ANALYSIS OF H-2 AND Ia MOLECULES BY TWO-DIMENSIONAL GEL ELECTROPHORESIS*

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The *H-2* gene complex, a cluster of tightly-linked genes on chromosome 17 of the mouse, controls a number of cellular properties important to the immune system (1). Genetic studies have mapped loci controlling some of these traits to regions within the complex, shown in Fig. 1.

The *H-2K* and *H-2D* loci determine the classical serologically-detectable alloantigens present on all cells in the adult mouse. 12 distinct sets of alleles, or haplotypes, exist for *H-2* among the inbred strains of mice, each marked by unique (private) antigenic specificities as well as by public specificities shared with other haplotypes (8). Between the *K* and the *D* regions lies the *I* region, which was defined initially by the existence of a series of *H-2*-linked immune response (Ir)[1] genes (9, 10). More recently, several laboratories have identified a series of lymphocyte alloantigens which also map to the *I*-region (11-15). The function of these *I*-region-associated (Ia) antigens is at present unclear. As has been summarized recently (16), Ia antigens seem to play a central role in mediating cellular interactions among T cells, B cells, and macrophages during immune responses. Conceivably, Ia antigens could represent the products of the *Ir* genes, exerting control over immune response capabilities at the level of cellular interactions.

Knowledge of the molecular structure of H-2 and Ia antigens has long been sought as a key to understanding the functions of these molecules and the complex genetics of the gene systems which control them. Analyses of proteins immunoprecipitated from detergent-solubilized preparations of radiolabeled cells have shown that *H-2* and *Ia* gene products are glycoproteins with mol wt of about 45,000 daltons and 25,000-35,000 daltons, respectively (17, 18). However, the existing technology has been unable to resolve the heterogeneous populations of H-2 and Ia obtained from lymphocytes into their individual molecular species. The one-dimensional sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoretic techniques most commonly used to characterize the immunoprecipitated proteins do not resolve different H-2 or Ia molecules which are similar in size.

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CSW, C3H.SW; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; IEF, isoelectrofocusing; Ia, *I*-region associated; Ir, immune response; NMS, normal mouse serum; NP-40, Non-Idet-P-40; PBS, phosphate-buffered saline; SaC, *Staphylococcus aureus*, Cowan I strain; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; 1-D, one-dimensional; 2-D, two-dimensional.
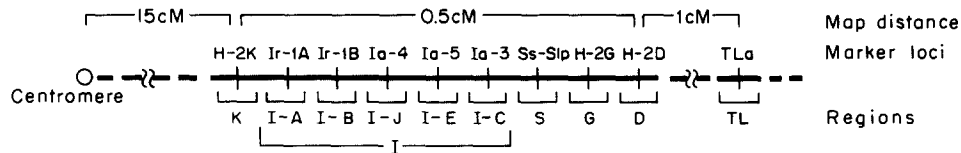
Fig. 1. Genetic map of the *H-2-Tla* gene complex on chromosome 17, showing the regions, marker loci, and map distances (adapted from references 1, 2, and 3). The *K*, *I*, and *D* regions and their products are discussed in the text. The *S* region controls the levels and testosterone-dependent structural markers of one or more serum proteins (1), including the fourth component of complement (4). A new region, *G*, which maps between *S* and *D*, contains the *H-2G* locus which determines alloantigens on erythrocytes (5, 6) and perhaps also on lymphoid cells (5). The *Tla* locus, located outside the *H-2* complex, controls the expression of thymic leukemia antigens which in some strains are normal differentiation antigens of thymocytes (7).

Recently, by combining isoelectrofocusing and discontinuous SDS polyacrylamide gel electrophoresis in a two-dimensional (2-D) separation system, O'Farrell was able to resolve the total cell proteins from both prokaryotic and eukaryotic cells into discrete spots on autoradiograms (19). This paper introduces the use of 2-D gel electrophoresis for the analysis of lymphocyte H-2 and Ia antigens. The results demonstrate that H-2 and Ia molecules exist in the cell as heterogeneous sets of distinct though related molecular forms. Perhaps more importantly the high resolution separation obtained with this technique makes available new approaches for probing the genetic polymorphisms and structural complexities of these cell surface antigens (Fig. 1).

Materials and Methods

Mice. BALB/c mice were purchased from Simonsen Laboratories, Gilroy, Calif. AKR/J mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. C3H/DiSn and B10.D2 mice were generously provided by Dr. H. O. McDevitt, Stanford University.

Antisera. The antisera used for immunoprecipitation and their potential reactivities, based on haplotype assignments given by Shreffler et al. (2), Murphy et al. (3), and Frelinger et al. (20), were as follows: (B10 × A)F₁ anti-B10.D2 (D31 pool 144, anti-K^d, I-A^d, I-B^d, I-J^d, and I-E^d) and (B10 × LP.RIII)F₁ anti-B10.A(5R) (D13 pool 66, anti-I-J^k, I-E^k, I-C^d, S^d, G^d, and D^d, TL) were obtained from Doctors M. Cherry and G. D. Snell of The Jackson Laboratory, under contract with the National Institutes of Health. Other antisera, generously provided by Dr. H. O. McDevitt, were: C3H.SW (CSW) anti-C3H (anti-K^k, I^k, S^k, G^k, and D^k), (A.TL × C3H.OL)F₁ anti-C3H (anti-K^k), and A.TH anti-A.TL (anti-I^k, S^k, and G^k). Normal CSW mouse serum was obtained from Dr. L. A. Herzenberg, Stanford University.

Preparation of Spleen Cell Suspensions. Mouse spleen cells were dissociated in Dulbecco's modified Eagle's medium (DME) (10× stock, Pacific Biological Co., Div. of Bio-Rad Laboratories, Richmond, Calif.) lacking sodium bicarbonate, methionine, and glutamine, supplemented with 2 mM Na₂HPO₄, 200 μM methionine, 4 mM glutamine, and brought to pH 7.2 with NaOH), containing 5% heat-inactivated fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N. Y.). After centrifugation the cells were resuspended in hemolytic Gey's solution (Gey's solution with 0.83% ammonium chloride replacing sodium chloride, 1 ml per spleen) and incubated for 2 min at room temperature to lyse the erythrocytes. After addition of FCS to 5%, the cells were passed through a 1-ml column of glass wool to remove cell debris, centrifuged, and washed once in DME plus 5% FCS. Cell viabilities determined by trypan blue dye exclusion ranged from 85 to 95%.

Radiolabeling of Cells. For internal labeling with ³⁵S-methionine, cells were suspended at 2–3 × 10⁷ viable cells/ml in DME minus methionine (prepared from 10x stock) buffered with 0.375% NaHCO₃ and supplemented with 4 mM glutamine and 5% heat-inactivated FCS. ³⁵S-methionine (New England Nuclear, Boston, Mass. or Amersham/Searle Corp., Arlington Heights, Ill.; 200–

500 Ci/mM) was added to 5×10^7 cells in 17×100-mm Falcon polystyrene tubes to a final sp act of 250 μCi/ml. The cells were incubated at 37°C in a 10% CO_2 incubator for 4–5 h with occasional mixing. Incorporation of ^{35}S-methionine into trichloroacetic acid (TCA)-precipitable material was linear over this period. Incorporation of label was stopped by addition of 5 ml cold Dulbecco's PBS containing 2 mM methionine, and the cells were centrifuged and washed twice in this medium. Lactoperoxidase-catalyzed radioiodination of cell surface molecules was done essentially as described by Kessler (21).

