THE EXPRESSION OF HISTOCOMPATIBILITY ANTIGENS ON CELLULAR AND SUBCELLULAR MEMBRANES

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Histocompatibility antigens are those tissue isoantigens involved in transplantation immunity. In the mouse, the histocompatibility antigens which have been most extensively studied are the H-2 antigens. These constitute a complex system of membrane-associated antigens determined by the pseudoallelic H-2 locus (1–3), which resembles in many respects the loci determining the Rh antigens in man (4) and similar blood group antigens in cattle (5) and chickens (6). The relationship between the H-2 locus and its antigens has aroused much interest (1–3, 7–9), and considerable attention has been given to the problem of the nature and biological significance of the H-2 antigens and of their location on and within cells (8, 10–20). The present communication concerns the results of further investigation of these problems.

Materials and Methods

Experimental Animals.—Mice weighing 17 to 25 gm were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, and were maintained on a diet of commercial mouse pellets and water *ad lib.* Mice used in tissue fractionation studies were fasted for 24 hours before being sacrificed. The strains of mice used in these studies are listed in Table I, together with their H-2 genotypes and phenotypes.

Antisera.—Antisera were obtained by isoimmunization of various strains of mice with spleen cells as described earlier (18).

Target Cells.—The indicator cell types used in the assay of cytotoxic antibodies were cells from the C57BL/6 ascites tumor EL-4 (18) or cells from lymph nodes of the desired mouse strain. Lymph node cells were obtained by teasing individual lymph nodes with two 22-gauge needles, suspending the lymphocytes and washing them twice in a buffered salt solution (medium 199, Microbiological Associates, Bethesda). The washed cells were finally suspended in medium 199, in a concentration of 5000 cells/mm^3.

Preparation of Antigens.—Animals were killed by cervical dislocation and the desired tissue removed, weighed, and homogenized with a Potter homogenizer with a teflon pestle to obtain a 20 per cent (w/v) suspension in either physiological saline solution or 0.25 M sucrose solution.

Titration of Antibody and Antigen.—The methods used to titrate cytotoxic antibodies and to assay the H-2 antigens by quantitative absorption of these antibodies were those of Basch and Stetson (17, 18).

Analytical Procedures.—Protein was determined by the method of Lowry *et al.* (21), acid phosphatase by the method of Shinowara *et al.* (22), adenosine triphosphatase by the method

of Schwartz (23), cytochrome oxidase by the method of Cooperstein and Lazarow (24), and TPNH oxidase by the method of Fouts (25).

EXPERIMENTAL

Tissue Distribution of H-2 Antigens.—As judged by various qualitative bioassay procedures, the H-2 antigens are present in most if not all of the tissues of the mouse. Medawar (11) postulated that the transplantation antigens are distributed more or less uniformly throughout all tissues except erythrocytes. Subsequent work has revealed large differences in the amounts of H-2 antigens in various tissues (7, 13, 17, 20), and this finding has been made the basis for a test of the physiological unity of the H-2 antigens by Pizarro *et al.* (7). These workers reasoned that if all of the antigens determined at the H-2 locus consti-

TABLE I

H-2 Genotypes and Corresponding Antigenic Specificities of Mouse Strains Used

Inbred mouse strain	H-2 allele	Antigenic specificities
A/HeJ	H-2^a	A C D E F H J K M N — — Y
C57BL/6	H-2^b	— — D^b E F — — K^b — N — V —
BALB/c	H-2^d	— C D E^d F H J — M N — — —
C3H	H-2^k	A C D^k E — H — K — — — — Y
DBA/1	H-2^q	— C — E F — — K M — Q — —

This table includes the major antigenic specificities for which each of these mouse strains has been tested. Capitals indicate the presence of a given specificity, while a (—) sign indicates its absence.

tuted a physiological unit, they should be distributed as a unit. If, however, the antigens were not a physiological unit but rather represented individual products of separate genes, they might be expected to be distributed differently in various tissues. The amounts of four H-2 antigens in a number of tissues were tested and found to be closely correlated (7). However, the assay used did not permit close quantitation, but sufficed to indicate only the presence of high or low concentrations of antigens. In the present study, with the use of a more quantitative assay for antigenic activity (17), the concentrations of a variety of H-2 antigens in various tissues have been examined in several mouse strains. The use of the A strain (genotype H-2^a) was considered particularly important, since this strain is believed to be the result of a crossover between genotypes H-2^d and H-2^k (1). There is a crossover frequency between antigens D and K of 1.4 per cent. From a study of this and other crossovers, Gorer and Mikulska (1) were able to construct a partial genetic map of the H-2 locus. From this, it is apparent that H-2^d anti-H-2^a and H-2^k anti-H-2^a antisera are directed against antigens determined at opposite ends of the H-2 locus. Thus, if these antigens are expressed independently in various tissues of the A mouse,

this should be demonstrable by the use of these two antisera. The results of such a study are shown in Table II. It can be seen that all of the H-2 antigens tested in the various tissues showed the same pattern of distribution. All were found in the highest concentration in spleen, with lesser amounts being found in liver, lymph nodes, lung, kidney, and skeletal muscle in descending order. No detectable amount of any of the antigens was found in brain. These results

