IMMUNOCHEMICAL EVIDENCE FOR AN ADDITIONAL *H-2* REGION CLOSELY LINKED TO *H-2D*

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The classical transplantation antigens (H-2 antigens) of the mouse are determined by information in two genetic regions, *K* and *D* located at opposite ends of the *H-2* complex. Products of each *K* and *D* region are characterized serologically by private specificities which are restricted to the *K* or *D* product of a single *H-2* haplotype or haplotypes presumed to be of a common origin, and by public specificities which are shared among several *H-2* haplotypes. The two locus *H-2* model postulates that there is a single gene product for each *K* and *D* region and that the genes coding for these two molecules evolved by gene duplication (1). Limited data corroborating the existence of one *K* or one *D* region product each expressing both private and public specificities has been reported in chemical studies (2) and in surface redistribution studies (3, 4), although other recent studies have shown that in one combination the *D* region molecules reactive with antisera detecting private and public *H-2* specificities do not co-cap on the lymphocyte membrane (5). Since capping depends on accessibility of antigenic determinants to the reagents used, a variety of interpretations of these capping studies are possible. We have therefore examined the reaction of pertinent *D* region alloantisera with solubilized cell surface antigens. In the studies reported here, we have distinguished two molecules of similar molecular weight determined by genes in or closely linked to the *D* region of each of two *H-2* haplotypes. In both cases one *D* region product reacted with both antisera to private and public specificities and the other with only antisera to public specificities.

Materials and Methods

Mice. The mouse strains used and their *H-2* haplotypes are shown in Fig. 1. All mice were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Alloantisera. Anti-H-2 sera that were used are listed in Table I. Reagents no. 2 and no. 3 were raised by immunizing with skin grafts followed by biweekly intraperitoneal injections of 2×10^7 live lymphoid cells beginning the 3rd wk after skin grafting. Mice were bled from the tail 1 wk after each injection and immune sera from successive bleedings were pooled. Reagents no. 1 and no. 4 were produced by The Jackson Laboratory under contract from NIAID.

Isolation and Analysis of Specific H-2 Antigens. Radiolabeled soluble H-2 antigens were prepared according to methods described recently in detail (6). Briefly, spleen cells were labeled in short-term tissue culture with ^{3}H-leucine, solubilized in buffer containing Nonidet P-40 (Particle Data, Inc., Elmhurst, Ill.), and purified by absorption to a lentil lectin affinity column and elution with alpha-methyl mannoside.

Specific H-2 antigens were isolated by indirect precipitation. Radiolabeled antigen was incubated with an excess of specific alloantisera, after which an excess of protein A-bearing *Staphylococcus aureus* Cowan I (SaCI) was added to precipitate antigen-antibody complexes. The SaCI-

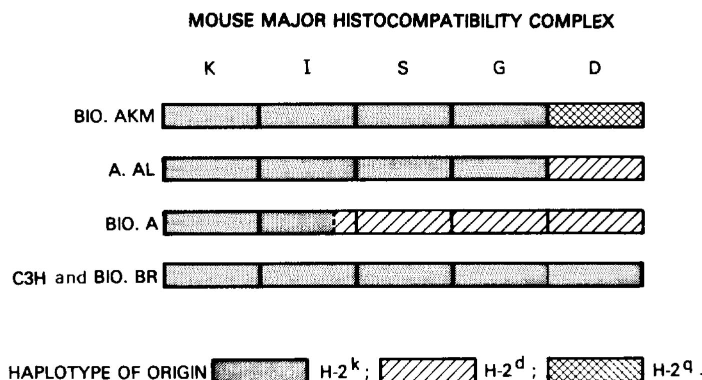

FIG. 1. Schematic representation of the origin of *H-2* regions carried by the mouse strains used. Note that since the antisera to public specificities (nos. 2 and 3, Table I) were produced in H-2^k strains (C3H and B10.BR) and since B10.AKM and A.AL differ from H-2^k only in the *D* region, these antisera-antigen combinations detect only *D* region or *D* region-linked products.

