Clin. exp. Immunol. (1976) 26, 491–504.

Leucocyte migration inhibition with inner and outer membranes of mitochondria and insoluble hepatocyte surface membranes prepared from rat liver in patients with chronic hepatitis and cirrhosis

GOROKU OHTA, AKITAKA NONOMURA & ISAO NISHIMURA *Department of Pathology, School of Medicine, Kanazawa University and Department of Medicine, National Kanazawa Hospital; Kanazawa, Japan*

(*Received* 5 *May* 1976)

SUMMARY

Patients with chronic liver disease were tested for delayed hypersensitivity to the outer and the inner membranes of mitochondria (OMM and IMM) and the insoluble hepatocyte-surface membranes (IHSM), prepared from rat livers, by means of leucocyte migration inhibition technique. Positive reaction to OMM was found in 37% of patients with chronic persistent hepatitis and 35% of those with chronic active hepatitis and 43% of those with liver cirrhosis ($P<0{\cdot}05$). That to IMM was 55%, 43% and 36% ($P<0{\cdot}05$) and to IHSM was 37%, 47% and 45% respectively ($P<0{\cdot}05$). IHSM was found to contain liver-specific components and patients with positive response to IHSM did not reveal at all a positive reaction to rat renal cell-surface membranes. The incidence of positive response to IHSM was significantly higher (54·2%) in patients with the present or previous infection with HBAg than in HBAg-non-infected patients (21·4%) ($P<0{\cdot}05$). And there seemed to be a good correlation between a degree of cellular response to purified HBsAg and that to IHSM in these HBAg-infected patients. No correlation, however, was found between that to purified HBsAg and that to OMM or IMM in the same patients. This suggested that the cellular response to either HBsAg or IHSM, both related closely, may play a role in the perpetuation of chronic liver disease.

INTRODUCTION

Increasing attention has been to a cellular immune mechanism of liver-cell destruction in chronic liver disease (Dudley, Fox & Sherlock, 1972; Eddleston & Williams, 1974; Sherlock, 1975). Recently liver-specific soluble lipoproteins (LSP) were isolated from human cadaver liver (Meyer zum Büschenfelde *et al.*, 1972) and active hepatitis was produced in rabbit with prolonged immunization with LSL (Meyer zum Büschenfelde, Kossling & Miescher, 1972). Subsequent studies of leucocyte migration with the antigens revealed the presence of cellular hypersensitivity to LSP in active chronic hepatitis and primary biliary cirrhosis (Miller *et al.*, 1972; Smith, Eddleston & Williams, 1975). A high frequency of positive cellular reactions to either HBsAg or LSP was found in HBAg-negative patients with chronic active hepatitis as well as in HBAg-positive cases (Lee *et al.*, 1975). This supported the hypothesis that cellular response to hepatitis B virus antigen can initiate an autoimmune reaction to liver-specific soluble lipoproteins on the hepatocyte surface in many cases of active chronic hepatitis and the autoimmune reaction may be important in the production of chronic liver damage (Lee *et al.*, 1975).

On the other hand delayed hypersensitivity to purified mitochondrial membranes from human or rat livers and other organs was found in many patients not only with primary biliary cirrhosis (Brostoff

Correspondence: Dr G. Ohta, Department of Pathology, School of Medicine, Kanazawa University, Kanazawa, Japan.

et al., 1969; Brostoff, 1970) but also non-hepatic diseases of immunological etiology, that is, Hashimoto thyroiditis (Brostoff, Roitt & Doniach, 1969; Calder *et al.*, 1972), Addison's disease (Nerup *et al.*, 1970), pernicious anaemia (Goldstone *et al.*, 1973) and diabetes mellitus (Richens *et al.*, 1973; Richens *et al.*, 1974) and so on. It seemed, therefore, likely that mitochondria responsible for the delayed reaction was neither organ-specific nor species-specific and the reaction with mitochondria did not play a major role in the production of damage to specific organs.

The present experiment was designed to look for possible correlations between cellular immunity to HBsAg and that to insoluble hepatocyte-surface membranes in chronic hepatitis and cirrhosis. The insoluble membrane is known to contain liver-specific proteins, as mentioned by Sheffield & Emmelot (1972), and are the site of direct contact with specifically functioning lymphocytes in diseased livers. Since mitochondrial membranes from the hepatocytes, however, are not the site of the contact, the results of the hepatocyte surface membrane experiments were compared with those using mitochondrial membranes in the same patients.

MATERIALS AND METHODS

Patients. Leucocyte-migration tests were performed on a total of seventy patients with chronic liver diseases which consisted of twenty patients with chronic persistent hepatitis, twenty-one patients with chronic active hepatitis and twenty-nine patients with post-necrotic cirrhosis. Diagnosis was made on liver-function tests, clinical course and histological features of needle liver biopsy specimens. Some of the patients with either chronic active hepatitis or liver cirrhosis were admitted to hospital and treated with immunosupressive drugs. Nineteen persons without previous history of either liver disease or blood transfusion, who were negative for HBsAg and HBsAb, served as controls. This group included five medical staff and two patients with essential hypertension. Leucocyte-migration tests with IHSM were performed on nineteen patients with chronic persistent hepatitis, nineteen chronic active hepatitis and twenty-nine post-necrotic cirrhosis. The same tests with HBsAg were performed on nineteen, nineteen and twenty-nine, those with IMM on twenty, twenty-one and twenty-eight, and those with OMM on nineteen, twenty and twenty-eight respectively.

Mitochondria. The method of Parsons, Williams & Chance (1966) was used. Briefly, perfused livers from Wistar rats were homogenized with 10 volumes of 1·0 mM Tris buffer at pH 7·2, containing 0·07 M sucrose, 0·21 M mannitol and 0·1 mM EDTA. The supernatants, obtained from centrifugation at 500 *g* for 10 min were centrifuged at 9,000 *g* for 10 min. Following further centrifugation at 500 *g* for 10 min of the suspended pellets, the supernatants were centrifuged at 9,000 *g* for 10 min and the suspensions of the pellets were recentrifuged at 9,000 *g* for 10 min. The sediments were added to 100 volumes of cold 20 mM phosphate buffer at pH 7·2, containing 0·02% bovine serum albumin, and left for 20 min in a cold room. The suspensions were centrifuged at 27,000 *g* for 30 min. The pellets were resuspended in 100 volumes of the same buffer and a centrifugation was made at 1,900 *g* for 15 min. The pellets, thus obtained, were used as the inner membranes of mitochondria. The supernatants were further centrifuged at 27,000 *g* for 30 min. The pellets were suspended with an appropriate volume of 20 mM phosphate buffer. The suspensions were gently placed over a sucrose density gradient column, followed by a centrifugation with Beckman SW-27 rotor at 25,000 rev/min for 2 hr. A fraction between sucrose density of 1·094 and 1·142 was obtained. It was mixed with 4 volumes of distilled water and centrifuged at 27,000 *g* for 60 min. The pellets, thus obtained, were quickly frozen at −20°C for storage. These were used as the outer membranes of mitochondria.

