Clin. exp. Immunol. (1981) **43**, 246–253.

Hepatitis B surface antigen circulating immune complexes (HBsAg-CICs) in patients with hepatitis B and asymptomatic HBsAg carriers

N. ANH-TUAN & E. NOVÁK *Hepatitis Research Laboratory, National Institute of Haematology and Blood Transfusion, Budapest, Hungary*

(Accepted for publication 22 August 1980)

SUMMARY

The PEG-trypsinization assay detected HBsAg-CICs in 31 out of 44 (70%) patients with acute hepatitis B, in five out of 107 (5%) asymptomatic HBsAg carriers and, in addition, in both patients with HBsAg-positive chronic liver disease. A close correlation between the levels of HBsAg-CICs and disease activity was observed. The clinical course, parameters of liver function tests and outcome of the disease in patients without HBsAg-CICs (group A) and in patients with transient HBsAg-CICs (group B) were essentially similar. In contrast, patients with persistent HBsAg-CICs (group C) had a poor prognosis, particularly those who received corticosteroids. The method appeared to be a valuable tool in monitoring disease activity and prognosis, and in evaluating the efficacy of corticosteroid treatment. The role of HBsAg-CICs in the pathogenesis of liver damage and clearance of circulating HBsAg is discussed.

INTRODUCTION

Circulating immune complexes (CICs) have been found in patients with hepatitis B, and much less frequently in clinically healthy carriers of HBsAg, by several authors using different methods. The majority of the methods that have been used are antigen-non-specific. In investigations into the causes of immune-complex-mediated diseases, however, the identification of the antigens involved is particularly important. Several attempts have been made in order to develop a suitable antigen-specific method for the detection of HBsAg-CICs (Shulman & Barker, 1969; Almeida & Waterson, 1969; Millman *et al.*, 1970; Bedarida, Zacchi & Tassi, 1971; Brzosko *et al.*, 1971; Madalinski, Sztachelska-Budkowska & Brzosko, 1974; Carella *et al.*, 1977; Santoro *et al.*, 1977; Fresco, 1978). However, none of the recently available methods has been found to be sufficiently sensitive, specific, easily reproducible and simple enough to allow them to be used widely in clinical situations. More recently, Pernice & Sedlacek (1979) have described a new method by which HBsAg can be identified in native soluble immune complexes (ICs). In a previous article, we reported a new assay, termed *polyethylene glycol-trypsinization* (PEG-trypsinization), by which HBsAg can be identified in dissociated ICs produced *in vitro* as well as those in sera from patients (Anh-Tuan & Novák, 1980).

In the present report, we wish firstly to provide a detailed assessment of the contribution of PEG-trypsinization to monitoring disease and evaluating response to corticosteroid therapy, and secondly to contribute to our better understanding of the role of HBsAg-CICs in the pathogenesis of liver injury and clearance of HBsAg from the circulation.

Correspondence: Dr N. Anh-Tuan, Hepatitis Research Laboratory, National Institute of Haematology and Blood Transfusion, Daróczi ú. 24, Budapest XI, Hungary H.1502.

0099-9104/81/0200-0246$02.00

MATERIALS AND METHODS

Patients. Serial blood samples were drawn from 44 HBsAg-positive acute hepatitis (19 men and 25 women, aged 11–84 years, mean 50) and, in addition, from one patient with HBsAg-positive chronic hepatitis (male, 54 years and one with HBsAg positive post-necrotic cirrhosis (female, 65 years). All patients were admitted to the Central Hospital for Infectious Diseases, László, Budapest, between August 1978 and February 1979. SGOT, SGPT activity and serum bilirubin concentration were determined in freshly-drawn sera by the routine methods used in the Department of Clinical Biochemistry at the László Hospital. The patients were followed up 3 to 10 months (mean 5 months) after discharge. At monthly intervals a physical examination was carried out in the out-patient department and the following laboratory tests were performed: SGOT, SGPT, serum bilirubin and thymol turbidity. Altogether 10 patients, including eight acute hepatitis (AH), one chronic hepatitis (CH) and one AH patient with underlying malignant disease (acute myelomonocytic leukaemia), received corticosteroids. Other immunosuppressive drugs were not administered to any of the patients with the exception of the one with leukaemia.

