DPM, and (c) materials similar in electrophoretic mobility to those formed in the presence of NH_2OH can be found after short incubation of high concentrations of PBG with Urogen I synthetase in the *absence* of NH_4^+ or NH_2OH.

Acknowledgments

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Biochemical Similarity of Papain-Solubilized *H-2*^{d} Alloantigens from Tumor Cells and from Normal Cells*

Kunio Yamane and Stanley G. Nathenson†

ABSTRACT: Papain-solubilized *H-2*^{d} alloantigens were purified from crude cell membranes of Meth-A tumor cells by $(NH_4)_2SO_4$ fractionation, Sephadex G-150, DEAE-Sephadex A-25 column chromatography, and 7.5% polyacrylamide disc gel electrophoresis. The purified preparations (class I and II) showed slightly broad single bands on disc gel electrophoresis at pH 9.3 and 4.3. The preparations were glycoprotein in nature having 85–90% protein, 3–4.5% neutral carbohydrate, 1.5–4% glucosamine, and 1–1.5% sialic acid. A comparison of peptide maps obtained by cellulose thin-layer chromatography for CNBr-treated and trypsin-digested class I and II glycoproteins showed that about half of the peptides of class II were identical with those found in the class I preparation.

The purified Meth-A materials and similarly prepared purified *H-2*^{d} alloantigens from DBA/2 normal mouse spleen cells, both of which carry the same *H-2* specificities, were found to have not only similar serological profiles by several criteria but also similar amino acid analyses, overall chemical analyses, molecular weight, chromatographic, and electrophoretic properties. Furthermore, a peptide analysis of CNBr-treated *H-2* antigens by column chromatography on PA-35 resin showed that the elution profiles of the two materials were very similar. Thus, *H-2* alloantigen preparations from normal and tumor cells sources which share the *H-2*^{d} genetic make-up but differ in several non-*H-2* properties as well as in cell type, were found to be exceptionally similar as far as could be discerned by our techniques.

Mouse *H-2* alloantigens are membrane-located products of the *H-2* genetic locus and carry the immunological determinants involved in tissue graft rejection. Of more than 15 histocompatibility loci, *H-1*, *H-2*, etc., the *H-2* locus and its products form the major system, and differences between individuals at this locus cause the most rapid graft rejection. Over 20 different alleles or "haplotypes" have been found for the *H-2* genetic region, and each allele determines a different mosaic of *H-2* specificities many of which are cross-reactive between the different alleles (*cf.* Snell and Stimpfling, 1966; Shreffler, 1966).

At the biological level, the *H-2* alloantigen is an unusually stable genetic product. Its expression in numerous cultured cell lines has shown complete resistance to loss even under conditions arranged to select against its presence (Klein,

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1967). A striking example of the continued expression of this product is demonstrated for the LM mouse cell line, a line in continuous culture for over 20 years, where all different sublines examined carried all the specificities originally found in the genotype of the mouse strain from which it arose (Drysdale *et al.*, 1967).

In recent studies utilizing papain digestion of cell membranes to release *H-2* alloantigenic activity, spleen cells were used as the tissue source, and the water-soluble fragments carrying the *H-2* antigenic sites were purified and characterized as glycoproteins (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970).

In the present work we examined the question of the relationship of the chemical structure of papain-solubilized *H-2* alloantigens from two different types of cell sources: cells of a fibrosarcoma (Meth-A, Old *et al.*, 1962) and cells from normal spleens of DBA/2 mice. These sources are of identical *H-2* type, but differ at many non-*H-2* loci, as well as in cell type. Such studies bear on the crucial problem of the extent of possible variation of the *H-2* product from cell to cell, as well as on the practical question as to the validity of the results arising from studies using cell culture lines, and in particular malignant tumor cell lines, for investigations on the chemical nature of histocompatibility alloantigens.

Materials and Methods

Mice and Tumor Cells. The following inbred mice, purchased from Jackson Laboratory, Bar Harbor, Maine, or obtained from the breeding colonies of Dr. Frank Lilly (Albert Einstein College of Medicine, Bronx, N. Y.), were used: B10.A ($H\text{-}2^a$); C57BL/6 ($H\text{-}2^b$); DBA/2, B10.D2, BALB/c ($H\text{-}2^d$); B10.BR ($H\text{-}2^k$); and I/st ($H\text{-}2^l$).

Meth-A cells from a fibrosarcoma ($H\text{-}2^d$) induced by methylcholanthrene in mice of BALB/c strain (Old *et al.*, 1962) were maintained in BALB/c mice.

Isoantisera and Test System. Alloantigenic activity was detected as described previously (Nathenson and Davies, 1966) by the method of inhibition of immune cytolysis (Sanderson, 1964; Wigzell, 1965). The alloantisera and target cells used in the detection of spleen cell antigens has been previously reported (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970).

