HEPATITIS B
A light microscopical and immunohistochemical study

(Een lichtmicroscopische en immunohistochemische studie)

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Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit te Rotterdam,
op gezag van de rector magnificus
Prof. dr. C.J. Rijnvos
en volgens besluit van het college van Dekanen.

De openbare verdediging zal plaatsvinden op
woensdag 20 september 1989 om 13.45 uur

door

Fiebo Jan Willem ten Kate
geboren te Rotterdam

1989

Grafische
verzorging
ROOS ECOR AVIDS
Alblasserdam

Promotiecommissie

Promotor:	Prof. dr. R.O. van der Heul
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voor mijn ouders

dankzij Christine
en de kinderen

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CHAPTER I

INTRODUCTION

A historical review

Hepatitis B virus infection, which shares many epidemiological characteristics with the Human Immunodeficiency virus infection, represents a major worldwide public health problem. At least 176 but probably 300 million people in the world (± 6 per cent of the world population) are persistent carriers of the hepatitis B virus (HBV) (Sobelavsky, 1978; Beasley, 1985). Chronic carriership can cause chronic liver disease, including cirrhosis and in the long term hepatocellular carcinoma, in at least 50 per cent of the cases. Chronic hepatitis B infection represents one of the highest relative risks (more than 200) known for a human cancer (Beasley, 1984, 1988).

This important pathogenic hepatitis virus was identified in 1968, when Blumberg's Australia antigen was by chance associated with hepatitis virus, type B. Before this discovery the distinction between at least two different types of viral hepatitis, currently designated as hepatitis A and B, was based on clinical observations (Findlay, 1939) and transmission studies of human volunteers only (MacCallum, 1944; Paul, 1945; Murray, 1954; Krugman, 1967). After it became possible to identify hepatitis type B, other cases of blood-borne hepatitis that could not be attributed to hepatitis B were detected. From this finding the existence of at least a third type of hepatitis virus (hepatitis non-A, non-B or hepatitis C) was presumed and, after a long search, recently verified (Houghton, 1988; Kuo, 1989; Choo, 1989) by a complementary DNA-hybridisation technique.

In the past hepatitis A, on the one hand, and hepatitis B and C, on the other, have been given various names, which were based mainly on either the suspected mode of transmission of the infectious agent or the clinical signs and symptoms of each of these types of hepatitis, e.g. "acute catarrhal jaundice", "short-incubation hepatitis", "infectious hepatitis" and "epidemic hepatitis" for hepatitis A and "long-incubation hepatitis", "serum hepatitis", "(post)transfusion hepatitis" and "postvaccinal hepatitis" for hepatitis B and/or hepatitis C.

Hepatitis A was known to occur in wide-spread outbreaks and was common among troops (Soldatengelbsucht; jaunisse des camps; campaign jaundice) during the second world war as well as earlier wars (Siegmund, 1942; Spooner, 1944). The disease was thought to be spread by droplets from the respiratory tract and/or food and water contaminated by excreta (Havens et al., 1945;

Findlay and Wilcox, 1945). Hepatitis B, on the other hand, was not recognized until more recent times although retrospectively it was probably first described by Lührman (1885). He reported an outburst of hepatitis in subjects vaccinated against smallpox, using the fluid collected from vesicles on the arms of previously vaccinated individuals. Later on, the occurrence of hepatitis was noted in individuals who had been given injections or infusions of human blood or blood products contaminated with virus, e.g. prophylactic serum against measles (McNalty, 1938; Propert, 1938), mumps (Beeson et al., 1944) and yellow fever (Findlay, 1943 and 1944). In addition, the large-scale introduction of blood transfusions in medical practice during World War II led to an increase in the incidence of hepatitis (Bradley, 1946). From these observations it was concluded that this type of hepatitis is transmitted parenterally via blood or blood products. By this time there was enough evidence to support the idea that two distinct agents were involved in the occurrence of hepatitis. In order to avoid confusion MacCallum suggested in 1947 that the two putative viruses should be called virus A, which gives rise to infectious hepatitis after a short incubation period, and virus B, which leads to serum hepatitis after a long incubation period.

Although a viral pathogenesis was postulated for both hepatitis A and hepatitis B (Findlay et al., 1939), all attempts to isolate and culture the virus failed and, as a result of the lack of serological and virological parameters, hepatitis research was severely hampered.

The studies of Blumberg and coworkers, however, resulting in identification of the "Australia antigen" (1965) and later the accidental discovery of its relation to viral hepatitis (1967), initiated extensive serological, epidemiological, virological, pathological and clinical research in the field of hepatitis.

During the above-mentioned studies directed initially toward a earch for serum polymorphisms, Blumberg identified an antigen by testing sera from poly-transfused patients against a panel of sera from healthy individuals from different geographical areas. Using the Ouchterlony immunodiffusion technique, one of the sera of this panel was found to give a single precipitation line with serum from a polytransfused American hemophilic patient. Since the serum that caused the precipitation reaction had been obtained from a healthy Australian aboriginal, the antigen involved was called the Australia antigen (Au-Ag).

Early epidemiological studies on this mysterious antigen indicated a relatively high prevalence of Au-Ag in sera from Oceanic, Oriental and Mediterranean populations, sometimes with family clustering. However, the antigen could only rarely be identified in sera from inhabitants of the United States (Blumberg, 1966).

Since a high prevalence of Au-Ag was noted for leukemia patients (Blumberg, 1965) as well as those with Down's syndrome (Blumberg, 1967), who are known to have a high risk for leukemia (Miller, 1964), the hypothesis that Au-Ag was

causally related to leukemia was put forward. By that time the presence of the antigen was considered to be important for the early diagnosis of leukemia.

A relationship between Au-Ag and hepatitis was first suspected when follow-up studies of a patient with Down's syndrome demonstrated the appearance of Au-Ag in the serum shortly before the development of an acute hepatitis. Later on, studies by Blumberg (1967), Sutnick (1968) and Okochi and Murakam (1968) confirmed the association between Au-Ag and hepatitis. Until that time, Au-Ag was considered to be associated with both hepatitis A and hepatitis B. However, the studies of Prince (1968) proved that Au-Ag was restricted to cases of hepatitis, type B only. This finding was confirmed by many authors and Au-Ag was established as a serological marker for hepatitis type B (Krugman et al., 1970).

In electron microscopical (EM) studies by Bayer et al.(1968), who investigated sera containing Au-Ag, particles with a diameter of about 23 nm were demonstrated. Originally considered to be similar to picornavirus or polioviruses, they were presumed to represent the morphological equivalent of the Au-Ag.

Further EM studies of Au-Ag-positive sera, however, revealed the presence of three different types of particles (fig.I.1):

1. Roughly spherical particles with a diameter of 16-25 nm.
2. Filamentous particles with a diameter of 16-25 nm and varying lengths (up to 700 nm).
3. Uniform, spherical particles with a diameter of 42 nm.

These latter particles, first described by Dane (1970), have a complex structure with a lipoprotein outer coat that surrounds a particulate, slightly hexagonal, inner core with a diameter of 27 nm (Almeida, 1971). These viral-like particles are commonly referred to as "Dane" particles. All three types of particle share common antigens on their surface that are identical with the Au-Ag. Hence, Au-Ag was renamed the "hepatitis B surface antigen" (HBsAg).

The inner core of the "Dane" particles, however, contains a distinct antigen, designated as hepatitis B core antigen (HBcAg) (Almeida, 1971).

Because of its ultrastructural architecture and the presence of a DNA polymerase (Robinson, 1974; Kaplan, 1973, 1974) and a small double-stranded circular DNA that serves as a primer template for the DNA polymerase (Robinson, 1974), the "Dane" particle was subsequently considered to be the complete virus.

Studies by Summers (1975) further defined the structure of the viral DNA and the way in which endogenous DNA polymerase uses this molecule as a template for DNA synthesis. It was concluded that the viral DNA was not entirely double-stranded but contained a large single-stranded gap of variable size. Thus, one of the two strands was complete whereas the other was, on the average, only half

complete; eventually endogenous DNA polymerase extends the incomplete strand, using the complete strand as a template. On the basis of the specific structure of the DNA genome (the smallest of all known DNA viruses) and the presence of an endogenous DNA polymerase, a new class of viruses was defined that were unrelated to any viruses previously described; they were called Hepadnaviridae (Marion, 1980). Despite evidence that the "Dane" particle represents the actual infectious virus of hepatitis B (HBV), all attempts to grow HBV in tissue culture systems have been unsuccessful and the upsurge in our understanding of the molecular biology of HBV resulted from Hepadnaviruses present in woodchucks (Summers), Beechey ground squirrels (Marion, 1980) and Peking ducks (Mason, 1980).

In addition to the "Dane" particle which represents the complete virus there are two other particles. These small spherical and filamentous particles are surplus viral coat material, produced by infected hepatocytes. Production of HBsAg by the hepatocytes of the host is coded by the HBV genome.

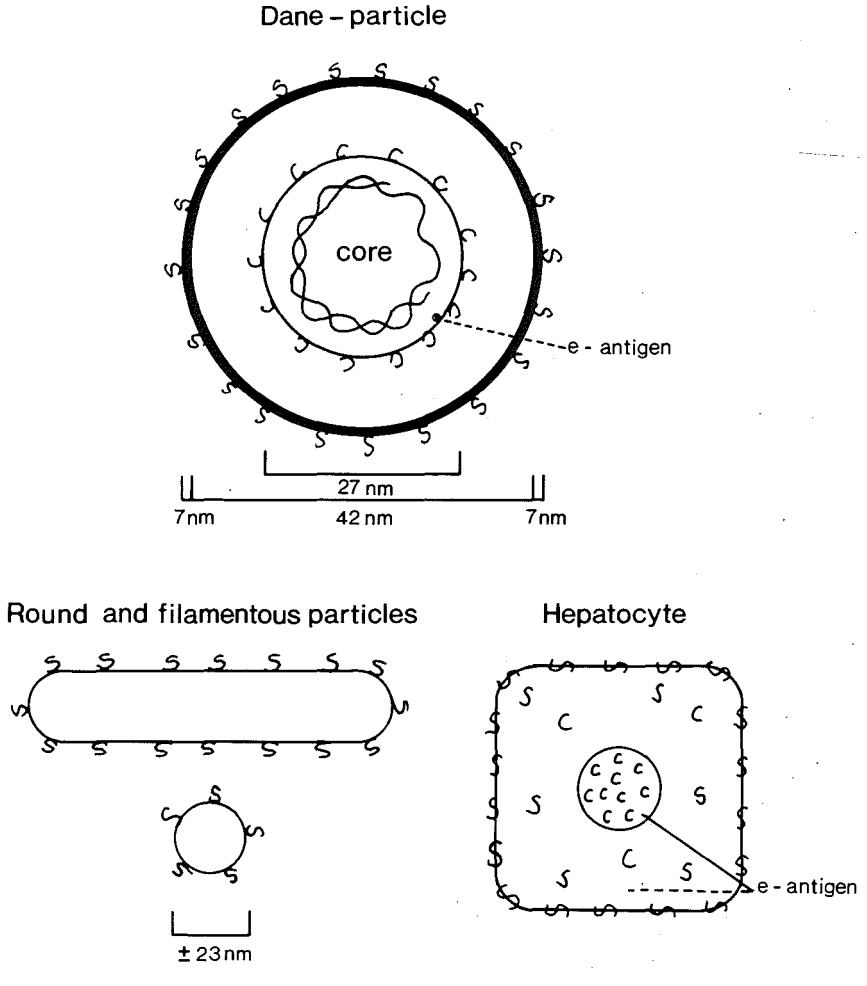
More detailed serological studies have resulted in the detection of several antigenic determinants of HBsAg, indicated as "a", "d", "y", "r" and "w", according to Le Bouvier (1971) and Bancroft (1972). While the "a" determinant is an obligatory group-specific determinant, the others represent two parts of mutually exclusive determinants: "d" and "y"; "r" and "w". Thus the following subtypes can be recognized: "adr", "adw", "ayr", "ayw". These subtypes define the HBV phenotypes and do not appear to be associated with intrinsic biological differences in terms of infectivity, virulence and type of liver disease. These antigenic determinants of HBsAg are located on the central hydrophilic region of HBsAg.

An additional antigen, designated as "e" antigen (eAg), was discovered by Magnius and Espmark (1972) in HBsAg positive sera. Although the precise characteristics were not yet known, eAg was not considered to be a subtype of the hepatitis B surface antigen. It differs in immunological properties as well as size and buoyant density (Magnius, 1975). Since the occurrence of "Dane" particles and eAg in serum seemed to be associated, it was initially suggested (Neurath, 1976; Okada, 1976) and later proven (Takahashi, 1979) that eAg is a component of the "Dane" particle. In accordance with this finding some authors (Okada, 1976) have demonstrated that HBV is transmitted by HBsAg seropositive patients with eAg more often than by HBsAg seropositive patients without eAg. As a result eAg is now considered a parameter for the infectivity of HBsAg positive serum. Moreover there appeared to be evidence that the presence of eAg may be associated with a more unfavorable type of hepatitis (i.e. chronic active hepatitis) (Nielsen, 1974; Trepo, 1976).

Ultrastructural and immunofluorescence studies on liver tissue from HBsAg seropositive patients demonstrated the presence of ± 27 nm particles in nuclei

of hepatocytes (Huang, 1971) and the occurrence of HBcAg in the particles (Brzosko, 1973), while HBsAg was discovered either in the cytoplasm or along the cellular membranes of hepatocytes (ten Kate, 1974; Roos, 1976; Ray, 1976).

Fig.I.1 *Schematic drawing of the different hepatitis B viral particles and the localization of the viral antigens on these particles and in the hepatocytes.*



Ultrastructurally the cytoplasm of the hepatocytes was found to contain filamentous and spherical particles in widened cisternae of the endoplasmic reticulum (Stein, 1972). These particles seemed to resemble the round and filamentous HBsAg particles in serum. Hepatocytes with these HBsAg-containing particles in the cytoplasm appear to be identical to the liver cells described as "ground-glass" hepatocytes in light microscopy. These ground-glass hepatocytes have special staining characteristics (Hadziyannis, 1973; Shikata, 1974). From immunofluorescence studies of liver tissue from HBsAg seropositive patients there is some evidence that eAg is present in the nuclei of hepatocytes (Arnold, 1977).

Studies of both experimentally infected monkeys (Barker, 1973) and human patients (Hoofnagle, 1973; Overby, 1982) revealed a complex pattern of appearance and persistence of HBV antigens and antibodies in serum following an HBV infection. After an infection HBsAg can be detected 5-24 weeks later in the serum of an infected individual. Four to ten weeks after the appearance of HBsAg, anti-HBcAg appears. This occurs about three weeks before onset of jaundice and at the height of liver dysfunction. HBsAg usually disappears slowly during recovery from hepatitis. The rate of disappearance of HBsAg may vary markedly. In patients who recover from acute hepatitis type B, a significant decrease in HBsAg titer can usually be observed within the first three weeks of clinical onset (Froesner, 1982). After the loss of HBsAg anti-HBs appears two to six weeks later, long after the appearance of anti-HBc. This HBsAg and anti HBs negative period is designated at the "window phase". Appearance of anti HBs indicates recovery from and immunity to HBV infection. However HBsAg antigenemia may persist for many years. After recovery from HBV infection anti-HBs usually disappears faster than anti-HBc.

In the meantime a new chapter in the history of hepatitis B was started by the discovery of Rizzetto (1977) of another hepatitis B-associated antigen-antibody system, which he designated δ/anti-δ. Initially this delta antigen was found by chance in the nuclei of hepatocytes when he was investigating the localization of hepatitis B antigens in frozen liver tissue from HBsAg positive patients by immunofluorescence methods. In this liver tissue HBcAg and delta antigen seemed to be present to the exclusion of the other in the nuclei of the hepatocytes. Subsequent studies of chimpanzees (Rizetto 1980) revealed that delta antigen was a component of a virus with unusual properties. The delta virus was unable to replicate autonomously; it could only replicate in the presence of the HBV virus. The delta virus is thus a defective virus that is dependent on helper functions provided by HBV. Its hybrid nature is reflected by the delta virion which is a particle composed of a RNA genome together with the delta protein surrounded by a shell of HBsAg derived from the HBV (Bonino 1984). This virus (500,000

dalton) is smaller than all known animal viruses, but its size is compatible with the dimensions of satellite viruses of plants, that are also unable to replicate autonomously. In view of the need for help from HBV, delta viruses can thrive only in individuals infected with HBV. This infection can occur as a coinfection simultaneously with the HBV infection, or it can be acquired as a superinfection in individuals who already carry the HBV. The two modes of infection result in different clinical pictures. Coinfection usually presents as a moderate to severe acute hepatitis, that runs a self-limiting course followed by complete recovery. Rarely is a fulminant hepatitis with massive liver necrosis or transition to chronic hepatitis seen. In contrast superinfection leads to a carrier state of the delta infection in over 70 per cent of the cases and aggravates the preexisting HBV disease or results in a new liver disease in previously healthy HBV carriers (Rizzetto 1983). In general, chronic delta infection has a tendency to progress more rapidly than infections of HBV only. In liver tissue delta antigen is located mainly in hepatocytic nuclei without detectable HBcAg.

The availability of serological markers for a hepatitis B and D infection, on the one hand, and the development of safe techniques for obtaining needle biopsy specimens (Knauer, 1978; Perrault, 1978) of the liver, on the other, have resulted in recognition of the wide range in the natural course of HBV with and without delta co/superinfection, both clinically and histologically (Peters, 1975; Ishak, 1976; Phillips, 1981).

The study of liver biopsy specimens by light microscopy is now considered to be the best and most constant method for classification of liver disease.

However, it has become evident that the value of the light microscopical study of liver tissue is limited, not only for determining the prognosis of the hepatitis, especially during the acute phase, but also for differentiating acute hepatitis from chronic hepatitis.

The purpose of the studies reported in this thesis is to investigate whether immunohistochemical and electron microscopical techniques, when performed in addition to light microscopical studies of liver biopsy specimens:

a. can contribute to a better understanding of the pathogenesis of hepatitis B and D.
b. are valuable for staging and determining the prognosis of hepatitis B and D.
c. are helpful in monitoring the effects of therapy.

For this purpose, the relationships between clinical and histological findings, viral expression patterns in the liver and serological viral parameters in a series of hepatitis B and D positive patients were investigated.

Highlights in hepatitis B story.

1885	Lührman:	First well-documented epidemic outbreak of a post-vaccination hepatitis, probably caused by hepatitis virus.
1939	Findlay:	Circumstantial evidence that hepatitis can be caused by an infective viral agent.
1940-1945	Findlay: Havens: Paul:	Evidence for the presence of two different types of infective hepatitis.
1947	MacCallum:	Suggestion to call the two putative viral agents: virus A and virus B.
1965	Blumberg:	Discovery of Australia antigen associated with leukemia and Down syndrome.
1967	Blumberg:	Australia antigen associated with hepatitis.
1970	Prince:	Australia antigen associated with hepatitis B.
1972	Magnius and Espmark:	Discovery of eAg/anti-e system.
1974	Robinson:	Demonstration of double-stranded circular DNA in the core of the "Dane"particles.
1975	Feinstone:	Proved evidence for the existence of at least a third hepatitis virus (non A- non B).
1975-1976	Purcell: Reesink: Buynak:	Development of first safe hepatitis B vaccins.
1977	Rizzetto:	Discovery of the delta-antigen, the marker for the hepatitis delta virus.
1981	Beasly:	Strong epidemiologic evidence for a close relation between chronic hepatitis B infection and the hepatocellular carcinoma.
1989	WHO:	Strategies for eradication of hepatitis B virus.

CHAPTER II

PATIENTS AND MATERIALS

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II.1 INTRODUCTION

A total of 1850 consecutive liver biopsy specimens, received for histological examination in the years 1973-1977, comprise the material for this study.

All 1850 liver biopsies were examined by light microscopy for evidence of hepatitis B infection. Those biopsies that exhibited light microscopical evidence for hepatitis B infection were further investigated. Of the 1850 biopsies, 252 biopsies were proffered fresh so that one part of each biopsy could be snap-frozen in liquid nitrogen and another part could be prepared for light microscopy studies. Both samples were investigated for the presence of HBV antigens by immunohistochemical methods. One of the major selection criteria for freezing part of a biopsy was a positive HBsAg serum reaction or clinical suspicion for a viral infection.

In addition a sample of each of the 252 biopsies was fixed in glutaraldehyde for electron microscopical examination.

In total, 165 of the 252 biopsies were obtained from 139 HBsAg seropositive patients and 87 from 84 HBsAg seronegative patients. The HBsAg seronegative patients represented a broad spectrum of liver diseases.

II.2 HBsAg SEROPOSITIVE PATIENTS

During the period of this study more than one biopsy was taken from several of the 139 HBsAg seropositive patients. The HBsAg seropositive patients can be divided into two groups:

Group A - 51 asymptomatic individuals who were found by chance to be HBsAg positive during a routine blood examination.

Group B - 88 patients who were found to be HBsAg positive during evaluation of liver disease.

II.2.1 GROUP A (ASYMPTOMATIC HBsAG CARRIERS)

In this group 41 individuals were "healthy" volunteers who had donated blood to the Red Cross Blood Transfusion Services of Rotterdam (Head: dr. Kothe) and were found to be HBsAg seropositive upon routine screening. Six other individuals in this group had visited an outpatient clinic for venereal diseases. In this clinic all patients are routinely checked for the presence of HBsAg in their serum. Two individuals were found to be HBsAg seropositive during a routine medical check-up and HBsAg was detected in the serum of two subjects when family members of HBsAg seropositive blood donors were screened. None of the 51 subjects had a history of jaundice; most of them, however, had experienced a period of nausea, malaise and/or complaints of abdominal distress. Three patients developed jaundice some time after the detection of HBsAg in their blood which suggests that the HBsAg was detec-ted during the incubation of a hepatitis B infection.

Volunteer blood donors

During this study all volunteers who donated blood to the Red Cross Blood Transfusion Services of Rotterdam (ranging in age from 18 to 65 years) were screened at the time of each donation for the presence of HBsAg in the serum. Initially HBsAg screening was performed by means of an agar-immuno-diffusion technique and later by a radioimmunoassay technique (Ausria; Abbott). Each volunteer donated blood once or twice a year.

Table II.1 lists per year:

a. the total number of blood donors tested for the presence of HBsAg.
b. the number of blood donors tested for the first time for the presence of HBsAg.
c. the number of blood donors found to be positive for HBsAg at the initial or a later screening.
d. the calculated prevalence of HBsAg in this population of blood donors, which may cautiously be considered to be representative of the entire "healthy" population (between the ages of 18 and 65 years).

