Detection of Antibody and the Antigen Classes in Circulating Immune Complexes Fractionated with the Sucrose Density Gradient Centrifugation Using an F(ab′)$_2$anti-C3 ELISA

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Circulating immune complexes (CICs) in sera from patients with liver cirrhosis (LC), primary biliary cirrhosis (PBC), hepatitis B type (BCH) and hepatitis not including BCH (nonBCH) were analyzed using F(ab′)$_2$anti-C3 ELISA, since CICs' functions are intimately associated with the composition and their sizes. Positive percentages for IgG-, IgA- and IgM-CIC were significantly increased in LC, PBC, BCH and nonBCH as compared to healthy specimens. The combination of F(ab′)$_2$anti-C3 ELISA and sucrose density gradient fractionation allowed us to detect the classes of antibodies and hepatitis B surface antigens in the fractionated CICs from sera of patients with liver diseases. Properties of the fractionized CICs are discussed in the text.

Key words —— immune complex, F(ab′)$_2$anti-C3 ELISA, sucrose density gradient fractionation, hepatitis B surface antigen, immunoglobulin

INTRODUCTION

Circulating immune complexes (CICs) are known to be associated with several human diseases including viral diseases,[1] autoimmune diseases,[2–4] malignancies[5] and so on. CICs are basically composed of antigens and antibodies toward the antigens, and the generated complement fragments attach to the complexes if the antigen-antibody complexes activate the complement system. Many detection methods for CICs, each based on different physicochemical, immunochemical and biological reactivities, have been developed.[6] The complement system is activated through the classical, alternative, and lectin pathways and the third component of the complement, C3, plays an important role in all pathways.[7] Antigen-antibody complexes initiate the activation of the classical pathway, resulting in the production of C3 fragment-bearing CICs. The alternative and lectin pathways are antibody-independently activated.

The F(ab′)$_2$anti-C3 radioimmunoassay was established by Pereira *et al.* in 1980.[8] Since then, enzyme-labeled antibodies have been used as a detecting reagent instead of radioisotope-labeled antibodies.[9–11] Combining the F(ab′)$_2$anti-C3 enzyme-linked immunosorbent assay (ELISA) with polyethylene glycol precipitation, they reported improved assay sensitivity.[12,13] F(ab′)$_2$anti-C3 assays are the most frequently used antigen non-specific quantitative type.[14] The enzyme-labeled anti-IgG is most frequently used in F(ab′)$_2$anti-C3 assays, detecting CICs which are dubbed IgG-CIC in this study. Using an enzyme-labeled anti-IgA, anti-IgM and anti-hepatitis B surface antigen (HBsAg) instead of the enzyme-labeled anti-IgG can detect so-called IgA-, IgM- and HBsAg-CIC, respectively. However, we must be aware of the fact that IgG, IgA, IgM and HBsAg in CICs are also detected when we use the F(ab′)$_2$anti-C3 ELISA format (Fig. 1).

It is important that CICs' composition and their sizes are analyzed, since their functions are intimately associated with the composition (complements, antigens, and classes, subclasses and types of the immunoglobulins); and their sizes.[15,16] For example, CICs of intermediate size are prone to cause such tissue injuries as glomerulonephritis.[17] It is suggested that IgA-CICs are related to IgA nephropathy.[10]

The combination of F(ab′)$_2$anti-C3 ELISA and sucrose density gradient fractionation allowed us to detect the classes of antibodies and the HBsAg in the fractionated CICs from sera of patients with liver diseases.

