ASSOCIATION OF THE H-Y MALE ANTIGEN WITH
\(\beta_2\)-MICROGLOBULIN ON HUMAN LYMPHOID AND
DIFFERENTIATED MOUSE TERATOCARCINOMA CELL LINES*

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The male specific antigen (H-Y) has been implicated in the rejection of male
skin grafts by syngeneic female mice and with the production of anti-H-Y
antibodies (1-4).

Antisera against the mouse H-Y antigen react with heterogametic cells in
mammals (including man), birds, and amphibians (5-8). Thus, the H-Y antigen
seems to be phylogenetically conserved, and it has been argued that the
serologically detectable H-Y antigen has an invariant function in testis induc-
during embryonic development (9).

It has also been suggested (10) that the H-Y antigen is associated with
antigens of the major histocompatibility complex (MHC) on the plasma
membrane, the MHC antigens \(\beta_2\)-microglobulin complex serving as the anchor-
age site for the H-Y antigen. By using rat anti-H-Y antisera, we have studied
the association of the H-Y antigen with the complex formed by the major
histocompatibility antigens and \(\beta_2\)-microglobulin on human lymphoid cells and
mouse teratocarcinoma cells.

The results reported here demonstrate that the H-Y antigen is associated
with \(\beta_2\)-microglobulin on the cell membrane.

Materials and Methods

**Immune Sera**

**Anti-H-Y Antisera.** Anti-H-Y antisera were prepared in 8- to 12-wk-old female inbred Lewis
rats by intraperitoneal injection of \(2 \times 10^6\) or \(4 \times 10^7\) spleen cells from male inbred Lewis rats.
After six to eight weekly injections, rats were bled and the sera were stored at \(-28^\circ\text{C}\). Before use,
the antisera were heat inactivated and absorbed with an equal volume of packed human female
AB erythrocytes for 1 h at 4°C. Normal female Lewis rat serum was used as control.

**Anti-\(\beta_2\)-Microglobulin.** Rabbit antimurine \(\beta_2\)-microglobulin serum (no. 7096) was kindly

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1 Abbreviations used in this paper: EC, embryonal carcinoma; MHC, major histocompatibility
complex; PBS, phosphate-buffered saline.
supplied by Dr. N. Tanigaki (11, 12). Two different rabbit anti-human \( \beta_2 \)-microglobulin sera were obtained from Doctors A. Colle and N. Tanigaki, respectively (13).

Anti-HLA sera. A rabbit anti-HLA serum was kindly provided by Dr. Y. Tanigaki. This xenogeneic antiserum was absorbed twice with an equal volume of Daudi cells, a human lymphoblastoid cell line lacking HLA antigen (14–16). Human anti-HLA sera were obtained from multiparous women. Only eluates (17) of anti-HLA sera from platelets were used. We had previously ascertained that neither the rabbit anti-\( \beta_2 \)-microglobulin nor the anti-HLA used was contaminated by anti-H-Y activity (the anti-\( \beta_2 \)-microglobulin or anti-HLA sera absorbed on female cell lines no longer react with Raji cells).

Cell Lines

Human cell lines. The human lymphoblastoid cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. They were obtained from Doctors J. Littlefield, G. Klein, W. Bodmer, P. Goodfellow, and P. Wernet and from our own laboratory.

The cell lines were derived either from Burkitt lymphomas: Daudi (14), Raji (18), Chevalier (unpublished data), Ramos (19), BJAB (20), Namalwa (21), or from leukemic patients: SKL-1 (22), RPMI 8866. The other lymphoid cell lines were derived from normal peripheral lymphocytes (23, 24).

Somatic hybrid cells. The origin and karyotypic characterization of the human 8 A Raji × Daudi hybrid have been described previously (25). The human hybrid Daudi × D 98/AH-2 was obtained by the Sendai virus fusion technique (26). D 98/AH-2, a line derived from HeLa female cells, is deficient in hypoxanthine phosphoribosyl transferase (27). The hybrid Daudi × D 98/AH-2 clone was selected in hypoxanthine, aminopterin, thymidine selective medium (28).