Insoluble hepatocyte-surface membranes. IHSM was prepared according to Berman's method. Briefly, perfused livers from Wistar rats of either sex, weighing approximately 150 *g*, were homogenized in 10 volumes of 0·25 M sucrose, 0·5 mM $CaCl_2$ and 5 mM Tris buffer (pH 7·4) with a Dounce homogenizer at 2°C. The homogenates were centrifuged at 150 *g* for 10 min and the supernatants were recentrifuged. Following further centrifugation at 2,000 *g* for 20 min of the supernatants the sediments were rehomogenized and recentrifuged in the same manner. Washing of the sediments with above-mentioned Tris buffer was repeated seven times. The sediments were put gently on a discontinuous sucrose gradient column and centrifuged with Beckman SW-27 rotor at 25,000 rev/min for 2 hr. The fraction between density of 1·16 and 1·18 was obtained and centrifuged at 27,000 *g* for 30 min with an addition of 4 volumes of distilled water. The final pellets were quickly frozen. A part of the pellets was fixed with 2·5% glutaraldehyde and 2% OsO_4, followed by dehydration and embedded in Epon 812. Thin sections, stained with uranyl acetate and lead citrate, were observed under Hitachi HU-IIB electron microscope.

Renal cell plasma membranes. The method of Fitzpatrick *et al.* (1969) was used. Briefly, perfused kidneys of Wistar rats were homogenized in 3 volumes of isotonic sucrose medium (0·25 M sucrose-1 mM EDTA) at 2°C. The homogenates were centrifuged at 1·475 *g* for 10 min. The sediments were suspended in 2 M sucrose with a ratio of 1 g of the original tissue per 1 ml and rehomogenized with three strokes of the homogenizer. The homogenates were centrifuged at 13,300 *g* for 10 min. The supernatants were diluted by an addition of 7 volumes of cold distilled water and then centrifuged at 35,000 *g* for 15 min. The upper layer of the pellets, consisting of two layers, was removed together with the supernatant and recentrifuged in the same manner. The pellets were washed several times and the final pellets were quickly frozen at −20°C.

Antigenicity of IHSM. Rabbits were injected once a week with IHSM, which were sonicated at 20 k cycles/min for 1 min prior to use, with Freund's complete adjuvant for 2 months. The antisera, obtained 2 weeks after the last injection and revealing a titre of 64 to the antigens on agar gel diffusion plate, were placed on a well of double agar diffusion plates were tested by double diffusion precipitation against rat organ homogenates: liver, kidney, heart, spleen, skeletal muscle, serum and IHSM. The homogenates were made with 10 volumes of 0·25 M sucrose–0·5 mM $CaCl_2$ and 5 mM Tris buffer (pH 7·4) filtered through four layers of gauze and sonicated at 20 k cycles/min for 1 min prior to use. Absorption of the antisera was performed with rat serum insolubilized with 2·5% glutaraldehyde with a ratio of 1 ml/100 mg and then with acetone powder of rat spleen with a ratio of 1 ml/5 mg and with that of rat kidney 1 ml/30 mg respectively. The absorbed antisera were tested again on double agar diffusion plates.

The absorbed antisera were coupled with FITC (Marshall, Eveland & Smith, 1958) and fractions with F/P ratio of 1·5 were obtained through column chromatography. Suspensions of isolated rat liver cells were prepared according to Sheffield & Emmelot (1972) and a drop of the suspension was stained with a drop of the fluoresceinated antisera in a tube for 30 min at 37°C. Following three washes with a cold FITC-labelled saline pH 7·2, a part of the suspension, mounted with buffered glycerine, was observed under Nikon fluorescence microscope.

Leucocyte migration test. The method of Bendixen & Søborg (1969) was used. Briefly, 20 ml of hepalinized blood were allowed to sediment in sterile tubes. The supernatants were centrifuged at 400 *g* for 5 min. The cell pellets were washed several times with Hanks's balanced salt solution. The pellets were suspended with appropriate volume of TC medium 199 (Difco Ltd) containing 10% of heat-inactivated foetal calf serum and packed into capillary tubes, followed by centrifugation at 1500 *g* for 10 min. The cell-packed tubes were cut at the level of fluid cell interface and placed in migration chamber containing the medium, 10% foetal calf serum, 100 u of penicillin/ml, 100 μg streptomycin/ml and 100 μg protein of the antigens per ml of medium. The antigens, IHSM, IMM and OMM, were sonicated at 20 k cycles/min for 1 min prior to use. Control chamber did not contain the antigens. All tests were performed in duplicate and two capillary tubes were placed in each chamber. The chambers were incubated at 37°C for 18 hr within an atmosphere containing 5% CO_2. The area of leucocyte migration was measured. A migration index was calculated by dividing the mean value of the test with the antigens to that of controls without the antigens. A migration index of less than 0·75 was taken to be significant.

HBsAg, HBsAb and cellular HBsAb. Purified HBsAg was kindly donated by Eisai Ltd. This was consisted of subtype both of *adr* and *adw* and the purification was made by the method of both CsCl-and sucrose-density gradient ultracentrifugation. Detection of HBsAg and HBsAb was made by solid-phase radioimmunoassay (DINABOT Ausria II and Ausab). Leucocyte-migration test with purified HBsAg was performed. 0·1 ml of TC medium 199 containing purified HBsAg, which was prepared by 180 times dilution of the original HBsAg fraction obtained from the ultracentrifugation, mentioned above, were applied per ml of the culture medium. The diluted HBsAg fraction revealed 1972 ct/min of HBsAg with solid-phase radioimmunoassay method when cut off value of 346 ct/min was taken.

RESULTS

Leucocyte migration with mitochondria

Fig. 1. indicates that seven out of nineteen patients with chronic persistent hepatitis (37%), seven out of twenty patients with chronic active hepatitis (35%) and twelve out of twenty-eight patients with liver cirrhosis (43%) showed a positive inhibition to OMM, while one out of sixteen control persons revealed positive inhibition (6%). Those were statistically significant, compared with control ($P<0·05$, $P<0·05$ and $P<0·02$ respectively). As shown on Fig. 2 an incidence of positive reaction to IMM in the same patients was somewhat different from that to OMM; the highest incidence of positive inhibition was seen in patients with chronic persistent hepatitis (55%) and the lowest in those with cirrhosis (36%). A positive reaction was seen in only one control subject, who also revealed positive inhibition to OMM as mentioned before. Each incidence of the positive reaction in different liver disease groups revealed statistically significant difference from that of control ($P<0·01$, $P<0·01$ and $P<0·05$ respectively).

Fig. 3 shows a correlation of migration index value in patients with chronic liver disease between the reaction to OMM and in those to IMM. There was a statistically significant correlation between them ($r = 0·335$, $n = 64$, $t = 2·80$, $P<0·01$).

