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STRUCTURAL CHARACTERIZATION OF H-2L ALLOANTIGENS

I. Complete Tryptic Peptide Map Analysis of H-2L^{a}[1]

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The complete tryptic peptide structure of the H-2L^{a} antigen is examined in this investigation by high-resolution, ion-exchange chromatography. H-2L^{a} was isolated by two different alloantisera: *k* × *r* anti-*h2* and *dm2* anti-*d*. An effective immunoadsorbant preclearing method for eliminating non-H-2, co-precipitating proteins is introduced, which greatly improved the specificity of these relatively weak alloantisera. The separate maps of the Arg-labeled and Lys-labeled tryptic peptides of H-2L^{a} are compared with the corresponding tryptic peptide maps of the H-2D^{a} and H-2K^{a} antigens isolated from RADA1 tumor cells. The peptide homology between any two of these antigens is only 30 to 40%, and it is concluded from this relatively extensive peptide map diversity that the primary amino acid sequences of all three of these antigens are probably less than 90% homologous to each other. Thus, H-2L^{a} is structurally as unrelated to H-2D^{a} or H-2K^{a} as any two H-2K and/or H-2D antigens are generally found to be unrelated (except for some of the mutant/parent H-2 antigen pairs). These results strongly support the proposal that a separate, MHC-linked *H-2L* genetic region encodes H-2L antigens; and they are consistent with the possibility that H-2L antigens comprise a third, structurally polymorphic class of alloantigens analogous to the highly polymorphic H-2K and H-2D classes of alloantigens. These results also formally demonstrate for the first time that *k* × *r* anti-*h2* and *dm2* anti-*d* both react with H-2L^{a} even though they recognize different sets of alloantigenic determinants.

As reviewed recently by Demant and Neauport-Sautes (1), a substantial body of evidence is consistent with the original proposal by Demant *et al.* (2) that there exists a third major histocompatibility complex- (MHC)[3] linked genetic region, designated *H-2L* (3), which is separate from the *H-2K* and *H-2D* regions of the MHC (4) but which encodes H-2L antigens having serologic, structural, and immunologic properties related to those of the H-2K and H-2D antigens themselves. Serologically, H-2L antigens are particularly unique, in that they apparently do not bear private antigenic determinants, in contrast to H-2K and H-2D antigens, but they do appear to share MHC-associated public antigenic determinants with H-2K and H-2D antigens (5). Specifically, H-2K, H-2D, and H-2L antigens all (6) appear to bear serologically defined public determinants of one or the other—but not both (2)—of two complex families of antigenic determinants—H-2.28 (7) or H-2.1 (8)—depending on the MHC halotype (4). Structurally, H-2L antigens are H-2-like, in that they are 45,000-dalton (9, 10), cell surface (5) glycoproteins apparently associated with a low-m.w. polypeptide (11; unpublished data cited in Reference 2) like β_2-microglobulin (β_2m), which is noncovalently associated with H-2 antigens in general (12). Immunologically, H-2L antigens are also H-2-like, in that they appear to serve as recognition structures for allogeneic and viral-specific cytotoxic killer T cells (13–15), although certain important differences have been noted with the apparent lack of involvement of H-2L in minor-histocompatibility-antigen-specific (13), certain viral-specific (13), and TNP-specific (16) immune responses. In spite of the more limited involvement of H-2L antigens in these various reactions, they do still appear to be "authentic" major histocompatibility antigens in the sense that allogeneic responses directed against them are highly specific and are not restricted by the accompanying H-2K and H-2D antigens on the same cells, as are the immune responses to other cell surface, non-H-2 antigens.

One intriguing property of H-2L antigens yet to be completely resolved is their genetic origin. Although prior data are entirely consistent with the possibility that *H-2L* itself is the structural gene for H-2L antigens, as Demant and Neauport-Sautes (1) document so persuasively, other possible interpretations of the data have still been possible, though admittedly less likely. For example, H-2L antigens could conceivably be modified H-2D antigens: some H-2D antigens could be post-translationally modified by an enzyme encoded by H-2L and thereby be antigenically altered, in a fashion somewhat analogous to the variations found with blood group antigens. The basic difficulty in resolving this point has been that no recombinant mice have as yet been identified in which the *H-2L* region is genetically—and, hence, functionally—separated from the *H-2D* region. However, a recent investigation from this laboratory by Sears and Polizzi (11) provides the strongest evidence to date that H-

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[1] This work was supported by Grant CA 24433-02, awarded by the National Cancer Institute, Department of Health, Education and Welfare.
[2] Recipient of a University of California President's Undergraduate Fellowship.
[3] Abbreviations used in this paper: MHC, major histocompatibility complex; β_2m, β_2-microglobulin; MuLV, murine leukemia virus; SWNMS, Swiss Webster normal mouse serum; NP-40, Nonidet P-40 detergent; SDS/PAGE, sodium dodecylsulfate/polyacrylamide gel electrophoresis; LcH, *Lens culinaris* hemagglutinin; PAH-agarose, polyacrylic hydrazideagarose; TSNP-40, 0.01 M Tris-HCl, 0.15 M NaCl, 0.25% NP-40, pH 7.4; TPCK, L-*p*-tosylamino-2-phenylethyl chloromethyl ketone.

2L antigens are indeed encoded by a locus that is distinct from *H-2D*: in comparing the tryptic peptide maps of H-2L^a and H-2D^a antigens, it was found that the tryptic peptide differences—and, hence, the primary amino acid sequence differences—between these two antigens were so extensive as to strongly implicate separate coding regions for these two antigens.

The purpose of the present investigation is to extend the initial observations of Sears and Polizzi (11) concerning the structure of the H-2L^a antigen. Although only the Arg-containing tryptic peptides were compared between the H-2L^a and H-2D^a antigens in the previous study, the Lys-containing tryptic peptides are now included in the comparison as well as the tryptic peptides of the H-2K^a antigen. Also, two different anti-H-2L antisera—$k \times r$ anti-*h2* and *dm2* anti-*d*—are compared in this study. Finally, a problem noted in the preceding investigation concerning the possibility that the anti-H-2 precipitates are contaminated with non-H-2 proteins is resolved in this study. An immunoadsorbent method for pretreating the RADA1 detergent extracts is described here that effectively eliminates co-precipitating, non-H-2 proteins.

MATERIALS AND METHODS

Mice. All homozygous inbred mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) except for the BALB/c-*H-2*^{dm2} mice, which were generously provided by Dr. H. I. Kohn (Harvard Medical School, Boston, Massachusetts). F_1's were provided by Dr. S. G. Nathenson (Albert Einstein College of Medicine, Bronx, New York). Swiss Webster outbred mice were obtained from Simonsen Laboratories, Inc., Gilroy, Calif.

