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Immunology

Structural similarities between a product of the *T/t*-locus isolated from sperm and teratoma cells, and H-2 antigens isolated from splenocytes

(*T/t* locus antigens/lactoperoxidase-catalyzed iodination)

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ABSTRACT **Molecules specified by the H-2-linked *T/t* locus in the mouse are expressed on H-2-negative cells such as early embryos, sperm, and teratoma cells. By means of enzymatic radioiodination of cells and immunoprecipitation of lysates with congenic antiserum, one of these molecules, known as F9, has been obtained from sperm and teratoma cells and compared to H-2 isolated from murine splenocytes. Our studies indicate that both H-2 and F9 have identical molecular weights and subunit structure, including the presence of a B2-microglobulin-like subunit. These findings, taken together with previous studies of TL alloantigens, suggest that a number of gene products of the 9th linkage group show structural homology with each other and, in addition, with immunoglobulin. The genes in question may therefore have arisen from a primitive gene concerned with cellular recognition.**

There is considerable evidence that preimplantation embryos such as eggs and morulae do not express serologically detectable H-2 antigens (1, 2). They express, however, another antigen (known as F9) which has been found also on primitive teratocarcinoma cells as well as on sperm (3). This antigen appears to be specified by a gene at the *T/t* locus (4) that is linked to H-2. It has been suggested, therefore, that molecules specified by the *T/t* locus may represent the embryonic analogues of H-2 molecules.

The purpose of these studies was to define the structure of this F9 antigen and to compare it with H-2. Thus, sperm and teratoma cells (both known to express F9 antigen) and spleen cells (which express H-2) were radioiodinated and the cell lysates were immunoprecipitated with hyperimmune sera specific for F9 and for relevant H-2 antigens.

MATERIALS AND METHODS

Mice. Adult 129/J and A/J mice were obtained from Jackson Laboratories, Bar Harbor, Me., and were maintained in the colony at Southwestern Medical School.

F9 Teratoma Cells. These primitive nullipotential teratocarcinoma cells isolated from 129/Sv mice have been described (3). They were grown in gelatin-coated 75 cm^2 (250 ml) Falcon flasks or 150 cm^2 (1000 ml) Corning flasks in Dulbecco's modified Eagle's medium containing 15% fetal calf serum (GIBCO, Grand Island, N.Y.). Cells were incubated at 37° in a moist 12% CO_2 incubator.

Antisera. (*1*) Anti-F9 serum, prepared in individual syngeneic 129/Sv mice and not pooled was assayed as described (3) and stored at −20° in aliquots which were frozen and thawed not more than twice prior to use. This serum had a cytotoxic titer of $1/3000$ against F9 cells.

(*2*) A.BY anti ASL 1 (anti H-2^a) (6). This antiserum contains antibodies to H-2^a (K and D) and TL 1, 2, 3. It had a cytotoxic titer of $1/2560$ against A strain splenocytes (representing its *anti H-2^a* activity).

(*3*) B6.H-2^k anti EL4 (anti H-2^b) (7). This serum had a cytotoxic titer of $1/640$ against C57BL/6 splenocytes.

(*4*) Normal mouse sera obtained from young 129/Sv male mice were individually tested against F9 cells. Seven such sera gave similar results and were used interchangeably.

(*5*) Rabbit anti mouse Ig. This antiserum, prepared against murine myeloma proteins, contained antibodies against μ, γ, α, κ, and λ chains (7).

(*6*) Goat anti mouse Ig. This antiserum was prepared against purified mouse IgG and contained antibodies against γ, κ, and λ chains. It was titrated against the mouse anti F9 serum and used in slight antibody excess.

(*7*) Goat anti rabbit Ig. This serum contained antibodies against rabbit γ and L chains and was titrated against the rabbit anti mouse Ig. It was used in slight antibody excess.

Radioiodination of Cells. Spleen cells were prepared as described previously (6). Sperm cells were prepared from minced epididymi as described (4). The cell suspension was diluted in 0.01 M phosphate-buffered saline, pH 7.3 and centrifuged for 5 min at 500 rpm in an IEC PRJ centrifuge to remove contaminating (epididymal) cells. The supernatant was centrifuged at 3000 rpm for 15 min at 4° to pellet the sperm which were then washed once in phosphate/saline. Teratoma cells were removed from the flasks by EDTA solution (3) and were washed twice in phosphate/saline. Cells (1 to 5×10^7) of each type were suspended in phosphate/saline at concentrations of 1 to 10×10^7/ml and were enzymatically radioiodinated as described (5, 6) with 1–2 mCi of $Na^{125}I$ (Amersham/Searle)/ 1 to 5×10^7 cells. After the labeling period, cells were centrifuged and washed once in phosphate/saline, lysed in 2 ml of 0.5% Nonidet P40 (NP40) (Shell), centrifuged at 3000 rpm, and dialyzed for 16 hr at 4° against 500 volumes of phosphate/saline.