TABLE II

Distribution of Various H-2 Antigenic Specificities in Tissues of Several Mouse Strains

Mouse strain tested	Antiserum used	Antigens detected	Antigenic activity (units/mg protein) of various tissues studied						
			Spleen	Liver	Lymph node	Lung	Kidney	Skeletal muscle	Brain
A	k-anti-a	DFJMN	4.8	4.0	2.4	1.0	0.60	0.09	<0.001
A	d-anti-a	AEKY	6.3	4.8	3.1	1.2	0.78	0.10	<0.001
A	d-anti-b	E	3.2	2.4	1.9	0.92	0.55	0.07	<0.001
A	k-anti-b	FN	3.7	2.6	1.7	0.70	0.57	0.09	<0.001
A	b-anti-d	CDHJM	5.3	3.6	2.8	0.86	0.65	0.08	<0.001
C57BL/6	d-anti-a	E	5.6	3.2	2.2	0.74	0.72	0.10	<0.001
C57BL/6	k-anti-a	FN	3.7	2.3	2.0	0.93	0.76	0.09	<0.001
C57BL/6	a-anti-b	D^bK^b	2.6	1.9	1.4	0.70	0.60	0.07	<0.001
BALB/c	k-anti-b	FMN	2.9	2.1	1.8	0.76	0.72	0.10	<0.001
BALB/c	k-anti-a	DFJMN	3.6	2.6	1.9	0.88	0.68	0.07	<0.001
C3H	d-anti-a	AEKY	5.6	3.7	2.2	0.94	0.88	0.07	<0.001
C3H	b-anti-d	CH	4.3	2.7	1.6	0.79	0.70	0.08	<0.001
DBA/1	a-anti-q	Q	3.0	1.6	1.4	0.60	0.80	0.09	<0.001

The cytotoxic antisera used for assaying antigenic activity are designated by the H-2 genotypes (see Table I) of the donor and recipient strains respectively. Thus, antiserum k-anti-a refers to an antiserum produced in C3H mice by immunization with splenic tissue from A/HeJ mice. Note that the pattern of distribution of each of the H-2 antigens or groups of antigens is the same, with the highest specific activity being found in spleen, and proportionately less activity in liver, lymph node, lung, and other organs in descending order.

indicate that all of the antigens determined by the H-2 genotype are probably expressed as a unit rather than independently, and thus give strong support to the Pizarro hypothesis (7). The distribution of these antigens in various subcellular fractions was next examined as a further test of the apparent physiological unity of the H-2 antigens.

Intracellular Localization of H-2 Antigens.—Previous studies of the intracellular localization of the H-2 antigens have yielded conflicting results. Billingham, Brent, and Medawar (10) found a nuclear localization and suggested that the antigens were associated with deoxyribonucleoprotein, but this has been subsequently shown not to be the case (26). Other workers have obtained data suggesting that the antigens are associated with the cell membrane (8, 15, 16)

while still others have found evidence of a microsomal localization (19, 20, 27); Basch and Stetson (18) presented evidence of a close association of a substantial fraction of the H-2 antigens with lysosomes. In the present study, fractionation of a liver homogenate was performed by the method of deDuve *et al.* (28). As can be seen in Table III, the H-2 antigens were present in all of the particulate fractions in approximately the proportions demonstrated by Basch and Stetson (18). There was high antigenic activity in the light mitochondrial fraction, which also showed high particle-bound acid phosphatase activity and therefore presumably contained a high concentration of lysosomes. The distribution of the various antigens examined was very similar, and these data are entirely consistent with the hypothesis that the H-2 antigens are expressed as a physiological unit.