To prepare radiolabeled cells for 2-D gel electrophoresis, 5×10^6 ^{35}S-methionine or ^{125}I-labeled cells were solubilized in 50–100 μl of isoelectrofocusing (IEF) sample buffer at room temperature (19). 1-μl samples were taken for determination of TCA precipitable radioactivity, and the remainder was stored at −70°C until use.

Preparation of Non-Idet-P-40 (NP-40) Extracts. Washed ^{35}S-methionine or ^{125}I-labeled cells were resuspended at 10^8/ml in 0.5% NP-40 (NP-40, Particle Data Inc., Elmhurst, Ill.) in Tris-buffered saline (150 mM NaCl, 50 mM Tris, 0.02% NaN_3, pH 7.0) and incubated 15 min at 4°C to extract membrane proteins. Insoluble material was removed by centrifugation at 45,000 g for 1 h. 1-μl samples of the supernatant fluid were precipitated with TCA to determine the total amount of label incorporated into NP-40 solubilized material. The extract was either used immediately for immunoprecipitation or stored at −70°C. For 2-D gel electrophoresis of NP-40-extracted proteins, crystalline urea was added to small volumes of the extract to a final concentration of 9 M, followed by an equal volume of IEF sample buffer. These samples were stored at −70°C before electrophoresis.

Immunoprecipitation of Radiolabeled Cell Proteins. Cell proteins were isolated by a two-step immunoprecipitation procedure with *Staphylococcus aureus* bacteria to bind the antigen-antibody complexes formed upon addition of alloantibodies to the cell extracts (21, 22). Cowan I strain of *S. aureus* (starting material provided by Dr. G. M. Iverson, Stanford University) was grown and processed as described by Kessler (21). The formalin-fixed, heat-killed, and washed *S. aureus* Cowan I (SaC) were resuspended as a 10% wt/vol suspension in phosphate-buffered saline (PBS) (0.2 M NaCl, 0.0125 M $K_3(PO_4)$, pH 7.6) containing 0.02% NaN_3 and stored at −70°C. Just before use, the SaC were centrifuged at 12,000 g for 10 min and then resuspended in SaC buffer (PBS containing 0.5% NP-40, 2 mM methionine, and 5 mM KI). After 15 min incubation the SaC were centrifuged, washed once more in this buffer, and resuspended to the original 10% wt/vol suspension in SaC buffer containing 1 mg/ml bovine serum albumin (BSA, 5× crystallized, Pentex Biochemical Co., Kankakee, Ill.).

Radiolabeled cell proteins were immunoprecipitated by addition of 10–20 μl of antiserum to 50–100 μl of NP-40 extract in 12×75 mm Falcon polystyrene tubes. After 15 min incubation at 4°C, 100–200 μl of SaC suspension in SaC buffer plus BSA was added and the mixture incubated for an additional 10 min at 4°C . This procedure has been found to be sufficient to bind all the antigen-antibody complexes generated by the amount of antisera used. The SaC were then diluted with SaC buffer, centrifuged for 10 min at 2,000 g, and washed three times with SaC buffer at 4°C. The SaC were transferred to fresh polystyrene tubes after the first two washes and to 3-ml conical glass centrifuge tubes for the final centrifugation. The bound antigen-antibody complexes were eluted by resuspending the SaC pellet in 30–50 μl of IEF sample buffer. The SaC were centrifuged at room temperature and the supernatant fluid carefully removed. 1-μl samples of these eluates were used for determination of TCA-insoluble radioactivity and the rest stored at −70°C before electrophoresis. Samples for 2-D gel electrophoresis consisted of 15–30 μl of the eluates.

Determination of TCA-Insoluble Radioactivity. 1-μl samples of ^{35}S-methionine or ^{125}I-labeled preparations were added to 0.5 ml PBS containing 0.5% FCS and precipitated with 0.5 ml of 10% TCA containing 2 mg/ml methionine and 0.3 mg/ml KI. The precipitates were collected on 0.45 μm Millipore nitrocellulose filters (Millipore Corp., Bedford, Mass.) and washed with 10 ml of 5% TCA. ^{35}S-methionine radioactivity was assayed in a Beckman LS-233 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.) by using a mixture of toluene (71%), Triton X-100 (25%), and water (4%) containing 4 g/liter of Omnifluor (New England Nuclear). ^{125}I radioactivity was counted in a Beckman Gamma 300 gamma counter.

Polyacrylamide Gel Electrophoresis

MATERIALS. Ampholines (LKB Instruments, Rockville, Md.); urea, ultra-pure grade (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.); SDS (BDH, Gallard-

Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.); acrylamide, N,N'-methylenebisacrylamide (Bis), N,N,N',N'-tetramethylenediamine and ammonium persulfate (Bio-Rad Laboratories, Richmond, Calif.); SeaKem agarose (Marine Colloids, Inc., Rockland, Maine); glycine and tris (TRIZMA base) (Sigma Chemical Co., St. Louis, Mo.).

2-D GEL ELECTROPHORESIS. Radiolabeled cell proteins were electrophoresed in two dimensions by the method developed by O'Farrell (19). Briefly, for separation in the first dimension, samples in IEF sample buffer (9.5 M urea, 2% (wt/vol) NP-40, 2% Ampholines (a mixture of 1.6% pH range 5-7 and 0.4% pH range 3.5-10), and 5% β-mercaptoethanol) were isoelectrofocused in thin glass tubes (130 mm × 2.5 mm inside diameter), by using a gel mixture consisting of 9 M urea, 2% (wt/vol) NP-40, 2% Ampholines (1.6% pH 5-7 and 0.4% pH 3.5-10), and 4% acrylamide. The IEF gels were extruded from the tubes and equilibrated for 2 h at room temperature in two 5-ml changes of SDS sample buffer (2.3% (wt/vol) SDS, 10% (wt/vol) glycerol, 5% β-mercaptoethanol, and 0.0625 M Tris-HCl, pH 6.8). The equilibrated gels were stored frozen in SDS sample buffer at −70°C before the second electrophoresis.

The second dimension electrophoresis was done as described by O'Farrell (19) utilizing the discontinuous Tris-glycine SDS gel system described by Laemmli (23) in 10% acrylamide slab gels. The equilibrated IEF tube gel was placed on top of the stacking gel, and anchored on with 1 ml of melted agarose. The slab gels were placed in individual slab gel tanks; up to eight slab gels were run at once, at 20 mA constant current per gel. The gels were fixed and stained in 0.1% Coomassie Blue in 50% TCA for 30 min and destained overnight in two changes of 7% acetic acid. They were then dried on Whatmann 3MM paper and exposed to Kodak NS2T No-Screen X-ray film (Eastman Kodak Co., Rochester, N. Y.) for autoradiography. Most autoradiograms presented below were the result of approximately 20 days of exposure.

ONE-DIMENSIONAL SDS GEL ELECTROPHORESIS. In some experiments samples were run on one-dimensional SDS slab gels. These gels were identical to the second dimension 10% acrylamide SDS gels of the 2-D procedure, except that sample wells were placed in the stacking gel by using a Teflon comb. Samples were prepared for SDS gels by mixing 1 vol of IEF sample with 1 vol 10% SDS and 2 vol of 2x SDS sample buffer. The samples were heated at 100°C for 2 min before being loaded on the SDS slab gel. The gels were stained, destained, and autoradiographed as described above. Molecular weight standards (rabbit IgG, BSA, ovalbumin, and chymotrypsin) were run on each slab gel, and the resulting Coomassie Blue-stained bands were used to determine the approximate molecular weights of bands on the autoradiograms.