The incidence of hepatitis B infection cannot be calculated from the number of blood donors with HBsAg positive serum following a negative test at the time of the previous donation. In fact, several blood donors proved to be positive for antibodies against HBsAg, whereas at the time of the previous donation neither HBsAg nor anti-HBsAg was detected. Because HBsAg antibody tests were not

Table II.1 *Volunteer blood donors*

	total number of blood donors	blood donors screened for HBsAg for the first time	HBsAg seropositive blood donors detected at the the first screening		HBsAg seropositive blood donors detected at subsequent screening		prevalence per cent
	n	n	n		n		%
1972	25.111	25.111	47		9		0.19
	♂ 17.621 ♀ 7.490		♂ 38	♀ 9	♂ 8	♀ 1	
1973	29.513	4.233	10		8		0.24
	♂ 20.615 ♀ 8.898		♂ 9	♀ 1	♂ 4	♀ 4	
1974	32.672	5.738	13		9		0.23
	♂ 22.476 ♀ 10.196		♂ 6	♀ 7	♂ 8	♀ 1	
1975	34.844	5.609	15		5		0.27
	♂ 23.640 ♀ 11.204		♂ 12	♀ 3	♂ 4	♀ 1	
1976	35.804	3.893	9		3		0.23
	♂ 24.095 ♀ 11.709		♂ 8	♀ 1	♂ 3	♀ 0	

performed routinely in all cases, the exact number of patients who exhibited conversion from anti-HBsAg negative to anti-HBsAg positive cannot be calculated. In addition, studies of individuals who were experimentally or accidentally infected with HBsAg have suggested that only transient viremia can exist without the formation of antibodies.

The annual prevalence of HBsAg positive blood donors in the years 1972/1977 ranged from 0.19 to 0.27, which is in agreement with the prevalence of HBsAg in other West European countries (Denmark 0.18: Banke, 1971; Germany 0.2: Rittner; United Kingdom 0.1-0.2) and North America (Taswell, 1972).

In the period 1973-1977 128 blood donors were found to be HBsAg positive. All of these 128 blood donors were referred to a physician for a medical check-up; in 41 of these 128 cases a liver biopsy was submitted to our department.

II.2.2 Group B (symptomatic HBsAg carriers)

Included in this group are 88 HBsAg seropositive patients, who were admitted to the hospital with clinical signs of liver disease. Four of these patients were taking immunosuppressive drugs at the time of the liver biopsy. Two patients had undergone a renal transplantation and one had a lymphoproliferative malignancy.

Follow-up

Except in 12 cases, all HBsAg seropositive patients were followed clinically and biochemically as long as their liver enzyme levels were abnormal and/or HBsAg was detectable in the serum.

During clinical follow-up a second, third and in some cases a fourth liver biopsy was obtained from 34 patients in the study period 1973-1977. However, only in 18 out of the 34 cases was there sufficient material to perform light microscopical as well as immunofluorescence and electron microscopical studies.

II.2.3 Sex and age

Sex

In the group of HBsAg seropositive subjects included in this study, a striking preponderance of males is noted.

Group A: of the 51 asymptomatic HBsAg seropositive individuals 10 were females and 41 males. The sex distribution in the group of 41 volunteer blood donors was 8 females and 33 males (table II.2). Of the 7,490 female and 17,621

male blood donors screened in 1972 for the presence of HBsAg, 9 females and 38 males were found to have HBsAg in their serum (the prevalence being 0.12 and 0.22, respectively). Therefore the female/male ratio for the whole group of HBsAg seropositive blood donors is approximately equal to the female/male ratio for the group of blood donors in this liver biopsy study. The 6 patients who were found to be HBsAg seropositive during a visit to a clinic for venereal diseases were all males.

In **group B** (88 HBsAg seropositive patients) the liver biopsies examined came from 23 females and 65 males (table II.2). The female/male ratio here is slightly altered in favor of the males.

Table II.2 *Female/male distribution in the different groups of HBsAg seropositive individuals*

	♀	♂
Group A	10	41
volunteer blood donors	8	33
Group B	23	65

Age

The age of the 139 HBsAg seropositive individuals ranged from 4 months to 81 years. No liver biopsies from HBsAg seropositive patients between the ages of 2 and 16 years were included in this study.

The age of the volunteer blood donors (group A) ranged between 18 and 65 years; only individuals in this age range are accepted by the blood transfusion services as blood donors. The ages (grouped in periods of 5 years) of the blood donors and the other 88 HBsAg seropositive patients are shown separately for females and males in figs.II.1 and II.2. The ages given in the figures are the ages of the patients at the time the liver biopsy was taken; when more than one biopsy was taken, the age at the time of the initial liver biopsy was noted.

II.2.4 Mode of infection/high risk groups

For approximately one-half of the 139 HBsAg seropositive patients included in this study, a possible cause of the HBsAg infection was found. For the other

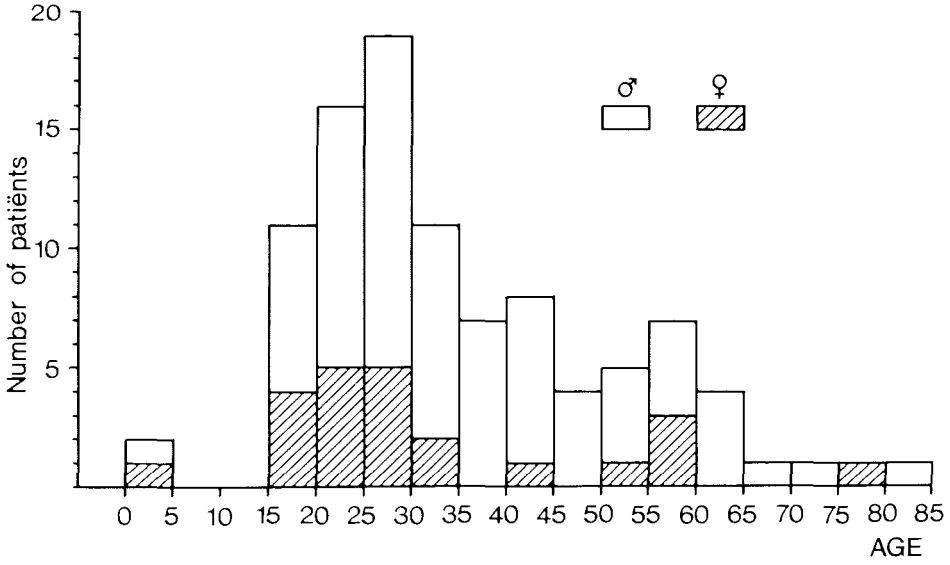
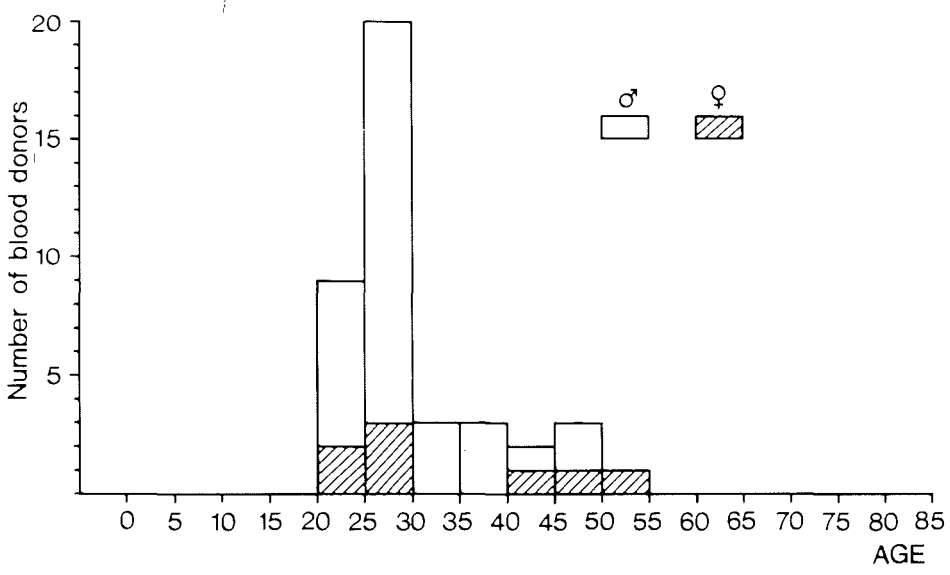


Fig.II.1 *Age distribution of the symptomatic HBsAg positive patients for males and females.*

Fig.II.2 *Age distribution of the HBsAg seropositive blood donors for males and females.*



half of the HBsAg seropositive patients, however, no risk factors or possible cause of the HBsAg infection could be established.

As far as the mode of infection is concerned, parenteral exposure of blood and blood products is considered the most important, probably the only, route of transmission of the hepatitis B virus (blood transfusion, (self)injections with improperly sterilized syringes, tattooing, razor blades) (Sherlock, 1975). However, many HBsAg seropositive patients, especially in the younger age groups, did not have a history of parenteral exposure or contact with other known hepatitis patients. Because HBsAg has been demonstrated in urine (Heathcote, 1974; Hourani, 1978; Di Bisceglie, 1985), stools (Grob, 1971; Villerejas, 1974), saliva (Macaya, 1979), semen (Karayiannis, 1985), milk (Boxal, 1974) and the saliva of insects; other modes of infection have been suggested for lomg times (venereal, enteral, aerogenous; Hersh, 1971).

Irrespective of the fact that the mode of transmission of the HB infection is uncertain in many cases, a high incidence of HBsAg has been found for special groups of individuals (high risk groups). In addition to immunologic deficiency, geography, ethnic factors and socio-economic status, several other factors appear to be associated with the risk of persistent HBsAg antigenemia, e.g. sex, age at testing, age at first exposure to infection, occupation, sharing a household with other carriers and sexual promiscuity (Szmuness, 1975).

Some of these groups consist of subjects who are more likely to become infected, e.g. doctors, nurses, laboratory technicians, hemophiliacs (exposed groups). In other groups close contact or socio-economic conditions may play a major role, e.g. people living in institutions (mentally retarded, prisoners, orphans), prostitutes and homosexuals. A variable, sometimes high, incidence of chronic hepatitis B infection has been reported for groups of patients with immune deficiencies (leukemia, renal transplant recipients) (Hersh, 1971; Grob, 1971; Perillo, 1981).

The numbers of patients belonging to the various high risk groups are shown in table II.3. It should be noted that some individuals belong to more than one high risk group (the number of such individuals is shown in parentheses). Twenty-four of the HBsAg seropositive patients were foreigners, chiefly from Mediterranean and South American (Surinam) countries where the incidence of HBsAg is known to be high. In the group of volunteer blood donors 14 (± 30 per cent) belonged to a high risk group: four were homosexuals (two of them had also been treated for a venereal disease and one was a drug addict), two were medical technicians, one had received a tattoo three months before and one had had a blood transfusion 5 months before. Three blood donors had undergone extensive dental treatment (3 to 5 months previously), one patient was under treatment for venereal disease and two patients had HBsAg seropositive individuals in their

Table II.3 *High risk groups*

	total n = 65	blood-donors n = 14
Professionals: doctors nursers, medical students butchers	5 3	2
Hemophiliacs	1	
Patients who have had a major operation	8 (3)	
(Poly-)transfusions	1	1
Patients treated for venereal disease	16 (2)	3 (2)
Homosexuals	10 (3)	4 (3)
Drug addicts	15 (3)	1
Infection possibly related to dental treatment	4	3
Individuals who have a tattoo	5 (1)	1
Patients with leukemia	1 (1)	
Renal transplant recipients	2	
Individuals with HBsAg positive family members	7	2

family. Amongst the HBsAg seropositive volunteer blood donors eight were foreigners from Mediterranean or South American countries.

From the available data, there is some evidence that sexual transmission is one of the most frequent routes of transmission of hepatitis B infection.

II.3 HBSAG SERONEGATIVE PATIENTS

From the 84 HBsAg seronegative patients (29 children and 55 adults) 87 liver biopsies were taken and processed for light microscopical, electron microscopical and immunofluorescence studies. HBsAg could not be detected in the sera of these patients by means of a radioimmunoassay technique (Ausria II, Abbott).

All kinds of liver disease were encountered in these biopsies: alcoholic liver disease, chronic active liver disease, biliary cirrhosis, sclerosing cholangitis, neonatal hepatitis, congenital biliary atresia, pancreatic fibrosis, α_1-antitrypsine deficiency, cholangitis, drug-induced liver disease, cytomegaly and luetic infections.

CHAPTER III

METHODS

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METHODS

III.1 SAMPLING OF LIVER BIOPSIES

The majority of liver specimens were obtained with a True-Cut® needle (Travenol Laboratories, Dearfield, Illinois, U.S.A.). A small number of liver biopsy specimens was taken with the Vim-Silberman needle, while some wedge biopsies were obtained during surgical intervention. About one third of all True-Cut needle biopsies were taken under laparoscopic control. Out of a total of 1850 biopsy specimens 1598 were totally and 252 were partly prepared for light microscopy studies. The remaining material of the 252 liver specimens was divided into pieces, using a razor blade. One piece of this material was snap-frozen for immunofluorescence studies, another was prepared for ultrastructural examination. If sufficient material was available, suspensions of isolated hepatocytes were made.

III.2 LIGHT MICROSCOPY

The liver biopsy sample for the light microscopy studies was fixed in 4 per cent buffered formaldehyde for at least 12 hours. In some cases part of the biopsy was fixed in 2 per cent paraformaldehyde. The fixed tissue was dehydrated in a series of alcohol baths of ascending concentration and embedded in paraplast (Sherwood Medical Industries).

After deparaffination serial 4μ-thick sections were sliced from the blocks and routinely stained using the following staining procedures:

1. Hematoxylin and azophloxin (H and A).
2. Hematoxylin and azophloxin with saffron (H.A.S.).
3. Periodic acid-Schiff (PAS).
4. Periodic acid-Schiff after glycogen digestion by diastases.
5. Lawson-von Gieson's technique for elastin (EG).
6. Gomori's technique for reticulin.
7. Aldehyde thionin (AT).
8. Rubeanic acid for copper.
9. Perls' stain for iron.

In addition Shikata's orcein staining method (Shikata, 1974) was used when the liver tissue came from HBsAg seropositive patients as well as those HBsAg seronegative patients with ground-glass hepatocytes on the hematoxylin-azophloxin stained slides or a positive aldehyde thionin stain.

Sections from most of the biopsies from the HBsAg seropositive patients and biopsies with ground-glass cells were used for the immunofluorescence studies as described below. The histopathological alterations present in the biopsies from the HBsAg seropositive patients were classified as described in chapter IV.

The degree of fibrosis in the liver tissue was scored as -, +, ++, +++; periportal fibrosis without the formation of porto-portal or porto-central septa was scored as +, periportal fibrosis with porto-portal or porto-central septum formation in some areas as ++, and peri-portal fibrosis with porto-portal or porto-central septum formation throughout the biopsy as +++. The iron content, as determined by Perls' stain, was scored as -, +, ++. Liver tissue with stainable iron in only some scattered Kupffer's cells or hepatocytes was scored as +, storage of iron diffusely throughout the liver or in groups of Kupffer's cells or macrophages as ++. Copper pigment was scored as absent or present.

III.3 IMMUNOHISTOCHEMICAL TECHNIQUES

III.3.1 SPECIMEN SAMPLING

From 252 needle biopsies one or two segments, at least 0.5 cm long, were sliced with a razor blade onto a dental wax plate. The biopsy segments were put on a small piece of cork covered with filter paper or in gelatin capsules and snap-frozen. The liver tissue was stored in aluminum film boxes in liquid nitrogen.

III.3.2 SECTIONING

Four μ-thick sections were cut from frozen liver tissue at -20°C in a cryostat and airdried at room temperature. After drying,the sections were tested with antisera (see below) the same day or stored at -20°C for staining the next day.

III.3.3 FIXATION

A series of sections from each biopsy was incubated with antisera without pretreatment of the slides. Another series was tested with antisera after washing with buffered saline (PBS pH 7.2) and a third series was incubated after dehydration for 10 minutes in 100 per cent acetone.

Moreover after sectioning various fixative solutions (para-formaldehyde, glutaraldehyde, alcohol) were applied to slides of a selected group of biopsies to study the effect of these fixatives on the morphology and the results of the immuno-histochemical procedures. Similarly deparaffinated sections of the formalin and paraformaldehyde-fixed, paraffin-embedded liver tissue were used for immunofluorescence staining.

III.3.4 STAINING

Direct and indirect immunofluorescence and immunoperoxidase staining techniques were applied. For the direct immuno-fluorescence and immuno-peroxidase techniques, the slides were first washed with phosphate-buffered saline (PBS pH 7.2) or fixed/dehydrated (see above). Subsequently the slides were incubated with labelled antisera for one hour, washed for 30 minutes in PBS at 20°C and mounted in a glycerin PBS mixture (9:1, pH 7.8).

For the indirect immunofluorescence and immunoperoxidase techniques, the slides were first incubated with unlabelled primary antisera for 30 minutes and washed in PBS for 20 minutes. Subsequently the slides were incubated with FITC, TRITC or peroxidase-labelled antisera for 30 minutes, washed again in PBS for 30 minutes and mounted in the glycerin PBS mixture. For immuno-fluorescence and immunoperoxidase staining of paraffin sections, the slides were deparaffinated and then incubated with the appropriate antiserum for one hour in a humid chamber at 37°C.

The mounted slides were read directly or stored at -20°C and examined within 24 hours using a fluorescence microscope.

III.3.5 MICRO-WAVE-STIMULATED INCUBATION

To shorten immunofluorescence and immunoperoxidase procedures, micro-wave irradiation was used in some cases during incubation of the tissue sections with the specific antisera (Hopwood, 1984; Chiu, 1987). Slides covered with excess of antiserum in usual dilutions were placed on the bottom of a commercially available household microwave oven (Miele Electronic M696). Microwave irradiation was performed at 150 W for 3 to 10 minutes.

III.3.6 FLUORESCENCE MICROSCOPE

The slides were evaluated in a Leitz orthoplan or a Zeiss fluorescence microscope with incident illumination, as described by Ploem (1967) and Hijmans et al. (1970). A Xenon lamp (Osram HBO 50W) was used for excitation.

III.3.7 ANTISERA

1. Antisera against immunoglobulins/complement factors.

For demonstration of IgG, IgM, IgA and complement, cryostat and paraffin sections of liver tissue were tested with commercially available anti-IgG, anti-IgM, anti-IgA and anti-complement antisera raised in rabbits (Central Laboratory of the Netherlands Red Cross Bloodtransfusion services, Amsterdam, the Netherlands and Kallestad Diagnostic Incorporation, Austin, Texas,USA).

2. Antisera against HBsAg.

A commercial HBsAg (subtype "ad") antiserum raised in rabbits was obtained from Behring Werke, Frankfurt, FRG). This serum was used in combination with the FITC labelled horse anti-rabbit antiserum, according to the method described by The and Feltkamp (1970) (CLB, the Netherlands).

A second HBsAg (subtype "ad") antiserum was raised in rabbits. These rabbits were hyperimmunized with HBsAg isolated from HBsAg positive human serum (Duimel, 1972). Complement in the antiserum was inactivated by heating (56°C) after incubation of the antiserum with human erythrocytes and sepharose beads coated with HBsAg-negative human serum (Avrameas, 1969). Neither hetero-agglutinins nor antibodies against human serum proteins were detectable in this antiserum by immuno-electrophoresis and agar double immunodiffusion (Ouchterlony, 1973).

Ig fractions of the anti-serum were conjugated with fluorescein isothio-cyanate, according to The and Feltkamp (1970). The molar F/P ratio for this serum was 2.3; it was free of Ig proteins with an F/P ratio of less than 1 and more than 4. The titer of this HBsAg antiserum found by the Ouchterlony tech-nique was 1:218; passive hemagglutination yielded a value of 1:500,000. The final working dilution of the antiserùm for cryostat sections was 1:50.

A third antiserum against HBsAg (subtype "ad") was obtained from a patient who had recently recovered from a hepatitis B infection. This serum contained antibodies against HBsAg, at a titer of 1:125,000 according to the passive hemagglutination technique. The Ig fraction of this serum was used for the indirect fluorescence technique, in combination with a FITC labelled horse anti-human immunoglobulin serum (CLB, the Netherlands) as secondary antibody. In addition a mouse monoclonal HBsAg antibody, kindly provided by Organon, was used on a part of the biopsy material.

All three polyclonal HBsAg (subtype "ad") antisera and the monoclonal (against determinant "a") antibody yielded a strong and identical fluorescence pattern for

liver tissue from HBsAg seropositive patients without a disturbing background. Minimal non-specific fluorescence of (eosinophilic) leukocytes in liver tissue from both HBsAg seropositive and HBsAg seronegative patients was abolished by absorption of the antisera with sonicated preparations of purified polymorphonuclear leukocytes. After absorption of the antisera with HBsAg positive serum all fluorescence was abolished. The fluorescence was not influenced by absorption with HBsAg-negative serum. Preincubation of HBsAg positive liver tissue with unlabelled anti-HBsAg antisera abolished the fluorescence of the labelled antiserum. As negative controls for the indirect fluorescence technique rabbit serum and normal HBsAg-negative human serum were used.

An antiserum against HBsAg (subtype "ay") was raised in guinea pigs hyperimmunized with a purified HBsAg (subtype "ay"). This antigen was obtained from the serum of an HBsAg (subtype "ay") positive patient by absorption of this serum with sepharose beads coupled to the purified rabbit antiserum against HBsAg subtype "ad" (see above). The guinea pig antiserum against HBsAg (subtype "ay") was purified of spurs of antibodies against human serum proteins by absorption with sepharose beads coupled to normal HBsAg-negative human serum (Avrameas, 1969).

The Ig fraction of this anti-serum showed the same fluorescence characteristics as the antisera containing antibodies against subtype "ad" HBsAg.

After incubation with this anti-serum, a FITC labelled horse anti guinea pig τ-globulin (Hyland, Ontario, Canada) was used as second fluorescence layer.

Antisera to viral coat determinants "d" and "y" were prepared by absorption of the HBsAg (subtype "ad") rabbit antiserum with human serum positive for HBsAg (subtype "ay") and by absorption of the HBsAg (subtype "ay") guinea pig antiserum with human serum positive for HBsAg (subtype "ad").