TABLE I
Alloantisera Used for Experiments

Reagent no.	Strain combination	Relevant specificities
1	(B10.A × LP.RIII)anti-B10.AKM*	Private for D^q (30)
2	C3H anti-C3H.SW	Public for D^q or D^d (6, 27, 28, 29)
3	(C3H × B10.BR) anti-B10	Public for D^q or D^d (6, 27, 28, 29)
4	(B10.AKM × 129) anti-B10.A*	Private for D^d (4)

* Produced by The Jackson Laboratory, under contract from the National Institute of Allergy and Infectious Diseases.

precipitates were washed and the radiolabeled antigens were eluted with 2% sodium dodecyl sulfate (SDS) and 2% 2-mercaptoethanol. The eluates were analyzed by discontinuous polyacrylamide gel electrophoresis in SDS (SDS-PAGE), under conditions that have been previously shown to differentiate H-2 antigens from other lymphocyte surface molecules such as Ia antigens and Ig heavy and light chains (6).

Results and Discussion

To determine whether or not private and public *H-2* antigenic specificities are expressed on the same molecules, sequential precipitations were performed. Labeled B10.AKM antigen was divided into three equal aliquots for the first precipitation step. The first aliquot was pretreated with normal mouse serum (NMS) as a control; the second was pretreated with an excess amount of antiserum specific for the private D^q specificity 30 (Table I, reagent no. 1); and the third aliquot was pretreated with an excess amount of antiserum specific for public D^q specificities 6, 27, 28, 29 (reagent no. 2). After incubation, precipitation was carried out by addition of an excess amount of SaCI. Supernates from each of these three aliquots were then subdivided and tested for residual antigen with the anti-private and anti-public antisera. The results of the second precipitation were analyzed on SDS-PAGE, and their electrophoretic migration patterns are shown in Fig. 2. In the control in which antigen was pretreated with NMS, antibodies specific for both the private and the public specificities precipitated D^q region molecules (panels 1 and 2, respectively). Complete removal of molecules reactive with the antiserum detecting public specificities caused concomi-