Isolation of Papain-Solubilized $H\text{-}2^d$ Alloantigens from Tumor Cells. The starting material was ascitic fluid which was harvested from the peritoneal cavity of mice which had been inoculated with 10^6 Meth-A cells/mouse 10 days earlier. The Meth-A cells were sedimented from the ascitic fluid by centrifugation at 700 rpm for 5 min to remove the majority of contaminating red blood cells, and then frozen and stored at $-50°$ until used. All subsequent steps, with the exception of the papain digestion step, were carried out at 0–4°.

The preparation of crude membrane fraction and solubilization of *H-2* alloantigens by papain digestion from tumor cell membranes was accomplished by a slight modification of the method used to prepare the *H-2* alloantigens from mouse spleens (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970). Frozen tumor cells were thawed at 4°, and were homogenized with a Potter Teflon homogenizer. The homogenate (about 30 mg/ml) was subjected to sonic oscillation (10 kc) for 1 min (Instrument Associates Co., N. Y.) since this treatment increased the yield of *H-2* active crude membrane. The sonicated suspension was centrifuged at low speed (1000*g*) for 10 min, and the pellet was resuspended at 100 mg/ml in 0.9% NaCl for 10 min, recentrifuged, resuspended at 100 mg/ml in 0.7% NaCl solution for 10 min, and then centrifuged again at low speed. The pooled supernatant of these extractions was centrifuged at 144,000*g* for 120 min, and the pellet—the crude membrane fraction—was resuspended in 0.05 M Tris-HCl buffer (pH 8.4) at a concentration of about 30 mg/ml as protein. The *H-2* alloantigenic activity was solubilized by limited papain digestion (37°, 60 min with ratio of papain to crude membrane fraction of 1:20, *cf.* Shimada and Nathenson, 1969, for details).

Disc Gel Electrophoresis. Preparative and analytic polyacrylamide disc gel electrophoresis (7.5% polyacrylamide, at pH 9.3 and 4.3) (Reisfeld *et al.*, 1962; Davis, 1964) were conducted under the same conditions as in previous papers (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970).

Amino acid analysis and other chemical analysis were carried out as previously described (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970). Protein was measured by the method of Lowry *et al.* (1951), total carbohydrate by a modification of the orcinol method (Francois *et al.*, 1962), and sialic acid by the method of Warren (1959). Glucosamine content was determined in the course of amino acid analysis or by a modification of the Elson and Morgan method (Reissig *et al.*, 1955). For glucosamine determinations calculated from the amino acid analyses a correction factor of 2.46 was applied since only 40.7% was recovered after samples were hydrolyzed with 5.7 N HCl at 110° for 24 hr, the conditions used to prepare samples for the amino acid analysis.

Peptide Mapping of H-2 Alloantigens. Freeze-dried *H-2* alloantigen (about 100 μg) was subjected to CNBr cleavage in 70% formic acid (about 300 μg of CNBr). Preliminary experiments suggested that treatment with CNBr rendered the protein portion of the alloantigen susceptible to trypsin digestion. After lyophilization, the treated protein was dissolved in 45 μl of 0.05 M ammonium bicarbonate buffer (pH 8.5) at a concentration of 2 mg/ml and TPCK[1]-treated trypsin was added to give a final enzyme to substrate ratio of 1:20. The mixture was incubated overnight at 37°, then freeze-dried, and redissolved in distilled water for application to a precoated 20 × 20 cm cellulose thin-layer glass plate (Brinkman Instruments, Inc., Westbury, N. Y.). Separation of peptides was achieved with two-dimensional ascending chromatography. The acidic solvent system used in the first dimension was 1-butanol–acetic acid–distilled water (4:1:2, v/v), and the neutral system used in the second dimension was 1-butanol–acetic acid–pyridine–distilled water (5:1:4:4, v/v) (Burns and Turner, 1967). After development, the plate was dried at room temperature and then heated at 100° for about 10 min. Peptides were detected by spraying with a sensitive Cd^{2+}-containing ninhydrin reagent (Heilman *et al.*, 1957). This peptide-mapping technique resolved about 80–90% of the expected number of peptides from an immunoglobulin light-chain digest as shown in another report (Shimada *et al.*, 1970).

Preparation of Radiolabeled Antigen from Meth-A Cells. A Meth-A suspension culture (500 ml) containing [^3H]arginine

[1] Abbreviation used is: TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone.

TABLE I: Purification of *H-2*^{d} Alloantigens from Meth-A Tumor Cells.