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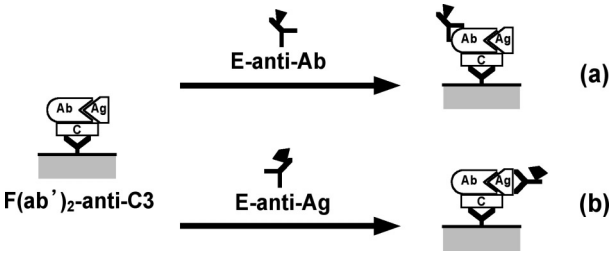

Fig. 1. Concept of the Detection of IgG-, IgA-, IgM-CIC (a) and HBsAg-CIC (b) in the F(ab′)$_2$anti-C3-Coated Multi-Well-Microplate Format
Ab, antibody; Ag, antigen; C, complement; E, enzyme.

MATERIALS AND METHODS

Materials —— The following materials were obtained from the sources as indicated: flat-bottomed multi-well plates (Immulon I) from Dynatech Laboratories, Inc., U.S.A.; goat F(ab′)$_2$anti-C3, Cappel, Organon Teknika Corp., U.S.A.; alkaline phosphatase (ALP)-labeled goat anti-human IgG (ALP-anti-IgG), ALP-anti-IgA, and ALP-anti-IgM from E-Y Laboratories, Inc., U.S.A.; human γ-globulin (Cohn fraction II) and bovine serum albumin (BSA) from Sigma Chemical Co., U.S.A.; horse radish peroxidase (HRP)-labeled monoclonal anti-HBsAg from Dynabot, Japan; and Sephacryl S300 from Pharmacia, Sweden. All other chemicals were of reagent grade or better. The water used was 17-Mohm grade.

Serum samples were obtained from healthy subjects (volunteers) and patients with liver cirrhosis (LC), primary biliary cirrhosis (PBC), hepatitis B type (BCH) and hepatitis not including BCH (nonBCH). The patients were classified according to the clinical diagnosis at Asahikawa Medical College Hospital.

Preparation of Standards for IgG-, IgA-, and IgM-CIC —— Aggregated human γ-globulin (AHG), C3-conjugated IgA (IgA-C3), and IgM-C3 were used as standards for IgG-, IgA-, and IgM-CIC measurements, respectively.

Human γ-globulin in Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} [phosphate buffered saline (PBS)] (20 mg ml^{-1}) was centrifuged at 30000 × *g*, 4°C for 30 min and the supernatant was heated at 63°C for 15 min. After the heated solution was left to stand at 30°C for 1 hr and then at 6°C overnight, the solution was centrifuged under the above conditions. The supernatant was applied to a Sephacryl S-300 column (equilibrated with PBS) and the peak fraction eluted at the void volume was used as the AHG. Fresh human serum was added to the AHG solution (1 : 1), the mixture was incubated at 37°C for 30 min and the aliquots were stored at –70°C. EDTA-serum (20 mmol l^{-1} EDTA:fresh normal human serum = 1 : 1) was added to the AHG solution before use and the mixture was used as a standard. We used EDTA-serum instead of heated normal human sera (56°C, 30 min), since the latter interfered with the F(ab′)$_2$anti-C3 ELISA.[18]

IgA- and IgM-C3 were prepared using the method previously reported by Hosoda *et al*.[19] Briefly, human complement C3 was purified by the method of Hammer *et al*.[20] and mercaptosuccinyl C3 was prepared by the method of Fujiwara *et al*.[21] Human serum IgA or IgM reacted with *N*-(*m*-maleimidobenzoyloxy)succinimide (MBS), forming MBS-acylated IgA or IgM. MBS-acylated IgA or IgM was mixed with mercaptosuccinyl C3. The mixture was fractionated with Sephacryl S-300 and the first peak was used as the IgA- or IgM-C3. EDTA-serum was added to the IgA- or IgM-C3 solution before use and the mixture was used as a standard.