Antisera. Anti-H-2.4 (private antigenic specificity of H-2D^a or H-2D^d): (B6 × C3H)F_1 anti-B10.A or $b \times k$ anti-*a*. Anti-H-2.23 (private antigenic specificity of H-2K^a or H-2K^k): C3H.0L anti-C3H or *01* anti-*k*. Anti-H-2.28 (public antigenic specificities (7) of H-2D^a, D^b, L^a, L^b (?) as well as the irrelevant private H-2.2 antigenic specificity of H-2D^b): (B10.BR × LP.RIII)F_1 anti-B10.A(2R) or $k \times r$ anti-*h2*, which is similar to the NIH contract antiserum D28b used in the previous investigation by Sears and Polizzi (11) except that thymocytes were not included in the production of the anti-H-2.28 antiserum here. Anti-H-2.64,65 (public antigenic specificities (17) of H-2L^a): BALB/c-*H-2*^{dm2} anti-BALB/c-*H-2*^{d} or *dm2* anti-*d*. Swiss Webster normal mouse serum (SWNMS) was obtained from unimmunized mice. The alloantisera used in this study were generously provided by Dr. S. G. Nathenson, and they were raised by hyperimmunizing mice with repeated i.p. injections of lymphocytes and splenocytes except that *dm2* anti-*d* was initiated by dorsal skin grafts.

Cells and the preparation of radiolabeled H-2 antigens. Two cell types were used in this investigation for the production of radiolabeled H-2 antigens: splenocytes from B10.A mice, and RADA1 lymphoblastoid cells derived from a radiation-induced leukemia in an A (*H-2*^{a}) strain mouse (18) and grown from an inoculum generously donated by Dr. E. A. Boyse, Memorial Sloan-Kettering Cancer Center, New York, New York. RADA1 cells were maintained in the authors' laboratory as described previously (11).

Radioactive amino acids were incorporated into growing RADA1 cells in culture as described before (11). The labeling medium was specially prepared Dulbecco's modified Eagle's medium (GIBCO)—lacking in either Arg, Lys, or Met—which was supplemented with fresh Gln, 10% (v/v) FCS, and one of the following: ^{3}H-L-Arg or ^{3}H-L-Lys at 0.1 mCi/ml (15 to 30 Ci/mmole, New England Nuclear); ^{14}C-L-Arg or ^{14}C-L-Lys at 0.01 mCi/ml (270 mCi/mmole, New England Nuclear); or ^{35}S-Met at 0.1 mCi/ml (>400 Ci/mmole, New England Nuclear). At the end of 8 hr in the labeling medium, the cell viability (always >90%) was checked by the trypan blue dye (GIBCO) exclusion test (19). The labeled cells were washed and lysed with Nonidet P-40 (NP-40, Shell Chemical Co.) as described before (11).

B10.A-*H-2*^{a} splenocytes were radiolabeled and lysed in an analogous fashion with variations in the labeling medium and procedure described previously (20).

Immunoprecipitation procedures. NP-40-solubilized H-2 antigens were isolated by immunoprecipitation with alloantibody where antibody-H-2 immune complexes were precipitated either by goat anti-mouse-Ig antiserum, for tryptic peptide maps; or by heat-inactivated, formaldehyde-cross-linked SaCI (*Staphylococcus aureus* Cowan I; 21) for sodium dodecylsulfate (SDS) gels, as described previously (11, 22).

Discontinuous polyacrylamide gel electrophoresis in SDS (SDS/PAGE). The alkaline discontinuous SDS/PAGE system of Laemmli and Favre (23) was employed with modifications for gradient polyacrylamide resolving slab gels (24). Samples were reduced with β-mercaptoethanol, prepared as described previously (11) and electrophoresed on discontinuous gels. Slab gels were made with 4% (w/v) acrylamide, 0.11% (w/v) bis-acrylamide stacking gels (pH 6.8), and with linear gradient 5 to 18% (w/v) acrylamide, 0.27% (w/v) bis-acrylamide resolving gels (pH 8.8). Cylindrical gels were made similarly, except the resolving gel was 10% (w/v) acrylamide. Slab gels were dried for fluorography (25). Cylindrical gels were sliced and counted as described previously (11).

Lectin affinity chromatography. Lens culinaris hemagglutinin (LcH) was extracted from lentil beans and covalently coupled to cyanogen-bromide-activated Sepharose 4B (Pharmacia Fine Chemicals) to make LcH-Sepharose lectin affinity columns as described by Hayman and Crumpton (26). Before immunoprecipitation, all cell detergent extracts were chromatographed on LcH-Sepharose as described by Freed *et al.* (22). The material fractionating in the 0.1 M α-methyl-D-mannoside, TSNP-40 (see Abbreviations) eluate (approximately 3% of the starting material) is referred to as the "glycoprotein" fraction, and it contained the H-2 antigens characterized in this investigation.

Immunoadsorbant chromatography. The glycoprotein fractions of RADA1 cell detergent extracts were precleared of the H-2.4 antigen by chromatography on immunoadsorbants prepared with anti-H-2.4 alloantiserum covalently coupled to glutaraldehyde-activated polyacrylic hydrazide-agarose (PAH-agarose, Miles Biochemicals), according to the procedure described by Wilchek and Miron (27). The adsorbed H-2.4 antigen was eluted from the immunoadsorbant column with 1 M NH_4OH, 0.25% NP-40 (pH 11.0), and the column was then recycled by gradually reequilibrating it with TSNP-40 buffer. As will be described in more detail elsewhere,[4] it was found that over 95% of the H-2.4 antigen in the glycoprotein fraction from 5×10^7 RADA1 cells could be eliminated by four successive passages through an immunoadsorbant prepared with 2 ml of anti-H-2.4 antiserum, recycled after each pass.

Except where noted, after LcH-Sepharose chromatography, glycoprotein extracts of RADA1 were chromatographed on immunoadsorbants of SWNMS coupled to PAH-agarose to remove non-H-2 antigens reactive with the alloantisera, as discussed below.

Gel filtration in SDS. As described elsewhere (11, 22), im-

[4] Manuscript in preparation: D. W. Sears. 1980. Rapid purification of antigenically active H-2 antigens by re-usable alloimmunoadsorbants.

munoprecipitates for peptide maps were reduced with dithiothreitol (DTT), alkylated with iodoacetamide (IAAm), and chromatographed on Bio-Gel A-1.5m (200-400 mesh, Bio-Rad Laboratories). A pool was made of the radioactive antigen eluting in the 45,000 m.w. range as determined by the position of the major radioactivity peak relative to the heavy (H) and light (L) chain peaks (monitored by their absorbance at 280 nm) of the mouse and goat Ig in the original immunoprecipitate. The column served the purpose of separating H-2 glycoproteins from other high and low m.w. proteins in the precipitate, such as β_2m. The pooled antigen was precipitated by TCA and washed in preparation for trypsin digestion (22).

Tryptic peptide mapping by ion-exchange chromatography. The washed TCA precipitate of the protein pooled from the SDS gel filtation column was digested by TPCK[3]-trypsin (Worthington Biochemical Co.) and chromatographed on a cation-exchange resin (Chromobeads Type P, Technicon Chemicals) eluted with a linear pyridine/acetate pH gradient (pH 3.13 to 5.00), all as described by Brown *et al.* (20). After completion of the gradient (~600 ml), each of the following buffers were passed through the column in the order given: 60 ml, 2 M pyr., pH 5.0; 30 ml, 2 M pyr., pH 5.5; 30 ml, 4 M pyr., pH 5.5; 30 ml, 8 M pyr., pH 7.4. Fractions of 3 ml were collected and heated to dryness.