The antiserum against the "d" determinant induced a fluorescence pattern similar to that of HBsAg (subtype "ad") antiserum for liver tissue derived from a HBsAg (subtype "ad") seropositive patient, while this antiserum caused no fluorescence in a liver biopsy obtained from a HBsAg (subtype "ay") seropositive patient. Similarly the antiserum against the "y" determinant induced the same fluorescence pattern as the guinea pig antiserum against HBsAg (subtype "ay") in liver tissue from a HBsAg (subtype "ay") seropositive patient. No fluorescence was seen in liver tissue from a HBsAg (subtype "ad") seropositive patient.

3. Antisera against HBcAg.

An antiserum against the viral core determinant (HBc) was obtained from a patient with an acute self-limiting HBsAg positive hepatitis two days after the HBsAg level just exceeded background levels, using the radioimmunoassay

technique (Ausria II, Abbott Laboratories, IL, USA) (Hoofnagle, 1973); HBeAg, anti-HBe and HBsAg were negative. This serum produced positive fluorescence of liver cell nuclei in those biopsies in which nuclear core particles were demonstrated ultrastructurally. No specific fluorescence was seen in liver biopsies from HBsAg seronegative patients. FITC labelled horse anti-human antiserum (CLB, see above) or peroxidase labelled anti-human antiserum (Dakopatts, Denmark) were used as second layer. Secondly, a mouse mono-clonal anti-HBcAg antibody was kindly provided by Organon. This antibody showed no anti HBs and anti HBe activity.

4. Antiserum against HBeAg.

A mouse monoclonal antibody against HBeAg was kindly provided by H.C.Thomas.

5. Antiserum against Delta antigen.

A monoclonal FITC and peroxidase labelled antibody against delta antigen was kindly provided by Rizzetto. This antibody showed no anti-HBe and anti-HBs activity.

III.4 ELECTRON MICROSCOPY

III.4.1 SPECIMEN SAMPLING

One or two small pieces of each of the 252 liver specimens (1 mm^3) were sliced (within 3 minutes of the biopsy procedure) onto a dental wax plate with a razor blade and placed in a Karnovski (1965) or glutaraldehyde fixative solution.

III.4.2 FIXATION

Fixation was carried out with 3 per cent glutaraldehyde in 0.1 mol. Na-cacodylate and 0.05 mol. $CaCl_2$ in distilled water (pH 7.4) or Karnovski's fixative at a temperature of 4°C. The specimens were kept in the fixatives for at least three days and then transferred for 24 hours to a buffer solution consisting of 0.1 mol. Na-cacodylate and 0.05 mol. $CaCl_2$ in distilled water (pH 7.4) before postfixation for 16 hours in 1 per cent OsO_4 in 0.1 mol. Na-cacodylate (pH 7.3) to which 0.05 mol. $K_3Fe(CN)_6$ had been added (De Bruijn, 1973).

III.4.3 DEHYDRATION

Dehydration was performed at room temperature in a series of acetone baths of ascending concentration, 2x10 minutes in 30 per cent acetone followed by 2x10 minutes in 50 per cent, 2x10 minutes in 70 per cent, 2x10 minutes in 90 per cent and 2x10 minutes in 100 per cent acetone.

III.4.4 INFILTRATION

After dehydration the tissue was immersed in a solution of 1.2 mmol epoxypropane for 2x10 minutes and then in a mixture of EPON 812 (Shell) and acetone (1:1) for one hour at room temperature.

III.4.5 EMBEDDING

Embedding was carried out in gelatine capsules containing Epon-C. The Epon-C mixture consisted of 38 ml Epon 812 (Shell), 26 ml dodecanyl succinic anhydride and 20 ml methyl nadix anhydride (FLUKA).

III.4.6 POLYMERIZATION

A suitable degree of hardness was achieved by keeping the capsule at 37°C for 18 hours and then at 60°C for 2x24 hours.

III.4.7 SECTIONING AND MICROSCOPY

One μm thick survey sections of the liver tissue blocks were cut on a LKB pyramiton, equipped with glass knives. They were then stained with toluidine-blue. Small areas (0.20x0.20 mm) of liver parenchyma were selected for further ultrathin sectioning. For this purpose, the blocks were trimmed into small pyramids and cut with an LKB ultramicrotome with glass knives. The sections were mounted on Formvar-coated copper grids and stained with uranyl acetate and lead citrate. The sections were examined and photographed with a Philips E.M. 200.

III.5 DETECTION OF ANTIGENS ON THE CELL SURFACE OF HEPATOCYTES

For the detection of Hepatitis B surface antigen, immunoglobulins and complement factors on the surface of hepatocytes, sedimentation and suspension

preparations of isolated hepatocytes from 50 of the biopsies from HBsAg seropositive patients were prepared.

III.5.1 Isolation of hepatocytes

A piece of liver tissue, the mass of which depended on the quantity of available liver material, was stored immediately after the biopsy procedure in a 5 per cent serum albumin solution in PBS (according to Hijmans, 1970) at 0-20°C. Within three hours the material was gently divided into fine fragments in some drops of Hijmans' solution on a glass slide using two preparation needles (temperature 4° C). The suspension was transferred with a plastic pipet into a plastic tube containing Hijmans' solution. The larger fragments of the suspended liver tissue, consisting chiefly of fibrous tissue, were removed. If larger liver tissue specimens were available (from surgical biopsies) 1 mm cubes of liver tissue were fragmented in Hijmans' solution (using a Borel bore). The suspension of liver and isolated hepatocytes was washed twice by centrifugation for 10 minutes at 1000 r.p.m. After the first washing in Hijmans' solution the resuspended liver cell pellet was divided into two equal portions. After centrifugation one portion was resuspended in 5 per cent bovine serum albumin for the sedimentation preparations and one portion was resuspended in 1 per cent bovine serum albumin for the suspension preparations of hepatocytes.

III.5.2 Immunofluorescence technique for sedimentation preparations of isolated hepatocytes

Six sedimentation preparations were made from the suspension of isolated hepatocytes in 5 per cent Hijmans' solution. Firstly, five drops of PBS (pH 7.4) were put in a sedimentation chamber (temperature 0°-2°C). A few minutes later, five to seven drops of the suspension were added to the sedimentation chamber, depending on the concentration of hepatocytes. The preparations were dried in the sedimentation chamber overnight. Two slides were incubated with a FITC labelled rabbit anti-HBsAg antiserum, one with FITC labelled anti-IgG (CLB) and another with a FITC labelled anti-complement antiserum (CLB); one slide was stained with Giemsa. The same day the slides were read under the fluorescence microscope.

III.5.3 Immunofluorescent technique for suspension preparations of isolated hepatocytes

The suspension of hepatocytes in a 1 per cent serum albumin solution was

divided into two portions, one for incubation with rabbit a-HBsAg, another for incubation with the FITC labelled a-IgG (CLB, the Netherlands).

The concentration of the fluorescein labelled antisera was twice as high as that used for the fluorescence technique for slides. One hour after incubation at 4°C, the suspensions were washed twice by centrifugation (1000 r.p.m.). The pellet was resuspended in a few drops of buffered glycerin solution (pH 7.8). This suspension was put on a glass slide and read immediately under a fluorescence microscope.

III.6 SEROLOGY

Patient sera were tested on the presence of HBsAg with standard radio-immunoassay (Ausria II, Abbott laboratories, II, USA). The presence of HBeAg and anti-HBe in serum were measured by double immunodiffusion technique and later on by radioimmunoassay (Abbott, USA). For the measurement of HBeAg by radioimmunoassay a constant serum dilution was used. This dilution was chosen such that a P/N (cpm patient sample/cpm negative controle sample) ratio of approximately 12 ± 2 would be obtained in pretreatment sera. A P/N ratio of less than 2.1 in undiluted serum was considered HBeAg negative.

DNA-polymerase activity was measured by the method of Howard as modified by Fang (1981).

Anti-delta agent antibodies were determined by Elisa (Abbott, laboratories, IL, USA).

CHAPTER IV

LIGHT MICROSCOPY OF VIRAL HEPATITIS, TYPE B

CHAPTER IV

LIGHT MICROSCOPY OF VIRAL HEPATITIS, TYPE B

IV.1 INTRODUCTION

Hepatitis, defined as a diffuse necrotizing inflammatory disease of the parenchymal cells of the liver, is known to have several etiological causes (e.g. drugs, alcohol, viruses) and multiple morphological variants. In man hepatitis of viral origin can occur as part of a systemic infection by herpes simplex virus type 1 (Flewett, 1969), Epstein-Barr virus, cytomegalovirus (Stern, 1972; Toghill, 1967; Snover, 1984) and Coxsackie virus (Sun, 1966) or by specific hepatotropic viruses (viral hepatitis).

Two types of viral hepatitis (hepatitis A and hepatitis B) are well known and can be distinguished by means of serological and epidemiological data (Krugman, 1976). The presence of one or more additional hepatitis viruses was implied by clinical and serological observations (Feinstone, 1975). In fact, after a long search, an additional virus is identified recently (Choo, 1989; Kuo, 1989; Houghton, 1988). However as long as tests for the presence of non-A, non-B virus infection are not available, the general term non-A, non-B hepatitis is to be preferred over a more definitive designation, such as hepatitis C.

The introduction of the liver needle biopsy, as a safe method for evaluation of liver disease, together with the availability of markers to identify hepatitis B virus (HBV) infection resulted in detailed knowledge of the histopathological features of HBV infections, in both man and chimpanzees. By means of a liver needle biopsy it is possible to obtain information regarding stage and prognosis of the disease (Bianchi, 1971).

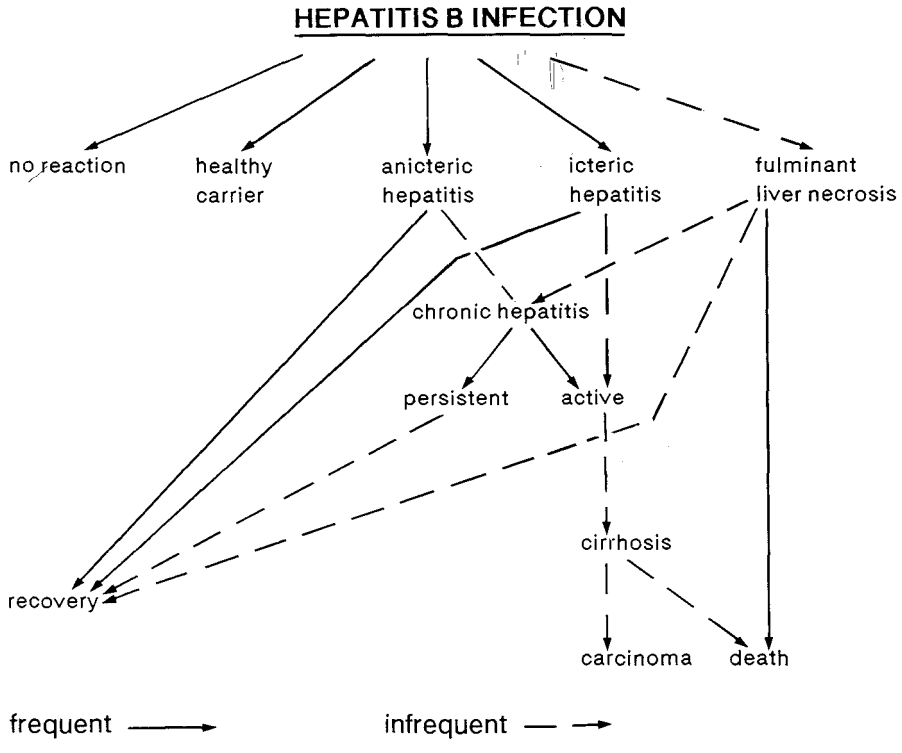
With the development of a variety of serological markers for hepatitis A infection (Miller, 1975; Purcell, 1976), reliable data on histopathological changes and the natural course of hepatitis A could be obtained (Dienstag, 1981; Lindberg, 1978; Mathiesen, 1980). In contrast to hepatitis type A, which is generally considered to be an essentially benign and self-limiting disease, hepatitis B runs a complex natural course with a wide range of clinical symptomatologies and histopathological changes (table IV.1).

Using chimpanzees experimentally infected with hepatitis type A or type B, Dienstag (1976) noted that the pattern of histo-pathological alterations in serial liver biopsies is basically the same for hepatitis A and hepatitis B. However, the

morphological changes are of shorter duration in hepatitis A; in hepatitis B they last much longer, showing fluctuations and slow regression. The location of parenchymal changes in hepatitis A is predominantly periportal in combination with a prominent portal round-cell infiltrate. In contrast, in hepatitis B the parenchymal alterations are found chiefly around the central veins while the portal infiltrate is less pronounced compared to that in hepatitis A. Later, another type of experimental hepatitis B was identified in chimpanzees; comparable with the manifestations of the carrier state in man. This infection has smoldering features, and is characterized histologically by a prominent portal inflammatory infiltrate, as seen in human chronic persistent hepatitis (Shikata, 1980; Shouval, 1980).

In contrast to the results obtained for chimpanzees, no distinct constant diagnostic differences in the morphological changes characterizing acute hepatitis caused by hepatitis virus type B or type A could be found for man (Peters, 1975; Ishak, 1976; Ludwig, 1977; Phillips, 1981). In addition to the histological picture of an acute hepatitis a wide range of clinicopathological manifestations can be seen following an HBV infection (fig.IV.1).

Fig.IV.1 *Clinicopathological reaction patterns after a hepatitis B infection*



Although the factors which determine the variable clinical and morphological aspects are not clearly understood, the interaction of the hepatitis B virus and the host produces roughly two clinico-histopathological types of lesion:

a. acute hepatitis, which in general corresponds to the morphological picture of lobular hepatitis.
b. chronic hepatitis, which in general has the morphological picture of a portal or periportal hepatitis.

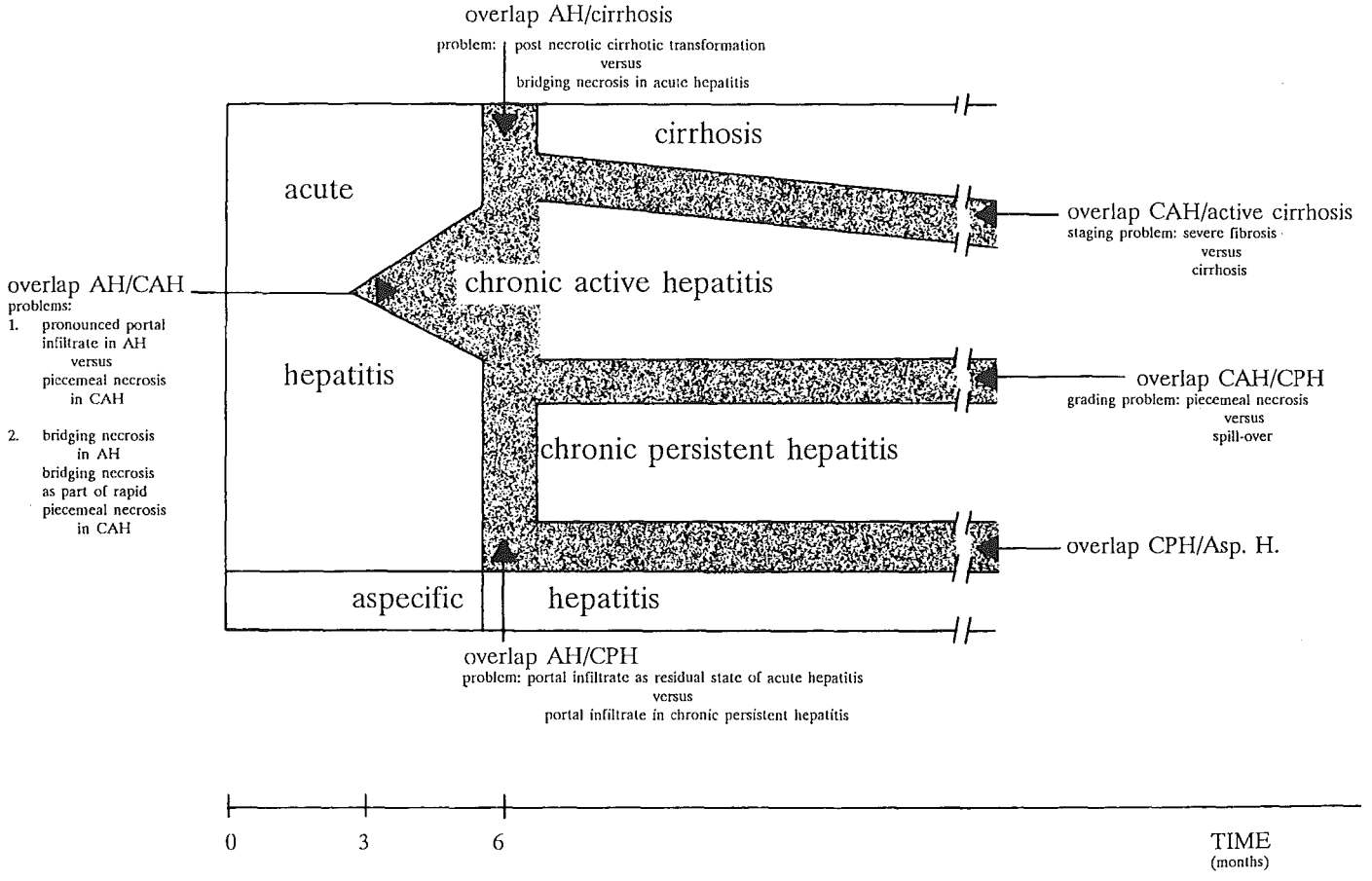
The histological definitions of classic acute and chronic hepatitis are rather simple and well defined (Bianchi, 1971). Nevertheless, the spectrum of the morphological pictures of both acute and chronic hepatitis B is broad and merge imperceptibly into one another. The morphological variations in acute and chronic hepatitis which are probably related to differences in the immune response of the host may cause diagnostic problems for the pathologist.

Clinically the differentiation between acute and chronic hepatitis may also be difficult, sometimes impossible. Hepatitis is arbitrarily considered chronic when biochemical changes are present for at least 10 to 12 weeks (Summerskill, 1974). However, some authors (Mistilis, 1970; Sherlock, 1974, 1976) demand a minimum time limit of six months before calling a hepatitis chronic, since in some instances self-limiting acute hepatitis can last this long.

These difficulties and the many efforts to create a single classification system that is both clinically and histologically valid have resulted (in the past) in an inextricable terminology (Conn, 1976). In addition, nomenclature is further confused by the use of many undefined temporal connotations such as acute, subacute (Boyer, 1970; Tisdale, 1963), subchronic (Björneboe, 1949) and chronic (Conn, 1976). Because the terminology applied by various authors differed, earlier reports on hepatic disease are exceedingly difficult to interpret and compare. It should be stressed that a uniform and reproducible classification system is indispensable for a comparison of the various studies on hepatic disease and for appraisal of the effects of therapeutic agents on these liver disorders. Histological evaluation of the effects of therapeutic agents on hepatitis B is particularly important, since several clinical trials have suggested that immunosuppressive and antiviral therapies (prednisone, azathioprine, interferon, acyclovir) (Cook, 1971; Soloway, 1972; Schalm, 1982; de Man, 1988) can prevent or at least slow down the progression from severe chronic hepatitis to lethal liver disease. The outcome of more benign types of hepatitis is not influenced by such therapy.

A newly introduced and widely accepted classification system for hepatitis has now led to considerable improvement in the sense that the same terminology is

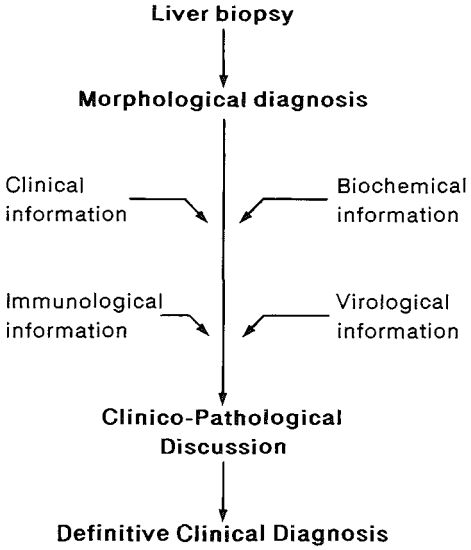
Fig.IV.2 *Representation of different histopathological expression patterns of hepatitis B infection and their mutual overlap in relation to duration of infection*



now used throughout the world, although all semantic problems have not yet been solved. This classification is based chiefly on histopathological criteria together with some clinical implications. However, histo-pathological classification is still prey to several everlasting difficulties (fig.IV.2) and so far, liver biopsies are only of moderate value for predicting the prognosis of the disease. This new classification system also uses temporal connotations such as acute, chronic and persistent. Because of the wide spectrum of histological changes in the various clinico-pathological categories, it would be of benefit to eliminate all temporal connotations in the histological description of liver disorders and to base classification on morphological changes only (lobular, portal, periportal hepatitis; Ludwig, 1977). The morphological alterations, supplemented with clinical, biochemical and pathogenetic information, should lead to the ultimate clinicopathological diagnosis. Therefore for a definite diagnosis, integration of clinical, biochemical, virological and (immuno)-histological data is indispensable (fig.IV.3).

In this chapter the histological variants of hepatitis B infection are described, using in principle the terminology propagated by the International Association for Study of the Liver and endorsed by the World Health Organization. In chapter V the material investigated will be grouped according to this classification system and discussed.

Fig.IV.3 *Scheme showing the path leading to a final diagnosis based on changes in a liver biopsy*



IV.2 CLASSIFICATION SYSTEM OF HEPATITIS.

The various categories of (viral) hepatitis are discussed under the following headings:

IV.2.1 ACUTE HEPATITIS

a. extent of necrosis
- acute hepatitis with focal necrosis
- acute hepatitis with bridging necrosis
- acute hepatitis with multilobular necrosis
- acute hepatitis with massive necrosis

b. stage of development
- early stage
- fully developed stage
- late stage
- residual stage

c cholestasis
- acute cholestatic hepatitis

IV.2.2 CHRONIC HEPATITIS

a. chronic persistent hepatitis

b. chronic active hepatitis
- mild
- moderate
- severe, including bridging hepatic necrosis

IV.2.3 CIRRHOSIS

IV.2.1 ACUTE HEPATITIS

In acute viral hepatitis there are hardly any diagnostic morphological features which distinguish it from other types of acute hepatitis (e.g. drug-induced hepatitis). Histopathological examination therefore does not play a significant role in the separation of acute viral hepatitis from other types of acute hepatitis. However, it can be of importance in the differentiation of acute hepatitis from other liver diseases that clinically resemble acute hepatitis (e.g. drug-induced cholestasis, biliary obstruction, acute exacerbation of chronic hepatitis).

