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—Original Article—

HEPATOCYTE PLASMA MEMBRANE ANTIGENS. I. DETERMINATION OF ANTIBODIES BOUND TO THE HEPATOCYTE PLASMA MEMBRANE BY ^{125}I-PROTEIN A BINDING ASSAY

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Summary

Anti-liver-specific membrane lipoprotein (anti-LP-1) and anti-Tamm-Horsfall glycoprotein (anti-THGP) rabbit antibodies were found to bind to Chang liver cells, a cultured human hepatocyte cell line, and PLC/PRF/5, a hepatoma cell line. The antibodies bound were determined by an immunofluorescence staining and a semiquantitative ^{125}I-protein A binding assay. The ^{125}I-protein A binding assay was successfully adapted to determine anti-hepatocyte plasma membrane antibodies in sera of patients with lupoid hepatitis and chronic active hepatitis. The percentage of ^{125}I-protein A bound in 10 normal subjects were 1.5 ± 0.4 (mean ± standard deviation) for PLC/PRF/5 and 1.6 ± 0.6 for Chang liver cell, while those in 2 patients with lupoid hepatitis were 7.2 ± 0.3, 5.9 ± 0.1, and those in 8 patients with chronic active hepatitis 3.9 ± 1.3, 3.2 ± 1.5, respectively. Furthermore, a blocking study revealed that LP-1 and THGP were partially involved in antigen sites recognized with anti-hepatocyte plasma membrane antibodies in sera of a patient with lupoid hepatitis. The retaining ability of antibody binding to the hepatocytes after the absorption with non-hepatocyte cells suggested the presence of antibodies specific for the hepatocyte plasma membrane in the patient's serum.

Key Words: *^{125}I-protein A binding assay, anti-LP-1, anti-Tamm-Horsfall glycoprotein, anti-hepatocyte plasma membrane antibody.*

Introduction

The presence of autoantibodies in sera of patients with a certain liver disease such as lupoid hepatitis has been well reviewed[1]. The antibodies binding to hepatocyte plasma membrane have been discussed to be involved in liver cell damage during the development of the disease. The membrane-bound antibodies have been detected with immunofluorescence staining using isolated rabbit hepatocytes[2] or Chang liver cells[3], as antibody adsorbents, though the method was less quantitative in its determination. Among the membrane-bound antibodies, antibodies reacting with liver-specific membrane lipoprotein (LP-1)[4], an

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antigen complex of the hepatocyte cell membrane prepared from normal liver tissue[5], and Tamm-Horsfall glycoprotein (THGP), a carbohydrate-rich urinary glycoprotein[6], were reported to exist with higher incidence in sera of patients with chronic active liver diseases than those of normal subjects[7~9]. In addition, using immunofluorescence staining, the antigens recognized with anti-LP-1 and anti-THGP rabbit antibodies were shown in isolated human hepatocytes[10] and hepatocytes of a section of human biopsied liver tissue[11,12]. On the other hand, it has been reported that a ^{125}I-protein A binding assay was a useful tool for the semiquantitative determination of plasma membrane-bound antibodies in other studies[13,14]. It was also known that establishing the assay may lead to the biochemical analysis of the antigen molecules on the membrane[14], though it requires the intact living cells of human liver origin as an antibody absorbent.

This paper reports determination of the anti-LP-1 and anti-THGP rabbit antibodies bound to the membrane of PLC/PRF/5, human hepatoma cell, and Chang liver cell, human hepatocyte by the ^{125}I-protein A binding assay, and that the assay was further adapted to the determination of anti-hepatocyte plasma membrane antibodies in sera of patients with lupoid hepatitis or chronic active hepatitis.

Materials and Methods

Patients: Sera from 2 patients with lupoid hepatitis, 8 patients with chronic active hepatitis (CAH), 3 with other diseases including ulcerative colitis with hypergammaglobulinemia, common variable immodeficiency with agammaglobulinemia and Sjögren's syndrome with a positive rheumatoid factor (RF), were included in the present study. Lupoid hepatitis was diagnosed according to the criteria of Mackay[15] and CAH was histologically diagnosed[16]. Six out of 8 patients with CAH were positive for hepatitis B surface antigen (HBsAg) by passive hemagglutination test. None of the patients had anti-HBs, which was shown by reversed passive hemagglutination test. Sera from 10 normal subjects were used as controls. None had a history of liver disease. Three normal subjects were positive for anti-HBs. These sera tested were inactivated at 56°C for 30 min and stored at −20°C until used.

