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Studies on the Distribution of Some Histocompatibility Antigens in Mouse Liver Plasma Membrane and Microsomal Fractions

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Summary. The plasma membrane fraction, its two major derivative subfractions, and a smooth-surfaced microsomal fraction have been prepared from mouse liver tissue homogenates and their purity has been assessed by using enzymic markers. The antigenicity of the fractions was compared using a lymphocytotoxicity inhibition assay. The results indicate that the vesicular subfraction of the plasma membranes, known to be enriched in certain marker enzymes, was the most active preparation, whereas the microsomal fraction was the least active. An approximately two-fold increase in antigenicity was observed when the assay was performed on deoxycholate-treated fractions. The results suggest that the *H-2* histocompatibility antigens may be present at higher concentrations in certain areas of the liver cell surface membrane.

INTRODUCTION

The *H-2* histocompatibility antigens are generally recognized to be associated with cellular membranes (Schreffler, 1967). Subcellular fractionation studies have pointed to a microsomal or lysosomal localization (Manson, Foschi and Palm, 1962, 1963; Basch and Stetson, 1963), but recent evidence has indicated a high degree of enrichment of these antigens in the surface or plasma membrane fraction (Herberman and Stetson, 1965; Haughton, 1966; Ozer and Wallach, 1967). The liver plasma membrane fraction, prepared by the method of Neville (1960) as modified by Emmelot, Bos, Benedetti and Rümke (1964) was shown by Herberman and Stetson (1965) to possess a high degree of antigenic activity in relation to other subcellular fractions. The plasma membrane fraction of mouse liver has now been prepared in high yield and purity using the zonal rotor, and the morphological, enzymic and chemical properties of these membranes have been subject to detailed examination (Evans, 1970). Furthermore, the isolated plasma membrane fraction has been resolved by sucrose-density centrifugation into two subfractions which differ in the specific activities of the accepted plasma membrane enzyme markers as well as in neuraminic acid and phospholipid content expressed relative to protein content (Evans, 1969, 1970). The two subfractions also differ morphologically in sections observed in the electron microscope, the light subfraction being vesicular and the heavy subfraction retaining the sheets of membranes and junctional complexes which characterize the liver plasma membrane fraction. The availability of these subfractions prompted us to examine

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by a lympho-cytotoxic inhibition assay their antigenicity in relation to unfractionated membranes, and the results are also compared with those obtained with a liver smooth-surfaced microsomal fraction prepared under conditions resulting in minimal contamination by fragments of the plasma membrane.

MATERIALS AND METHODS

Animals

Two inbred strains of mice, CBA and C57BL/6, of either sex, and weighing 10–15 g were used. Animals starved for 16 hours were used for preparation of the microsomal fraction, and fed animals for the plasma membrane fractions.

Preparation of subcellular fractions

The preparation of the liver plasma membrane fractions has been described (Evans, 1969, 1970). Briefly, mouse livers were dispersed in 1m-mole $NaHCO_3$ using a loose-fitting Dounce homogenizer, radial clearance 0·006 in., and the filtered homogenate centrifuged at 1000 g for 10 minutes. The crude pellet was gently resuspended in 1 m-mole $NaHCO_3$ and introduced into the centre of the 'A' type zonal rotor (M.S.E. Ltd, Buckingham Gate, London) in an M.S.E. 'Mistral' 6L centrifuge. The zonal rotor contained a sucrose gradient of 6–54 per cent (w/v) sucrose, and following centrifugation at 4000 rev/min for 50 minutes the plasma membrane fraction was observed as a discrete band at sucrose concentration 40–44 per cent (w/v). After unloading the rotor, the membranes were collected by pooling and centrifuging the appropriate fractions, and gently resuspending in 0·25 M sucrose. The plasma membranes were further purified by density gradient centrifugation in a continuous sucrose gradient 30–50 per cent (w/v) in a Beckman S.W.27 rotor for 3 hours after which time the plasma membrane fraction banded at sucrose density 1·17 g/cm^3. The plasma membranes were resuspended in 0·25 M sucrose using twenty-five strokes of a tight-fitting Dounce homogenizer, radial clearance 0·002 in. and then fractionated into light and heavy subfractions of density 1·12 and 1·18 g/cm^3 by density gradient centrifugation in sucrose. The pooled membrane fractions were collected, centrifuged and resuspended in 0·25 M sucrose for enzymic analysis, or in a phosphate-buffered saline of the following composition for serial dilution for the antigen assay: NaCl, 8·09 g; KCl, 0·29 g; KH_2PO_4, 0·29 g; K_2HPO_4, 1·159 g; $CaCl_2$, 0·19 g; $MgCl_2.6H_2O$, 0·19 g, dissolved in 1 litre H_2O.