The morphological picture of acute viral hepatitis, as described by many authors (Bianchi, 1971; Peters, 1975; Ishak, 1976; Phillips, 1981), is with rare exceptions (see below) predominated by changes in the hepatic lobuli. The characteristic light microscopic feature is that of a lobular hepatitis. In fact, from a functional point of view, acinar hepatitis would be a more appropriate designation (Rappaport, 1976) but, because it is not widely used, it will not be applied here. Although changes in the portal tracts are always present, they are in most cases far less evident than the changes in the lobuli. These lobular changes consist of:

a. degeneration and necrosis of liver parenchymal cells.
b. inflammatory infiltration.
c. mesenchymal reaction.

The mutual relationships and degree of these different aspects of acute hepatitis are related to the duration of the disease; they are helpful in roughly determining the stage of viral hepatitis (Bianchi, 1971). On the basis of these features we can distinguish an early, a fully developed, a late and a residual phase of acute hepatitis although delineation from one phase to the next is vague. In addition, based on the extent of necrosis of the liver parenchymal cells, some morphological variants of acute hepatitis are recognized:

- acute viral hepatitis with necrosis of solitary scattered hepatocytes.
- acute viral hepatitis with bridging necrosis.
- acute viral hepatitis with multilobular necrosis.
- acute viral hepatitis with massive necrosis.

Thus, two factors can be considered for the subdivision of acute hepatitis: the extent of necrosis and the stage of development (duration).

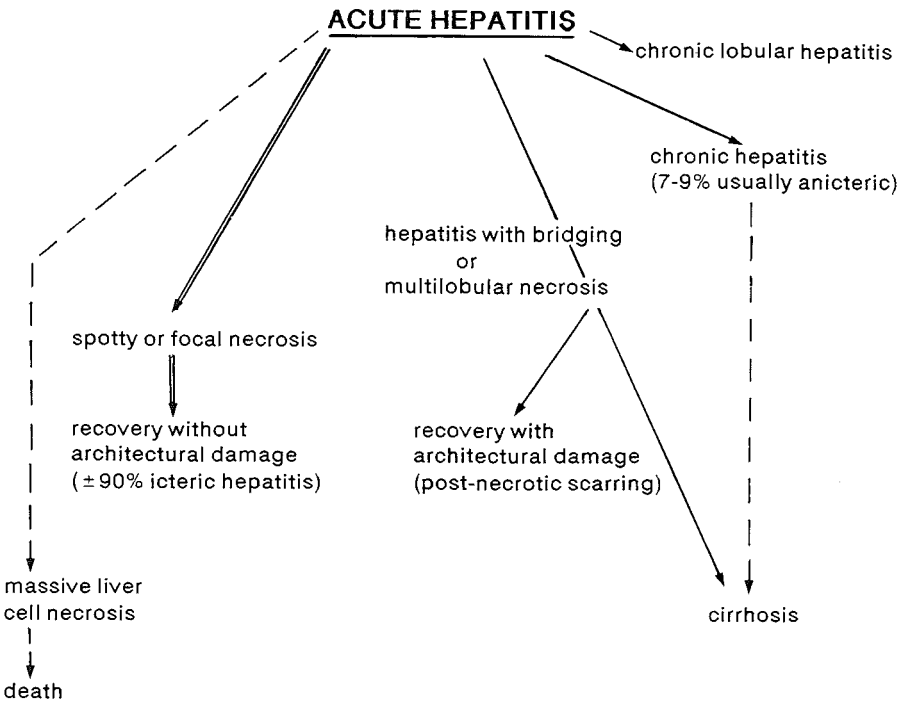
It will be clear that the degree of necrosis can determine the short-term prognosis of acute hepatitis as well as the post-hepatic sequelae. It is doubtful

whether extensive necrosis of the liver parenchyma in acute hepatitis is in some way associated with conversion into chronic hepatitis (Boyer and Klatskin, 1970). On the contrary, Sherlock (1976) demonstrated that lobular hepatitis without jaundice, i.e. hepatitis with a low degree of necrosis, is related to the development of a chronic hepatitis. The natural course of lobular (acute) hepatitis is variable, as shown in fig.IV.4.

The general morphological changes in acute viral hepatitis will be described separately for the lobules and portal tracks, followed by a short description of the various histopathological categories of acute hepatitis with respect to:

a. the extent of necrosis.
b. the stage of development.
c. cholestasis.

Fig.IV.4 *Scheme illustrating the various courses of an acute hepatitis B*



Lobular changes

a. Degeneration and necrosis of liver parenchymal cells.

An increased mitotic activity with the appearance of numerous binucleate hepatocytes is considered to be the earliest light microscopic feature of viral hepatitis (Bianchi, 1971).

Subsequently but preceding jaundice as a clinical sign of acute hepatitis, degeneration and necrosis of the liver cells dominate the morphological picture. Liver cell damage occurs partly in the form of hydropic swelling and partly in the form of eosinophilic shrinkage of hepatocytes. These changes are seen in all lobules, although to a variable degree.

The hydropic swelling of hepatocytes, the most striking feature of classical fully developed acute hepatitis, is found particularly around the hepatic venous radicles. At low power magnification it causes a typical picture of extreme centrilobular anisomorphism of the hepatocytes (fig.IV.5). These swollen cells, which are sometimes two to three times larger than normal, contain a finely granular, almost homogeneous, pale eosinophilic cytoplasm and a large nucleus with a prominent nucleolus (fig.IV.6). This aspect of the hepatocytes is known as "ballooning". It is believed that these changes arise from imbibition of fluid due to a disturbed membrane pump function and are, up to a point, reversible (Trump, 1975). However, hydropic degeneration often causes disruption of the cellular membrane, resulting in spillage of cell organelles into Disse's spaces and the sinusoidal lumen. These ballooned hepatocytes must be differentiated from so-called "feathery degenerated" hepatocytes (fig.IV.7). This feathery degeneration is due to cholestasis and may be found also in viral hepatitis, especially the cholestatic variant.

Eosinophilic degeneration, the other type of hepato-cellular damage, is characterized by cell shrinkage and deeply eosinophilic aspect of the cytoplasm (Kerr, 1971). In contrast to hepatocytes with ballooning degeneration, which are localized predominantly around the venous radicles, hepatocytes with eosinophilic degeneration are dispersed throughout the liver lobule. They lose their position in the hepatic cord (apoptosis) and are found in the sinusoids or Disse's spaces, partly phagocytized by Kupffer's cells, as rounded deep eosinophilic structures with or without nuclear remnants. They are known as acidophilic, apoptotic or free hyaline bodies (fig.IV.8). These bodies were first described by Councilman (1890) in livers from patients with yellow fever and since then often referred to as Councilman-like or Councilman bodies. Klion and Schaffner (1966) attributed these changes to dehydration and Trump (1976) explained them by loss of cellular water due to selective loss of potassium in the event of excess sodium gain.

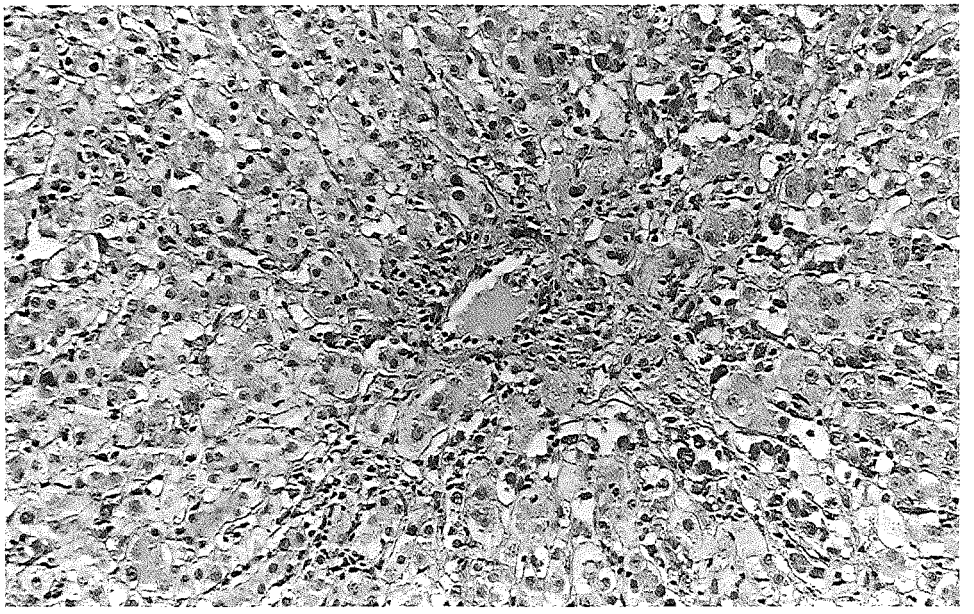
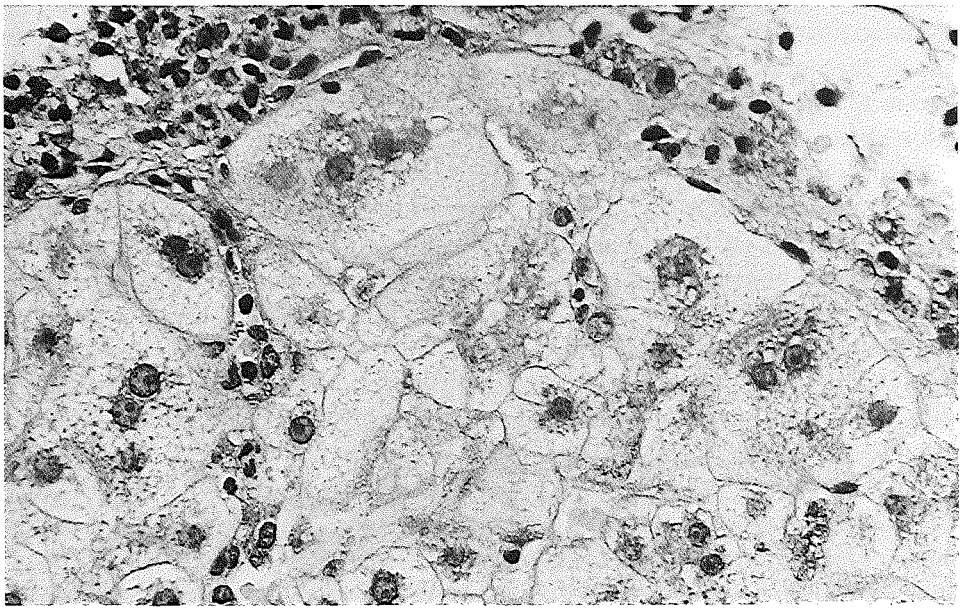


Fig.IV.5 *Acute hepatitis in the fully developed/late stage. Note the essentially centrilobular changes with small foci of collapse, the round-cell infiltrate and the hepatocellular disarray (H and A, 160x).*

Fig.IV.6 *Acute hepatitis. Hepatocytes with ballooning degenerative changes. Nuclei are large and reveal a prominent nucleus (H and A, 380x).*



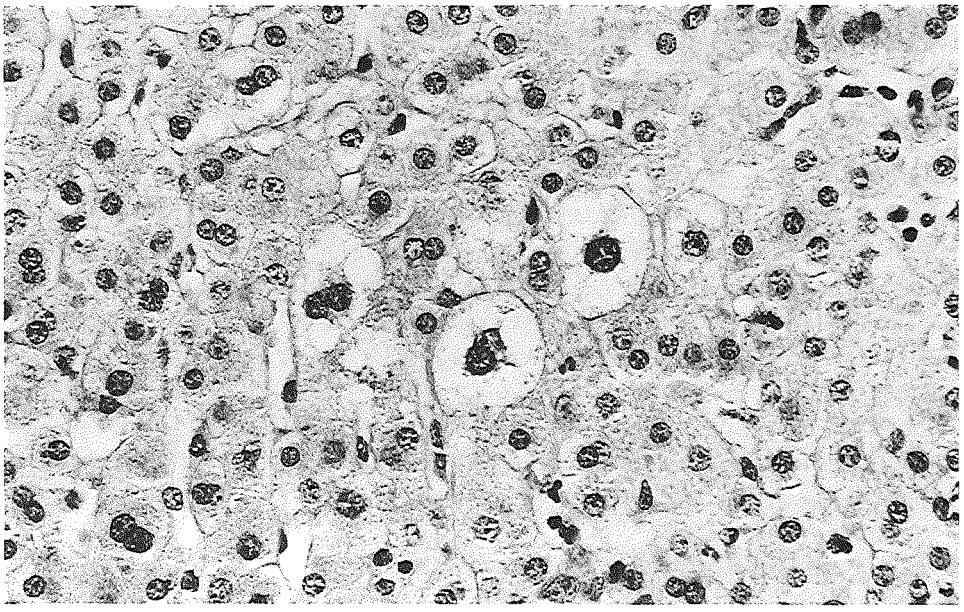
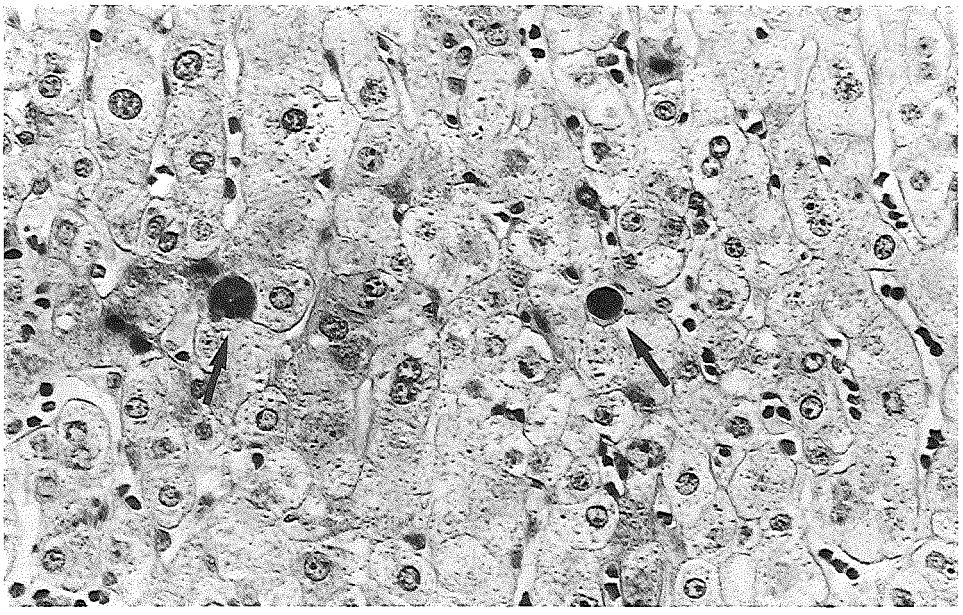


Fig.IV.7 *Liver tissue containing three hepatocytes with feathery degeneration of the cytoplasm (H and A, 380x).*

Fig.IV.8 *Acute hepatitis: scattered Councilman bodies (arrows) (H and A, 380x).*



Although most authors stress the frequent occurrence of Councilman bodies in acute viral hepatitis (Cavalli et al., 1968), they are not specific for viral hepatitis. However, the combination of Councilman bodies and hepatocytes with ballooning degeneration around the venous tributaries in particular is highly indicative of viral hepatitis. The hydropic swelling and the necrosis together with the regenerative activity of hepatocytes cause a distortion of the architecture of hepatic lobules due to loss of uniformity and thickening of the liver cell plates, particularly in the centrilobular regions. Often rearrangement of hepatocytes in acini can be found in these regions (fig.IV.9). In the event of necrosis of solitary hepatocytes minimal condensation of the reticulin skeleton occurs focally.

Necrosis of groups of hepatocytes in a relatively short time interval results in collapse of the reticulin framework in these areas (fig.IV.10). The degree of liver cell necrosis has implications for the post-necrotic sequelae e.g. cirrhose. No relation has been demonstrated between the degree of liver cell necrosis and transition to chronic liver disease.

b. Inflammatory infiltration.

In acute hepatitis the infiltrate within the lobuli is usually mild to moderate and consists of small aggregates of lymphocytes and monocytes, especially in the centrilobular regions (fig.IV.11). Polymorphonuclear leukocytes are scanty, in contrast, for example, to the many seen in alcoholic and drug-induced acute hepatitis. In the event of confluent necrosis the inflammatory infiltrate is not impressive: it is usually less marked than that seen in classical lobular hepatitis (Peters, 1975).

c. Mesenchymal reaction.

In classical acute hepatitis, liver cell necrosis is followed by a significant mesenchymal reaction, which comprises activated macrophages, Kupffer's cells and endothelial lining cells. These cells occur as solitary elements or as aggregates together with some lymphocytes and polymorphonuclear leukocytes in the vicinity of a collapsed reticulin skeleton. Both the enlarged Kupffer's cells and macrophages and the swollen endothelial cells may virtually occlude the sinusoidal lumen, thereby presumably causing changes in the microcirculation. The macrophages, situated chiefly in the centrilobular areas, are loaded with irregular granules of yellowish brown pigment, representing the desintegrated and phagocytized parts of necrotic liver cells (fig.IV.11). Sometimes necrotic hepatocytes or acidophilic bodies are seen in the cytoplasm of these macrophages. This pigment, one of the lipofuchsin pigments (glycoproteins), is known as ceroid

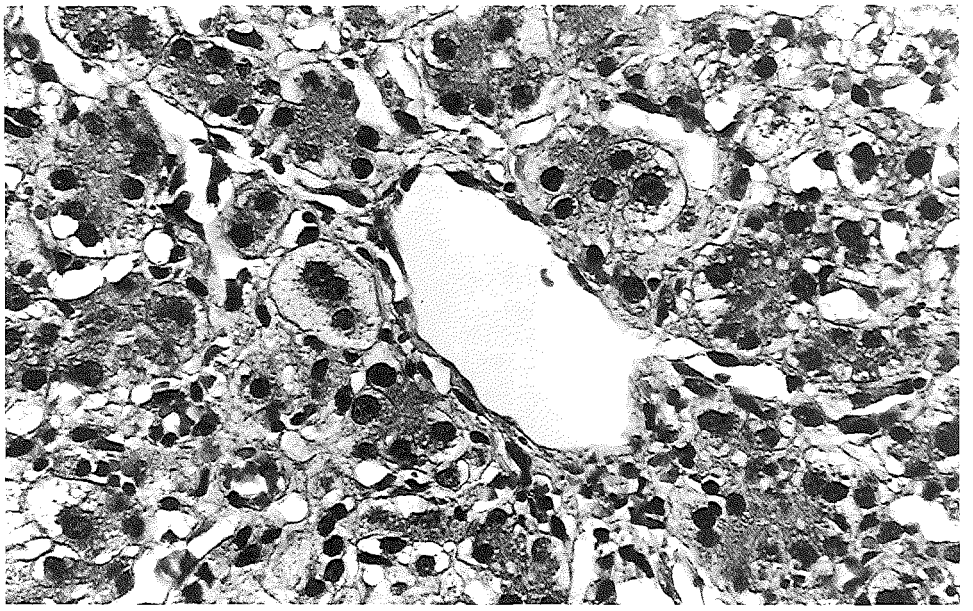
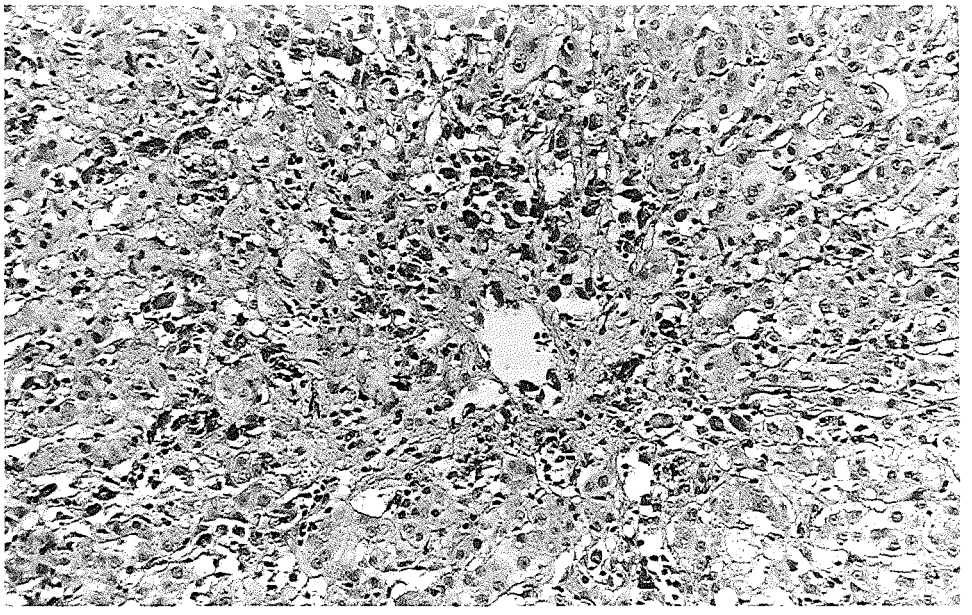


Fig.IV.9 *Acute hepatitis: centrilobular area with marked hepatocellular disarray; cytoplasmic and nuclear anisomorphism, multinucleation and acinar rearrangement; mononuclear infiltrate (H and A, 380x).*

Fig.IV.10 *Acute hepatitis: centrilobular necrosis of hepatocytes. Note accumulation of ceroid-containing macrophages (PAS after diastase, 160x).*



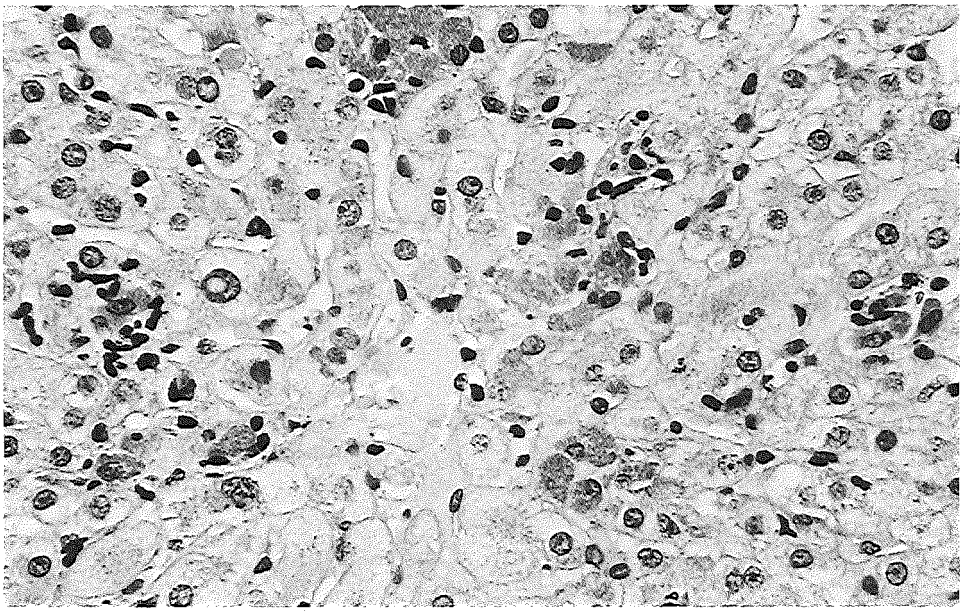


Fig.IV.11 *Acute hepatitis in the fully developed/late stage. Note the large clusters of macrophages, Kupffer's cells loaded with ceroid pigment and lymphocytes (PAS after diastase, 380x).*

pigment. The periodic acid Schiff reaction after diastatic pretreatment and aldehyde thionin stain demonstrate the presence and quantity of this pigment very well. Under the fluorescence microscope this ceroid pigment exhibits a bright auto-fluorescence. At a later stage, pigment-loaded macrophages are believed to move from the site of liver cell necrosis to the portal areas, where they can be found in large quantities.