Nature of association of H-2 antigenic activity with lysosomes: In view of the reported association of H-2 antigens with cellular membranes, either those of the cell surface (8, 15, 16) or of microsomes (19, 20), it seemed possible that the apparent association of high antigenic activity with lysosomes might be due to antigenic activity of the lysosomal membranes rather than to antigen contained within the lysosomes. In order to investigate this possibility, lysosomes were disrupted by suspension of the light mitochondrial fraction in distilled water and incubated for 30 minutes at 4°C, a method known to cause release of the lysosomal enzymes by osmotic shock (29). The suspension was then centrifuged in a Spinco model L centrifuge at 100,000 *g* for 30 minutes to sediment the lysosome membranes and other particulate material. The resultant supernate and sediment were then assayed for antigenic activity and for acid phosphatase activity. As can be seen in Table IV, all of the acid phosphatase activity was found in the supernate after osmotic lysis of the lysosomes, while all of the antigenic activity remained in the sediment. There was, in fact, some increase in specific antigenic activity in the sediment, presumably due to the loss of the non-antigenic soluble lysosomal proteins. Thus, the association of the H-2 antigens with the lysosomes must be of a different nature than that of the lysosomal enzymes: the antigens appear to be firmly associated with the sedimentable membrane material in these preparations.

Antigenicity of "cell membrane" fractions: The possibility was next considered that the high antigenic activity reported in various fractions (cell membrane, light mitochondria, and microsomes) might be due to the presence of H-2 antigens as an integral component of all the membrane material of the cell, including that of the cell surface as well as that of intracellular organelles. This type of distribution has been suggested previously (14, 18) but there has been little direct evidence to support the hypothesis and an attempt was therefore made in the present study to obtain data bearing on this point. The preparation of a cell membrane fraction, relatively free of contamination by intracellular organelles, presented considerable difficulty. Of the many attempts reported in the literature, the method of Neville (30) appears to have been most successful.

TABLE III

Distribution of H-2 Antigens in Various Subcellular Fractions Derived from Mouse Liver Homogenates

Mouse strain tested	Antiserum prepared		Antigens detected	Homogenate fraction tested	Antigenic activity	Acid phosphatase	ATPase	Cytochrome oxidase
	In	Against			*units/mg*	*μg Pi/mg/10 min.*	*μg/Pi/mg/15 min.*	*units/mg*
C57BL/6	BALB/c	C57BL/6	D^bEK^bV	Whole homog.	3.3	2.1	9.2	0.19
				Nuclear	3.5	1.2	5.8	0.07
				Dense mitochond.	3.1	1.8	14.4	0.32
				Light mitochond.	5.2	3.7	10.0	0.28
				Microsomal	1.9	1.0	13.0	0.03
				Supernatant	0.0		8	0.00
A/HeJ	BALB/c	A/HeJ	AEKY	Whole homog.	2.2	2.6	8.5	0.14
				Nuclear	1.3	1.1	3.4	0.10
				Dense mitochond.	1.8	2.0	15	0.37
				Light mitochond.	8.4	5.6	12	0.30
				Microsomal	4.5	0.9	16	0.02
				Supernatant	0.0		4.3	0.00
A/HeJ	C3H	A/HeJ	DFJMN	Whole homog.	2.6	2.3	9.7	0.16
				Nuclear	2.1	1.8	6.3	0.09
				Dense mitochond.	2.7	2.3	14	0.39
				Light mitochond.	6.3	4.8	13	0.36
				Microsomal	3.0	0.7	15	0.03
				Supernatant	0.0		5.8	0.00
DBA/1	A/HeJ	DBA/1	Q	Whole homog.	2.8	2.8	9.1	0.15
				Nuclear	2.7	1.7	4.4	0.09
				Dense mitochond.	2.8	2.2	13	0.31
				Light mitochond.	6.9	6.3	15	0.38
				Microsomal	3.8	1.1	15	0.04
				Supernatant	0.0		3.9	0.00

In each of the antigenic situations studied, the highest H-2 antigenic activity appears in the "light mitochondrial" fraction, which contains most of the lysosomes as judged by its high acid phosphatase activity. The ATPase activity of each of the pellets is membrane-associated ATPase, and gives a rough index of the amount of membrane material in each fraction. Cytochrome oxidase activity was measured as an index of the presence and relative numbers of mitochondria in each of the fractions. In this and subsequent tables, these enzyme activities serve as markers for judging the adequacy of the fractionation procedures; specific activities are expressed as activities per milligram of protein.

The main objection to the procedure is that its validity rests entirely on evidence obtained by phase contrast and electron microscopy, there being no biochemical demonstration of lack of contamination by mitochondria. Emmelot and Bos (31) found evidence of mitochondrial contamination in a fraction prepared by this method, but were able to remove the mitochondria by interposing a third layer of sucrose solution of density 1.20 between the layers of densities 1.16 and 1.22. The cell surface membranes accumulated at the interface between the layers of densities 1.16 and 1.20, and the mitochondria at the interface between the layers of densities 1.20 and 1.22. High ATPase activity was found to be associated with cell membrane fractions prepared in this way.