Liquid scintillation counting procedures. Dried tryptic peptide map samples were counted in 0.1 ml H_2O plus 5 ml Aquasol (New England Nuclear). Immunoprecipitates were dissolved in 0.5 ml Protosol (New England Nuclear) and counted in 4.5 ml toluene ("Scintrex" grade, J. T. Baker) containing 0.4% (w/v) Omnifluor (New England Nuclear).

RESULTS

Immunoadsorbant preclearing of non-H-2 antigens precipitated by anti-H-2 alloantisera. All the anti-H-2 alloantisera used in this investigation were found to precipitate several antigens, apparently unrelated to H-2, from NP-40 extracts of radiolabeled RADA1 tumor cells, as illustrated in Figure 1. Lane 3 in this fluorogram shows the electrophoretic pattern of an anti-H-2.4 immunoprecipitate of ^{35}S-Met-labeled RADA1 glycoprotein (eluted from LcH-Sepharose), which was electrophoresed on a discontinuous, 5 to 18% linear gradient gel in SDS. In addition to the H-2D^a (H-2.4) antigen and β_2m, several other relatively minor bands are visible in this precipitate, the most intense of these being marked by open arrows at the right. The m.w. of the various components in this gel have been roughly estimated from a semi-log plot of their mobilities relative to the heavy (H) and light (L) chain immunoglobulin markers shown in lane 1.

The non-H-2 components in the anti-H-2 immunoprecipitates were found to migrate in SDS polyacrylamide gels in parallel with protein components precipitated (from the same radiolabeled antigen preparations) by normal mouse serum, such as preimmune (B10.BR × LP.RIII)F_1 serum (data not shown) or unimmunized SWNMS as shown in lane 4 of Figure 1. In addition to the bands co-migrating with the non-H-2 bands noted above, one component in lane 4, being 45,000 in m.w., co-migrates with H-2D^a. It is thought that most of these non-H-2 components are murine leukemia virus- (MuLV) related, because 1) RADA1 tumor cells are known to express MuLV antigens (28); 2) mouse antisera generally contain anti-MuLV antibodies (19); and 3) the basic pattern (relative mobilities in SDS gels) of the non-H-2 bands observed in the immunoprecipitates approximates that found for MuLV antigens in general (30). However, it is also likely that some actin is present in the immunoprecipitates as well (31, 32).

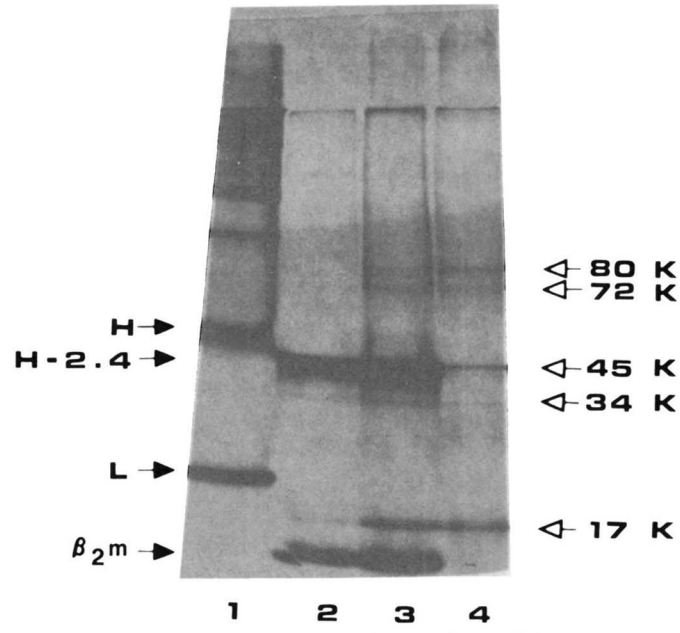

Figure 1. Fluorogram of a discontinuous, 5 to 18% linear gradient, SDS polyacrylamide slab gel of anti-H-2.4 and SWNMS immunoprecipitates of ^{35}S-Met-labeled, RADA1 glycoprotein. Electrophoresed in lane 3 is a direct anti-H-2.4 precipitate; in lane 4 is a direct SWNMS precipitate; in lane 2 is an anti-H-2.4 precipitate of the supernatant remaining after SWNMS precipitation; and in lane 1 is reduced, ^{14}C-labeled, mouse MPC 11 immunoglobulin included as a m.w. standard. The positions of H-2D^a (H-2.4), β_2m and the immunoglobulin heavy (H) and light (L) chains are indicated at the left by solid arrows. The open arrows at the right mark the positions of other prominent bands also observed in the immunoprecipitates. The m.w. (e.g., 70 K = 70,000 daltons) of these bands were estimated by a semi-log plot of their mobilities relative to the mobilities of H (55 K), H-2D^a (45 K), L (22.5 K), and β_2m (11.5 K).

Although the SDS gel filtration step (before trypsin digestion of the samples for peptide maps) served to separate, and thereby eliminate, most of the high and low m.w. extraneous proteins from the 45,000-dalton H-2 antigens, this would not be true of the co-migrating component such as that observed in the SWNMS precipitate or actin that might also be present but obscured in the anti-H-2 precipitates. Assuming the intensity ratio of the 80 K (or 72 K) component relative to the 45 K component is the same in the anti-H-2.4 precipitate as it is in the SWNMS precipitate in Figure 1, contamination of the H-2D^a antigen by the 45 K "impurity" would not be expected to be significant, since the intensity of the H-2.4 antigen band greatly overshadows that of the parallel 45 K band. However, the levels of contamination of the anti-H-2.28 precipitates by the 45 K impurity might be significant, because approximately 10 times more radiolabeled extract and 10 times more alloantiserum were required to isolate the H-2L^a antigen with a radioactivity level comparable to that of the H-2D^a antigen.

Thus, in order to reduce the possibility of contamination, most glycoprotein pools from the LcH-Sepharose column were chromatographed through an immunoadsorbent prepared from SWNMS covalently coupled to polyacrylic hydrazide-agarose, as described in *Materials and Methods.* The effectiveness of SWNMS preclearing is illustrated by the sample electrophoresed in lane 2 of Figure 1. Here, the radiolabeled glycoprotein pool was pre-precipitated by SWNMS before precipitation by

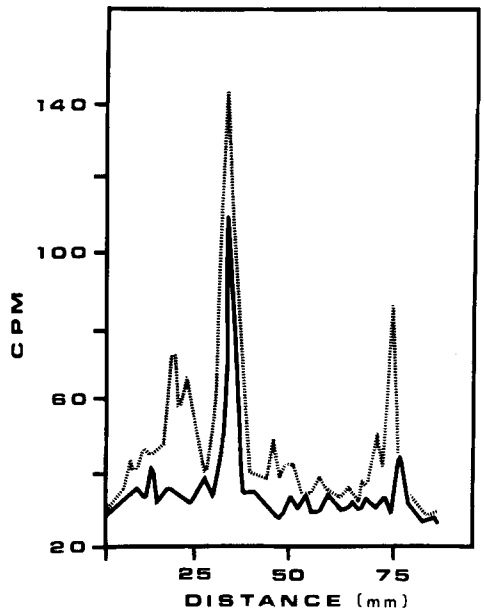

Figure 2. Discontinuous SDS/PAGE of anti-H-2L (*dm2* anti-*d*) immunoprecipitates of RADA1 extracts before and after immunoadsorbent chromatography. ^{3}H-Arg-RADA1 glycoprotein was precipitated with anti-H-2.64,65 before (-----) and after (——) immunoadsorbent pretreatment as described in the text. The precipitates were electrophoresed separately on discontinuous cylindrical gels and their profiles are superimposed here. The major peak at 34 mm corresponds to m.w. of 45,000 daltons relative to immunoglobulin heavy and light chain markers (not shown). A similar result was obtained with the $k \times r$ anti-*h2* anti-H-2.28 antiserum (data not shown).