Serial specimens were taken usually within a few days after admission and then at weekly intervals until the patients were discharged or died.

Asymptomatic HBsAg carriers. One hundred and seven clinically healthy adult blood donors positive for HBsAg were examined at the National Institute of Haematology and Blood Transfusion between June 1978 and June 1979. Freshly-drawn sera were examined for SGOT activity by the quick method of Kőszeghy & Novák (1973).

Single blood specimens from 100 HBsAg-negative AH patients and 200 healthy blood donors negative for both HBsAg and anti-HBs seen during the same period served as controls.

Handling of serum samples. Blood samples obtained by venepuncture were allowed to clot at room temperature in glass tubes for about 2 hr and separated by centrifugation at 2,000 r.p.m. for 5 min at room temperature. Sera were aliquoted and tested immediately in reversed passive haemagglutination (RPHA), counter-immunoelectrophoresis (CIEP) and PEG-trypsinization, or after storage at −20°C for a period varying from a few weeks to 9 months in radioimmunoassay (RIA).

Methods for detection and quantitation of HBsAg and anti-HBs. Quantitative determination of HBsAg was carried out by RPHA (Hepanosticon Organon, Oss, The Netherlands) according to the micromethod of Novák & Kőszeghy (1977). The basic dilution was 1:66 and titration was performed by serial two-fold dilutions. To check the results, all samples positive for HBsAg were absorbed with Hepanosticon Absorbent. In addition, CIEP according to Pesendorfer, Krassnitzki & Wewalka (1970) and RIA (AUSRIA-II) were used.

Anti-HBs was determined in freshly separated sera by CIEP and inhibition of RPHA (RPHA-I), and in frozen sera by RIA (AUSAB) according to the instruction booklet of Abbott Laboratories, USA.

PEG-trypsinization assay for detection and measurement of HBsAg-CICs. The details of the method are published elsewhere (Anh-Tuan & Novák, 1980). Briefly, CICs were precipitated with 3·5% PEG, then PEG precipitates were treated with trypsin at 2 mg/ml for 30 min at 37°C; finally, the liberated HBsAg was detected and titrated. The level of HBsAg-CICs was expressed as the titre of HBsAg released from the complexes.

Statistical methods. Results were analysed by Student's *t*-test, the chi-square test and by calculation of the correlation coefficient (*r*).

RESULTS

Prevalence of HBsAg-CICs in different disease groups

HBsAg-CICs were detected in 31 out of 44 (70%) patients with acute hepatitis B, in five out of 107 (5%) asymptomatic HBsAg carriers, and in both patients with HBsAg-positive chronic liver disease, but in none of the 100 HBsAg-negative AH patients and 200 normal blood donors.

In acute hepatitis B, the prevalence of HBsAg-CICs gradually declined during the first 6 weeks after the onset of jaundice (68, 56, 50, 34, 27 and 20% respectively).

HBsAg-CICs, disease activity and outcome

We were able to monitor HBsAg-CICs, HBsAg, anti-HBs and other indices of disease activity in all 46 patients (Tables 1, 2 and 3). On the basis of the results of serial testing for HBsAg-CICs, 44 AH patients were categorized into three groups.

AH patients without detectable HBsAg-CICs (group A, n = 13). HBsAg-CICs could not be detected throughout the period of observation in 13 patients. All had essentially similar clinical features. These were characterized by rapid resolution of clinical symptoms, a sharp fall in the levels of SGOT, SGPT and serum bilirubin (Fig. 1a). Transient rash and arthralgia in the prodromal period were observed in one case. By the time of discharge the patients had essentially recovered after a course of symptomatic and supportive therapy varying from 19 to 54 days with a mean of 35

Table 1. Characteristics of 44 patients with acute hepatitis B, according to HBsAg-CIC status

Group	HBsAg-CICs status	No.	Sex		Age (years)		
			Male	Female	Range	Mean ± 1 s.d.	Difference
A	Negative	13	7	6	22–76	46·7 ± 20	
B	Transiently (+)	24	7	17	11–75	45·0 ± 20	n.s. ($P > 0·8$)
C	Persistently (+)	7	5	2	54–84	69·0 ± 8·3	$P < 0·005$

n.s. = Not significant.