Results

2-D Electrophoresis of ^{35}S-Methionine-Labeled Lymphocyte Proteins. By separating proteins according to two independent parameters — their isoelectric point and molecular weight — each molecular species in a mixed population can be resolved from all the others as a single spot with very little overlap (19). Thus, autoradiography of 2-D gels of cell extracts prepared from radiolabeled lymphocytes makes it possible to examine all the cell proteins that have been labeled. Fig. 2 shows the pattern generated from an 0.5% NP-40 extract of B10.D2 splenic lymphocytes that had been labeled for 4 h with ^{35}S-methionine. Since both the IEF and SDS separations were done under reducing and denaturing conditions, each spot on the 2-D gel represents a single polypeptide chain.

This autoradiogram contains more than 900 spots, which represent a major fraction of the total cell proteins, though not all. The pH gradient for the IEF gels is restricted to pH 4.5 to pH 7, and some proteins do not focus within this range. In addition to these proteins, any polypeptide chains with mol wt below about 20,000 daltons migrate with the dye front in the 10% acrylamide SDS slab gels.

As described by O'Farrell the autoradiographic procedures used in conjunction with 2-D gel electrophoresis increase the sensitivity of detection of proteins
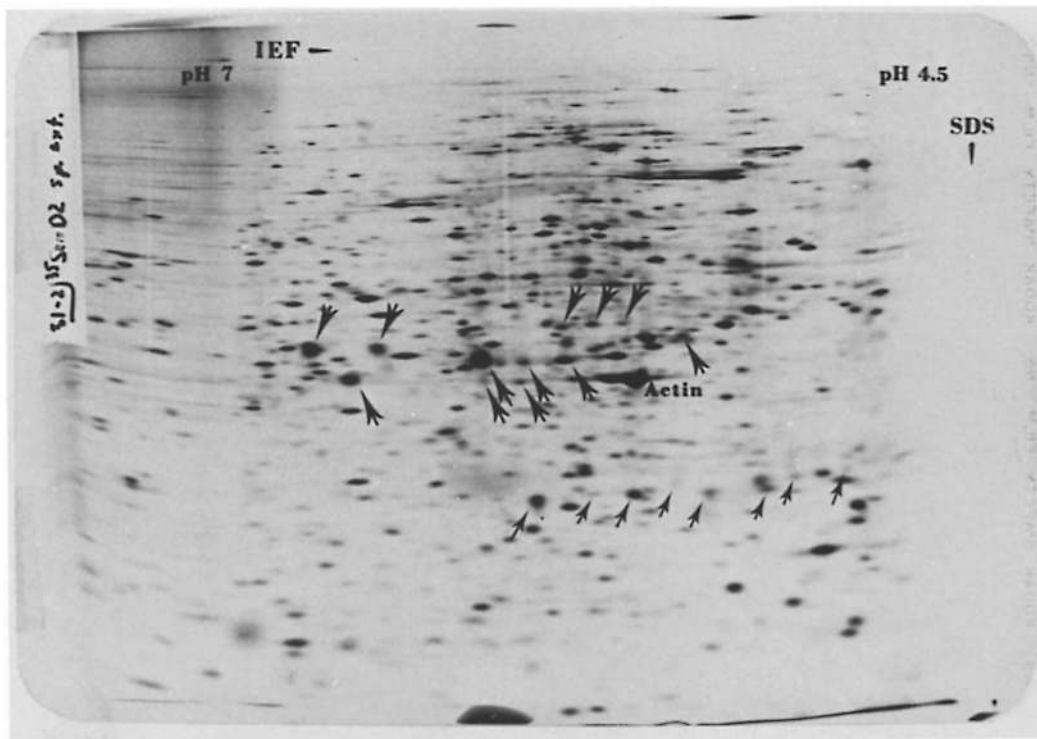
FIG. 2. Autoradiogram of 2-D gel of an 0.5% NP-40 extract of ^{35}S-methionine-labeled
B10.D2 spleen lymphocytes. The sample for this gel contained 400,000 cpm in 6 μl of
extract, and the gel was exposed to film for 20 days. The basic end of the IEF gel is on the
left, and the direction of SDS electrophoresis was from top to bottom. Actin, the major spot
in the center, serves as an internal mol wt marker of 44,000 daltons. The arrows indicate
those spots that were shown by immunoprecipitation (see below) to be H-2K (large arrows
pointing down), H-2D (large arrows pointing up), and Ia (small arrows pointing up).

greatly over conventional staining procedures. The 20-day exposure used for
the gel shown in Fig. 2 should be sufficient to allow detection of spots
containing as little as 1 cpm (19). Since the sample run on this gel contained
400,000 cpm, such barely detectable spots would constitute 2.5 × 20^{-4} of 1% of
the total cell protein. At the other end of the intensity spectrum is the major
spot in the center of the gel (mol wt 44,000), shown to be actin by its similarities
in electrophoretic mobility to purified muscle actin (P. Rubenstein, personal
communication). Actin makes up several percent of the total cell protein.
Thus, these autoradiograms reveal proteins whose relative abundances span
about four orders of magnitude.

*Identification of H-2^d and Ia^d Molecules from ^{35}S-Methionine-Labeled
B10.D2 Spleen Cells.*   To identify which spots shown in Fig. 2 correspond to
H-2 and Ia polypeptide chains, portions of the same NP-40 extract were
immunoprecipitated with antisera directed against the products of the *H-2^d*
region. The autoradiograms shown in Fig. 3 represent, for these immunoprecipi-
tates, composites of the 2-D gel patterns and the corresponding lanes cut from
a one-dimensional (1-D) SDS slab gel. Fig. 3a shows that very few proteins
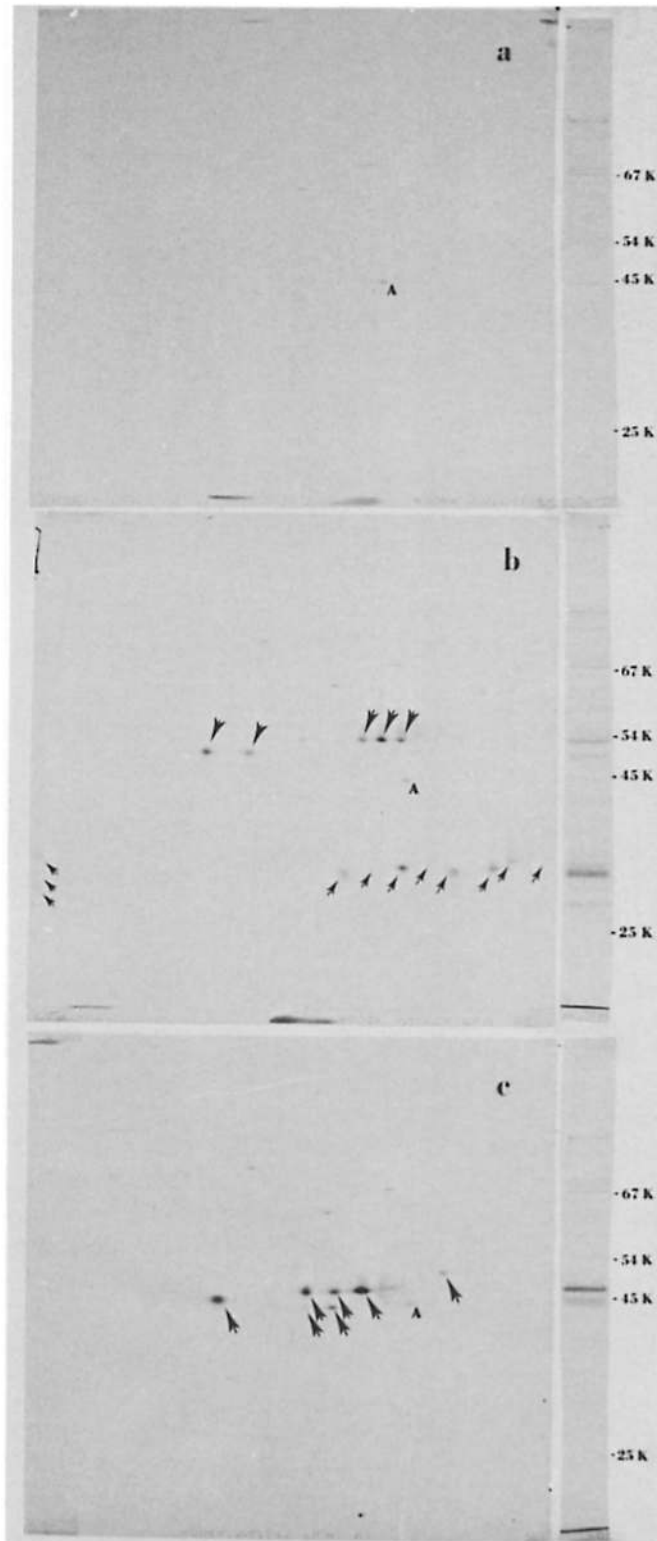
were immunoprecipitated by the normal mouse serum (NMS) control. Coelectrophoresis of trace amounts of cell extracts with the immunoprecipitates has shown that one of the proteins in the NMS precipitate is actin, a common though variable contaminant of immunoprecipitates. Actin probably is the 44,000-dalton polypeptide that others have found in similar precipitates (24, 25).