Hepatocyte surface membranes

As shown in Fig. 4, a pellet of IHSM showed a vesicular appearance under the electron microscope. This was similar to the figure shown by Berman, Gram & Spirtes (1969), suggesting that the pellet consisted mostly of cell membranes. Immunological examination of IHSM, as shown on the left side of Fig. 5, indicated that IHSM [1] contained components common with the other organ homogenates. Absorption of the antisera with rat serum, spleen and kidney respectively resulted in complete

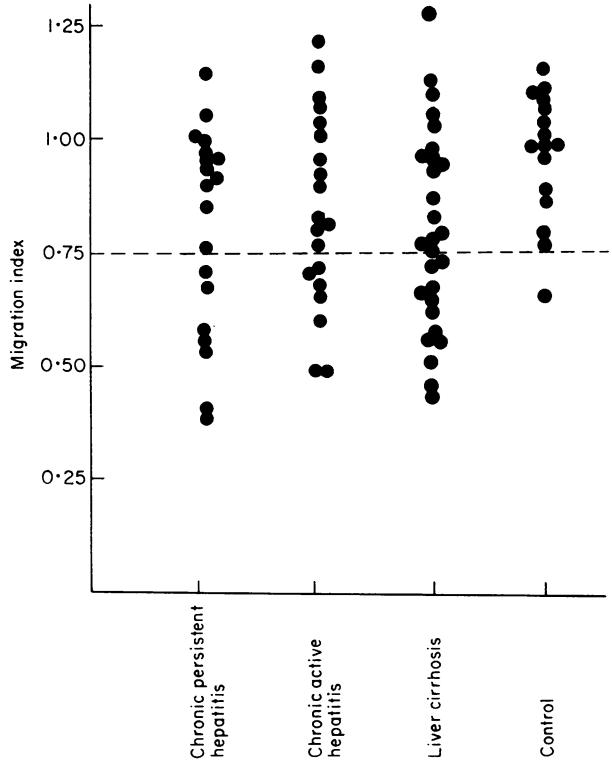

FIG. 1. Leucocyte migration test with purified outer membranes of mitochondria, prepared from rat liver, in patients with chronic liver diseases.

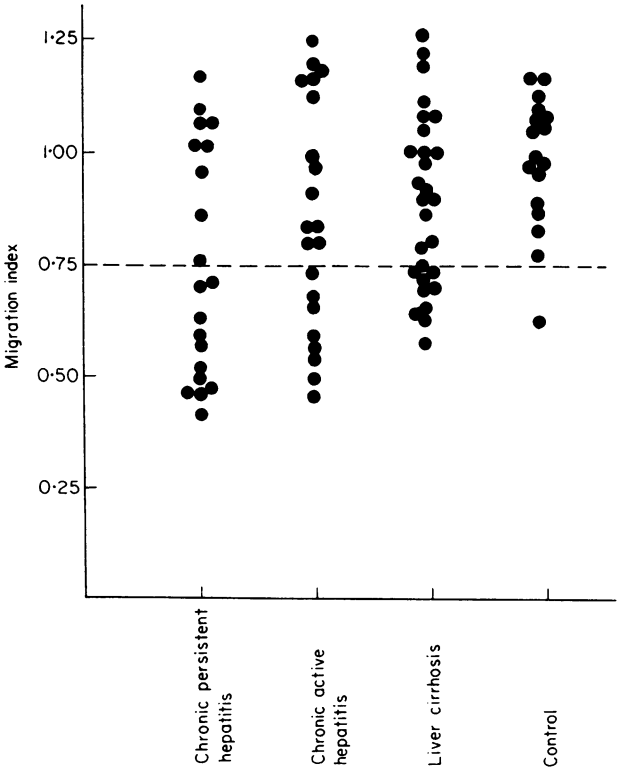

FIG. 2. Leucocyte migration test with purified inner membranes of mitochondria, prepared from rat liver, in patients with chronic liver diseases.

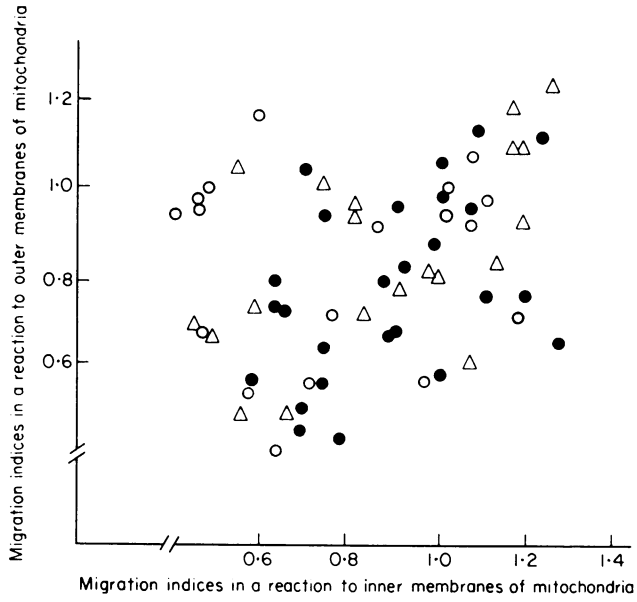

FIG. 3. Correlation of migration indices between reactions to inner membranes and to outer membranes of mitochondria in patient with chronic liver diseases. $r = 0{\cdot}335$; $n = 64$; $t = 2{\cdot}80$; $P = < 0{\cdot}01$. (○), Chronic persistent hepatitis; (△), Chronic active hepatitis; (●), Liver cirrhosis.

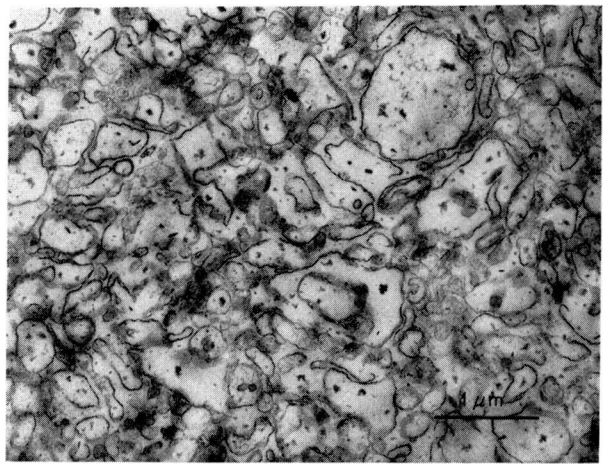

FIG. 4. Note vesicular appearance of rat hepatocyte-surface membranes under electron microscope. The surface membranes were prepared according to Berman's method and a part of the membrane fraction was fixed with 2·5% glutaraldehyde and 2% OsO_4, and embedded in Epon 812. The thin sections were stained with uranyl acetate and lead citrate.

elimination of the common components and two precipitin lines between the antisera and either IHSM [1] or liver homogenate [2] were demonstrated on the right side of Fig. 5. The absorbed antisera did not react with the soluble lipoproteins [3], prepared from rat liver using the method of Meyer zum Büschenfelde & Miescher (1972), as shown in Fig. 6. When isolated rat liver cells were stained with the absorbed antisera labelled with FITC, specific fluorescence observed was confined to the cell surface membranes, as shown in Fig. 7.