Table 2. Summary of data for SGPT levels and serum bilirubin concentrations on admission in three groups of patients with acute hepatitis B

Group	No.	SGPT (iu/l)			Serum bilirubin (mg/100 ml)		
		Range	Mean ± 1 s.d.	Difference	Range	Mean ± 1 s.d.	Difference
A	13	450–1,620	1,130 ± 425·77		5·3–19·3	11·0 ± 4·3	
B	24	220–2,000	1,057 ± 596·18	n.s. ($P > 0·6$)	3·6–17·2	8·8 ± 3·7	n.s. ($P > 0·1$)
C	7	250–580	402 ± 143·30	$P < 0·001$	3·4–10·0	7·1 ± 2·3	$P < 0·02$

n.s. = Not significant

Table 3. Summary of data for hospital days and outcome of the disease in three groups of patients with acute hepatitis B (mean follow-up of 5 months)

Group	No.	Hospital days			Outcome			No. of cases positive for anti-HBs	No. of cases converted to HBsAg-negative
		Range	Mean ± 1 s.d.	Difference	Recovered	Progressed to CH	Died		
A	13	19–54	35·5 ± 13·0		13	0	0	3	1
B	24	15–78	40·0 ± 15·5	n.s. ($P > 0·3$)	23	0	1*	16	16
C	7	31–72	48·5 ± 14·7	$P < 0·025$	2	1†	4	3	0

* Patient with concurrent leukaemia.

† Died within the follow-up period.

n.s. = Not significant, CH = chronic hepatitis.

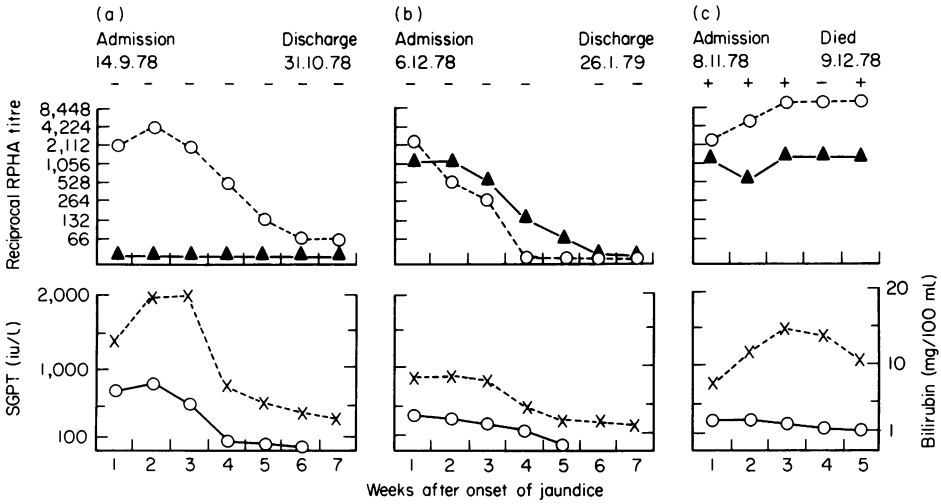

Fig. 1. Serial determinations of HBsAg-CICs (▲——▲), HBsAg (o - - - o), anti-HBs (+, −), SGPT activity (o——o) and serum bilirubin concentration (× - - - ×) in three representative patients. (a) Group A, patient BJ 40 (female, 76 years), (b) group B patient GJ 340 (female, 67 years), (c) group C, patient NG 231. (female, 84 years).

days. During follow-up all were essentially asymptomatic and had normal values of liver function tests (SGPT < 40 iu/l, bilirubin < 1 mg/100 ml).