The distribution and location of these pigment-loaded macrophages play an important role not only in determination of the stage of acute hepatitis but also in differentiation from so-called reactive hepatitis. Reactive hepatitis is believed to be the result of extra-hepatic events, in which the mesenchymal compartment of the liver (Kupffer's cells, endothelial lining cells) plays a role in the clearance and breakdown of necrotic material and polluting elements transported in the blood from outside the liver, especially from the intestinal tract.

In cases of reactive hepatitis Kupffer's cells are diffusely scattered throughout the lobuli in contrast to acute viral hepatitis in which pigment macrophages occur in clusters (fig.IV.12). In acute viral hepatitis the macrophages may also contain some iron pigment in addition to the ceroid pigment. Although the presence of iron pigment is considered by some authors to be an essential feature of acute

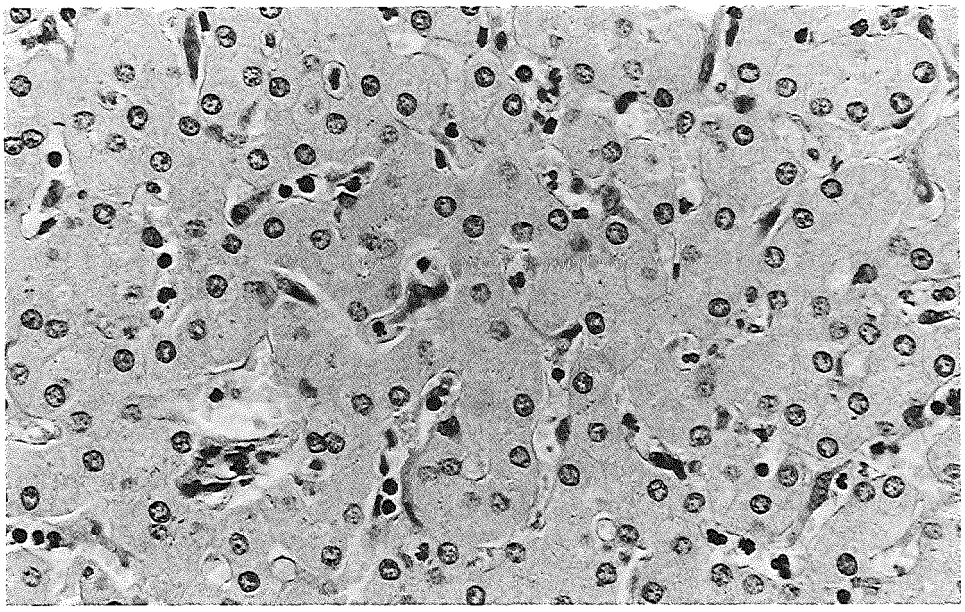


Fig.IV.12 *Reactive non-specific hepatitis. Diffuse activation and swelling of Kupffer's cells. Increase in inflammatory infiltrate in sinusoids. Only minimal changes in hepatocytes (PAS after diastase, 380x).*

viral hepatitis (Scheuer, 1980), the quantity differs widely from case to case. In most cases of acute viral hepatitis in our series, no iron pigment could be detected at all. The number of activated macrophages in classic lobular hepatitis is striking, but the degree of hepatocellular necrosis is not always proportional. In some cases of extensive necrosis, relatively few pigment macrophages are seen. Probably the degree and rate of necrosis in the liver is so great that before the macrophages are sufficiently activated, the cell debris is carried off in the blood stream, which can be a factor in cerebral dysfunction.

Portal changes

In all types of acute viral hepatitis, portal changes are present but less prominent than the lobular changes. The portal tracts show some expansion due to edema of the portal connective tissue and the presence of an inflammatory infiltrate. This infiltrate consists of lymphocytes, histiocytes, some plasma cells and polymorphonuclear leukocytes. Eosinophilic granulocytes, which are considered by most authors to be an indication of drug-induced hepatitis, are sometimes present in strikingly large quantities. Although the bile ducts are

normally intact, changes in the epithelium of the interlobular bile ducts may be present as well (fig.IV.13). Poulsen (1969) pointed out changes in the bile ducts in some cases of viral hepatitis, which transform into chronic active hepatitis. These bile duct changes sometimes cannot be distinguished from the changes seen in early primary biliary cirrhosis. In contrast, Schmid (1972) noted bile duct changes resembling those of mechanical obstruction of the extra-hepatic bile ducts in nine of 109 cases of viral hepatitis with "submassive" necrosis. In acute cholestatic viral hepatitis (see below) marked proliferation of the cholangioles is sometimes seen. The lumen is often dilated and may contain bile, and the wall is infiltrated with neutrophils.

In acute hepatitis the portal infiltrate can sometimes extend deep into the lobular parenchyma which then exhibit the features of periportal (chronic) hepatitis (fig.IV.14). In these cases a diagnosis of chronic hepatitis can mistakenly be made by the pathologist. However, the simultaneous presence of lobular changes and the lack of portal fibrosis in these cases should prevent the pathologist from making this wrong diagnosis. In drug-abusers, in particular, a prominent portal infiltrate can be seen.

Various categories of acute hepatitis

IV.2.1.a **Extent of necrosis**

The degree and rate of liver cell necrosis in acute hepatitis vary from patient to patient. The cause of this variability is not known exactly, but it may be the result of variations in either the virulence of HBV or immunological defense mechanisms against the virus or viral products. In general it appears that the stronger and faster a patient reacts to the virus or viral products, the faster the virus is eliminated with less chance of the development of a chronic hepatitis. On the other hand an explosive reaction to the virus infection, reflected in extensive necrosis and fulminant hepatitis, can result in life-threatening situations. Moreover the extent of necrosis is certainly of significance for the short-term prognosis which then depends on the regenerative capacity of the liver parenchyma.

IV.2.1.a.1 Focal necrosis

In focal necrosis, multiple small foci of liver cell necrosis are seen, usually concentrated around the central venous tributaries, with some condensation of the reticulin framework in this area. This type of acute hepatitis, together with the type in which necrosis of only scattered solitary liver cells is seen, is known as classical lobular hepatitis. This histological type of acute hepatitis usually runs a

favorable self-limiting course with few or no residual sequelae after recovery.

Clinically this type of acute hepatitis will run an icteric or anicteric course. A small number of cases with icteric acute hepatitis turn into a chronic form of hepatitis (7-9 per cent; Redeker, 1975). The percentage of patients with acute anicteric hepatitis who develop a chronic form of hepatitis is unknown because anicteric or subclinical cases unusually remain unrecognized. Sherlock (1976), however, stressed that an anicteric acute hepatitis is more often followed by chronic hepatitis than an icteric acute hepatitis, and indeed many patients with chronic hepatitis have no history of jaundice.

IV.2.1.a.2 Bridging necrosis

In bridging necrosis, described by Boyer and Klatskin (1970) as subacute hepatic necrosis, the degree of confluent necrosis is such that portal tracts and central veins as well as mutual central veins are linked together by necrotic areas: septa of collapsed reticulin (so-called passive septa) run from central veins to portal tracts (central-portal bridging) and from central veins to central veins (central-central bridging). Theoretically these septa of collapsed reticulin form a scaffold for the later development of fibrous septa. This histological type of hepatitis sometimes follows a fatal course, especially in older patients. In younger patients the outcome is usually quite favorable with or without so-called post-hepatic scarring or cirrhosis (Boyer and Klatskin, 1970). It should be mentioned that bridging necrosis can also be a phenomenon of a severe type of chronic active hepatitis. In these cases the hepatitis generally follows an insidious clinical course. To differentiate between the collapse of liver parenchyma seen in acute hepatitis and the development of active septa with new fiber formation in chronic hepatitis, orcein and special collagen staining methods can be helpful (Scheuer, 1980).

IV.2.1.a.3 Multilobular necrosis

In multilobular necrosis all, or nearly all, hepatocytes in adjacent lobuli are destroyed (fig.IV.15). The reticulin skeleton in these lobuli is condensed but the architecture remains in principle intact. The condensed reticulin skeleton shows the location of the pre-existing liver cell plates and provides the framework for a complete regeneration of the lobulus. The portal tracts stand close together, separated by the collapsed lobuli that contain variable amounts of Kupffer's cells and macrophages. The portal tracts show changes, the degree of which depends on the rate of liver cell necrosis. The essentially intact liver architecture is best visualized by reticulin stains. In addition to hepatic lobuli with a complete absence of hepatocytes, lobuli with varying degrees of liver cell necrosis can be found.

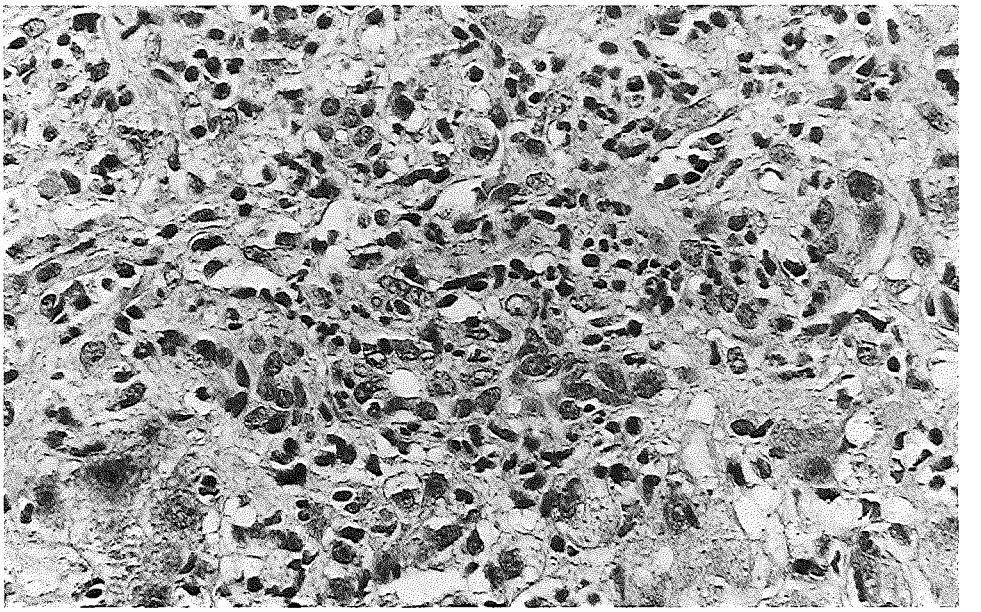
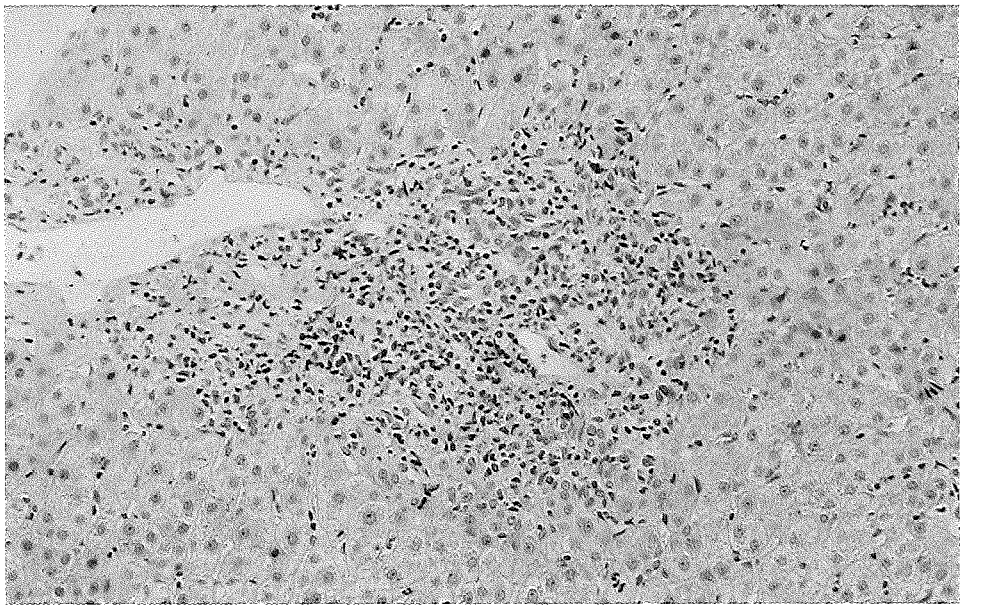


Fig.IV.13 *Acute self-limiting hepatitis: portal tract with cholangiolitis and slight bile duct proliferation (H and A, 380x).*

Fig.IV.14 *Acute self-limiting hepatitis: lobular changes and an unusual pronounced portal infiltrate (H and A, 150x).*



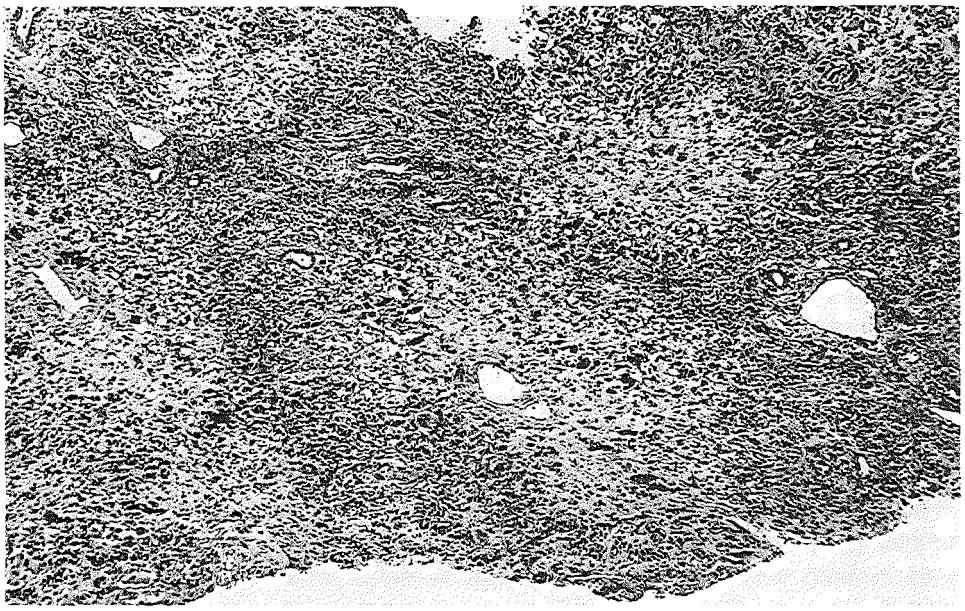
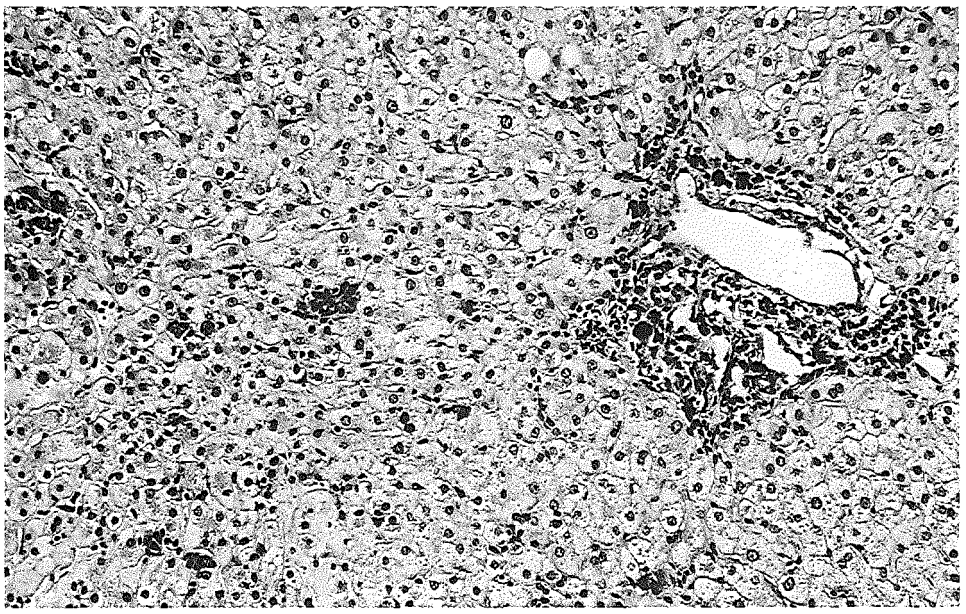


Fig.IV.15 *Multilobular necrosis; architecture is in principle intact. Note portal tracts with extensive periportal fibrosis; centrilobular collapse (H and A, 60x).*

Fig.IV.16 *Acute hepatitis (late stage): lobular changes with clusters of ceroid-containing macrophages; portal tracts with lymphoplasmacellular infiltrate and ceroid-containing macrophages (H and A, 150x).*



Sometimes duct-like structures emanating from the portal areas in particular can be seen. They are regarded as proliferating bile ducts or as pseudobile ducts.

Clinically the multilobular type of viral hepatitis often presents as a fulminant hepatitis. Fulminant hepatitis is a syndrome of hepatic failure and coma within eight weeks of the onset of the illness, as defined according to the criteria of the fulminant hepatic failure surveillance study (Trey, 1972). It must be stressed that most patients with fulminant hepatitis die from complications (hemorrhage, cerebral edema, sepsis) and that in only a minority of the cases death can be attributed solely to hepatic failure (Gazzard, 1975). A substantial proportion of all cases of fulminant hepatitis are non-A non-B hepatitis (Gimson, 1983). Acute fulminant hepatitis accounts less than 1 per cent of all cases of acute hepatitis caused by HBV and has a slightly better prognosis compared to both acute fulminant hepatitis caused by toxic dyes and hepatitis non-A non-B virus (Gimson, 1983). In addition to a fatal outcome, cirrhosis is also frequently encountered (Redeker, 1974). In this type of acute viral hepatitis the prognosis is slightly better for younger patients than for older patients. Peters (1975) stressed that younger patients who survive fulminant hepatitis rarely, if ever, develop cirrhosis or chronic hepatitis. Older patients appear to have an impaired regeneration.

IV.2.1.a.4 Massive necrosis

The differential diagnosis between acute hepatitis with multilobular necrosis and acute hepatitis with massive necrosis is based only on the number of lobuli with complete hepatocellular necrosis. Massive necrosis is not compatible with life. The diagnosis massive necrosis can be assumed on the basis of a liver biopsy but not established with certainty, because a liver with extensive pan-lobular necrosis may include some circumscribed areas that have remained intact.

IV.2.1.b **Stages of development of acute viral hepatitis**

This information is based on the presence and mutual relationships of liver cell necrosis and mesenchymal reaction. The histological pattern, can provide information on the stage of development of acute hepatitis (Bianchi, 1971). However, the stage of development cannot be translated into an exact duration of the acute hepatitis because the rate of progression of hepatitis varies markedly between patients. Early, fully developed, late and residual stages of acute hepatitis can be distinguished.

IV.2.1.b.1 Early stages

Early stages of acute hepatitis may be seen occasionally. The histological changes have been studied in experimentally infected monkeys (Dienstag, 1976) and are sometimes encountered in biopsies obtained from HBsAg seropositive individuals during the incubation period of hepatitis B, before the onset of clinical acute hepatitis (see chapter XI). Liver cell necrosis, especially around the central or terminal veins, together with some proliferation and swelling of sinusoid lining cells and Kupffer's cells are the hallmarks of this stage.

IV.2.1.b.2 Fully developed stage

Fully developed stage acute hepatitis is characterized by the simultaneous presence of liver cell necrosis and focal aggregations of macro-phages and Kupffer's cells loaded with ceroid pigment (pigment macrophages) within the areas of necrosis.

IV.2.1.b.3 Late stage

In the late stages the accumulations of swollen, pigment-loaded Kupffer's cells and/or macrophages are more impressive than the prevalence of necrotic liver cells, which are scarce. The Kupffer's cells and macrophages are seen both adjacent to earlier liver cell necrosis, as indicated by focal reticulin collapse, and in portal tracts (fig.IV.16). Later on, pigment macro-phages in the portal areas dominate the histological picture. During this stage the remaining inflammatory infiltrate is more impressive, partly due to the lack of necrotic hepatocytes and the decreased number of pigment macrophages in the lobuli. It must be noted that in acute hepatitis genuine piecemeal necrosis (see CAH) is a rare event and proliferation of fibroblasts is inconspicious.

IV.2.1.b.4 Residual stage

The residual stage is characterized by a slight round-cell infiltrate together with a few macrophages in the portal tracts. These changes persist for a long time, up to a year. Aspecific reactive hepatitis and sometimes chronic persistent hepatitis must be included in the differential diagnosis for this stage. If the portal infiltrate of the residual stage of acute hepatitis is marked, the possibility of transition to chronic hepatitis must be considered and careful follow-up, eventually with a liver biopsy, is indicated.

IV.2.1.c Cholestasis

Acute cholestatic viral hepatitis is in principle a clinical variant. Differences in cholestasis in liver biopsies are difficult to interpret and are dependent on the mode and duration of fixation of the liver tissue. Moreover the severity of jaundice and the quantity of bile pigment in liver biopsies do not always run in parallel. However, cholestasis in liver biopsies is sometimes striking and biliary obstruction must be included in the differential diagnosis. As a rule, the diagnosis is not too difficult, because there are usually few pigment macrophages in biliary obstruction and bile ducts are more severely affected by polymorphonuclear leukocytes. In later stages of biliary obstruction extensive liver cell necrosis can also be caused by the storage of toxic bile pigments. In that case the number of macrophages is more striking. Differentiation of cholestatic viral hepatitis from drug-induced cholestasis may cause more often problems. Bile plugging in the canaliculi is more prominent in drug-induced cholestasis which may help in the differential diagnosis.

