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Ryoji Fujiwara[*] Kazuo Tobe[†]

Hideo Nagashima[‡]

[*]Okayama University,

[†]Okayama University,

[‡]Okayama University,

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Ryoji Fujiwara, Kazuo Tobe, and Hideo Nagashima

Abstract

Cellular immunity against human bile proteins was investigated by the leukocyte migration inhibition test (LMIT) with 13 primary biliary cirrhosis (PBC) patients, 10 chronic aggressive hepatitis (CAH) patients and 21 healthy adults. Hepatic bile taken from patients operated on for lithiasis of the biliary tract was fractionated into five fractions with Sepharose 6B gel. A subtoxic dose of each fraction was determined in the healthy adults, and used as the antigen for LMIT. Out of the 5 fractions, only the third fraction led to an LMIT positive response in 8 out of 11 (73%) PBC patients and in 1 out of 10 (10%) CAH patients. The difference between PBC and CAH was significant (p less than 0.005). The remaining 3 PBC patients with LMIT negative responses were all under D-penicillamine treatment. Antibody to each fraction was prepared in rabbits. Using the antibodies after absorption with human serum, the localization of the antigens which were present in each fraction was investigated immunohistochemically using human liver sections. The antigen to the anti-first fraction antibody was detected specifically in the epithelial cells of the bile ducts and the ductules, and the antigen to the anti-third fraction antibody was detected specifically on the membrane of the bile canalicules. The third fraction was fractionated into three fractions by Sephadex G-200 gel. Only the first of the 3 fractions showed an LMIT positive response in 3 PBC patients, and its molecular weight was determined to be about 500,000. It is concluded that PBC patients develop cellular immunity against canalicular-antigen-containing fractions but not ductal-antigen-containing ones.

KEYWORDS: primary biliary cirrhosis, leukocyte migration inhibiton test, bile protein, canalicular antigen, ductal antigen.

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Studies on Cellular Immunity against Bile Proteins in Primary Biliary Cirrhosis by the Leukocyte Migration Inhibition Test (Microdroplet Method)

Ryoji Fujiwara, Kazuo Tobe and Hideo Nagashima

First Department of Internal Medicine, Okayama University Medical School, Okayama 700, Japan

Cellular immunity against human bile proteins was investigated by the leukocyte migration inhibition test (LMIT) with 13 primary biliary cirrhosis (PBC) patients, 10 chronic aggressive hepatitis (CAH) patients and 21 healthy adults. Hepatic bile taken from patients operated on for lithiasis of the biliary tract was fractionated into five fractions with Sepharose 6B gel. A subtoxic dose of each fraction was determined in the healthy adults, and used as the antigen for LMIT. Out of the 5 fractions, only the third fraction led to an LMIT positive response in 8 out of 11 (73%) PBC patients and in 1 out of 10 (10%) CAH patients. The difference between PBC and CAH was significant ($p < 0.005$). The remaining 3 PBC patients with LMIT negative responses were all under D-penicillamine treatment. Antibody to each fraction was prepared in rabbits. Using the antibodies after absorption with human serum, the localization of the antigens which were present in each fraction was investigated immunohistochemically using human liver sections. The antigen to the anti-first fraction antibody was detected specifically in the epithelial cells of the bile ducts and the ductules, and the antigen to the anti-third fraction antibody was detected specifically on the membrane of the bile canalicules. The third fraction was fractionated into three fractions by Sephadex G-200 gel. Only the first of the 3 fractions showed an LMIT positive response in 3 PBC patients, and its molecular weight was determined to be about 500,000. It is concluded that PBC patients develop celluar immunity against canalicular-antigen-containing fractions but not ductal-antigen-containing ones.

Key words : primary biliary cirrhosis, leukocyte migration inhibition test, bile protein, canalicular antigen, ductal antigen.

Primary biliary cirrhosis (PBC) is a chronic intrahepatic cholestasis caused by chronic destruction of intrahepatic bile ducts, and is associated with various immunological disorders, such as increases in serum IgM and immune complex levels, the presence of anti-mitochondrial antibody in the serum, and the dysfunction of suppressor T cells (1).