In the present study, mouse liver cell membrane fractions were therefore

TABLE IV

Retention of H-2 Antigenic Activity in "Light Mitochondrial" Pellet after Lysis by Osmotic Shock

Material assayed	Antigenic activity	Acid phosphatase
	units/mg	*μg Pi/mg/10 min.*
Pellet before lysis	6.3	14
Pellet after lysis	10.0	0
Supernatant after lysis	0	20

Osmotic lysis of the light mitochondrial pellet in distilled water leads to release into the supernatant of acid phosphatase but not of H-2 antigens. The sedimentable material which remains has no detectable acid phosphatase activity, but retains H-2 antigenic activity. The higher specific antigenic activity of the pellet after lysis is presumably a reflection of the loss of antigenically inert protein into the supernatant.

prepared in the manner described by Emmelot and Bos (31). ATPase and cytochrome oxidase activities were measured as tests of purity of the fractions, which were also subjected to electron microscopy and found to be free of mitochondria. As may be seen in Table V, the cell membrane fractions had high antigenic activity, high ATPase activity, and virtually no cytochrome oxidase activity. The membrane fraction, when gently resuspended in saline, showed particles or aggregates of considerable size and did not give a completely uniform suspension; when this suspension was then further homogenized by a few gentle strokes of the Potter homogenizer, the antigenic activity and ATPase activity both increased considerably. These results appear to confirm the presence of a large amount of antigenic activity in the cell membranes. The data also provide further evidence of the close association of the various H-2 antigens, as determined in the three different antigenic situations. The mitochondria which were isolated during this procedure were also quite pure, with high cytochrome oxidase activity but no acid phosphatase activity. It was of great interest to note that little H-2 antigenic activity could be detected in this mitochondrial fraction. This was consistent with the demonstration (18)

that the antigenic activity of the fractions of liver homogenate did not correlate well with the cytochrome oxidase activity, but rather correlated with the acid phosphatase activity.

Antigenicity of the smooth endoplasmic reticulum of liver: The endoplasmic reticulum has been pictured as a series of membranes continuous with the cell membrane and the nuclear membrane (32, 33). If these membrane systems are

TABLE V

H-2 Antigenic Activity of Plasma Membrane and Mitochondrial Fractions of Mouse Liver Homogenates

Mouse strain tested	Antiserum prepared		Antigens detected	Fraction tested	Antigenic activity	ATPase activity	Cytochrome oxidase activity
	In	Against					
					units/mg	*μg Pi/mg/15 min.*	*units/mg*
C57BL/6	BALB/c	C57BL/6	D^bEK^bV	Whole homog.	3.8	9.6	0.19
				"Cell membrane"	167	80.0	0.01
				Mitochondria	0.55	43.0	1.07
A/HeJ	BALB/c	A/HeJ	AEKY	Whole homog.	4.3	9.4	0.17
				"Cell membrane"	100	62.8	0.00
				Mitochondria	0.29	58.2	1.14
A/HeJ	C3H	A/HeJ	DFJMN	Whole homog.	3.0	8.6	0.15
				"Cell membrane"	111	71.4	0.00
				Mitochondria	0.34	51.6	1.02

The "cell membrane" material exhibits a specific H-2 antigenic activity which averages 35-fold higher than that of the crude homogenate and more than 300-fold higher than that of the mitochondrial fractions. In this and other experiments, the most highly purified mitochondrial preparations showed the lowest specific H-2 antigenic activity, suggesting that the mitochondria themselves probably possess no H-2 antigens.In the three antigenic situations tested here, the various H-2 antigens appear to be present in high concentration in the plasma membrane.

indeed continuous and anatomically similar, it might be expected that they will be found to be similar biochemically and antigenically. Manson *et al.* (20) have shown that a fraction obtained from microsomes contains high H-2 antigenic activity. We therefore isolated a smooth endoplasmic reticulum fraction from mouse liver homogenate to see whether it had H-2 antigenic activity comparable to that of the cell membrane fraction. This fraction was prepared by the method of Peters (34). The extent of separation of the smooth-surfaced microsomes from the rough was tested by electron microscopy and by means of assays for ATPase and TPNH oxidase, enzymes which are associated with the smooth-surfaced microsomes (23, 25). As shown in Table VI, the smooth

endoplasmic reticulum displayed high antigenic activity, high ATPase activity, and high TPNH oxidase activity, while the rough endoplasmic reticulum showed much lower activity for each. Neither the antigenic activity nor the ATPase activity, however, were as high as in the purified cell membrane fraction, perhaps because of a greater degree of purification of the cell membrane

TABLE VI

H-2 Antigenic Activity of Microsomes and of Smooth and Rough Endoplasmic Reticulum of Mouse Liver Cells