	Class I (H-2.4,10,13Activity)			Class II (H-2.31 Activity)		
	Total Units	Units/mg of Protein	Purification	Total Units	Units/mg of Protein	Purification
1. Cell extract	2800×10^4	495	1	960×10^4	178	1
2. Crude membrane preparation	2500×10^4	1,156	2.34	720×10^4	338	1.90
3. Papain digest	840×10^4	480	0.97	120×10^4	69	0.39
4. $(NH_4)_2SO_4$ fraction	610×10^4	3,000	6.05	118×10^4	528	2.96
5. Sephadex G-150						
Class I	330×10^4	5,000	10.0			
Class II				27×10^4	1,250	7.03
6. DEAE-Sephadex A-25	216×10^4	72,500	146.5	16×10^4	8,720	46.4
7. Disc gel electrophoresis						
A	9×10^4	90,000	182	3.7×10^4	36,200	204
B	87×10^4	163,000	330	2.8×10^4	28,100	158
C	46×10^4	106,000	214			

(703 mCi/mmole), [^3H]lysine (1.2 Ci/mmole), and [^3H]methionine (450 mCi/mmole) was incubated for 16 hr at 37° in media and conditions described elsewhere (Cullen and Nathenson, 1969). The alloantigen in the cell membrane was solubilized by papain digestion and purified by $(NH_4)_2SO_4$ fractionation, Sephadex G-150, and DEAE-Sephadex A-25 column chromatography by the previously described techniques (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970).

Peptide Column Chromatography. Freeze-dried preparations of the alloantigens (about 300–500 μg of protein) were treated at 37° for 18 hr with dithiothreitol (final concentration 0.02 M) in 8 M urea. Iodoacetic acid (buffered to pH 8.4 and at a final concentration of 0.08 M) was then added and the solution was incubated for 15 min at 37°. The reduced and alkylated preparations were then treated with sufficient glacial acetic acid to reduce the pH to 2 and then subjected to Sephadex G-10 column chromatography in 30% acetic acid. The eluate in the excluded volume contained all the protein reactive material and this was lyophilized *in vacuo* and subjected to CNBr cleavage (0.8–1.2 mg of CNBr in 70% formic acid, overnight in a sealed test tube at room temperature). After removal of the CNBr and formic acid by lyophilization, the CNBr-treated peptides were dissolved in a small amount of 70% formic acid (about 0.1 ml); 1.5 ml of 0.2 M pyridine acetate (pH 3.1) was added, and the solution was applied to a 0.9 × 22 cm column of Spinco PA-35 resin (Hill and Delaney, 1967) which had been equilibrated with 0.2 M pyridine acetate buffer (pH 3.1). The peptides were eluted by a linear gradient from 0.2 M pyridine acetate (pH 3.1) to 2 M pyridine acetate (pH 5.0). A final fraction was eluted with 8 M pyridine acetate (pH 6.5). The chromatography was carried out at 55° with a flow rate of 10 ml/hr. The contents of each tube was dried in an air oven at 90–100°. After addition of 3 drops of 20% NaOH, the peptide mixtures were autoclaved for 20 min, neutralized, and analyzed with ninhydrin (Hirs, 1967).

Chemicals. Papain (twice crystallized) was purchased from Sigma Chemical Co., and TPCK-treated trypsin (trypsin-TPCK) was purchased from Worthington Biochemical Co. Sephadex and DEAE-Sephadex were purchased from Pharmacia Chemical, Inc. All other chemicals were of reagent grade.

Results

Purification Steps and Recovery of Alloantigens from Tumor Cells. The *H-2* alloantigens were solubilized from the cell membranes of the Meth-A (*H-2*^{d}) tumor cells by papain digestion. Purification was carried out by the method outlined in Table I, which is the same method used for the *H-2* alloantigens from mouse spleens of the *H-2*^{d} strain (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970). The *H-2* alloantigenic activity in the papain digest was recovered in the fraction precipitating between 50 and 75% saturation with ammonium sulfate. Column chromatography on Sephadex G-150 separated the H-2.4,10,13 activity from that of the H-2.31 activity as reported for the *H-2* alloantigens from mouse spleens. The peaks of *H-2* activity were pooled into two fractions; the material of higher molecular weight was called class I and was rich in H-2.4,10,13 activity, while the lower molecular weight material rich in H-2.31 activity was termed class II. These two fractions were subjected to further purification on DEAE-Sephadex A-25 and disc gel electrophoresis on polyacrylamide gels. Three major bands of activity coinciding with protein staining material were noted for the class I alloantigens (A, $R_{BPB} = 0.36$, B, $R_{BPB} = 0.40$, and and C, $R_{BPB} = 0.40$) (R_{BPB} = migration distance of measured material/migration distance of the bromophenol blue marker). The class II material was separated into two fractions: A, $R_{BPB} = 0.53$ and B, $R_{BPB} = 0.58$. At this final stage of purification the highest specific activity for the class I and class II fragments showed that the class I preparation, B, was about 330 times more active than the starting material, with recovery of about 3.1% of the activity units found in the cell extract and 10.4% of those in the papain digest. The class II prepara-

