*ASSOCIATION OF H-2 ANTIGENS WITH THE CELL MEMBRANE FRACTION OF MOUSE LIVER**

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Of the fifteen or more genetic loci in mice that determine antigens capable of eliciting homograft rejection,[1] only one, the H-2, has been shown also to control erythrocyte antigens detectable by hemagglutination.[2] It recently has become feasible, with the development by Stimpfling of an easy, reproducible hemagglutination method for mouse red blood cells,[3] to use the absorption of hemagglutinins as an index of H-2 antigen throughout a purification procedure.

Using this method, we demonstrate below the isolation of most of the H-2 antigenic activity of mouse liver in a lipid- and protein-containing fraction which appears to consist principally of nuclear and cellular membranes.

Materials and Methods.—Mouse strains:

DBA/2J	$H\text{-}2^d$	Retired breeders via Simonsen Laboratories, Gilroy, California.
C3H/Bi	$H\text{-}2^k$	From Simonsen Laboratories.
C57Bl/6J	$H\text{-}2^b$	Retired breeder females from incross, Jackson Memorial Laboratories, Bar Harbor, Maine.
C57Bl/10J	$H\text{-}2^b$	From Jackson Memorial Laboratories.
C57Bl/10-H-2^d (B10.D2)	$H\text{-}2^d$	Courtesy of Dr. G. D. Snell.
DBA/2JTu	$H\text{-}2^d$	From the colony of the late Dr. J. W. Turner.

Solutions: Phosphate buffered saline (PBS) contains 7.65 gm NaCl, 0.725 gm Na_2HPO_4, and 0.212 gm KH_2PO_4 in 1 liter distilled water, pH adjusted to 6.5.

PVP-PBS contains 15 gm polyvinylpyrrolidone (PVP) (33 gm of 45% aqueous solution of type NP-K60 obtained from Antara Chemicals, Division of General Aniline Corporation) in 1 liter PBS. It was autoclaved and stored at room temperature.

Sucrose-phosphate contains 85.8 gm sucrose per liter $M/75$ phosphate buffer pH 7.0 (Sorensen's phosphate buffer diluted $1/6$).

Citrate saline contains 1 part of 3.4% sodium citrate to 4 parts 0.85% NaCl.

Red blood cells: Red blood cells from the appropriate mouse strain were collected in citrate-saline, washed 3 times with 0.85% NaCl, resuspended in PBS to 10% and stored in the refrigerator. Before use, a further dilution to 2% with PBS was made.

Hemagglutination: The method of hemagglutination used was a slight modification of the method developed by Stimpfling.[3] Each 10 mm tube contained 0.1 ml of a serial dilution of antiserum in PVP-PBS plus 0.05 ml of 2% red blood cell suspension in PBS. After 30 minutes, the tubes were centrifuged for 30 seconds at approximately 250 × *g*, 0.85% NaCl (0.3–0.5 ml) was layered over the PVP-PBS, and then, with a Pasteur pipette, a jet was gently directed at the pellet. If agglutination occurred, the cells came up as: one large clump (scored as 4), several large clumps (scored as 3), small clumps and loose cells (scored as 2), or mainly loose cells with some persistent clumps (scored as 1). In the absence of agglutination, there was a cloud of loose cells, no clumps (scored as negative). The titer was taken as the last serum dilution in the series to be scored as 1 or more.

Absorption: Material to be used to absorb antiserum was packed by centrifugation, then resuspended in antiserum diluted $1/20$ in PVP-PBS. After 30 minutes at 8°C, the absorbing material was once again centrifuged, this time in the cold, and the antiserum was removed and titrated.

Antiserum production: $H\text{-}2^b$ (C57Bl/6J) female breeders (retired from incrossing) were injected with DBA/2JTu cell suspension six times at weekly intervals, (1 spleen and thymus per 30

animals per injection). Blood was collected from the tail artery, pooled, and allowed to clot overnight in the refrigerator. The clot was removed and small aliquots of serum were frozen in sealed ampoules.

No diminution of titer of antiserum has been noted due to freezing or storage for over 6 months at −20°C.

Protein determinations: Protein was determined by the method of Lowry *et al.*[4] Crystalline bovine serum albumin (Armour) was used as standard.