Mouse strain tested	Antiserum prepared		Antigens detected	Fraction tested	Antigenic activity	ATPase activity	TPNH oxidase
	In	Against			*units/mg*	*μg/Pi/ mg/15 min.*	*μmoles/ mg/10 min.*
C57BL/6	BALB/c	C57BL/6	D^bEK^bV	Whole homogen.	4.3	8.9	n.d.
				Microsomal	3.2	14	n.d.
				Smooth E.R.	22	34	3.2
				Rough E.R.	1.5	7.5	0.63
A/HeJ	BALB/c	A/HeJ	AEKY	Whole homogen.	3.8	9.4	n.d.
				Microsomal	2.7	15	n.d.
				Smooth E.R.	24	37	3.4
				Rough E.R.	1.2	6.3	0.61
A/HeJ	C3H	A/HeJ	DFJMN	Whole homogen.	4.0	9.1	n.d.
				Microsomal	3.3	14	n.d.
				Smooth E.R.	21	32	3.1
				Rough E.R.	1.3	6.8	0.64

The microsomal and rough endoplasmic reticulum fractions studied do not show a higher specific antigenic activity than that of the whole homogenate. Preparations of smooth endoplasmic reticulum, on the other hand, show a specific antigenic activity which is several-fold higher. ATPase and TPNH oxidase activities of the smooth and rough endoplasmic reticulum fractions indicate that the former is considerably richer in membrane material.

fraction. If the ATPase activity, which is membrane-associated (23), is considered to be an index of purity of the membrane fraction, then the amount of antigenic activity should be correlated with ATPase activity. Fig. 1 shows that such a correlation was in fact found.

Antigenicity of various membrane fractions as compared to that of whole liver homogenate: Since the membrane fractions which were isolated represented only very small fractions of the whole homogenate, it might be argued that these account for only a portion of the total antigenicity and that some non-membrane material might contain appreciable H-2 antigenicity. Therefore, an attempt was made to determine whether the antigenicity of the whole homoge-

nate could be accounted for on the basis of the antigen content of the several cell membrane fractions. If it is assumed that the cell membrane and mitochondrial fractions were relatively pure, as appeared to be the case as judged by enzyme assays and electron microscopy, then the approximate amount of antigenic activity that would be expected in the whole homogenate from the

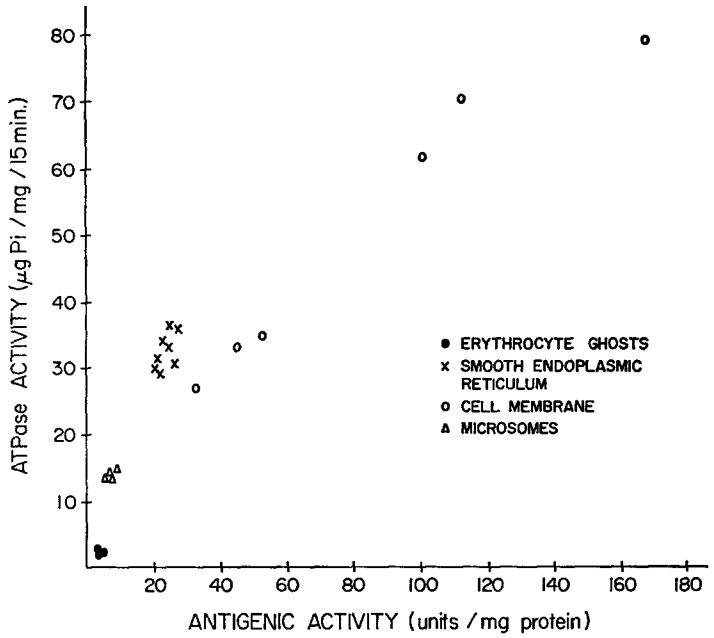

FIG. 1. Relationship between H-2 antigenic activity and ATPase activity of fractions derived from various mouse cells. This figure shows the general correlation between ATPase activity and H-2 antigenic activity of various membrane-containing fractions. The data are taken from Tables III, V, VI, VIII, and IX. Those fractions containing the highest proportion of membrane material, as judged by their specific ATPase activity, exhibit highest H-2 antigenic activity.

amount of membrane material present can be calculated (Table VII). Membrane-associated ATPase activity is a good indicator for the amount of membrane present, but this is of course obscured by mitochondrial ATPase when both mitochondria and membranes are present together. While the ATPase activity of the membranes can be distinguished since it is much more sensitive to inhibition by ouabain than is mitochondrial ATPase (23), a complete dissociation cannot be made by the use of ouabain. It was therefore necessary to determine the relationship between the ATPase and cytochrome oxidase activities of the mitochondrial fraction, and then to calculate the amount of ATPase activity due to the mitochondria in the whole homogenate. This value

was subtracted from the total ATPase activity to yield the non-mitochondrial, or membrane, ATPase activity. From this estimation of the total membrane ATPase in the whole homogenate, the amount of membrane-associated antigenicity was calculated. In view of the many assumptions necessary to make these approximate calculations, it is rather surprising that the calculated antigenicity (Table VII) corresponded so closely to the actual antigenic activity.