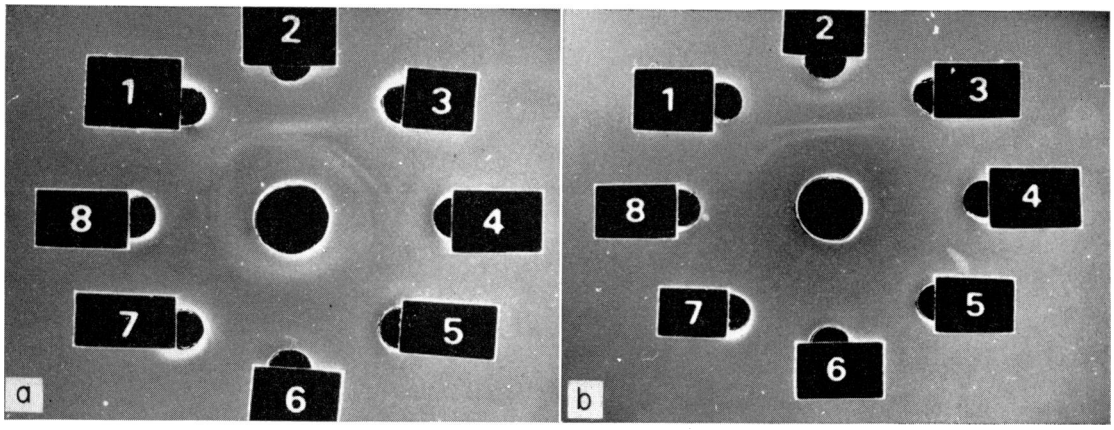

FIG. 5. (a) Shows a reaction of non-absorbed rabbit antisera against IHSM in the central large well and homogenates of rat organs on agar gel diffusion plate. (b) Represents the same reaction with the antisera, absorbed with 2·5% glutaraldehyde-treated rat serum and acetone powder of rat spleen and kidney respectively. 1, a fraction of IHSM; 2, homogenates of rat liver; 3, homogenates of rat kidney; 4, a fraction of rat renal cell-surface membranes; 5, homogenates of rat skeletal muscle; 6, rat serum; 7, homogenates of rat heart; 8, homogenates of rat spleen. In Fig. 5(b) there are two precipitin lines between the absorbed antisera and either IHSM (1), or liver homogenates (2), but no reaction with the other organ homogenates (3–8).

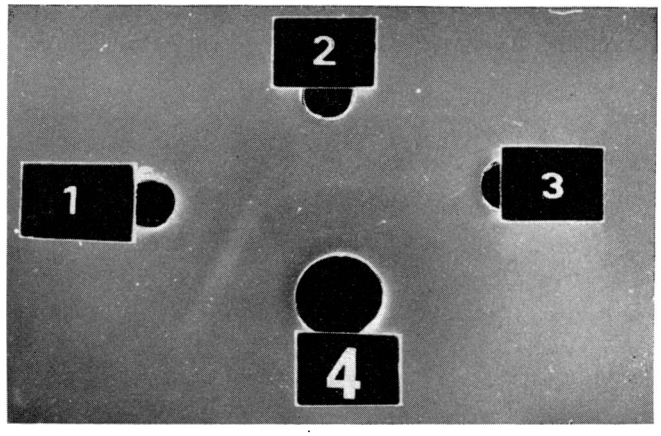

FIG. 6. Precipitin lines are observed between either rat liver homogenates (1), or a fraction of IHSM (2), and absorbed antisera (4) against IHSM. No line, however, is found between LSP 100-1, prepared according to the method of Meyer zum Büschenfelde *et al.* (1974) (3), and the antisera (4).

Leucocyte migration with IHSM

Fig. 8 shows the effect of IHSM on leucocyte migration. Significant migration inhibition was observed in seven out of nineteen patients with chronic persistent hepatitis (37%), in nine out of nineteen patients with chronic active hepatitis (47%) and in thirteen out of twenty-nine patients with liver cirrhosis (45%), while one out of eighteen control persons revealed positive reaction. The incidence of the positive reaction in each group of liver diseases was statistically significant ($P<0·05$, $P<0·01$ and $P<0·01$ respectively) in comparison with that of the control. Migration inhibition, however, to rat renal cell plasma membranes was not detected in the same patients with chronic liver disease except one patient with chronic persistent hepatitis showing migration index of more than 1·25, as shown in Fig. 9. None of the patients tested suffered from renal diseases and some of the control persons received therapy for essential hypertension.

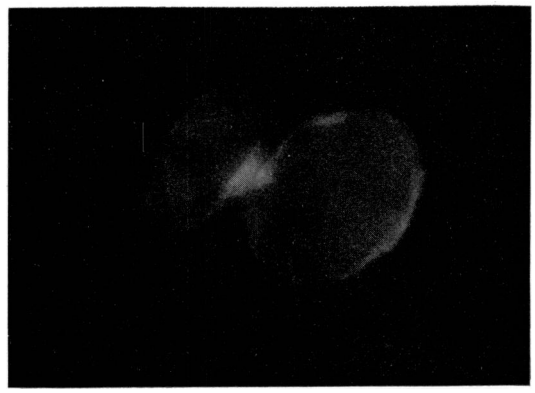

FIG. 7. Membrane immunofluorescence of liver cells. Specific fluorescence is seen in the surface membrane of isolated liver cells.

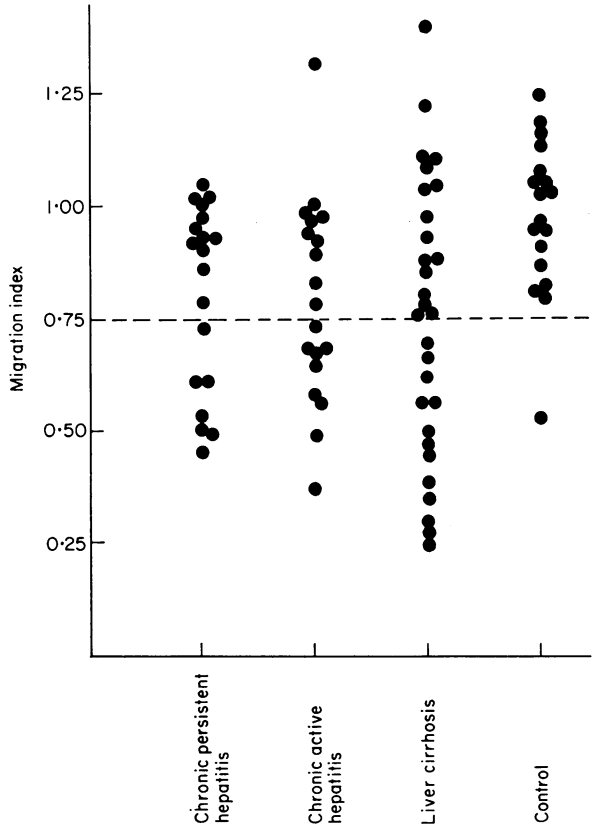

FIG. 8. Leucocyte migration test with insoluble liver-cell-surface membranes in patients with chronic liver diseases.