Experimental Results.—Isolation: Livers from exsanguinated H-2^d (DBA/2J) and H-2^k (C3H/Bi) mice were ground with a Duall[5] homogenizer in 5 volumes of sucrose-phosphate and centrifuged for 3 minutes at 250 × *g* (Fig. 1). The sediment, consisting mainly of nuclei, cell membranes and debris, was resuspended in

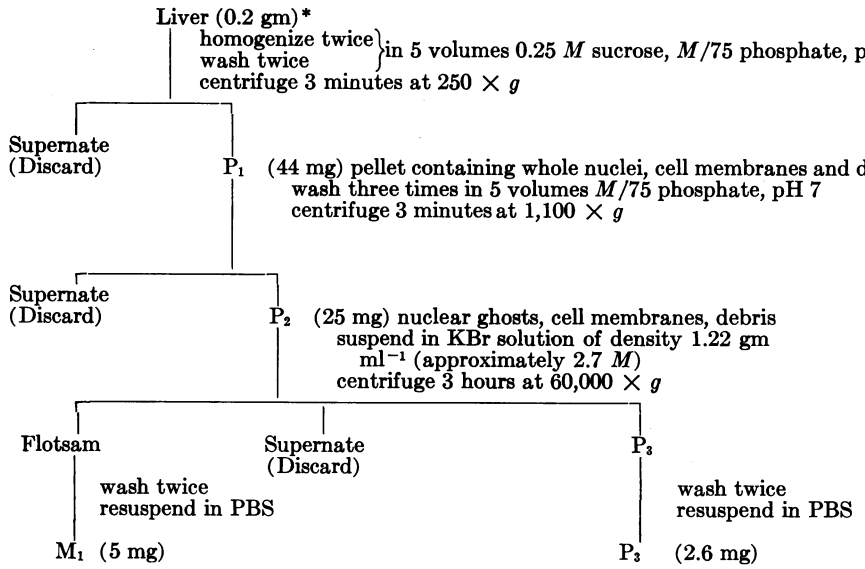

FIG. 1.—Isolation flow sheet. * The figures in parentheses give protein content expressed per gm fresh weight of liver.

sucrose-phosphate, homogenized again and washed twice (P_1). It was then suspended in *M*/75 phosphate buffer, pH 7.0 (which lysed the nuclei), centrifuged for 3 min at 1100 × *g* and washed twice in the same buffer (P_2). Next it was suspended in potassium bromide solution at a final density of 1.22 gm ml^{-1} (approximately 2.7 *M*, or 317 gm KBr per liter solution) and centrifuged 3 hr at 60,000 × *g*.

After centrifugation the material in the tube was distributed as (1) a compacted sheet of flotsam at the top, which was lifted off with a 60-gage stainless-steel-screen spatula, washed twice, and resuspended in PBS (M_1), (2) a clear viscous supernatant which was discarded, and (3) a pellet, which was washed twice and resuspended in PBS (P_3). The M_1 fraction contained 2 per cent of the protein of the original liver homogenate.

After several hours in the refrigerator, M_1 expanded and became fluffy. When observed microscopically, it appeared to consist mostly of cellular and nuclear membranes.

Immunological characterization: Incubation of 4.0 mg M_1 with 0.2 ml of a $^1/_{20}$ dilution of an H-2^b anti H-2^d antiserum, which has a titer of $^1/_{1280}$ to $^1/_{2560}$, for 30

TABLE 1

ABSORPTION OF HEMAGGLUTININS BY MEMBRANE FRACTION*

M_1 (Membrane fraction) Mg Protein	Antiserum dilution (after absorption) (1/20)	(1/40)	(1/80)	(1/160)	(1/320)	(1/640)	(1/1280)	(1/2560)
4.0	†1	1	±	—	—	—	—	—
3.0	1	1	1	—	—	—	—	—
2.0	2	3	2	±	—	—	—	—
1.0	2	4	2	2	1	—	—	—
0.5	3	3	2	2	1	—	—	—
0.25	2	4	4	4	3	2	1	—
Unabsorbed antiserum	4	3	3	2	3	3	2	1
Normal serum	—	—	—	—	—	—	—	—

* Antiserum used: H-2^b anti H-2^d. 0.2 ml of serum was absorbed at 1/20 dilution for 30 minutes at 8°C.
† 4, 3, 2, 1 indicate agglutination, 4 being the greatest, 1 being the weakest; ± indicates questionable agglutination; — indicates no agglutination. Also see *Materials and Methods* for further explanation.

TABLE 2

ANTI H-2^d ACTIVITY OF C57Bl/6J ANTI-DBA/2JTu SERUM

Source of erythrocytes	H-2 allele	Antiserum dilution (1/40)	(1/80)	(1/160)	(1/320)	(1/640)	(1/1280)	(1/2560)
C57Bl/ 10-H-2^d	H-2^d	*4	4	4	2	1	1	—
C57Bl/10J	H-2^b	—	—	—	—	—	—	—

* 4, 3, 2, 1 indicate agglutination, 4 being greatest, 1 being weakest; ± indicates questionable agglutination; — indicates no agglutination. Also see *Materials and Methods* for further explanation.

