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ELECTRON MICROSCOPIC OBSERVATIONS ON HEPATITIS B ANTIGEN-ASSOCIATED PARTICLES IN THE SERA OF PATIENTS WITH VARIOUS LIVER DISEASES

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Abstract: To analyze the appearance of three forms of hepatitis B antigen-associated particles (HB Ag particles) and antigen-antibody (Ag-Ab) complexes in the sera of patients with various liver diseases, electron microscopic observations with the combinations of a variety of immunological assays were made at first on the HB Ag and Ab mixed *in vitro* in various ratios, and then on the samples from the sera of each patient. The number of patients observed were 64 in total, which consisted of various types of hepatitis, Hodgkin's disease, Down's syndrome and an asymptomatic carrier. For the detection of HB Ag-Ab complexes a modified method of ALMEIDA was used, and for the isolation of large HB Ag particles (Dane particles) DANE's method was employed. Electron microscopy proved to be a useful method for detecting HB Ag and the Ag-Ab complexes when the ratio of HB Ag to Ab was in the equivalence. Large aggregates of Ag-Ab complexes were frequently observed in the attacks of acute hepatitis and the recrudescences of chronic aggressive hepatitis. The aggregates were also observed in fulminant hepatitis but the ratio of HB Ag to Ab was different from each other among 3 cases examined. The large HB Ag particles were not observed in more than half of the cases in the attacks of acute hepatitis, but appeared in the majority of cases in chronic aggressive hepatitis, even massively during the period with transiently elevated levels of serum glutamic pyruvic transaminase. A few large particles were also found in sera of an asymptomatic carrier, Hodgkin's disease, and Down's syndrome.

Hepatitis B antigen-antibody complexes in sera of patients with hepatitis B were suspected to be a causative factor by electron microscopy (1). Large particles among the three particles already described in association with HB Ag (3, 4) were thought to be complete virus particles (2), while we do not know clearly the role of HB Ag in viral hepatitis. WRIGHT (5) and NIELSEN *et al.* (6) have described about the relationship between three typical forms of HB Ag particles and various liver diseases. We also reported the appearance of HB Ag-Ab complexes and Dane particles in the course of some cases with chronic aggressive hepatitis (7, 8). In this article we have studied the aggregates and forms of HB Ag particles in sera of patients with various liver

diseases. In order to know pathogenetic role of immune complexes and Dane particles in necrosis of the liver, we have examined the morphological changes of aggregates by a modified method of ALMEIDA *et al.* (1) and the number of large particles by a modified method of DANE *et al.* (2), before and after liver cell damages as indicated by a raised level of serum glutamic transaminase (SGPT) during the course of chronic aggressive hepatitis.

PATIENTS AND METHODS

1. *In vitro experiment*

Sera were obtained from a blood donor with elevated transaminase levels in whom HB Ag had been detected by immuno-electro-osmophoresis (IEP) (9). Whole serum was spun at 2,000 rpm for 10 min and then the supernatant was centrifuged at 23,000 g for one hr. This supernatant (the titer of HB Ag : about 1,000 Okayama units (10) in single radial immuno-diffusion = 52 mg/dl) was diluted up to 1,024-fold with 0.005 M phosphate buffered saline (PBS) at pH 7.2 and used as the serum containing free HB Ag particles. A rabbit anti-HB Ag serum supplied from the Eisai Co., Ltd. was purified to gamma-globulin by saturated ammonium sulphate. An optimum ratio of HB Ag to HB Ab (gamma-globulin) was one with IEP. Purified antiserum was also diluted with PBS.

In antigen excess zone, one ml of serum containing free HB Ag particles was mixed with 0.1 ml of various dilutions of purified antiserum. In antibody excess zone, one ml of various dilutions of serum containing free HB Ag particles was mixed with 0.1 ml of purified antiserum. The mixtures were kept for one hr at 37°C, then overnight at 4°C. HB Ag in them was detected with IEP and single radial immuno-diffusion (SRID). The HB Ag-Ab complexes were subsequently centrifuged at 23,000 g for one hr. The pellets were washed in PBS by centrifuging at 23,000 g, negatively stained with phosphotungstate, and then observed with an electron microscope.

2. *Patients*

Blood samples from 64 subjects were studied. The subjects consisted of 12 patients with acute hepatitis, 6 fulminant hepatitis, 3 subacute hepatitis, 38 chronic aggressive hepatitis (11), and one each postnecrotic scar (12), Hodgkin's disease (50-year-old female), Down's syndrome (7-year-old female with slightly elevated SGPT levels), chronic hepatitis complicated by malignant lymphoma (61-year-old female) and an asymptomatic carrier. All of the patients with acute hepatitis had clinical and biochemical features compatible with diagnosis. All the patients with fulminant hepatitis, subacute hepatitis, chronic aggressive hepatitis and postnecrotic scar were diagnosed by characteristic clinical and histopathological features. A HB Ag-positive case with Hodgkin's disease had no history of hepatitis, nor abnormal findings in liver function tests and physical examinations. A patient diagnosed as chronic aggressive hepatitis, activity moderate (11) according to histology 6 years ago, was admitted again because of malignant lymphoma. In this case SGPT levels were

still 80-100 Kunkel units (K. u.). An asymptomatic carrier was defined as a subject in whom HB Ag had been present in the serum for periods longer than 18 months without any history of hepatitis nor abnormal liver function tests.

3. *Methods for immunological and electron microscopic observations*

a) Immunological assays

Sera obtained from each patients were stored at −70°C. The presence of HB Ag and HB Ab was determined by IEP (9) or radioimmunoassay (13) and titers were measured by SRID (Okayama units) (10) or radioimmunoassay. Assays of SGPT, and serum gamma-globulin were carried out serially at a few weeks' intervals. The serum concentration of anticomplementary (AC) activity (14) was measured in 12 specimens among 4 cases with chronic aggressive hepatitis.

b) Electron microscopic observations

i. A modified method of ALMEIDA *et al.* (1) for the preparation of HB Ag-Ab complexes in sera.

Whole sera were spun at 2,000 rpm for 10 min and then one ml of the supernatant was spun at 23,000 g for one hr. The pellet was washed twice in PBS at a similar speed and observed electron microscopically for HB Ag-Ab complexes. Free HB Ag particles were mainly present in the supernatant obtained after the centrifugation at 23,000 g for one hr. In order to examine the quantity of free HB Ag, 0.1 ml of a rabbit anti-HB Ag serum (Behringwercke Lab.) was added to the supernatant. The mixture was incubated for one hr at 37°C, kept overnight at 4°C, and spun at 3,000 rpm for 30 min. The pellet was centrifuged with PBS twice as before, and observed with an electron microscope.

ii. A modified method of DANE *et al.* (2) for the preparation of large particles in sera.