Cells and culture: PLC/PRF/5[17] was kindly donated by Prof. Nakao Ishida, Tohoku University School of Medicine, Hep-3B[18], human hepatoma cell, by Dr. P. Aden, Wistar Institute, University of Pennsylvania, and Scuthola[19], human kidney cancer cell, by Dr. Ryuzo Ueda, Aichi Cancer Center. Chang liver cell[20] was obtained from Dai Nihon Seiyaku Inc., Osaka, and FL[21], human amnion cell from Hayashibara Biochemical Laboratories Inc., Okayama. Both Raji[22] and Molt-3[23] were supplied by Prof. George Klein, Karolinska Institute, Stockholm. PLC/PRF/5, Hep-3B, Chang liver cell, Scuthola and FL were grown in Eagle's minimum essential medium (MEM; GIBCO Lab., New York), Raji and Molt-3 were in RPMI-1640 (GIBCO Lab.) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO Lab.), 100 U/ml penicillin and 100 μg/ml streptomycine at 37°C in humidified 95% air-5% CO_2.

Preparation of LP-1 and THGP: LP-1 was prepared from normal autopsy liver by the method of McFarlane et al.[24], with a minor modification. Briefly, tissue was sliced into small pieces, washed for 5 hours with 0.25 M sucrose and homogenized in ice cold 0.25 M sucrose with a Polytron homogenizer (Kinematica, Luzern, Switzerland). The homogenate was centrifuged at 4°C for 1 hour at 105,000×g in a 65T rotor (Hitachi, Tokyo). Then the clean supernatant was applied to a 2.2 × 90 cm column of Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) pre-

viously equilibrated with 0.1 M Tris-HCl, pH 8.0, containing 1 mM ethylenediaminetetraacetate (EDTA) and 0.15 M NaCl (Tris-EDTA buffer). The column was eluted with the same buffer. A peak appeared in the void volume was collected, concentrated and designated LP-1.

THGP was prepared from normal human urine according to the method of Fletcher et al.[6]. Ten liters of pooled human urine was mixed with sodium chloride to give a concentration of 0.58 M. The precipitate collected by centrifugation at 20,000×g at 4°C for 30 min was washed twice with 0.58 M sodium chloride and dissolved in distilled water. After dialysis against distilled water, the solution was clarified by centrifugation. The clarified supernatant was liophilized and designated THGP.

Protein concentration was determined by the method of Lowry et al.[25].

Preparation of antisera: Anti-LP-1 and anti-THGP antisera were raised in rabbits by immunization with LP-1 and THGP, respectively. The rabbits received 5 mg of LP-1 or THGP in an equal volume of Freund's complete adjuvant (DIFCO Lab., Detroit) and subsequently the same dose of the antigens without adjuvant every 2 weeks for a total of 6 weeks. These rabbits were bled 1 week after the final injection. The antisera were inactivated at 56°C for 30 min and stored at −20°C until used.

Immunofluorescence staining: Cells grown on a 5 × 5 mm cover glass (Matsunami glass industries, LTD., Osaka) in a well of a culture plate (Falcon 3008, Div. Becton, Dickinson and Co.) were washed three times with Dulbecco's phosphate buffered saline (PBS; GIBCO Lab.) and incubated with 20 μl of diluted rabbit antisera or a patient's serum for 45 min at room temperature. Then the cells were washed three times with PBS and incubated with 20 μl of fluorescein-isothiocyanate (FITC)-conjugated anti-rabbit γ-globulin goat immunoglobulins (Boehringerwerke AG.) for a further 45 min at room temperature. After three washings with PBS, the cells were examined for membrane fluorescence by ultraviolet microscopy (OLYMPUS, model BH-RFL). A control test was run with normal rabbit serum instead of rabbit antisera.