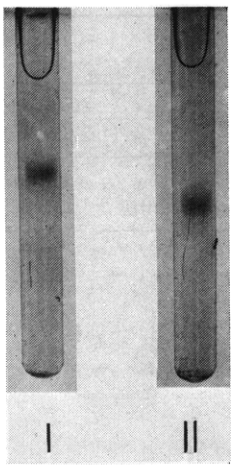

FIGURE 1: A photograph of polyacrylamide gels (class I on the left and class II on the right) electrophoresed at pH 9.3 according to the method of Davis (1964), and stained with coomassie blue. Direction of electrophoresis is toward the positive pole at the bottom of the photo.

tion, A, was about 200 times more active than the starting material with a recovery of about 0.4% of the total H-2.31 units in the cell extract and 3.1% of those in the papain digest.

The eluted materials from the disc gel electrophoresis, when reelectrophoresed in the original system at pH 9.3 showed single bands of protein staining material (Figure 1). The protein-staining material coincided with alloantigenic activity. Electrophoresis of these eluates at pH 4.3 showed single but rather broad bands of protein-staining material.

Some Physical Properties of the Purified H-2^d Alloantigens from the Meth-A Tumor Cells. An estimate of the molecular

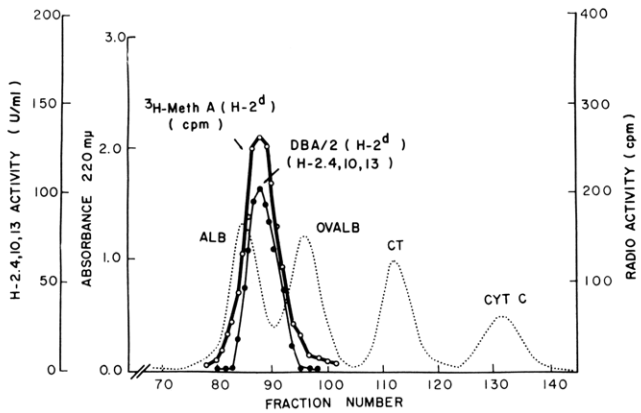

FIGURE 2: Comparison of molecular weights of normal and tumor *H-2* alloantigens (class I) on Sephadex G-75 column chromatography. Class I *H-2* alloantigens (containing [^3H]arginine, [^3H]lysine, and [^3H]methionine) from Meth-A tumor cells (2000 cpm, 50 units of H-2.4,10,13 activity) unlabeled *H-2* alloantigen (class I) from DBA/2 normal spleen cells (2500 units of H-2.4,10,13 activity), and molecular weight markers (albuminin, mol wt 67,000; ovalbumin, mol wt 45,000; chymotrypsinogen A, mol wt 25,000; cytochrome *c*, mol wt 12,400) were applied to Sephadex G-75 column (1.5 × 118 cm) which had been equilibrated with 0.15 M Tris-HCl buffer (pH 8.4) containing 0.15 M NaCl and eluted with the same buffer. Flow rate, 3 ml/hr; absorbance 220 mμ (---), cpm O——O, and *H-2* inhibitory activity for H-2.4,10,13 ●——● were monitored on each tube.

TABLE II: General Chemical Analyses of Papain-Solubilized *H-2^d* Alloantigens.

	Meth-A Tumor Cells		DBA/2 Normal Mouse Spleen Cells[a]	
Class	I	II	II	II
Protein as BSA[b]	100	100	100	100
Neutral carbohydrate[c]	4.5	3.5	3.9	4.3
Glucosamine[d]	4.1	1.3	3.2	1.4
Sialic acid[e]	1.5	1.2	1.1	0.4

[a] Data taken from Shimada and Nathenson; Yamane and Nathenson (1970). [b] Protein value was determined as the milligram equivalent to bovine serum albumin (BSA) by the method of Lowry *et al.* (1951). [c] Determined by the orcinol method of Francois *et al.* (1962) using mannose as standard. [d] Determined by method of Reissig *et al.* (1955) using glucosamine as a standard after hydrolysis in 4 M HCl at 100° for 4 hr or determined from amino acid analysis using a factor of 2.46 to compensate for different hydrolysis conditions. [e] Determined by the method of Warren (1959).

weight of the purified class I and class II materials was determined by use of Sephadex G-75 column chromatography (1.5 × 118 cm) in 0.01 M Tris-Cl–0.15 M NaCl (pH 8.4). The column was standardized with proteins of known molecular weight, and a direct relationship of the logarithim of the molecular weight to elution volume was obtained which allowed us to estimate a molecular weight of 57,000 plus or minus 2000 for the class I fraction and 37,000 plus or minus 1000 for the class II preparation. A confirming value for the molecular size of the class I fragments could be estimated from their ultracentrifugal behavior. Although the ultracentrifugation was carried out only at one pH (*i.e.*, 8.4, 0.005 M Tris-HCl) a single peak was seen with an approximate sedimentation coefficient of 3.6 S. Using values for diffusion coefficient and partial specific volume of either albumin (Oncley *et al.*, 1947) or the glycoprotein from fetuin (Spiro, 1960), one could estimate molecular weight to be 50,000 and 55,000, respectively. Such estimates are in the same range as were obtained from molecular seiving.