Antigenicity of smooth endoplasmic reticulum fractions from other tissues: The specific H-2 antigenic activity seen in the smooth endoplasmic reticulum frac-

TABLE VII

Calculation of Proportion of Total H-2 Antigenic Activity of Homogenates Attributable to "Membrane"-Associated Antigen

	H-2 antigenic specificities detected		
	D^bEK^bV	AEKY	DFJMN
(*a*) Mitochondrial protein in homogenates, calculated from specific activities of cytochrome oxidase in homogenates and in mitochondrial fractions	14.1 mg/ml	12.8 mg/ml	12.5 mg/ml
(*b*) Total ATPase activities of homogenates	768 μgPi/ml/15 min.	782 μgPi/ml/15 min.	715 μgPi/ml/15 min.
(*c*) Mitochondrial contribution to total ATPase activities of homogenates	606 "	691 "	605 "
(*d*) "Membrane"-associated (nonmitochondrial) ATPase activity, obtained by subtracting values in C above from those in B above	162 "	91 "	110 "
(*e*) "Membrane" protein required to yield ATPase activities shown in D above	2.03 mg/ml	1.45 mg/ml	1.54 mg/ml
(*f*) Total H-2 antigenic activities in the amounts of "membrane" protein shown in E above	338 units/ml	145 units/ml	172 units/ml
(*g*) Total H-2 antigenic activities of whole homogenates	304 "	368 "	255 "
(*h*) Proportion of total H-2 antigenic activities attributable to "membrane"-associated antigen	111 per cent	39.5 per cent	67 per cent

These calculations are based on the data of Table V, and involve the assumptions that all of the cytochrome oxidase activity of a homogenate is associated with mitochondria, that all of the ATPase activity is associated either with mitochondria or with cell membrane material, and that the specific H-2 antigen activity of the membrane material recovered in the "cell membrane" fraction is representative of that of the entire cell membrane. The calculations show that a very substantial fraction of the total H-2 antigenic activity of mouse liver homogenates is referable to the antigens associated with the cell-membrane material.

tions, as compared to that of the whole homogenate or even that of microsomal fractions, was quite high. If the H-2 antigens are present in the membranes of all cells, those cells with only a small amount of endoplasmic reticulum might appear to have little or no antigenic activity. If the smooth-surfaced endoplasmic reticulum fractions were isolated from such cells, however, it seemed possible that they might show comparable specific activity to those derived from the hepatic smooth endoplasmic reticulum. The microsomal fractions of various tissues were, therefore, isolated by the method of Jarnefelt (35) and the smooth-surfaced reticulum was then isolated (34). As shown in Table VIII, the smooth endoplasmic reticulum fractions of all the tissues tested gave comparably high ATPase activity. However, the smooth endoplasmic reticulum

TABLE VIII

H-2 Antigenic Activity of Smooth Endoplasmic Reticulum of Various Mouse Tissues

Mouse strain tested	Antiserum prepared		Antigens detected	Smooth E.R. fraction tested from	Antigenic activity	ATPase activity
	In	Against				
					units/mg	*μgPi/mg/ 15 min.*
C57BL/6	BALB/c	C57BL/6	D^bEK^bV	Liver	22	34
				Spleen	25	30
				Kidney	21	29
				Brain	<0.38	28
				Testis	<0.32	42
				Skeletal musc.	<0.45	38
				Cardiac musc.	<0.5	35
A/HeJ	BALB/c	A/HeJ	AEKY	Liver	24	37
				Spleen	23	33
				Kidney	20	30
				Brain	<0.31	36
				Testis	<0.36	45
				Skeletal musc.	<0.42	39
				Cardiac musc.	<0.34	36
A/HeJ	C3H	A/HeJ	DFJMN	Liver	21	32
				Spleen	22	34
				Kidney	23	38
				Brain	<0.31	36
				Testis	<0.36	45
				Skeletal musc.	<0.42	39
				Cardiac musc.	<0.34	36

While the smooth endoplasmic reticulum fractions derived from each of the tissues studied contain comparable amounts of membrane material, as judged by their ATPase activities, those derived from liver, spleen, and kidney exhibit high H-2 antigenic activity while no activity was found in the fractions derived from the other tissues.

fractions of brain, testes, and muscle showed no detectable antigenic activity. These results would seem to indicate that the cells of these tissues have little or no H-2 antigen on their membranes.