The human Y chromosomes have been identified on all human cell lines by the "atebrin fluorescence technique" (29) and a C banding technique (30). A minimum of 10 metaphases were analyzed for each cell line.

Mouse teratocarcinoma. A number of different embryonal carcinoma cell lines (EC) and their differentiated derivatives were used. The culture and differentiation of embryonal carcinoma cells have been described previously (31). After 10–14 or 20 days in culture, differentiated cells were replated 48 h before testing. The cells were detached by treatment with EDTA solution (2 mM in phosphate-buffered saline [PBS]) and replated at 1/3 the initial density.

Two of the cell lines were derived from testicular teratocarcinomas OTT 6050 of mouse strain 129/Sv: the nullipotential F9 line (32) and the multipotential PCC3/A/1 line (31). In neither of these lines could a Y chromosome be detected by cytogenetic techniques (31, 33, 34).

The multipotential EC line PCC7-S and its azaguanine-resistant clone PCC7-S-AzaR1 were isolated from a spontaneous testicular teratocarcinoma arising in a recombinant (129 × B6) inbred line (F7). PCC7-S is a typical EC line in respect of morphological (i.e. low cytoplasm/nucleus ratio) and immunological characteristics (presence of F9 antigen according to the method previously described) (32). It gives tumors that contain well-differentiated tissues belonging to the three embryonal germ layers. Differentiation in vitro gives preferentially nervous derivative types (S. Pfeiffer, unpublished results).

The cytogenetic analysis of PCC7-S as well as of its azaguanine-resistant clone is apparently euploid: 40 chromosomes with X and Y chromosomes easily identifiable (Fig. 1). The Y chromosome can be shown to be present in tissues of the tumors even after continuous exponential growth for more than 2 mo. No preferential Y chromosome loss was observed under our in vitro culture conditions.

Sperm cells were prepared following the technique described previously (13, 35).

Serological Techniques. A microlymphocytotoxicity test was used according to Mittal (36). Selected rabbit complement was diluted 1:2 with fresh human AB serum before use. Raji cells were used as target cells in the standard assay when quantitative absorptions were performed as follows: 20 µl of undiluted antiserum was mixed with variable amounts of cells for 1 h at 4°C. After centrifugation, the supernate was tested for cytotoxic activity.

The indirect immunofluorescence technique was used on human and mouse cells in culture.

Goat anti-rat IgG (Hyland Diagnostic Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) labeled with fluorescein or rhodamine were absorbed (vol/vol) at 4°C by human or mouse fibroblasts.

Aliquots of the absorbed antiglobulin were stored at −40°C. Sequential binding of anti-H-Y
and antiglobulin sera was performed as follows: 100 μl of anti-H-Y at various dilutions was added to a pellet of 5 x 10⁶ cells. After incubation for 1 h at 4°C with occasional shaking, the suspension was diluted with PBS and 4% bovine serum albumin and washed twice. The pellet was resuspended in 100 μl of antiglobulin at a dilution of 1:30, incubated for 40 min at 4°C, and washed three times. The final pellet was spread on a microscope slide, air dried, and fixed in the cold with methanol. The smear was then mounted in 80% glycerol in PBS and observed with both an epi-illumination fluorescence and a phase contrast microscope.

For HLA and β₂-microglobulin labeling we followed the same procedure; goat anti-human IgG and goat anti-rabbit IgG labeled with fluorescein (Hyland Laboratories) or rhodamine were absorbed on human or mouse fibroblasts and stored at -40°C before used. The relationship between H-Y, β₂-microglobulin and HLA antigen has been studied by the differential redistribution immunofluorescence method (37).

Results

Presence of the H-Y Antigen on Raji Cells. Raji is a male lymphoid cell line established from a Burkitt lymphoma (18). The results of cytotoxicity tests performed on Raji cells using a rat anti-H-Y serum (properly absorbed, see Materials and Methods) are given in Fig. 2. 90-100% of Raji cells were killed by anti-H-Y serum at 1/1 to 1/8 dilution. This cytotoxic activity was completely abolished after absorption by 10 different male lymphoid lines or by absorption with peripheral lymphocytes from 5 different male donors. The cytotoxic activity was not changed after absorption with female lymphoid cell lines or peripheral lymphocytes from female donors.