the anti-H-2.4 antiserum, and it is evident that the contaminating bands are now greatly diminished. The antigen preparations used for isolating H-2L^a were chromatographed through SWNMS and/or anti-H-2.4 immunoadsorbents before precipitation by anti-H-2L. These steps greatly diminished or completely eliminated the SWNMS-reactive antigens, including the 45 K component (data not shown). Figure 2 illustrates the improvement in the anti-H-2L immunoprecipitates resulting from immunoadsorbent pretreatment.[5]

Comparative arginine peptide maps of the H-2D^a antigens of RADA1 tumor cells and B10.A splenocytes. As an additional test that the H-2 antigens precipitated from RADA1 tumor cells were not significantly contaminated, the peptide maps of tumor and splenocyte H-2D^a antigens were compared. Figure 3 is the paired-label tryptic peptide map of the anti-H-2.4 reactive antigens isolated from ^{3}H-Arg-labeled B10.A splenocytes and ^{14}C-Arg-labeled RADA1 cells. In both cases, the antigens were precipitated directly from LcH-Sepharose glycoprotein pools without pretreatment by immunoadsorbents. The precipitates were then combined, reduced, heat denatured, alkylated, chromatographed in SDS, and finally precipitated by TCA before trypsin digestion, as described in *Materials and Methods.* The peptide profiles of the antigens from these two sources are very nearly identical, not only in the corresponding positions of the peaks but also in the relative sizes of the peaks. The most prominent differences between the profiles are a few minor peptide peaks (indicated by arrows) in the RADA1 H-2D^a map that do not have corresponding peaks in the B10.A H-2D^a map. These peaks most likely represent peptides from contaminating, co-migrating proteins rather than from the H-2D^a antigen itself, because 1) the magnitudes of these peaks have been found to vary with the size of the pool actually made from the SDS Bio-Gel column, as shown previously (11); and 2) these peaks are virtually absent in maps of the H-2D^a antigen isolated from precipitates of RADA1 glycoprotein extracts pre-chromatographed through SWNMS immunoadsorbants (see Fig. 4). It is concluded that the H-2D^a antigens isolated from RADA1 tumor cells and from B10.A splenocytes are probably structurally identical, there being no apparent differences in their peptide map profiles. A similar conclusion is reached concerning the H-2L^a antigen (see Fig. 7).

Comparative arginine peptide maps of H-2L^a and H-2D^a. Illustrated in Figure 4 is the paired-label peptide map of ^{3}H-Arg-H-2L^a and ^{14}C-Arg-H-2D^a precipitated by anti-H-2.28 and anti-H-2.4, respectively, from RADA1 NP-40 extracts chromatographed on LcH-Sepharose and on SWNMS immunoadsorbants, as described above. The ^{3}H-Arg-glycoprotein was also precycled through an anti-H-2.4 immunoadsorbant (until less than 5% of the anti-H-2.4-reactive antigen remained) before precipitation of H-2L^a by the anti-H-2.28 antiserum, which also reacts with H-2D^a, as shown previously (11). The two precipitates were combined and treated as outlined in the preceding section.

The Arg-peptide profiles of H-2L^a and H-2D^a are clearly different, there being only five coincident peaks (which co-elute at identical pH values) out of a total of 27 prominent peptide peaks for the two antigens—12 from H-2L^a and 15 from H-2D^a. This represents a peptide homology between the two antigens of approximately 37%, as discussed further below (see *Discussion*). Note that the H-2L^a map here is very similar but not quite identical to the Arg-peptide map of H-2L^a published previously (11). In the former study, detergent extracts of RADA1 were pre-chromatographed only through LcH-Sepharose and anti-H-2.4 immunoadsorbants but not SWNMS immunoadsorbants as they were in this study. The latter step appears to have resulted in the virtual elimination of three intermediate-sized peaks— at pH's 4.50, 4.59, and 4.74—that were previously identified with H-2L^a but that probably represent peptides from other contaminating, non-H-2 proteins. Note also that the H-2D^a map in Figure 4 more closely resembles the splenocyte H-2D^a map shown in Figure 3 than it does the tumor cell H-2D^a map shown in the same figure. Presumably, the SWNMS immunoadsorbant has further reduced the level of contaminating, non-H-2 protein in this case also.

Comparative lysine tryptic peptide maps of H-2L^a and H-2D^a. Figure 5 shows the paired-label tryptic map of the Lys-containing peptides of H-2L^a isolated from ^{3}H-Lys-RADA1 and of H-2D^a isolated from ^{14}C-Lys-RADA1. The antigen preparations were chromatographed through immunoadsorbants, and the precipitates were treated identically to the samples analyzed in Figure 4. As was the case with the Arg maps, the Lys maps for the two antigens are distinct. Of the 10 major peptide peaks from the two molecules—five peaks from each antigen—only one peak from each co-elutes at an identical pH value, indicating that there is approximately a 20% peptide homology between the two molecules, as discussed below.

Peptide map comparison between H-2L^a and H-2K^a anti-

[5] Subsequent, unpublished studies by S. Y. and D. S. have shown that the peptide maps of the 45 K, SWNMS-reactive antigen and the 72-80 K antigens in the alloantisera precipitates are very complex and are not obviously related to the peptide maps of either the H-2L^a or H-2D^a antigen. The 72-80 K contaminants, and probably the 45K contaminant, are present to a significant but not a predominant extent in straight anti-H-2L^a immunoprecipitates of RADA1 glycoprotein. Their removal by the SWNMS or anti-H-2.4 immunoadsorbants before immunoprecipitation considerably reduces the background complexity (or "noise") of the H-2L^a maps.