The clinical symptoms of acute cholestatic hepatitis last in general much longer (up to 5 months) than those of classical acute hepatitis, sometimes causing the clinician to worry if he is not familiar with this type of hepatitis. Although cholestasis may be marked in the liver biopsy, the histological picture includes the features of classical lobular hepatitis. The prognosis for this type of hepatitis is good and transition to chronic hepatitis occurs only sporadically.

Finally it must be stressed that simultaneous occurrence of acute viral hepatitis and other cholestatic liver diseases may be present; this event must be considered especially in liver grafts in hepatitis B positive recipients.

IV.2.2 CHRONIC HEPATITIS

Chronic hepatitis can be defined as an unresolved inflammation of the liver. This inflammation can be initiated by several processes, including a reaction to hepatitis viruses. The term chronic hepatitis covers a wide spectrum of clinical, morphological, etiological and immunological aspects. Lacking a better criterion, hepatitis is arbitrarily regarded as "chronic", when clinical and/or biochemical changes are present for *more than six months* (Sherlock, 1974). In the event of a hepatitis B infection the duration of the hepatitis can be determined by the presence of hepatitis B surface antigen (HBsAG) in serum.

However, it is well known that HBsAg can persist in serum without the biochemical changes and the clinical signs of a liver disease. In these cases the

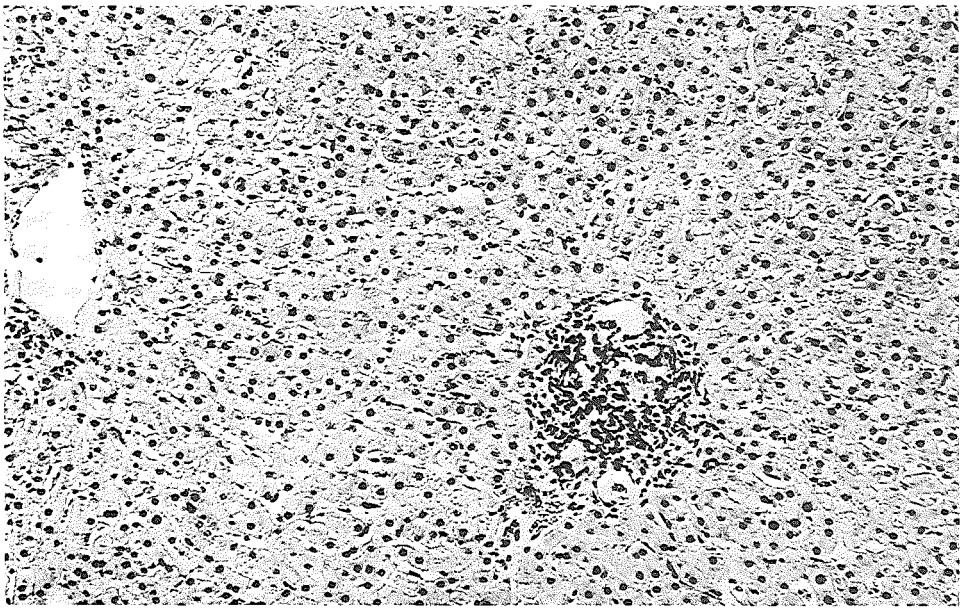
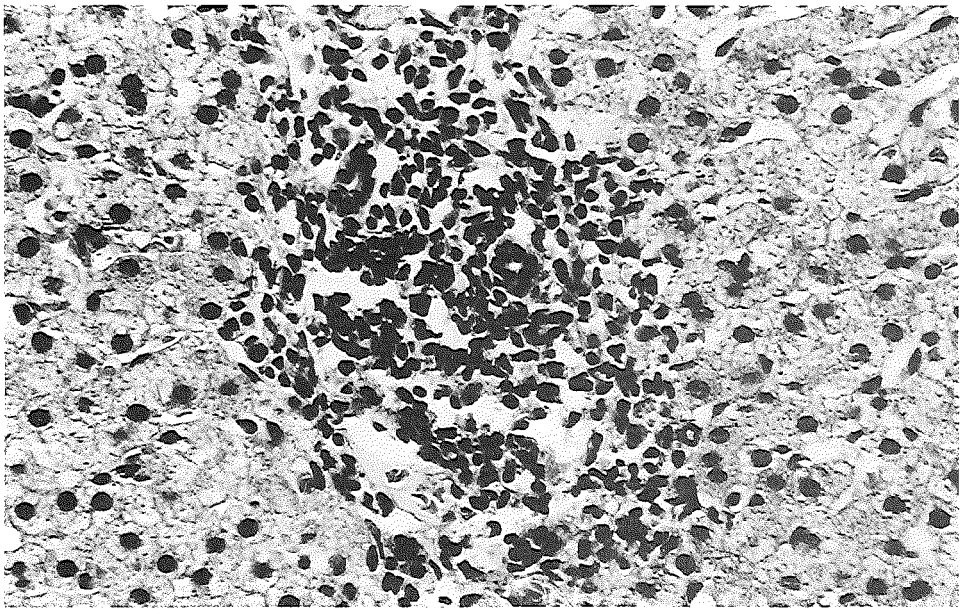


Fig.IV.17 *Chronic persistent hepatitis: dense compact portal infiltrate. Minimal lobular changes (H and A, 90x).*

Fig.IV.18 *Chronic persistent hepatitis; no fibrosis; spill-over (H and A, 380x).*



infection can be associated with two different microscopic features:

a. Minimal lesions without an inflammatory reaction.
b. A portal inflammatory infiltrate consistent with the diagnosis of aspecific reactive hepatitis or chronic persistent hepatitis.

Chronic viral hepatitis (type B) may also be defined as an inflammatory liver disease with persistence of HBsAg in the serum for more than 6 months. This chronic inflammation can occur with or without biochemical changes. The definition of chronic hepatitis is easy to use when the illness starts with an acute attack but awkward when the onset is insidious. In these latter cases a careful 6-month follow-up is indicated, especially because the morphological features of both the residual phase of acute viral hepatitis and acute viral hepatitis in drug-abusers and children resemble those of chronic hepatitis in many aspects.

In general, chronic hepatitis is regarded as a difficult disease entity. This difficulty is both indicated and partly caused by the complex and constantly changing terminology. Desmet (1972) listed, for example, 47 different terms related to chronic hepatitis. In the past each investigator in this field introduced his own nomenclature, based sometimes on special views concerning the etiology and pathogenesis of the disease and sometimes on special aspects of the clinical presentation. Classification systems and subdivisions are based on various concepts, using intermingling combinations of etiological, clinical, morphological and immunological parameters. Most of these parameters are highly variable during the course of the disease and as a rule exhibit only a partial overlap. Therefore it will be impossible to create a single classification system that is valid for clinical, biochemical, etiological and morphological parameters. Although some authors therefore prefer not to split chronic hepatitis into subgroups, the variable natural course of chronic hepatitis and the resulting variations in the need for and type of therapeutic intervention make classification of chronic hepatitis inevitable.

In 1968 a new, widely accepted and simplified classification system was introduced by de Groote et al. Classification is based on morphological features as well as some clinical aspects and prognostic implications.

Chronic hepatitis with its wide spectrum of morphological features is essentially divided into two categories:

Chronic persistent hepatitis (CPH), which is characterized by portal inflammation (portal hepatitis).
Chronic aggressive hepatitis (CAH), which is characterized by periportal inflammation (periportal hepatitis).

The prefix "aggressive" was later changed to "active", not least because of the anxiety which the term "aggressive" evoked in patients when they heard or read the diagnosis of their disease. **Chronic active hepatitis** in turn was subdivided into the moderate and severe types. Later Popper and Schaffner (1971) added another subgroup to the classification of chronic hepatitis also defined by its histological changes: **chronic lobular hepatitis** (CLH). This type of hepatitis is characterized by the same lobular changes seen in acute hepatitis but now on a chronic time-scale. Nevertheless the international group for standarization of the definitions, nomenclature and diagnostic criteria for liver disease (1976) did not include CLH in their classification scheme since they regarded CLH as a protracted acute hepatitis. This interpretation of CLH is however in conflict with the above-mentioned definition of chronic hepatitis, i.e. an inflammatory liver disease of more than 6 months duration. This definition must then be extended to include: "with the histological features of portal or periportal hepatitis". More recently, the importance of the presence of lobular changes, especially necrosis, for the prognosis of chronic hepatitis has been stressed (Sheuer, 1986).

In contrast to acute hepatitis B, which exhibits no pathognomonic light microscopic features, chronic hepatitis caused by hepatitis B virus can be characterized by changes in the cytoplasm of hepatocytes known as "ground-glass hepatocytes" (see chapter VII) (Hadziyannis, 1973; Shikata, 1974). Using immunochemical techniques these hepatocytes have been proven to contain abundant HBsAg (see chapter VI). The appearance of these "ground-glass" hepatocytes and their significance for the diagnosis of hepatitis B will be discussed in chapter VII.

IV.2.2.a **Chronic persistent hepatitis (portal hepatitis)**

Chronic persistent hepatitis (CPH) is characterized by an inflammatory infiltrate, usually confined to somewhat enlarged portal tracts (portal hepatitis, fig.IV.17). This infiltrate consists of lymphocytes and histiocytes, admixed with some plasma cells and eosinophilic and neutrophilic polymorphonuclear leukocytes. The degree of infiltration varies from portal tract to portal tract and when dense, extension of the inflammatory infiltrate into periportal liver parenchyma can be seen (spill-over, fig.IV.18). Although the boundary between portal areas and parenchyma is sometimes blurred by the overlying inflammatory infiltrate, the so-called limiting plate is basically intact. Periportal liver cell necrosis should not be present in CPH. Some macrophages, loaded with ceroid pigment, can be found in the portal tracts (Scheuer, 1986). Lobular changes are minimal and consist of some small foci of mesenchymal cells, including Kupffer's cells and macrophages loaded with ceroid pigment. Sporadically a degenerating

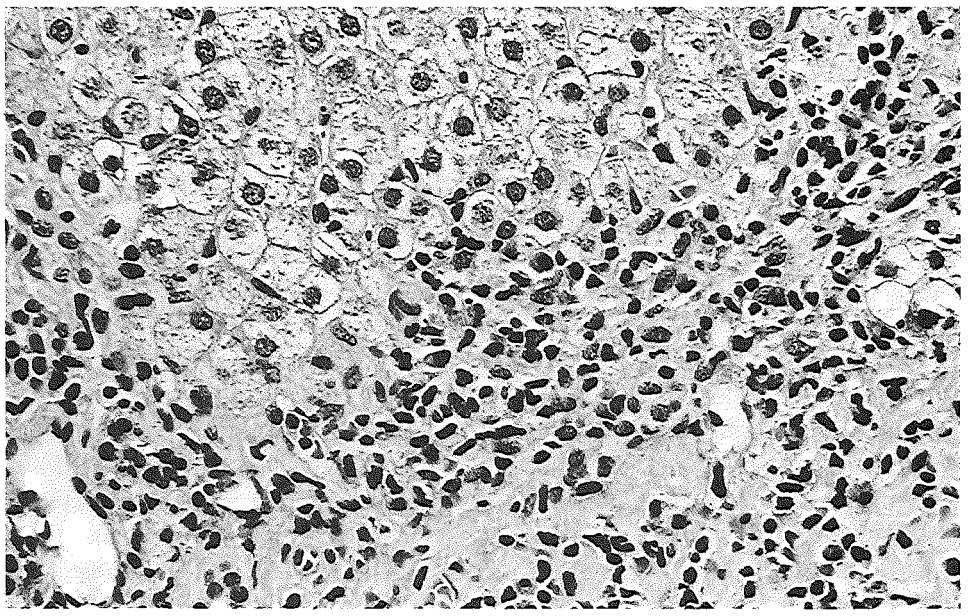
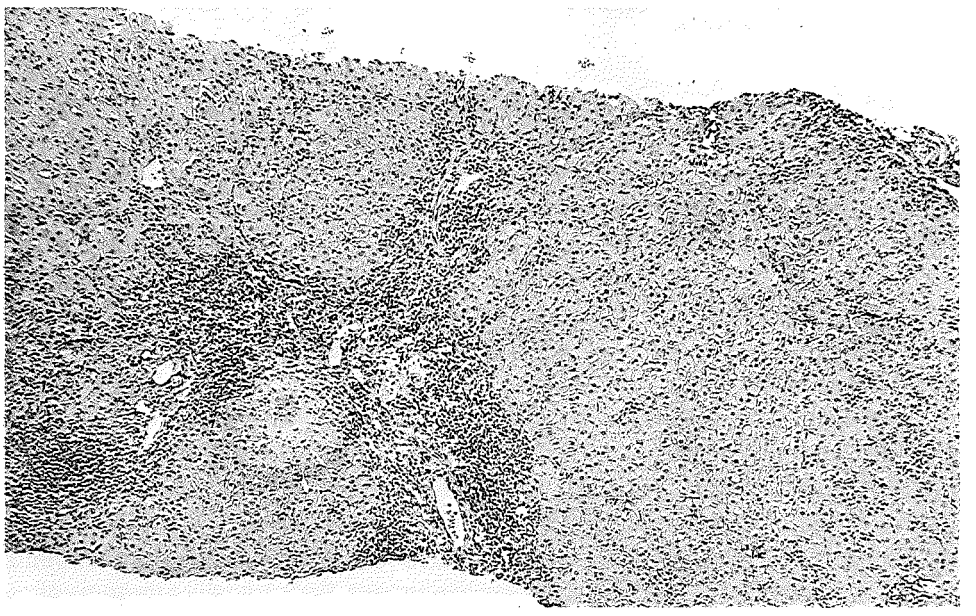


Fig.IV.19 *Chronic active hepatitis: irregular boundaries of the enlarged portal tracts (H and A, 380x).*

Fig.IV.20 *Chronic active hepatitis: architectural distortion with active septum formation and porto-portal septa (H and A, 60x).*



hepatocyte can be seen. In general fibrosis is absent or minimal. The presence of a larger degree of fibrose indicates a more severe type of hepatitis in the past. The good prognosis for CPH is the justification for distinguishing it from CAH (Chadwick et all., 1979). The disease requires no therapeutic intervention. The lesion remains static over long periods; in many cases the inflammatory infiltrate subsides after HBe seroconversion (Sánchez-Tapias, 1984). Occasionally spontaneous transition to chronic active hepatitis has been reported. However, transition of liver abnormalities that resemble CPH during treatment (immunosuppressive; anti-viral) to those of chronic active hepatitis after withdrawal of the therapeutic agent often takes place (Vido, 1969). Acute exacerbation of chronic HBV infections, with the histological features of chronic persistent hepatitis, can occur:

1. during transition of a high to a low rate of viral replication with serum conversion from HBeAg to anti-HBe (Liaw et all., 1983; 1985; Sánchez-Tapias, 1984.).
2. during reactivation of hepatitis B infection (Davis and Hoofnagle, 1985).

It must be stressed that the histological picture of portal hepatitis, as seen in CPH, covers several clinicopathological entities (table IV.1) (Ludwig, 1977).

Table IV.1 *Differential diagnoses for portal hepatitis.*

Morphological diagnosis	Definite clinicopathological diagnosis
portal hepatitis	- chronic persistent hepatitis - aspecific reactive hepatitis - residual of acute hepatitis - chronic active hepatitis (light) - primary biliary cirrhosis - lymphoproliferative malignancy

The spectrum of liver changes in CPH, borders upon CAH on one side, on the other side upon reactive hepatitis and residual phase of acute hepatitis, which can be persist for long times (fig.IV.2). Differentiation between CPH and CAH, reactive hepatitis and residual phase of acute hepatitis may sometimes be difficult. This difficulty is aggravated by the occasional finding that some portal tracts or some areas of the liver show the typical histological features of CAH, whereas other portal tracts or other areas of the liver are characterized by the

the features of CPH (Dudley, 1972). Small liver biopsies in particular, can therefore give rise to sampling errors and may result in the wrong diagnosis.

If specific features of hepatitis B (ground-glass cells) are lacking, leukemic infiltration and primary biliary cirrhosis stage I also have to be included in the differential diagnosis.

IV.2.2.b **Chronic active hepatitis (periportal hepatitis)**

On one side of the histological spectrum of chronic hepatitis, chronic active hepatitis (CAH) borders CPH and on the other side cirrhosis (fig.IV.2). Sharp demarcations between these various groups do not exist, leading to problems in diagnosis as well as differences in interpretation between different observers. CAH can be distinguished from CPH by the presence of "piecemeal necrosis", a widely accepted term introduced by Paronetto et al. (1962). The characteristics of this phenomenon are a periportal inflammatory infiltrate combined with periportal liver cell necrosis, resulting in erosion of the limiting plate. This periportal inflammatory infiltrate, which mainly consists of lymphocytes and plasma cells, bulges irregularly into the liver parenchyma and surrounds single and small groups of hepatocytes (fig.IV.19). During this process hepatocytes may show eosinophilic and sometimes hydropic degeneration. The periportal areas also exhibit the aspect of a liver cell-"eating" infiltrate. This "piecemeal necrosis" is the hallmark for diagnosis of chronic active hepatitis.

The term piecemeal necrosis was coined by Cohen (1960) in one of the first immunohistochemical studies on the liver in which τ-globulin was visualized; he associated τ-globulin with the necrosis of single and small groups of hepatocytes, which were replaced by lymphocytes, plasma cells and histiocytes. In view of the composition of the infiltrate and the presence of τ-globulins, Paronetto (1962) suggested that immunological processes caused the necrosis of the hepatocytes. In contrast to the inert infiltrate in CPH, the behavior of the inflammatory infiltrate in CAH towards the liver parenchyma is "aggressive" or "active". As a result, in CAH most of the inflammatory infiltrate is found at the boundary between the connective tissue of the portal tracts and the liver parenchyma. In the larger portal tracts in particular this typical localization is easily visualized because the central areas of these portal tracts are nearly free of infiltrate. However, sometimes lymphoid follicles may be present in these larger portal tracts. Following periportal liver cell necrosis, the necrotic areas are replaced by connective tissue. In this process, known as "active septum formation", portal tracts show irregular enlargement due to the newly formed periportal connective tissue which radiates out into the liver parenchyma, ultimately giving rise to pronounced architectural changes (fig.IV.20). During the periportal increase of connective tissue, enlarging

the pre-existing portal tracts, solitary and small groups of anisomorphic hepatocytes may become isolated within this connective tissue. As a result of regeneration these isolated groups of hepatocytes can form so-called regeneration nodules. Together active septa formation, that links portal tracts together, and regeneration of groups of isolated hepatocytes initiate the process of cirrhotic transformation of the liver.

Although the periportal events are the most striking feature in CAH, centrilobular changes are always present to varying degrees. So far as, the outcome of chronic hepatitis is concerned the centrilobular changes in particular are assumed to be important (Popper, 1983). In the hepatic lobuli hepatocytes show cellular and nuclear anisomorphism and acinar transformation, mainly in the periportal areas. The liver cell plates become irregular and broadened. In contrast to acute hepatitis only small numbers of pigmented macrophages are present.

Distinction of subgroups of CAH is based on the spread of the inflammatory infiltrate into the hepatic lobuli and the degree of periportal and lobular liver cell necrosis. In rapidly progressing chronic hepatitis the formation of connective tissue does not keep pace with the rate of liver cell necrosis. In these cases marked periportal and lobular reticulin collapse can occur, sometimes in combination with either porto-portal or porto-central bridging necrosis. In the event of necrotic bridge formation, known as "bridging hepatic necrosis" (BHN) or "subacute hepatic necrosis" (SHN), differentiation between chronic hepatitis with bridging necrosis and acute hepatitis with bridging necrosis is difficult. In such cases the diagnosis CAH can only made when other features of chronic hepatitis, such as piecemeal necrosis and periportal fibrosis, are present. As a result of these problems indifferential diagnosis bridging hepatic necrosis has been considered a phenomenon of both acute and chronic hepatitis. While Boyer and Klatskin (1970) first described bridging necrosis as a feature of acute viral hepatitis in a series of patients with a poor prognosis (hepatic failure; cirrhotic transformation), bridging necrosis was later considered to be a feature of CAH (Baggenstoss, 1972; Boyer, 1976).

A international working party on standardization of definitions, nomenclature and diagnostic criteria in liver disease included bridging necrosis in the entity CAH (US Government Printing Office 1976). Clinically, bridging necrosis is associated with either overt liver disease with jaundice or insidious grumbling liver disease without jaundice.

Categories of chronic active hepatitis

In their report, de Groote et al. (1968) subdivided CAH into two categories, designated as moderate and severe. Later (1976) BHN was presumed to be a

feature of CAH and subdivisions were based on this: CAH with and CAH without bridging necrosis. On the basis of prognostic and therapeutic consequences, in this thesis CAH is divided into three categories:

1. chronic active hepatitis (mild).
2. chronic active hepatitis (moderate).
3. chronic active hepatitis (severe) with or without bridging necrosis.

It must be stressed that CAH runs an undulating course with transient marked changes in activity. Moreover different areas in the liver can show differences in CAH activity, resulting in underestimation or (less likely) overestimation of the real activity of the underlying chronic hepatitis.

IV.2.2.b.1 Chronic active hepatitis (mild)

In chronic active hepatitis (mild), there are few or no changes in lobular architecture. However, in several places destructionand irregularities of the limiting plate are present. The periportal inflammatory infiltrate, consisting of plasma cells and lymphocytes, reaches not deeper into the liver parenchyma than five layers of hepatocytes. The portal tracts are slightly distended in a web-like manner due to periportal fibrosis. In many places the pattern of chronic persistent hepatitis can be seen. The hepatic lobuli exhibit only slight liver cell changes. Because of the mild degree of periportal liver cell destruction this type of CAH has a relatively good prognosis. The need for therapeutic intervention is disputable.

Histological differentiation from CPH with spill-over is sometimes difficult but usually has no therapeutic consequences. Careful follow-up is indicated in such cases.

