ISOLATION OF A CHROMATOGRAPHICALLY UNIQUE GLYCOPEPTIDE FROM MURINE HISTOCOMPATIBILITY-2 (H-2) MEMBRANE ALLOANTIGENS LABELLED WITH H^3-FUCOSE or H^3-GLUCOSAMINE

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Murine H-2 alloantigen glycoproteins from cultured mast cell tumor cells labelled with H^3-glucosamine or H^3-fucose, were solubilized by papain digestion and purified by a combination of conventional techniques and an antibody-antigen complex step. Sephadex G-50 chromatography of pronase digested radiolabelled H-2 alloantigen showed a single major glycopeptide of around 3,300 molecular weight, whereas glycopeptides from pronase digested unpurified membrane glycoproteins were heterogeneous upon Sephadex chromatography.

Glycoproteins, as integral components of mammalian membrane systems, are of great interest because of their probable role in specific functional features of the cellular surface such as cell association, contact phenomena, recognition of foreign or tumor cells, etc. However, since very few specific membrane glyco proteins have been purified and analyzed, there is relatively little information about the chemical nature of their carbohydrate portion.

The murine H-2 alloantigens form the major transplantation system in the mouse, and the products of the well-characterized H-2 genetic region are membrane located (cf. review by Snell and Stimpfling, 1966 and Shreffler, 1967). Soluble glycoprotein fragments (66,000 to 75,000 M.W.) carrying the H-2 alloantigenic sites have been isolated after digestion of cell membranes by papain, and have been purified by a series of fractionation techniques. They are homogeneous by disc gel electrophoresis, and contain 85 to 90% protein, about 5% neutral sugar, 3-4% glucosamine and 1-2% sialic acid.

The availability of these specific purified glycoproteins offered the unique opportunity to examine the chemical architecture of the carbohydrate portion of such membrane located macromolecules. This information might reveal features which membrane-located glycoproteins share generally, and also might reveal structural elements of the H-2 alloantigen relevant to its

immunological specificity.

Because of the very small quantities of purified H-2 alloantigen glycoproteins available, we utilized H^3-fucose or H^3-glucosamine as tracers to label the carbohydrate chains, and an antibody-antigen binding procedure as a final, rapid purification step. The preliminary findings of our studies on these products are presented here.

Materials and Methods: The inbred mouse strains, alloantisera preparation and measurement of alloantigenic activity by the method of inhibition of immune cytolysis have been described previously (Nathenson and Davies, 1966; Shimada and Nathenson, 1969). Three antisera or immunoglobulins (IgG) isolated from these antisera (gifts of A. Shimada) were used. Antiserum one contained activity against H-2 specificities 4,10,13 and 31; antiserum two against H-2 specificities 3,4,8,10,13 and 31; and antiserum three against H-2 specificities 2,5, 22 and 33. The Mastocytoma tumor ($H\text{-}2^d$; DBA/2) was a gift from Dr. Ted Brunner, Geneva, Switzerland and carries the following $H\text{-}2^d$ specificities: 3,4,6,8,10,13, 14,27,28,29 and 31. The cells were maintained in DBA/2 mice and cultured in suspension in minimum essential medium (Joklik-modified, Grand Island Biological Co.) with a two-fold enrichment of amino acids and 10% fetal calf serum. Thyroglobulin glycopeptide (Unit B) was kindly given by Drs. T. Arima and R. G. Spiro. Repurified pronase (type VI) was purchased from Sigma Chemical Co.

Results: Preliminary purification of the H^3-glucosamine and H^3-fucose labelled H-2 alloantigen glycoproteins was carried out by previous methods through the Sephadex G-150 chromatography stage (Shimada and Nathenson, 1969). The Sephadex eluate was electrophoresed in a 7.5% polyacrylamide gel (0.9 x 15 cm) according to the method of Davis (1964). Slices (0.5 cm) were eluted with 0.05M NaCl 0.01M Tris-HCl, pH 8.4, and the fractions containing the H-2 alloantigen were then pooled and further purified by immunological means to assure complete homogeneity with respect to H-2 alloantigenic properties.