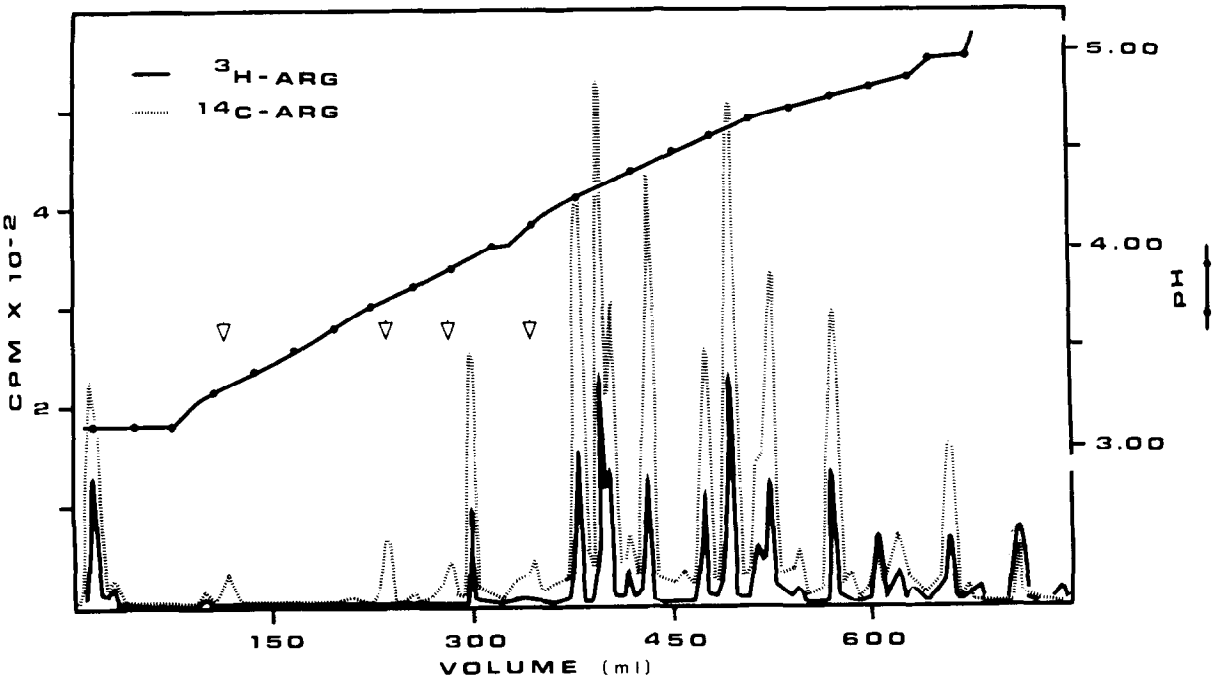

Figure 3. Comparative tryptic Arg-peptide maps of H-2D^a antigens from B10.A splenocytes and RADA1 tumor cells. The 45,000 dalton H-2D^a antigens immunoprecipitated from ^{3}H-Arg-B10.A and ^{14}C-Arg-RADA1 extracts by anti-H-2.4 were isolated together by SDS gel filtration on a high resolution, ion-exchange column eluted with a pyridine/acetate pH gradient (●——●) as described in *Materials and Methods.* The resulting peptide map chromatogram shows the ^{14}C-Arg (-----) narrow cpm and ^{3}H-Arg (——) narrow cpm (corrected for ^{14}C-spill) plotted *vs* the elution volume. The arrows point to regions of significant, but relatively minor, peptide differences between the two maps.

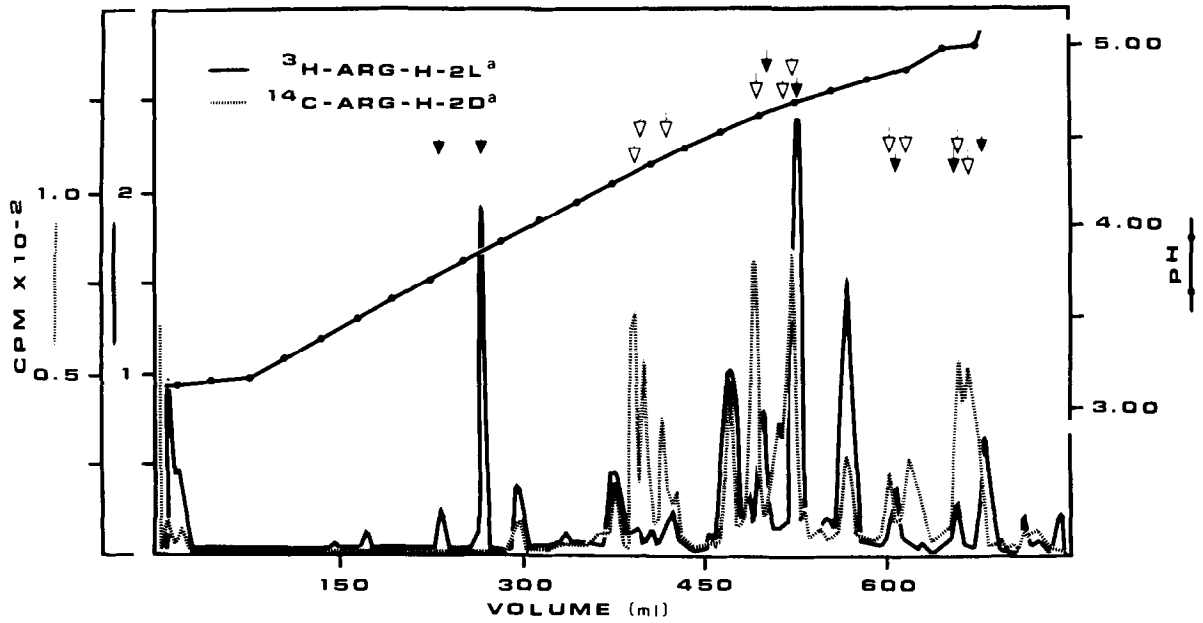

Figure 4. Comparative tryptic Arg-peptide maps of H-2L^a and H-2D^a. The 45,000-dalton ^{3}H-Arg-H-2L^a and ^{14}C-Arg-H-2D^a antigens immunoprecipitated from RADA1 glycoprotein extracts by anti-H-2.28 and anti-H-2.4, respectively, were isolated together for tryptic peptide map analysis as summarized in the legend of Figure 3. The resulting peptide map chromatogram shows the ^{14}C-Arg (-----) narrow cpm and ^{3}H-Arg (——) narrow cpm (corrected for ^{14}C-spill) plotted *vs* the elution volume. The solid and open arrows identify peaks exclusively associated with H-2L^a and H-2D^a, respectively. The remaining peaks of the two antigens are coincident.

gens. The tryptic Arg- and Lys-peptide profiles of the H-2L^a antigen were also compared with those of the H-2K^a antigen isolated from RADA1 glycoprotein extracts by alloantiserum directed against its H-2.23 private specificity. Table I summarizes the results of the tryptic peptide map comparisons between H-2L^a, H-2K^a, and H-2D^a. It is found that 40% of the major tryptic peptide peaks from H-2L^a and H-2K^a co-elute at identical pH values. A similar percentage (42%) of co-eluting tryptic peptide peaks is also found in the comparison of H-2D^a with H-2K^a.

Peptide map comparison between antigens precipitated by k × r anti-h2 and dm2 anti-d. Compared in Figure 6 are the tryptic peptide maps of the 45,000-dalton antigens immunoprecipitated by the *k* × *r* anti-*h2* (6A) and *dm2* anti-*d* (6B) alloantisera, both reacted with NP-40 detergent, glycoprotein extracts of RADA1 tumor cells internally labeled with ^{3}H-Arg.

