Differential Regulation of Mouse H-2 Alloantigens†

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ABSTRACT: The expression of H-2K and H-2D antigens of the major histocompatability complex was examined in different tissues of mice sharing the *H-2*^{k} haplotype. Mouse H-2K^k and H-2D^k alloantigens are cell-surface glycoproteins that are heterogeneous with respect to size and charge, and they can be distinguished from each other by two-dimensional gel electrophoresis of immunoprecipitates. Hepatocytes, lymphocytes, and macrophages of mice contain a set of *H-2*^{k} alloantigens that also are distinguishable among the different cell types. The total set of either H-2D or H-2K glycoproteins differs in charge among each of these cell types but not in molecular weight. The difference in charge appears to be due to differences in extent of sialylation among the entire set of alloantigens expressed by each cell type. The extent of sialylation is highest in liver, relative to lymphocytes and macrophages. Precursors for the H-2 glycoproteins were identified in pulse–chase experiments with [^{35}S]methionine and by electrophoretic analyses of immunoprecipitates in two-dimensional polyacrylamide gels. The precursor forms of these glycoproteins can be distinguished from the mature forms mainly by differences in content of sialic acid residues. The H-2K and H-2D glycoproteins in the C3H mouse with the *H-2*^{k} haplotype appear to be regulated in a type of coordinate manner. They are always expressed at a constant ratio of 1:2 (H-2K:H-2D) regardless of cell type, and this ratio is preserved among congenic strains of mice sharing the same haplotype as the C3H strain. In spleen cells, the mature glycoprotein of H-2K accumulates at a faster rate than the mature H-2D glycoprotein. There also exists an intracellular pool of H-2 alloantigens consisting of at least 80% of the total cellular content of H-2K and H-2D. The constant ratio of 2:1 (H-2D:H-2K) was not exhibited in cells isolated from thymus of C3H/HeHa mice whereas their counterparts from thymus of AKR/Sn do retain this 2:1 ratio. The turnover of cell H-2 alloantigens was examined and compared among the different cell types of the *H-2*^{k} mice. In macrophages, turnover was biphasic with a half-life for turnover of total cellular H-2 glycoproteins of about 6 h in the rapid phase. In isolated hepatocytes the turnover of total cellular H-2 glycoproteins also exhibited biphasic kinetics with the fast components having a half-life of about 12–14 h for H-2D and H-2K. Although the overall rate of turnover of the total H-2 glycoproteins appears to vary among the two cell types, the H-2D and H-2K glycoproteins turn over in a relatively synchronous fashion relative to each other, again suggesting some type of coordinancy in the regulation of concentration of the two membrane glycoproteins in hepatocytes and in macrophages.

Alloantigens of the major histocompatability complex (MHC) or *H-2* complex of the mouse are glycoproteins located in the cell plasma membrane. They are present in relatively large amounts in various tissues of the mouse, including lymphocytes, thymocytes, macrophages, and hepatocytes. These cells can be easily obtained as single cell suspensions in which the H-2 glycoproteins can be labeled both by metabolism and by external methods. Furthermore, these proteins are very antigenic, and antibodies to them, both monoclonal and conventional, are already available or can be readily prepared. Moreover, the *H-2* complex is a genetically well-defined region with a large degree of polymorphism (Hyafil & Strominger, 1979; Klein, 1979). Many of the variants are on congenic backgrounds and are available for biochemical analysis. In fact, much attention is being directed toward the biochemical analysis of the H-2K and H-2D proteins themselves and the structural genes that encode them (Klein, 1975). All of these properties make the *H-2* system a good one to study the mechanisms, both genetic and biochemical, used by the cell to regulate the concentration of glycoproteins in its plasma membrane.

The synthesis of the H-2 glycoprotein antigens has already been examined by labeling cells in primary culture with radioactive amino acid precursors. The majority of H-2 antigens are synthesized in microsomal fraction on membrane-bound polysomes (Wernet et al., 1973), suggesting that the H-2 cell-surface antigens eventually are incorporated into the surface membrane by fusing of microsomally derived membrane material with the plasma membrane. By a combination of isoelectric focusing and discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis in a two-dimensional separation system, lymphocyte H-2 and Ia antigens were shown to consist of heterogeneous sets of related molecular forms (Jones, 1977). The different molecular forms may exist in different membrane compartments of different cells. Studies on the time required to regenerate these molecules at the cell surface after they have been perturbed either with enzyme or with specific antibody suggest that they exist in a highly dynamic state. For example, tumor cells treated with papain were able to regenerate the degraded antigen beginning at about 1.5 h, and the cells attained their full antigenicity 6 h later (Schwartz & Nathenson, 1971). Also, modulated cells in which H-2 antigens were "down regulated" by antibodies were able to recover their full antigenicity within 2 h. Whether the cell accomplishes the replacement of these antigens after perturbation by de novo synthesis or by recruitment from intracellular compartments is not known.

In this paper, we examine the expression of the two related membrane glycoproteins encoded by the D and K region of the mouse MHC. To help elucidate the mechanism used by the cell to regulate the concentration of these proteins in the plasma membrane, we determined whether there is any synchrony in the synthesis and insertion of these proteins in the plasma membrane and, if so, whether this synchrony is specified at the level of the gene. Using two-dimensional electrophoresis in polyacrylamide gels, we show the existence of differential secondary modifications among the H-2 antigens

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0006-2960/82/0421-5730$01.25/0

of different cell types. Pulse–chase experiments with labeled methionine indicate that the D and K polypeptides appear at the cell surface at different rates. Furthermore, there appears to be an imbalance in the expression of H-2 alloantigens in thymocytes of C3H/HeHa mice compared to those of spleen cells, macrophage, and hepatocytes from the same animal. Moreover, this imbalance was not observed in AKR/Sn mice. However, both glycoproteins appear to turn over in a relatively synchronous fashion. The turnover of these polypeptides in hepatocytes and macrophages is biphasic in nature. In contrast, H-2K^k antigens were found to be rapidly turned over and shed by CBA/J spleen cells whereas the turnover of H-2D^k antigens was extremely slow (Emerson et al., 1980).

Experimental Procedures

Cells. Mouse spleen cells were isolated as described by Jones (1977). Peritoneal macrophages were collected from mice 4 days after the intraperitoneal injection of 1 mL of thioglycollate broth (Difco) (Cohn & Benson, 1965). Cells were seeded at 10^7 cells/25-cm^2 flask in Dulbecco's modified minimal essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum (Gibco) and were washed extensively for 2 h and again 15 h later with this medium to remove nonadherent cells. Primary mouse hepatocytes were isolated by a modified method of Seglen (1976).

