefore indicate that only one of several H-Y antigenic specificities recognized by mouse H-Y antiserum is shared by the two species. Such a positive result would be compatible with the existence of a major H-Y antigen peculiar to each species in addition to the common mammalian H-Y antigen recognized by the MHA.HA direct test.

On the other hand, the form of the cytotoxicity test used in this study is indirect, i.e., the cells of the species being tested for crossreactivity with mouse H-Y are used to absorb the mouse H-Y antiserum, the absorbed antiserum then being tested on mouse cells (sperm). A positive reaction (i.e., positive absorption) in this test therefore indicates that at least most of the cytotoxic H-Y antibody has been removed, and hence that the major H-Y specificities recognized by the mouse H-Y antiserum must be shared by the two species. So the fact that the cytotoxicity (absorption) test was positive for all species tested, including man, favors the view that the major H-Y antigen or antigens are those that are widely represented, and perhaps ubiquitous, in mammals. The data do not reveal to what extent H-Y may be a complex of several antigens, rather than a single antigen, and they do not exclude the occurrence of H-Y antigen limited to particular species or even the existence of intraspecies polymorphism of H-Y antigen (11, 12), both of which might be superimposed on a background of H-Y antigen common to mammals. [The stronger reaction of rat male leukocytes in the MHA.HA test as compared with guinea pig, rabbit, or human male leukocytes (Table 1) may indicate greater similarity between the H-Y antigens of rat and mouse, but this remains to be substantiated.]

It is uncertain whether the cell-surface component recognized in the male as H-Y antigen is related to any specific characteristic that functionally distinguishes males from females, or whether possession of H-Y antigen by the male is a fortuitous result of lack of crossing-over between X and Y chromosomes, which would perpetually confine Y-determined characters to the male. In either case, with the establishment of the X/Y mode of sex determination in the course of mammalian evolution, it is apparent that an already existing gene (or set of genes) that codes for H-Y antigen was incorporated in the Y chromosome and has been inherited by mammals generally (assuming that the hypothesis of ubiquitous mammalian H-Y antigen will be substantiated). So it may be informative to look for the same H-Y antigen in other classes of vertebrates to see whether these cell-surface components can be traced back to a time before mammals evolved.

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