^{125}I-Protein A binding assay: Protein A (Pharmacia Fine Chem.), 100 μg in 10 μl PBS, was labelled with 1.0 mCi of Na ^{125}I (New England Nuclear) by the Chloramin-T method[26]. The specific activity of the labellled protein A was about 8,000 cpm/ng. A ^{125}I-protein A binding assay was performed according to the method of Spira et al.[14], with a minor modification. Twenty thousand cells were cultivated in a well of a microtestplate (Falcon 3034) with MEM containing 10% FCS for 72 hours in a humidified 95% air-5% CO_2 atmosphere at 37°C. A subconfluent monolayer of cells was washed three times with PBS and subsequently incubated with 50 μl of serum diluted with PBS containing 1% bovine serum albumin (BSA) for 45 min at 37°C with periodic gentle agitation. After incubation, the cells were washed three times with PBS and incubated with 50,000 cpm/25 μl of ^{125}I-protein A for another 45 min at room temperature with occasional gentle shaking. After the following third washing with PBS, the cells were lysed with 150 μl of 1 N NaOH, transferred to a test tube and the radioactivity in the lysate was counted with a gamma counter (Aloka, model ARC-500, Tokyo). The percentage of ^{125}-protein A bound was calculated according to the following formula.

Percentage of ^{125}I-protein A bound = {(cpm in cell lysate treated with test serum − cpm in cell lysate treated with PBS containing 1% BSA)/cpm of ^{125}I-protein A added} × 100.

The assay was done in duplicate. The viability of these cells during the experiment was greater than 90% as determined by a trypan

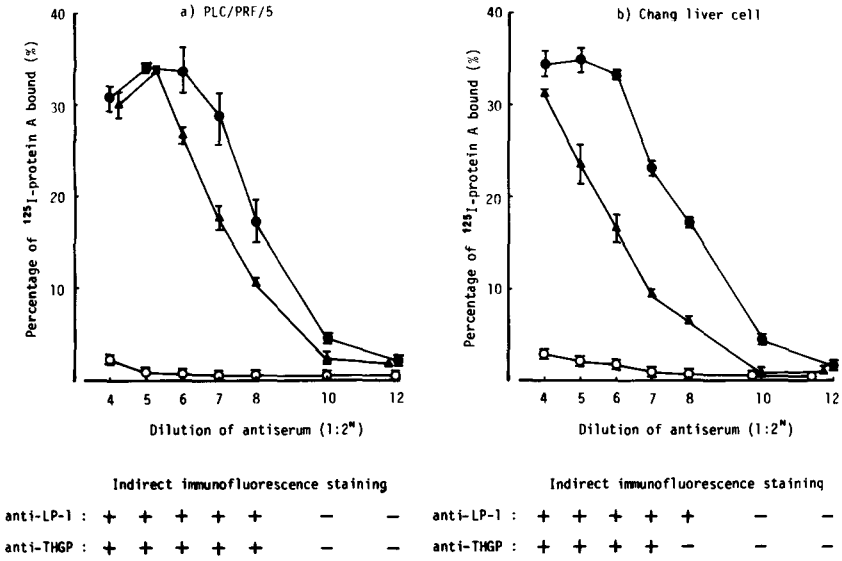

Fig. 1. Effect of rabbit antiserum diluton on ^{125}I-protein A binding and immunofluorescence staining. PLC/PRF/5 (a) and Chang liver cells (b) were incubated with serially diluted rabbit anti-liver-specific membrane lipoprotein (LP-1; ●—●), anti-Tamm-Horsfall glycoprotein (THGP; ▲—▲) antiserum or normal rabbit serum (○—○). Cell-bound antibodies were examined by the ^{125}I-protein A binding assay or indirect immunofluorescence technique.

blue dye exclusion test.

Detection of Fc receptors: Human IgG prepared from pooled normal plasma according to the method of Onoue et al.[27] was aggregated by heating at 63°C for 20 min. Soluble aggregated human IgG (AHG) was collected by centrifugation at 1,500×g for 30 min. Cells were incubated with serially diluted AHG for 45 min at 37°C and washed three times with PBS and tested for the binding of AHG with the ^{125}I-protein A binding assay as described above. Raji derived from Burkitt's lymphoma[22] and Molt-3 derived from T cell leukemia[23] were used for Fc receptor positive and negative control, respectively.

Blocking study: The cells were incubated with 1:2^5 diluted serum of a patient with lupoid hepatitis together with serially diluted LP-1 or THGP prior to adding ^{125}I-protein A. BSA was added instead of LP-1 or THGP in a control study.