Radiolabeling of Cells. Lactoperoxidase-catalyzed iodination of cell-surface molecules was done essentially as described by Kessler (1975). Spleen cells (10^7/mL), macrophages (3×10^6/mL), and hepatocytes (5×10^6/mL) were also labeled metabolically with [^{35}S]methionine in Dulbecco's modified minimal essential medium, containing only 10% of the original concentration of methionine required for growth, buffered with 25 mM Hepes[1] and supplemented with 4 mM L-glutamine, penicillin, streptomycin, and 10% heat-inactivated fetal calf serum. [^{35}S]Methionine (1000 Ci/mmol) was added to a final specific activity of 250 μCi/mL. The cells were incubated at 37 °C in a 10% CO_2 incubator for 15 h. Incorporation of labels was stopped by removal of medium, and the cells were washed 3 times with Earles' balanced salt solution.

Preparation of Nonidet P-40 (NP-40) Extracts. Washed [^{35}S]methionine- or ^{125}I-labeled cells were resuspended in 50 mM phosphate buffer, pH 7.6, containing 1% NP-40 from Particle Data Laboratories, Ltd. (10^8/mL for spleen, 10^7/mL for macrophages and hepatocytes) and were incubated for 30 min at 4 °C to extract membrane proteins. Insoluble material was removed by centrifugation at 150000g for 1 h. All three extracts were first combined with protein A–Sepharose (100 μL of packed beads/mL of NP-40 extract) for 1 h at 4 °C and then used immediately for immunoprecipitation.

Digestion of H-2 Glycoproteins with Neuraminidase. Membrane proteins of [^{35}S]methionine-labeled spleen cells used for neuraminidase digestion were extracted in 50 mM Tris-HCl, pH 6.5, containing 1% NP-40. Insoluble materials were removed by centrifugation, and the supernatant fluid was treated with protein A–Sepharose as described above. To this extract was added 4 units/mL neuraminidase (Sigma type VI), and the mixture was incubated at 37 °C for 45 min. At the end of the incubation period, the pH was raised to 8.0 to stop the reaction, and the H-2 glycoproteins were immunoprecipitated as described below. As a control, labeled spleen cells were also subjected to the same procedure except that neuraminidase was omitted during the 37 °C incubation period.

In another experiment, whole cells were also treated with neuraminidase. Briefly, [^{35}S]methionine-labeled spleen cells were incubated with 25 units/mL neuraminidase (Calbiochem) for 1 h at 37 °C in serum-free DMEM, pH 6.5. At the end of the incubation period, the cells were washed 3 times with Earles' balanced salt solution and then processed for immunoprecipitation as described above.

Immunoprecipitation of Radiolabeled Cell Proteins. Radiolabeled cell proteins were immunoprecipitated by the addition of 5 μL of antiserum to 250 μL of NP-40 extract in an Eppendorf polystyrene tube. After an overnight incubation at 4 °C, the mixture was added to 50 μL of packed protein A–Sepharose beads and was reacted for another 1 h at 4 °C. This procedure was found to be sufficient to bind all the antigen–antibody complexes generated by the amount of antiserum used. The Sepharose beads were then sedimented for 1 min at 12000g and washed twice with 50 mM phosphate buffer, pH 7.4, containing 0.1% $NaDodSO_4$ and 1% NP-40 and twice with 50 mM phosphate buffer, pH 7.4, containing 1 M NaCl and 1% NP-40. They then were transferred to a new polystyrene tube for the final wash in 50 mM phosphate buffer, pH 7.4, containing 1% NP-40. Bound antigen–antibody was eluted from the beads with 50–100 μL of isoelectric focusing sample buffer. After a 30-min incubation at room temperature, the protein A–Sepharose beads were again sedimented for 2 min at 12000g, and supernatant fluid was carefully removed for analysis.

Polyacrylamide Gel Electrophoresis. Immunoprecipitates were analyzed by the two-dimensional electrophoretic system developed by O'Farrell (1975).

Peptide Mapping by Limited Digestion of H-2 Molecules. The procedure for peptide mapping by limited proteolysis described by Cleveland et al. (1977) was followed.

Mice. AKR/Sw and CBA/Sw mice were from West Seneca Laboratories, Buffalo, NY, and C3H/HeHa mice were kindly provided by Dr. V. Chapman, Roswell Park Memorial Institute. BALB.K mice were generously provided by Dr. F. Lilly, Albert Einstein University.

Antisera. The antisera used for immunoprecipitation were obtained from the Immunobiology and Immunochemistry Branch of the National Institutes of Health. Their potential reactivities were as follows: [B10.A (2R) × C3H.SW] F_1 anti-C3H (D-32, anti D^k); (C3H.JK × HTG) F_1 anti-C3H-H-2° (D-5b-GS, anti D^k) [D-5b-GS was found to have the potential to react with both D^k and K^k]; and (B10 × LP-RIII) F_1 anti-B10.A (2R) (D-23, anti K^k).

Results

Differential Secondary Modification of H-2 Glycoproteins in Different Mouse Cells. H-2 alloantigens give a characteristic multispot pattern (Figure 1d) when resolved by electrophoresis on two-dimensional polyacrylamide gels. The H-2K^k glycoprotein immunoprecipitated from an NP-40 extract of labeled C3H spleen cells exhibits a set of four or more clearly visible, regularly spaced spots or forms whose molecular weights appear to be uniform. In some cases, the K polypeptide exists as doublets differing slightly in molecular weight (data not shown). Under the conditions of electrophoresis, the molecular weight of the K glycoprotein was estimated as 45000, in agreement with the values reported by others (Jones, 1977; Nathenson & Cullen, 1974). On the other hand, H-2D^k glycoprotein immunoprecipitated from spleen cells labeled as described above clearly exhibited two separate sets of regularly spaced forms (Figure 1d). One set consisted of only two resolvable forms focusing just above actin, a common, though

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic aid; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

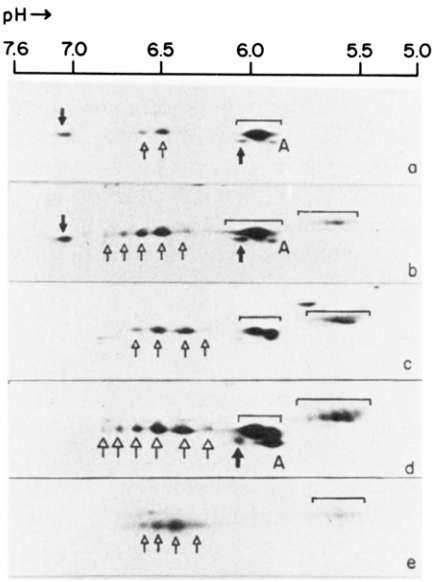

FIGURE 1: Kinetics of labeling of H-2K and H-2D and identification of their metabolic precursors. Proteins immunoprecipitated from C3H/HeHa spleen cells labeled with [^{35}S]methionine under pulse and chase conditions as well as surface ^{125}I-labeled proteins as resolved by two-dimensional gel electrophoresis are shown. The immunoprecipitates were from cells as follows: (a) 45-min [^{35}S]methionine pulse; (b) 2-h [^{35}S]methionine pulse; (c) 2-h [^{35}S]methionine pulse followed by a 15-h chase; (d) labeled continuously for 15 h; and (e) ^{125}I lactoperoxidase-catalyzed cell-surface radioiodination. The samples loaded per gel contained 33 600, 71 900, 48 570, 41 700, and 32 050 cpm, respectively. The basic end is on the left of the figure, and the direction of dodecyl sulfate–polyacrylamide gel electrophoresis was from top to bottom. This and all subsequent gels were impregnated with 2,5-diphenyloxazole prior to processing for autoradiography and exposure was for 10–14 days. Only the relevant portion of the two-dimensional gel is shown. The arrows pointing up indicate H-2K, and brackets indicate H-2D polypeptides as identified by monospecific alloantisera. Actin is denoted by A. The precursors are indicated by heavy arrows, pointing down for H-2K and pointing up for H-2D. The antiserum used was D.5b·GS.