AH patients with transient HBsAg-CICs (group B, n=24). HBsAg-CICs fell to undetectable levels within the first 6 weeks after the onset of jaundice in 24 patients (seven men and 17 women, aged 11–75 years, mean 45). The clinical features were essentially similar to those observed in group A. No statistical difference was found between the mean ages, SGPT, serum bilirubin levels and hospital days of the two groups (Tables 1, 2 and 3). Highly significant correlation was found between the HBsAg-CIC status and early anti-HBs response ($\chi^2 = 8{\cdot}277$, $r = 0{\cdot}47$, $P < 0{\cdot}005$), and between the HBsAg-CIC status and numbers of cases converted to HBsAg-negative ($\chi^2 = 14{\cdot}302$, $r = 0{\cdot}62$, $P < 0{\cdot}005$) in the two groups. Transient rash and/or arthralgia in the prodromal period were observed in three cases. By the time of discharge essential recovery had been noted in all patients with a course of symptomatic and supportive therapy averaging 40 days. Corticosteroids were administered to five patients.

Fig. 1b depicts the temporal relationship among HBsAg-CICs, free HBsAg, anti-HBs, SGPT and serum bilirubin levels in a patient representing this group.

AH patients with persistent HBsAg-CICs (group C, n=7). HBsAg-CICs remained detectable for more than 6 weeks after the onset of jaundice in seven patients (five men and two women, aged 65–84 years, mean 71). The mean age and hospital day of the group were statistically higher (Tables 1 and 3), whereas the levels of SGPT and serum bilirubin were significantly lower (Table 2) than those of the above two groups. Transient rash and arthralgia were observed in one case. No patient later became HBsAg-negative. The outcome of the disease is presented in Table 3 (two recovered, one progressed to CH, four died). Four patients received corticosteroids.

Fig. 1c depicts the temporal relationship among HBsAg-CICs, HBsAg, anti-HBs, SGPT and serum bilirubin levels of one representative patient.

HBsAg-CICs in two patients with HBsAg-positive chronic liver disease

HBsAg-CICs were detectable throughout the period of observation in both patients with HBsAg-positive chronic liver disease and the persistency of HBsAg-CICs was accompanied by persistent HBs antigenaemia. The CH patient had a history of AH 4 years earlier. He received prednisolone throughout the period of study. After 7 weeks' hospital course he was discharged with clinical improvement (Fig. 2a). SGPT and bilirubin levels remained abnormal (150 iu/l and 3 mg/100 ml) $1\frac{1}{2}$ months after discharge.

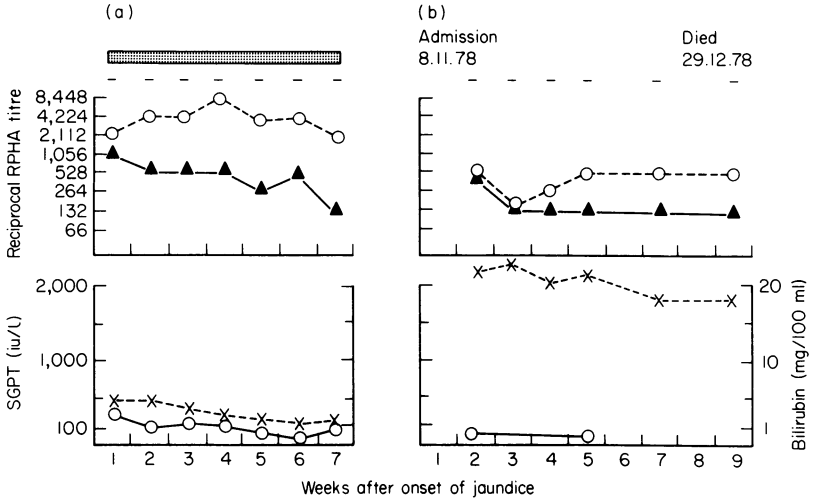

Fig. 2. Serial determinations of HBsAg-CICs, HBsAg, anti-HBs, SGPT activity and serum bilirubin concentration in (a) patient ZZ 56 (male, 54 years) with HBsAg-positive chronic hepatitis, and (b) patient TM 236 (female, 65 years) with HBsAg-positive post-necrotic cirrhosis. Note: dotted area refers to the course of corticosteroid therapy. (See legend to Fig. 1 for key.)