Absorption of serum with cells of non-liver origin: Fifty microliters of serum from the patient with lupoid hepatitis diluted to 1:2^5 with PBS containing 1% BSA was incubated with approximately 4×10^4 cells of monolayered Scuthola and/or FL in the well of the microtestplate for 1 hour at 4°C. This process was repeated three times.

Statistical analysis: "Analysis of variance" was used for statistical analysis.

Results

Anti-LP-1 and anti-THGP antibodies bound to the cells: anti-LP-1 and anti-THGP antibodies bound to PLC/PRF/5 and Chang liver cells were semiquantitatively determined by the ^{125}I-protein A binding assay (**Fig. 1**). Results of immunofluorescence are also shown in the figure. Sensitivities of the two methods in determining anti-THGP and anti-LP-1 were almost the same, though the protein-A binding assay showed more quantitative results. The radioactivities bound to the cells after preincubation

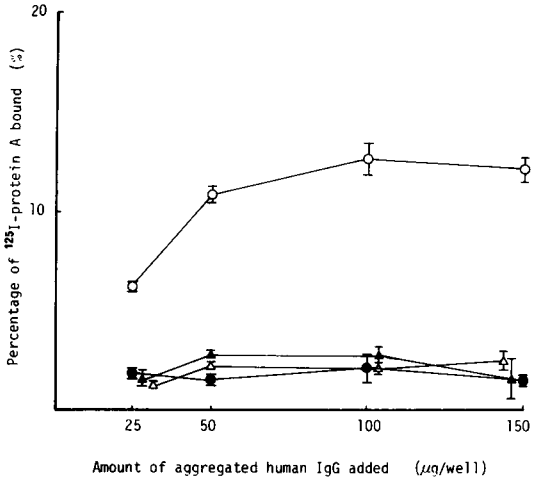

Fig. 2. Aggregated human IgG binding. PLC/PRF/5 (●—●), Chang liver cell (▲—▲), Molt-3 (△—△) and Raji (○—○) were incubated with serially diluted aggregated human IgG (AHG) and the bound AHG was measured by the ^{125}I-protein A binding assay.

with the antisera were higher than that with normal rabbit serum, which was as low as the background radioactivity. The radioactivities bound to these cells decreased proportionally with the amount of antisera added at certain ranges of serum dilution. The lineally proportional ranges were between 1:2^6 and 1:2^{10} for anti-LP-1 antiserum, 1:2^5 and 1:2^{10} for anti-THGP antiserum, when PLC/PRF/5 were adsorbents. The ranges, when Chang liver cells were used, were 1:2^6 and 1:2^{10} for anti-LP-1 antiserum, 1:2^4 and 1:2^{10} for anti-THGP antiserum. It was also found that the percentage of ^{125}I-protein A bound was reduced to the background level of radioactivity when 50 μg LP-1 or 25 μg THGP was added at the preincubation stage.

Furthermore, the binding of AHG to PLC/PRF/5 and Chang liver cells was examined by the ^{125}I-protein A binding assay (**Fig. 2**), in order to clarify whether these cells have Fc receptors on their plasma membrane or not. Raji, Fc receptor positive control cells, reacted with AHG, while PLC/PRF/5 and Chang liver cells showed low reactivities with AHG as Fc receptor negative Molt-3 did.