In view of the previously reported values of approximately 66,000 for the molecular weight of the DBA/2 alloantigen (Shimada and Nathenson, 1969), a direct comparison on the same Sephadex G-75 column was carried out using DBA/2 class I fragments mixed with class I Meth-A material which had been labeled with tritiated methionine, lysine, and arginine. As shown in Figure 2, the radioactivity profile and the specific immune assay profile are identical for these two substances. This result establishes that the molecular size of these fragments by Sephadex chromatography is identical, and possibly that previous estimates of somewhat higher molecular weight for DBA/2 materials were either in error, as they were carried out on less highly purified material, or different from present measurements due to the use of Sephadex G-150 instead of Sephadex G-75.

Some Chemical Properties of Meth-A Alloantigens. The

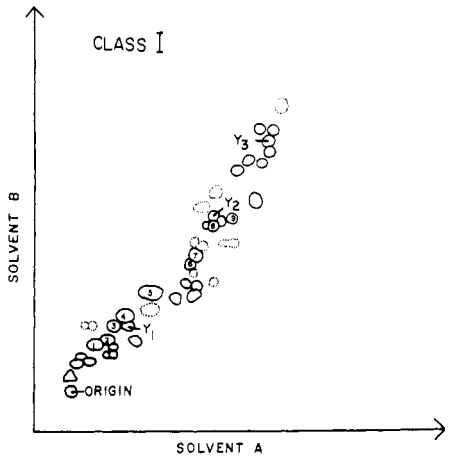

FIGURE 3: Peptide mapping of class I alloantigens by cellulose thin-layer chromatography according to the procedure outlined in the Materials and Methods section. Orange or yellow spots, Y_1–Y_3; deep red spots, solid outlines; weak red spots, dashed outlines. Numbered peptides, 1–9, are identical for class I and class II patterns.

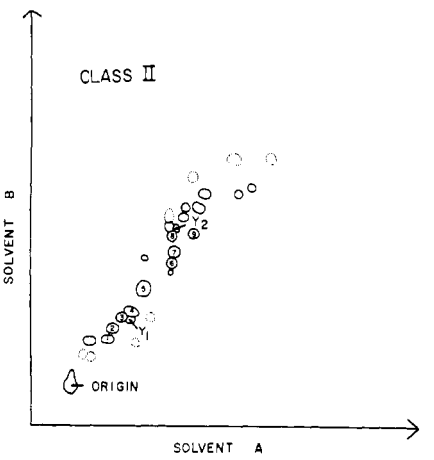

FIGURE 4: Peptide mapping of class II by cellulose thin-layer chromatography according to procedures outlined in the Methods section. Orange or yellow spots, Y_1–Y_2; deep red spots, solid outline; weak red spots, dashed outline. Numbered peptides, 1–9, are identical for class I and class II patterns.

overall chemical characteristics of purified Meth-A *H-2* alloantigen fragments are shown in Table II. For these data we arbitrarily set the protein content equal to 100.0 and related to this the neutral carbohydrate, glucosamine, and sialic acid values. Included for comparison are the chemical analyses of glycoprotein fragments from normal DBA/2 spleen cells (class I, Shimada and Nathenson, 1969; class II, Yamane and Nathenson, 1970), for which approximately 95% of the dry weight was found to be accounted for by the protein, neutral carbohydrate, and amino sugar. Since the ratios of these constituents for the tumor antigen fragments are very similar to the values reported the antigen fragments from normal cells it would appear that the tumor alloantigens are also glycoproteins consisting mainly of protein. The neutral carbohydrate for both class I and class II material from both sources is about 4% of the protein. Glucosamine is around 3–4% for class I whereas the class II fragments carry only about 1 or 1.5% glucosamine. The sialic acid values range between 0.5 and 1.5%.

Amino acid analyses of the class I and class II glycoprotein fragments from the Meth-A tumor cells showed that these preparations had amino acid compositions similar to the comparable preparations from normal DBA/2 spleen cells with differences in any amino acid not exceeding ±1–2 residues/100 residues of total amino acid.