Labeling with [^{3}H]Leucine. Splenocytes (1 to 3×10^8) were washed in Eagle's minimal essential medium lacking leucine and containing 10% fetal calf serum. Cells were suspended in the same medium containing 20 μCi/ml of L-[4,5-^{3}H]leucine (38 Ci/mmol) (Amersham/Searle) at 10^7 cells per ml and were incubated in Falcon flasks for 6 hr in a moist 5% CO_2 incubator. After the incubation period, cells were centrifuged, washed, and lysed as described above. Teratoma cells were labeled in monolayer. The Dulbecco's medium was decanted and the cells were incubated with 10

Table 1. Percent of cell-associated protein that is F9 or H-2*

Cells	Strain	Isotope	Acid-precipitable radioactivity (cpm × $10^7/10^7$ cells)	% Acid-precipitable radioactivity immunoprecipitated by			
				anti F9	anti H-2^a	anti H-2^b	NMS†
F9	(129/SV) (H-2^{bc})	[^{3}H] Leucine	7.2	2.4	0.27	0.29	0.21
		^{125}I	1.0	4.1	0.43	0.48	0.33
Sperm	129/J (H-2^{bc})	^{125}I	0.95	9.7	0.97	1.20	0.86
	A/J (H-2^a)	^{125}I	0.88	8.8	1.31	1.29	1.21
Spleen	129/J (H-2^{bc})	^{125}I	0.94	0.49	0.51	4.20	0.42
	A/J (H-2^a)	^{125}I	1.20	0.51	3.60	0.59	0.57

* The table shows data from one representative experiment of several.
† NMS, normal mouse serum.

ml of the same medium described above for spleen cells for 6 hr in a moist 12% CO_2 incubator. After the incubation period, the medium was decanted, the monolayer was rinsed in phosphate/saline, and the cells were lysed in 3 ml of 0.5% Nonidet P40/75 mm^2. Lysates were transferred to 5-ml plastic Falcon tubes and centrifuged for 15 min at 3000 rpm, and the lysates were dialyzed as described above.

Immunoprecipitation. After dialysis, lysates from ^{125}I-labeled or [^{3}H]leucine-labeled cells were centrifuged at 10,000 × *g* for 30 min, and small aliquots were precipitated with 10% trichloroacetic acid to determine protein-associated radioactivity. Ig was removed from the spleen cell lysates by immunoprecipitation using a "sandwich" technique with rabbit anti mouse Ig and goat anti rabbit Ig (7). Ig-depleted lysates from spleen cells or untreated lysates from sperm or teratoma cells were divided into four aliquots and treated with optimal amounts of anti F9 (20 μl/10^7 cells), anti H-2^a (20 μl/5 × 10^7 cells), anti H-2^b (30 μl/10^7 cells), or normal mouse serum (control; 40 μl/10^7 cells). Immune complexes were precipitated by a slight excess of goat anti mouse Ig (7) and the precipitates were washed four times in phosphate/saline. After the last wash, precipitates were transferred in a small volume of phosphate/saline to fresh tubes and pelleted. Precipitates were dissolved by boiling for 3 min in 0.1 M Tris buffer, pH 8.6, containing 1% sodium dodecyl sulfate and 8 M urea. Aliquots to be reduced were treated for an additional 30 min at 56° with 0.1 M 2-mercaptoethanol. The radioactivity in the dissolved precipitate was determined in a small aliquot in either the Beckman gamma counter or the Beckman LS 350 liquid scintillation counter

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis. All samples were mixed with internal markers prior to electrophoresis. For ^{125}I-labeled samples, these included [^{3}H]leucine-labeled mouse μ and L chains (8), and for [^{3}H]leucine-labeled samples, ^{125}I-labeled human μ and L chains. Samples were prepared so that there was a 3- to 5-fold excess of ^{3}H cpm to ^{125}I cpm. In instances where [^{3}H]F9 and ^{125}I-labeled H-2 (or ^{125}I-labeled F9 and [^{3}H]H-2) were mixed, additional aliquots of each were also electrophoresed in separate gels with the labeled marker proteins described above. All samples were electrophoresed on 12.5-cm 5% or 7.5% sodium dodecyl sulfate gels (5, 8). In the first instance, samples were electrophoresed for 2.5 hr at 15 mA per gel and in the latter instance for 15–17 hr at 5 mA per gel. Gels were fractionated (5) and the double label was counted in a Beckman LS 350 liquid scintillation counter with appropriate discriminators (8). Counts per min were corrected for spillover using standard quench correcting curves.