Smooth microsomal membranes were prepared essentially according to the method of Glaumann and Dallner (1968). A 25 per cent (w/v) homogenate of livers in 0·25 M sucrose was prepared by using five to seven strokes of the loose-fitting Dounce homogenizer. The post-mitochondrial supernatant, prepared by centrifugation of the homogenate at 9000 $g_{av.}$ for 20 minutes was made 15 m-moles in CsCl by addition of 1 M CsCl and layered over a one-third volume of 1·3 M sucrose, 15 m-moles CsCl. Following centrifugation for 2 hours at 95,000 $g_{av.}$ in a SW27 rotor the material collected at the interphase, designated the smooth microsomal fraction, was collected, washed by centrifugation and resuspended in sucrose for enzyme analysis or phosphate buffered saline for antigen tests.

Enzyme determinations

Glucose-6-phosphatase, 5′-nucleotidase and succinic dehydrogenase activities were estimated at 37° as described previously (Evans, 1969). Protein content of membrane and

cell suspensions was measured by the method of Lowry, Rosebrough, Farr and Randall (1951) with bovine serum albumin (Armour & Co., Eastbourne) as standard.

Antigen assay

The antigen content of the various fractions was determined by assaying their inhibitory activity on the cytotoxic reaction of allo-antisera against lymph node lymphocytes labelled with ^{51}Cr (Sanderson, 1964; Wigzell, 1965). Antisera were raised in the two mouse strains against each other by repeated intraperitoneal injections of spleen cell suspensions. Two-fold or 1·6-fold serial dilutions of the liver preparations were mixed with equal volumes of an antiserum diluted to obtain lysis of 90 or 100 per cent (experiments 1 and 3) or a lower value (experiment 2) of the labelled target cells. After incubation for at least 2 hours at room temperature and overnight storage at 4°, the suspensions were centrifuged at 1000 ***g*** for 20 minutes. Portions (0·1 ml) of the supernatant were transferred to test tubes, and 5×10^5 lymphocytes in 0·1 ml VBS-BSA (veronal-buffered saline tablets (OXOID), dissolved in 0·1 per cent (w/v) bovine serum albumin) were added. After incubation at 37° for 30 minutes the suspension was diluted with 1 ml VBS-BSA, and centrifuged at 600 ***g*** for 12 minutes. The sedimented cells were resuspended in 0·2 ml VBS-BSA, and 0·1 ml of absorbed rabbit C, which had been diluted five-fold with VBS-BSA was added. The degree of cytolysis occurring after incubation at 37° for 30 minutes was determined after stopping the reaction with 1 ml of ice-cold saline and centrifuging at 1000 ***g*** for 10 minutes. Portions of the supernatant were then counted in a Packard scintillation counter for released ^{51}Cr. The inhibiting activity was expressed as units per mg protein, in which one unit represents the decrease in lysis from 85 to 35 per cent of the cells (Basch and Stetson, 1962).

RESULTS

The subcellular fractions used for antigenic studies were first examined for marker enzymes in order to determine their relative purity (Table 1). A plasma membrane enzyme marker, 5′-nucleotidase, was shown to be present at high specific activities in the isolated plasma membranes, and the light subfraction prepared showed a further increase in activity. The yield of plasma membrane was 4·5 mg protein/g liver protein. The microsomal fraction, consisting of elements of the smooth endoplasmic reticulum showed enrichment of the marker enzyme glucose-6-phosphatase, whereas the low activity of 5′-nucleotidase present indicates the low degree of contamination by fragments of the surface membrane. Succinic dehydrogenase assays indicate the low extent of contamination of the fractions by mitochondria.