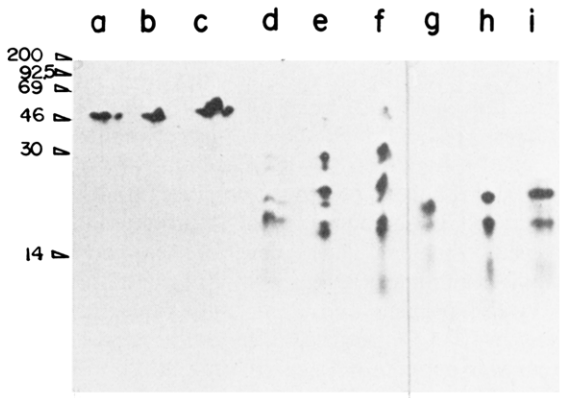

FIGURE 2: Peptide mapping of H-2 alloantigens immunoprecipitated from [^{35}S]methionine-labeled C3H/HeHa spleen extract. Immunoprecipitated proteins were resolved by two-dimensional gel electrophoresis, corresponding spots were cut out, and protease digestion was carried out as described under Experimental Procedures. Into lanes a, d, and g were loaded spots corresponding to H-2K^k. Into lanes b, e, and h were loaded spots corresponding to the smaller and more basic subset of H-2D^k, and into lanes e, f, and i were loaded spots from the larger and more acidic subset of H-2D^k. α-Chymotrypsin (35 μg) was added to lanes d–f and *S. aureus* (1 μg) was added to lanes g–i. No protease was added to lanes a–c.

variable, contaminant of the immunoprecipitates as also found by others (Jones, 1977). Toward the acidic end of the gel, a series of larger molecular weight and more acidic forms can be seen. Although the most acidic forms in these gels are difficult to resolve discretely, this more acid series of the H-2D^k antigens consisted of five or more forms.

To explore possible precursor–product relationships between the two subsets of the H-2D glycoproteins in more detail, we examined the sequence of labeling of the different forms of the glycoprotein antigen in a series of labeling experiments with [^{35}S]methionine. C3H spleen cells were labeled for 0.75, 2, and 15 h with [^{35}S]methionine. After the labeling period, the cells were washed free of label. Those that were labeled for 2 h were then cultured for 15 h in complete medium containing unlabeled methionine. In another experiment, C3H spleen cells were labeled with ^{125}I iodination catalyzed by lactoperoxidase. After iodination, only a subset of the H-2 glycoproteins are labeled in agreement with earlier reports by Jones (1977). Comparison of the patterns obtained from radioiodinated cells (Figure 1e) with those obtained from cells labeled continuously for 2 (Figure 1b) and 15 (Figure 1d) h with [^{35}S]methionine reveals that the ^{125}I becomes incorporated into all but one of the total H-2K molecules but only one subset of the H-2D series. The H-2K series isolated from cells labeled continuously for a short period (0.75 and 2 h) instead of the usual 15 h reveals a basic spot of slightly lower molecular weight than the mature H-2K polypeptides that was absent from the 15 h labeled H-2K series and also was not accessible for radioiodination (Figure 1e).

In the two-dimensional polyacrylamide gel electrophoretic pattern of the glycoproteins obtained from spleen cells that were labeled for up to 2 h, the larger and more acidic subset of H-2D molecules is missing or greatly reduced (Figure 1b). The H-2D molecules immunoprecipitated during the short labeling period could represent potential precursors of the mature H-2D glycoproteins. If so, as [^{35}S]methionine is chased with cold methionine, the label should move progressively into the larger and more acidic mature forms. This is in fact the case as shown by labeling cells for 2 h with [^{35}S]methionine followed by a chase for up to 15 h (Figure 1c). The finished product of H-2D biosynthesis could be detected only after a 2-h pulse (Figure 1b). In comparison, mature forms of H-2K glycoproteins are already present at the end of the 45-min pulse (Figure 1a). However, the subset of labeled molecules that we believe to be the precursors of H-2D glycoproteins still shows significant amounts of [^{35}S]methionine radioactivity at the end of a 15-h chase (Figure 1c). Furthermore, this subset has at this time been resolved into a doublet. Since only the larger and more acidic subset of the H-2D glycoproteins is accessible to cell-surface radioiodination, the other subset that shows significant incorporation of [^{35}S]methionine even after a short labeling period is believed to be an intracellular precursor of the mature more acidic series of H-2D and also may represent a reservoir of this glycoprotein on intracellular membranes.

To show that the two subsets of H-2D glycoprotein were homologous in primary sequences, we examined the digestion patterns of the two subsets of H-2D produced by different proteases. The peptide patterns generated by α-chymotrypsin and *Staphylococcus aureus* protease are shown in Figure 2. α-Chymotrypsin digestion clearly generates three major and one minor band with apparent molecular weights ranging from 32 000 to 13 000. Digestion with *S. aureus* protease generates two major bands with apparent molecular weights of 22 000 and 17 000. In each case, the bands are all shared by the subsets of the H-2D series. The difference in molecular weight between the bands in lanes e and f is not surprising since it reflects the same difference in molecular weight between the two undigested subsets of H-2D^k. In contrast, digestions of H-2K^k by the same protease did not generate the same pattern.