TABLE 3

SPECIFICITY OF HEMAGGLUTININ ABSORPTION*

Material used to absorb	Equiv. gm fresh wt. liver	Reciprocal of hemagglutination titer with erythrocytes from H-2^d	H-2^k
H-2^d P_2 (lysed homogenate)	1.0	40	—†
H-2^b P_2 (lysed homogenate)	1.0	640	—
H-2^d M_1 (membrane fraction)	1.0	<40	<40
H-2^k M_1 (membrane fraction)	1.0	160	<40
Unabsorbed serum		640	320

* Antiserum used: H-2^b anti H-2^d. 0.2 ml of serum was absorbed at 1/20 dilution for 30 minutes at 8°C.
† (—) indicates not tested.

minutes at 8°C reduced the titer of the serum to 1/40 (Table 1). (The unabsorbed serum contains anti H-2^d antibodies as it agglutinates erythrocytes from C57Bl/10-H-2^d but does not agglutinate erythrocytes from the coisogenic strain C57Bl/10 (H-2^b) (see Table 2). The decrease in titer is roughly proportional to the amount of antigen used over the range 4.0 to 0.25 mg protein (Table 1).

The specificity of absorption was demonstrated in two ways. A fraction (P_2) prepared from H-2^d livers removed nearly all the anti H-2^d activity from the serum, while a similar fraction prepared from H-2^b liver (the strain of the serum donor) left the hemagglutinins intact (Table 3, lines 1 and 2).

A more critical test of specificity was based on the selective absorption of antibodies from a serum, utilizing the immunological cross-reactivities of H-2 antigens (Table 4). Thus, an anti H-2^d serum prepared in H-2^b animals would be expected to cross-react with antigens in the H-2^k strain, and therefore to agglutinate H-2^k erythrocytes. An H-2^k fraction should remove hemagglutinins directed against the cross-reacting components (C, H) of H-2^k but should leave behind the hemag-

TABLE 4

RELEVANT H-2 COMPONENTS AND EXPECTATION OF ANTIBODY SPECIFICITIES[6]

Serum	Strain	H-2 allele	Relevant antigenic components									Erythrocytes agglutinated
	C57Bl/6	H-2^b		–	D^b	E	F	–	–	–	N	Z	
	DBA/2	H-2^d		C	D	E^d	F	H	J	M	N	Z	
H-2^b anti H-2^d			*anti*	*C*	*D*	*E^d*	–	*H*	*J*	*M*	–	–	H-2^d, H-2^k
	C3H	H-2^k		C	–	–	–	H	–	–	–	±	
H-2^b anti H-2^d absorbed with H-2^k			*anti*	–	*D*	*E^d*	–	–	*J*	*M*	–	–	H-2^d

TABLE 5

RECOVERY OF HEMAGGLUTININ ABSORBING ACTIVITY DURING FRACTIONATION*

Fraction used to absorb	Equivalent fresh weight of liver (gm)	Protein (mg)	Antiserum dilution (after absorption) (1/20)	(1/40)	(1/80)	(1/160)	(1/320)	(1/640)	(1/1280)	(1/2560)
P_1 (homogenate before lysis)	0.6	26	†±	–	–	–	±	–	–	–
P_2 (lysed homogenate)	0.6	15	±	–	–	–	–	–	–	–
S_1 (KBr sediment)	0.6	1.6	2	2	2	2	2	±	–	–
M_1 (membrane fraction)	0.6	3.0	1	1	1	–	–	–	–	–
Unabsorbed serum	—	–	4	3	3	2	3	3	2	1

* Antiserum; H-2^b anti H-2^d. 0.2 ml of serum was absorbed at 1/20 dilution for 30 minutes at 8°C.
† Same as † footnote in Table 1.

TABLE 6

ANALYSIS OF MEMBRANE FRACTION

Material	Dry weight (mg)	Protein (mg)	Carbohydrate (mg)[7] (α-Napthol)	(Indole)	Hexosamine[8] (mg)
M_1 total	6.45	4.95	0.05	0.04	<0.01
Lipid soluble[9]	2.65*	0.55	–	–	–
Lipid insoluble[9]	3.80	3.77	–	–	–

* Figures indicate mg per 6.45 mg dry weight of M_1. Each value is the average of 2 determinations. 60–80 mg dry weight of M_1 is a usual yield from 10 gm fresh weight of liver.

glutinins directed against the remaining H-2^d components. H-2^d M_1 should, of course, remove all the hemagglutinins.

The results conformed to the above predictions when the appropriate absorptions were performed (see Table 3, lines 3 and 4). Absorption of the H-2^b anti H-2^d serum with H-2^d M_1 removed all hemagglutinating antibody (including that which could have agglutinated H-2^k erythrocytes). Absorption with H-2^k M_1 removed only the cross-reacting antibodies, removing therefore all the titer against H-2^k erythrocytes but leaving a substantial titer against H-2^d erythrocytes.