TABLE 1

ENZYME ACTIVITIES OF MOUSE LIVER SUBCELLULAR FRACTIONS

Fraction	5′-nucleotidase	Glucose-6-phosphatase	Succinic dehydrogenase
Homogenate	0·53	0·88	0·070
Plasma membranes	15·2	0·55	0·012
Light subfraction	28·2	ND	ND
Heavy subfraction	9·0	ND	ND
Smooth-surfaced microsomes	0·86	2·60	0·029

Activities are expressed as μ-moles product liberated per mg of protein per hour at 37°.
ND, Not determined.

In three experiments, the indicated subcellular fractions were prepared from liver homogenates of either C57BL/6 or CBA inbred strains of mice and assayed for their antigen content (Table 2). In experiment 2, the fractions were also diluted in saline containing 0·012 per cent deoxycholate in an attempt to assess whether more antigenic activity could be made available for assay. The results show that deoxycholate caused an approximately two-fold increase in activity in all the fractions examined.

TABLE 2

H-2 ANTIGENIC ACTIVITY OF MOUSE LIVER PLASMA MEMBRANE AND MICROSOMAL FRACTIONS

Expt No.	Mouse strain tested	Antiserum prepared		Fraction	Antigenic activity (units/mg protein)*	
		In	Against		Without DOC†	With DOC†
1	C57BL/6	CBA	C57	Plasma membrane	8	–
				Light subfraction	16	–
				Heavy subfraction	4	–
2	CBA	C57	CBA	Plasma membrane	0·6	1·5
				Light subfraction	6	10
				Heavy subfraction	2·5	4
3	CBA	C57	CBA	Plasma membrane	10	–
				Light subfraction	20	–
				Heavy subfraction	12	–
				Smooth microsomes	6	–

* The unit of antigenic activity is defined in Methods; in experiment 2, a lower anti-serum dilution was used.
† DOC, deoxycholate.

In specificity control experiments preparations from C57BL/6 mice were incubated with C57BL/6-anti-CBA sera and preparations from CBA mice were absorbed with CBA-anti-C57BL/6 sera. Antigen suspensions with the same concentrations as used in the experimental series never showed any non-specific absorption of cytotoxic antibodies from the allo-antisera. Because of the two-step modification of the ^{51}Cr-release assay applied, the anti-complementary activity of the antigen preparations did not interfere with the test. In no instance was a smaller percentage of the test cells lysed than could be lysed by the addition of complement alone.

The main feature which emerges is that the light plasma membrane subfraction exhibits a higher antigenic activity than the other fractions examined. The low activity of the unfractionated plasma membranes observed in experiments 2 and 3 when compared with its two derivative light and heavy subfractions may be explained in terms of the unavailability of large areas of the membraneous components present because the membranes were only gently dispersed. The intensive homogenization used in the subfractionation procedure breaks up the membrane into smaller discrete units which are more amenable to antigen assay.

The smooth-surfaced microsomal fraction (experiment 3) is less active than the plasma membrane fractions, but still contains a relatively high degree of antigenic activity when compared with the low 5′-nucleotidase activity of this fraction. Furthermore, the difference in antigenicity between the microsomal and plasma membrane fractions is not so great as the six-fold difference reported by Herberman and Stetson (1965).