F(ab′)$_2$anti-C3 ELISA —— Multi-well plates (96 wells) were coated with F(ab′)$_2$anti-C3 by adding 100 μl of 280 μg ml^{-1} F(ab′)$_2$anti-C3 in PBS and 0.02% NaN_3 (PBS-N) to each well, then incubating overnight at 6°C. After the wells were washed 5 times with PBS-N, 400 μl of 1% BSA in PBS-N was added to each well. The plates were incubated for 2 hr at 30°C and then washed 3 times with PBS-N. One hundred microliters of test sera dilutions or standards were added to each well. The plates were incubated for 2 hr at 30°C and washed 5 times with PBS-N. The next procedure was based on the idea that we can detect antibodies or antigens in CICs (Fig. 1). First of all, for the detection of IgG-, IgA- or IgM-CIC, 100 μl of ALP-anti-IgG, ALP-anti-IgA or ALP-anti-IgM was added to each well. After incubating overnight at 30°C, the plates were washed 5 times with PBS-N. One hundred microliters of 1 mol ml^{-1} *p*-nitrophenyl phosphate in diethanolamine buffer (pH 9.8, 0.5 mmol ml^{-1} $MgCl_2$) was added to each well and the plates were incubated for a fixed time at 30°C. The reaction was stopped by the addition of 25 μl of 3 mol ml^{-1} NaOH. Absorbance at 405 nm was then measured in a microplate reader (Maltiskan MC, Flow Labs., U.S.A.). Secondly, to detect HBsAg-CIC, 100 μl of HRP-anti-HBsAg was added to each well. After incubating overnight at 30°C, the plates were washed 5 times with PBS. One hundred microliters of 400 μg ml^{-1} *o*-phenylenediamine in a citrate buffer (pH 5.2, 0.01% H_2O_2) was added to

Table 1. The Level and Positive Percentage of IgG-, IgA- and IgM-CICs in the Sera of Patients with Various Liver Diseases

	n	IgG-CIC			IgA-CIC		
		Range	Positive		Range	Positive	
		/μgAHG ml^{-1}	n	Percentage/%	/μgIgA-C3 ml^{-1}	n	Percentage/%
Normal	67	$<$ 1.0–17.5	4	6.0	$<$ 0.70–19.0	3	4.5
LC	34	$<$ 1.0–240	23	67.6*	$<$ 0.70–112	20	58.8*
PBC	13	24.0–660	13	100*	9.9–77.0	10	76.9*
BCH	45	1.1–255	22	48.9*	0.70–54.0	18	40.0*
nonBCH	100	1.0–710	73	73.0*	$<$ 0.70–91.0	41	41.0*

	n	IgM-CIC		
		Range	Positive	
		/μgIgM-C3 ml^{-1}	n	Percentage/%
Normal	67	$<$ 0.88–9.5	3	4.5
LC	34	$<$ 0.88–16.3	7	20.6**
PBC	13	2.1–11.0	7	53.8*
BCH	45	$<$ 0.88–18.2	8	17.8**
nonBCH	100	$<$ 0.88–13.2	26	26.0*

*$p < 0.01$, **$p < 0.05$.

each well and the plates were incubated for a fixed time at 30°C. The reaction was stopped by the addition of 100 μl of 2.5 mol ml^{-1} H_2SO_4. Absorbance at 492 nm was then measured in the microplate reader.

Sucrose Density Gradient Fractionation —— The size of CICs in the sera was generally determined by sucrose density gradient (SDG) ultracentrifugation. One hundred μl of serum was centrifuged at 190000 $\times$ *g* for 15 hr in an RPS 50-2 rotor (Hitachi Koki Co., Ltd., Japan) through 5 ml of 5–50% linear sucrose gradient in PBS. Fractions of 3 drops were collected from the bottom of each centrifuge tube and each fraction was analyzed for the antibodies and antigens in the CICs by the F(ab′)$_2$anti-C3 ELISA. Radioiodinated BSA, human IgG, catalase, and human IgM were used as 4.5, 7, 11 and 19S markers, respectively. These proteins were labeled with ^{125}I using Hunter's iodination method.[22]

Statistics —— Statistical significance for the positive percentage was examined by Fisher's exact probability test.