Because the H-Y antigen has previously been found on sperm (5-8) of several mammalian species including man but not on chicken spermatozoa, absorption of the anti-H-Y activity by sperm cells was studied (Fig. 3). The cytotoxic
activity of the rat anti-H-Y sera on Raji cells was removed after absorption with mouse, pig, and human spermatozoa. On the contrary, chicken spermatozoa did not remove this anti-H-Y activity. Moreover, in a quantitative absorption experiment, $2 \times 10^7$ human or pig spermatozoa completely removed the anti-H-Y activity of 20 µl of undiluted serum, whereas $10^8$ chicken spermatozoa had no effect.

The cytotoxic activity of nonimmune female Lewis rat sera was studied on Raji cells; two out of six sera were found to be cytotoxic on Raji cells at a dilution of 1:2. However, this activity was equally well absorbed by male and female lymphoid cell lines. Several different anti-H-Y sera were analyzed. Sera

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**Fig. 2.** Cytotoxic activity of anti-H-Y sera on Raji cells. Curve (●), unabsorbed serum; curve (□), serum absorbed with three different human male lymphoid lines; curve (○), serum absorbed with three different human female lymphoid lines. For absorption, see Materials and Methods.

**Fig. 3.** Cytotoxic activity of anti-H-Y serum on Raji cells. Curve (●), unabsorbed serum. The anti-H-Y serum is absorbed with human sperm (△); mouse spermatozoa (○); pig spermatozoa (□); chicken spermatozoa (▲). For absorption, see Materials and Methods.
obtained after weekly injection of $2.10^6$ spleen cells had generally a higher activity than those obtained by injecting $4.10^7$ cells.

The presence of H-Y antigen on Raji cells could also be demonstrated by indirect immunofluorescence. The anti-H-Y activity was revealed with a fluorescein-conjugated goat anti-rat IgG. 80–90% of cells were labeled (Fig. 4). The labeling was removed after absorption with male lymphoid cells but not with female cells. When these experiments were performed at 37°C, caps were observed on 60% of the labeled cells.

Presence of H-Y Antigen on Different Human Lymphoid Cell Lines. The expression of H-Y antigen was examined on 12 lymphoid lines established from male donors and 10 lymphoid lines from female donors by direct lymphocytotoxic or absorption technique (Table I). None of the 10 female cell lines absorbed the anti-H-Y activity. On the contrary, 10 out of 12 male cell lines expressed H-Y antigen by absorption criteria. Of the H-Y positive cell lines, one, Chevalier, lacks HLA antigens but is $\beta_2$-microglobulin positive (Fellous et al. Manuscript in preparation). The two exceptional male lines that lacked H-Y antigen were Ramos (19), which also lacks the Y chromosome, and Daudi, which already lacks HLA and $\beta_2$-microglobulin. By direct lymphocytotoxic and indirect immunofluorescence assay, the H-Y antigen was detected on Raji, Chevalier, SKL-1 but not on the other seven male lymphoid lines. It should be noted that the presence of H-Y antigen could be detected only by absorption on most male lymphoid lines, as it has been already found on normal lymphocyte from other species (6). In this context Raji and Chevalier obtained from Burkitt lymphoma seem to be rather exceptional.