Recently, Eddleston *et al.* (2) and other investigators (3, 4, 5) have shown by the leukocyte migration inhibition test (LMIT) that cellular immunity is formed against human bile proteins in PBC, and have suggested that cellular immunity plays an important role in bile duct destruction.

We attempted to identify the bile antigens against which the cellular immunity is formed in PBC by the microdroplet LMIT method using bile protein fractions and antisera to respective fractions.

Materials and Methods

Patients. The subjects for the LMIT were 13 patients with PBC, 10 with chronic aggressive hepatitis (CAH), both diagnosed by liver biopsies, and 21 healthy adults as controls. Among the PBC patients, only one was icteric, and 7 complained of occasional pruritus. The rest were in the asymptomatic stage of the disease. Of the CAH patients, none had jaundice, and histologically 4 were CAH 2A, while the others were CAH 2B. Healthy controls were used for the determination of the subtoxic dose of each bile fraction and the normal range of the LMIT.

Bile fractions. Hepatic bile samples of 200 ml each were collected from the T-tubes of patients operated on for biliary tract stones, concentrated about ten-fold with an Amicon PM 10 microfilter, and then subjected to gel filtration through a Sepharose 6B(Pharmacia Fine Chemicals, Uppsala, Sweden) column (2.6 ×90cm) using 0.1 M Tris-HCl buffer (1mM EDTA, 0.2M NaCl, pH 8.0). Five-ml aliquots of the eluted solution were collected, and their optical densities at 280 nm and protein concentrations by Lowry's method were measured. The gel filtration gave 5 fractions. Each fraction was reconcentrated about ten-fold and dialysed against PBS (−) for 24 hours. Identical fractions obtained from the 5 patients were pooled. Each pooled fraction was used as an added antigen in the following LMIT. The 3rd fraction obtained with Sepharose 6B from 3 different patients was subjected to an additional gel filtration with a Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (2.6 × 60cm) to give 3 fractions. After dialysis against PBS (−), identical fractions were pooled to use as 3 different added antigens in another LMIT.

For approximation of the molecular weight of the eluted bile protein fractions, a Gel Filtration Calibration Kit (Pharmacia Fine Chemicals, Uppsala, Sweden) was used. For the Sephadex G-200 column, ferritin, MW 440,000 ; catalase, MW 232,000 ; aldolase, MW 158,000 ; albumin, MW 67,000, and chymotrypsinogen A, MW 25,000, were used as standards.

Microdroplet LMIT. The tests were carried out according to the method of McCoy *et al.* (6). Heparinized peripheral blood collected from the subjects was mixed in a syringe with a 6% dextran solution at a ratio of 9 : 1, and allowed to stand at room temperature for 60 minutes. The supernatant, the leukocyte layer, was recovered, washed 3 times with McCoy's 5A medium, and adjusted to a volume of 1 ml. After hemolysis of the contaminating erythrocytes with 4 ml of distilled water for 20 seconds, 4 ml of double-strength McCoy's 5A medium was added to the leukocyte suspension, which was then washed 3 times with McCoy's 5A medium. The trypan blue exclusion test showed that more than 80% of the leukocytes were alive. The suspension was centrifuged, and the pellet was mixed with a 0.2% agarose solution in medium 199 containing 10% FCS and 100 μg/ml gentamicin. Two-μl microdroplets containing 4×10^5 leukocytes were placed into wells of a Micro Test Plate II (Falcon Labware, Oxnard, USA) and allowed to stand at room temperature for 5 minutes. Then, 0.1-ml aliquots of McCoy's 5A medium containing 10% FCS, 100 μg/ml gentamicin and subtoxic doses of the bile antigen were gently added to the wells. Wells with no addition of antigens were used as controls. More than 8 wells with or without antigen were used for each test. The wells were maintained under the usual conditions (5% CO_2, 95% air, 100% humidified atomosphere, and 37℃) for 24 hours. Thereafter, diameters of the outer cell migration areas (A) and those of the microdroplets (B) were microscopically measured, and the migration index (MI) was calculated using the following equation:

$$\mathrm{MI} = \frac{\text{Mean migration area with antigen}}{\text{Mean migration area without antigen}},$$

$$\text{where migration area} = 3.14 \times \left(\left(\frac{A}{2}\right)^2 - \left(\frac{B}{2}\right)^2\right)$$