Figs. 3b and c represent immunoprecipitates from the B10.D2 cell extract. Serum (B10 × A)F_1 anti-B10.D2 potentially can react with K^d, I-A^d, I-B^d, I-J^d, and I-E^d, but not I-C^d or D^d. The 47,000-52,000 dalton proteins in Fig. 3b therefore must correspond to H-2K molecules. The 28,000-37,000 dalton molecules are I-region products; these include not only the spots at the acidic (right) end of the gel but also the three streaks at the basic (left) end. The smallest of these, mol wt 28,000, corresponds to a major band on the 1-D SDS gel but has no matching spots in the body of the 2-D gel. This molecule most likely has an isoelectric point > pH 7.

Antiserum (B10 × LP.RIII)F_1 anti-B10.A(5R) has potential reactivities for I-J^k, I-E^k, I-C^d, S^d, G^d, D^d, and TL. Since lymphocytes do not express proteins coded for by the S region (1), and since there is negligible expression of H-2G (5, 6) and TL (7) on splenic lymphocytes, it is unlikely that any of the precipitated molecules are derived from these loci. Although this antiserum possibly could react with some I-region determinants, Fig. 3c shows that no 25,000-35,000 dalton molecules were precipitated. Similar results were obtained with strains BALB/c (H-2^d, see below) and A/J (H-2^a, P. P. Jones, unpublished observations), suggesting that this antiserum has little or no anti-I-region activity detectable in this assay. This antiserum potentially could have activity against Qa-1 and Qa-2, two recently discovered lymphocyte alloantigens coded for by loci that map between H-$2D$ and Tla (26, 27). It is unlikely that any of the precipitated molecules are Qa-1 because, as will be shown later, this antiserum precipitates the same molecules from BALB/c which is Qa-1-negative (26). In addition, the antiserum used for identification of Qa-1, (B6 × A-Tla)F_1 anti-ASL1, does not precipitate any molecules from radiolabeled cell extracts (J. Michaelson and T. Stanton, personal communication). It is also unlikely that the molecules seen on these gels are Qa-2. Although Qa-2 is the same size as H-2, Qa-2 precipitated from cell extracts contains less than 5% of the amount of radioactivity present in H-2 (28). The 44,000-46,000 dalton spots in Fig. 3c therefore probably represent products of the H-$2D$ region.

The spots that were shown in Fig. 3 to be H-2 and Ia by immunoprecipitation

Fig. 3.   Composite 2-D gel and 1-D SDS gel patterns of immunoprecipitated proteins from ^{35}S-methionine-labeled B10.D2 spleen extracts. The sera used for immunoprecipitation, their potential reactivities, and the percentage of input radioactivity that was precipitated by each serum were as follows: (a) NMS, 0.33%; (b) (B10 × A)F_1 anti-B10.D2 (anti-K^d, I-A^d, I-B^d, I-J^d, and I-E^d), 1.1%; (c) (B10 × LP.RIII)F_1 anti-B10.A(5R) (anti-I-J^k, I-E^k, I-C^d, S^d, G^d, and D^d), 0.71%. The samples of SaC eluates for 2-D gels contained (a) 5,640 cpm, (b) 18,240 cpm, and (c) 12,060 cpm; samples for 1-D SDS slab gels contained one-third as many counts. The gels were exposed to film for 20 days. The indicated molecular weights correspond to the positions of the Coomassie Blue-stained bands of the following marker proteins, which were electrophoresed in a parallel lane on the 1-D gel: bovine serum albumin (67,000 daltons), rabbit γ-chain (54,000 daltons), ovalbumin (45,000 daltons), and chymotrypsin (25,000 daltons). The arrows mark the H-2K, H-2D, and Ia spots similarly labeled in Fig. 2. In this and subsequent figures actin is indicated by the letter "A".
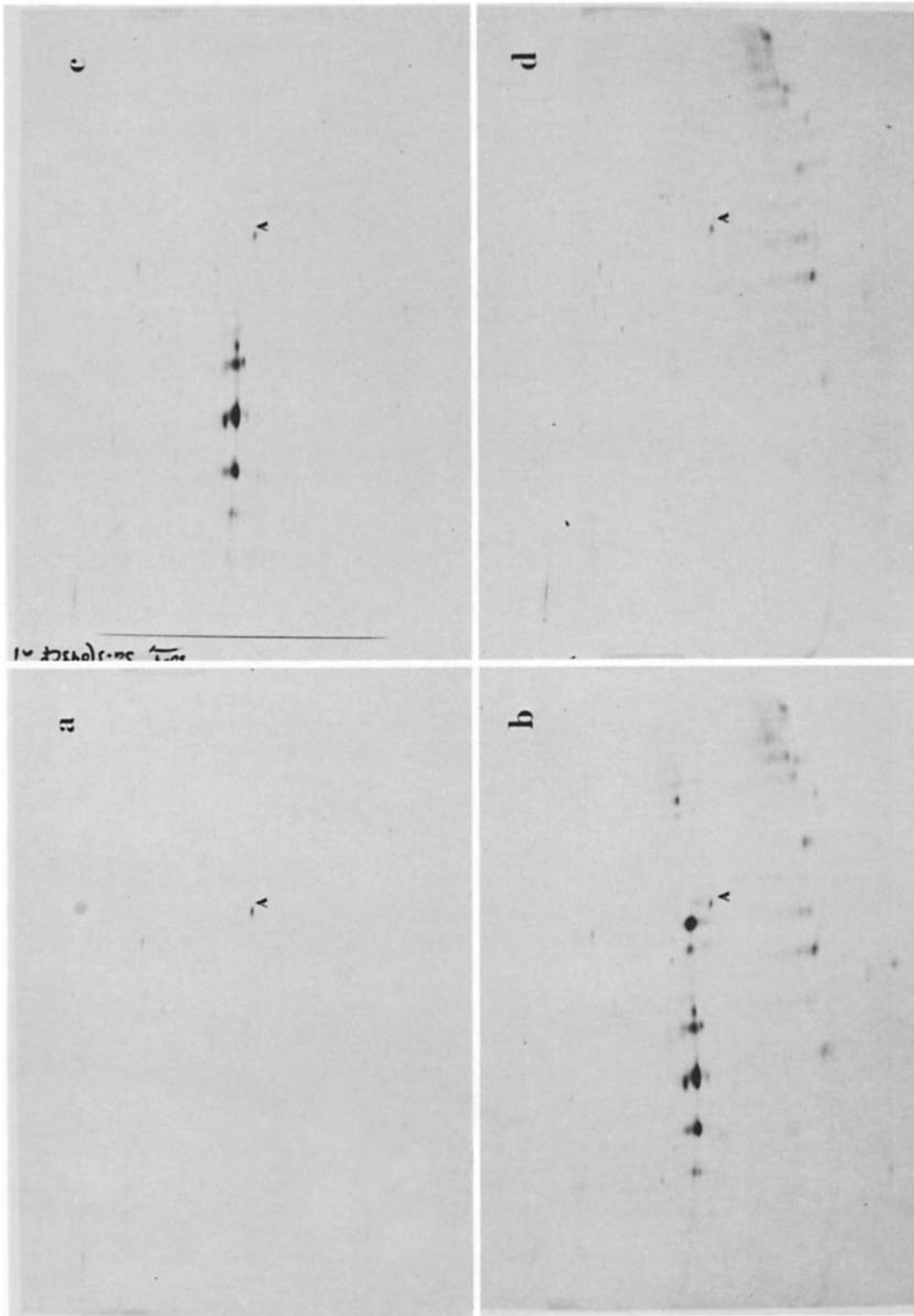
can be identified in the pattern generated by the NP-40 extract itself. As indicated by the arrows in Fig. 2, H-2 and Ia represent major groups of proteins in splenic lymphocytes.