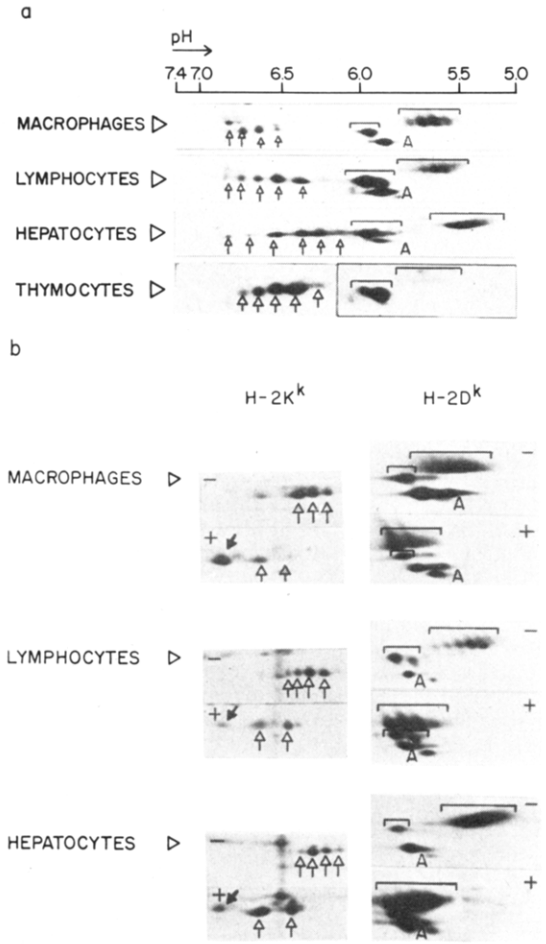

FIGURE 3: (a) Expression of H-2D and H-2K antigens in C3H/HeHa spleen cells, macrophages, hepatocytes, and thymocytes. Immunoprecipitated proteins from [^{35}S]methionine-labeled macrophage, lymphocyte, hepatocyte, and thymocyte extracts are shown. The samples applied to the gel contained 39 000 cpm of incorporated radioactivity for macrophages, 46 000 cpm for lymphocytes, 25 000 cpm for hepatocytes, and 20 000 cpm for thymocytes. Heterogeneity in size and charge of the resolved glycoproteins is preserved between tissues, but there is a major difference between tissues by virtue of the presence of one or more units of charge (presumably due to sialic acid residues) on the glycoproteins rendering them more or less acidic. Sialylation is extensive in hepatocytes and lymphocytes, less so in macrophages. In thymocytes the larger and more acidic subset of H-2D is virtually absent. (b) Neuraminidase digestion of NP-40 extracts of [^{35}S]methionine-labeled spleen cells, macrophages, and isolated primary hepatocyte from C3H/HeHa mice. The digestions were done as described in the text. Heavy arrows pointing up show precursors for H-2D, and heavy arrows pointing down show precursors for H-2K.

These data taken together show that these subsets of H-2D glycoprotein are indeed related even though they appear to be distinct in their cellular localization.

When the two-dimensional electrophoretic pattern of the H-2 alloantigens from C3H spleen cells was compared with its counterpart from macrophages of the same strain, the H-2K^k and the acid subset of the H-2D^k glycoproteins of the spleen cells appear to be more acidic by at least two charge units (Figure 3a). The sets of H-2^k glycoproteins of hepatocytes were even more acidic. However, there was little difference in charge among the different cell types in one subset of the H-2D series, the more basic set with p*I* between 5.8 to 6.0. When this comparison was extended to the other two strains sharing the *k* haplotype discussed above, the same relative differences in charge heterogeneity among the resolved glycoproteins of the different cell types was found (data not shown).

To ascertain whether covalently bound sialic acid contributes to the heterogeneity of the H-2 glycoproteins, we treated detergent extracts with neuraminidase prior to immunoprecipitation and electrophoresis. In each case, this treatment altered the electrophoretic mobility of both the H-2K and H-2D glycoproteins in the two-dimensional gel system (Figure 3b). In no case did these molecules convert into a single electrophoretic spot as a result of neuraminidase treatment. Removal of sialic acid residues from H-2K molecules not only causes a shift toward the more basic end of the two-dimensional gel but also generates additional basic spots as seen in Figure 3b. One of these spots is very similar to the single basic form of this series identified earlier as the possible intracellular precursor for H-2K. On the basis of the labeling kinetics with methionine and lack of accessibility for lactoperoxidase-catalyzed iodination, our interpretation is that the more basic spot represents an intracellular precursor of the H-2K series that becomes modified as H-2K moves out to the cell surface. The shift in distribution of label with time toward the acidic end of the gel would be due to the addition of one or more sialic acid residues to this core sugar.

Treatment with neuraminidase also causes the disappearance of the larger more acidic forms of the H-2D series (Figure 3b). As expected, there was a shift in mobility toward that of the more basic H-2D subset or the subset that showed a signficant incorporation of [^{35}S]methionine following a labeling period of only 45 min. Under the conditions of neuraminidase digestion, only an incomplete removal of sialic acid residues was achieved, thereby generating a new subset of molecules migrating in about the same area of the gel as the intracellular H-2D^k forms. A difference in molecular weight of about 3000 could be attributed to the presence of a whole carbohydrate side chain. Furthermore, a faint spot (heavy arrow pointing up in Figure 1b,d) to the immediate left of the cytoplasmic H-2D becomes more intense after neuraminidase treatment, indicating that a significant number of the larger and more acidic molecules were converted to this spot (result not shown). The H-2D tentatively identified as the intracellular form appears to represent only an intermediate product between actual precursor and mature H-2D.

Differential Expression of H-2 Glycoproteins in Different Mouse Tissues. The amount of H-2D^k relative to H-2K^k polypeptides in spleen cells can be estimated from relative quantitation of the total methionine incorporated into the spots on the gel after elution. In spleen cells, the total amount incorporated into the H-2K series was always about half that incorporated into the H-2D series. The total amount incorporated in the four or five clearly differentiated members of the H-2K series was equal to that of each subset of the H-2D series, and in the H-2D series, the total methionine incorporated into the basic subset migrating around actin was equal to that incorporated into the more acidic subset of H-2D glycoproteins. It is possible that the difference in the relative amounts of H-2D and H-2K observed here is not due to an imbalance in the expression of the H-2 antigens but rather to a difference in the number of methionines in each molecule. This is not the case since similar results were obtained when an ^{3}H-labeled amino acid mixture was used instead of [^{35}S]methionine. Furthermore, this relative ratio of methionine incorporated into various subsets of the H-2K and H-2D glycoproteins is also maintained in macrophages and isolated primary hepatocytes (Table I) of C3H/HeHa mice. Similar results were obtained with the same tissues in AKR/Sn and in BALB.K mice (results not shown). The latter is a congenic line to C3H in which the H-2 region of C3H *H-2^k* haplotype

Table I: Relative Ratio of H-2D to H-2K in Different C3H/HeHa Tissues[a]

	K^k (cpm)	D^k (cpm)	D^k/K^k
macrophages	2156	5080	2.36
lymphocytes	2422	6161	2.54
hepatocytes	1996	4216	2.11
thymocytes	1925	1541	1.25

[a] Corresponding spots from the autoradiograms were cut from the gels shown in Figure 3a, and the radioactivity was eluted from the gel slices with Protosol (NEN). [^{35}S]Methionine radioactivity was then counted in a Beckman LS-7000 liquid scintillation counter.