Prior to carrying out the above tests, the optimal type of agarose for leukocyte migration was evaluated using Seakem LE, Seakem Seaplaque (both of Marine Colloid Inc., Rockland, USA) and Agarose A-45 (Nakarai Chemicals, Kyoto, Japan). Microdroplets (2 μl) in 0.2%, 0.3% and 0.4% solutions of different agaroses containing leukocytes (4×10^5) collected from controls were cultured in the same medium for 24 hours. Thereafter, preservation of the agarose droplets was examined and the migration ratio, $A^2 : B^2$, was calculated to determine which agarose yielded adequate leukocyte migration.

Subtoxic doses of each fraction obtained by Sepharose 6B and Sephadex G-200 gel filtration were determined according to the following procedures : The fractions were measured as to their protein concentration, diluted with McCoy's 5A medium containing 10% FCS and 100 μg/ml gentamicin into concentrations of 500, 200, 100, 50, 10, 5, 1, 0.1, 0.01, 0.001 and 0.0001 μg/ml, and then subjected to the LMIT using leukocytes from controls. Migration indices for different concentrations were calculated, and the highest concentrations among those with migration indices exceeding 0.8 were defined as the subtoxic doses.

To determine the normal range of the LMIT for each fraction, LMITs were carried out on 17 healthy adults using the subtoxic doses of each fraction as an added antigen. The normal range was defined as the mean migration index $\pm$2SD, and values outside this range were defined as LMIT positive.

Anti-sera. The five Sepharose 6B fractions 5 mg each were combined and subcutaneously administered with an equal amount of Freund's complete adjuvant into rabbit foot pads 4 times at one-week intervals. After the same interval, a 5th injection of only one of the fractions was administered without the adjuvant. One week after the final injection, the rabbits were sacrificed and their blood was collected. The anti-sera were absorbed on gelatinized human plasma proteins (7) prior to their use.

Immunodiffusion and Immunohistochemistry. The precipitation reactions between each Sepharose 6B fraction and their respective anti-sera were investigated according to the Ouchterlony method using a 0.7% agarose gel in 0.01M Tris-HCl buffer, pH 7.4.

Localization of the antigen corresponding to the anti-sera was investigated on paraffin sections of human liver according to Sternberger's peroxidase anti-peroxidase complex (PAP) method (8). The liver tissues were obtained from hepatobiliary disease patients during surgery. As primary antibody, the rabbit anti-sera to the fractions were used. Swine anti-rabbit IgG for the second antibody and PAP solution were purchased from DAKOPATTS A/S (Glostrup, Denmark). To confirm the specificity of the reaction, the rabbit anti-sera were absorbed with respective fractions and used instead of the primary antibody.

Results

Bile fractions. The elution pattern of human hepatic bile obtained by Sepharose 6B gel filtration is shown in Figure 1. The sharp peak near the void fraction was defined as Fraction 1, the successive plateau was defined as Fraction 2, and the 3 peaks appearing in the later stage were defined respectively as Fractions 3, 4 and 5. Frac-

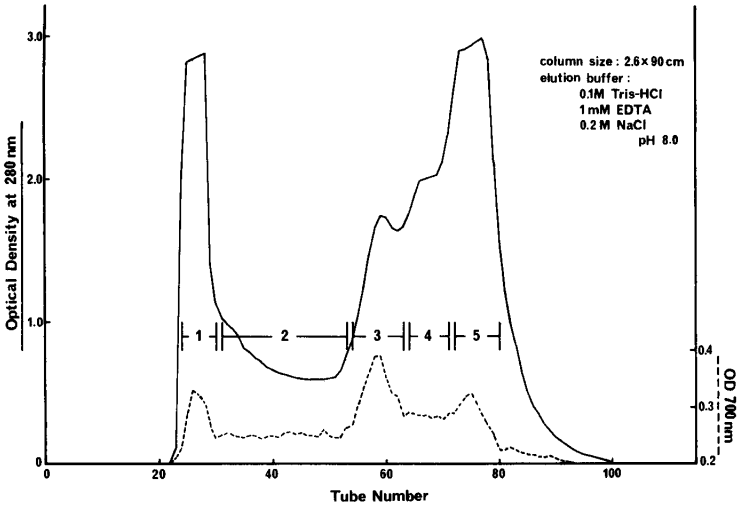

Fig. 1 Elution pattern of concentrated human hepatic bile with Sepharose 6B gel filtration. Four peaks and a plateau were defined as Fractions 1 thru 5 according to the elution order. The continuous and dotted lines indicate OD at 280 nm and at 700 nm (Lowry's method), respectively.