Re-electrophoresis of Samples Extracted from Gels. In instances where samples were to be extracted from the gels, the peak was localized by determining the ^{125}I, the fractions were pooled, and the gel was removed by filtration through a 200 mesh stainless steel screen. When necessary, the eluate was concentrated by pervaporation and the extract adjusted to 1% sodium dodecyl sulfate and 8 M urea. Samples were boiled, an aliquot was reduced in 0.1 M 2-mercaptoethanol, and the samples were electrophoresed with appropriate internal markers.

RESULTS

Detection of radiolabeled F9 and H-2 antigens

As seen in Table 1, approximately 2% of the total [^{3}H]leucine-labeled protein from F9 cells can be specifically immunoprecipitated with anti F9 serum. In contrast, only 0.2% is immunoprecipitated by anti H-2^b or the control sera (anti H-2^a or normal mouse serum). A similar situation was seen when ^{125}I (surface)-labeled protein was immunoprecipitated with anti F9. About 4% of total labeled protein was precipitated as compared with 0.3–0.5% with either anti H-2^b or the control sera. In the case of radioiodinated sperm, the anti F9 serum brought down approximately 8–10% of the protein-associated radioactivity, whereas the anti H-2 sera and the normal mouse serum brought down 0.8–1.3%. The results with F9 and sperm were in marked contrast to those obtained with radioiodinated splenocytes, in which the specific anti H-2 brought down 5- to 10-fold more radioactivity than the anti F9, the reciprocal anti H-2, or normal mouse serum (3.6–4.2% as compared to 0.4–0.6%). These data indicate the presence of F9 on both F9 cells and sperm but not on spleen cells. H-2 is not detectable on either sperm or F9 cells but is present on spleen cells. It is noteworthy that in initial experiments, small amounts of H-2 were sometimes detected on sperm. It was subsequently found that the sperm preparations were contaminated with 10% of other cell types; when these were removed (by low-speed centrifugation) H-2 was consistently absent from the sperm in the resulting cell preparation, but was detected in the contaminating cells removed by low-speed centrifugation.

These data indicate that if F9 is present on splenocytes, it is present in less than 10% of the concentration found on F9 cells and sperm. In contrast, H-2 is present on splenocytes and is undetectable on F9 and sperm (<10%).

Similarity in the structure of F9 and H-2 molecules

As seen in Figs. 1–3, electrophoresis of reduced H-2 and F9 immunoprecipitates from sperm, F9 cells, and spleen indi-

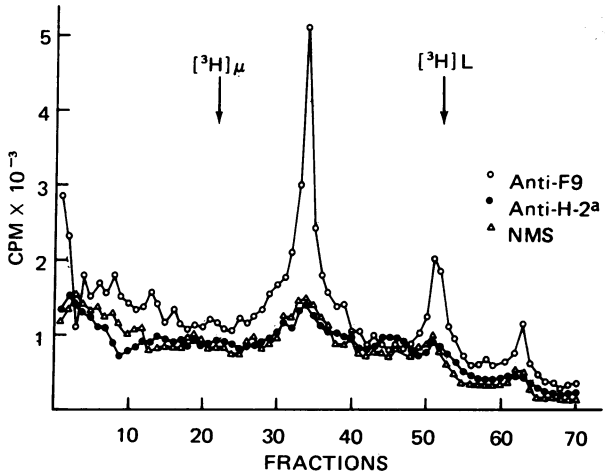

FIG. 1. F9 and H-2^a immunoprecipitated from lysates of radioiodinated A/J sperm. Immunoprecipitates were mixed with [^{3}H]IgM, reduced, and electrophoresed for 15 hr on sodium dodecyl sulfate/7.5% acrylamide gels. In plotting the three gel patterns, the positions of the internal markers (μ and L) were aligned.