Whole sera were centrifuged at 2,000 rpm for 10 min, then 0.5 ml of its supernatant was mixed with 9.5 ml of PBS, and spun at 150,000 g for 5 hr in a Hitachi 'RP50' rotor. The pellet was washed in PBS at a similar speed and observed with an electron microscope. The quantity of DANE particles was classified into 4 groups by the number of DANE particles per grid opening of 400-mesh specimen screen; O: without any particles, I: a few particles in all grid openings until 10 particles per grid opening, II: 10 to about one thousand particles per grid opening, III: above one-thousand particles per grid opening or large aggregates of DANE particles.

The pellets in a few cases were used to determine the sedimentation rate of HB Ag particles in CsCl by DANE's method (2). The pellet was carefully laid onto a CsCl solution at a density of 1.24 g/ml and centrifuged at 96,000 g for 42 hr in a Hitachi 'RPS' rotor. The density of fractions was determined with a Zeiss Abbe refractometer. HB Ag activity of each fraction was examined with SRID. HB Ag particles of them and also HB Ab added to some fractions were observed by electron microscopy.

iii. Preparation of specimens for electron microscopy.

The pellet was suspended in about 0.1 ml of distilled water and a drop of this suspension was placed on a 400-mesh carbon coated collodion grid. After one min, the excess fluid was drawn off by touching the edge of the grid with a piece of a filter paper. A drop of 4% phosphotungstate (PTA) at pH 6.5 diluted with same volume of distilled water was added to the grid. After 15 to 20 sec the drop of PTA was removed by a filter paper and the grid was air dried. Specimens were examined with an electron microscope (Hitachi Hu-12), using 75 KV at a magnification of 20,000 to 40,000.

RESULTS

1. *In vitro experiment*

Electron microscopy was more useful than IEP or SRID in the zone of antigen-antibody with the relative equivalence (TABLE 1).

TABLE 1 HB ANTIGEN (Ag) AND HB ANTIBODY (Ab) ARE MIXED IN VARIOUS RATIOS *in vitro*. SENSITIVITY OF IMMUNO-ELECTRO-OSMOPHORESIS (IEP) AND SIGLE RADIAL IMMUNO-DIFFUSION (SRID) IS COMPARED WITH THAT OF ELECTRON MICROSCOPIC METHOD (EM) IN DETECTING HB ANTIGEN AND HB ANTIGEN-ANTIBODY COMPLEXES.

Ratio of Ag/Ab.		IEP	SRID	E M	
Antigen dilution	Antibody dilution	HB-Ag	HB-Ag	Free antigen	Ag-Ab complex
1	8	++	++	+	±
1	4	++	++	+	+
1	2	++	++	+	++
1	1	++	++	+	++
2	1	+	+	+	++
4	1	−	−	−	++
8	1	−	−	−	++
16	1	−	−	−	++
32	1	−	−	−	++
64	1	−	−	−	++
128	1	−	−	−	++
256	1	−	−	−	+*
512	1	−	−	−	+*
1024	1	−	−	−	+*

* shows antibody excess picture.

The morphological changes from antigen excess to antibody excess are shown in Fig. 1. In an extreme antigen excess, a few unattached spherical forms and small aggregates were demonstrated (Figs. 1-1A, 1B). When the ratio of antigen to antibody was equivalent, large complexes composed mainly of 20 nm particles were observed (Fig. 1-2). In antibody excess zone, the complexes became smaller, the distance between HB Ag particles became

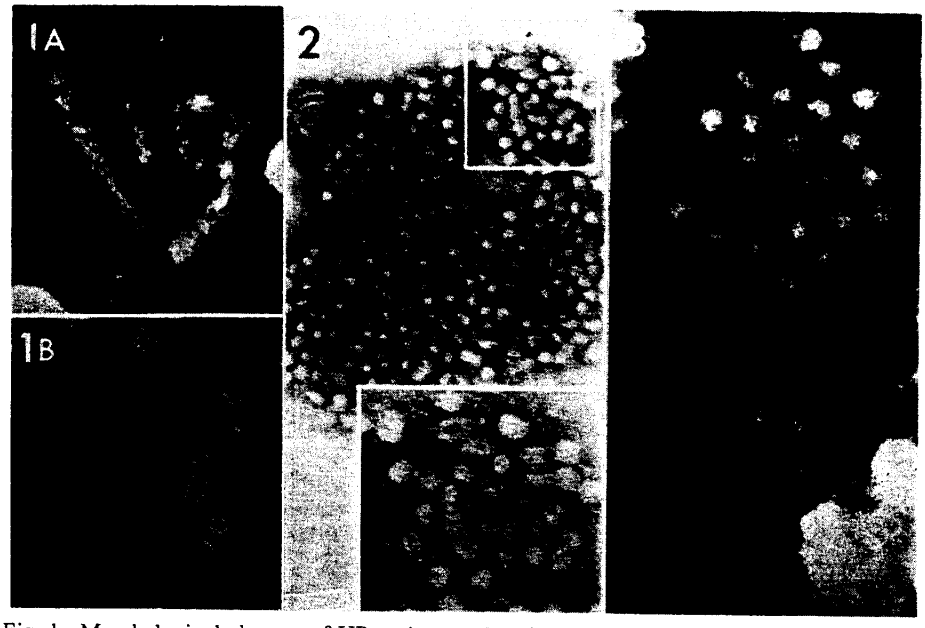

Fig. 1. Morphological changes of HB antigen and antibody complexes mixed in various ratios *in vitro*.

1. Extreme antigen excess
   A. A small antigen-antibody complex. Such is also observed in serum alone without addition of antiserum. (×128,000)
   B. A few unattached spherical forms and small aggregates of only spherical forms. (×128,000)
2. The ratio of antigen to antibody in the equivalence. A large aggregate is composed mainly of small particles. (×64,000, ×128,000)
3. Antibody excess
   The distance between HB antigen particles is wider. Particles can be seen linked with each other by many elongated rods. (×128,000)

wider, and the particles were seen linked with each other by many elongated rods, which were presumed to be antibodies (Fig. 1-3).