tion 3 showed the highest protein concentration among the 5 fractions. The elution pattern of Fraction 3 after additional gel filtration with Sephadex G-200 is shown in Figure 2. Three peaks were obtained, and defined as Fractions (1), (2) and (3) according to the order of elution. The approximate molecular weights of the peaks were respectively 500,000, 210,000 and 42,000.

LMIT. Optimal agarose : Migration ratios obtained with three different agaroses are shown in Table 1. The use of 0.2% Seakem LE showed the highest ratio, with no destruction of agarose microdroplets. Thus, 0.2% Seakem LE was used in subsequent tests.

Subtoxic doses : The subtoxic doses for Fractions 1 thru 5 were respectively 0.01, 10, 10, 50 and 50 μg/ml, while those for Sephadex G-200 Fractions (1) thru (3) were respectively 100, 50 and 50 μg/ml.

Normal ranges of LMIT : The ranges for Fractions 1 thru 5 were 0.83-1.20, 0.85-1.13, 0.86-1.16, 0.83-1.20 and 0.75-1.26 (Fig. 3), whereas those for Sephadex G-200 Fractions (1) thru (3) were 0.83-1.09, 0.80-1.26 and 0.75-1.13 (Fig. 4).

LMIT in patients : In the LMIT using Sepharose 6B fractions as antigens, only Fraction 3 gave a positive response in 8 out of 11 PBC cases, 7 cases with migration inhibition and 1 case with migration stimulation. Only one out of 10 CAH cases was LMIT-positive (Fig. 3). The LMIT-positive ratio in PBC was higher than in CAH ($p < 0.005$). Photomicrographs of leukocyte migration inhibition and stimulation are shown in Figure 5. The 3 PBC patients who showed negative responses were all under D-penicillamine treatment, while the 8 positive patients were not. As for CAH cases, no clinical or histological differences were noted between LMIT-positive and LMIT-negative cases. In the LMIT using Sephadex G-200 fractions as antigens, only Fraction (1) gave a positive response in 3 PBC patients, none of whom were receiving D-penicillamine (Fig. 4).

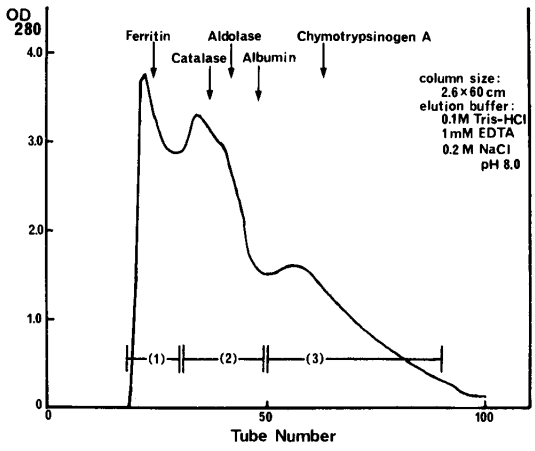

Fig. 2 Elution pattern of Sepharose 6B Fraction 3 with Sephadex G-200 gel filtration and molecular weight calibration. Three peaks were defined as Fractions (1) thru (3) according to the elution order.

Table 1 Comparison of leukocyte migration areas with different types of agarose in various concentrations.

Agarose (concentration)	Migration Ratio[a] (mean ± SD)		
	0.2%	0.3%	0.4%
Seakem, LE	2.45 ± 0.19	2.13 ± 0.10	2.16 ± 0.11
Seakem, Sea Plaque	1.73 ± 0.13	1.63 ± 0.15	1.90 ± 0.09
Agarose, A-45	ND[b]	ND	ND

a: $\frac{\text{Inner droplet area} + \text{Outer migration area}}{\text{Inner droplet area}}$

b: ND = Not determined because of fragility.