In Table 5, the recovery of H-2 antigen throughout the purification procedure is estimated. Most of the activity is recovered in the M_1 fraction. Reworking of the sediment (P_3) gave additional active M_1 fraction and an inactive sediment.

Chemical characterization: As has been pointed out earlier, the M_1 fraction consists principally of cellular and nuclear membranes, and therefore is most decidedly not pure antigen. However, chemical characterization of the fraction is of interest with respect to both the chemistry of membranes and the chemical nature of the H-2 antigens.

The material in M_1 is almost entirely lipid and protein (Table 6). There is less than 1 per cent total carbohydrate, and hexosamine is not detectable.

The antigenic activity is stable for a week or more in the refrigerator but was not detectable after exposure to 56–60°C for 1 hr or 100°C for 5 min.

Discussion.—Serological methods as assays for transplant antigens must be used cautiously due to the uncertainty that antigens which react with hemagglutinins are identical to those which elicit a transplantation immunity.[10, 11] However, the evidence that hemagglutinins are also enhancing antibodies,[12] which act by blocking the passage of antigens from a graft to the sites where they can evoke the cellular immune response,[13] encourages the use of methods involving humoral antibodies for isolation of transplantation antigens, even though a material cannot be finally accepted as a transplant antigen until its injection can be shown to cause an accelerated homograft reaction.

Using hemagglutinin absorption as an index of antigen offers certain advantages in the mouse beyond the rapidity with which tests can be made. It immediately restricts consideration to the antigen (or antigens) controlled by the H-2 locus, as agglutination of erythrocytes by isoantisera other than those directed against H-2 antigen (or antigens) is not readily demonstrable.[14] This permits the use of standard mouse stains such as C57Bl/6 and DBA/2 which differ at a number of histocompatibility loci without losing the selectivity offered by Snell's (less readily available) isogenic resistant pairs, which differ only at a single locus.

Histocompatibility antigens have been found in the nuclear, mitochondrial and microsomal fractions.[15, 16] This is not inconsistent with our finding of most of the H-2 antigen in the cell membranes, since nuclear fractions usually contain cell membranes; and when cells are disrupted by sonic oscillation, membrane fragments become sedimentable only by high-speed centrifugation[17] and will thus contaminate the mitochondrial and microsomal fractions.

Most of the antigen present in the extract after the first homogenization is recovered in the fraction which on centrifugation floats in the high-density potassium bromide solution. This material was observed microscopically to consist of folded, membranous structures suggestive in appearance of empty liver parenchymal cells. Electron microscope studies on a rat liver membrane preparation obtained using a similar fractionation scheme[18] (which differed from our own essentially in the use of high-density sucrose solution instead of a potassium bromide solution for the flotation step) showed that the structures isolated are nuclear and cell membranes.

The amino sugar and carbohydrate content of our preparation is lower than that reported by Kandutsch and Reinert-Wenck[16] for their preparations of enhancement factor, but as the data do not permit an estimation of the percentage of H-2 material in a fraction, the small carbohydrate content could still be a major constituent of the H-2 component. In fact, as long as the antigen resides in an insoluble cell fraction, where its purity cannot be determined, conclusions based on stability to treatment with various agents (e.g. heat, solvents, enzymes) must be constantly under review. Any effect may be due either to a change in the antigen or to a change in the material surrounding the antigen, rendering the antigenic activity undetectable.

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THE SOLUBILIZATION OF MICROSOMAL ANTIBODY ACTIVITY BY THE SPECIFIC INTERACTION BETWEEN THE CRYSTALLIZABLE FRACTION OF γ-GLOBULIN AND LYMPH-NODE MICROSOMES

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In a previous study,[1] it was shown that when 2,4-dinitrophenyl bovine γ-globulin is injected into guinea pigs there appears in the microsomal fraction of regional lymph nodes an antibody activity which is specific for the 2,4-dinitrophenyl group. The present work is concerned with the questions: (1) is the antibody activity of lymph-node microsomes[2] due to conventional serum antibody linked to microsomes, and, if so, (2) what kind of linkages are responsible? In order to examine these questions it was necessary to determine the conditions required to separate antibody activity from microsomes and to compare the solubilized material with serum antibody of the same specificity.

The recent work of Porter[3] describing three fractions of rabbit γ-globulin and antibodies, obtained by papain digestion and chromatography, has led to a significant advance in understanding the structure of antibodies and has provided methods which may prove applicable to studies of antibody synthesis. In the present report, evidence is presented that microsomal antibody activity is the result of a specific association between lymph-node microsomes and that segment of γ-globulin which corresponds to Porter's fraction III.