Identification of H-2^k and Ia^k Molecules from ^{35}S-Methionine-Labeled AKR Spleen Cells. Fig. 4 presents the results of similar 2-D gel analyses of H-2^k and Ia^k molecules from AKR spleen cells. The pattern shown in Fig. 4b represents proteins that were precipitated by CSW anti-C3H antiserum, which can react with the entire *H-2* region of the *k* haplotype. The H-2K spots were defined by precipitation with (A.TL × C3H.OL)F$_1$ anti-C3H, which reacts only with H-2K (Fig. 4c). By difference, the other 43,000–48,000 dalton proteins should be H-2D products. This was confirmed by using an anti-H-2D^k antiserum (results not shown). In Fig. 4d are shown the *I* region products, identified with A.TH anti-A.TL. Aside from the H-2D molecules, the restricted anti-H-2K and anti-*I*-region antisera detect all of the molecular species recognized by the antisera made against the whole *H-2* region.

One of the most impressive features of the 2-D gel profiles of H-2 and Ia proteins is their marked heterogeneity. Both H-2 and Ia are glycoproteins (17, 29), and some of the modification patterns shown in Figs. 3 and 4 might reflect shifts in mobility caused by sequential glycosylations or by other post-translational modifications. The heterogeneity of H-2K, H-2D, and Ia antigens may also reflect the existence of multiple genes coding for distinct though related proteins. A series of experiments was initiated to probe the origins and physiological significance of these complex patterns.

Comparison of C3H Lymphocyte Proteins Labeled with ^{35}S-Methionine and ^{125}I. If intracellular modification of H-2 and Ia proteins contributes to the observed molecular heterogeneity, then it might be expected that those molecules that are on the cell surface would be most heavily modified. Comparing the autoradiograms from cells internally labeled with ^{35}S-methionine with those from cells surface-labeled with ^{125}I with lactoperoxidase should reveal which molecules are on the cell surface. Figs. 5a and d show patterns generated by C3H spleen cells labeled, respectively, with ^{35}S-methionine and ^{125}I. Only a minor population of the total ^{35}S-methionine spots also labels with ^{125}I, as expected, since relatively few cell proteins are on the surface.

The molecules precipitated from the ^{35}S-methionine C3H extract with CSW anti-C3H (Fig. 5c) produce essentially the same pattern as that from AKR, also *H-2^k* (Fig. 4b). Thus the H-2 and Ia patterns are haplotype-specific and are independent of the genetic background. The identification of H-2K, H-2D, and Ia molecules was done as for AKR (Fig. 4), by using antisera of restricted specificity, and gave similar results (not shown). By comparing the spots precipitated from the ^{35}S-methionine and ^{125}I-labeled extracts, it can be seen that only subsets of the total H-2 and Ia ^{35}S spots also are labeled with ^{125}I. For

Fig. 4. Immunoprecipitated proteins from ^{35}S-methionine-labeled AKR spleen extracts. The sera used for precipitation, their potential reactivities, and the percentage of input radioactivity precipitated by each were as follows: (a) NMS, 0.58%; (b) CSW anti-C3H (anti-K^k, I^k, S^k, G^k, and D^k), 2.2%; (c) (A.TL × C3H.OL)F$_1$ anti-C3H (anti-K^k), 1.1%; (d) A.TH anti-A.TL (anti-I^k, S^k, and G^k), 1.3%. The samples for the gels contained (a) 9,740 cpm, (b) 35,940 cpm, (c) 18,900 cpm, and (d) 22,280 cpm. The gels were exposed to film for 23 days.
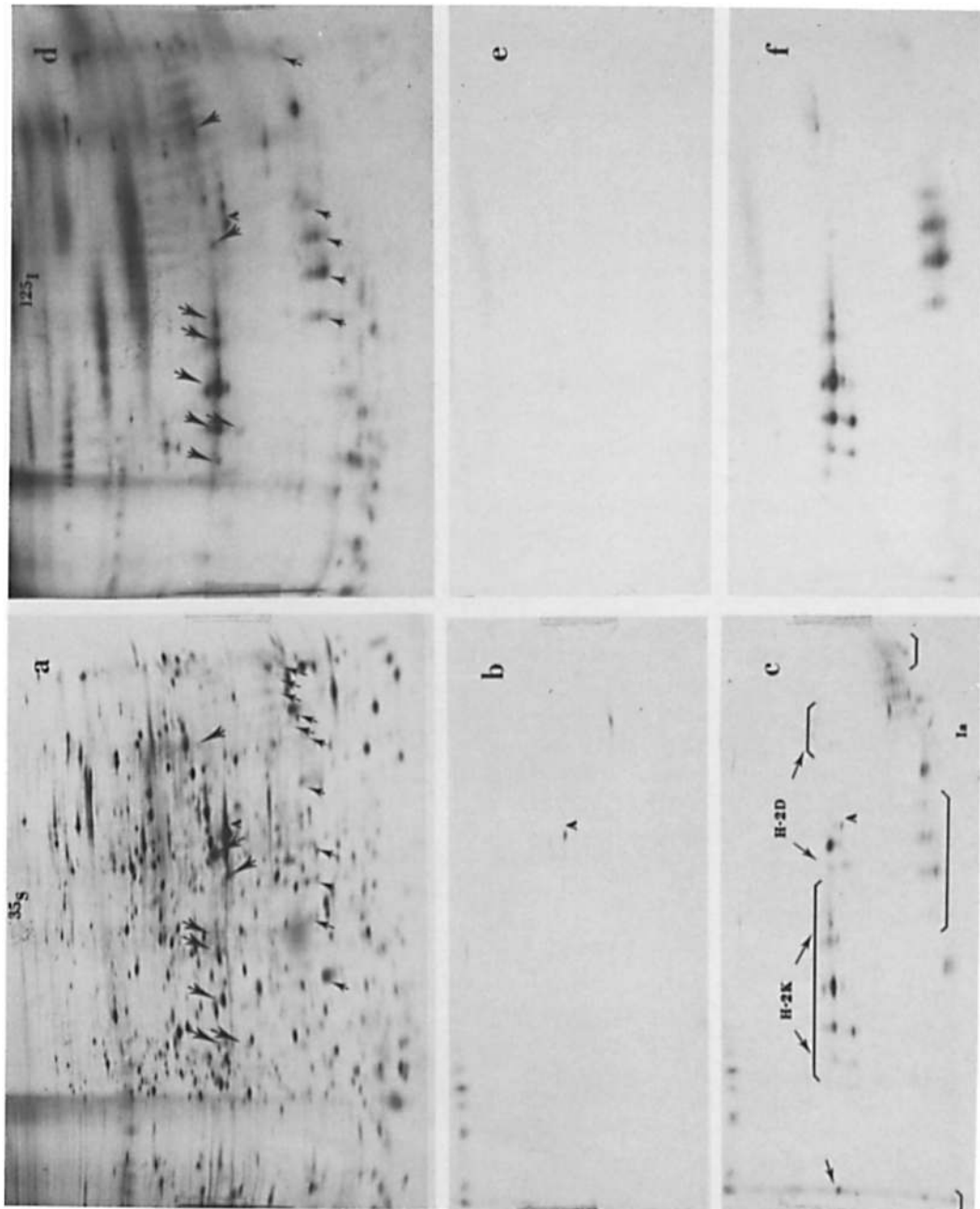
H-2D, the major iodinated species are those that are larger and more acidic. All of the H-2K molecules that migrate in the main part of the gel carry both ^{35}S and ^{125}I markers. However, there is an ^{35}S spot at the very basic end of the gel which is not iodinated. The observation that the iodinated spots tend to be the larger and more acidic of the ^{35}S spots lends weight to the hypothesis that at least some of the spot heterogeneity arises from modification of the poly- peptide chains as they are transported to the membrane. The H-2 patterns, in which the larger and more acidic molecules are on the surface, are consistent with those representing more extensively glycosylated molecules; the shift towards more acidic isoelectric points could be attributed to the addition of more sialic acid residues. For Ia, the relationship of ^{125}I to ^{35}S-labeled spots is less clear than for H-2. The most acidic spots, not well resolved in these gels, do show some ^{125}I-labeling. The major iodinated species migrate between sets of other spots that are not iodinated.