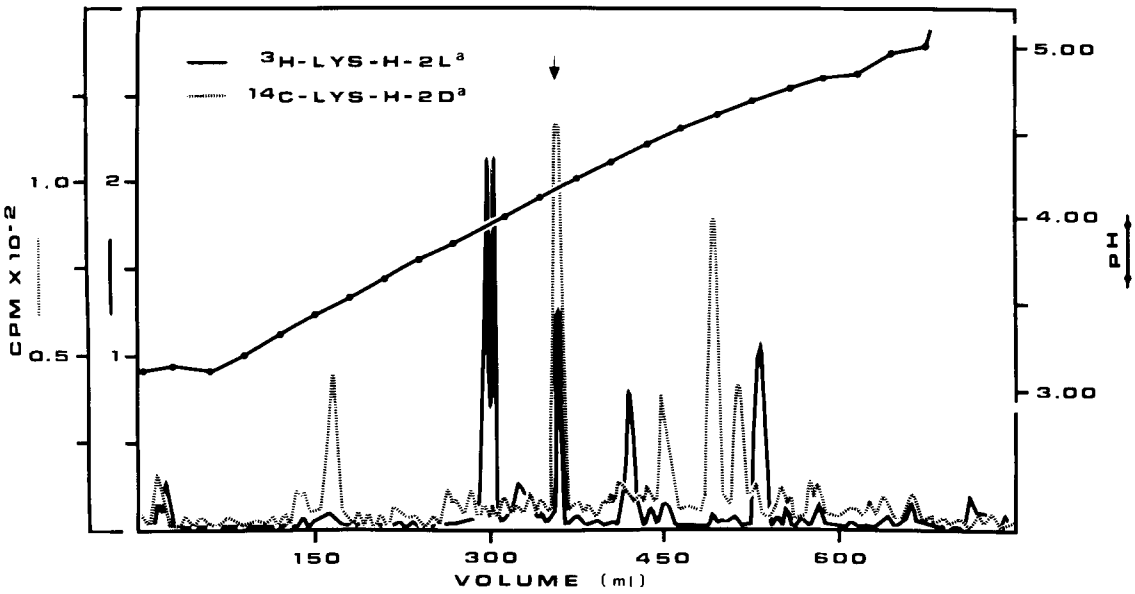

Figure 5. Comparative tryptic Lys-peptide maps of H-2L^a and H-2D^a. The 45,000-dalton ^{3}H-Lys-H-2L^a and ^{14}C-Lys-H-2D^a antigens immunoprecipitated from RADA1 glycoprotein extracts by anti-H-2.28 and anti-H-2.4, respectively, were isolated together for tryptic peptide map analysis as summarized in the legend of Figure 3. The resulting peptide map chromatogram shows the ^{14}C-Lys (-----) narrow cpm and ^{3}H-Lys (——) narrow cpm (corrected for ^{14}C-spill) plotted *vs* the elution volume. The solid arrow points to the only coincident peaks of the two antigens.

TABLE I

Summary of tryptic peptide map comparisons between H-2L^a, H-2D^a, and H-2K^a

Labeled Amino Acid	No. of Major Peaks			Coincident Peaks		
	H-2L^a	H-2D^a	H-2K^a	H-2L^a: H-2D^a	H-2L^a: H-2K^a	H-2D^a: H-2K^a
					%	
Arg	12	15	16	37	55	44
Lys	5	5	11	20	12	38
Arg + Lys	17	20	27	32	40	42

The pH gradients of the two maps are virtually identical at all points, and the maps can therefore be compared directly, since they are in complete register with one another.[6] Clearly, the overall profiles of the tryptic peptides are very similar in the two maps, although certain qualitative and quantitative differences are noted. Every single major peak in the $k \times r$ anti-*h2* map is paralleled in the corresponding *dm2* anti-*d* map. Several relatively minor and intermediate-sized peaks are also evident in the latter but not in the former map. Quantitatively, the sizes of the peaks in relation to each other are also comparable in the two maps except for the exceptionally large peak at pH 3.87 in the $k \times r$ anti-*h2* map. However, other $k \times r$ anti-*h2* peptide maps (data not shown) show that this peak and several of the trailing peaks at the end of the map all vary considerably in size compared with the rest of the peaks in the map. Thus, it appears that variations in these peaks are probably not significant indicators of structural differences. Because a dominant and corresponding profile of peptides runs in parallel through both of these two maps, it is concluded (see *Discussion*) that both antisera react predominantly with the same antigen, the H-2L^a antigen.

The data in Figure 7 support this conclusion. Shown here are the ^{3}H-Lys-peptide profiles of H-2^a antigens precipitated by these two antisera. An important distinction between the *dm2* anti-*d* map in Figure 7*B* compared with the corresponding map in Figure 6*B* is that the *dm2* anti-*d* antiserum was reacted with glycoprotein from a B10.A splenocyte extract rather than a RADA1 tumor cell extract. As before, a dominant and corresponding profile is found to run in parallel through both maps. Additional peaks are also evident in the *dm2* anti-*d* map, but these probably correspond to non-H-2 proteins, since the very large immunoprecipitate for this map exhibited a substantial 70–80 K component on the SDS Bio-gel column (data not shown). It is concluded that the predominant antigen recognized by both antisera is the H-2L^a antigen and that the H-2L^a antigen isolated from B10.A splenocytes appears to be structurally identical to that isolated from RADA1 tumor cells.

DISCUSSION

The results of this investigation extend the structural analysis of the H-2L^a antigen initiated by Sears and Polizzi (11), who compared the tryptic peptide maps of the Arg-labeled peptides of the H-2L^a and the H-2D^a antigens of RADA1 tumor cells. Here, both the Arg- and Lys-labeled, tryptic peptides are compared between the H-2L^a, H-2D^a, and H-2K^a antigens. Because the alloantisera directed against these antigens were found to precipitate significant levels of other non-H-2 proteins (see Fig. 1), an immunoadsorbant preclearing step was developed in this study in order to remove these additional components from the RADA1,NP-40 extracts before immunoprecipitation. Immunoadsorbants of SWNMS covalently coupled to PAH-agarose

[6] Note that it was not possible to compare the *dm2* anti-*d*-reactive and $k \times r$ anti-*h2*-reactive antigens directly by simultaneously chromatographing them, as done previously in comparisons between H-2L^a and H-2D^a (11), because the anti-H-2L antisera were weak (titer <1/30) and the total amount of mouse and goat immunoglobulin in the large anti-H-2L precipitates would have exceeded the capacity of the ion-exchange column. However, the single-label comparisons presented here are considered to be extremely reliable, because the ion-exchange tryptic maps are found to be highly reproducible: 10 different tryptic peptide maps of the H-2D^a antigen have now been analyzed in the laboratory, and the only significant variations observed between any two of them have been attributed to variations in the amounts of co-precipitating, non-H-2D^a components. The largest variations occur at the end of the maps, and variable peaks have been found to increase with the amount of protein in the immunoprecipitate.