Table II: Relative Ratio of H-2D to H-2K from Cells Isolated from Spleen and Thymus of AKR/Sn and C3H/HeHa Mice[a]

	D^k/K^k	
	AKR/Sn	C3H/HeHa
lymphocytes	1.89 ± 0.35	2.17 ± 0.26
thymocytes	1.90 ± 0.24	1.37 ± 0.15

[a] The values are the mean ± SEM of seven measurements for lymphocyte from AKR and C3H and four measurements for thymocyte from AKR and C3H.

is present on a BALB/c background. However, this ratio was found to be 1.37 ± 0.15 to 1 (H-2D to H-2K) for cells isolated from the thymus of C3H/HeHa mice. In contrast, a ratio of 1.90 ± 0.24 to 1 was obtained from the same cells isolated from AKR/Sn mice (Table II).

Cellular Distribution of H-2 Glycoproteins. The existence of an intracellular pool of H-2D comprising at least 50% of the total cellular H-2D prompted us to look for the existence of a similar pool for the H-2K glycoproteins. Neuraminidase treatment of soluble H-2 glycoproteins generates a new set of H-2K and H-2D glycoproteins that differ from the nontreated proteins in electrophoretic mobility on two-dimensional gel electrophoresis. Hence, neuraminidase treatment of labeled whole cells prior to analysis of the immunoprecipitated H-2K and H-2D on two-dimensional gels can show that the H-2 alloantigens exist in two distinct cellular compartments, one of which is accessible to the enzyme. Figure 4 shows the difference in electrophoretic pattern on two-dimensional gels of cell-surface H-2D and H-2K immunoprecipitated from neuraminidase-digested whole cells. In these cells, the cell-surface components were labeled with ^{125}I. As expected, there was a significant shift of both H-2D and H-2K from neuraminidase-treated whole cells toward the more basic end (Figure 4a) of the gel. Furthermore, this treatment was sufficient to remove the maximum number of sialic acid residues from all iodinated cell-surface H-2 glycoproteins while cell viability remained unaltered. Since the presumptive intracellular pool of H-2D and H-2K will not be affected by this type of treatment, and if such a pool does exist, the expected pattern of H-2D and H-2K from neuraminidase-treated whole cells would appear as a composite of gels a and b of Figure 4 but different from the pattern generated by neuraminidase digestion of soluble H-2 glycoproteins as shown in Figure 3. The two-dimensional pattern of H-2 glycoproteins immunoprecipitated from [^{35}S]methionine-labeled, neuraminidase-treated, and untreated cells is shown in Figure 4c,d. Again, as expected, the H-2 glycoproteins isolated from the neuraminidase-treated cells appear to be very complex. Although the basic pattern is similar to that of the untreated cells, there are noticeable differences. The more acidic spots of either the H-2K or H-2D series now appear less intense on the autoradiogram, while the somewhat fainter basic spots now appear more intense. Moreover, neuraminidase digestion of cell-surface glycoproteins has also generated several new more basic spots in both the H-2D and H-2K series. These data taken together suggest that a proportion of the more acidic H-2D and H-2K series has been converted to their more basic counterparts due to the loss of one or more sialic acid residues thereby generating the new pattern shown in Figure 4c,d. If this is indeed the case, the remaining acid spots should in part represent those that belong to the intracellular pool that is not accessible to neuraminidase. By measuring the loss of radioactivity asociated with the more acidic spots and by comparing this loss to the unmodified H-2K and H-2D series, one can estimate the size of the internal pool. It was found that in AKR/Sn spleen cells, at least 80% of the total H-2 glycoproteins represent the intracellular pool (Table III) whereas the internal pool for H-2K and H-2D in cells isolated from the thymus of the same animal was estimated to be about 75–85% of the total H-2K and H-2D present in the cell. This is not surprising, since in the case of H-2D we suggested earlier from the pulse-chase and cell surface labeling experiments that at least 50% of the total H-2D resides in some intracellular compartments of the cells.

Turnover of H-2 Alloantigens in Different Mouse Tissues. Previous studies on the time required to regenerate surface

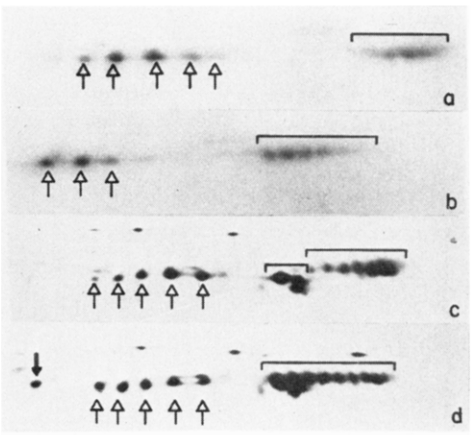

FIGURE 4: Identification of an intracellular pool of H-2 alloantigens by selective removal of sialic acid from their cell-surface counterpart. Labeled spleen cells were treated with neuraminidase at 25 units/mL in DMEM serum-free medium at pH 6.5. The cells were washed 3 times with Earles' balanced salt solution and then lysed with 1% NP-40 in 50 mM Tris-HCl, pH 7.5. Insoluble materials were sedimented by high-speed centrifugation. Immunoprecipitation and two-dimensional gel electrophoresis analysis of H-2K and H-2D were as described in the text. Shown are the two-dimensional patterns of H-2K and H-2D from iodinated cells (a) not treated with neuraminidase and (b) treated with neuraminidase for 60 min at 37 °C and from [^{35}S]methionine-labeled spleen cells (c) not treated with neuraminidase and (d) treated with neuraminidase for 60 min at 37 °C.

Table III: Percent Total Cellular H-2 Antigens[a]

		AKR/Sn (%)	C3H/HeHa (%)
spleen:	cell-surface D^k	20.3	nd[b]
	cell-surface K^k	20.2	nd
thymus:	cell-surface D^k	13.9	5.7
	cell-surface K^k	25.6	13.8

[a] Cell-surface H-2 antigens were determined by measuring the amount of radioactivity lost from the more acidic spots from the H-2K or H-2D series in cells that were treated with neuraminidase. This value was compared to the radioactivity in the glycoprotein sets in the untreated cells as shown in Figure 4 and is expressed as a percentage of the total methionine incorporated into the whole H-2K or H-2D series, respectively. [b] nd, not determined.

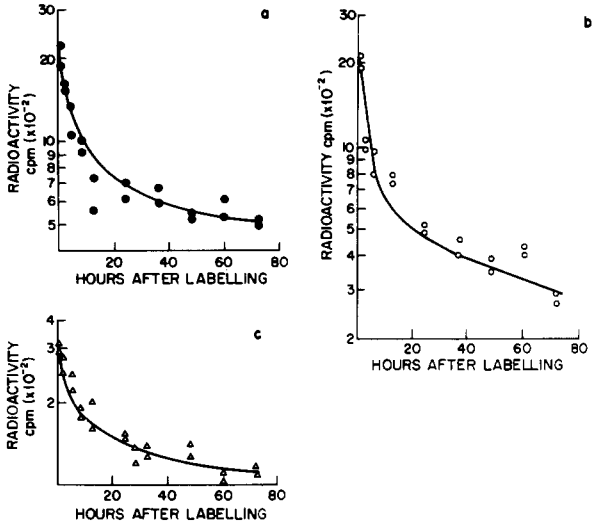

FIGURE 5: Loss of radioactivity from [^{35}S]methionine-labeled proteins from macrophages during a prolonged chase in culture. The macrophages were isolated from mice previously injected intraperitoneally with 1 mL of thioglycollate broth, plated at 10^7 cells/25-cm^2 flask, and labeled for 15 h (time 0): (a) (●) loss of [^{35}S]methionine-labeled total H-2K; (b) (○) loss of H-2D; (c) (△) loss of total [^{35}S]-methionine-labeled protein. The data shown are a representative example of one of four similar experiments.