For this latter step, we employed techniques identical to those used by Cullen and Nathenson (1969), except for the fact that our materials were la-

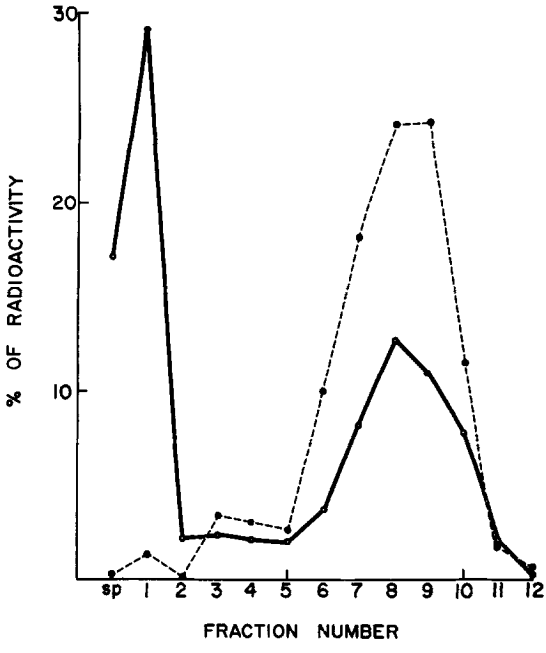

FIGURE 1: Disc gel electrophoresis of antigen-antibody reaction mixture. Disc gel electrophoresis was carried out by the method of Cullen and Nathenson (1969). Conditions for the electrophoresis were the same as described by Davis (1964) except the bridging reagent, bis-acrylamide, was replaced by ethylene-diacrylate. After the run, the gels (0.6 x 11 cm) were cut into 0.5 cm slices, dissolved in 1 ml of conc. ammonium hydroxide, and counted for radioactivity. ●——●: 3.8 μg of partially purified H-2 alloantigen labelled with H^3-fucose + γ-globulin (470 μg) from an anti-H-2.3,4,8,10,13,31 serum. o---o: 3.8 μg of partially purified H-2 alloantigen labelled with H^3-fucose + γ-globulin (1,400 μg) from an anti-H-2.2,5,22,33 serum. "Sp" represents the spacer gel.

belled in the carbohydrate portion rather than in the protein portion. Since H-2 alloantisera are non-precipitating, the antibody-antigen complex was obtained free from unreactive material by gel filtration or disc gel electrophoresis. An example of such complex formation is demonstrated in Fig. 1. The radioactive profile of two disc gel electrophoretic runs are plotted. One profile is of the partially purified H^3-fucose labelled antigen fraction reacted with an IgG fraction containing antibodies against specificities 4,10,13 and 31, which are found in this $H\text{-}2^d$ antigen. About 45% of the radioactivity has been complexed with antibody and now migrates in the spacer gel and first fraction, and an equivalent amount is lost from the region of fractions 6 to 10 (purified IgG electrophoresed under identical conditions is found in the spacer gel and

TABLE I: Specificity of Complex Formation Between H-2 Alloantigen and Antibody

H-2 Alloantiserum	Percent radiolabel in complex	
	H^3-fucose labelled partially purified H-2 alloantigen (3.8 μg)	H^3-glucosamine labelled partially purified H-2 alloantigen (3.6 μg)
Anti-H-2 Specificities 4,10,13,31	31.8	12.5
Anti-H-2 Specificities 4,10,13,31 preincubated with unlabelled H-2 alloantigen (18.7 μg)	0.9	0.5
Anti-H-2 Specificities 2,5,22,33	1.5	0.4

Partially purified H-2 alloantigen fractions labelled with H^3-fucose or H^3-glucosamine were prepared as follows:

For the H^3-fucose experiment 8.77×10^8 mast cell tumor cells were cultured in 900 ml of culture medium with 800 μc H^3-fucose (21.4 mc/mg, New England Nuclear Corp.). After 24 hrs an additional 900 ml of culture medium was added. After 48 hrs the cells (3.54×10^9 cells) were collected by centrifugation and washed once with 0.9% NaCl. 9.6% of the total H^3-fucose was incorporated in the cells. H-2 alloantigen from these cells was solubilized by papain digestion from crude membranes and purified by Sephadex G-150 column chromatography, followed by disc gel electrophoresis. The partially purified preparation contained 7,620 units of H-2.4,10,13 activity, 1.85×10^5cpm of radioactivity and 334 μg of protein.

For the H^3-glucosamine experiment 4.22×10^9 mast cell tumor cells were cultured for 48 hrs in 1.8 liters of culture medium with 1 mc of H^3-glucosamine (D-glucosamine-6-H^3, 5.25 mc/mg, New England Nuclear Corp.). The H-2 alloantigen was partially purified by the same procedure used for preparing the H^3-fucose labelled H-2 alloantigen. The partially purified preparation contained 10,600 units of H-2.4,10,13 activity, 5.42×10^5cpm of radioactivity and 420 μg of protein.

H^3-fucose or H^3-glucosamine labelled alloantigen was mixed with antiserum and subjected to disc gel electrophoresis. The radioactivity of the antigen-antibody complex was determined as described for Fig. 1.