Relationship Between the Expression of $\beta_2$-Microglobulin and of H-Y Antigen

H/Y antigen is absent on Daudi cell but is expressed after cell fusion. The Daudi cell line carries the Y chromosome but lacks the H-Y antigen. It is already known that Daudi lacks the human $\beta_2$-microglobulin and HLA antigens (15, 16, 38). It was therefore of particular interest to determine
### Table I

**Expression of H-Y Antigen on Human Lymphoblastoid Lines from Male and Female Donors**

| Cell lines | Sex chromosomes | Direct cytotoxic test | Immunofluorescence test | Absorption* |
|------------|-----------------|----------------------|-------------------------|-------------|
|            |                 | %                    | %                       | Presence    |
| From male donors |                 |                      |                         |             |
| Raji‡      | XY              | 100                  | 98                      | Present     |
| Chevalier‡ | XY              | 100                  | 70                      | Present     |
| SKL‖       | XY              | 60                   | 60                      | Present     |
| JOST§      | XY              | 0                    | 0                       | Present     |
| ScTa§      | XY              | 0                    | 0                       | Present     |
| REMB1§     | XY              | 0                    | 0                       | Present     |
| H2LcL§     | XY              | 0                    | 0                       | Present     |
| RPMI 8866‖ | XY              | 0                    | 0                       | Present     |
| PGw1§      | XY              | 0                    | 0                       | Present     |
| Dauid†     | XY              | 0                    | 0                       | Not present |
| Ramos‡     | XY              | 0                    | 0                       | Not present |
| From female donors |                 |                      |                         |             |
| BJAB, Namalwa | XX            | 0                    | 0                       | Not present |
| T51, Brel, SCBMB georget§ | XX | 0 | 0 | Not present |
| WT50, Maja, AUR Letule§ | XX | 0 | 0 | Not present |

* Anti-H-Y activity test on Raji cell after absorption on lymphoid line.
† Line from Burkitt lymphoma.
§ Line from normal peripheral blood.
‖ Line from leukemic patient.

whether or not the absence of HLA, H-Y, and β₂-microglobulin on Daudi were related (Table II).

Two types of somatic cell hybrids involving Daudi have been analyzed: (a) one hybrid (Raji × Daudi) clone which expresses β₂-microglobulin and HLA (17, 39); this hybrid also expresses the H-Y antigen (Table II); (b) one hybrid (Daudi × D98 AH-2) clone, D98 AH-2, being a human female cell line. This hybrid, which has a Y chromosome derived from Daudi, expresses both β₂-microglobulin and H-Y antigen.

**Expression of H-Y Antigen on Mouse Teratocarcinoma Cell Lines.** The mouse embryonal carcinoma lines, PCC7-S and PCC7-S-Aza³, which carry the Y chromosome were studied (Table III). They both lack β₂-microglobulin and the H-Y antigen. After 3-4 wk of culture under conditions allowing in vitro “differentiation,” β₂-microglobulin becomes detectable by indirect immunofluorescence on about ½ of the cell population (Table III). Absorption experiments also showed the presence of H-Y antigen on these cells. An eventual correlation between the appearance of H-Y antigen and of β₂-microglobulin was investigated by double labeling immunofluorescence experiment (see Materials and Methods). All the cells which expressed H-Y antigen were also found to be β₂-microglobulin positive; the reverse was not true. Similarly, the mouse embryonal carcinoma cell lines F9, PCC4, and LT (Table III), which are XO or
**TABLE II**

Relationship between the Expression of Human $\beta_2$-Microglobulin HLA and H-Y Antigen on Human Cell Lines

| Cell line          | Sex chromosomes | Presence of | HLA | $\beta_2m^*$ | H-Y |
|--------------------|-----------------|-------------|-----|-------------|-----|
| Raji†              | XY              | +           | +   | +           | +   |
| Daudi‡             | XY              | −           | −   | −           | −   |
| Raji × Daudi§      | XY, XY          | +           | +   | +           | +   |
| D 98               | XX              | +           | +   | −           | −   |
| Daudi × D 98§      | XX, XY          | +           | +   | +           | +   |
| Chevalier†         | XY              | −           | +   | +           | +   |

* $\beta_2$-microglobulin.
† Line from Burkitt lymphoma.
§ Hybrid cell.