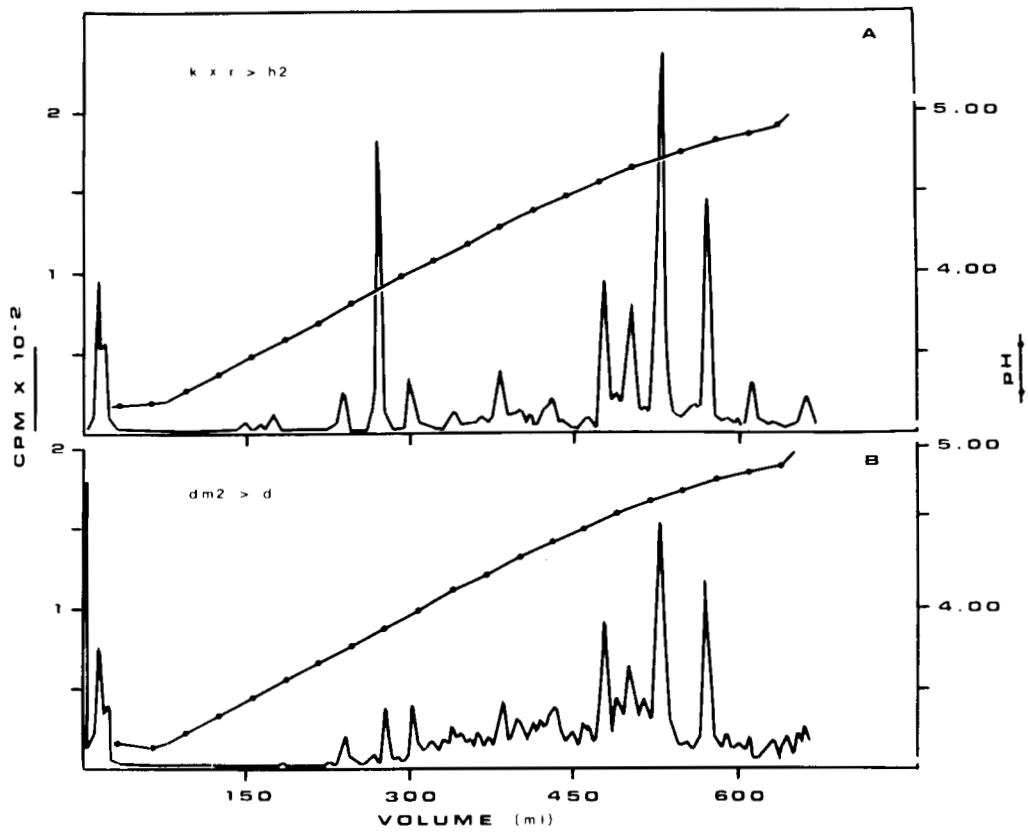

Figure 6. Tryptic Arg-peptide comparison of the H-2L^a antigens isolated by $k \times r$ anti-*h2* and *dm2* anti-*d* alloantisera. *A*, tryptic peptide map of the 45,000 dalton antigen immunoprecipitated by $k \times r$ anti-*h2* from a ^{3}H-Arg-RADA1 glycoprotein extract precleared of H-2D^a by anti-H-2.4 immunoadsorbent chromatography. *B*, tryptic peptide map of the 45,000-dalton antigen immunoprecipitated by *dm2* anti-*d* from a ^{3}H-Arg-RADA1 glycoprotein extract. The acid-soluble peptides in the two tryptic digests were fractionated on a high-resolution, ion-exchange column eluted with a pyridine/acetate pH gradient (●——●). The resulting radioactivity elution profiles (——) are shown relative to the elution volumes. *Note:* The maps are nearly in register because their respective pH gradients are nearly identical.

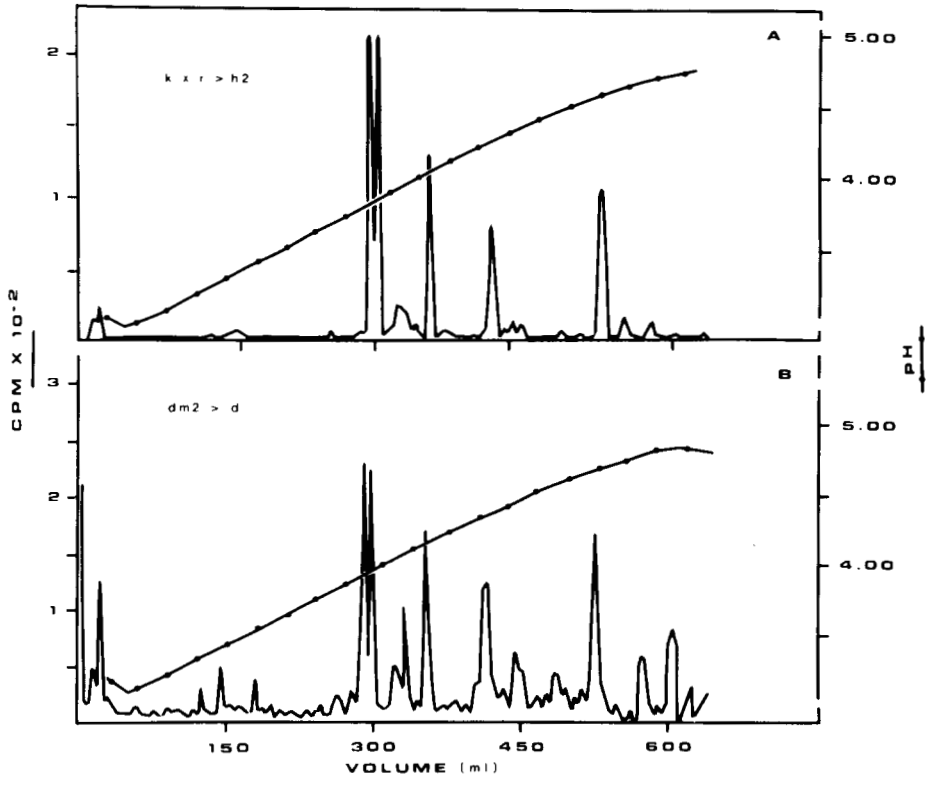

Figure 7. Tryptic Lys-peptide comparison of tumor cell and splenocyte H-2L^a antigens isolated by $k \times r$ anti-*h2* and *dm2* anti-*d* alloantisera, respectively. *A*, tryptic peptide map of the 45,000-dalton antigen immunoprecipitated by $k \times r$ anti-*h2* from a ^{3}H-Lys-RADA1 glycoprotein extract precleared of H-2D^a by anti-H-2.4 immunoadsorbent chromatography. *B*, tryptic peptide map of the 45,000-dalton antigen immunoprecipitated by *dm2* anti-*d* from a ^{3}H-Lys-B10.A glycoprotein extract. The pyridine/acetate pH gradients (●——●) and radioactivity profiles (——) are as described in the legend of Figure 6.

effectively eliminated these non-H-2 co-precipitating proteins, and this step resulted in the elimination of a few tryptic peptide peaks previously identified with the H-2L^a and H-2D^a antigens (see Figs. 3 and 4 and Reference 11). However, the basic conclusion reached earlier by Sears and Polizzi remains unchanged here: H-2L^a and H-2D^a appear to share approximately 37% of their tryptic Arg-peptides. If the Lys-labeled tryptic peptides are now included in this comparison, the overall tryptic peptide homology between these two antigens is approximately 32%. A similar result obtains in the comparison between H-2L^a and H-2K^a, in that there appears to be an overall tryptic peptide homology of 40% between these two antigens.[7]

The primary conclusion to emerge from this study is that the amino acid sequence of the H-2L^a antigen is significantly different from that of either the H-2D^a or the H-2K^a antigen. The extent of the peptide differences between all three of these antigens (~60% peptide heterogeneity; see Table I) is within the range of differences generally found for any two H-2K and/or H-2D antigens compared by the same technique (20). By drawing a comparison to the tryptic peptide differences and the corresponding primary amino acid sequence differences of immunoglobulin light chains, a peptide map homology of 20 to 40% is likely to correspond to a primary amino acid sequence homology of less than 90%, as discussed by Sears and Polizzi (11). This estimate agrees well with the partial amino acid sequence data thus far obtained for several H-2K and H-2D antigens themselves (33, 34); over 10% of the amino acids at corresponding positions in the sequences of any two of these molecules are found to differ (except in cases of certain mutant H-2 antigens compared with their respective parental antigens; 35). Thus, because the degrees of peptide difference between the three antigens studied here are approximately the same, we conclude that the sequences of H-2L^a, H-2D^a, and H-2K^a are all probably less than 90% related to one another. H-2L^a appears to be as structurally polymorphic in its relationship to H-2K^a and H-2D^a as H-2 antigens are in general.