cate that F9 antigen is present on sperm and F9 cells but not on spleen. In contrast, H-2 was demonstrable on spleen but not on F9 cells or sperm. Both H-2 and F9 precipitates from the respective positive cells, under reducing conditions, appear similar, with three peaks of radioactivity corresponding to molecules of 44,000, 22,000, and 12,000–14,000 daltons. To examine more critically this implied similarity in structure, F9 precipitates containing one label (^{3}H or ^{125}I) were mixed with H-2 precipitates containing the other label, and the samples were boiled and electrophoresed under both reducing and nonreducing conditions. Fig. 4 shows the results of electrophoresis of a mixture of unreduced ^{125}I-labeled F9 and [^{3}H]H-2. Peaks of molecular weight of approximately 90,000, 150,000, 180,000, and 13,000 were observed. Boiling the precipitates for longer periods of time favored the appearance of the 90,000 molecular weight peak at the expense of the larger two, although all three peaks were consistently present. It has already been suggested that H-2, like HLA, consists of a disulfide-bonded dimer of 88,000 attached noncovalently to two B2-microglobulin subunits (9–11). The peaks of 90,000 and 12,000–14,000 therefore probably represent the dimer and the B2-microglobulin-like subunits which dissociate under conditions that break noncovalent bonds. The larger peaks may represent disulfide-bonded trimers and tetramers of the 44,000 subunit or noncovalently bound aggregates of material. In several experiments, considerable amounts of the 44,000 subunit were observed. It is not clear whether the monomer is present as such on the cell surface or represents a breakdown product from the intact molecule. Monomeric units of HLA have also been observed (9).

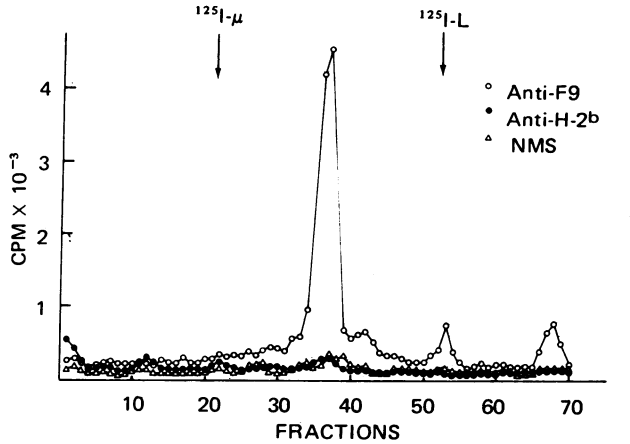

FIG. 2. F9 and H-2^b immunoprecipitated from lysates of [^{3}H]leucine-labeled F9 cells. The internal marker consisted of ^{125}I-labeled μ and L chains. See legend of Fig. 1 for details.

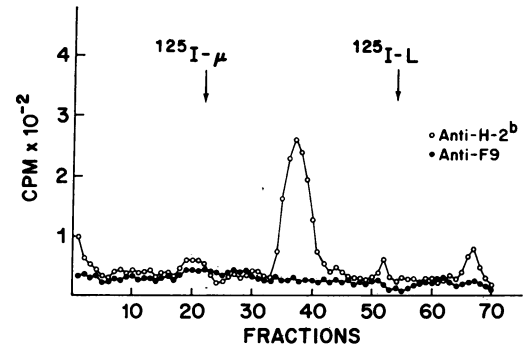

FIG. 3. F9 and H-2^b immunoprecipitated from lysates of [^{3}H]leucine-labeled 129/J splenocytes. See legends of Figs. 1 and 2 for details.

The most striking aspect of these experiments was that both the F9 and H-2 molecules appeared to be identical in structure in any given experiment. In all double-label experiments they co-electrophoresed, e.g., when monomers of H-2 were present, monomers of F9 were also present. When the ^{125}I and [^{3}H]leucine labels were reversed (^{125}I-labeled H-2 and [^{3}H]F9), or when F9 and H-2 were electrophoresed separately with internal markers, the results were identical.

After reduction of a mixture of differentially labeled H-2 and F9, coelectrophoresis revealed three subunits (Fig. 5). The major one of 44,000 daltons was present in both H-2 and F9. The 22,000 subunit was consistently more prominent on F9 antigen than on H-2, although it was always observed on both. This subunit also varied in relative amounts in different experiments. It has been suggested that this is a breakdown product of H-2 (and presumably F9) (11), although its exact nature is as yet unclear. For example, it is also possible that the 90,000 dalton subunit is composed of one 44,000 unit and two 22,000 units, although from studies of others we consider this unlikely (9–11). It is of interest that in previous studies of H-2 on *thymus*, the 22,000 peak was not observed (6). In addition, since it was not present on the gel of the unreduced precipitate it is unlikely that it is Ia, since this antigen has been reported to exist as monomers and dimers of a 30,000–35,000 peptide (12, 13). However, based on these experiments, we cannot definitively exclude the possibility that this peak represents Ia or an embryonic analogue of Ia.