**TABLE III**

Presence of H-Y Antigen on Cell Mouse Teratocarcinoma Lines

| Embryonal carcinoma line | Presence of differentiated type cells | Sex chromosomes | Presence of embryonal carcinoma line | Absorption test* H-Y | Mouse $\beta_2$-microglobulin, labeled cells | Immunofluorescence test |
|--------------------------|---------------------------------------|-----------------|-------------------------------------|----------------------|----------------------------------------------|------------------------|
| F9                       | No                                    | XO              | %. %                                | 0                    | 0                                            | 0%                     |
| PCC4                     | No                                    | XO              | %. %                                | NT†                  | 0                                            | 0%                     |
| LT1                      | No                                    | XX              | %. %                                | NT                   | 0                                            | 0%                     |
| PCC7-S, 0 day            | No                                    | XY              | %. %                                | 0                    | 0                                            | 0%                     |
| PCC7-S, 28 days§         | Yes                                   | XY              | %. %                                | 35                   | 24                                           | 24%                    |
| PCC7-S Aza,h, 0 day      | No                                    | XY              | %. %                                | 0                    | 0                                            | 0%                     |
| PCC7-S Aza,h, 24 days§   | Yes                                   | XY              | %. %                                | NT                   | 18                                           | 18%                    |
| PCC3/A/1, 0 day          | No                                    | XO              | %. %                                | 0                    | 0                                            | 0%                     |
| PCC3/A/1, 30 days        | Yes                                   | XO              | %. %                                | 36                   | 0                                            | 0%                     |

* Anti-H-Y activity test on Raji cell after absorption on teratocarcinoma line.
† Not tested.
§ Differentiation appears under conditions of differentiation in vitro.

XX, lacked both H-Y antigen and $\beta_2$-microglobulin. PCC3/A/1 cells which are XO can differentiate in vitro and then express $\beta_2$-microglobulin but still do not express H-Y antigen.

**Relation between H-Y and Human $\beta_2$-Microglobulin on Raji Cell.** Redirect experiments were performed on Raji cells to analyze whether H-Y and $\beta_2$-microglobulin were associated on the cell membrane (Table IV). After the first labeling with rabbit anti-$\beta_2$-microglobulin (revealed by fluorescein or rhodamine conjugated anti-rabbit Ig) and incubation at 37°C, capping was observed on 70–80% of the labeled cells. If the second staining was performed at 0°C with anti-H-Y sera (revealed by rhodamine or fluorescein-conjugated anti-mouse Ig), 90% of the cells doubly stained (Fig. 5) showed...
### Table IV
**Effect of Redistribution of Human β₂-Microglobulin on the Distribution of H-Y Antigen on Raji Cell**

| First labeling | Second labeling | Cells labeled for H-Y | Cells labeled for β₂m* | Percent of cells where labeling of human β₂m is aggregated and | H-Y aggregated at the same place | H-Y has a diffuse labeling |
|----------------|-----------------|-----------------------|-------------------------|---------------------------------------------------------------|-------------------------------|----------------------------|
| 37°C           |                 |                       |                         |                                                               |                               |                            |
| Rabbit anti-β₂m 1/200 and FITC goat anti-rabbit Ig | Anti H-Y followed by TRITC goat anti-mouse Ig | 90                     | 99                      | 92                                                             | 8                             |                            |
| Rabbit anti-β₂m 1/200 and TRITC goat anti-rabbit Ig | Anti-H-Y followed by FITC goat anti-mouse Ig | 85                     | 100                     | 96                                                             | 4                             |                            |
| Anti H-Y and FITC goat anti-mouse Ig | Rabbit anti-β₂m and TRITC goat anti-rabbit Ig | 87                     | 99                      |                                                               |                               |                            |
| Rabbit anti-β₂m 1/200 and FITC goat anti-rabbit Ig | Rabbit anti-β₂m 1/50 and TRITC goat anti-rabbit Ig | 100                    | 95                      | β₂m TRITC aggregated at the same place | 95                             | 5                           |

*Abbreviations used in this table: β₂m, β₂-microglobulin; FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate.

Cocapping of β₂-microglobulin and H-Y antigen. However, in 4-8% of the cells the redistribution of β₂-microglobulin was not associated with a correspondent capping of H-Y antigen. These latter results can tentatively be explained by the fact that in 5% of the cells where β₂-microglobulin caps (last line of Table IV), there is still β₂-microglobulin incompletely redistributed and left outside of the caps. No such cocapping between H-Y and β₂-microglobulin was observed when H-Y antigen is the first to be aggregated.