The autoradiograms shown in Fig. 5 also provide evidence against the possibility that artifactual modification of proteins during the NP-40 extraction or immunoprecipitation procedures might contribute to the observed spot heterogeneity. The samples shown in Figs. 5a and d were prepared by solubilizing radiolabeled cells directly in IEF sample buffer, which apparently does not result in protein modification (19). All of the H-2 and Ia spots apparent in the gels of the immunoprecipitates in Fig. 5 are visible in the IEF sample buffer preparations.

It is also of interest that in this and all other radioiodination experiments performed actin is a major iodinated species. It is possible that actin released from dead cells in vivo or in vitro binds to lymphocyte surfaces. Alternatively, actin might be close enough to the cell surface to be iodinated directly.

Biosynthetic Labeling and ^{125}*I-Labeling of H-2 and Ia Molecules from BALB/ c Spleen Cells.* To explore possible precursor-product relationships among the H-2 and Ia molecules in more detail, the sequence of labeling of the spots with ^{35}S-methionine was examined in pulse-chase experiments. BALB/c spleen cells were labeled for 15 min with ^{35}S-methionine (1 mCi/ml). After the labeling period the cells were washed free of label and cultured for 0, 1, and 4 h in complete medium. Another culture was continuously labeled with ^{35}S-methio- nine for 4.5 h, and a final portion of the same cell preparation was labeled with ^{125}I to iodinate the surface proteins.

Fig. 6 presents the patterns generated by antisera reacting with H-2K and Ia (Figs. 6a–e) and with H-2D (Figs. 6f–j). Comparing the patterns obtained from radioiodinated cells (Figs. 6 e and j) with those obtained from cells labeled

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Fig. 5. ^{35}S-methionine- and ^{125}I-labeled C3H spleen cell proteins. The samples and percentage of radioactivity precipitated (where appropriate), were, for ^{35}S: (a) spleen cells in IEF sample buffer; (b) NMS precipitate, 0.58%; (c) CSW anti-C3H precipitate, 1.2%; and for ^{125}I: (d) spleen cells in IEF sample buffer; (e) NMS precipitate, 0.52%; and (f) CSW anti- C3H, 1.7%. The samples loaded per gel contained: (a) 300,000 cpm; (b) 11,220 cpm, (c) 22,740 cpm; (d) 300,000 cpm; (e) 4,100 cpm; and (f) 13,260 cpm. The gels were exposed to film for 18 days. The arrows in (a) and (d) mark those spots identified in the immunoprecip- itates as H-2K (large arrows pointing down), H-2D (large arrows pointing up), and Ia (small arrows pointing up). The brackets in (c) indicate those ^{35}S-methionine spots which have corresponding ^{125}I spots.
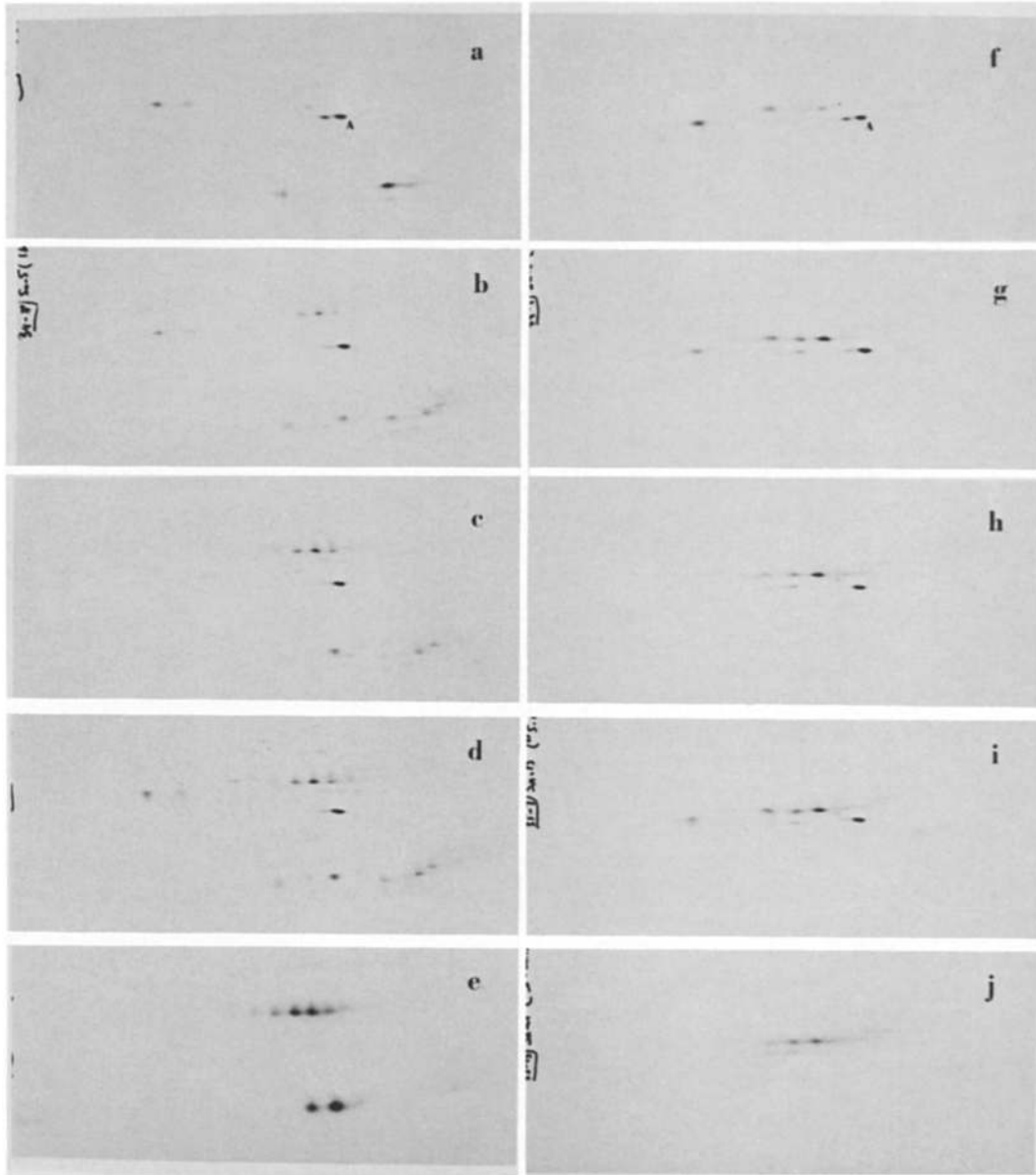
FIG. 6.   Proteins immunoprecipitated from BALB/c spleen cells labeled with ^{35}S-methio-
nine during pulse and chase: comparison with ^{125}I-labeled proteins. The radiolabeling of
cells was done as described in the text. Amounts of extracts containing 10^6 cpm of ^{35}S or
^{125}I radioactivity were immunoprecipitated with 10 μl of antisera and 100 μl of SaC. Bound
proteins were eluted with 40 μl of IEF sample buffer; 30 μl were loaded per gel. The
antisera used for immunoprecipitation were: (a–e) (B10 × A)F$_1$ anti-B10.D2; and (f–j) (B10
× LP.RIII)F$_1$ anti-B10.A(5R). The extracts were derived from cells labeled as follows: (a, f)
15 min ^{35}S-methionine pulse; (b, g) 15 min ^{35}S-methionine pulse followed by 1 h chase; (c,
h) 15 min ^{35}S-methionine pulse followed by 4 h chase; (d, i) 4.5 h ^{35}S-methionine continuous
label; and (e, j) ^{125}I surface label. The samples loaded per gel contained: (a) 10,500 cpm; (b)
13,380 cpm; (c) 9,030 cpm; (d) 14,940 cpm; (e) 6,270 cpm; (f) 8,760 cpm; (g) 10,350 cpm; (h)
9,420 cpm; (i) 9,930 cpm; and (j) 5,130 cpm. The gels were exposed to film for 28 days.