Patient TM 236 (Fig. 2b) progressed to cirrhosis 15 years after an acute hepatitis. She died of hepatic failure with terminal coma and hepatorenal syndrome 51 days after admission.

Corticosteroid therapy and the level of HBsAg-CICs

The response to corticosteroid therapy in altering HBsAg-CIC levels was found to be heterogeneous.

In five AH patients that proved to be well responsive to the treatment, HBsAg-CICs promptly decreased to undetectable levels simultaneously with the improvement of clinical and biochemical

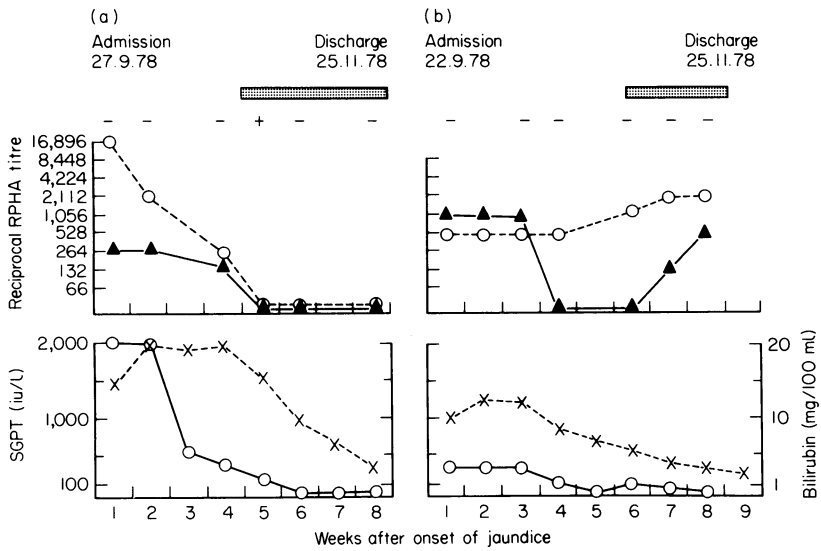

Fig. 3. Effect of corticosteroids on HBsAg-CIC levels. Note: (a) patient KL 85 (male, 58 years) responsive case and (b) patient SE 62 (male, 76 years) unresponsive case. Dotted areas refer to the course of corticosteroid therapy. (See legend to Fig. 1 for key.)

markers of the disease activity (Fig. 3a). By the time of discharge three patients had converted to HBsAg-negative. During the follow-up period all patients recovered fully, with the exception of the one with concurrent leukaemia.

In the other five cases (four AH and one CH), HBsAg-CICs continued to be detectable during the course of steroid therapy and persistent HBs antigenaemia was observed. Among the four AH patients one developed a CH with a rising concentration of HBsAg, one recovered and two died. An interesting example of the failure of corticosteroids to reduce HBsAg-CICs is shown in Fig. 3b, that is, HBsAg-CICs reappeared after administration of prednisolone then increased in titre during the course of therapy. The patient died 3 months after discharge.

HBsAg-CICs and the carrier state

The range of HBsAg titres measured by RPHA was from 1:66 to 1:67,584. None of the 107 carriers had anti-HBs detectable by CIEP and RPHA-I. Thus, HBsAg-CICs found in the five carriers were those in excess of antigen. None of the five carriers with HBsAg-CICs had elevated SGOT levels. All were symptom-free at the time of blood testing.

DISCUSSION

Using PEG-trypsinization we found a high incidence of HBsAg-CICs in acute hepatitis B and, in striking contrast, a very low incidence in asymptomatic HBsAg carriers.