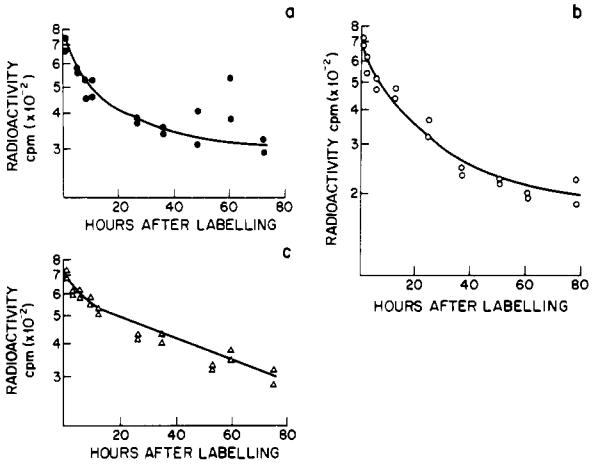

FIGURE 6: Loss of radioactivity from [^{35}S]methionine-labeled hepatocytes during a prolonged chase in culture. Hepatocytes were isolated by a modified method of Seglen (1976), plated at 5×10^6 cells/25-cm^2 flask precoated with biomatrix isolated from rat liver, and labeled for 15 h (time 0): (a) (●) loss of [^{35}S]methionine-labeled H-2K; (b) (○) loss of H-2D; (c) (△) loss of total [^{35}S]-methionine-labeled protein. The data shown are a representative example of one of two similar experiments.

H-2 molecules after perturbation (Schwartz & Nathenson, 1971) suggested that these molecules exist in a highly dynamic state. For example, after removal of cell-surface antigens with papain, replacement of the lost antigens was completed by 6 h. We have also found that H-2D and H-2K differ in the time required for posttranslation modifications, suggesting a different rate of biogenesis for the H-2D and H-2K molecules. To explore reasons for the differences in biogenesis in more detail, we measured and compared the rate of turnover of these two proteins in macrophages and hepatocytes. We chose to conduct these studies in thioglycollate-elicited macrophages because they can be obtained as biochemically and morphologically homogeneous populations of cells that can be cultured in vitro under conditions promoting steady-state levels of total protein for up to 8 days (Skudlarek & Swank, 1979). Similarly, isolated mouse primary hepatocytes can also be obtained as a homogeneous population and, when cultured on rat liver biomatrix, show increased attachment, survival efficiencies, and retention of some hepatocyte specific functions (Rojkind et al., 1980).

Macrophages or primary hepatocytes from mice were isolated and then maintained for 24 h under the same conditions in which the experiment was carried out. They were then metabolically labeled with [^{35}S]methionine for 15 h, washed 3 times with serum-free medium, and then cultured for up to 72 h in medium that consisted of DMEM containing 10% fetal calf serum. At intervals, samples were removed for analysis of cell number, viability, and radioactivity in a detergent extract of the cells and in the H-2 alloantigens precipitated from these extracts with alloantibodies. Indirect immunoprecipitation of the antigen with specific alloantisera was carried out, and immunoprecipitates were analyzed by two-dimensional gel electrophoresis. The gels were exposed to fluorography on Kodak X-Omat film, and the radioactive spots were quantitated by measuring the total methionine incorporated into the spots on the gel after elution. The results are shown in Figures 5 and 6 in which the relative radioactivity is expressed as a function of time after labeling. These data indicate that H-2D and H-2K in macrophage were lost with biphasic kinetics (Figure 5). The rapid phase had a half-life of approximately 6 h for both H-2D and H-2K. On the other hand, the slow component had a half-life well beyond 100 h. Similarly, total cellular proteins in macrophages were shown to decay also with biphasic kinetics with half-lives of the fast and slow components of about 6 and over 100 h, respectively. Others have reported biphasic turnover for the surface proteins of macrophage with a value of 110 h for the slow phase and a half-life of about 15 h for the fast phase (Kaplan et al., 1979). We also examined the loss of labeled H-2 alloantigens from isolated primary hepatocytes in which cellular proteins were metabolically labeled with [^{35}S] methionine. As shown in Figure 6, the loss of labeled H-2 alloantigens also exhibited biphasic kinetics, with a fast component of 12 and 14 h for H-2D and H-2K, respectively, compared to approximately 10 h for total cellular proteins. However, in each case, H-2D and H-2K appear to turn over in a relatively synchronous fashion. Moreover, during the course of the experiment, which lasted over 5 days, cell viability was always greater than 90%.

Discussion

The overall goal of the studies presented in this paper is to unravel the mechanism by which different mouse cells regulate the concentration of two structurally related externally oriented glycoproteins in their plasma membrane. These two glycoproteins, H-2D and H-2K, are products of the *D* and *K* structural genes, which delineate the right and left ends of the major histocompatibility (MHC) or *H-2* complex on chromosome 17 of the mouse. This complex is quite large in the mouse, and there is sufficient DNA between the *D* and *K* regions of the complex to accommodate a large number of genes (Klein, 1979). The *D* and *K* regions of this complex most likely arose via a gene duplication(s) followed by separation and some divergence on chromosome 17. An obvious and important question arises of whether the *D* and *K* regions function independently or whether in the terminology of Klein (1975) they collaborate with each other in the way in which their expression is regulated in the cell. However, to eventually work out the mechanism of regulation of expression by this complex locus, more basic knowledge about the structure of the glycoproteins, their tissue distribution, and their concentration relative to each other in different cell types of the mouse is needed. Hence, in this paper, we have employed immuno-

logical methods relying on different alloantisera to isolate the H-2 glycoproteins from three different cell types. Two-dimensional electrophoretic methods in combination with metabolic and in situ surface labeling were then used to resolve and to quantitate the different components of the H-2D^k and H-2K^k glycoproteins. Finally, the turnover behavior of the two glycoprotein sets was assessed and compared in the different cell types.

