H-Y ANTIGEN
Cell Surface Mapping and Testosterone-induced Supramolecular Repatterning*

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The widespread phylogenetic occurrence of the H-Y antigen, and the correlation of its expression with the presence of testicular tissue (1-3), led to the proposition that this serologically detectable cell membrane component is the product of highly conserved testis-determining genes (3-6). This proposition is supported by recent experimental data in vitro. In dissociation reaggregation cultures, H-Y antibody blocks testicular organization of dispersed Sertoli cells (7, 8) whereas molecules of free H-Y antigen induce testicular organization in cells of the presumptive ovary (9). Ohno (10) has postulated that cell-surface products of the major histocompatibility complex (MHC) components, conjoined with β2-microglobulin, may serve as carriers or anchorage sites for the H-Y molecules.

Because of the likely significance of H-Y in male development, and its postulated association with MHC products (10), we have been exploring its location on the cell surface, using the thymocyte as a model because the surface of this cell has already been extensively mapped with respect to several immunogenetic markers (11, 12).

Materials and Methods

Mice. Mice were obtained from colonies maintained at the New York State Department of Health, Albany or at Memorial Sloan-Kettering Cancer Center, New York. B6-Tla° mice were used as a source of thymocytes.

Antisera. Descriptions of anti-TL, anti-D°, anti-K°, anti-Lyt-1,2, and anti-Lyt-2,2 are given elsewhere (12). Some of these sera were generously provided by Doctors F-W. Shen and E. A. Boyse of Memorial Sloan-Kettering Cancer Center. Anti-H-Y serum was produced according to procedures described elsewhere (2) and absorbed with female C57BL/6 (B6) thymocytes to insure specificity for H-Y. Anti-H-2L° (anti-L°), made by immunizing BALB/c-H-2° mice
Step 1: Fixation

Step 2: Incubation with 1st antibody

Step 3: Incubation with 2nd test antibodies

Step 4: Cytotoxicity testing

of residual antibody activity

Fig. 1. Procedure for the blocking assay.

with BALB/c cells and cross-reactive with the L\textsuperscript{b} molecule, was a gift from Dr. Ted Hansen, National Institutes of Health.

Fixation. Cells were fixed with paraformaldehyde according to Parr and Oei (13, 14). In brief, thymocytes were incubated with 1% paraformaldehyde, made in isotonic media, for 1 h on ice, and washed three times in M-199-containing 10% GG-free fetal calf serum (Grand Island Biological Co., Grand Island, New York) before use in the blocking assay.

Blocking Assay. This was performed according to Boyse et al. (11) with fixed as well as unfixed cells (12). Briefly, the assay consists of the four basic steps schematized in Fig. 1.

The percent blocking was calculated according to the formula:

\[
\frac{N_{06} \text{ (blocked thymocytes)} - N_{06} \text{ (normal mouse serum [NMS]-treated thymocytes)}}{N_{06} \text{ (NMS-treated thymocytes)}} \times 100;
\]

where \(N_{06} \) = the number of thymocytes which will reduce the cytotoxic index by 50%. The cytotoxic index is calculated by the formula:

\[
\frac{A - B}{100 - B'}
\]

where \(A \) = percent lysis with antibody plus complement and \(B \) = percent lysis with complement alone.

All tests were coded. Incubations with blocking antisera were performed at room temperature.

Hormones. Testosterone and 17\( \beta \)-estradiol were purchased from Steraloids and used at a concentration of \(10^{-6} \text{ M}\).

Results

Position of H-Y on the Thymocyte Cell Surface in Relation to the Markers TL, \(D^b\), \(K^b\), \(L^b\), Lyt-1.2, and Lyt-2.2. As Table I (Exp. 1) and Fig. 2 show, blocking with anti-H-Y serum on fixed thymocytes impedes the subsequent binding of anti-TL but not of anti-K\textsuperscript{b}, anti-D\textsuperscript{b}, anti-L\textsuperscript{d}, anti-Lyt-1.2, or anti-Lyt-2.2. Thus, H-Y lies close to TL and relatively distant from K\textsuperscript{b}, D\textsuperscript{b}, L\textsuperscript{b}, Lyt-1.2, and Lyt-2.2.
**H-Y ANTIGEN**