Thirteen patients without HBsAg-CICs (group A) had a normal, uncomplicated course of acute hepatitis. The HBsAg-CIC levels in 24 patients of group B closely correlated with the disease activity, that is, a fall in the HBsAg-CIC concentration was regularly associated with a fall in SGPT, serum bilirubin and free HBsAg levels and with a subsidence of clinical symptoms. Conversely, the clinical picture and outcome of the disease in seven AH patients of group C and two patients with chronic liver disease appeared to be serious. All deaths, with the exception of the one with leukaemia, and a progression from acute to chronic hepatitis occurred among these patients. The correlation between the persistence of HBsAg-CICs and poor prognosis, therefore, was evident. The mean age of the group was significantly higher than that of the other two groups. It is well known that older patients in whom fulminant hepatic failure develops secondary to viral hepatitis have a much worse prognosis (reviewed by Gaines & Sorrel, 1979). Thus, measurement of HBsAg-CICs proved to be useful in monitoring disease activity and prognosis. A simultaneous persistence of HBsAg and HBsAg-CICs seems to contribute to predicting a chronic outcome, particularly because the clinical symptoms and biological signs are usually very poor in the great majority of AH patients with an evolution into a CH (De Groote, Fevery & Lepontre, 1978).

The assumption that liver cell damage in hepatitis B is caused by the immune system rather than the virus itself has led to corticosteroid therapy in some forms of hepatitis B. However, there have been no antigen-specific tests so far developed for monitoring HBsAg-CIC levels in corticosteroid-treated patients and hence evaluating the immunosuppressive effect of corticosteroids. PEG-trypsinization revealed that HBsAg-CIC levels in five AH cases decreased and then disappeared, while simultaneously clinical and biochemical benefits were observed. Among the four AH cases with persistent ICs, three died and prednisolone induced only a short-term remission in the CH case with persistent HBsAg-CICs. The effect of corticosteroid treatment may then be assessed by immune complex monitoring, using the PEG-trypsinization assay. The method may also facilitate selecting individual unresponsive cases for various types of therapy.

Our observations seem to support the hypothesis that the immunopathogenesis of hepatitis B likely involves a complex interaction of cellular and humoral mechanisms (Eddleston & Williams, 1974). More recently, Edgington & Chisari (1975) suggested that the outcome of an attack of acute hepatitis B may be most dependent on the immune responses of the host rather than the cytotoxic capacity of the infectious agent or the generation of ICs. HBsAg-CICs themselves alone seem unlikely to play a prime role in initiating hepatic injury because: (1) no clinical symptoms and biochemical signs of hepatitis were observed in the five HBsAg carriers possessing complexes. (2) Clinical and biochemical markers, reflecting the extent of liver damage, and outcome of the disease

were statistically indistinguishable in patients with acute hepatitis B transiently positive (group B) or negative (group A) for HBsAg-CICs. (3) HBsAg-CICs were present in AH patients both with and without arthritis. Conversely, arthritis was observed in AH patients both with and without HBsAg-CICs. (4) A persistent HBs antigenaemia was found in all AH patients with persistent HBsAg-CICs. Therefore, the high correlation between the persistence of ICs and unfavourable outcome may be explained as follows: (a) persistent source of the antigen, which may reflect the failure of the host to eliminate HBsAg, or perhaps, a sustained virus replication; (b) continuation of anti-HBs synthesis to a certain degree; or (c) failure of the host in clearing ICs. Thus, the transient or persistent presence of HBsAg-CICs may be most dependent on whether the host is able to eliminate the antigen and the complexes or not. The persistence of HBsAg-CICs, therefore, may be an epiphenomenon and an indicator rather than a 'cause' of severity and poor prognosis of the disease. By contrast, it is possible that the persistent presence of HBsAg-CICs can contribute to perpetuating the inflammatory process in the liver of CH patients.

Though the episode of AH in patients transiently positive or negative for HBsAg-CICs was similarly self-limited, there was a significant correlation between the HBsAg-CIC status and early anti-HBs response, and between the HBsAg-CIC status and the number of cases converted to HBsAg-negative in these two groups. Consequently, it seems likely that an early anti-HBs response, which resulted in the formation of HBsAg-CICs, favours the elimination of HBsAg from the circulation.

Ageing is associated with decreased immune responsiveness (Gross, 1965). This impaired immunosurveillance mechanism may partly explain why the patients of group C failed to clear HBsAg.

In conclusion, AH patients with a persistently positive PEG-trypsinization test seem likely to have a poor prognosis and to need particularly careful follow-up so that an adequate therapy regimen may be given in time.