2. *Fulminant hepatitis and subacute hepatitis*

The specimens in fulminant hepatitis were obtained from the patients in mental stage 3 or 4 (15), *i. e.* semicoma or coma stage before exchanging blood. In 3 of 7 cases HB Ag was positive with IEP or RIA. HB Ag was strongly positive with IEP in Case 1, positive only with RIA in Case 2 and weakly positive with IEP in Case 3. Electron microscopically, HB Ag particles were observed in these 3 cases, but HB Ag structures were clearly different from each other among 3 cases. Aggregates in the pellets and free HB Ag particles in the supernatant were examined by a modified method of ALMEIDA. In the pellets small aggregates composed of three different forms

were observed in Case 1, small aggregates mainly of tubular forms in Case 2 and large aggregates of only small particles in Case 3 (Figs. 2-1A, 2A, 3A). The quantity of free HB Ag particles in these sera was estimated according

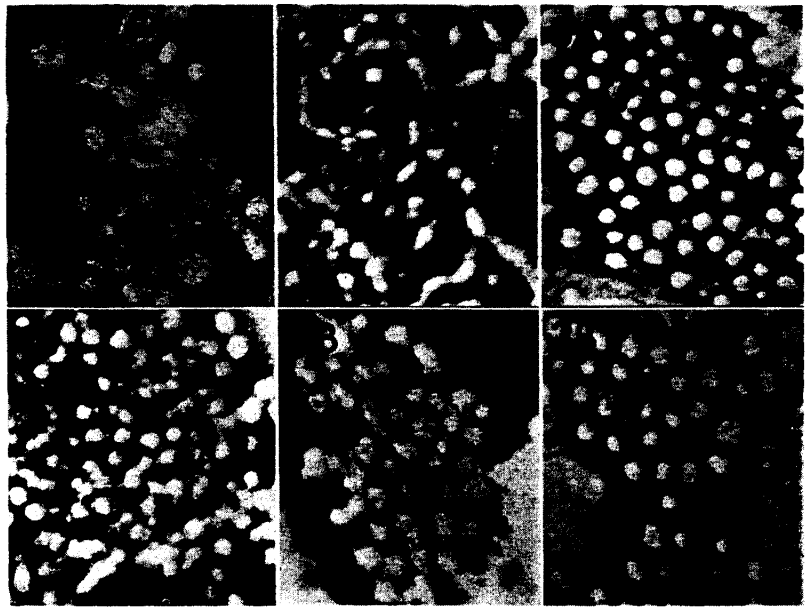

Fig. 2. HB Ag particles in sera of patients with fulminant hepatitis by a modified method of ALMEIDA. (A: in the pellets, B: in the supernatants by addition of antiserum) (×90,000)

1. A 68 year-old-male, HB Ag strongly positive with IEP.
   A. Small aggregates of three different forms.
   B. A part of large aggregate similar to aggregates in the ratio of HB Ag-Ab antibody in the equivalence as shown in Fig. 1-2.
2. A 53 year-old-male, HB Ag negative with IEP.
   A. A small aggregate of tubular forms.
   B. A small aggregate similar to antibody excess picture as shown in Fig. 1-3.
3. A 40 year-old-male, HB Ag weakly positive with IEP.
   A. A part of large aggregate composed of small particles.
   B. A small aggregate similar to antibody excess picture as shown in Fig. 1-3.

to the shape of the complexes formed by addition of antiserum to the supernatant. In Case 1 the existence of many free particles was suggested by the formation of large aggregates, while in two other cases the existence of a few free particles was suspected by the formation of aggregates similar to antibody excess pictures as shown in *in vitro* experiment (Figs. 2-1B, 2B, 3B). Case 1 and Case 2 died but Case 3 is healthy now. In one of three cases with subacute hepatitis, small aggregates composed of small particles and tubular forms were observed.

3. *Acute hepatitis*

All cases developed jaundice with SGPT above 1,000 K. u. All specimens were obtained during the period after the peak of raised SGPT levels.