for 4.5 h with ^{35}S-methionine (Figs. 6 d and i) reveals that ^{125}I labels only subsets of the total H-2 and Ia molecules, as was found for C3H. Again, only the larger and more acidic of the ^{35}S-methionine-labeled H-2K and H-2D molecules can be labeled with [125]I. The labeling pattern for Ia also is similar to that obtained with C3H. The major iodinated species migrate between others that are not iodinated. In addition, the most acidic Ia molecules also label with [125]I.

For both the H-2K and Ia patterns (Figs. 6 a–e) it can be seen that none of the spots showing significant amounts of ^{35}S radioactivity after the 15-min-labeling period correspond to those available for radioiodination. The presence of trace amounts of ^{35}S-label in these spots after 15 min does indicate, however, that this time period is sufficient to allow some newly-synthesized molecules to reach the cell surface. For H-2D (Figs. 6 f–j) ^{35}S can be detected in some but not all of the ^{125}I spots after the 15-min labeling period. As the ^{35}S-methionine is chased with cold methionine, the label moves progressively into those spots that are iodinated. These shifts in distribution of label with time are consistent with the early-labeled spots representing cytoplasmic precursor molecules that become modified as they move out to the cell surface. The Ia patterns show the highest degree of complexity. From the relatively few spots that are labeled during the 15-min exposure to ^{35}S-methionine is generated an array of molecular forms showing considerable size and charge heterogeneity. Despite their marked modification few of the Ia spots are iodinated by the lactoperoxidase technique. As will be discussed below, the two sets of iodinated spots correspond to the products of different *I*-region loci.

It should be noted that the patterns generated from immunoprecipitates of extracts of BALB/c cells labeled for 4.5 h with ^{35}S-methionine (Figs. 6 d and i) are nearly identical to those obtained with the same antisera from extracts of B10.D2 cells, also *H-2^d* (Fig. 3). These patterns, in turn, are different from those of the *H-2^k* strains AKR (Fig. 4) and C3H (Fig. 5).

Discussion

An impressive feature of the 2-D gel profiles of the H-2 and Ia proteins is their molecular complexity. The molecules immunoprecipitated from 0.5% NP-40 extracts of cells internally labeled with ^{35}S-methionine migrate as groups of spots that show variability in both size and isoelectric point. The molecular basis for the observed heterogeneity is potentially of great importance, since intracellular modification of H-2 and Ia proteins might regulate their function. A number of observations indicate that much of the spot heterogeneity is the result of intracellular modification of H-2 and Ia polypeptide chains, perhaps involving extensive glycosylation. Both H-2 and Ia are glycoproteins, containing about 7% (17) and 10% (30), respectively, of their total mass in carbohydrate, probably in the form of one oligosaccharide side chain (30). By labeling cells continuously with ^{35}S-methionine in culture, all of the biosynthetic intermediates should be labeled and should generate discrete though related spots on the autoradiograms. Glycosylation of proteins should cause very characteristic shifts in electrophoretic migration; the sequential addition of neutral sugars, such as fucose, should produce small-step increases in molecular weight, while

the addition of a sialic acid residue would result in both a shift to a more acidic isoelectric point as well as a slight increase in molecular weight.

Changes in mobility of the kind described above are evident in the 2-D gels of H-2 and Ia. That the observed shifts in migration are caused by glycosylation is supported by several observations. First, comparisons of ^{35}S-methionine internally-labeled preparations with those from ^{125}I surface-labeled cells has shown that for H-2K and H-2D proteins of both the k and d haplotypes, surface iodination labels subsets of ^{35}S spots that are larger and more acidic than those spots that are labeled only with ^{35}S-methionine. This is consistent with the shifts in migration being the result of the addition of neutral and charged sugars. The kinetics of labeling of the H-2K and H-2D spots in the pulse-chase experiment (Fig. 6) suggest that the more basic spots that are labeled with ^{35}S-methionine but not ^{125}I are, in fact, the precursors of the molecules that are on the surface and available for iodination. Finally, preliminary experiments have shown that neuraminidase treatment of ^{125}I-labeled lymphocytes eliminates the largest, most acidic H-2 spots and generates slightly smaller and more basic spots that normally are not labeled with ^{125}I (P. P. Jones, unpublished observations). This result would be expected if the enzyme removes sialic acid residues.

It is somewhat more difficult to interpret the complexities of the Ia patterns because at this point the number of loci that determine the proteins recognized by the antisera is uncertain (3, 31). In addition, some immunoprecipitated Ia molecules from both d (Fig. 3 b) and k (Fig. 4 d) haplotypes seem to be too basic to be resolved by the pH range of these IEF gels and hence are difficult to analyze. Nonetheless, major features of the Ia-labeling patterns, including the distribution of iodinated spots, shifts of ^{35}S-methionine radioactivity in pulse-chase experiments, and effects of neuraminidase, resemble those of H-2 and suggest that a significant part of the Ia heterogeneity is caused by glycosylation.