Peptide Analysis of the Meth-A H-2^d Alloantigens. We carried out several types of peptide analyses on *H-2* alloantigens isolated from the Meth-A tumor cell membranes. Because of the availability of only small amounts of class II fragments, a peptide-mapping technique was used to compare the class I and class II fragments from the Meth-A tumor source. Figure 3 shows a line drawing of an average of four separate maps of CNBr-treated and trypsin-digested peptides from the class I glycoprotein fragment. This includes 31 peptides (solid line circles) which gave intense red spots, and 3 peptides which stained yellow with ninhydrin (Y_1 through Y_3). As derived from amino acid analysis, the theoretical maximum number of peptides which could be present in class I material is about 60–65, and the number detected was about 40 (including both strong and weakly reacting spots), or about 70% of the theoretical maximum. A line drawing of the map for class II glycoprotein peptides produced by CNBr cleavage and trypsin digestion is shown in Figure 4. The theoretical maximum number of peptides calculated from the amino acid composition was about 40, but 28 peptides were seen experimentally. Two orange, (Y_1 and Y_2), 21 deep red spots, and 5 fainter red spots were detected.

A comparison of the peptide maps from the class I and class II, glycoprotein fragments shows that the smaller class II, as expected, had only about two-thirds of the number of peptides found for the class I fragment. Of considerable interest is the finding that 11 of the peptides of class II appear identical with those found in the class I molecule (*e.g.*, Y_1, Y_2, and 1–9).

CNBr Peptide Analysis Using Column Chromatography. Peptide column chromatographic analyses of cyanogen bromide fragments of *H-2* class I fragments from Meth-A and normal DBA/2 spleen sources were also carried out. As shown in Figure 5 the elution pattern of the peptides produced by reduction and alkylation followed by cyanogen bromide cleavage are quite similar between the two sources. Nine ninhydrin positive peaks were separated (each chromatography was performed in triplicate). Except the peaks shown by the arrow, all the more prominent peaks were found in material from both sources. The one peptide peak distinguishing antigen from normal cells was eluted at fraction 25, while that specific for the tumor-derived alloantigen was eluted at fraction 42.

Immunological Specificity Profile of H-2 Alloantigenic Glycoproteins. Each of the class I and class II alloantigen fragments from the DBA/2 and Meth-A tumor cells was examined for its reactivity in the inhibition of immune cytolysis assay. As can be seen in Table III there is a very similar pattern of reactivity between the normal and tumor cell alloantigens of the class I and the class II types.

The class I and class II preparations were, as expected, negative in control tests for H-2.2,22, H-2.5, and H-2.33, specificities which are not genetically determined by the *H-2^d*

TABLE III: Comparison of Specificity Profiles of DBA/2 and Meth-A Tumor Cell Purified Fractions.

H-2 Specificity Detected in Assay System[a]	Class I		Class II	
	DBA/2 Normal Spleen Cells (U/mg of Protein)	Meth-A Tumor Cells (U/mg of Protein)	DBA/2 Normal Spleen Cells (U/mg of Protein)	Meth-A Tumor Cells (U/mg of Protein)
3	46,000	42,700	0	0
4, 10, 13	190,000	163,000	0	0
4	59,000	57,500	0	0
10	54,000	57,500	0	0
8	810	8,950	500	845
31	8,630[b]	9,700[b]	31,800	37,400
6, 14, 27, 28, 29	4,700	1,410	500	545
2, 22	0	0	0	0
5	0	0	0	0
33	0	0	0	0

[a] *H-2* activities assayed by inhibition of immune cytolysis as described under Methods section. [b] In our previous studies (Shimada and Nathenson, 1969; Yamane and Nathenson, 1970), under the usual papain digestion procedures, most of the specificity 31 has been recovered in the class II fraction after Sephadex chromatography. A certain proportion of 31 activity also chromatographed with the class I fraction, but could be converted into the smaller size by a more vigorous treatment with papain. In the present study, our class I preparations contained a certain amount of 31 activity as demonstrated here. However, the actual content of the 31 fragment in the class I fraction was determined to be 7% by direct reaction of antibody with similarly purified alloantigen fragments labeled with [^3H]arginine, [^3H]lysine, and [^3H]methionine.

genotype. The reactivity patterns of the preparations show that the immunological specificities of the *H-2* alloantigens from the two sources, tumor cells and normal mouse spleen cells, were qualitatively similar. Furthermore, the specific activity (units per milligram of protein) of *H-2* alloantigens from tumor cells was also at the same level as that from the mouse spleens. For example, the maximum specific activity for H-2.4,10,13 was 190,000 units/mg for DBA/2 spleens and 163,000 units/mg for Meth-A tumor. And the specific activity for H-2.31 was 31,800 units/mg for DBA/2 material and 37,400 units/mg for Meth-A tumor material.