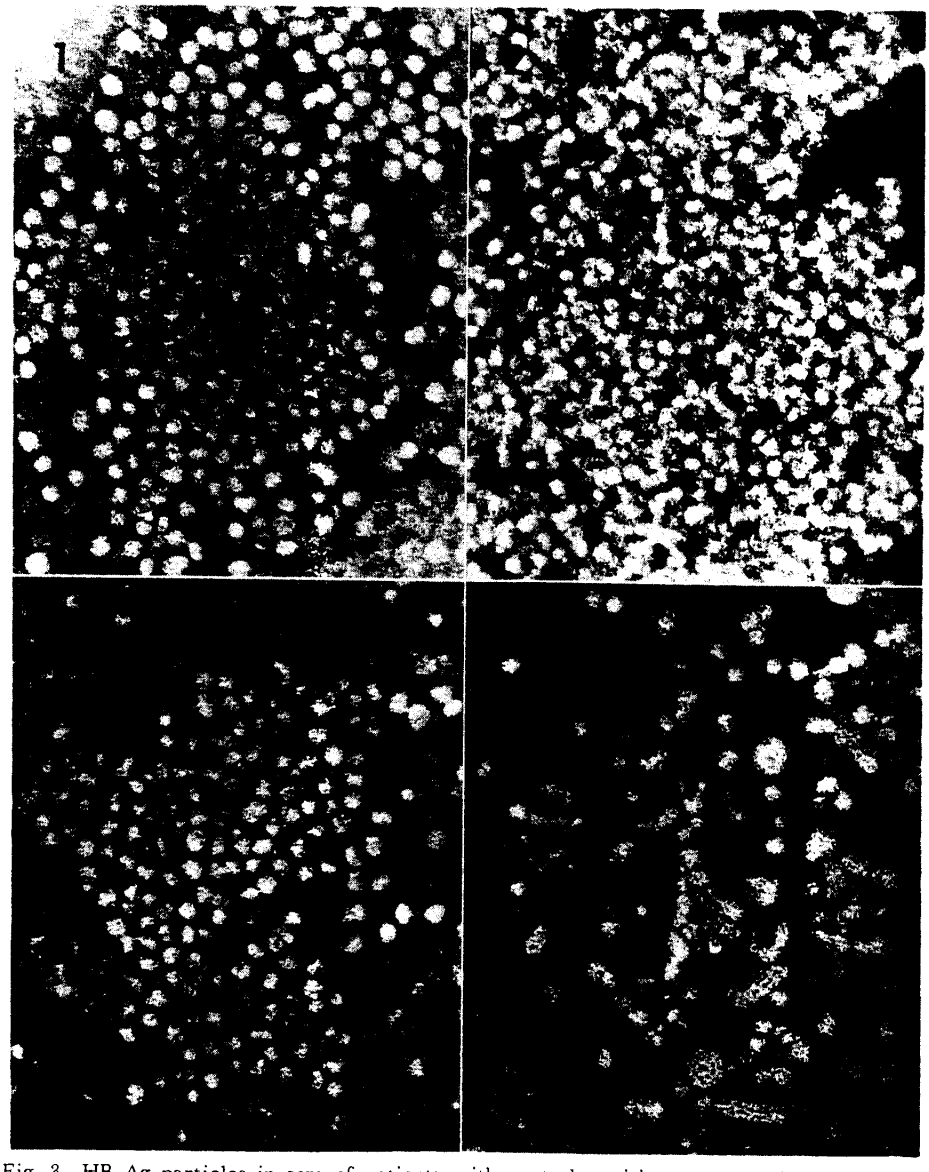

Fig. 3. HB Ag particles in sera of patients with acute hepatitis.
1, 2, 3: Large aggregates of small particles in 3 different cases. 4: In this case large aggregates are not observed. A few Dane particles and tubular forms appeared. (×103, 500)

HB Ag was positive with IEP in 7 cases. In 6 of them HB Ag became negative with IEP within one to 6 weeks. HB Ag particles were not found in any of 5 patients consistently negative for HB Ag with IEP, but large aggregates of small particles were observed in 6 of 7 HB Ag positive cases (Fig. 3). Large aggregates persisted for several weeks even after HB Ag became negative with IEP. A few Dane particles were observed in 3 cases; two cases recovered favorably but one case had the second attack in 6 months from onset. Dane particles were not found in 4 other HB Ag positive cases with good clinical course.

4. *Chronic aggressive hepatitis*

HB Ag particles were observed in all specimens of 22 HB Ag positive cases by modified methods of ALMEIDA or DANE, though in 14 cases of them HB Ag was sometimes negative with IEP. In only one of 16 cases with HB Ag consistently negative with IEP, HB Ag particles (small and tubular forms) were found by electron microscopy and also ascertained with RIA.

Modified method of ALMEIDA: Forty-six specimens in 11 HB Ag positive cases were examined by this method. The specimens were serially observed before and after a transient elevation of SGPT levels above 500 K.u. in 6 cases. In 4 of them the large aggregates similar to HB Ag-Ab complexes as shown in *in vitro* experiment appeared transiently around the peak of SGPT. In accordance with the appearance of large aggregates HB Ag often became transiently negative with IEP, quantitatively also decreased using SRID, and serum gamma-globulin was increased. However, in one case no large aggregates were observed, and in another case these aggregates were always observable. These large aggregates were mainly composed of small particles but in a few cases many large particles and tubular forms were observed in aggregates. In 4 cases AC activity was elevated above 16 units when aggregates were observed by electron microscopy, but there was no relationship between the AC activity and the size of aggregates. In two specimens without elevation of AC activity HB Ag particles were not aggregated. Aggregates accompanied by elevated AC activity were sometimes observed in the stable levels of SGPT. In the case examined weekly (Figs. 4 & 5), massive Dane particles were observed for 3 weeks before the peak of raised SGPT levels. At the peak of SGPT Dane particles were hardly found, but large aggregates of small particles appeared, HB Ag became negative with IEP, and the titer of HB Ab increased with RIA at the same time.

Modified method of DANE: Eighty specimens in 27 cases were examined. Dane particles were not found in all of 12 cases of HB Ag always negative with IEP. In HB Ag-positive cases Dane particles were observed in 13 of 15 cases (TABLE 2) and more frequently and numerously in chronic

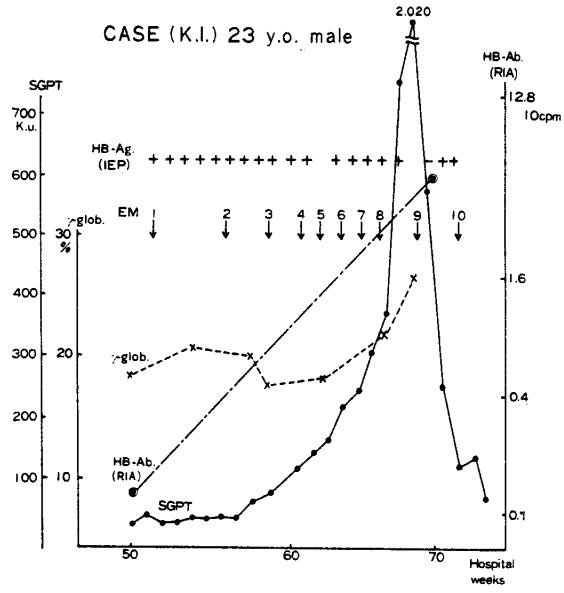

Fig. 4. Hospital course of a 23 year-old-male with chronic aggressive hepatitis.
Free or small aggegates of particles in EM-1～5.
Massive appearance of Dane particles in EM-6～8.
Large aggregates composed mainly of small particles in EM-9. Small aggregates of tubular forms and small particles in EM-10. ("EM-1" denotes first observation electron microscopcially)

TABLE 2 THE APPEARANCE RATE OF DANE PARTICLES IN SERA OF PATIENTS WITH VARIOUS LIVER DISEASES.