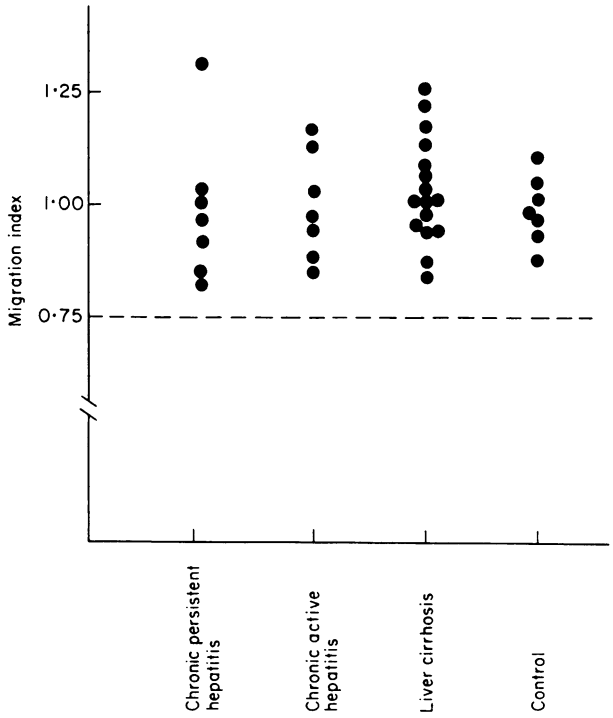

FIG. 9. Leucocyte migration test with renal cell-surface membranes in patients with chronic liver diseases

TABLE 1. Incidence of HBAg-infected patients among patients with positive LMT* activity to insoluble liver-cell membranes in chronic liver diseases

Diseases		No. of HBAg-infected† patients/ no. of patients with LMF		
Chronic hepatitis	Persistent	6/7‡ (85·7)	13/16¶ (81·3)	23/29¶ (79·3)
	Active	7/9‡ (77·8)		
Cirrhosis		10/13‡ (76·9)		

Figures in parentheses are percentages.

* Leucocyte migration inhibition test.

† Patients with either HBsAb, HBsAg, or cellular HBsAb are included into HBAg-infected group.

‡ $P<0·01$.

¶ $P<0·001$. Significant difference from the incidence of HBAg-infection in control persons.

TABLE 2. Percentage of positive LMT* activity to insoluble liver-cell membranes in each of HBAg-infected and HBAg-noninfected patients with chronic hepatitis

	HBAg-infected group	HBAg-noninfected group
Percentage of patients with the positive LMF	54·2 (13/24)	21·4 (3/14)

$P<0·05$.

* Leucocyte migration inhibition test.

Leucocyte migration with IHSM in HBAg-infected group and non-infected group

Patients with either HBsAg, HBsAb in the blood or positive migration reaction to purified HBsAg were included in the HBAg-infected group and patients without any of these in the HBAg-non-infected group, though it is difficult to exclude present or previous infection with HBAg. A surprisingly high incidence (70%) of HBAg-infected patients was found in chronic liver disease, partly due to the presence of cellular response to HBsAg in many HBAg-negative patients.

Table 1 shows that approximately 80% of patients, who were suffering from chronic liver disease and had a cellular immunity to IHSM, belonged to the HBAg-infected group. This was statistically significant from the incidence of HBAg-infection in control persons ($P<0·001$). In cases of chronic hepatitis, as shown in Table 2, a positive migration reaction to IHSM was found in 54% of HBAg-infected patients and in 21% of HBAg-noninfected patients ($P<0·05$).

Correlation of leucocyte migration reaction with HBsAg and with IHSM in patients with chronic hepatitis

Table 3 illustrates that the positive response to IHSM was found in 64·3% of patients with a positive response to HBsAg but in 29·2% of patients with a negative response to HBsAg in cases of chronic

TABLE 3. Percentage of patients with positive LMT activity to insoluble liver-cell membranes in group of positive cellular response to HBsAg and of negative one in chronic hepatitis

	Group with positive response to HBsAg	Group with negative response to HBsAg
Percentage of patients with the positive LMT	64·3 (9/14)	29·2 (7/24)

$P<0·05$.

TABLE 4. Percentage of patients with positive LMT activity to purified HBsAg in group of positive cellular response to IHSM* and of negative one in chronic hepatitis

	Group of positive cellular response to IHSM*	Group of negative cellular response to IHSM*
Percentage of patients with the positive LMF to HBsAg	56·3 (9/16)	22·7 (5/22)

$P<0·05$.

* Insoluble hepatocyte-surface membranes.

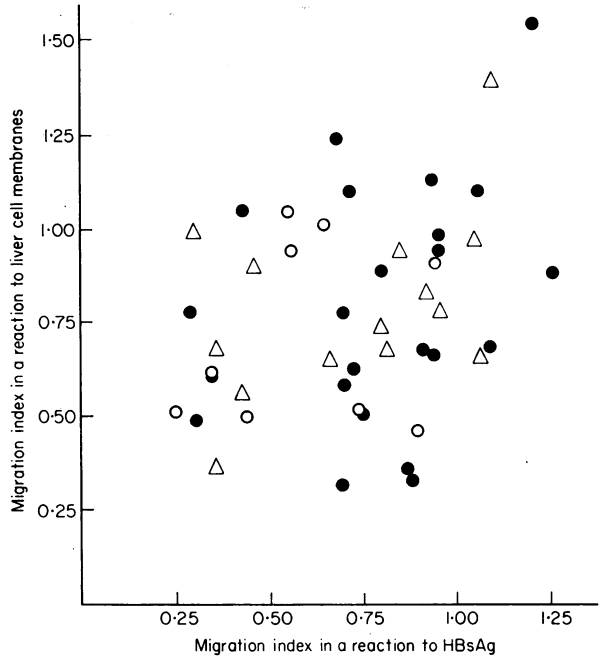

FIG. 10. Correlation of migration indices to purified HBsAg and to insoluble liver-cell membranes in HBAg-infected patients with chronic liver diseases. In chronic liver disease: $r = 0.326$; $n = 46$; $t = 2{\cdot}289$; $P < 0{\cdot}05$. In chronic hepatitis: $r = 0{\cdot}423$; $n = 24$; $t = 2{\cdot}190$; $P < 0{\cdot}05$. (○) Chronic persistent hepatitis; (△), chronic active hepatitis; (●), liver cirrhosis.