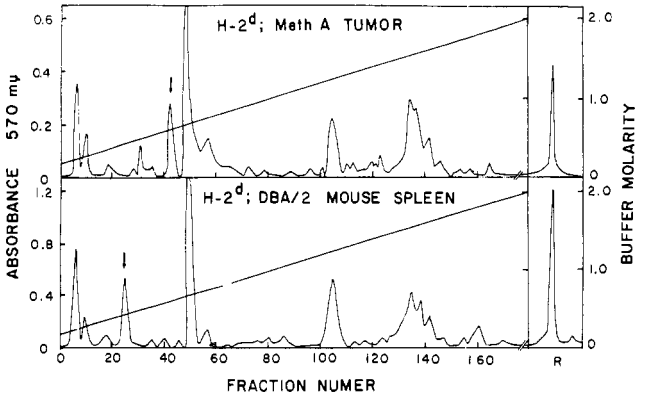

FIGURE 5: Peptide column chromatography on PA-35 resin of *H-2* alloantigens (class I) from Meth-A tumor cells (upper) and from DBA/2 normal mouse spleen cells (lower). *H-2* alloantigens (class I) from tumor cells (270 μg) and from mouse spleen cells (420 μg) were reduced, alkylated, and treated with CNBr. Then the samples were applied to the PA-35 column. Each peptide was eluted and detected by the procedures outlined in Materials and Methods.

Further analysis of the specificity profile of these two preparations was carried out by inhibition of radioactive alloantigen–alloantibody complex formation (Table IV). In these studies alloantigen labeled with [^3H]methionine, [^3H]lysine, and [^3H]arginine residues was purified to the DEAE-Sephadex stage of fractionation. Antibody in excess as shown in column I was added to the partially purified antigen, allowed to complex, and then the antibody–antigen complex removed by discontinuous gel electrophoresis and counted. Between 60 and 70% of the radioactivity was so complexed.

Use of *H-2*^{d} class I alloantigen from DBA/2 mouse spleen cells (*H-2*^{d}) for preincubation with the alloantibody abolished the complex formation between the *H-2*^{d} antiserum and the labeled *H-2*^{d} tumor alloantigen. A similar experiment carried out with the Meth-A tumor cell antigen likewise abolished the complex formation. The specificity control serum (anti-*H-2*^{b} specificities 2,5,22,33) showed a nonspecific background of 1.5%. These data demonstrate, therefore, the identical capacity of the normal DBA/2 mouse spleen antigen and of the Meth-A tumor cell antigen to compete for radiolabeled tumor alloantigen. This result shows the similar specificity of the tumor and normal cell derived antigen preparations.

Discussion

The purpose of the work reported in this paper was to examine the chemical nature of *H-2* alloantigens extracted and purified from tumor cells in order to compare these properties with properties of antigen from normal cells.

The estimated molecular weight from gel filtration of the class I materials from the Meth-A fibrosarcoma was about 55,000, a value corresponding to the estimate from the ultracentrifugation sedimentation analysis. Since an identical

TABLE IV: Similarity of *H-2*^{d} Alloantigens From DBA/2 and Meth-A Sources as Determined by the Effect of Preincubation on Specific Immune Complex Formation.[a]

H-2 Specificities Detected by Antiserum	Unlabeled Alloantigen Used for Preincubation	% Radioactivity in the Complex Region
3, 4, 8, 10, 13, 31 (*H-2*^{d})	None	66
3, 4, 8, 10, 13, 31 (*H-2*^{d})	Class I fraction from DBA/2 spleen cells (*H-2*^{d})	−0.9
3, 4, 8, 10, 13, 31 (*H-2*^{d})	Class I fraction from Meth-A tumor cells (*H-2*^{d})	0
3, 4, 8, 10, 13, 31 (*H-2*^{d})	Class I fraction from C57BL/6 cells (*H-2*^{b})	52
2, 5, 22, 33 (*H-2*^{d})	None	1.5

[a] [^3H]Arginine-, [^3H]lysine-, [^3H]methionine-labeled *H-2*^{d} alloantigen (2000 cpm/50 units of H-2.4,10,13 activity/1.6 μg of protein) from Meth-A tumor cells was reacted with anti-*H-2* serum (150–200 units/2.4 mg of protein) or with anti-*H-2* serum preincubated with unlabeled alloantigen as noted. The antigen–antibody complex formed was separated by disc gel electrophoresis and the per cent radioactivity in complex was measured by the method described elsewhere (Cullen and Nathenson, 1969; Muramatsu and Nathenson, 1970). For inhibition experiments the anti-*H-2* serum (150–200 units/2.4 mg of protein) was preincubated with unlabeled *H-2*^{b} or *H-2*^{d} antigen (1200–1600 units/20–30 μg of protein). The units of activity for the *H-2* antigen were determined as described previously (Nathenson and Davies, 1966). The units for the *H-2* antisera were defined as the reciprical of the final dilution of sera needed to give 80% lysis in the standard cytotoxic assay.

elution volume for these Meth-A class I fragments and for DBA/2 class I fragments was obtained when these materials were rechromatographed on Sephadex G-75 (Figure 2), our previous value of 66,000 for the DBA/2 class I alloantigen fragments appears to be too large (Shimada and Nathenson, 1969).