Antigenicity of erythrocytes and erythrocyte ghosts: The antigenicity of the erythrocyte has been a subject of considerable controversy. The presence of

TABLE IX

H-2 Antigenic Activity of Erythrocytes and Erythrocyte Ghosts

Mouse strain tested	Antiserum prepared		Antigens detected	Preparation tested	Antigenic activity	ATPase activity
	In	Against			*units/mg*	*μgPi/mg/15 min.*
A/HeJ	BALB/c	A/HeJ	AEKY	Erythrocytes	0.67	0.42
				Ghosts (pH 7.1)	3.1	2.1
				Ghosts (pH 8.1)	3.8	3.3
				Ghosts (pH 7.1–8.1)	0.00	8.6
A/HeJ	C3H	A/HeJ	DFJMN	Erythrocytes	0.87	0.58
				Ghosts (pH 7.1)	3.2	2.0
				Ghosts (pH 8.1)	3.8	2.7
				Ghosts (pH 7.1–8.1)	0.00	9.4
C57BL/6	BALB/c	C57BL/6	D^bEK^bV	Erythrocytes	0.073	0.25
				Ghosts (pH 7.1)	0.20	1.6
				Ghosts (pH 8.1)	0.18	1.2
				Ghosts (pH 7.1–8.1)	0.00	5.2
C57BL/6	C3H	C57BL/6	D^bFK^bNV	Erythrocytes	0.060	0.46
				Ghosts (pH 7.1)	0.14	1.9
				Ghosts (pH 8.1)	0.15	1.8
				Ghosts (pH 7.1–8.1)	0.00	9.9

Erythrocyte ghosts prepared at pH 7.1 or at pH 8.1 show higher specific activity of ATPase and of H-2 antigens, indicating that these are membrane-associated. Ghosts prepared at pH 7.1 and then exposed to pH 8.1 show a still further rise in specific ATPase activity, but no longer exhibit H-2 antigenic activity.

H-2 antigens on erythrocytes has been clearly indicated by the occurrence of hemagglutination in the presence of specific isoantisera and Möller (36) has shown that red cells have some ability to absorb isoantibodies, but erythrocytes are generally believed to be incapable of eliciting transplantation immunity (2, 11). Barrett *et al.* (37, 38), however, produced immunity to tumor transplants by injections of erythrocytes. On the basis of the high antigenic activity found to be associated with other cell membranes, one might expect the erythrocyte membrane to have comparable specific activity. The low antigenic activity of intact erythrocytes might then be explained by the small amount of mem-

brane present, compared to the extensive systems of membranes in other cells. Erythrocyte ghost preparations were made, therefore, with the aim of eliminating soluble non-antigenic proteins and thereby concentrating the antigenic activity of the red cells. The ghosts were prepared by the methods of Post *et al.* (39). The ghosts of A/HeJ erythrocytes, prepared in buffered water at pH 7.1, showed (Table IX) an increase both in antigenic activity and in ATPase activity over the intact erythrocytes. When these ghosts were then suspended in buffered water at pH 8.1 and washed several times, they showed no detectable antigenic activity. To test the possibility that the alkaline pH had in itself an adverse effect on the H-2 antigens on red cells, ghosts were prepared by lysis of erythrocytes directly in buffered water at pH 8.1. These had considerable antigenic activity. On microscopic examination, the ghosts prepared either at pH 7.1 or at pH 8.1 had easily discernible morphology. However, the ghosts which had been prepared at pH 7.1 and then brought to pH 8.1 showed only very small fragments of membrane material. These observations may be relevant to experiments by Barrett (37, 38) which showed that disruption of erythrocyte ghosts into very small fragments resulted in loss of antigenic activity.

As can be seen in Table IX, A/HeJ erythrocytes showed much more antigenic activity than did cells of the C57BL/6 strain. This is consistent with the older observation that erythrocytes of the A strain are agglutinated by isoantibody in saline while red cells of other strains are agglutinated only in the presence of dextran and normal human serum (40). Yet, even the A strain cells showed much lower specific antigenic activity than was found in membrane preparations from other cells of the same strain. This may be related to the relative impurity of the red cell membrane preparations, since the ATPase specific activity was also relatively low (see Fig. 1).