Anti-hepatocyte plasma membrane anti-

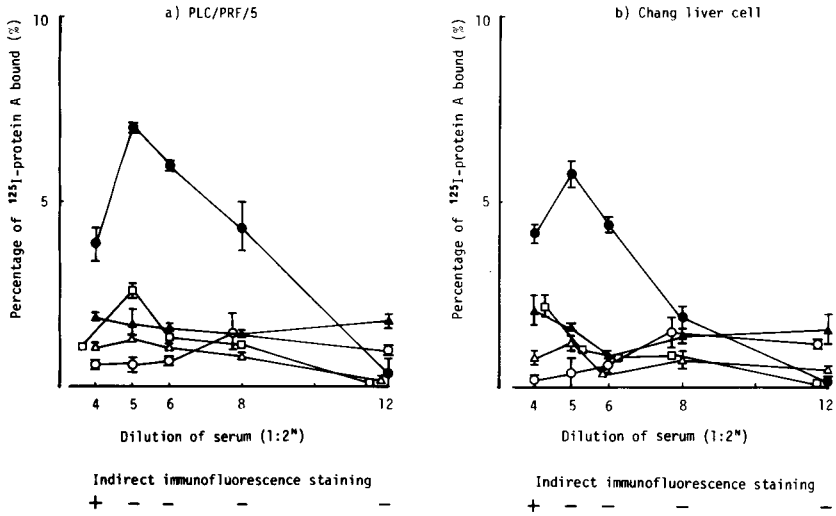

Fig. 3. Effect of patient's serum dilution on ^{125}I-protein A binding. PLC/PRF/5 (a) and Chang liver cells (b) were incubated with serially diluted sera of patients with lupoid hepatitis (●—●), ulcerative colitis with hypergammaglobulinemia (△—△), common variable immunodeficiency with agammaglobulinemia (○—○), Sjogren's syndrome (□—□) and normal subject (▲—▲). Cell-bound antibodies were measured by the ^{125}I-protein A binding assay.

bodies in patients' sera: The ^{125}I-protein-A binding assay used for the determination of anti-LP-1 and anti-THGP rabbit antibodies was adapted to the measurement of anti-hepatocyte plasma membrane antibodies in the serum of a patient with lupoid hepatitis (**Fig. 3**). ^{125}I-protein A bound was high in binding percentage and was lineally proportional to the mount of the serum added, at a range of serum dilution of $1:2^4$ and $1:2^{12}$, when compared to those of the agammaglobulinemia patient and normal subjects. Indirect immunofluorescence staining with the patient's serum gave a weak membrane fluorescence outlining both PLC/PRF/5 and Chang liver cells only at a dilution point of $1:2^4$. The two other test sera from patients with ulcerative colitis with hypergammaglobulinemia (5.0 g/dl) and Sjögren's syndrome with a positive RF were examined in order to investigate whether the high percentage value of ^{125}I-protein A bound was due to high γ-globulin level or additional positiveness of RF, since the serum of the patient with lupoid hepatitis had a γ-globulin level of 5.2 g/dl and positive RF. The percentages of ^{125}I-protein A bound of two sera were low and almost equal to those of normal subject's sera.

A blocking study by adding LP-1 or THGP was performed. The percentage of ^{125}I-protein A bound was reduced when either LP-1 or THGP was added in the preincubation stage with serum of the patient with lupoid hepatitis. LP-1 had a higher potency to inhibit the anti-hepatocyte plasma membrane antibody binding than that of THGP (**Fig. 4**). Cells of non-liver origin, Scuthola and FL were examined for the ability to absorb the anti-hepatocyte plasma membrane antibody. These cells had lower but significantly more antibody adsorbing ability than PLC/PRF/5, Chang liver cells and another hepatoma cells, Hep-3B. The pre-absorption of the serum with Scuthola reduced the 58%, 66% and 64% of antibodies bound

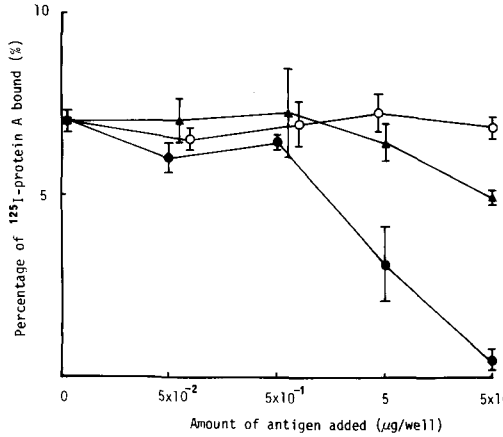

Fig. 4. Effect of adding liver-specific membrane lipoprotein (LP-1; ●—●), Tamm-Horsfall glycoprotein (THGP; ▲—▲) and bovine serum albumine (BSA; ○—○) on ^{125}I-protein A binding of serum from a patient with lupoid hepatitis. Dilution of the serum was $1:5^5$. PLC/PRF/5 was used as adsorbent.

for PLC/PRF/5, Chang liver cells and Hep-3B, respectively, FL still retained 18.3% (**Fig. 5**). The serum adsorbed with Scuthola followed by FL showed no more ability of the antibody binding for Scuthola and FL, but the same levels for PLC/PRF/5, Chang liver cells and Hep-3B as those after absorbation with Scuthola only.