	HB antigen (IEP)	The appearance rate of Dane particles
Acute hepatitis	+	3/7
	−	0/5
Fulminant hepatitis	+	1/3
	−	0/4
Subacute hepatitis	+	0/1
	−	0/3
Chronic aggressive hepatitis	+	13/15
	−	0/12
Postnecrotic scar	+	0/1
Asymptomatic carrier	+	1/1
Hodgkin's disease	+	1/1
Down's syndrome	+	1/1
Chronic hepatitis & malignant lymphoma	+	1/1

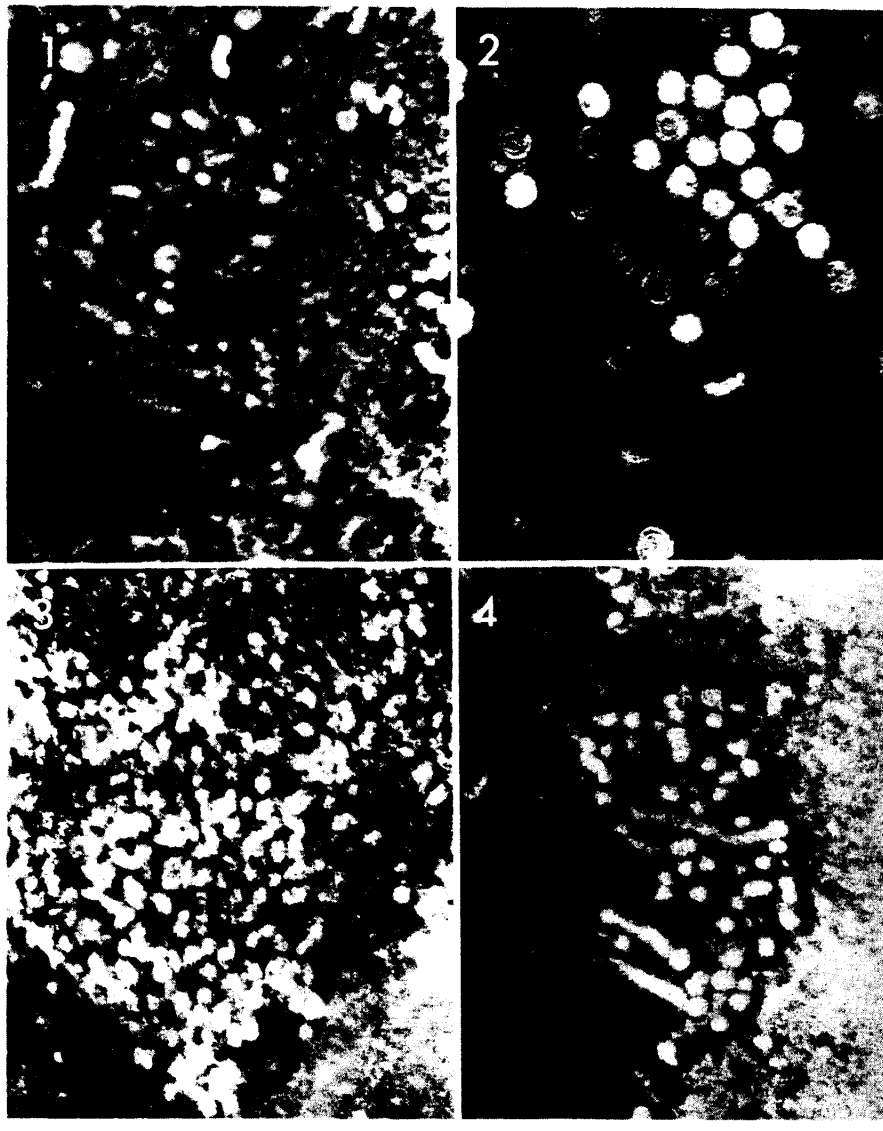

Fig. 5. Serial observations on HB Ag particles in sera of the patient (Fig. 4) by a modified method of ALMEIDA. (×103,500)
1. EM-1, 2. EM-8, 3. EM-9, 4. EM-10

aggressive hepatitis than in other forms of hepatitis cases (TABLE 3). Five cases were examined weekly or monthly for 40 weeks (Fig. 6). In the first case as shown in Fig. 6, a few free or small aggregated HB Ag particles were observed on admission, and the first histological findings revealed chronic

TABLE 3 GRADING OF DANE PARTICLES IN SERA OF HB Ag-POSITIVE (IEP) PATIENTS WITH VARIOUS LIVER DISEASES.

	No. of specimens	Grading of Dane particles			
		0	I	II	III
Acute hepatitis	16	12	3	1	
Fulminant hepatitis	3	2		1	
Subacute hepatitis	3	3			
Chronic aggressive hepatitis	68	12	24	25	7
Postnecrotic scar	1	1			
Asymptomatic carrier	3		3		
Hodgkin's disease	1			1	
Down's syndrome	1			1	
Chronic hepatitis & maligmant lymphoma	1			1	

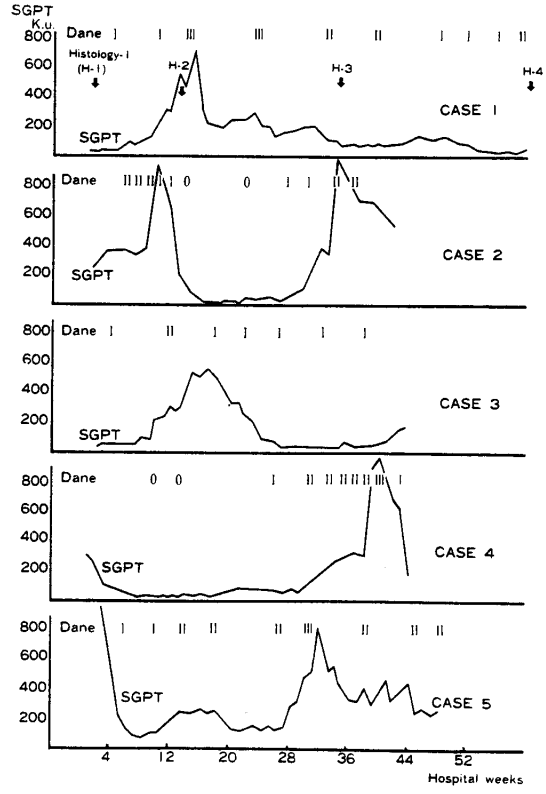

Fig. 6. Serial observations on grading of large particles before and after the recrudescence shown by SGPT levels in 4 cases with chronic aggressive hepatitis. Histology-1 (H-1): chronic aggressive hepatitis, activity moderate. H-2: chronic aggressive hepatitis, activity severe. H-3, H-4: early picture of liver cirrhosis.

aggressive hepatitis, activity moderate (Fig. 7-1). Many Dane particles were observed during three months around the recrudescence which was indicated