IV.2.2.b.2 Chronic active hepatitis (moderate)

In chronic active hepatitis (moderate), piecemeal necrosis is marked and present in almost all lobuli. Architectural changes exist and the inflammatory infiltrate penetrates deeper into the lobuli. Around all portal tracts there is formation of new connective tissue (collagen type III) which penetrates the liver lobuli in many places. In the newly-formed connective tissue irregular bile ductules may be found. It is disputable whether the genesis of these bile ductules can be attributed to hepatocellular transformation, proliferation of pre-existent bile ducts or both. Periportal hepatocytes can show acinar formation, the lumen of which may contain accumulation of bile pigment. The centrilobular hepatocytes usually show some cellular and nuclear anisomorphism.

The prognosis of this type of CAH may vary. The architecture alters slowly and cirrhosis develops late, if at all. Immunosuppressive treatment may be considered, although in cases of viral CAH the use of immunosuppressive treatment has proven to be of little benefit (Schalm,1982). Specific antiviral treatment is preferable (de Man,1988).

IV.2.2.b.3 Chronic active hepatitis (severe)

In chronic active hepatitis (severe), liver cell degeneration and necrosis are not confined to the periportal areas. Due to the degenerating and necrotizing hepatocellular process collapse of the reticulin skeleton with or without bridging is initially more evident than increase of connective tissue (active septa formation). The inflammatory infiltrate extends throughout the liver lobuli. Reticulin and collagen stains sometimes visualize the contours of intact, pre-existing portal spaces. The presence of thick collagen type I fibers in these pre-existing portal tracks contrasts with the recently produced type III collagen. The pre-existing portal areas are surrounded by a rim of collapsed and fibrosed pre-existing parenchyma (Popper, 1977). There is a marked pan-lobular aniso-morphism of the hepatocytes with "ballooning" degeneration and acinar transformation. Cholestasis is sometimes pronounced.

This type of CAH has to be distinguished from acute hepatitis with confluent necrosis. The presence of piecemeal necrosis and the use of orcein staining (Scheuer, 1980) for differentiation between active and passive septa is helpful. The prognosis for severe CAH is worse than that for acute hepatitis with bridging or confluent necrosis, which will often heal completely if the patient survives the acute episode (Peters, 1975).

IV.2.3 Cirrhosis

The question of the incidence of and pathways by which viral hepatitis will lead to cirrhosis remains controversial (Popper, 1983; Sherlock, 1974).

Cirrhosis caused by viral hepatitis, which is often of the macronodular or mixed macro-micronodular type, is presumably the result of both active and passive septum formation.

The incidence of cirrhosis after viral hepatitis varies in the literature from less than one to about three per cent (Björneboe, 1974). Some authors reported no evidence of cirrhosis after acute viral hepatitis (Beebe, 1970) in extensive follow-up studies. However, others have presented evidence, documented by serial liver biopsies, that acute viral hepatitis can progress to cirrhosis. This is especially true for patients with extensive hepatocellular necrosis. Peters (1975) stressed that

cirrhosis rarely, if ever, develops in younger patients with acute viral hepatitis, even if extensive confluent necrosis is present. On the other hand in older patients acute hepatitis with extensive necrosis frequently leads to cirrhosis. If acute hepatitis progresses to cirrhosis, the latter will occur as a rule within 12 to 18 months, sometimes even within 5 months (Krieg, 1969, this thesis chapter XI) after onset of the disease. However, in most cases, cirrhosis appears to develop slowly from chronic active viral hepatitis, whereby accompanying lobular events play a major role (Popper, 1983). Liaw (1988) reported on a cohort of 684 patients with chronic hepatitis B who exhibited an annual incidence of cirrhosis of 2.4 per cent for HBeAg sero-positive patients and 1.3 per cent for anti-HBe positive patients. In addition to viral replication, reflected by the HBe/anti-HBe and HBV-DNA status, age of the patient and the extent, severity, duration and frequency of lobular hepatic changes were important factors for the development of cirrhosis. In this study hepatitis delta superinfection was not directly related to the development of cirrhosis, in contrast to the results of the long-term follow-up study of Fattovich (1988). He reported that cirrhosis developed in eight out of ten hepatitis delta-positive patients.

In the course of cirrhosis there is a stage which separates the tendency toward morphological repair from progression to life-threatening disease; in this stage the differentiation between chronic active hepatitis and cirrhosis is difficult or even impossible (Scheuer, 1977) (fig.IV.2). Because, in these cases, cirrhosis is the scarred end-stage of chronic inflammation of the liver, it can be accompanied by features similar to those seen in chronic active hepatitis. This type of cirrhosis is interchangeable designated as "cirrhosis with features of chronic active hepatitis", "chronic active hepatitis with cirrhosis" or "active cirrhosis". The term "active cirrhosis" covers all types of cirrhosis with morphological signs of liver parenchyma damage from which the cirrhosis can be the end result. In contrast, inactive cirrhosis shows no signs of necrosis and inflammation. Although the pathogenesis of cirrhosis remains an enigma, host factors appear to play an important role; studies on hepatic fibrogenesis may provide a contribution by solving some of the pathogenetic problems (Popper, Review 1977).

In view of the prognostic and therapeutic implications, it must be stressed that restrictive histological criteria should be used for the diagnosis of cirrhosis. In this context Schlichting et al (1981) realized significant prognostic differences, sub-dividing severe architectural changes in the liver biopsies in:

a. a group with definite cirrhosis.
b. a group with probably cirrhosis.
c. a group compatible with but not diagnostic for cirrhosis.

IV.3 HEPATOCELLULAR CARCINOMA

Epidemiological and clinical studies (Blumberg, Review 1982; Beasley, 1988) have provided a substantial body of evidence for the hypothesis that persistent hepatitis B virus infection is the main cause of the development of primary cancer of the liver. This cancer is probably one of the most common malignant tumors in the world. However, the relative frequency differs markedly in different parts of the world. This evidence is based on:

1. the high incidence of hepatocellular carcinoma in areas of prevalent HBV infection, especially in sub-Saharan Africa and Southeast Asia, while hepatocellular carcinoma is a rare tumor in areas with a low prevalence of hepatitis B.
2. the high incidence of hepatocellular carcinoma in HBV carrier families.
3. the presence of HBsAg in many patients with hepatocellular carcinoma (Nayak et al., 1977).
4. Infection of animals with their species-specific hepatitis B-like virus has been proven to cause hepatocellular carcinoma (Marion, 1986).

In cases of hepatocellular carcinoma hepatitis B surface antigen and core particles can be found in non-cancerous areas of the liver, but only sporadically in malignant hepatocytes. However, integrated viral DNA can be detected in hepatocellular carcinoma cells (Prasanta et al., 1980; Brechot et al., 1980). This integration of viral DNA in the genome of the host cells is consistent with findings in animal virus models, in which this integration also takes place during oncogenic transformation.

In a study by Beasley et al. (1981) it appeared that the relative risk of acquiring hepatocellular carcinoma is more than 250 times greater for HBV carriers than for non-HBV carriers. In this study a group of 3500 HBV carriers and 3500 non-carriers, matched for age and place of origin, were followed for 2-4 years. During this period 41 subjects died from a hepatocellular carcinoma, 40 of whom belonged to the HBV carrier group. Thus, 98 per cent of the cases of hepatocellular carcinoma occurred in HBV carriers, which implies that chronic hepatitis B infection is probably the highest risk factor known for any of the more frequent occurring cancers. It is believed that the period between the occurrence of HBV infection and clinically demonstrable carcinoma is at least 20 years. The shortest well-documented period within which hepatocellular carcinoma occurred was 6 years and 2 months; the patient was a 6-year-old child (Shimoda et al., 1980).

Histologically hepatocellular carcinoma (HCC) in HBV-positive patients does not differ from HCC in HBsAg-negative patients. As mentioned above only in

sporadic cases can HBsAG and HBcAg can be detected in the neoplasm. In nearly all cases the liver parenchyma outside the HCC shows cirrhotic transformation. In this cirrhotic liver parenchyma the hepatocytes are usually characterized by remarkable cellular atypia. From these observations hepato-cellular atypia is considered to be a pre-neoplastic feature in cirrhotic livers.

IV.4 CONCLUDING REMARKS

The histological definitions of the various categories of liver disease, as seen in HBsAg seropositive patients, are rather simple so that classification is rational and reproducible. Classification has prognostic implications as far as the development of cirrhosis is concerned, particularly for chronic persistent and chronic active hepatitis. In contrast the prognostic value of the histo-pathological classification of acute hepatitis is, as far as the transition to chronic hepatitis is concerned, limited and at the least uncertain. In fact, multiple causes may give rise to difficulties in classification of the liver abnormalities, leading to an incorrect diagnosis. These difficulties can be grouped under the following headings:

- Difficulties in the delineation of the various categories of hepatitis (histopathological overlap) (fig.IV.2).
- Errors due to biopsy sampling.
- Clinicopathological liver diseases (entities) that resemble one of the histological types of viral hepatitis B.
- Interference in a liver disorder caused by the hepatitis virus by liver disorders of other etiologies (heterogeneity of liver disease).

IV.4.1 HISTOPATHOLOGICAL OVERLAP OF THE VARIOUS CATEGORIES OF VIRAL LIVER DISEASE

With regard to the separation of histological subdivisions of viral hepatitis problems are inevitable since the continuous spectrum of viral hepatitis has been subdivided artificially. Despite correct histological definition, the subdivisions exhibit a certain overlap, leading to inter-observer "errors" and, to a lesser degree, difficulties in reproducibility (intra-observer errors).

Problems in the delineation of hepatitis are not confined to the subdivisions of the two basic groups, acute and chronic hepatitis, but also apply to the subgroups of acute and especially chronic hepatitis in themselves (fig.IV.2). A well-known problem is bridging necrosis which is a feature of both acute and chronic hepatitis. Also differential diagnostic problems may arise between the diagnoses

of residual liver changes, acute hepatitis and chronic (persistent) hepatitis, and between acute hepatitis with pronounced portal inflammation (e.g. acute hepatitis in drug-abusers) and chronic active hepatitis. For the group of patients with chronic hepatitis differentiation between chronic persistent and chronic active hepatitis is based on the phenomenon of "piecemeal necrosis". So-called "spill-over", seen in chronic persistent hepatitis, must not erroneously be interpreted as piecemeal necrosis. On the other side of the spectrum of chronic active hepatitis, it is sometimes difficult to separate chronic active hepatitis with extensive fibrosis from cirrhosis with features of chronic active hepatitis. To solve this problem the group with definite cirrhosis must be separated from those with "probable" or "compatible with but not diagnostic for" cirrhosis.

IV.4.2 Sampling errors

Although all types of viral hepatitis can be considered basically as diffuse liver disorders, variations in the histopathological features from area to area and from lobule to lobule are not uncommon. When liver biopsy specimens are small, sampling errors due to these local differences will occur with increasing frequency. Of all categories of hepatitis, chronic persistent hepatitis has the greatest chance of being diagnosed incorrectly or not at all. It has been shown that in wedge biopsies some portal tracts will exhibit the alterations typical of chronic persistent hepatitis while others will be characteristic of chronic active hepatitis; in many cases, the latter will be the ultimate diagnosis because of its less favorable prognosis. Similarly some areas of the liver may show changes consistent with aspecific reactive hepatitis, while others will be indicative of chronic persistent hepatitis. In acute hepatitis necrosis is not equally distributed throughout the liver and depends on local differences in the microenvironment. Therefore the degree of necrosis cannot always be determined exactly in needle or even wedge biopsies; for example, liver cell necrosis throughout a needle biopsy need not be representative of the entire liver.

IV.4.3 Clinicopathological liver diseases resembling or identical to one of the histological types of viral hepatitis B

On histological grounds alone acute hepatitis B cannot be distinguished easily and reliably from acute hepatitis with a different etiology. The ground-glass hepatocyte, typical for chronic hepatitis B infection, is usually not found in acute hepatitis. As far as the hepatitis viruses are concerned, only hepatitis non-A non-B causes features during acute hepatitis that are helpful in diagnosing this viral infection (Kuroo, 1980 and Popper, 1980). In hepatitis non-A non-B the reported

to give a markedly sinusoidal mononuclear infiltrate resembles that seen in patients with infectious mononucleosis. Early in the evolution of acute non-A non-B hepatitis some accumulation of fat can be seen in the hepatocytes. This feature is unusual in hepatitis A and hepatitis B. Proliferation of bile ductules covered with abnormal epithelium and a pronounced cholestasis are reported to be more frequent in non-A non-B viral infections. Acute hepatitis that is not caused by viral agents, such as drug-induced hepatitis, is the most difficult as far as differential diagnosis is concerned. Abundant eosinophils, bile duct lesions and fatty changes in the hepatocytes support a diagnosis of drug-induced acute hepatitis. Acute alcoholic hepatitis can be distinguished from acute viral hepatitis by the presence of extensive fatty changes, Mallory's hyaline bodies, giant mitochondria and a predominance of polymorphonuclear leukocytes. Moreover longstanding biliary obstruction may sometimes mimic the cholestatic type of acute viral hepatitis. Both chronic persistent and chronic active hepatitis can occur as reaction patterns in other liver diseases, for example hepatitis non-A non-B, the early stages of primary biliary cirrhosis, drug-induced liver disease and the residual stage of acute hepatitis. However, light microscopically hepatitis B infection can often be diagnosed by the presence of ground-glass hepatocytes (see chapter VII). Chronic active hepatitis is seen not only during a hepatitis B infection but also in the more advanced stages of primary biliary cirrhosis, Wilson's disease, α_1 antitrypsin deficiency, drug-induced liver disease, non-A non-B virus infection, hemochromatosis and "lupoid" hepatitis. In most of these diseases the chronic active hepatitis is accompanied by special concomitant histological features, which lead to the correct diagnosis. In a considerable number of biopsies (see chapter VII) chronic active hepatitis B was indicated by the presence of ground-glass cells. Since cirrhosis is usually the end-stage of chronic active hepatitis, in case of a chronic hepatitis B infection, the special histological features of this chronic active hepatitis B can also be found in this cirrhotic liver parenchyma. When cirrhosis develops from an acute hepatitis with extensive necrosis, it is often not possible to establish the etiology of the cirrhosis. After an acute hepatitis the cirrhosis may be of the incomplete septal type, whereby very slender septa incompletely surround liver parenchymal nodules (Vido and Wildhirt, 1969).

IV.4.4 Heterogeneity of liver disease

When more than one etiological factor is involved in a liver disorder, it is often very difficult or impossible to determine which changes are caused by which factor. May be more than in any other liver disease, patients with hepatitis B infection are exposed to other factors that complicate the viral liver disease, e.g.

venereal infections, alcohol abuse and other viral infections. The combination of several pathogenetic factors plays an important rôle especially in livers grafted in HBsAg positive patients e.g. recurrence of hepatitis B infection and rejection (see chapter XI). It should therefore be noted that not all liver changes in HBsAg seropositive patients are the result of infection with hepatitis B virus.

CHAPTER V

RESULTS OF LIGHT MICROSCOPICAL ANALYSIS OF LIVER BIOPSIES FROM PATIENTS WITH HEPATITIS B VIRAL INFECTION.

CHAPTER V

RESULTS OF LIGHT MICROSCOPICAL ANALYSIS OF LIVER BIOPSIES FROM PATIENTS WITH HEPATITIS B VIRAL INFECTION.

V.1 SUMMARY

The histopathology of acute and chronic infections associated with viral hepatitis B was analyzed in a series of 165 consecutive liver biopsies from 139 HBsAg seropositive subjects; special attention was focussed on the spectrum of the histo-pathologic changes and problems in their classification as well as the relation between the histological changes and the (longterm) prognosis.

Out of 139 subjects 51 were asymptomatic HBsAg carriers identified by chance during routine screening, and 88 were patients with symptomatic liver disease. In these 88 cases HBsAg was detected during evaluation of the liver disease. Clinical and histopathological aspects of both groups were analyzed together and separately, and compared. In the asymptomatic HBsAg seropositive group most subjects were found to have persistent HBsAg serology. In 32 out of 51 cases, the liver changes were classified as minimal changes (11), reactive hepatitis (8) and chronic persistent hepatitis (13). Eight subjects had a chronic active hepatitis, and one an inactive cirrhosis. Surprisingly in 10 cases the diagnosis was an acute hepatitis which, in seven cases (70 per cent), subsequently transit into a histologically proven chronic hepatitis. In 3 asymptomatic blood donors an acute self-limiting hepatitis was detected.

In the group of patients with symptomatic liver disease a relatively large proportion had acute hepatitis (33), four of which transformed into chronic hepatitis: two had an anicteric course. A substantial proportion of the symptomatic patients with chronic HBsAg antigenemia had active liver disease (38), 17 with cirrhosis.

It is concluded that:

a. a substantial proportion of the asymptomatic HBsAg seropositive individuals will exhibit quite severe liver changes. For these individuals abnormal liver tests are the criterion for careful evaluation, including a liver biopsy.
b. an icteric acute HBV infection rarely transforms into a chronic HBV infection, which mainly develops from an anicteric acute infection.

c. piecemeal necrosis and bile duct lesions can be present in acute self-limiting hepatitis.
d. the prognosis for hepatitis B seropositive individuals with minimal and aspecific liver alterations is excellent.
e. HBV-associated cirrhosis, found mainly in the older age group, has a poor prognosis due to decompensation of liver functions and development of hepatocellular carcinoma in some cases.

V.2 INTRODUCTION

After discovery of the Australia antigen as a marker for hepatitis B infection many reports on the liver histology of the hepatitis B infection (HBV) were published (Peters, 1975; Ishak, 1976; Phillips 1981). The natural course of a hepatitis B infection was characterized by the wide range of light microscopical liver changes all with their own clinical and therapeutical implications. This fact and the broad acceptance of the liver biopsy as an safe and important tool for evaluation of liver disease, stimulated the development of a uniform classification system in which the same criteria and the same terminology would be used.

Previously, as a result of the lack of uniform classification and because of the complex and confusing nomenclature, studies on liver disease and the effects of therapeutic treatment could not be compared.

Now "The standardization of nomenclature, diagnostic criteria and methodology for disease of the liver and biliary tract", prepared jointly by the Fogarty International Center and the International Association for Study of the Liver, (1976) provides a basis for classifying liver disease. In this classification system liver histology plays a key role.

Using the principles of this classification and the histological criteria given in chapter IV, a series of 165 liver biopsies, received over a five-year period (1973-1977) was analyzed. Special attention was directed to problems encountered in the classification of the liver changes. The subsequent course of the disease in the different hepatitis groups was followed.

V.3 RESULTS

V.3.1 HISTOLOGICAL CLASSIFICATION

The results of the classification of the histological changes present in 165 biopsies from 139 HBsAg seropositive patients, using the criteria given in the

previous chapter, are listed in table V.1 and described below. The histological subdivision of the light microscopical liver changes seen in group A, those individuals in whom HBsAg was found accidentally (e.g. volunteer blood donors), and group B, the patients with symptomatic liver disease, will be described separately. In this chapter "ground-glass" hepatocytes are not used for the classification of viral hepatitis. The role of these ground-glass cells in the classification of viral hepatitis is discussed in chapter VII.

Table V.1 *Results of the evaluation of the histological changes in 165 liver tissue specimens from HBsAg seropositive patients.*

acute hepatitis	47		
fully developed type		18	
with focal necrosis			16
with passive septa			1
with multilobular necrosis			1
late-phase type		27	
with focal necrosis			26
with bridging necrosis			1
residual phase		2	
chronic persistent hepatitis	30		
chronic active hepatitis	43		
mild		30	
moderate		12	
severe		1	
cirrhosis	20		
active		17	
inactive		3	
minimal lesions	14		
aspecific reactive hepatitis	11		

V.3.1.1 Acute hepatitis

The diagnosis of acute hepatitis was established for 47 liver biopsies. These 47 biopsies were taken from 43 patients (15 females, 28 males), ranging in age from 16 to 70 years (mean: 32 years).

Early or fully developed type of acute hepatitis

Early or fully developed acute hepatitis, characterized by the histological picture typical of lobular hepatitis, was demonstrated in 18 of 47 biopsies. These biopsies were taken from 18 patients (9 females, 9 males) with a mean age of 32 years (range: 16-58 years). In 16 of these 18 biopsies only spotty necrosis was present. In one case this spotty necrosis was accompanied by passive septa; the biopsy came from a patient of the older age group (57 years). In all 18 biopsies moderate to large quantities of Councilman bodies were detected (1-3 Councilman bodies per lobulus). There were numerous macrophages loaded with ceroid pigment lying mainly in clusters, especially in the centrilobular zones. Sometimes a few ceroid macrophages were also present in the portal triads.

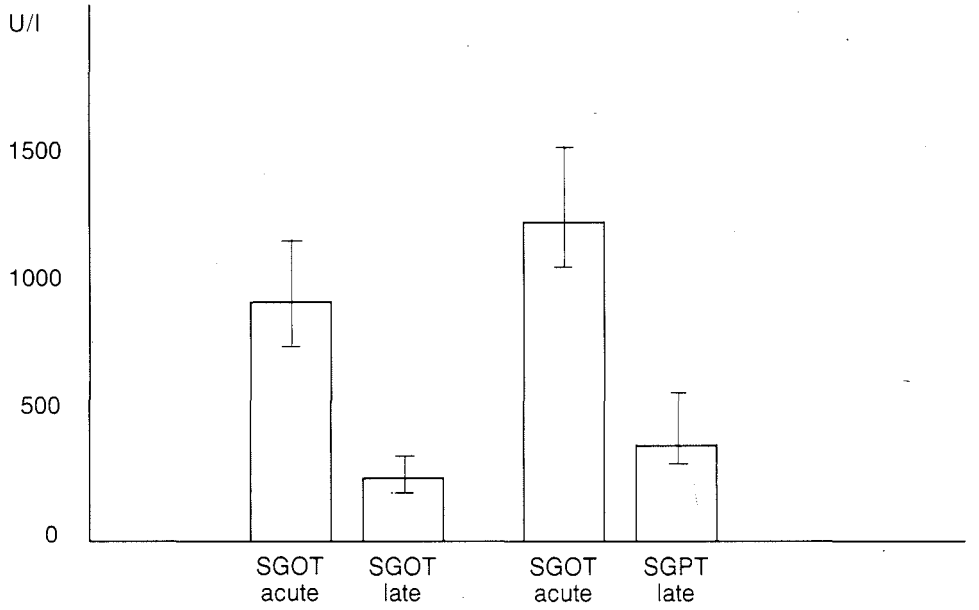
In all 18 biopsies the predominant lobular changes were accompanied by changes in the portal triads, consisting of some edema and inflammatory infiltrates that varied in density. In 3 out of 18 biopsies the portal infiltrate was pronounced and contained many lymphocytes and plasma cells. Locally the infiltrates extended into the surrounding liver parenchyma, blurring out the border between the liver parenchyma and the portal tract and mimicking the phenomenon of piecemeal necrosis. On the basis of this pronounced portal infiltrate the possibility of a transition to the chronic type of hepatitis was suggested. Periportal fibrosis was not present in any of the biopsies. The mean value and standard deviations of SGPT (serum glutamate pyruvate transaminase) and SGOT (serum glutamic oxaloacetic transaminase) levels (normal level both < 30 IU/L) in serum on the day the biopsy was taken are shown in fig.V.1.