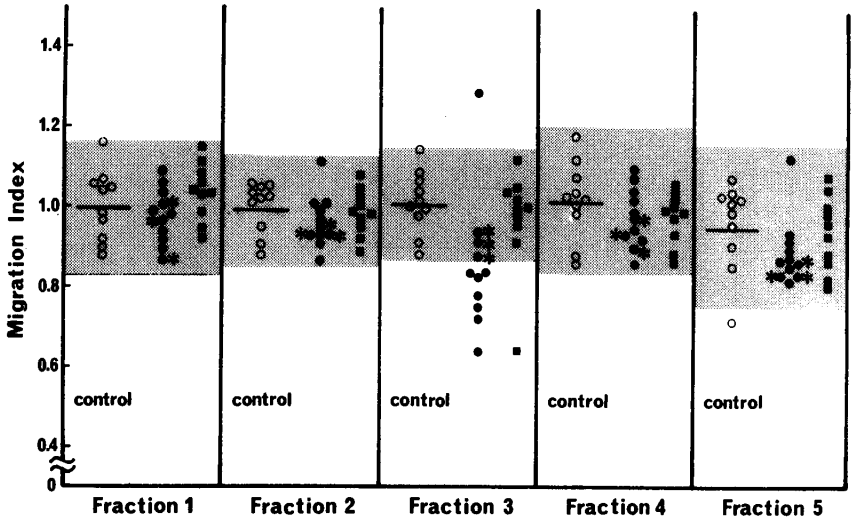

Fig. 3 Results of leukocyte migration inhibition test (LMIT) with Sepharose 6B fractions in healthy adults (○) and in patients with primary biliary cirrhosis (● PBC) and chronic aggressive hepatitis (■ CAH). Eight out of 11 PBC patients and 1 out of 10 CAH patients show LMIT positive responses with Fraction 3. ▨ normal range of LMIT ; * patients under D-penicillamine treatment.

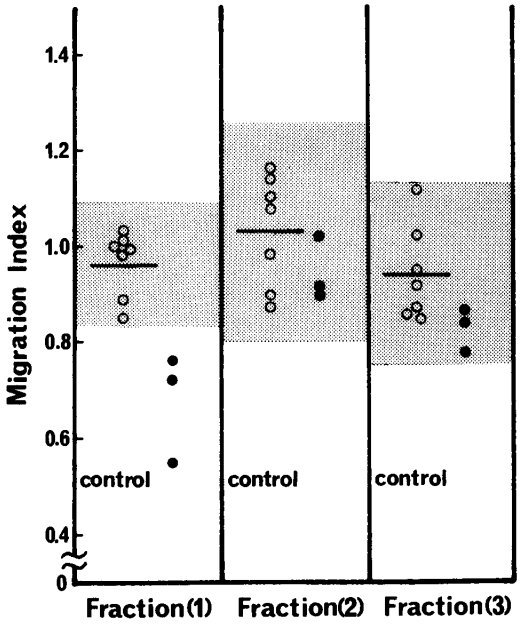

Fig. 4 Results of leukocyte migration inhibition test (LMIT) with Sephadex G-200 fractions in healthy adults (○) and 3 primary biliary cirrhosis patients (● PBC). All 3 PBC patients show LMIT positive responses with Fraction (1). ▨ normal range of LMIT.

Immunodiffusion and immunohistochemistry. Immunodiffusion studies revealed a precipitation line between Fractions 1, 2 and 3 and respective anti-sera. The precipitation line of Fraction 1 fused with that of Fraction 2, but the precipitation line of Fraction 2 did not seem to fuse with the Fraction 3 line in cross immunodiffusion.

Immunohistochemistry using anti-Fraction 1 antibody clearly demonstrated that the peroxidase products were localized on the epithelial cells of bile ductules and bile ducts (Fig. 6), not only of the interlobular bile ducts but also of larger bile ducts including septal ones. The products were diffusely distributed in the cytoplasm of the bile duct epithelial cells. The products obtained with anti-Fraction 3 antibody were clearly localized on the membrane of the bile canalicules (Fig. 7). With anti-Fraction 2 antibody, the products were observed on both sites, and no products were present in anti-Fraction 4 or 5 antibody.