RESULTS AND DISCUSSION

It is well known that the F(ab′)$_2$anti-C3 ELISA can measure the level of C3 fragment-bearing CIC [Fig. 1(a)]. Radioisotope- or enzyme-labeled anti-IgG are generally used as the detecting reagent in the final step of the method, since CICs consist of antibodies, antigens and complement components. Enzyme-labeled anti-IgA and IgM in addition to the enzyme-labeled anti-IgG were used in this study because the serum levels of IgA and IgM are also high besides IgG.

CICs in 67 sera from healthy volunteers were analyzed using F(ab′)$_2$anti-C3 ELISA (Table 1). The levels of IgG-, IgA- and IgM-CIC were expressed as microgram equivalents of AHG, IgA-C3 and IgM-C3. The reference value was defined as mean + 2[standard deviation (S.D.)], and the values of IgG-, IgA- and IgM-CIC were 13.5 μg AHG ml^{-1}, 14.6 μg IgA-C3 ml^{-1} and 6.2 μg IgM-C3 ml^{-1}, respectively. The samples with the levels lower than the limit of detection were excluded for determining the reference value.

The ranges and positive percentages for IgG-, IgA- and IgM-CIC are listed in Table 1. Positive percentages for IgG-, IgA- and IgM-CIC were significantly increased in LC, PBC, BCH and nonBCH as compared to healthy specimens ($p < 0.01$ or 0.05). Positive percentages for IgG-, IgA- and IgM-CIC decreased in the order, IgG-, IgA-, IgM-CIC, in LC, PBC, BCH and nonBCH. Significant correlations were not found by regression analysis between IgG- and IgA-CIC, IgG- and IgM-CIC, or IgA- and IgM-CIC in LC, PBC, BCH and nonBCH (data not shown).

Using the enzyme-labeled anti-HBsAg instead of the enzyme-labeled anti-Igs enabled detection of HBsAg-CIC [Fig. 1(b)]. HBsAg-CIC in the sera derived from 43 healthy volunteers, 33 BCH patients and 12 LC patients which were positive for hepatitis B [LC(B)] were analyzed using F(ab′)$_2$anti-C3

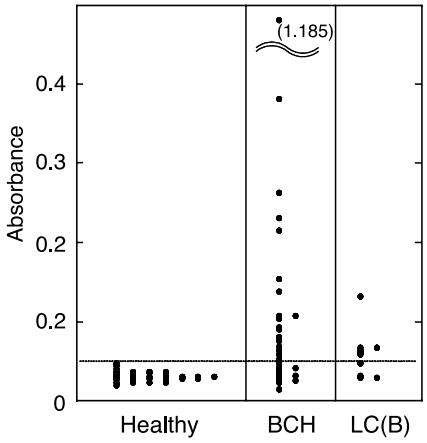

Fig. 2. Detection of HBsA-CIC in the Sera of Healthy Humans and Patients with BCH and LC(B) Using F(ab′)$_2$anti-C3 ELISA

BCH and LC(B) indicate patients with hepatitis B and hepatitis B-positive liver cirrhosis, respectively.

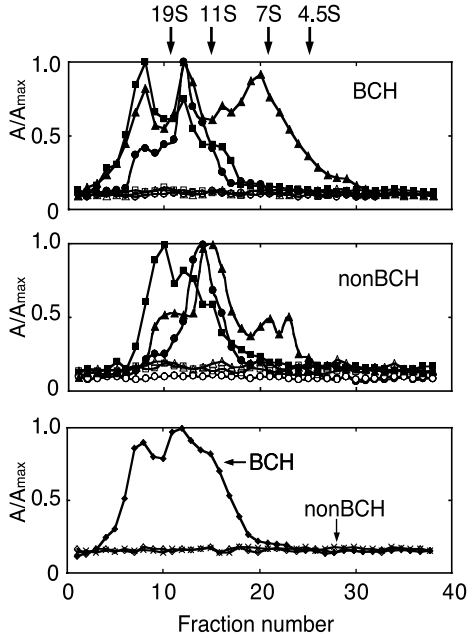

Fig. 3. Detection of IgG-, IgA-, IgM- and HBsAg-CIC in the CICs Fractionated with SDG Ultracentrifugation Using F(ab′)$_2$anti-C3 ELISA