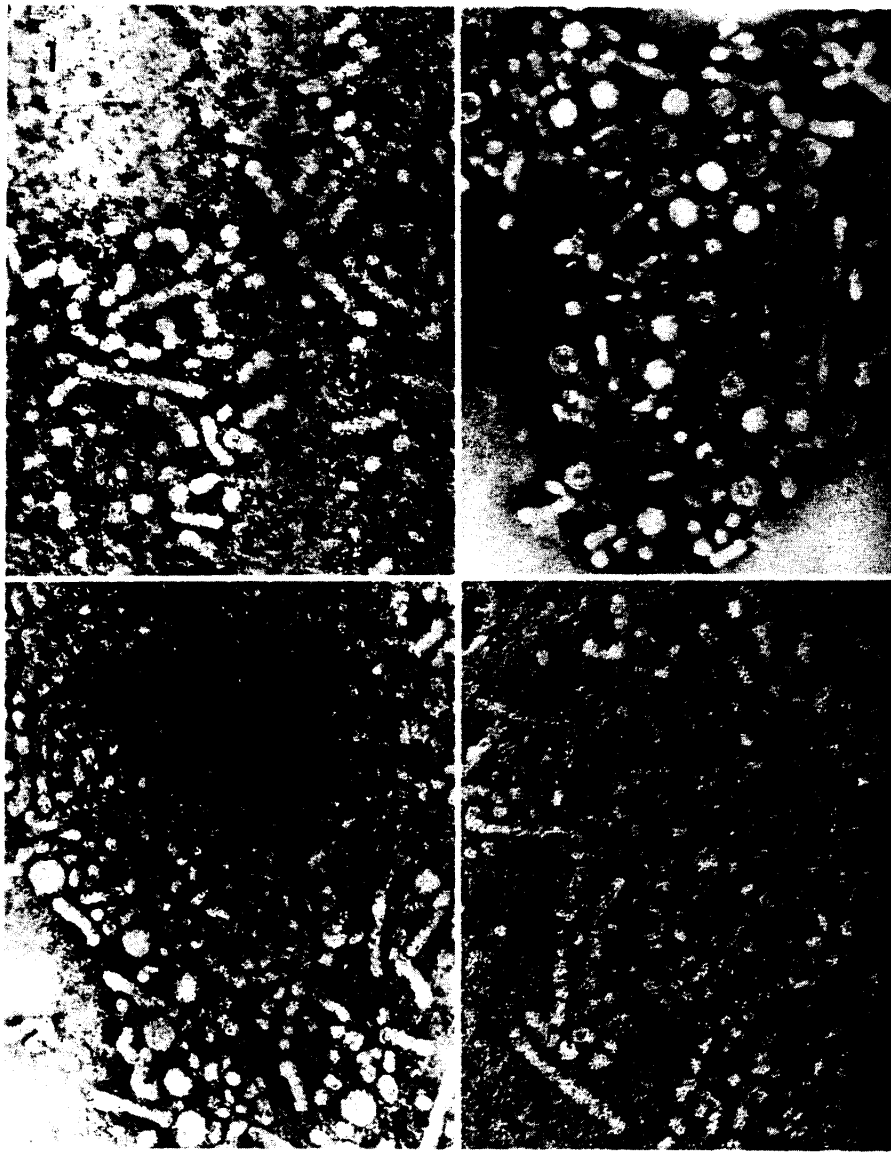

Fig. 7. Large particles in serial observations on sera of Case 1 in Fig. 6 by a modified method of DANE. (×103, 500)
1. Large particles in grade I at 3rd hospital weeks.
2. Large particles in grade III at 13th hospital weeks.
3. Large particles in grade II at 33rd hospital weeks.
4. Large particles in grade I at 55th hospital weeks.

by a raised SGPT and chronic aggressive hepatitis, activity severe on the second histological finding (Fig. 7-2). Within half a year SGPT levels had gradually dropped down, although histologically it progressed towards early picture of liver cirrhosis. Dane particles were decreased and large aggregates of small particles and tubular forms were observed (Fig. 7-3). Within 10 months after the recrudescence, SGPT levels were below 100 K. u. and a few Dane particles were still observed in small aggregates consisting of small particles and tubular forms (Fig. 7-4). Three different forms counted serially in the 4th case of Fig. 6 are shown in TABLE 4. Dane particles and tubular forms increased in parallel with elevation of SGPT levels, but decreased suddenly after the peak of a raised SGPT. In other 3 cases aggregates of

TABLE 4 DISTRIBUTION OF 3 DIFFERENT FORMS OF A THOUSAND HB Ag PARTICLES BY A MODIFIED METHOD OF DANE SERIALLY IN SERA OF CASE 4 OF Fig. 6.

Hospital weeks	SGPT (K.u.)	Grading of Dane particles	Forms of particles		
			small	large	tubular
8	39	0	998	0	2
12	33	0	988	0	12
26	78	I	991	2	7
30	92	II	973	4	23
34	260	II	939	14	47
35	260	II	858	23	119
38	310	II	721	40	239
41	1,090	III	791	131	78
44	650	I	975	1	24

Dane particles also appeared in small and tubular forms at the time of recrudescence. The relationships of Dane particles to SGPT levels in 68 specimens of 5 cases as shown in Fig. 6 and 10 cases who were examined once are shown in TABLE 5. Dane particles were increased in the period of elevated SGPT levels ; especially in the ascending stage of the recrudescence.

TABLE 5 LARGE PARTICLES AND SGPT LEVELS IN 68 SPECIMENS OF FIFTEEN HB Ag-POSITIVE CASES WITH CHRONIC AGGRESSIVE HEPATITIS.

SGPT K.u. / Dane particle	≦ 100	101 - 200	201 - 500			500 <
			ascending period	descending period	continuously	
0	8	1		1		2
I	12	5		3	1	3
II	3	4	9		4	4
III			1		2	4

In a case where these large particles were examined by DANE's method, each fraction of CsCl was observed by electron microscopy. One thousand particles were counted in each fraction (TABLE 6). The majority of small

TABLE 6 HB Ag WITH SRID AND FORMS OF A THOUSAND HB Ag PARTICLES BY ELECTRON MICROSCOY (EM) IN EACH DENSITY FRACTIONS OF CsCl ARE EXAMINED IN A CASE WITH CHRONIC AGGRESSIVE HEPATITIS.

Fraction No.	SRID HB Ag	E M		
		small	large	Tubular
37	−	++	−	+
35 (1.204)	±	987	2	11
33	+	978	1	21
31 (1.222)	++	905	8	87
29	+	886	5	109
27 (1.240)	−	857	41	107
25	−	+	++	+

particles and Dane particles appeared in 1.204 g/ml and 1.240 g/ml, respectively. Dane particles were aggregated to small particles when a purified antiserum was added to the fraction (Fig. 8-3). Internal structures were seen in about half of large particles. Five or six-sided symmetrical inner bodies were observed in some particles (Fig. 8-1). Core-like components were also suggested in some inner bodies.