**Table I**

*Effects of Blocking with Anti-H-Y on Unfixed and Fixed Cells*

| Experiment No. | Source of test thymocytes* | Anti-H-Y absorbed with‡ | Specificity of test antibody§ | Unfixed | Fixed before blocking | Fixed after blocking |
|----------------|-----------------------------|-------------------------|--------------------------------|---------|----------------------|---------------------|
| 1              | Male                        | Female                  | K<sup>b</sup>                  | <5      | <5                   | <5                  |
|                |                             |                         | D<sup>b</sup>                  | 10      | <5                   | 5                   |
|                |                             |                         | L<sup>a</sup>                  | <5      | <5                   | <5                  |
|                |                             |                         | Lyt-1.2                       | <5      | <5                   | <5                  |
|                |                             |                         | Lyt-2.2                       | <5      | <5                   | <5                  |
|                |                             |                         | TL                            | >90     | 44                   | >90                 |
| 2              | Male                        | Female                  | K<sup>b</sup>                  | <5      | <5                   | <5                  |
|                |                             |                         | D<sup>b</sup>                  | <5      | 65                   | 65                  |
|                |                             |                         | TL                            | >90     | >90                  | >90                 |
| 3              | Male                        | Female                  | D<sup>b</sup>                  | 17      | 57                   | 57                  |
|                |                             |                         | TL                            | 57      |                      |                     |
|                |                             | Male                    | D<sup>b</sup>                  | <5      |                      |                     |
|                |                             |                         | TL                            | <5      |                      |                     |
| 4              | Female                      |                        | D<sup>b</sup>                  | <10     | >90                  | <10                 |
|                |                             |                         | TL                            | 70      |                      |                     |
|                |                             | Male                    | D<sup>b</sup>                  | 80      |                      |                     |

* Cells were from B6-Tla<sup>a</sup> mice.
‡ Anti-H-Y and NMS (control) were used at a 1/5 dilution. Anti-H-Y was absorbed with female or male thymocytes for 30 min on ice at a ratio of five parts diluted serum to one part packed cells.
§ Test antibodies were used at the following dilutions: anti-K<sup>b</sup> (B6-H-2<sup>a</sup> anti-B6.AK1), 1/400; anti-D<sup>b</sup> (B6.AK1 anti-B6.K1), 1/100; anti-Lyt-1.2 (C3H anti-CE), 1/100; anti-Lyt-2.2 (C3H × B6-Lyt-2<sup>a</sup> anti-B6 leukemia, ERLD), 1/50; anti-L<sup>a</sup> (BALB/c-H-2<sup>me</sup> anti-BALB/c), 1/25; anti-TL (B6 × A-Tla<sup>a</sup> anti-A strain leukemia, ASL1), 1/3200.
∥ Percentage of blocking was calculated as described in Materials and Methods.

**Relative Movement of H-Y and D<sup>b</sup> Induced by H-Y Antibody. Comparison of Fixed and Unfixed Thymocytes.** There was a noteworthy difference between unfixed and fixed cells in blocking with anti-H-Y (Exp. 1, Table I). On fixed cells anti-H-Y did not hinder binding of anti-D<sup>b</sup>. But on unfixed cells anti-H-Y did diminish binding of anti-D<sup>b</sup>. Controls in which anti-H-Y was absorbed with male or female cells or controls where anti-H-Y was tested on male or female cells show that the proximity of H-Y and D<sup>b</sup> induced by anti-H-Y is not a result of an unrecognized cross-reactive, cross-linking anti-D<sup>b</sup>:H-Y antibody (Exps. 3 and 4, Table I). To test the possibility that D<sup>b</sup> and H-Y are normally adjacent and that treatment with paraformaldehyde causes D<sup>b</sup> and H-Y to diverge, we compared the effect of fixation before and after incubation with anti-H-Y. The results were identical (Exp. 2, Table I and Fig. 2). Thus, fixation does not alter the capacity of anti-H-Y to block D<sup>b</sup>. We conclude that incubation of unfixed cells with anti-H-Y, in step 2 of the blocking procedure, triggers a prescribed rearrangement involving H-Y, and that this is prevented by fixation.

**Relative Movement of H-Y and D<sup>b</sup> Induced by Testosterone.** Some controversy persists regarding the effects of male sex steroids on expression of H-Y antigen (15, 16). In view of this controversy and because there are claims that testosterone can influence the expression of cell surface antigens in general (17), we wished to determine whether
testosterone might affect the relative position of H-Y on the thymocyte. Accordingly, a suspension of viable washed thymocytes was divided into two portions and incubated either with or without testosterone. The two portions were washed again and fixed with paraformaldehyde to arrest movements of components of the cell surface. After such treatment, reaction with anti-H-Y should reveal any changes in position induced by the hormone. As shown in Fig. 3 and Table II, incubation with testosterone affected the ability of anti-H-Y to block D^b (but not K^b or TL). These results imply that testosterone, like anti-H-Y, alters the relative position of H-Y to D^b. This movement was not induced by 17β-estradiol (Exp. 3 and 4, Table II).