A indicates the absorbance at 492 and 405 nm for IgG-, IgA- and IgM-CIC, and HBsAg-CIC, respectively. A_{max} indicates the maximum absorbance. Patient serum: ▲, IgG-CIC; ●, IgA-CIC; ■, IgM-CIC; ◆, ×, HBsAg-CIC; healthy serum: △, IgG-CIC; ○, IgA-CIC; □, IgM-CIC; ◇, HBsAg-CIC.

ELISA (Fig. 2). The level of HBsAg-CIC was expressed as the absorbance at 492 nm in this study. The maximum value of HBsAg-CIC for healthy specimens was 0.047, thus, the value was accepted as a reference value. Positive percentages for HBsAg-CIC in BCH and LC(B) were 51.5 and 33.3%, respectively, and the percentages were significantly increased as compared to those for healthy specimens ($p < 0.01$). The results indicated that HBsAg-CIC was frequently detected in BCH and LC(B).

A typical example of SDG fractionation analysis is shown in Fig. 3. The technique is important to estimate the size of CICs. The classes of antibody and antigens in the SDG-fractionated CICs were detected with F(ab′)$_2$anti-C3 ELISA. IgG-, IgA-, IgM- and HBsAg-CICs were not found in the fractionated samples from healthy sera. IgG-CICs distributed from about 7S to more than 19S for BCH and nonBCH. IgA-CICs distributed from 11S to more than 19S for BCH and from 11S to 19S for nonBCH. Almost all IgM-CICs biphasicaly distributed around 19S for BCH and nonBCH. HBsAg-CICs were not found in the nonBCH samples, but distributed from about 11S to more than 19S for BCH. These results indicated that the antigen was bound to the antibodies in the CICs. The structure of the hepatitis B virus is spherical and/or filamentous and the envelope carries HBsAg. HBsAg contains a group determinant (a) and subtype determinants (d, y, w and r). Since the HRP-labeled monoclonal anti-HBsAg used in the study recognizes the group determinant a, four serotypes (adr, adw, ayr and ayw) can be detected by the labeled antibody. The envelope contains three proteins that are designated small, middle and large.[23,24] The small protein exists in a glycosylated (GP27) form and a non-glycosylated (P24) form. The middle protein is a glycoprotein in two forms called as GP33 and GP36. The large protein is present in two forms, glycosylated (GP42) and non-glycosylated (P39). HBsAg is found on all three of the proteins. It appeared that these proteins and/or glycosylated proteins made the complexes with various classes of antibody in different ratios. It was noted that the molecular mass of human serum IgG, IgA and IgM are 150, 150–600 and 900 kD, respectively.[25]

Figure 3 shows that some of the IgG-CICs distributed from 7 to 8S, indicating that they might be C3 fragment-bearing IgG molecules, but not complexes. It is known that the complement system in fresh serum dissolves immune complexes, resulting in the release of many IgG molecules, which bind C3 fragments, from CICs.[26,27] In addition, HBsAg was not found in IgG-CICs distributed around 7S, indicating that the IgG-CIC in about the 7S position

could be monomeric IgG with C3 fragments apart from immune complexes. Pereira *et al*.[8] made ^{125}I-BSA-^{131}I-anti-BSA complexes and subsequently dissolved the complexes with fresh normal human serum following SDG fractionation analysis. Some of the dissolved antibodies appeared at around the 7S position, whereas a negligible amount of antigen was found in the same position.

The combination of F(ab′)$_2$anti-C3 ELISA and SDG fractionation allowed the detection of antibody classes and HBsAg in the fractionized CICs from the sera of patients with liver diseases.

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