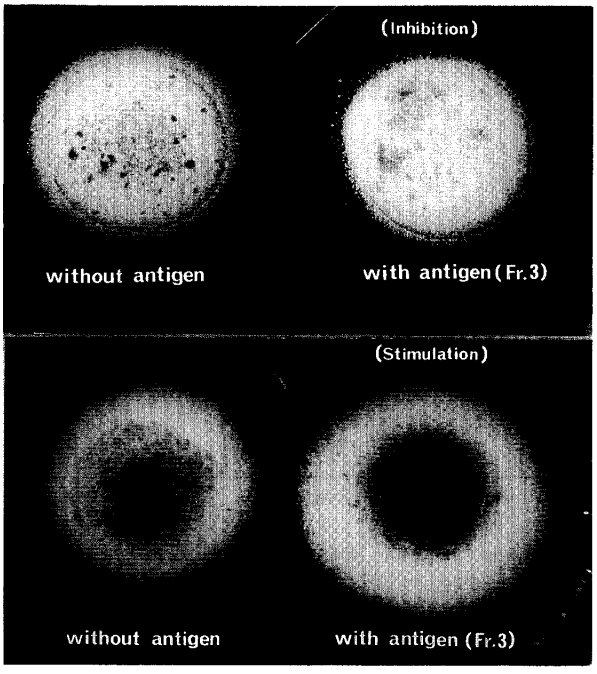

Fig. 5 Microphotographs of leukocyte migration inhibition and stimulation with Sepharose 6B Fraction 3 as the antigen.

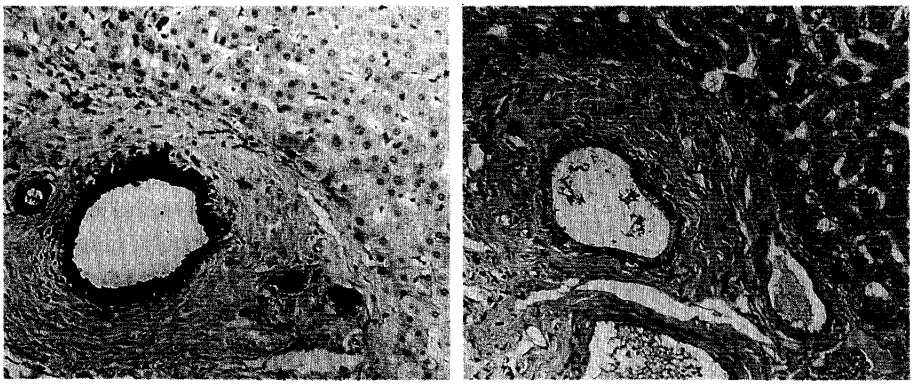

Fig. 6 Immunohistochemical staining of human liver sections using anti-Fraction 1 antibody. The peroxidase products are clearly localized on the epithelial cells of the bile ducts. Right photo, control using non-immunized rabbit serum. ×33

Discussion

The present study demonstrated that cellular immunity in PBC is formed against Fraction 3 which contains canalicular antigen, but not against Fraction 1 which contains ductal antigen.

It is known that human hepatic bile contains 3 or 4 bile specific proteins, in addition to several serum proteins (9, 10). Using a crude protein fraction of bile, Eddleston *et al.* (2) have reported that cellular immunity was formed against bile proteins in PBC. Although similar observations followed

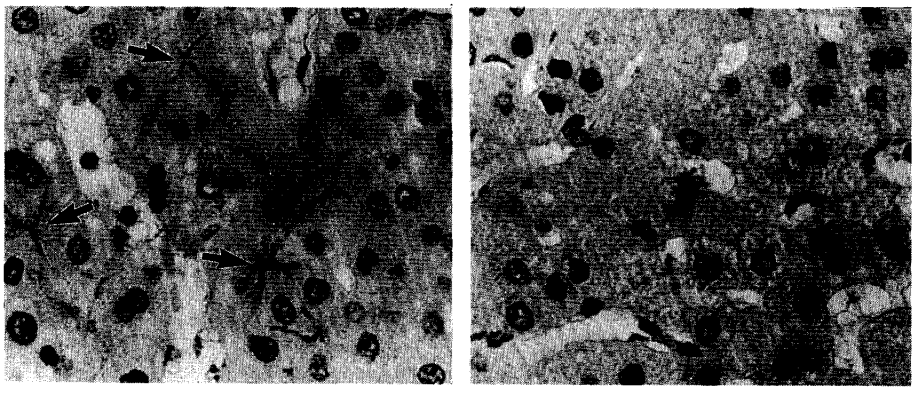

Fig. 7 Immunohistochemical staining of human liver sections using anti-Fraction 3 antibody. The peroxidase products are clearly localized on the membrane of the bile canalicules (arrows). Right photo, control using non-immunized rabbit serum. ×132