In addition to the 44,000 and 22,000 dalton subunits, both F9 and H-2 contained a small subunit of 12,000–14,000 daltons which was also seen in the unreduced precipitate, as mentioned previously. This subunit was particularly prevalent on the F9 antigen isolated from F9 cells and H-2 isolated from splenocytes. It represented a much smaller peak on the gel of the F9 antigen precipitated from sperm. The relevance of these quantitative differences is not clear.

To confirm the relationship between the 90,000 dalton peak obtained under nonreducing conditions and the 44,000 and 22,000 subunits seen after reduction, the 90,000 dalton peak was eluted from the gel of the unreduced H-2 and F9 mixture (where only the ^{125}I was counted), reduced, and re-

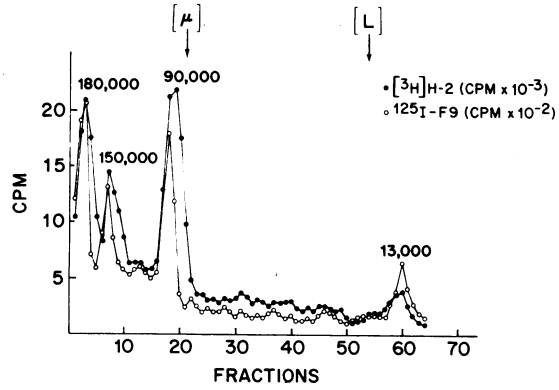

FIG. 4. F9 and H-2 immunoprecipitated from lysates of radioiodinated F9 cells and [^{3}H]leucine-labeled splenocytes, respectively. Precipitates were mixed, boiled, and electrophoresed as described in the legend of Fig. 1. The resultant counts were corrected for spillover. Separate aliquots of each precipitate were also mixed with μ and L chains obtained from reduced and alkylated ^{3}H- or ^{125}I-labeled IgM and electrophoresed in order to determine the molecular weights of the peaks shown in the figure.

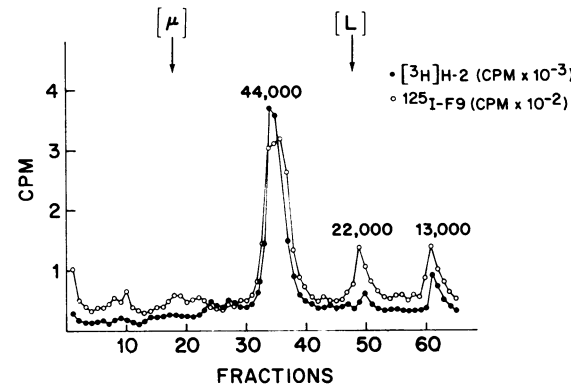

FIG. 5. F9 and H-2 immunoprecipitated from lysates of radioiodinated F9 cells and [^{3}H]leucine-labeled splenocytes, respectively. Precipitates were mixed, reduced, and electrophoresed as described in the legend of Fig. 4.

electrophoresed (not shown). After electrophoresis, peaks of 44,000 and 22,000 daltons were present. As expected, the 12,000–14,000 subunit was absent.

DISCUSSION

Although the antigenic determinants of H-2 and F9 are not immunologically crossreactive in the defined systems used for immunization (mouse anti-mouse), the molecules on which they are carried resemble one another in molecular weight(s) and subunit composition. The major intact molecule in each case appears to consist of a disulfide-bonded dimer of 90,000 daltons made up of two 44,000 subunits (or alternatively one 44,000 and two 22,000 units) bound noncovalently to one or more B2-microglobulin-like subunits. The probable structure is two large chains and two B2-microglobulin molecules (112,000 daltons) analogous to HLA (9–11). Trimers and tetramers of the 44,000 dalton subunit bound to an unspecified number of B2-microglobulin units may also be present. After reduction of the whole immunoprecipitate or the 90,000 dalton peptide, peptides of 44,000 and 22,000 are generated; it is not clear whether the 22,000 moiety is a breakdown product of the 44,000 unit or a discrete subunit. Although we have consistently found this subunit in both H-2 (from spleen) and F9 (from F9 or sperm), it appears variable in quantity from experiment to experiment. It was not observed in previous experiments of H-2 isolated from thymocytes. Rask *et al.* (11) have also reported this subunit for H-2, and based on peptide mapping, also suggest that it might be a breakdown product generated during extraction. The exact nature of the 22,000 dalton subunit awaits further study. We emphasize that although there is uncertainty about the precise molecular composition of surface F9 and H-2, in all instances the two molecules coelectrophoresed in sodium dodecyl sulfate gels.