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the first fraction). As shown in the second profile, purified IgG containing antibodies against H-2 specificities 2,5,22,33 (specificities which these $H\text{-}2^d$ tumor cells lack) complexed only 1% of radioactivity. Table I shows the specificity of such complex formation for both H^3-fucose and H^3-glucosamine labelled alloantigen preparations. Antisera against specificities 4,10,13 and 31 complexed 31.8% of H^3-fucose material and 12.5% of H^3-glucosamine material, whereas two specificity controls, one using antisera pre-incubated with unlabelled $H\text{-}2^d$ antigen, and one using antisera with activity against specificities not found in the antigen preparations (anti 2,5,22,33) complexed

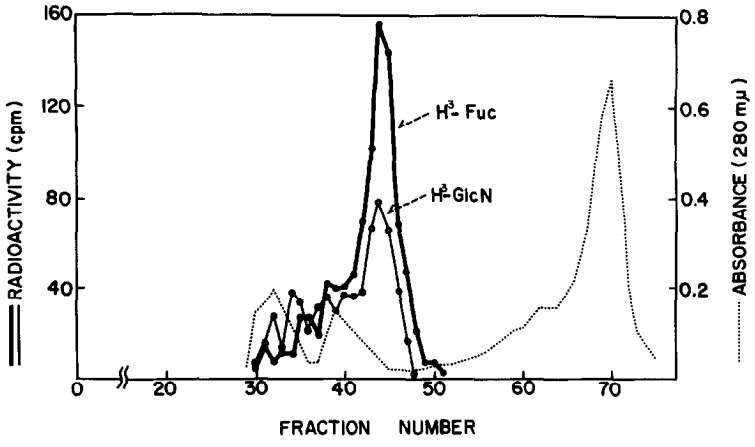

FIGURE 2: Gel filtration of glycopeptide preparations from H-2 alloantigens. a) H^3-fucose labelled purified H-2 alloantigen (1040 cpm) was hydrolyzed with 5 mg of pronase in 0.1 M Tris-HCl, pH 8.4 containing 0.135 M NaCl and 0.01 M $CaCl_2$ at 37°C with a small amount of toluene. After 24 hrs an additional 5 mg of pronase was added. After 48 hrs the digest was applied to the top of a column of Sephadex G-50, fine (0.9 x 110 cm) equilibrated with 0.05 M NaCl, 0.0033 M Tris-HCl, pH 8.4 buffer and eluted with the same buffer. b) H^3-glucosamine labelled purified H-2 alloantigen (550 cpm) was hydrolyzed with pronase and applied to the column of Sephadex G-50, in the same way as above. The elution pattern for both H^3-fucose and H^3-glucosamine labelled glycopeptides are plotted. In the other runs, blue dextran ($MW > 10^6$), fetuin glycopeptide (MW = 4,500), throglobulin glycopeptide (Unit B) (MW = 4,100), ovalbumin glycopeptide (MW = 1,550) appeared at fractions 28,41,42 and 51, respectively.

negligible amounts. Thus, we can conclude that the antibody-purified H-2 alloantigen which we have characterized contained only H-2 reactive material.

We prepared glycopeptides by pronase digestion of the purified H^3-labelled H-2 alloantigen and also of a crude membrane fraction from the cells. Analysis by Sephadex G-50 chromatography of the H^3-fucose and H^3-glucosamine labelled glycopeptides from the H-2 alloantigens showed essentially a single peak of identical elution volume for both preparations (Fig. 2). In contrast, a highly heterogeneous pattern was seen when glycopeptides of the crude membranes of the tumor cell were examined (Fig. 3).

For each allelic form of the H-2 gene, at least two glycoprotein fragments are released by papain digestion of the cellular membrane (Shimada and Nathenson, 1969; Yamane and Nathenson, submitted for publication). In the cells of $H\text{-}2^d$ genotype, one glycoprotein apparently carries at least the H-2