Anti-hepatocyte plasma membrane antibodies in sera of patients with CAH and lupoid hepatitis: The ^{125}I-protein A binding assay was performed in sera from 8 patients with CAH and 2 with lupoid hepatitis (**Fig. 6**). A serum dilution of $1:2^5$ was chosen for the survey with the assay. Mean ± standard deviation of the percentage of ^{125}I-protein A bound to PLC/PRF/5 were 1.5 ± 0.4 for normal subjects, 3.9 ± 1.3 for CAH and 7.2 ± 0.3 for lupoid hepatitis. The values among these three groups were significantly different by the analysis of variance ($p<0.01$). Six out of the eight patients with CAH and 2 out of the 2 patients with lupoid hepatitis were positive for anti-hepatocyte plasma membrane antibody, when a percentage of >2.3%, which was the value of

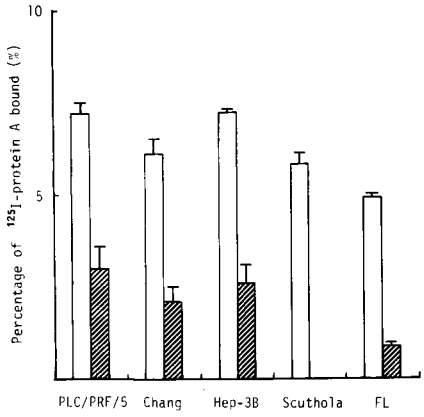

Fig. 5. Cross-reactivity with various cells of serum from a patient with lupoid hepatitis and effect of absorption with Scuthola. Hep-3B, Scuthola and FL besides PLC/PRF/5 and Chang liver cells were incubated with serum non-absorbed (□) or absorbed with Scuthola (▨) from the patient with lupoid hepatitis. Dilution of the serum both non-absorbed and absorbed was 1:2^5. Cell-bound antibodies were quantitated by the ^{125}I-protein A binding assay.

the mean plus two standard deviations of 10 normal subjects, was taken to be positive. The values with Chang liver cell were 1.6 ± 0.6 for normal subjects, 3.2 ± 1.5 for CAH and 5.9 ± 0.1 for lupoid hepatitis ($p<0.01$). Four out of the eight patients with CAH and 2 out of the 2 patients with lupoid hepatitis were positive for anti-hepatocyte plasma membrane antibody, as their values obtained were higher than 2.8, which was a value of mean plus two standard deviations of 10 normal subjects. There was no correlation between the positiveness for the anti-hepatocyte plasma membrane antibody and HBsAg.

Discussion

In the present study, it was found that anti-LP-1 and anti-THGP antibodies bound to PLC/PRF/5 and Chang liver cells were semi-quentitatively determined by a method of ^{125}I-protein A binding assay. Furthermore, anti-hepatocyte plasma membrane antibodies in sera of patients with a certain liver disease could be determined by the assay. Previous methods for determining anti-liver-cell-membrane antibody were performed by isolated rabbit hepatocytes[2]. This adsorbent may trap non-specific heterophilic antibodies in the