(3, 4), only the report of Wojcicka *et al.* (5) is available, except for ours, on the use of individual fractions of bile proteins as antigens for the LMIT. Wojcicka *et al.* obtained three fractions by procedures different from ours : "Fraction B" containing "Antigen 1" of unknown localization, "Fraction C" containing canalicular antigen which was localized on the membrane of bile canalicules, and "Fraction D" containing both canalicular and ductal antigens, the latter of which was localized in the epithelial cells of the bile ducts. Using each of the three fractions as an added antigen for the LMIT, they obtained positive responses with all three fractions in PBC, and speculated that cellular immunity was formed against canalicular antigen and Antigen 1, because they were unable to separate the ductal antigen from the canalicular antigen. Our results were unexpectingly coincident with their speculation : cellular immunity was formed against the canalicular-antigen-containing fraction, but not against the ductal-antigen-containing fraction in PBC. The ductal antigen was detected immunohistochemically not only in the epithelial cells of interlobular bile ducts but also in those of larger bile ducts including a bile duct 3 mm in diameter. PBC is characterized by the destruction of interlobular bile ducts. Thus, it is reasonable that cellular immunity is not formed against the ductal-antigen-containing fraction. Wojcicka *et al.* (5) prepared anti-Antigen 1 antibody, but we were not able to identify the antibody in the present study.

PBC is a chronic intrahepatic cholestasis induced by the destruction of intrahepatic bile ducts, and was designated histologically as chronic non-suppurative destructive cholangitis (CNSDC) by Rubin *et al.* (11). Transmission electron microscopy of CNSDC suggested the existence of T-cell cytotoxicity against bile duct epithelial cells in PBC (12, 13). However, our study demonstrated the formation of cellular immunity against canalicular antigen, but not ductal antigen. In view of the descrepancy between our results and the histological results of other investigators, the study of Ogasawara *et al.* (14) deserves attention. They have reported their observation on serial liver sections in pre-cholestatic PBC patients. The bile secreted from bile canalicules flows down to the leading duct of septal bile ducts through the collecting duct of interlobular bile ducts. CNSDC was found in the latter dile ducts, *i.e.* interlobular bile ducts, but not in the

septal bile ducts. The findings suggest the presence of an intimate relation between the functional difference of bile ducts and the localization of ductal damage in PBC. Canalicular antigen is possibly absorbed only through the interlobular bile ducts and causes CNSDC. Further studies on bile duct functions are necessary to correlate our results to the histological characteristics, as well as those on purification of canalicular antigen and localization of Antigen 1.

Another interesting fact is that the three PBC patients who showed LMIT negative responses were all under D-penicillamine treatment. D-penicillamine is well known to depress cellular immunity, as well as to promote copper excretion and to lower the serum immunoglobulin and immune complex levels (15, 16). This drug has been used in PBC mainly to decrease the copper level in the liver, and our results suggest the beneficial effect of the drug in PBC. On the other hand, treatment with Azathioprine, an immunosuppressant, has been reported to have no influence on the LMIT in PBC (4) and is known to be ineffective in prolonging the survival of PBC patients (17). The effect of D-penicillamine on survival of PBC patients is not conclusive and requires further elucidation.

The microdroplet method of McCoy *et al.* (6) used in the present investigation has certain advantages over the capillary tube method (18) and the agarose plate method (19). The advantages are that the method is less complicated and the requirements of blood and added antigen are less. This method would be useful in investigating cellular immunity.

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Reprint requests to :
Ryoji Fujiwara
First Department of Internal Medicine
Okayama University Medical School
2-5-1 Shikata-cho Okayama 700, Japan