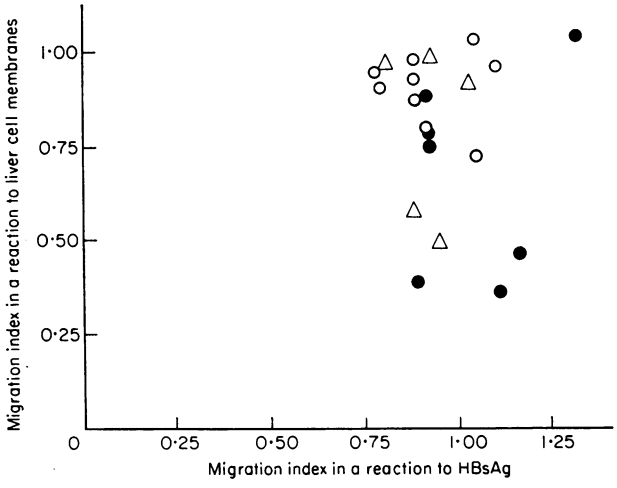

FIG. 11. Correlation of migration indices between to purified HBsAg and to insoluble liver-cell membranes in HBA-non-infected patients with chronic liver diseases. In chronic liver diseases: $r = 0{\cdot}115$; $n = 21$; $t = 0{\cdot}502$; $P > 0{\cdot}5$. In chronic hepatitis: $r = 0{\cdot}056$; $n = 14$; $t = 0{\cdot}193$; $P > 0{\cdot}5$. (○), chronic persistent hepatitis; (△), chronic active hepatitis; (●), liver cirrhosis.

hepatitis ($P < 0{\cdot}05$). Conversely a positive response to HBsAg was seen in 56·3% of patients who responded to IHSM compared with 22·7% of those with a negative response to IHSM in the same cases ($P < 0{\cdot}05$), as shown on Table 4. Thus it seems likely that the development of a delayed reaction with IHSM in many patients with chronic hepatitis was closely related with that of a cell-mediated reaction to HBsAg. Further observation was made as to any correlation in degree of the cellular response between that to purified HBsAg and to IHSM in HBAg-infected patients with chronic hepatitis and cirrhosis. As

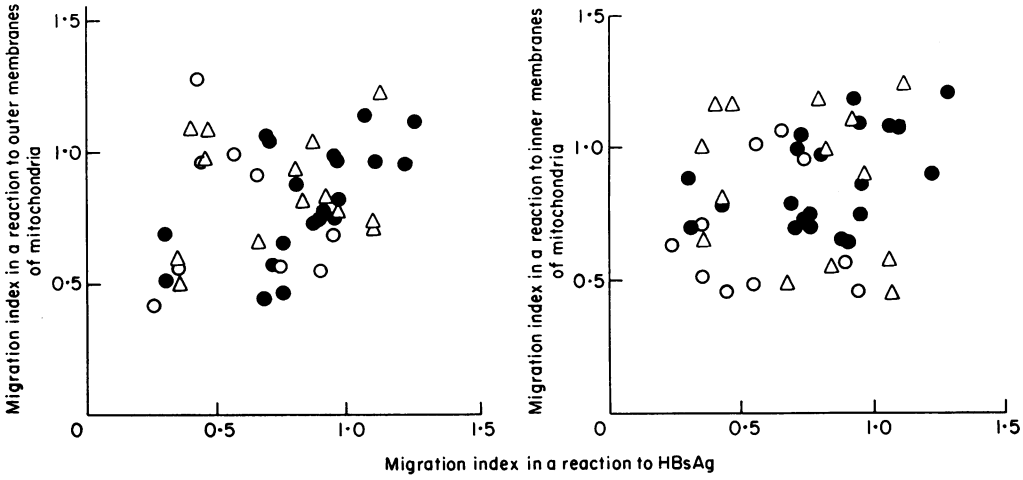

FIG. 12. Correlation of migration indices to purified HBsAg and to outer membranes of mitochondria, and to purified HBsAg and inner membranes of mitochondria in HBAg-infected-patients with chronic liver diseases. Outer membranes: $r = 0{\cdot}248$; $n = 44$; $t = 1{\cdot}659$; $P > 0{\cdot}05$. Inner membranes: $r = 0{\cdot}223$; $n = 45$; $t = 1{\cdot}501$; $P > 0{\cdot}05$. (○), chronic persistent hepatitis; (△) chronic active hepatitis; (●), liver cirrhosis.

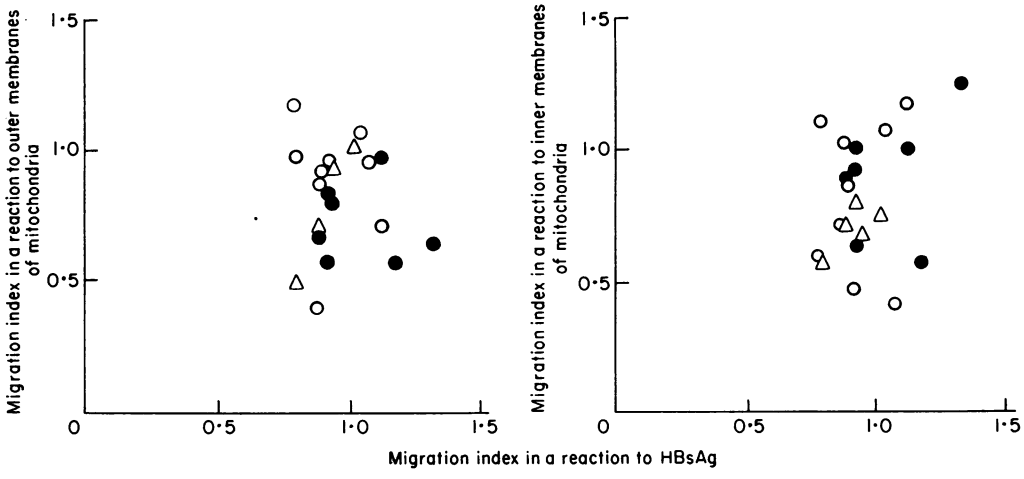

FIG. 13. Correlation of migration indices between to purified HBsAg and to outer membranes of mitochondria, and to purified HBsAg and to inner membranes of mitochondria in HBAg-noninfected patients with chronic liver diseases. Outer membranes: $r = 0{\cdot}091$; $n = 20$; $t = 0{\cdot}386$; $P > 0{\cdot}05$. Inner membranes: $r = 0{\cdot}3188$; $n = 21$; $t = 1{\cdot}466$; $P > 0{\cdot}05$. (○), chronic persistent hepatitis; (△), chronic active hepatitis; (●) liver cirrhosis.

seen in Fig. 10, there was a significant correlation in HBAg-infected patients either with chronic hepatitis ($r = 0{\cdot}423$, $n = 24$, $t = 2{\cdot}190$, $P < 0{\cdot}05$) or with chronic hepatitis and cirrhosis ($r = 0{\cdot}326$, $n = 46$, $t = 2{\cdot}289$, $P < 0{\cdot}05$). In HBAg-non-infected patients, however, no correlation was found between these parameters ($r = 0{\cdot}115$, $n = 21$, $t = 0{\cdot}502$, $P > 0{\cdot}5$ in chronic liver disease; $t = 0{\cdot}056$, $n = 14$, $t = 0{\cdot}193$, $P > 0{\cdot}5$ in chronic hepatitis), as illustrated in Fig. 11.