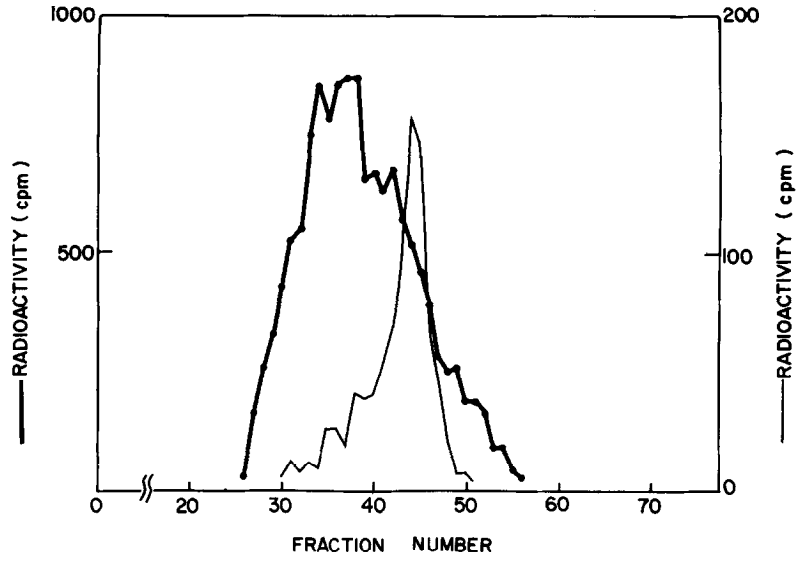

FIGURE 3: Comparison of glycopeptides from crude membrane fractions and from the purified H-2 alloantigen. The crude particulate membrane fraction of the mast cell tumor cell (250 µg) labelled with H^3-fucose was hydrolyzed with 5 mg of pronase as described for Fig. 2. After 72 hrs the reaction mixture was extracted with chloroform-methanol (2:1), the aqueous layer was evaporated to dryness, dissolved in 0.5 ml of distilled water and applied to the column of Sephadex G-50 as described in Fig. 2. For comparison, the chromatographic glycopeptide profile from the purified H^3-fucose labelled H-2 alloantigen shown in Fig. 2 is also plotted. •——•: Glycopeptides from crude membrane fraction. ——: Glycopeptides from H-2 alloantigen.

specificities 3 and 4 and probably H-2 specificities 10,13 and 28. The other glycoprotein apparently contains the antigenic site or sites of the H-2 specificity 31 (Cullen and Nathenson, 1969). Thus an antisera against H-2 specificities 3,4,8,10,13,31 would complex with both major glycoproteins. In the data of Figs. 2 and 3 we analyzed glycopeptides from a preparation containing both fragments. However, we also analyzed the H^3-labelled glycopeptides of the isolated specificity 31 fragment and found a single glycopeptide peak with the same chromatographic behavior as that obtained from the mixture of both H-2 alloantigen fragments from this $H\text{-}2^d$ tumor cell (data not shown).

By calibration of the Sephadex G-50 column with the glycopeptides from ovalbumin, from fetuin, and the unit B glycopeptide from thyroglobulin, we estimated by the method of Andrews (1964) that the molecular weight of the H-2 glycopeptide was about 3,300, with a range of error of the order of $\pm$ 500.

In the H^3-fucose experiments, the radioactivity was proven to be in fucose by acid hydrolysis and paper chromatography. In the H^3-glucosamine experiment 15% of the radioactivity was in sialic acid and 85% in glucosamine. About 70% of the sialic acid was N-glycolylneuraminic acid and the remainder N-acetylneuraminic acid.

Discussion: Our data show that fucose, glucosamine and sialic acid are present in the glycopeptides from H-2 alloantigenic glycoproteins which are purified by antibody complex formation. These results support and extend our previous findings of glucosamine and sialic acid in H-2 preparations purified by conventional techniques (Shimada and Nathenson, 1969).

From the estimate of 3,300 as the molecular weight of each glycopeptide, one can roughly estimate that there are about 12 to 15 carbohydrate residues, assuming that very few amino acids are still present due to the severe conditions for the pronase digestion. Since the molecular weight of the H-2 glycoproteins is about 66,000, of which 10% is carbohydrate (Shimada and Nathenson, 1969) each H-2 molecule would have approximately two carbohydrate chains.

The glycopeptides obtained by pronase digestion of the cell membrane glycoproteins are quite heterogeneous when examined by Sephadex G-50 chromatography. However, as we have shown, the glycopeptides from the pronase digests of the purified H-2 alloantigens are of a unique molecular size as judged by their Sephadex behavior. Thus, the glycopeptide portion of this specific membrane component is different in size from the glycopeptides of nearly all the other membrane components containing carbohydrate with fucose, glucosamine or sialic acid.

The finding of a uniquely sized glycopeptide as part of the H-2 alloantigen of the cell membrane suggests an important function for this carbohydrate moiety, in some way related to the physiologic role of the antigen. Further studies are in progress to compare the glycopeptides of the H-2 alloantigens of different mouse strains, as well as to analyze the more detailed

fine structure within the carbohydrate chain itself, in an attempt to shed information of the role of carbohydrate in the H-2 alloantigen molecule.

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