The peptide maps by thin-layer two-dimensional chromatography revealed that as expected, the smaller Meth-A class II fragment showed about two-thirds of the number of peptide spots as the larger class I fragment. Of the peptides from the class II fragment, about 50% migrated in identical fashion to peptides from the class I fragment. Such a finding suggests some common primary amino acid sequences between these two fragments. However, since we are analyzing fragments proteolytically derived from natural membrane polypeptide molecules, at present any speculation is tenuous. These preliminary studies are being extended presently in order to establish the relationship of the papain-derived fragments to the *in situ H-2*-bearing macromolecules.

In the present paper, the evidence suggests that both the class I and class II alloantigen bearing fragments solubilized by papain from the fibrosarcoma, Meth-A, are nearly identical with those isolated from an *H-2*^{d} identical normal source (DBA/2) with respect to purification properties, molecular size as judged by Sephadex G-75 chromatography, electrophoretic mobility, overall chemical properties, and amino acid composition.

Comparison of the class I fragments of Meth-A and DBA/2 by peptide chromatographic analysis showed overall similarity, but a small but reproducible difference. One peptide (Figure 5) was eluted in a slightly different position in the tumor and normal preparations, although all the other major peptides appeared to be shared by the alloantigens from the two sources. Such a difference is rather small when compared with the results of peptide chromatographic analysis of CNBr fragments of class I fragments from spleens of DBA/2 (*H-2*^{d}) and C57BL/6 (*H-2*^{b}), where we found differences of at least three ninhydrin-positive peaks out of the nine resolved by this technique (K. Yamane, A. Shimada, and S. G. Nathenson, in preparation). The findings by column chromatography are supported by a peptide map comparison of CNBr-trypsin-digested class I fragments of the Meth-A and DBA/2 origin (data not shown) which showed identity except for one peptide.

Overall, the finding of very great chemical similarity of the *H-2*^{d} alloantigens from the two sources examined provide chemical evidence for one of the widely held tenets of transplantation immunology that *H-2* alloantigens are essentially identical or at least very similar on cells from different tissues. The minor peptide differences which were found in our studies, might possibly be ones which distinguish the tumor from the normal spleen cells. Of importance to future studies is support for the notion that, within limits, information gained from studies on the chemical structure and on the biosynthetic properties of the *H-2* alloantigen in certain selected cell lines (*e.g.*, tumor lines, or "normal tissue culture cell lines") may be validly extrapolated to normal tissues.

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Spectrophotometric Observations on the Oxidation–Reduction Cycle of the Respiratory Chain-Linked Reduced Nicotinamide–Adenine Dinucleotide Dehydrogenase*

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ABSTRACT: Bois and Estabrook have described a cycle of absorbance changes occurring in inner membrane preparations of heart mitochondria which is initiated by addition of reduced nicotinamide–adenine dinucleotide (NADH) and may be followed at 470 mμ with 500 mμ as the reference wavelength. There is a rapid bleaching followed by an incomplete, slower return of color. The time of the cycle, the rate of reoxidation, and the amount of irreversibly bleached chromophore are increased by rotenone. These observations are extended in the present study and interpreted in terms of the oxidation–reduction of components associated with the respiratory chain-linked NADH dehydrogenase. In particular, the effect of specifically and unspecifically bound rotenone and piericidin A, of mercurials, and of a combination of mercurials and piericidin on the various parameters of the cycle have been investigated.

The findings provide strong support for the applicability of this experimental procedure, using the particular wavelength pair mentioned, to the study of the redox cycle of the dehydrogenase as an enzyme unit.

There have been numerous attempts to devise experimental procedures for measuring the redox state of the various components of the NADH dehydrogenase of the respiratory chain during electron transport. Initially Chance (1956) suggested that the absorbance changes at the 465 mμ minus 510-mμ wavelength pair are indicative of oxidation–reduction of the flavin moiety. The method was adopted by many investigators but was later questioned on the grounds that the nonheme iron-labile S components of the dehydrogenase and of other enzymes in the respiratory chain as well as cytochromes would be expected to interfere with absorbance changes at this wavelength pair (Singer, 1961; Minakami *et al.*, 1963; Machinist and Singer, 1965; Nicholls and Malviya, 1968; Gutman *et al.*, 1970b). Further, in view of the occurrence of interchain electron transport and the fact that NADH dehydrogenase flavin is a very small part of total mitochondrial flavin (Cremona and Kearney, 1964), it was likely that flavin components of other mitochondrial enzymes might contribute to the absorbance changes attributed to NADH dehydrogenase flavin.

Hatefi (1968) ascribed the absorbance changes at the wavelength pair 460 minus 510 mμ to the nonheme iron components of NADH dehydrogenase. However, objections based on interference by cytochromes (Nicholls and Malviya, 1968), by the nonheme iron of other enzymes, and possibly by other

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