**Discussion**

As compared with spermatozoa or epidermal cells, the Raji cell line turns out to be a useful target cell for studies of the H-Y antigen (5, 40): it is easy to grow and killable to 100% by rat anti-H-Y sera at a dilution up to 1:8.
Serological screening of male and female human lymphoid cell lines strongly suggests that the serologically detectable H-Y antigen is associated with the presence of the Y chromosome. The H-Y antigen is expressed on all male lymphoid cell lines with only two exceptions: (a) the Ramos cell line which was established from a male donor and has lost the Y chromosome in culture, and (b) the Daudi cell line which has a Y chromosome. Recently, Ohno (10) has suggested that H-Y antigen used the major histocompatibility $\beta_2$-microglobulin antigens complex as an anchorage site on the cell membrane. The experiments reported here were designed to test this hypothesis. The first striking result is the absence of H-Y antigen on Daudi cell, known to lack both $\beta_2$-microglobulin and HLA antigens. Cell hybrids between Daudi and D98 (a female cell line that expresses $\beta_2$-microglobulin and HLA antigens) express the H-Y antigen. More-
over, Chevalier, a cell line that lacks HLA antigens but expresses β2-microglobulin, also expresses H-Y antigen.

To test a possible association between HLA, β2-microglobulin, and H-Y antigen, redistribution experiments between H-Y and β2-microglobulin or between H-Y and HLA were performed on Raji cells. In the redistribution experiments between β2-microglobulin and H-Y antigen, 90% cocapping was observed when β2-microglobulin was redistributed first, but not when the H-Y antigen is capped first. These results show that H-Y antigen is associated with β2-microglobulin on the cell membrane of Raji. No cocapping has been observed however when redistribution experiments with HLA and H-Y antigens were carried out. These results show clearly that in the cell membrane H-Y antigen is associated only with β2-microglobulin but not with HLA antigen (as suggested by Ohno).

The association between H-Y antigen and β2-microglobulin has also been found on mouse teratocarcinoma cells. The male pluripotent embryonal carcinoma lines PCC7-S and PCC7-Aza, carrying the Y chromosome lack both H-Y and β2-microglobulin. During in vitro differentiation correlated appearance of both antigens was observed. Moreover, the cells that are H-Y positive are always β2-microglobulin positive. On the contrary, β2-microglobulin positive, H-Y negative cells were found, a result that suggests that during in vitro differentiation β2-microglobulin might appear before H-Y antigen.

The presence of H-Y antigen has also been described on mouse morulae embryos (41). However, at this stage of embryonic development, we were unable to detect β2-microglobulin (12). Although the presence of β2-microglobulin is apparently required for H-Y expression on adult cells, embryonic cells appear to express H-Y without expressing adult β2-microglobulin. On the embryo, H-Y antigen might be associated with an eventual embryonic form of β2-microglobulin which has already been postulated (42).

The association between H-Y and β2-microglobulin must be compared to the already described association between β2-microglobulin and other membrane antigens related by sequence similarities and coded by closely linked genes: major histocompatibility antigens (37), Tla antigen (43), or Qa antigen (44).

β2-Microglobulin might behave as a regulatory element in controlling the expression of a series of cell membrane antigens during development and differentiation.

**Summary**

The expression of the H-Y antigen has been tested on several human lymphoid lines and mouse teratocarcinoma cell lines during differentiation. The human male lymphoid cell line Raji is a very useful target for studies of the H-Y antigen by lymphocytotoxicity test with rat anti-H-Y sera. With a few exceptions, all cells carrying the Y chromosome were H-Y positive. One of the exceptions is the human Daudi cell line which, besides lacking H-Y antigen, also lacks β2-microglobulin. We have studied a possible association between the H-Y antigen, β2-microglobulin, and HLA antigen with redistribution experiments. The results strongly suggest that H-Y antigen is not associated with HLA antigens but with β2-microglobulin.
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