By the radioiodination technique, we were unable to detect H-2 on uncontaminated preparations of sperm. Considering the limitations of our techniques, it is estimated that if sperm do possess H-2 it must be at less than 10% of the concentration found on splenocytes. The data showing absence of H-2 on F9 cells are also consistent with our failure to detect it serologically by absorption (14). To recapitulate, F9 teratoma cells do not absorb anti H-2 antibody, they grow progressively in mice of foreign H-2 types, and, as will be shown in another paper (15), they cannot sensitize or act as targets in assays for cell-mediated lymphocytotoxicity directed against H-2.

The evidence that products of *T/t* and *H-2* genes are similar in molecular weight and subunit structure, and also are both associated with a B2-microglobulin-like moiety in the cell membrane, lends credence to current hypotheses that the *T/t* and the *H-2* regions are related in an evolutionary sense (16–18). Thus, four loci within about 20 centimorgans of the centromere of chromosome 17 have now been shown to specify molecules that: (*i*) reside in the plasma membrane, (*ii*) are similar in molecular structure, and (*iii*) govern the surface constitution of cells at particular phases of the overall program of development. The four relevant loci are, in order from the centromere, *T/t*, *H-2K*, *H-2D*, and *Tla*.

Presumptions for some relationship between the *T/t* and *H-2* loci were based on both genetic and developmental criteria: the two loci are linked on chromosome 17, and the fact that wild mice are polymorphic for recessive lethal *t*-alleles that in some undefined way inhibit recombination into the *H-2* region led to speculation that the *T/t* – *H-2* region might be maintained as a "super gene" (19), i.e., that there might exist some relationship in the expression of the genes. It has been known for some time that *T/t* – locus genes specify antigens at the cell surface (20) and are in this respect functionally similar to *H-2* genes. Also, like *H-2*, the *T/t* region appears to be separable by recombination into at least two loci (21, 4) (unpublished data). Furthermore, although H-2 products are present on all normal adult somatic cells and are probably essential for their viability (22), H-2 is not found on early embryonic cells, where *T/t*-gene products are found instead (4). This developmental reciprocity is reflected in findings that populations of teratoma cells differentiating *in vitro* lose T/t-antigen and acquire H-2 with time (23).

On the basis of these facts it can be envisaged that a set of genes on the 17th chromosome arose to govern cell-cell recognition and interaction and that subsequently a duplication or series of duplications of this region occurred, allowing for more complex cellular distinction.

Evidence for a structural and functional relationship between TL and *H-2* has been available for some time. The two gene loci are linked (24); TL and H-2 antigens have similar molecular weights (6, 7, 10, 25), and both TL (6, 26) and H-2 (6, 27, 28) are associated with a B2-microglobulin-

like subunit in the cell membrane. Furthermore, the expression of TL is inversely correlated with expression of H-2D, the presence of TL on a cell reducing the manifestation of D (but not K) antigen (29–31). This reciprocal interaction between TL and *H-2D* suggests that the two molecules may make functionally related contributions to the plasma membrane. This suggestion is bolstered by the fact that the reciprocal expression of TL and D occurs in both *cis* and *trans*. Since this type of interaction is limited to the D end of the H-2 complex, and since the chromosomal locus of *T1a* is closer to *H-2D* than to *H-2K*, it may be that *T1a* arose as a duplication of *H-2D*.

The finding that four linked gene loci that may all be involved in cell-cell recognition and are functionally interrelated, are all associated with a B2-microglobulin-type molecule is of particular interest in view of the report of sequence homologies between B2-microglobulin and a domain in the constant region of the immunoglobulin molecule (32–34). The conclusion is almost inescapable that devices in the cell surface involved in certain types of cellular recognition and response phenomena are very old, very conserved, and based on evolutionary transitions involving a limited set of ancestral genes.

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