5. *Carriers and others*

In one case with postnecrotic scar, aggregates of small particles and tubular forms were found without Dane particles.

An asymptomatic carrier and one case each of Hodgkin's disease and Down's syndrome had no aggregates similar to HB Ag-Ab complexes as observed in *in vitro* experiment. The quantity of Dane particles was classified to the first grading in an asymptomatic carrier and the second grading in one case each of Hodgkin's disease and Down's syndrome. By counting 3 different forms, Dane particles were observed remarkably more in the case with chronic aggressive hepatitis complicated by malignant lymphoma than in 3 other cases, but quantitatively the case with chronic aggressive hepatitis complicated by malignant lymphoma contained the second grading of Dane particles because all particles were not so many in total (TABLE 7, Fig. 9).

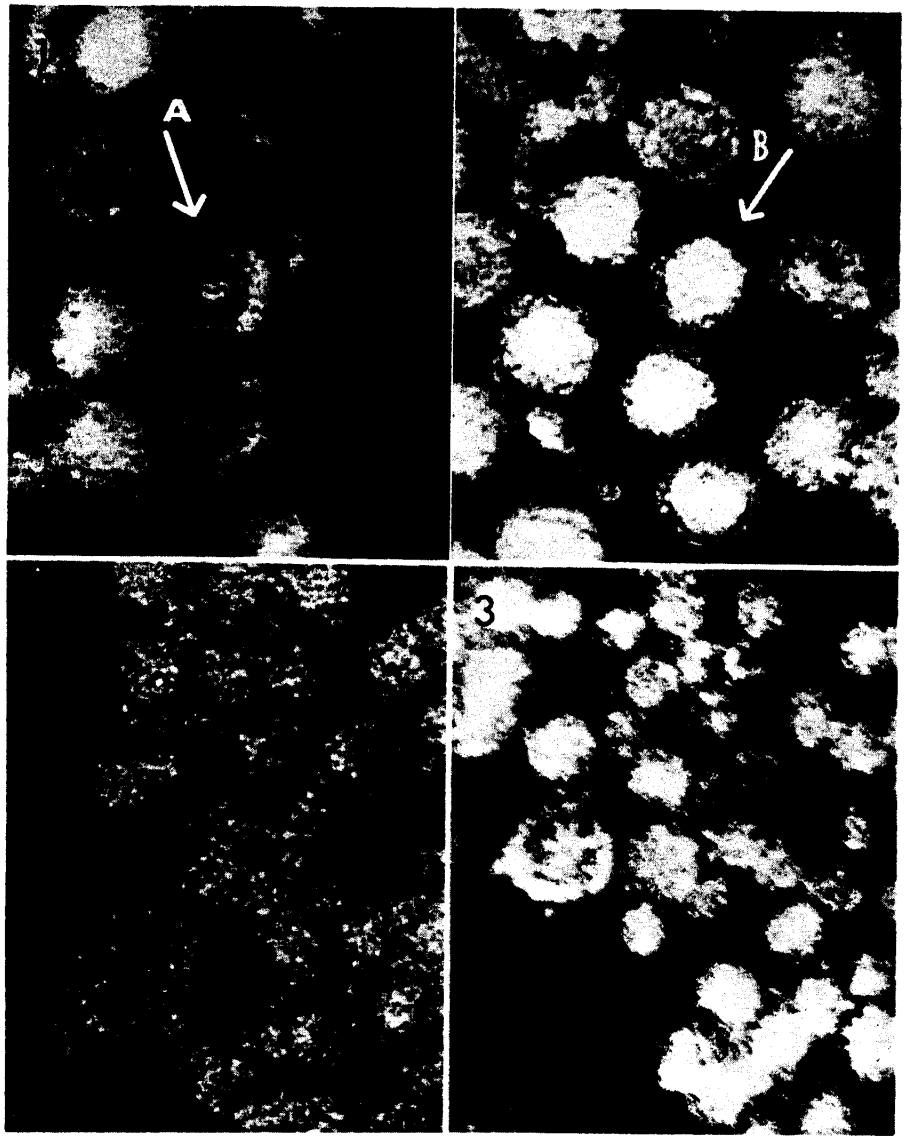

Fig. 8-1. Dane particles in serum of a patient with chronic aggressive hepatitis by a modified method of ALMEIDA. Arrow A shows a Dane particle containing a six-sided inner body. Arrow B shows a Dane particle containing a five-sided inner body. (×405,000)

2. By addition of antiserum a Dane particle is aggregated with small particles in the supernatant obtained by a modified method of ALMEIDA. (×405,000)

3. By addition of antiserum a Dane particle aggregated with small particles is observed in a 1.22 g/ml fraction of CsCl. (×405,000)