The H-2 antigens immunoprecipitated from detergent extracts of cells metabolically labeled with [^{35}S]methionine migrate in two-dimensional gel electrophoresis as groups that show variability in both size and isoelectric point. The heterogeneity in charge observed on the autoradiogram of these molecules is attributed mainly to the addition of one or more sialic acid residues, since treatment with neuraminidase causes a shift toward the more basic end. Heterogeneity in charge exhibited by HLA and HLB was also attributed to different degrees of sialylation (Parham et al., 1974). In addition to the multiple electrophoretic forms seen in each tissue, there are major differences between tissues, notably between lymphocytes and macrophages. In lymphocytes, the entire multiple-spot pattern is shifted more toward the anode after isoelectric focusing. The tissue differences are apparently due to differential sialylation of the alloantigens and are nongenetic in origin. These results also suggest that all of the multiple forms of H-2 alloantigens, both within and between tissues, are derived from a common gene(s) whose products undergo modification. This modification is tissue specific and is preserved among inbred lines sharing the same haplotype, including a line carrying the same *H-2^k* haplotype on a totally different genetic background [the BALB.K line (data not shown)].

We were able to clearly show that the H-2D molecules exist as two distinct subsets in which only the larger and more acidic molecules were accessible to radioiodination at the cell surface in agreement with Jones (1977). We propose that the less acidic subset comprises an intracellular membrane compartment(s) of H-2D molecules. In pulse–chase experiments with methionine, the H-2D^k molecules immunoprecipitated from cells labeled for 0.75 and 2 h with [^{35}S]methionine exist only as the smaller less acidic forms. Following a 15-h chase with cold methionine, the label progressively moved toward the larger and more acidic spots that were also accessible to lactoperoxidase-catalyzed iodination. Since this method only labels cell-surface proteins, the iodinated subset reflects cell-surface H-2D whereas the other subset is oriented in such a way that they are not accessible for the iodination probe (or for digestion with neuraminidase), which we consider as not likely, or they must reside on intracellular membrane(s) as do many other plasma membrane proteins (Baumann & Doyle, 1979; Doyle & Baumann, 1979). This distribution is also consistent with what is known about the biosynthesis of the H-2 alloantigen (Wernet et al., 1973). Furthermore, both subsets of H-2D still show quantitatively signficant amounts of [^{35}S]methionine radioactivity following a 15-h period in chase medium. The presence of two related subsets of H-2D glycoproteins was confirmed by using a different anti-H-2D^k antiserum [(C3H·KK × HTG) F_1 anti-C3H-H-2^0], which reacts only with H-2D^k. The pulse–chase and peptide mapping experiments also support the interpretation that the more basic subset of H-2D antigen is indeed related to the H-2D subset composed of the larger and more acidic molecules.

From the neuraminidase digestion of [^{35}S]methionine-labeled whole cells and analysis of the immunoprecipitated H-2 by two-dimensional gel electrophoresis, we were able to show that at least 40% of the larger and more acidic subset of H-2D^k (or 20% of the total H-2D^k series) represents the cell-surface H-2D^k. In a similar experiment, we have also identified two populations of H-2K^k in which the cell-surface population represents only 20% of the total cellular H-2K. It could be argued from these experiments that the estimations of cell-surface H-2 alloantigens only represent a specific population of cell-surface H-2D and H-2K that is easily accessible to lactoperoxidase-catalyzed iodination and also sensitive to neuraminidase digestion. The true cell-surface population has been reported to consist of about 80% of the total cellular H-2 alloantigen (Wernet et al., 1973; Vittetta & Uhr, 1975). However, a major drawback in these reports is that the distribution of H-2 alloantigens among various cells organelles has been studied by immunolabeling and cell fractionation. It is almost impossible to obtained a pure fraction of any one organelle; the fractions are always contaminated, and this contamination makes interpretation of the results obtained by this method difficult. In the present studies, the data from the pulse–chase experiment taken together with those from the neuraminidase-digestion experiment argue in favor of the existence of an intracellular pool of H-2D^k that represents between 60% and 80% of the total cellular H-2D. In this respect, the presence of an intracellular pool of H-2K in addition to the H-2D pool is not unlikely.

Most significantly there appears to be some basic differences in the biosynthesis of the H-2D and H-2K region gene products. One difference is apparent at the posttranslational modification level. Although H-2D and H-2K proteins appear to be synthesized intially at the same rate, sialic acid residues are added to H-2K proteins at a much faster rate. From autoradiograms of the pulse–chase experiment (Figure 1), it takes less than 1 h for the cell to synthesize the complete product of H-2K. At least 2 h was required before any detectable level of the mature form of H-2D appeared on the autoradiogram. This difference in the biosynthesis of H-2K and H-2D is consistent with findings reported by others (Morre et al., 1979; Croze & Morre, 1981) that different membrane proteins have different flow rates in the same tissue.

H-2D was found to be always present in the cell at about twice the concentration of H-2K as assayed by the total amount of [^{35}S]methionine or ^{3}H-labeled amino acid mixture incorporated into the two sets of glycoproteins. While this ratio is maintained in splenocytes, macrophages, and hepatocytes isolated from C3H/HeHa mice, the same ratio was not observed in thymocytes from the same strain. C3H/HeHa thymocytes exhibited a ratio of 1.37 ± 0.15 to 1 (H-2D to H-2K). In contrast, AKR/Sn thymocytes do not show this ratio difference among the four tissues studied. This AKR/Sn inbred strain, which was developed by selecting for high incidence of leukemia, shows characteristic modifications of H-2 and Thy-1 in relation to age and leukemia development. In contrast to the high Thy-1/low H-2 levels on 2-month-old AKR thymocytes, thymocytes from 6-month-old mice and thymic leukemia cells frequently show low Thy-1/high H-2 surface phenotype (Kawashima et al., 1976). Normal thymocytes from mouse strains with a low incidence of leukemia do not show these changes. The quantitative variation between T and B cells in the expression of total cellular H-2 antigens presented here as well as the variation in cell-surface expression of H-2 antigens, which is believed to be allele specific (O'Neill & McKenzie, 1980), suggests that the regulation of these antigens is at the level of the gene. Moreover, the difference in expression of H-2D alloantigens between T cells of AKR/Sn and CoH/HeHa mice also suggests that this regulation may

be confined to just the K or D region.

However, other regulation could exist at the level of the cell membrane including cleavage, glycosylation, or H-2 binding affinity for β2-microglobulin. It is unlikely that these post-translational processes could account for this variation. Inhibition of glycosylation by tunicamycin did not affect the surface expression of the unglycosylated MHC antigens (Black et al., 1981; Ploegh et al., 1981). Both polypeptides had similar rates of turnover in hepatocytes and macrophages, indicating that neither of these two molecules are more resistant to degradation. Synthesis and insertion of the H-2 antigens into the membrane of the endoplasmic reticulum are not dependent on the expression or the concomitant synthesis of β2-microglobulin. Experiments with the Daudi cell line show that the antigen heavy chains are synthesized and processed normally in the absence of β2-microglobulin although these cells do not express these antigens at the cell surface (Ploegh et al., 1979). However, the present study reports a variation in the quantitative cellular expression of H-2D^k and H-2K^k and not a variation in cell-surface expression of these antigens. It could be argued here that the regulation of the expression of total cellular H-2 antigens may not be at the level of the cell membrane. In the light of recent evidence in the variation in the number of molecules expressed by different K and D alleles (O'Neill & McKenzie, 1980) along with the existence of mutants that no longer express a simple allele (Rayan & Flores, 1977; Vittetta et al., 1974), it appears, therefore, that one allele can be expressed independently of the other and may involve H-2-linked regulator genes. However, care should be taken in interpreting these results since antisera are used to follow antigenic determinant and not actual molecules. Small changes as a consequence of a mutation could result in an altered antigenic determinant, therefore, escaping detection by specific antisera.