An additional order of complexity in the 2-D gel patterns would exist if the H-2K, H-2D, or Ia spots represent multiple gene products. Proteins coded for by tightly-linked genes never separated by recombination might behave serologically like a single gene product. For the K and D regions most of the information available from immunoprecipitation (32) and sequencing (33, 34) analyses supports the contention that each region contains a single locus that codes for a protein bearing all the serologically-detectable antigenic specificities. However, a recent study by Hansen et al. (35) has provided immunochemical evidence for an additional locus, closely linked to H-$2D$ in d and q haplotype strains, that also codes for a 45,000-dalton glycoprotein.

If H-2K and H-2D molecules actually were coded for by multiple genes, one would expect considerable heterogeneity in the 2-D gel patterns obtained from immunoprecipitates. However, multiple gene products would not necessarily be recognizable as such from the ^{35}S-methionine spot patterns alone. The results presented in this paper for the k and d haplotypes, as well as other studies with the b haplotype (P. P. Jones, unpublished observations), have demonstrated that not only do the H-2K or H-2D molecules from different haplotypes resemble each other in their electrophoretic properties, but the H-2K and H-2D molecules within a given haplotype have overlapping distributions of isoelectric points and molecular weights. The molecular similarities among all

H-2 proteins displayed on the autoradiograms apparently reflect the primary structural similarities of these molecules that have been revealed by sequencing analyses (36). Thus it would be expected that if lymphocytes do synthesize multiple H-2K or H-2D proteins, they would also be very similar in their electrophoretic mobilities.

Since the 2-D gels allow one to observe the labeling behavior of each molecular species by surface iodination and during pulse-chase experiments, it may be possible to identify groups of spots within the total array precipitated by an antiserum that show precursor-product relationships appropriate for a single gene product. For example, the complex labeling patterns of H-2D^d with ^{35}S-methionine and ^{125}I (Figs. 6 f–j) conceivably may be resolved into two overlapping sets of spots representing the two distinct *H-2D* gene products described by Hansen et al. (35).

Comparisons of patterns generated by different labeling procedures may also help to sort out the complexities of the Ia antigens apparent on the 2-D gel autoradiograms. Other studies have shown that these molecules are B-cell proteins (37), and loci in the *I-A, I-E,* and perhaps also *I-C* subregions are known to code for B-cell Ia (1, 2). The assignment of 2-D gel spots to these subregions currently is in progress. Preliminary results, however, suggest that for the *k, d,* and *b* haplotypes loci in the *I-A* subregion code for the most acidic of the Ia spots as well as the streaks at the basic end of the 2-D gels; the remainder of the Ia spots, in the central part of the gels, apparently are controlled by the *I-E* subregion (37, and P. P. Jones, unpublished observations). The relationships of the ^{125}I-labeled spots to the total ^{35}S-methionine-labeled array suggest that both the *I-A* and *I-E* subregions may contain multiple loci coding for Ia antigens. First of all, the *I-A* subregion seems to control two sets of spots with very distinct electrophoretic mobilities. The spots in the first set are very acidic and have mol wt ranging from 33,000 to 37,000 daltons. The second set consists of very basic molecules, resolved poorly as streaks at the basic end of these gels, which range in size from 28,000 to 33,000 daltons. Both groups contain species that can be surface iodinated. These two sets of spots probably correspond to the two peaks seen by others on SDS gels of I-A immunoprecipitates (30).

The labeling behavior of the Ia spots in the central region of the gel, tentatively assigned to the *I-E* subregion (37), suggests that they also may represent two gene products. For both *d* and *k* haplotypes the only ^{125}I-labeled spots in this group are intermediate in size and charge compared to species which do not label with ^{125}I (Figs. 5 and 6). It is somewhat surprising that the most acidic molecules in this group are not iodinated, because they show extensive heterogeneity of the kind anticipated from addition of both neutral and charged sugars. One possible explanation, supported by the kinetics of labeling of these Ia spots in the pulse-chase experiment, is that some of the spots represent molecules which actually are on the cell surface but are not available for iodination by the lactoperoxidase technique. Fig. 6 shows that the 15-min pulse of ^{35}S-methionine labels spots just to the left and right of the spots which can be iodinated. During the chase with cold methionine the label moves progressively from these molecules into more acidic ones; after 4 h of chase there is no ^{35}S label left in the original spots. Because the sequence of labeling

of spots within each group seems to be in the direction of higher molecular weight, more acidic molecules, it may be that each group represents a different gene product. The left-hand group would contain, then, a precursor in the cytoplasm which, after modification, would be inserted into the membrane. The right-hand group also contains a rapidly-labeling precursor which also becomes modified, but these new species either are not expressed on the cell surface or are expressed but are not susceptible to radioiodination. A similar phenomenon has been noticed for guinea pig Ia antigens (38); SDS gel electrophoresis of proteins immunoprecipitated from extracts labeled with radioactive amino acids generated two peaks of 25,000 and 33,000 daltons. The smaller component also labels with ^{125}I by using the lactoperoxidase technique, but the larger one does not.

The molecular weights, haplotype distributions, and reactivities with more restricted antisera of the molecules detected on 2-D gels are consistent with their being H-2 and Ia gene products. Potentially, however, some of the spots could represent polypeptide chains that are not recognized by the antisera but which are precipitated because they are bound physically to H-2 or Ia molecules which do carry the appropriate antigenic determinants. For example, alloantisera against human Ia bind complexes of noncovalently joined 28,000 and 33,000 dalton glycoproteins (39). Only the 33,000-dalton polypeptide chain carries detectable Ia alloantigenic determinants. In fact, the 28,000-dalton chain may not even be coded for by genes in the HLA complex. Further studies with mouse preparations should reveal whether any non-H-2-linked gene products are present in immunoprecipitates of mouse alloantigens.

As has been documented above, the electrophoretic separations in this 2-D system generate spot patterns that are so reproducible from sample to sample and from experiment to experiment that they actually represent haplotype-specific molecular fingerprints of the individual proteins in the sample. The ability to compare ^{35}S-methionine-labeled cells with those surface-labeled with ^{125}I, and to examine the kinetics of labeling of spots in pulse-chase experiments, should continue to yield information about the biosynthesis and expression of alloantigens. In addition, such analyses may provide new ways of determining how many H-2 and I-region loci code for cell surface proteins. Comparative peptide mapping or sequencing of individual spots or sets of spots cut from 2-D gels should be a simple and sensitive approach for quantitating numbers of distinct gene products.

Summary

Mouse lymphocyte H-2 and Ia glycoproteins have been analyzed with a two-dimensional (2-D) acrylamide gel electrophoresis technique, in which proteins are separated first according to their charge in isoelectrofocusing gels and then according to their size in sodium dodecyl sulfate gels. Individual polypeptide chains from radiolabeled cells are resolved as discrete spots on autoradiograms of the gels, forming patterns which are characteristic of the proteins in the sample.

2-D gels of H-2K, H-2D, and Ia glycoproteins immunoprecipitated from ^{35}S-methionine-labeled cells reveal that these proteins exist in the cells as complex

arrays of molecules heterogeneous in both size and charge. Lactoperoxidase-catalyzed radioiodination of lymphocyte surfaces labels only subsets of the total H-2 and Ia molecules with ^{125}I, indicating that some of the molecules may represent cytoplasmic precursors of the cell surface proteins. This theory is supported by the kinetics of labeling of various spots in ^{35}S-methionine pulse-chase experiments. The 2-D gel patterns obtained for both H-2 and Ia antigens have also been shown to be haplotype-specific and independent of the genetic background.

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