Correlation of leucocyte migration reactions with HBsAg and with OMM or IMM in patients with chronic liver disease

In contrast to the results of the response to IHSM, as mentioned above, a degree of response to either OMM or IMM was not correlated with that to HBsAg in HBAg-infected patients with chronic

liver diseases, as shown in Fig. 12. The same was true of HbAg-non-infected patients with chronic liver disease, as shown on Fig. 13.

DISCUSSION

Recent arguments as to cellular immune mechanism of liver-cell destruction in HBAg-positive or -negative chronic liver diseases suggested the importance of the hepatocyte-surface membranes as a site of interaction of specifically sensitized or killer lymphocytes (Dudley *et al.*, 1972; Eddleston & Williams, 1974).

Smith *et al.* (1975) reported that leucocyte migration to the soluble lipoproteins from human liver was inhibited in approximately 59% of patients with active chronic hepatitis and 40% of patients with primary biliary cirrhosis. Immunological and immunofluorescent study of the lipoproteins indicated that they were liver-specific but not species-specific and derived from the hepatocyte-surface membranes (Hopf, Meyer zum Büschenfelde & Freudenberg, 1974). The surface membranes, prepared according to Berman's method (1969) and used here, are insoluble and very stable, unlike the lipoproteins of Meyer zum Büschenfelde & Miescher (1972). Electronmicroscopical observation of the membranes revealed vesicular appearance similar to that of Berman's report, suggesting pure membrane components. Immunological investigation of the membranes confirmed that they contained liver-specific components, as mentioned by Sheffield & Emmelot (1972), which differed antigenically from the Meyer's zum Büschenfelde's lipoprotein. The present experiment indicated that cellular hypersensitivity to IHSM was present in 42% of patients with chronic hepatitis and in 45% of those with liver cirrhosis. An absence of cellular hypersensitivity to the renal cell-surface membranes in patients with chronic liver diseases and without renal diseases suggested strongly that the active components of IHSM responsible for the migration inhibition may be liver-specific.

There were several evidences, obtained in the present experiment, suggesting a close relationship between present or previous HBAg infection and the development of cellular immunity to IHSM in many cases of chronic liver disease: (1) incidence of the positive cellular reactions to IHSM was significantly higher in the HBAg-infected group (54%) than that in the HBAg-non-infected group (21%) ($P<0{\cdot}05$). (2) This was also higher in the cellular HBsAb-positive group (64%) than in the cellular HBsAb-negative group (29%) ($P<0{\cdot}05$). (3) Inversely the incidence of cellular HBsAb was higher in the cellular IHSM responsive group (56%) than in the negative group (22%) ($P<0{\cdot}05$). (4) There was a good correlation in migration index value in individual patients with chronic liver diseases between that to purified HBsAg and that to IHSM in the HBAg-infected group but not in the HBAg-non-infected group ($P<0{\cdot}05$). Thus it would appear that there is a link between the appearance of the cellular hypersensitivity to HBsAg and that to IHSM, and the sensitization to IHSM is induced by hepatitis B virus infection. The virus, coming into contact with the cell-surface membranes following entry into the hepatocyte, may alter the liver-specific proteins of the surface membranes by immune response (Sherlock, 1975) or incorporation of the viral antigens into the surface membranes (Edington, *et al.*, 1975). Development of the altered proteins or the neoantigen on the surface may stimulate either the activation of T cells to be specifically sensitized or the activation of B cells to produce the circulating surface antibodies in the presence of helper T-cell function (Eddleston & Williams, 1974). Even after complete elimination of hepatitis-B virus from the liver, the immune response to the altered membranes might persist in the absence of suppressor T-cell function to play some role in continuation of liver-cell damage. Thomson *et al.* (1974) suggested that the continuing liver-cell damage, whether or not HBsAg is detectable in the blood at the time of examination, was the result of cytotoxic function of T cells sensitized with the liver-specific lipoprotein. In view of this concept it would appear that the cell-mediated immune response to IHSM is important in production of chronic liver disease.

Recent observations showed that the frequency of cytophilic antibodies both to HBsAg and IHSM in HBAg-infected patients in the active stage of acute viral hepatitis was very low without significant difference from that of the controls (Nonomura, 1976). Knolle *et al.* (1973), however, reported that 30% of patients with acute viral hepatitis revealed positive cellular responses to the liver-specific lipoproteins

of Meyer zum Büschenfelde and approximately 80% of the same patients showed an abnormal cellular reaction with HBsAg. The reason for the disagreement between Knolle's and our results may be due to differences in the liver antigens used, in the species of the antigens and in the stage of acute viral hepatitis at the time of the leucocyte migration test between them. The fact that a delayed reaction with IHSM was found mostly in patients with chronic liver disease and more frequently in the HBAg-infected group than in the HBAg-non-infected group suggested strongly that sensitization to IHSM may play a major role in continuation of liver disease initiated by hepatitis virus infection.

Our recent investigation as to the species-specificity of IHSM revealed that a migration of the peritoneal exudate cells from guinea-pigs immunized with IHSM from rat liver was inhibited in the presence of either rat liver IHSM or guinea-pig liver IHSM (in preparation). This suggested strongly that the antigen responsible for cellular reaction with IHSM was not species-specific, as in the case of the liver-specific lipoprotein of Meyer zum Büschenfelde & Miescher (1972). Whether or not IHSM, prepared from human cadaver liver, reacts similarly *in vitro* with leucocytes from patients with chronic liver diseases remains to be tested.

Some patients in the HBAg-non-infected group of chronic liver diseases did reveal a sensitization to IHSM and not to HBsAg. This can be explained by the assumption that hepatitis viruses other than HBsAg, if present in the liver, might act in a similar fashion.

The present experiment of cellular response to mitochondria indicated that 36% of patients with chronic hepatitis and 43% of those with cirrhosis revealed a delayed type of hypersensitivity to OMM, while 49% and 36% respectively showed hypersensitivity to IMM. Richens *et al.* (1974) mentioned that the antigenic components of mitochondria responsible for the migration inhibition in patients with diabetes mellitus were exclusively in IMM. The present experiment, however, indicated the presence of definite correlation in activity of the cellular response between that to OMM and IMM in patients with chronic liver diseases ($P<0{\cdot}01$). This was not in accordance with Richen's emphasis. The antigenic components of mitochondria responsible for the migration inhibition have been thought to be non-organ and non-species specific (Calder *et al.*, 1972; Brostoff, 1970). It seems, therefore, not reasonable that T cells sensitized with non-organ-specific mitochondria should produce cell-mediated damage to specific organs. Occurrence of cellular responses to non-organ-specific mitochondria in many patients with chronic liver diseases may be a result of a destruction of liver cells rather than a cause of the destruction. This was supported by the present experiment indicating a lack of correlation in migration inhibition activity of individual patients for HBsAg between that for OMM or IMM either in the HBsAg-infected group or in the HBsAg-noninfected group. The cellular response to mitochondria is merely a marker of a delayed hypersensitivity state in immunological disorders.

We are grateful to Professor Hattori, 1st Department of Internal Medicine, Kanazawa University Hospital and to Dr Sugioka, Department of Internal Medicine, National Kanazawa Hospital for permission to examine the blood from patients under their care. The present experiment was supported by Grant No. 948083 from Japanese Educational Ministry.