Despite the difference in expression of H-2D and H-2K polypeptides, these two polypeptides are turned over in a relatively synchronous manner. They exist in a highly dynamic state with a rapid turnover and rate of biosynthesis in hepatocytes and macrophages. The half-life of between 6 and 15 h for the turnover of the fast component of the H-2 alloantigens in macrophages and hepatocytes, respectively, is consistent with the average turnover of total membrane proteins in these cell types. Similarly, β2-microglobulin exhibits the same rate of turnover as the H-2 antigen heavy chain (result not shown). Again, it should be stressed that in this case one is following antigenic determinant and not molecules. In this respect, it is possible that the antibodies used can only recognize the H-2 antigens as a heavy–light chain complex whereas as an individual subunit, they may escape detection. The possibility that the heavy and light chains may have totally different kinetics of turnover than the complex is not unlikely. Therefore, it is not yet clear as to what role β2-microglobulin might play in the turnover of the H-2 antigens except for its implication in the transport of the antigens to the cell surface. This rapid turnover could help explain the rapid reappearance of H-2 antigens on the cell surface after tumor cells had been partly denuded by papain digestion (Schwartz & Nathenson, 1971). However, we do not yet know whether this replacement is due entirely to the appearance of newly synthesized alloantigens or from a preexisting pool. An interesting question remaining to be answered is whether the loss of radiolabeled H-2 antigens is due to actual molecular degradation, sequestration within intracellular compartments, or shedding and whether different mechanisms for removal are used by the same cell, resulting in the biphasic kinetics of turnover seen among the different cell types. We are presently studying these possibilities. However, there appears to be some controversy over the mechanism by which H-2 antigens turn over in spleen cells. On the one hand, Vittetta & Uhr (1975) reported that H-2 antigens are not readily chased from the plasma membrane and are not shed or internalized during short-term culture. Similarly, Wernet et al. (1973) also found that H-2 antigens are neither secreted nor shed from the cell surface. Moreover, Vittetta et al. (1974) found that Thy-1 is rapidly shed from cultured thymocytes whereas H-2 alloantigens are not. On the other hand, H-2K^k antigens were found to be rapidly turned over and shed by CBA/J spleen cells, whereas the turnover of H-D^k antigens was extremely slow. Furthermore, there is variation in the shedding rates of H-2K and H-2D antigens, which is allele specific (Emerson et al., 1980). If this is indeed the case, the findings that the H-2 alloantigens turn over in a relatively synchronous fashion in macrophages and hepatocytes but not in spleen cells suggest that different mechanisms may exist for the removal of specific cell-surface proteins and may be controlled by specific genes mapping in the major histocompatibility complex (Klein, 1975). In the event that these molecules are not shed as reported by Emerson et al. (1980), the presence of a large intracellular compartment for H-2D that account for 50–80% of the total cellular H-2D points to a possible mechanism of sequestration and recycling of H-2D. Antibodies to membrane proteins as well as certain plant lectins such as concanavalin A can bring about a redistribution of the protein in the membrane and internalization of the membrane containing the pertubed proteins (Baumann & Doyle, 1980; Eldeman, 1976; Jacobson et al., 1976). There is evidence that the surface receptor for serum glycoproteins terminated in galactose is reutilized many times to deliver the galactose-terminated glycoproteins to the lysosome to be degraded (Warren & Doyle, 1980; Tanabe et al., 1979). In contrast, membrane receptors for polypeptide hormones and the hormone itself (Carpenter & Cohen, 1976; Kosmakos & Roth, 1978) may be degraded and replaced either by de novo synthesis or from a preexisting pool of membrane protein inside the cell. If the turnover of specific H-2 antigens is regulated at the level of the gene, it would be of interest to know whether an antibody-induced redistribution of the protein in the membrane and internalization of the membrane are controlled by the same genes. If not, what is the exact fate of the membrane and protein after antibody-induced internalization, and what is the mechanism by which the cell replaces specific proteins in its plasma membrane after ligand-induced interiorization? On the basis of the studies presented here on the regulation of the H-2 alloantigens in different cell types, we are presently attempting to answer these questions.

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Antibody Interaction with a Membrane-Bound Fluorescent Ligand on Synthetic Lipid Vesicles†

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ABSTRACT: The interaction of membrane-bound ligand with bivalent and monovalent fragments of monoclonal antibody was studied by fluorescence and precipitation analysis using synthetic lipid vesicles. The ligand N^{ϵ}-[5-(dimethylamino)-naphthyl-1-sulfonyl]lysine was linked to the hydrophobic anchor dipalmitoylphosphatidylethanolamine and ranged between 0.01 and 1 mol % of the membrane components. The effects of cholesterol on the specific interaction were observed over the range of 0-50 mol %. A precipitation assay was developed to evaluate various factors related to the cross-linking of small unilamellar vesicles by bivalent antibody. The cholesterol content was critical for this process as demonstrated by the increased efficiency of precipitation over the range of 0-40 mol % of this component. Fluorescence analysis yielded the parallel finding of increased accessibility of the ligand to the antibody with greater cholesterol content. Increased surface density of the ligand also was found to enhance the intervesicle interaction. Finally, a comparison of the kinetics by fluorescence analysis of the binding of monovalent and bivalent fragments indicated that the bivalent interaction involved primarily the cross-linking of vesicles in accord with published findings of the interaction of monoclonal antibody with cell membrane antigens.

Synthetic lipid vesicles (liposomes) provide model membranes for the analysis of antibody interactions at surface membranes. The defined composition of such membranes and their characterization with respect to the motion and distribution of the components of the membrane (Brûlet & McConnell, 1977) render feasible the effort to relate the quantitative aspects of antibody interaction to individual properties of the membrane. An early and useful demonstration of the utility of liposomal membranes for immunological study emerged from the finding that hapten-sensitized liposomes were damaged by interaction with specific antibody and complement (Kinsky, 1972; Six et al., 1973). This observation has been effectively exploited by McConnell and colleagues with liposomal membranes bearing a spin-label hapten reactive with specific antibody [e.g., see Parce et al. (1978)]. The present study was designed to compare bivalent and monovalent antibody fragments in bulk phase with respect to their interaction with a fluorescent ligand attached to a

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0006-2960/82/0421-5738$01.25/0