DISCUSSION

A number of criteria has been used for evaluating the purity of the plasma or surface membrane fraction of animal cells (Wallach, 1967). Membrane-bound enzymic activities accepted to be located more or less exclusively in the liver plasma membrane fraction include 5′-nucleotidase, β-leucylnaphthylamidase and Na^+-stimulated adenosinetriphosphatase whereas assays for markers for other subcellular fractions monitor the extent of cross-contamination (Evans, 1969, 1970; Emmelot and Bos, 1969). Morphologically, the appearance in the electron microscope of areas containing sheets of membranes with junctional complexes (tight junctions, desmosomes) and bile canaliculi indicate the derivation of the membranes from the liver cell surface. The sialic acid and cholesterol content, although these constitutents are not confined to the plasma membrane fraction, can also function as additional markers. Immunochemical markers for the liver cell surface have not been explored in such detail. Emmelot *et al.* (1964) using heterologous antisera found the rat liver plasma membrane fraction to contain three non-serum antigens which, however, were released by saline extraction, thus throwing some doubt on their role as membrane antigens, since they could be absorption artefacts. Neville (1968) has isolated an organ specific protein antigen from rat liver plasma membranes which could not be detected in extracts of liver nuclei, mitochondria, endoplasmic reticulum or cell sap. The present results demonstrate by a semi-quantitative cytotoxic inhibition assay, that the liver cell surface fraction possesses extremely high *H-2*-antigenic activity and may, therefore, provide an additional criterion for the surface derivation of the membranes prepared by this method. The smooth-surfaced microsomal fraction possesses lower antigenic activity. However, microsomal fractions are generally accepted to contain fragments of the surface membrane, and this is often invoked to account for the presence of alloantigens in such fractions (Boyle, 1968). Since the highly antigenic plasma membrane light subfraction is vesicular, and has a lower sedimentation rate than unfractionated membranes, contamination of liver microsomes by this plasma membrane subfraction could contribute to the antigenicity of microsomal fractions. The major mouse transplantation antigens can be isolated from a variety of sources as insoluble lipoprotein complexes of buoyant density in sucrose of 1·13–1·14 (Palm and Manson, 1965), and the plasma membrane light subfraction examined in the present work also has a similar density. The continuity of the endoplasmic reticulum and the surface membrane occurring in the intact cell, and the unanswered question of the site of biosynthesis of the surface membrane and its antigenic components indicate, however, that caution should be exercised in the interpretation of subcellular distribution studies of the transplantation antigens.

In measuring antigenic activity which appears to form an intrinsic part of the membrane structure, the question of availability of the antigenic sites for assay is clearly an important one. The use of detergents represents an attempt to overcome the problem of masking of groups, and the low concentration of deoxycholate used (0·012 per cent) resulted in an increase in the antigenicity measured in all three plasma membrane fractions. Higher concentrations of detergent could not be used in the cytotoxic assay because of the instability of the target cells. A further point deserving comment is the problem of the 'masking' of antigenic sites due to their possible localization on the inner sides of vesicular subcellular structures. Homogenization of the liver results in the breakage of the membrane systems of the endoplasmic reticulum into fragments with subsequent fusion of the free membrane edges to form vesicles of various sizes. The isolated plasma membrane fraction

contains vesicles attached to membrane strips and present inside the bile canaliculi. The plasma membrane heavy subfraction contains large numbers of membrane strips, and it would be expected that any antigens present would be favourably orientated for detection in contrast to the vesicular light subfraction or the smooth microsomal fraction examined in which antigenic sites could be located on the inner or outer, or on both surfaces of the vesicles.

The morphological entities present in the heavy subfraction give a clear indication that many of the membranes are derived from the areas of the parenchyma cell surface that are in contact with neighbouring cells. The high antigenicity of the light subfraction, together with its relatively high content of neuraminic acid and marker enzymes, adds credence to the surface derivation of the vesicular membranes prepared from the liver plasma membrane fraction. It has been suggested (Evans, 1970) that the vesicular light subfraction is derived from the area of the surface membrane which invaginates to form the microvilli of the bile canaliculus, since this interpretation would be in agreement with histochemical results. The light subfraction is enriched in phosphate-hydrolysing enzymes, and Novikoff, Essner, Goldfischer and Heus (1962) have shown by histochemical studies that there is intense nucleoside phosphatase activity in the region of the bile canaliculi of intact liver sections. The results presented therefore suggest that the *H*-2 antigens are not uniformly distributed over the whole of the liver cell surface, but are present at higher concentration in certain parts. Such an interpretation would be in agreement with results showing the selective distribution of surface antigens in other cell types (Boyse, Old and Stockert, 1968; Hämmerling, Aoki, Wood, Old, Boyse and de Harven, 1969).

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