REFERENCES

BENDIXEN, G. & SØBORG, M. (1969) A leucocyte migration technique for the *in vitro* detection of cellular hypersensitivity in man. *Dan. med. Bull.* **16**, 1.

BERMAN, H.M., GRAM, W. & SPIRTES, M.A. (1969) An improved, reproducible method of preparing rat liver plasma cell membranes in buffered isotonic sucrose. *Biochim. Biophys. Acta*, **183**, 10.

BROSTOFF, J., ROITT, I. & DONIACH, D. (1969) Leucocyte-migration inhibition in autoimmune diseases. *Lancet*, **i**, 1212.

BROSTOFF, J. (1970) Migration inhibition studies in human disease. *Proc. roy. Soc. Med.* **63**, 905.

CALDER, E.A., MCLEMAN, D., BARNES, E.W. & IRVINE, W.J. (1972) The effect of thyroid antigens on the *in vitro* migration of the leucocytes from patients with Hashimoto thyroiditis. *Clin. exp. Immunol.* **12**, 429.

DUDLEY, F.J., FOX, R.A. & SHERLOCK, S. (1972) Cellular immunity and hepatitis-associated, Australia antigen liver disease. *Lancet*, **i**, 723.

EDDLESTON, A.L.W.F. & WILLIAMS, R. (1974) Inadequate antibody response to HBAg or suppressor T-cell defect in development of active chronic hepatitis. *Lancet*, **ii**, 1543.

EDGINGTON, T.S. & CHISARI, F.V. (1975) Immunoligcal aspects of hepatitis B virus infection. *Amer. J. Med. Sci.* **270**, 213.

FITZPATRICK, D.F., DAVENPORT, G.R., FORTE, L. & LANDON, E.J. (1969) Characterization of plasma membrane

proteins in mammalian kidney. I. Preparation of a membrane fraction and separation of the protein. *J. biol. Chem.* **244**, 3561.

GOLDSTONE, A.H., CALDER, E.A., BARNES, E.W. & IRVINE, W.J. (1973) The effect of gastric antigens on the *in vitro* migration of leucocytes from patients with atrophic gastritis and pernicious anaemia. *Clin. exp. Immunol.* **14**, 501.

HOPF, U., MEYER ZUM BÜSCHENFELDE, K.H. & FREUDENBERG, J. (1974) Liver-specific antigens of different species. II. Localization of a membrane antigen at cell surface of isolated hepatocytes. *Clin. exp. Immunol.* **16**, 117.

KNOLLE, J., MEYER ZUM BÜSCHENFELDE, K.H., BOLTE, J.P. & BERGER, J. (1973) Celluläre Immunreaktionen gegenüber dem hepatitisassoziierten Antigen (HAA) und homologen leber-spezifischen protein (HLP) bei akuten HAA-positiven Hepatitiden. *Klin. Wschr.* **51**, 1172.

LEE, W.M., REED, W.D., MITCHELL, C.G., GALBRAITH, R.M., EDDLESTON, A.L.W.F., ZUCKERMAN, A.J. & WILLIAMS, R. (1975) Cellular and humoral immunity to hepatitis-B surface antigen in active chronic hepatitis. *Brit. med. J.* **i**, 705.

MARSHALL, J.D. JR., EVELAND, W.C. & SMITH, C.W. (1958) Superiority of fluorescein isothiocyanate (Riggs) for fluorescent antibody technique with a modification of its application. *Proc. Soc. exp. Biol. (N.Y.)*, **98**, 898.

MEYER ZUM BÜSCHENFELDE, K.H. & MIESCHER, P.A. (1972) Liver specific antigens. Purification and characterization. *Clin. exp. Immunol.* **10**, 89.

MEYER ZUM BÜSCHENFELDE, K.H., KÖSSLING, F.K. & MIESCHER, P.A. (1972) Experimental chronic active hepatitis in rabbits following immunization with human liver proteins. *Clin. exp. Immunol.* **11**, 99.

MILLER, J., SMITH, M.G.M., MITCHELL, C.G., REED, W.D., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1972) Cell-mediated immunity to a human liver-specific antigen in patients with active chronic hepatitis and primary biliary cirrhosis. *Lancet*, **ii**, 296.

NERUP, J., ANDERSEN, V. & BENDIXEN, G. (1970) Anti-adrenal cellular hypersensitivity in Addison's disease. IV. *In vivo* and *in vitro* investigations on the mitochondrial fraction. *Clin. exp. Immunol.* **6**, 733.

NERUP, J. & BENDIXEN, G. (1969) Anti-adrenal cellular hypersensitivity in Addison's disease. *Clin. exp. Immunol.* **4**, 355.

NONOMURA, A. (1976) Cell-mediated immunity in the evolution of chronic liver diseases—lymphocyte response to purified HBsAg and insoluble liver cell surface membranes. *J. Juzen Med. Soc.* **85**, 272.

PARSONS, D.F., WILLIAMS, G.R. & CHANCE, B. (1966) Characteristics of isolated and purified preparations of the outer and inner membranes of mitochondria. *Ann. N.Y. Acad. Sci.* **137**, 643.

POPPER, H. & MACKAY, I.R. (1972) Relation between Australia antigen and autoimmune hepatitis. *Lancet*, **i**, 1161.

POPPER, H. (1975) The immunologic basis of liver disease. *Bull. N.Y. Acad. Med.* **51**, 519.

RICHENS, E.R., ANCILL, R.J., GOUGH, K.R. & HARTOG, M. (1973) Cellular hypersensitivity to mitochondrial antigens in diabetes mellitus. *Clin. exp. Immunol.* **13**, 1.

RICHENS, E.R., IRVINE, W.J., WILLIAMS, M.J., HARTOG, M. & ANCILL, R.J. (1974) Cellular hypersensitivity to mitochondrial antigens in diabetes mellitus and its relationship to the presence of circulating autoantibodies. *Clin. exp. Immunol.* **17**, 71.

SHEFFIELD, T.B. & EMMELOT, P. (1972) Studies on plasma membranes XVI. Tissue specific antigens in the liver cell surface. *Exp. Cell. Res.* **71**, 97.

SHERLOCK, S. (1975) Immunological reactivity and viral hepatitis. *Bull. N.Y. Acad. Med.* **51**, 528.

SMITH, M.G.M., GOLDING, P.L., EDDLESTON, A.L.W.F., MITCHELL, C.G., KEMP, A. & WILLIAMS, R.S. (1972) Cell-mediated immune responses in chronic liver diseases. *Brit. med. J.* **i**, 527.

SMITH, M.G.M., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1975) Immunological factors in the evolution of active chronic hepatitis and other autoimmune liver diseases. *Clin. Gastroenterol.* **4**, 297.

THOMSON, A.D., COCHRANE, M.A.G., MCFARLANE, I.G., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1974) Lymphocyte cytotoxicity to isolated hepatocytes in chronic active hepatitis. *Nature (Lond.)*, **252**, 721.