Conceivably, testosterone is a nonspecific inducer of cell-surface repatterning, i.e., it will cause the approximation of other pairs of molecules in addition to H-Y:D^b. To test this hypothesis, we examined the effects of testosterone on the approximation of TL and D^b. Because this pair is known to approximate when triggered by either anti-TL or anti-D^b (12, Table III), it would seem a likely choice for testing this hypothesis. Because no difference was observed between control and testosterone-treated female cells in their absorption capacity of anti-TL when the cells were incubated with anti-D^b as a blocking antibody (Exp. 5, Table II), we have no evidence for any nonspecific effects of testosterone.

Discussion

By application of the blocking technique, on unfixed and on paraformaldehyde-fixed cells, relative map positions have been established for D^b, K^b, TL, Lyt-1.2, and
Lyt-2.2 on the B6-Tla\textsuperscript{a} thymocyte (11, 12; see Table III for summary). For example, D\textsuperscript{b} maps near Lyt-2.2 and K\textsuperscript{b} maps near Lyt-1.2 on both fixed and unfixed cells. Comparisons of fixed and unfixed cells imply that specific migration of two of these surface components, TL and D\textsuperscript{b}, occurs from exposure of cells to anti-TL or anti-D\textsuperscript{b} sera (12). Thus, on cells fixed before the addition of blocking antibody, TL and D\textsuperscript{b} are too far apart to cause mutual interference in the attachment of TL and D\textsuperscript{b} antibodies. But when cells are unfixed, or are fixed after incubation with TL or D\textsuperscript{b} antibody, interference occurs thus implying approximation of TL and D\textsuperscript{b}.

The experiments reported here indicate that H-Y (or D\textsuperscript{b}) also undergoes directed migration. With prefixed cells H-Y antigen appears close to TL, but not to D\textsuperscript{b}, K\textsuperscript{b}, L\textsuperscript{b}, Lyt-1.2, or Lyt-2.2. But with cells fixed after incubation with anti-H-Y, then H-Y appears close to D\textsuperscript{b}. Because of difficulties in quantifying expression of H-Y antigen on thymocytes, we were unable to perform experiments which involve blocking with anti-D\textsuperscript{b}, K\textsuperscript{b}, L\textsuperscript{d}, Lyt-1.2, or Lyt-2.2 and testing in a quantitative absorption assay for residual anti-H-Y activity.

This is a second example of directed movement detected by the mapping of fixed and unfixed cells; thus, supramolecular repatterning, which has been called phenotypic adaptation (18), involving cell surface components identified as alloantigens is not a phenomenon peculiar to TL. It is perhaps worth noting that these studies were performed on B6-Tla\textsuperscript{a} (or B6) thymocytes. Conceivably different associations may
# Table II

**Effects of Testosterone and Estradiol on Cell-surface Repatterning**

| Experiment No. | Hormone* | Blocking antibody† | Sex of test thymocyte | Percentage of blocking of§ |
|---------------|----------|---------------------|------------------------|---------------------------|
|               |          | Anti-K<sub>b</sub>  | Anti-D<sub>b</sub>     | Anti-TL                   |
| 1             | Control  | Anti-H-Y abs.       | Male                   | <5                        | <5                        | 58 |
|               | Testosterone | Anti-H-Y abs.       | Male                   | <5                        | 31                        | >90 |
| 2             | Testosterone | Anti-H-Y abs.       | Male                   | <5                        | 75                        | >90 |
| 3             | Control  | Anti-H-Y abs.       | Male                   | <5                        | <5                        | 51 |
|               | 17β-Estradiol | Anti-H-Y abs.       | Male                   | <5                        | <5                        | 54 |
| 4             | 17β-Estradiol | Anti-H-Y abs.       | Male                   | <5                        | <5                        | 54 |
|               | Testosterone | Anti-D<sub>b</sub> abs. | Female               | <5                        | >90                       | <5 |
| 5             | Control  | Anti-D<sub>b</sub> abs. | Female               | <5                        | <5                        | <5 |

* Hormones were used at a final concentration of $10^{-6}$ M. They were first dissolved in ethanol (100 µl) and then in 10 ml of M-199. Control cells were incubated in 10 ml of M-199 that contained 100 µl of ethanol but without hormone.