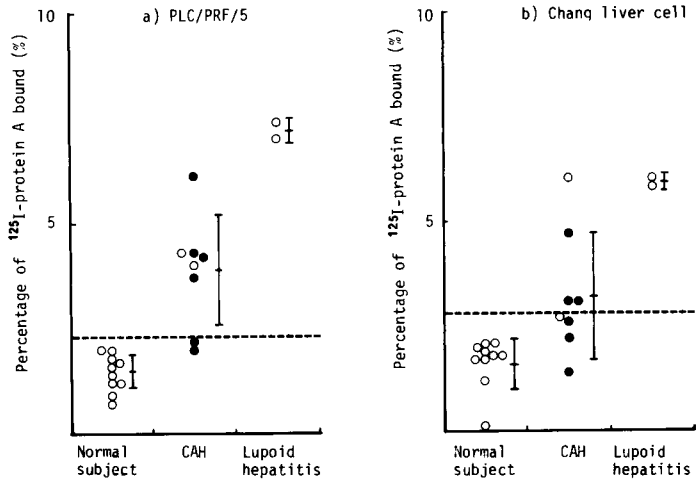

Fig. 6. Percentage of ^{125}I-protein A bound in 8 patients with chronic active hepatitis (CAH), 2 with lupoid hepatitis and 10 normal subjects (●: HBsAg positive, ○: HBsAg negative). Dilution of the sera was 1:2^5. PLC/PRF/5 (a) and Chang liver cells (b) were used as adsorbents. Horizontal dotted lines indicate the value of mean plus two standard deviations of 10 normal subjects. The ranges of mean ± standard deviation of the respective disease groups were indicated.

damaged membrane. The author developed a ^{125}I-protein A binding assay to determine anti-hepatocyte 'intact' plasma membrane antibodies using human cultured cells as adsorbent. The assay required the adsorbent cells to retain the expression of well differentiated antigens on the membrane as they expressed in the tissue. PLC/PRF/5 and Chang liver cell were used as the adsorbents. PLC/PRF/5 is a cell line derived from hepatocellular carcinoma[17], and has been shown to retain the property of producing 20 nm HBsAg particles[28] and 7 species of serum protein into the medium[29], which is likely carrying well differentiated liver antigens on the membrane also. Chang liver cell is a cell line derived from normal human liver tissue[20].

In this study, both PLC/PRF/5 and Chang liver cells were shown to exhibit the antigens reacting with anti-LP-1 and anti-THGP rabbit antibodies on their plasma membrane. Chisari et al.[30] reported that PLC/PRF/5 expressed LP-1, while Chang liver cells did not. On the contrary, another laboratory showed that Chang liver cells retained LP-1 on the plasma membrane[3]. Possible explanation of these conflicting results leads to the conclusion that cell lines of Chang liver cell used in their studies might be different[31]. THGP is a urinary glycoprotein[6] and has been detected in kidney cells[32]. In addition, using the immunofluorescence technique and anti-THGP rabbit antiserum, immunologically cross-reacting THGP was shown on the hepatocyte membrane of a tissue section of normal human liver [12]. The author found immunologically cross-reacting THGP on the plasma membrane of the two cultured hepatocytes used in this experiment.

It was shown in this paper that anti-LP-1 and anti-THGP rabbit antibodies bound to PLC/PRF/5 and Chang liver cell via non-Fc region, since these cells did not have Fc receptors (**Fig. 2**), though it was reported that isolated rabbit hepatocyte did[33]. The antibodies bound could be semiquantitatively determined by the ^{125}I-protein A binding assay. Thereafter, the assay was adapted to determine the anti-hepatocyte plasma membrane antibodies in the sera of patients with lupoid hepatitis and CAH. Meyer zum Büschenfelde[3] demonstrated by immunofluorescence technique that the antibodies bound to Chang liver cells were present in 7 out of 7 "CAH autoimmune" cases and in 14 out of 19 "CAH HBsAg positive" cases. It was also found in this study, that the anti-hepatocyte plasma membrane antibodies detected by the ^{125}I-protein A binding assay using PLC/PRF/5 and Chang liver cells were present in 2 out of 2 patients with lupoid hepatitis and, out of 8 patients with CAH, 6 with PLC/PRF/5 and 4 with Chang liver cells. The similarity of the results in the two expriments may indicate the usefulness of the ^{125}I-protein A binding assay for the survey of hepatocyte membrane bound antibodies. Furthermore, the binding to these cells of the antibodies in serum of a patient with lupoid hepatitis may be specifically subject to the hepatocyte in part, since the ability of the antibody binding to the hepatocyte was retained after extensive absorption with non-hepatocyte cells (**Fig. 5**). It was also found that the binding of the anti-hepatocyte plasma membrane antibody was partially blocked by LP-1 and THGP added in preincubation (**Fig. 4**). This result is consistent with the report from our laboratory that anti-LP-1 and anti-THGP existed in sera of patients with lupoid hepatitis[9]. Thus, the antigens reacting with anti-hepatocyte plasma membrane antibodies may contain LP-1 and THGP in part. The ^{125}I-protein A binding assay developed in this study may lead to further biochemical approaches to analyze those antigens recognized in other studies[14].

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