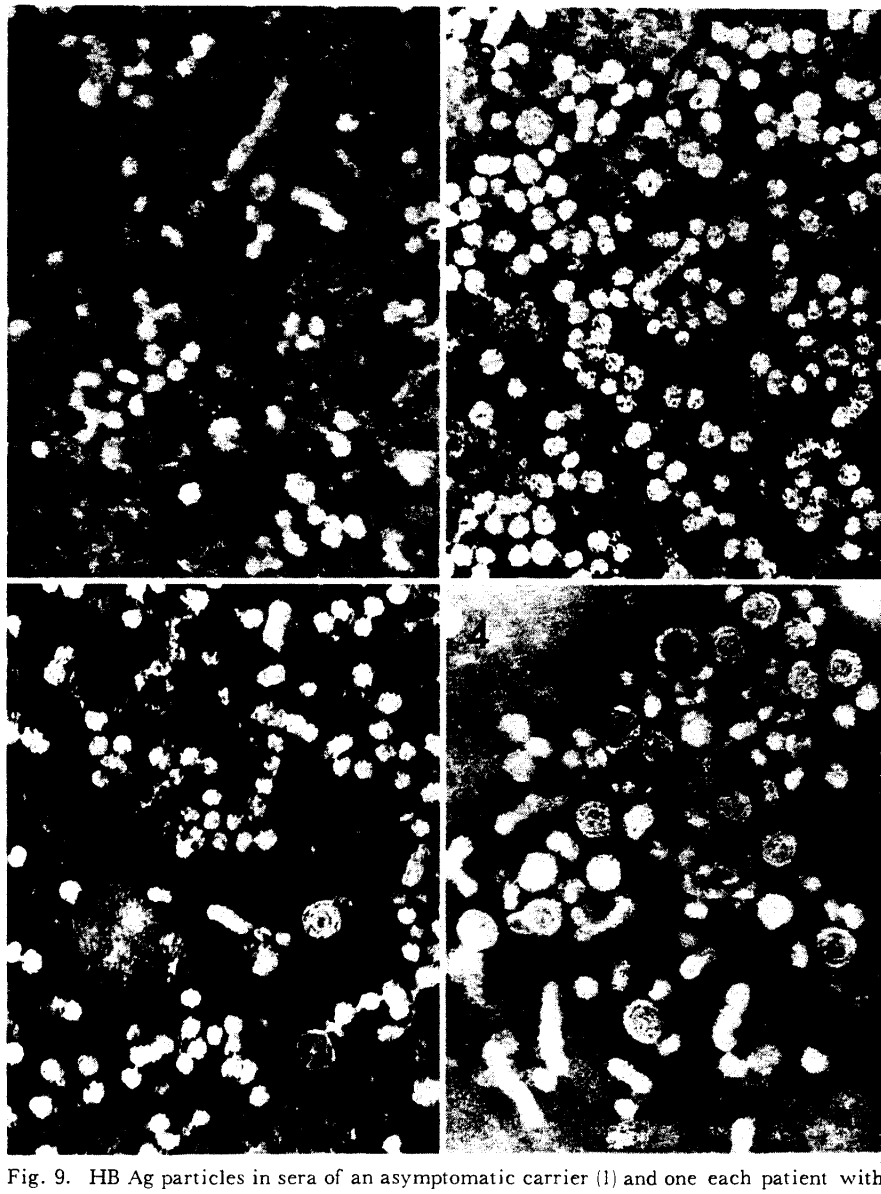

Fig. 9. HB Ag particles in sera of an asymptomatic carrier (1) and one each patient with Down's syndrome (2), Hodgkin's disease (3) and chronic hepatitis complicated by malignant lymphoma (4). In 1, 2 and 3, free or small aggregated particles are observed but aggregates similar to HB antigen-antibody complexes as shown in Fig. 1 are not observed. (×103, 500)

TABLE 7 THE QUANTITY OF DANE PARTICLES AND DISTRIBUTION OF 3 DIFFERENT FORMS OF A THOUSAND HB Ag PARTICLES IN SERA OF AN ASYMPTOMATIC CARRIER AND EACH ONE CASE WITH HODGKIN'S DISEASE, DOWN'S SYNDROME AND CHRONIC AGGRESSIVE HEPATITIS COMPLICATED BY MALIGNANT LYMPHOMA.

Case	Grading of Dane particles	Forms of particles		
		small	large	tubular
Asymptomatic carrier	I	985	3	12
Hodgkin's disease	II	951	1	48
Down's syndrome	II	945	8	47
Chronic hepatitis & malignant lymphoma	II	822	63	115

DISCCUSION

The electron microscopy proves to be excellent in demonstrating HB Ag in sera of patients negative for the antigen with IEP or SRID when the antigen and antibody exist in the equivalence. The antigen-antibody ratio in the patient sera was determined by either electron microscopic observation on changes of shapes of HB Ag-Ab complexes or IEP.

In sera of fulminant hepatitis cases the aggregates were observed without addition of antiserum. In one of 3 cases antigen excess was rather suspected because of electron microscopically small aggregates composed of 3 different forms in the pellet, many free antigens suggested by the formation of large aggregates in the supernatant with addition of antiserum, and HB Ag clearly positive with IEP. This finding indicates that the ratio of HB Ag-Ab in sera of fulminant hepatitis cases is not always antibody excess as previously reported by ALMEIDA *et al.* (1). In one surviving case only small spherical particles were obseved, but it was not clear whether or not the difference in the forms of HB Ag particles among 3 cases had any bearing on prognosis.

Large aggregates similar to HB Ag-Ab complexes as shown in *in vitro* experiment were observed in the course of acute hepatitis and chronic aggressive hepatitis, as previously described (1, 16, 17, 18, 19). When large aggregates appeared around the recrudescence in HB Ag-positive chronic aggressive hepatitis, HB Ag often became transiently negative with IEP, quantitatively decreased with SRID, and serum gamma-globulin increased. Elevation of AC activity and detection of HB Ab with RIA in accordance with the appearance of large aggregates were shown in some cases. These immunological assays also suggested that large aggregates were HB Ag-Ab complexes. But these complexes in sera were not interpreted to be triggers of transient recrudescence, because the serial observations on cases with chronic aggres-

sive hepatitis clarified that the large aggregates often appeared at the peak of SGPT levels or after it rather than before it, and also even in the stable levels of SGPT.

Large particles (Dane particles) have not yet been clarified whether these are complete virus particles or assembly of proteins (21, 22). These large particles may have common antigen to small spherical particles and tubular forms (2), because they are aggregated with small particles and tubular forms on the addition of antiserum, and have never been found in sera of hepatitis patients consistently negative for HB Ag with IEP. Our five or six-sided inner bodies and core-like components in some particles suggested that large particles might be true viruses, as reported by several investigators (4, 23).

Dane particles were observed more numerously in sera of patients with chronic aggressive hepatitis than in those of other diseases. In chronic aggressive hepatitis, the number of Dane particles changed in the disease course; in general, Dane particles were a few in the period of stable SGPT levels but increased around the recrudescence as shown by transiently elevated levels of SGPT. And in some cases these large particles transiently appeared in mass during the period of ascending SGPT levels in the recrudescence. However, we have not yet elucidated whether or not the appearance of these particles in sera is related to liver cell damages. Because in an asymptomatic carrier and a case with Hodgkin's disease a few Dane particles were observed, though both cases were not examined histologically. This observation in carriers differs from the result reported by NIELSEN *et al.* (6); furthermore, no complexes may be related to abnormal immune responses in hosts (24).

In the sera of patients with acute hepatitis, Dane particles were a few or not observed, as previously described (5, 6). And it is difficult to state that these large particles are the indicator of the progression to chronicity (6), because we have not been able to observe sera in acute stages of our cases with chronic aggressive hepatitis and neither have we had the chance to examine sera in the incubation period of acute hepatitis cases.

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