† Anti-H-Y was preabsorbed with female B6 thymocytes for 30 min on ice at a ratio of five parts diluted serum to one part packed cells. It was used at a 1/5 dilution. Anti-D<sub>b</sub> was preabsorbed with female B6-H-2<sup>a</sup> thymocytes for 30 min on ice at a ratio of five parts diluted serum to one part packed cells. It was used at a 1/100 dilution.

§ Percentage of blocking was calculated according to the formula in Materials and Methods. For dilutions of test antisera see Table I.

arise when other H-2 molecules (or Ly molecules) specified by alternative haplotypes are involved.

Approximation of H-Y and D<sub>b</sub> under the influence of testosterone is a welcome indication that such molecular reorganization, previously seen only in the artificial setting of exposure to antibody, has physiological relevance, and it is perhaps worth emphasizing once again that the female hormone β-estradiol had no such effect, nor did testosterone cause TL-D<sub>b</sub> approximation in the absence of H-Y (i.e., on female cells); rearrangement is precise in regard to position. Concentrations of testosterone in vitro were 100-fold higher than the normal plasma levels in B6-Tla<sup>a</sup> males (L. Flaherty, unpublished results). It is open to question whether smaller elevations in testosterone levels will also induce D<sub>b</sub>:H-Y approximation.

Certainly, the in vitro action of testosterone may reflect a more general situation, and in fact there are several examples of D:H-Y associations. (a) Survival of H-Y-incompatible grafts is influenced by H-2 haplotype of the donor (19). (b) T-cell-mediated lysis of H-Y-incompatible lymphocytes is H-2 restricted, requiring D-end compatibility of killer and target cells (20; a similar phenomenon is reported in man, 21). Recent evidence indicates that this T-cell-mediated lysis can be blocked by anti-D but not anti-K sera (22). (c) H-2D is more weakly expressed than H-2K on testicular cells of the newborn mouse, an observation consistent with the notion of preferential masking by H-Y (9). (d) Daudi cells (from a cultured male Burkitt lymphoma that has lost Histocompatibility leukocyte antigen (HLA) and β<sub>2</sub>-microglobulin (β<sub>2m</sub>)), absorb considerably less anti-H-Y than cells from cultured male lymphomas that have retained β<sub>2m</sub> and HLA; restoration of β<sub>2m</sub> and HLA (by hybridization with HeLa D96) is associated with a 10-fold increase in H-Y expression (23). Perhaps H-Y depends on association with the D molecule for some part of its expression or function on the cell surface and that one of the inducers of this association is the male hormone, testosterone.
Table III

Summary of Results

A. Blocking units or domains

| Name of domain | Members | Reference |
|----------------|---------|-----------|
| 1 D b D b, Lyt-2.2 | D b, Lyt-2.2 | 11, 12 |
| 2 K b | K b, Lyt-1.2 | 11, 12 |
| 3 TL | TL, H-Y | 11, Table I |

B. Inducible repatterning

| Inducer | New association between | Reference |
|---------|------------------------|-----------|
| 1 Anti-TL | TL and D b | 12 |
| 2 Anti-D b | TL and D b | 12 |
| 3 Anti-H-Y | H-Y and D b | Table I |
| 4 Testosterone | H-Y and D b | Table II |
| 5 Estradiol | None yet detectable | Table II |

* Mutual blocking of members within a domain is observed both on fixed and unfixed cells.

‡ Blocking is only observed on unfixed cells or cells fixed after incubation with the inducing agent.

Summary

Previous work with the antibody-blocking technique showed that the map of surface components for thymocytes prefixed with paraformaldehyde is the same as the map for unfixed thymocytes, with the following exception: after exposure to anti-TL or anti-D b, TL and H-2D b occupy adjacent positions on unfixed cells but not on fixed cells. This was interpreted as an indication that activation of particular components of the surface phenotype initiates ordered changes in the display of cell-surface molecules, approximation of TL and D b in this instance.

These studies have now been extended to the H-Y component on the surface of male cells. On fixed male mouse thymocytes, H-Y lies adjacent to TL and relatively distant from H-2D b, H-2K b, H-2L b, Lyt-1.2, and Lyt-2.2. However, on unfixed male mouse thymocytes, similarly exposed to H-Y antibody, H-Y and H-2D b are adjacent. Presumably, this engagement of H-Y sites by H-Y antibody brings H-Y and H-2D b together. Evidence that this change in pattern may be physiologically relevant comes from the finding that testosterone, but not estradiol, caused the same selective approximation of H-Y and H-2D b.

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