Whether H-2L antigens as a class of molecules are as structurally polymorphic as the H-2K and H-2D antigen classes is still an open question. Like H-2K and H-2D antigens, H-2L antigens are serologically diverse (1, 7, 17) and are therefore probably structurally diverse. One way of examining this serologic diversity at the structural level is to compare the tryptic peptide composition of H-2L antigens isolated by different anti-H-2L antisera. In this study, two potentially unrelated anti-H-2L antisera—anti-H-2.28 and anti-H-2.64,65—were employed to isolate *H-2^a* glycoproteins. As shown in Figures 6 and 7, both antisera are found to react with the H-2L^a antigen as defined by the anti-H-2.28 antiserum, since a dominant and parallel peptide profile runs through both the Arg-labeled and Lys-labeled tryptic maps. (Although the corresponding maps are not completely identical, it is extremely likely that the differences arise from other co-precipitating, non-H-2 proteins, because in one case—Fig. 7B—an immunoadsorbent step was not applied, and in both cases exceptionally large amounts of protein were present in the immunoprecipitates due to the low titer of the *dm* anti-*d* antisera.) The importance of this observation is 2-fold: First, it establishes a consistent serologic and structural definition of the H-2L^a antigen and, second, it formally demonstrates that these two antisera, which have been used independently by different groups of investigators (10, 17) to identify and isolate H-2L antigens, both predominantly react with the same antigen of the *a* haplotype (with H-2D^a cleared).

A question that now comes to mind concerns the exact relationship between the antigenic specificities (or determinants, or epitopes) detected by the two antisera and the structure of H-2L^a itself. Are the same or different structural epitopes on H-2L^a recognized by the two antisera? According to the H-2 chart (4), the $k \times r$ anti-*h2* antiserum should detect antigen specificities H-2.27,28,29 of the *a* haplotype, and because H-2L molecules have been isolated with this antiserum, it has naturally been assumed that one or more members of this antigen family reside on the H-2L^a molecule. However, as discussed previously (11), it is still possible that this antiserum reacts with an undefined antigenic specificity. In fact, in a recent study where the H-2.64,65 antigenic specificities (as defined by *dm2* anti-*d*) were added to the H-2 chart (17), it was suggested that $k \times r$ anti-*h2* reacts with H-2.64 (although the *r* haplotype was not tested directly). Thus, even though the two antisera are quite different in origin, it is possible that they both recognize H-2L^a through the H-2.64 determinant as well as through other potentially unrelated determinants.

Whether the antigenic epitopes on the H-2L^a antigen are identical or are very similar to public epitopes on the H-2D^a antigen is also a matter of interest. As noted in the previous study from this laboratory (11), $k \times r$ anti-*h2* precipitates the H-2D^a antigen, although it is apparently cytotoxically silent toward this antigen on the cell surface. Thus, in contrast to suggestions by others (10, 17), we find this antiserum is not exclusively directed toward H-2L^a (or H-2L^d) antigens with H-2D^a (or H-2D^d) also present. If $k \times r$ anti-*h2* reacts exclusively with specificity H-2.64 on H-2L^a, then the determinants on the two molecules would have to be different (i.e., recognized by different antibodies in the antiserum). This is so because H-2.64 cannot be an H-2D^a determinant, since *dm2* anti-*d*, which defines this specificity, was raised in the *dm2* mutant, which has an H-2D^d antigen identical to the parent's (36). Data from other investigators (6) show that identical antigenic determinants of the H-2.28 family exist (in relevant haplotypes) on all three types of H-2 antigens (K, D, and L), including H-2L^a and H-2D^a. However, the anti-H-2.28 antiserum used in that study (*k* anti-*b*) may be different in its overall reactivity from $k \times r$ anti-*h2*. Clearly, further information about the fine aspects of the serologic reactivities of these antisera and the epitope structures and sequences of these antigens are necessary before a definitive relationship can be drawn between structure and serology.

A final point that should be reemphasized is the effectiveness of the normal mouse immunoadsorbents in eliminating the numerous non-H-2 proteins that co-precipitate with the H-2 antigens from RADA1 extracts. In those cases where the immunoadsorbents were not applied (e.g., Figs. 3 and 7*B*), variable additional peaks were frequently seen in the peptide maps. SWNMS antiserum was considerably more reactive against these non-H-2 contaminants than other normal mouse sera tested. As discussed above, SWNMS serum probably has a particularly high concentration of antibodies against MuLV-related antigens (this possibility is currently being tested),

[7] These estimates of peptide homology are only approximate. Some tryptic peptides are not included in these comparisons, such as those that are not soluble in the most acidic buffer (~pH 3.1) used to construct the pH gradient (~30 to 40% of the cpm of the tryptic digests were acid-insoluble) and those that do not adhere to the ion-exchange resin (~5 to 10% of the acid-soluble cpm passed directly through the ion-exchange column). Moreover, if the C-terminal amino acid is not Arg or Lys, the C-terminal peptide will not be identified by this method. Finally, in calculating the percentage peptide homology, it was assumed that each major peak in the peptide maps corresponds to just one peptide, an assumption that cannot always be true because the $^3H/^{14}C$ cpm ratios for peaks co-eluting at identical pH values are not constant in value for all coincident peaks.

thereby accounting for its effectiveness. The methods for preparing immunoadsorbents with serum are easy and rapid, as will be described in a future publication.[4] Because mouse antisera are generally contaminated with anti-MuLV antibodies (29), because actin reacts with immunoglobulin (31) and is found in immunoprecipitates of lymphocyte extracts (32), and because most studies of H-2 antigens continue to rely on immunoprecipitation reactions with such sera, the methods employed here may be of general value for improving the specificity of such reactions.

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Note added in proof. At the 1979 I.C.N.-U.C.L.A. Molecular and Cellular Biology Symposium in Keystone, Colorado, at which the first account of the work by Sears and Polizzi (11) was given, Rose *et al.* presented preliminary data indicating that the peptide maps of H-2L^d and H-2L^q are similar but not identical. More recently, Coligan *et al.* (1980. Proc. Natl. Acad. Sci., U.S.A., in press) have found by microsequencing that the partial primary amino acid sequence of the H-2L^d antigen is only 80% homologous to the corresponding sequences of H-2K^b, H-2D^d, and H-2K^d, thus confirming our prediction that the H-2D^a and H-2L^a, or H-2D^d and H-2L^d are less than 90% homologous in sequence.

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