DISCUSSION

The results of the present study indicate that the H-2 antigens are closely associated with cell membrane material. The apparently conflicting reports of localization on cell surface membranes (15, 16), microsomes (19, 20, 27), and lysosomes (18) may thus be resolved. The membranes of the endoplasmic reticulum are believed to be continuous with the surface and nuclear membranes (32, 33), and may also be continuous with the Golgi apparatus (41). It is not surprising therefore to find the H-2 antigens present in or on the membrane material in these various locations. Consistent with this interpretation is the remarkable lack of antigenic activity in mitochondria. The membranes of the mitochondria are thought not to be continuous with other membranes of the cell and therefore might not be expected to share the same antigens. The observations of Easton *et al.* (42) are probably relevant to this point. These workers immunized rabbits against mouse ascites tumor cells and used the ferritin-conjugated antisera to study the localization of antigens in the tumor

cells. Antigen was detected in the plasma membranes and smooth endoplasmic reticulum, but not in the mitochondrial membranes. The findings of Manson *et al.* (20) that non-microsomal lipoproteins have little or no antigenic activity might be explained on the basis of the small amount of surface membrane and other membrane material in the residual lipoprotein. The bulk of this residual lipoprotein may well have been derived from the mitochondria and, in view of the results of the present study, would therefore not be expected to show antigenic activity.

It is quite possible that not only H-2 antigens but all of the histocompatibility antigens are localized on these intracellular and surface membranes. An indication of this is to be found in the report of Davies (43) that both H-2 and non–H-2 antigens are found in highly purified extracts from ascitic fluid. Davies has argued that there is no reason to expect that the products of different genes should be found in the same cellular fraction. This consideration is undoubtedly valid in cases in which the gene products have different functions. However, the association of various histocompatibility antigens in the same fractions suggests that all of these antigens may have a similar biological function. Perhaps all of the histocompatibility loci determine the formation of important structural or functional elements in the cellular membranes. The findings that the H-2 antigens do not appear to be present in the membranes of the endoplasmic reticulum of brain, testis, and muscle are somewhat surprising, since if the H-2 antigens do play some important functional role one might expect them to be present on all such membranes. A reasonable hypothesis is that the histocompatibility antigens do play an important role on or in these membranes, but that different histocompatibility loci express their products in different tissues. This interpretation is consistent with what little is now known of the differences in tissue distribution of the various histocompatibility antigens. For example, Amos *et al.* (44) have shown that antigens determined at the locus H-5A are primarily present in erythrocytes, kidney, testis, and lung, while antigens determined at H-6A are primarily present in erythrocytes, testis, brain, and spleen. Our working hypothesis predicts that the distribution of non–H-2 antigens in subcellular fractions will be found to be the same as that of the H-2 antigens, and a study to obtain information on this point is now under way.

In the present study, an attempt was also made to gain further insight into the genetic function of the H-2 locus. Two theories have been proposed to explain the complexity of the antigenic system determined by this locus: either there are multiple alleles determined at the locus with all of the antigens being the product of a single gene; or the antigens are the products of a series of closely linked genes. Most of the evidence for both theories has been discussed in detail (45). Pizarro *et al.* (7) have presented some evidence for the physiological unity of the H-2 system. The present study provides further evidence of this unity: in tissue distribution and in intracellular localization, all the

experiments have shown a parallel distribution of all of the H-2 antigens tested. This is in marked contrast to the findings of Shaw and Stone (5) in their investigation of the ontogeny of the B blood group antigenic system of cattle, in which the individual antigenic components did not develop simultaneously but rather varied considerably in their time of appearance. In this respect, and in the absence of crossing-over, the function of the B locus in cattle seems quite different from that of the H-2 locus in mice. The B locus in cattle has not been shown to determine histocompatibility, however, and it may not be meaningful to compare these two loci.

On the basis of the analyses of the fine structure of the genetic region of pseudoalleles in bacteriophage (46), the following construction is offered as an explanation of the behavior of the H-2 system: the H-2 locus, because of the occurrence of crossing-over, should be considered to be composed of several recons; the occurrence of mutations in the H-2 locus is considered to be evidence for the existence of two or more mutons; and the apparent physiological unity of the antigens determined at the H-2 locus suggests that this large area of genetic material constitutes a single cistron.

SUMMARY

The mouse isoantigens determined at the major histocompatibility locus known as H-2 have been found to be closely associated with the cellular surface membranes, with the membranes of the endoplasmic reticulum, and probably with those of the lysosomes as well. Mitochondrial membranes, on the other hand, show little or no H-2 antigen activity. Membrane material prepared from certain tissues, including brain and muscle, have no detectable H-2 antigenic activity. Evidence is presented which indicates that all of the H-2 antigens of the genome are expressed as a unit, supporting the hypothesis that the complex H-2 genetic locus consists of a single cistron. It is postulated that these histocompatibility antigens form some structural or functional unit in the membranes of cells.

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