We wish to thank Dr B. László and Dr J. Kéri for generously providing sera from patients with hepatitis and for making clinical information available. We also acknowledge the co-operation of the many physicians and nurses in the László Hospital.

We are very grateful to Professor S. R. Hollán, Dr E. Farkas and Dr G. Füst for their helpful discussion.

REFERENCES

ALMEIDA, J.D. & WATERSON, A.P. (1969) Immune complexes in hepatitis. *Lancet*, **ii**, 983.

ANH-TUAN, N. & NOVÁK, E. (1980) Detection and quantitation of hepatitis B surface antigen immune complexes (HBsAg-ICs) by an antigen-specific method. *J. Immunol. Methods*, **33**, 293.

BEDARIDA, G., ZACCHI, T. & TASSI, G.C. (1971) Preliminary observation on Au–anti-Au immune complex research by a modified electrosyneresis method. *Boll. Ist. Sieroter. Milan.* **50**, 74.

BRZOSKO, W.J., MADALINSKI, K., KRAWCZYNSKI, K., SKAWARSKA, H. & NOWOSLAWSKI, A. (1971) Australia antigen immune complexes in patients with different forms of hepatitis. *J. infect. Dis.* **123**, 251.

CARELLA, G., DIGEON, M., FELDMANN, G., JUNGERS, P., DROUET, J. & BACH, J.F. (1977) Detection of hepatitis B antigen in circulating immune complexes in acute and chronic hepatitis. *Scand. J. Immunol.* **6**, 1297.

DE GROOTE, J., FEVERY, J. & LEPONTRE, L. (1978) Long-term follow-up of chronic active hepatitis of moderate severity. *Gut*, **19**, 510.

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

EDGINGTON, T.S. & CHISARI, F.V. (1975) Immunological aspects of hepatitis B virus infection. *Am. J. med. Sci.* **270**, 213.

FRESCO, G.F. (1978) Detection of circulating immune complexes in hepatitis by means of a new method employing ^{125}I-antibody. *Acta Hepatogastroenterol. (Stuttg.)*, **25**, 185.

GAINES, K.C. & SORREL, M.F. (1979) Host resistance in liver disease. Its evaluation and therapeutic modification. *Med. Clin. N. Am.* **63**, 495.

GROSS, L. (1965) Immunological defect in aged population and its relationship to cancer. *Cancer*, **18**, 201.

KŐSZEGHY, Zs. & NOVÁK, E. (1973) A quick-method for determining serum transaminase in donors. *Transfusio (Budapest)*, **7**, 43.

MADALINSKI, K., SZTACHELSKA-BUDKOWSKA, A. & BRZOSKO, W.J. (1974) DEAE-cellulose chromatography: a method for dissociation of soluble immune complexes of hepatitis B antigen. *J. infect. Dis.* **129**, 371.

MILLMAN, I., LONDON, W.T., SUTNICK, A.I. & BLUM-

BERG, B.S. (1970) Australia antigen–antibody complexes. *Nature*, **226,** 83.

NOVÁK, E. & KŐSZEGHY, Zs. (1977) Detection of hepatitis Bs antigen by reversed passive haemagglutination. *Transfusio (Budapest)*, **10,** 88.

PERNICE, W. & SEDLACEK, H.H. (1979) Antigen specific detection of soluble immune complexes by a solid phase specific antibody system. *J. Immunol. Methods*, **28,** 33.

PESENDORFER, F., KRASSNITZKI, O. & WEWALKA, F. (1970) Immunoelektrophoretischer Nachweis von 'Hepatitis associated antigen' (Au/SH antigen). *Klin. Wochenschr.* **48,** 58.

SANTORO, F., WATTRE, P., DESSAINT, J.-P. & CAPRON, A. (1977) Hepatitis B circulating immune complexes. Characterization by radioimmunoprecipitation-PEG assay (RIPEGA). *J. Immunol. Methods*, **15,** 201.

SHULMAN, N.R. & BARKER, L.F. (1969) Virus-like antigen, antibody and antigen–antibody complexes in hepatitis measured by complement-fixation. *Science*, **165,** 304.