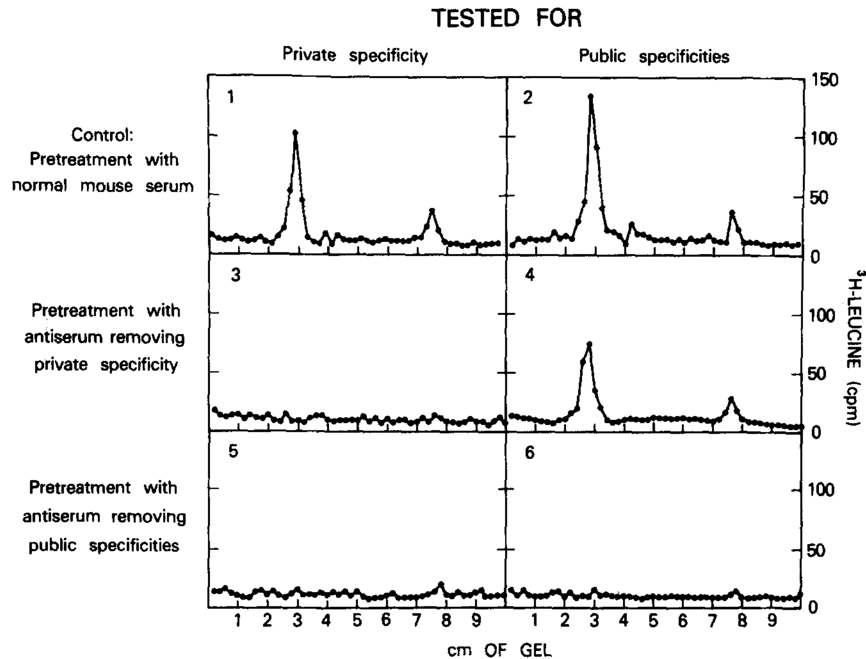

FIG. 2. Electrophoretic patterns from B10.AKM antigen sequentially precipitated with antisera to private and public *H-2* specificities. ^{3}H-leucine-labeled antigen was pretreated by precipitation with the reagent indicated on the left and the supernates from this first precipitation step were then tested for residual reactivity with the same or alternate reagent. The test precipitates were solubilized with SDS and subjected to disc electrophoresis on 10% polyacrylamide gels. Using this gel system, the *H-2* molecules migrate to about 3 cm, indicating a mol wt of 45,000 daltons by comparison to the migration of ^{14}C-labeled IgG heavy and light chains in comparable gels. A small nonspecific peak appears at the running front of the gels (about 7^1/$_2$ cm). The top panels show the *H-2D* molecules precipitated by antisera to private (panel 1) and public (panel 2) specificities. The middle panels show that while pretreatment with the antiserum to the private specificity removes all molecules bearing the private specificity (panel 3), the antiserum to public specificities still precipitates some molecules of the same molecular weight (panel 4). In contrast, the bottom panels show that pretreatment with the antiserum to public specificities removes molecules bearing public (panel 6) and private (panel 5) specificities. Therefore, there are two distinct classes of molecules associated with the *D* end of B10.AKM, those that react with both the antisera (panel 1) and those that react only with the antisera to public specificities (panel 4).

tant removal of molecules positive for the private specificity (panels 5 and 6), indicating that all of the molecules that expressed the private antigen of the D^q region also expressed at least one of the public antigens. However, the complete removal of molecules positive for the private specificity (panel 3) did not remove all molecules reactive with the antiserum to public specificities (panel 4), indicating that there is a population of molecules which reacted with the antiserum to public but not private *H-2* specificities. Therefore, there must be at least two molecules defined by these antisera, both of similar size (45,000 daltons) by migration in the SDS-PAGE gels.

Co-precipitation patterns identical with those shown in Fig. 2 were obtained

in two other strains. Antigen preparations from B10.A and A.AL (Fig. 1) were tested using antiserum no. 4 and no. 2 or no. 3 (Table I). These combinations are specific for the *D* region which originated in the $H\text{-}2^d$ haplotype (D^d). The D^d and D^q regions share public specificities 6, 27, 28, 29, which are detected by antisera no. 2 and no. 3. Therefore, using the same antiserum to public specificities, multiple gene products have been demonstrated for both the D^d and D^q regions.

We envisage two possible interpretations of these results. First, there could be two categories of *H-2D* region-associated molecules, one bearing private and public alloantigenic specificities and the other only public specificities, as has been suggested previously on the basis of co-capping studies (5). The data could also be explained if antibodies specific for another gene product were present in the antisera to public and not in the antisera to private specificities. Our experimental results require that such molecules, determined by this putative additional gene, be present in significant quantities in our radiolabeled spleen cell antigen preparation and be of similar molecular weight to that of the *H-2* molecules. Our choice of antisera and mouse strain combinations precludes the possibility that such additional antibodies were reacting with the gene products of non-*H-2* loci or of *H-2* loci to the left of *H-2D* (Fig. 1). Furthermore, the pattern of reactivity of these hypothesized additional antibodies is not correlated with known Qa[1] or Tla (7) types. Therefore, it is most probable that such antibodies detect the products of a second gene, closely linked to the *D* region gene. Indeed, both alternative explanations imply that there is more than one *H-2* product determined by genes in or adjacent to the *D* region. In man, a third segregant series of HLA antigens (HLA-C) has recently been defined serologically (8), by co-capping (9) and chemically (10). It is intriguing to speculate that the second *D* region product may be the murine homologue of HLA-C.

Summary

Anti-H-2 reagents have been tested on solubilized spleen cell preparations in combinations expected to be specific for *D* region products. Two different types of molecules were detected. One showed the expected reactivity with both antisera to private and antisera to public specificities. However, an additional molecule was detected which reacted only with antisera to public specificities. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis migration patterns indicated that both products have a similar molecular size of approximately 45,000 daltons. The data therefore present chemical evidence for the existence of a third *H-2*-associated gene product of 45,000 mol wt in addition to the classical *H-2K* and *H-2D* antigens.

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