An epithelioid cell granuloma was present in one of the biopsies. In 7 biopsies eosinophilic granulocytes were frequently detected in the inflammatory infiltrates both in the portal tracts and in the liver parenchyma. In 12 biopsies bile pigment was seen, mainly in the centrilobular zones. This bile pigment was located in the cytoplasm of hepatocytes and macrophages as well as the somewhat dilated bile canaliculi. In 7 out of the 18 biopsies some irregularity and/or proliferation of the bile ductules was present. In 5 cases these biliary changes were accompanied by accumulation of bile pigment, mainly in the liver parenchyma. The amount of bile pigment accumulated did not correlate with the severity of the bile duct changes.

In 5 out of 18 biopsies Perls' stain for iron revealed iron pigment, mainly in swollen Kupffer's cells and macrophages and always together with ceroid pigment. In 2 biopsies the iron deposits were minimal, in 2 slight and in one more pronounced.

Fifteen of the 18 biopsies were taken 8 to 25 days after the onset of jaundice (average: 12 days). One biopsy was not taken until 8 weeks after the onset of jaundice. In none of these cases did the hepatitis follow an anicteric course.

Fig.V.1 *Mean SGPT and SGOT serum values in fully developed acute hepatitis and late phase type of acute hepatitis. SGPT p = 0,002 SGOT p = 0,004 (two-sided Wilcoxon Rank-Sum test).*



One out of the 18 biopsies showed the picture of an acute hepatitis with multilobular necrosis. Hepatocytes were not found in this biopsy. The reticulin skeleton had collapsed but was in principle intact (fig.V.2). The portal tracts were located close together, separated by the collapsed liver parenchyma which contained only a mild mixed polymorphonuclear and lymphoplasmacellular inflammatory infiltrate. In relation to the severity of the liver cell damage there was very little ceroid pigment. Macrophages and Kupffer's cells were inconspicuous. Iron pigment could not be demonstrated. This biopsy was taken from a 28-year-old woman, 5 weeks after the onset of jaundice. The patient survived and HBsAg cleared from the serum within 3 months. Follow-up by laparoscopy revealed a circumscribed area of liver parenchyma in the right lobe of a liver that had otherwise collapsed.

Late-phase type of acute hepatitis

The histological changes in 27 of the 47 biopsies classified as acute hepatitis were of the late-phase type. These biopsies were taken from 23 patients (5 females and 18 males), ranging in age from 19 to 70 years (mean: 32 years).

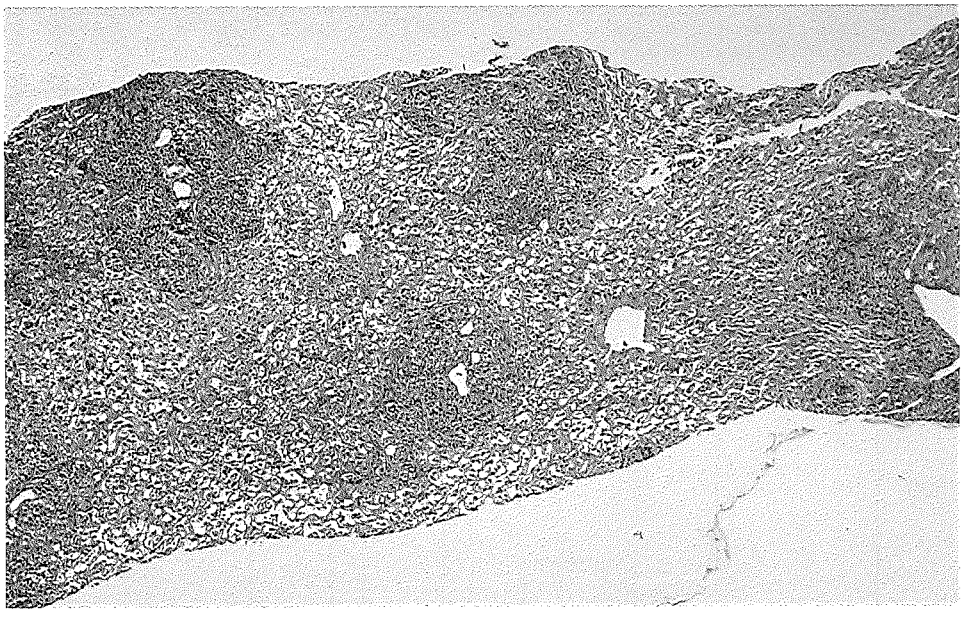


Fig.V.2 *Acute hepatitis with multilobular necrosis: portal tracts are located close together, separated by collapsed liver parenchyma, in which no hepatocytes are left (H and A, 60x).*

In 9 patients the acute hepatitis was neither accompanied nor followed by jaundice (anicteric acute hepatitis). The other 14 patients were jaundice at the time of the biopsy. The duration of the jaundice before the biopsy was obtained varied considerably among these 14 patients, from 7 days to 7 weeks, with a mean of 23 days.

During follow-up a second biopsy from 4 of these 23 patients still showed late-type acute hepatitis. The time interval between the first and second biopsies ranged from 2½ to 9 months. In all biopsies the histological picture was characterized by changes in the hepatic lobuli. The predominant feature in these biopsies was a marked mesenchymal reaction as indication of liver cell necrosis which occurred some time ago; this mesenchymal reaction exceeded far the less frequent occurrence of necrotic hepatocytes and consisted of swollen macrophages and Kupffer's cells, both loaded with ceroid pigment. These ceroid-loaded macrophages were scattered throughout the hepatic lobuli as solitary cells and small clusters, mainly in the centrilobular areas. In this phase of acute hepatitis the ceroid macrophages were also found in large quantities in the portal tracts. The number of necrotic hepatocytes varied from low in 18 (less than 1 Councilman body per lobulus on the average) to moderate in 9 of the 27 biopsies (1-2 Councilman bodies per lobulus on the average). In one of the 27 biopsies the

phenomenon of bridging necrosis was present.

In all biopsies the lobular changes were accompanied by portal inflammatory infiltrates. These inflammatory infiltrates which were in general more pronounced than in the fully developed type of acute hepatitis, consisted mainly of lymphocytes and plasma cells and sometimes also eosinophilic leukocytes. Pronounced inflammatory infiltrates in the portal tracts were seen in 18 out of the 27 biopsies. These 18 biopsies were taken from 14 patients. In 7 of these 18 biopsies the infiltrate also extended into the surrounding liver parenchyma, exhibiting the features of chronic active hepatitis. In all cases it appeared likely that the acute hepatitis would transform into a chronic type of hepatitis.

In 2 biopsies lymph follicles had formed in some of the portal tracts. In several biopsies changes in the bile ductules were also present. In addition to erosion of bile ductules by the portal inflammatory infiltrates, proliferation of bile ductules was also seen in some biopsies.

Iron pigment was found in 6 of the 27 biopsies: a small amount in 3 and more pronounced amounts in the other 3. The iron pigment was located mainly in the cytoplasm of swollen Kupffer's cells and macrophages.

Fibrosis was not an important feature in these biopsies. However, in 9 out of the 27 biopsies some periportal fibrosis was detected; it was scored as mild in 7 cases and as mild to moderate in 2 cases. The mean value with two-sided standard deviation of SGPT and SGOT levels are shown in fig.V.1.

Residual phase of acute hepatitis

An end-stage acute hepatitis was diagnosed in 2 out of the 47 cases. This diagnosis was based on the presence of numerous ceroid macrophages, whereas degeneration and necrosis of the hepatocytes were rare. The macrophages loaded with ceroid pigment were present mainly in the portal tracts; only small scattered groups were detected in the hepatic lobuli. Indicating previous hepatocellular necrosis and subsequent regeneration, the plates of the hepatocytes showed a mild disarray and broadening. Frequently binucleated hepatocytes were seen. In addition the hepatocytes exhibit a mild nuclear and cytoplasmic anisomorphism. One patient underwent biopsy 4 weeks after onset of jaundice. The other patient revealed no jaundice at all. SGPT and SGOT levels were within normal limits.

Iron deposition

Of the 47 liver biopsies from patients with acute hepatitis, only 12 gave a positive iron stain. In 2 cases the amount of stainable iron was minimal, in 6 slight

to moderate (+). Only 4 biopsies contained more pronounced Fe deposits (++). The iron pigment was located mainly in Kupffer's cells and macrophages, usually in combination with deposits of ceroid pigment in the same cells. Only a small amount of iron pigment could be detected in the cytoplasm of hepatocytes.

Five of the 12 iron-positive biopsies exhibited the histological picture of a fully developed acute hepatitis. These biopsies were taken from 5 patients, all of whom had had icterus for a period of 3 to 14 days (mean: 9 days).

The picture of late-phase acute hepatitis was seen in 6 of the 12 biopsies taken from 6 different patients. Five of these patients had icterus at the time of the biopsy. The icterus had been present for a period of 7 days to 6 weeks (mean: 30 days). The hepatitis in the other patient ran an anicteric course. This patient had undergone kidney transplantation, thus receiving many blood transfusions.

Cholestasis

Cholestasis was found in 26 out of 47 biopsies from patients with acute hepatitis. Of the 18 biopsies from patients with the fully developed type of acute hepatitis 12 (66 2/3 per cent) showed cholestasis; it was minimal in 2, mild to moderate in 9 and more extensive in 1 case. Of the group of 27 patients with late-phase acute hepatitis 14 (52 per cent) exhibited cholestasis; it was minimal in 2, mild to moderate in 7 and extensive in 4 cases; in one case it was one of the most striking features of the biopsy.

Bile pigment accumulation was visualized in the cytoplasm of hepatocytes partly as "feathery" or xanthomatous degeneration, partly as small droplets of greenish or brownish bile pigment. These cytoplasmic changes in the hepatocytes were seen in combination with varying numbers of dark-green bile thrombi in somewhat dilated bile canaliculi. Some bile pigment could also be found in the cytoplasm of Kupffer's cells, together with ceroid and sometimes iron pigment.

A rough correlation existed between bile pigment accumulation in the biopsies and the level of bilirubin in serum. In only one case, the course of the acute hepatitis was anicteric; the other patients had icteric acute hepatitis.

During follow-up 2 out of 26 biopsies, initially exhibiting cholestasis, underwent transition from acute hepatitis to chronic hepatitis; in the group of 21 biopsies without detectable cholestasis 13 from 9 patients transformed from acute hepatitis into a chronic type of viral infection. Clinically 8 of these 9 patients exhibited no icterus at all, whereas 1 patient had had icterus 3 months beforehand.

Bile duct changes

Although nearly all biopsies from patients with acute hepatitis showed focal

changes in the bile ductules, in most cases these changes were inconspicuous and consisted of some irregularity and disarray of the bile duct epithelium together with mild cellular and nuclear anisomorphism. The cytoplasm of the epithelial cells showed mild vacuolization.

However, 18 out of the 47 biopsies exhibited more marked bile duct changes, which were always segmental in character and occurred in only a few of the portal tracts. These ductular changes could be subdivided into 2 groups: a cholangiolitis group and a group characterized by an increase in bile ductules (bile duct proliferation). Cholangiolitis was seen in 7 biopsies. The bile ductules in these cases were invaded focally by lymphocytes and plasma cells, which were found between the individual epithelial cells, of these bile ducts. In 13 cases proliferation of irregular bile ductules had occurred. These bile ductules were found along the periphery of the, sometimes enlarged, portal tracts together with slightly irregular limiting plates (biliary piecemealing). In 3 cases the degree of bile duct proliferation was striking; in the rest (10) it was mild to moderate. In 2 biopsies bile duct proliferation was seen together with cholangiolitis.

Cholestasis was seen in 11 of the 18 biopsies with bile duct changes, mild cholestasis in 7 biopsies and more marked cholestasis in 4 biopsies. Cholestasis was demonstrated in all but 1 of the 7 biopsies with cholangiolitis and in 7 of the 13 biopsies with bile duct proliferation.

Eosinophilic leukocytes

In 27 out of 47 biopsies with the histological picture of acute hepatitis, eosinophilic leukocytes were detected in the inflammatory infiltrates. These eosinophils were localized mainly in the portal tracts. In 10 cases the number of eosinophils was marked. The inflammatory infiltrate contained only few eosinophils in the other cases. Four of the 10 patients with large numbers of eosinophils were drug-addicts. Thirteen of the 27 biopsies exhibited the picture of late-phase acute hepatitis, whereas 14 showed a fully-developed type of acute hepatitis.

Steatosis

Steatosis was detected in 7 of the 47 biopsies. These 7 biopsies were taken from 5 patients. The steatosis was of the coarse vacuolar type and was scored mild in 5 cases and moderate in the other 2. In all 7 cases the acute hepatitis was of the late phase type. None of the biopsies with the picture of a fully developed hepatitis exhibited steatosis.

Prominent portal inflammatory infiltrates

Pronounced inflammatory infiltrates in the portal areas were seen in 21 out of the 47 biopsies with a histological picture of an acute hepatitis. These biopsies came from 17 patients (5 females and 12 males). In those cases (7 biopsies) in which the dense inflammatory infiltrates were accompanied by piecemeal necrosis, transition of the acute hepatitis to chronic hepatitis was considered a serious possibility. The histological changes in the 21 biopsies were classified as fully developed acute hepatitis in 3 cases, as late-phase acute hepatitis in 17 and as the residual-phase of acute hepatitis in 1 case. Jaundice was present at the time of the biopsy in 10 out of the 17 patients, including all 3 with fully developed acute hepatitis. One patient had suffered an icteric period 3 months before the biopsy was taken, while jaundice was not seen at all in 6 out of 17 patients (anicteric acute hepatitis).

Follow-up

All 43 patients with acute hepatitis were followed clinically. In 31 of the 43 patients serum liver enzymes normalized and serum HBsAg disappeared spontaneously within 3 months of the biopsy. Out of this group of 31 patients, only the patient with acute hepatitis with multilobular necrosis was followed histologically. Repeat biopsies were taken 27 days and 6½ months after the first biopsy. The last biopsy from this patient showed extensive post-collapse fibrosis and some circumscribed areas of intact liver parenchyma. Despite the fact that the HB virus markers had become negative and the serum liver enzymes had normalized a mild lymphoplasmacellular infiltrate was still present in the collapsed and fibrosed parts of the liver and in the portal tracts of the intact parts of the liver.

In contrast 11 of the 43 patients remained HBsAg positive for many years. Two of these 11 patients no longer exhibited HBsAg 6 and 8 years after discovery of the acute HBV hepatitis. The other nine patients were still HBsAg-positive at least 12 years after the initial biopsy. And yet 5 of these patients showed HBeAg seroconversion 4, 5, 5, 6 and 8 years (mean 5.8) after the first biopsy, respectively.

One of the 43 patients died 5 weeks after the biopsy was taken; his liver enzyme levels were markedly elevated. The biopsy from this 70-year-old man showed late-phase acute hepatitis and extensive bridging necrosis.

Various distinct histological and clinical features of the two biopsy groups (one with acute self-limiting hepatitis and the other with acute hepatitis with persistent HBsAg antigenemia) are analyzed in tables V.2, V.3 and V.4.

Table V.2 shows the histological stages of acute hepatitis over the two groups.

Table V.2 Histological characteristics of biopsies from patients with acute self-limiting hepatitis and acute hepatitis which transformed into chronic hepatitis.

	n = 43	fully developed type n = 18	late type n = 23	residual n = 2
acute self-limiting hepatitis	n = 31	18/18	11/23	2/2
acute hepatitis that transformed into chronic	n = 11	0/18	11/23	0/2
not evaluable	n = 1	0/18	1/23	0/2

Table V.3 *Frequency of jaundice in patients with acute self-limiting hepatitis and acute hepatitis which transformed into chronic hepatitis.*

	n = 43	icteric n = 34	anicteric n = 9
acute self-limiting hepatitis B	31/43	31/34	0/9
acute hepatitis that transformed into chronic	11/43	2/34	9/9
not evaluable	1/43	1/34	0/9

Table V.4 *Portal infiltrate in biopsies from patients with an acute self-limiting hepatitis and acute hepatitis which transformed into chronic hepatitis*

	pronounced portal infiltrate n = 17	pronounced portal infiltrate with piecemeal necrosis n = 7	inconspicuous portal infiltrate n = 26
acute self-limiting hepatitis B	8/17	2/7	23/26
acute hepatitis transformed into chronic	8/17	4/7	3/26
not evaluable	1/17	1/7	0/26

None of the patients with the fully developed type of acute hepatitis showed persistent HBsAg antigenemia. In contrast 11 of the 23 patients with the late-type of acute hepatitis remained HBsAg-positive. Nine of the 11 patients in whom the acute hepatitis transformed into chronic hepatitis were anicteric. Only two patients had an icteric hepatitis (table V.3). A prominent portal infiltrate was seen in 8 of the 11 patients who developed chronic hepatitis, whereas 8 of the 31 patients with an acute self-limiting hepatitis exhibited a dense inflammatory infiltrate in the portal tracts (table V.4). Characteristics that mimic piecemeal necrosis were seen in 4 of the 11 patients who underwent transition of the acute hepatitis to chronic liver disease and in only 2 of the 31 with acute self-limiting hepatitis.

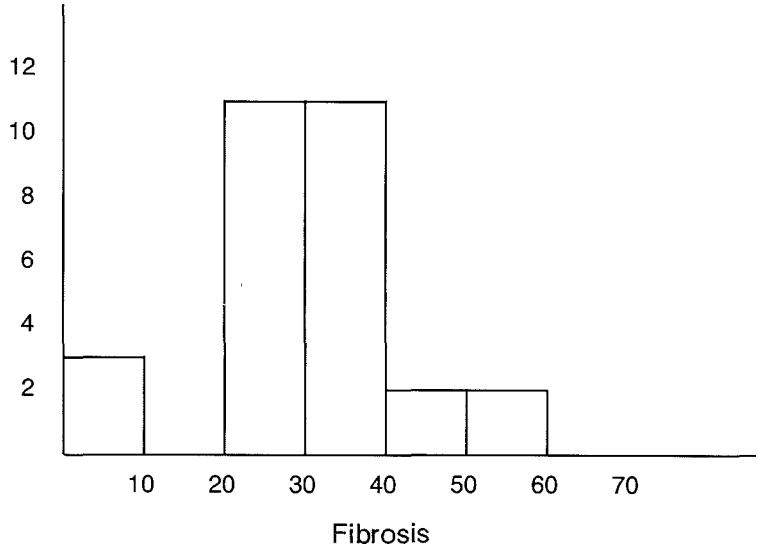
V.3.1.2 Chronic persistent hepatitis

The diagnosis chronic persistent hepatitis was established for 30 biopsies. These biopsies were taken from 29 patients (24 males, 5 females), ranging in age from 2 months to 57 years (mean 29 years). The age distribution pattern for these patients is given in table V.5.

Portal hepatitis was the basis for diagnosis. In 10 of the 30 cases the diagnosis chronic persistent hepatitis was established only after extensive differential diagnostic considerations. For 8 of these 10 biopsies the problems consisted of

the differentiation between non-specific reactive hepatitis and chronic persistent hepatitis. Two of these biopsies showed some fibrosis, possibly a rest of a more severe liver disease (residual phase of an acute hepatitis). In 2 cases the differential diagnosis was chronic persistent or chronic active hepatitis. Six biopsies with chronic persistent hepatitis were one out of a series of follow-up biopsies. In 4 cases the other biopsies in the series showed chronic active hepatitis, while in 1 case the other biopsy showed aspecific reactive hepatitis. In 1 case the biopsy with chronic persistent hepatitis was preceded by a biopsy with the features of the late-phase type of acute hepatitis. Two biopsies of chronic persistent hepatitis were taken from patients who were being treated with prednisone. The other patients were not being treated for their liver disease.

Table V.5 *Age distribution of patients with chronic persistent hepatitis.*



Architectural changes were in general inconspicuous in 24 biopsies. In addition to the 2 biopsies mentioned above, fibrosis was seen in 6 other biopsies. The degree of fibrosis ranged from mild to severe: mild in one, moderate in one and severe in 4 biopsies.

Iron deposition

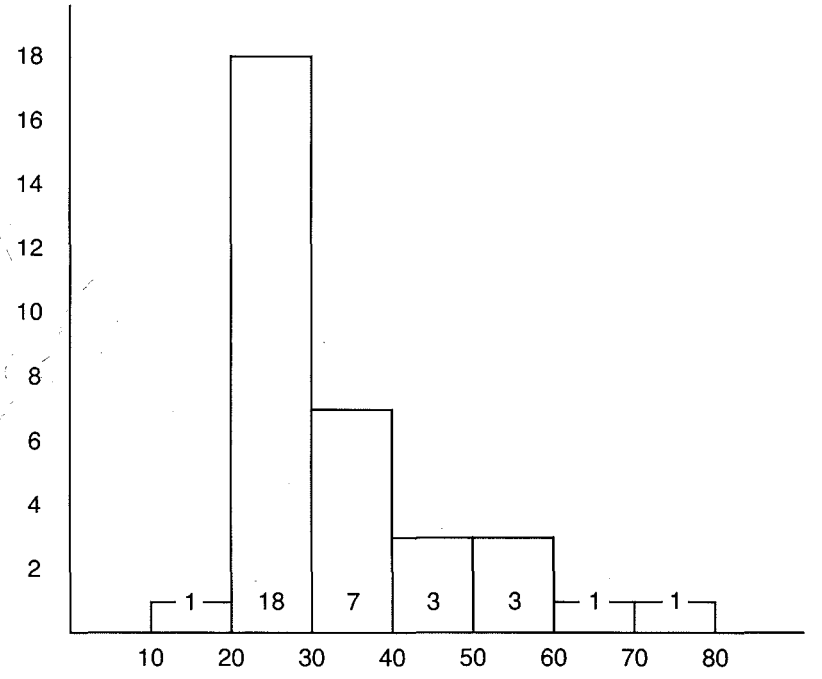
In 4 biopsies ion stain was positive. The amount of iron was slight in 2 biopsies and moderate in the other 2. Iron was present mainly in the cytoplasm of Kupffer's cells and macrophages.

V.3.1.3 **Chronic active hepatitis**

Chronic active hepatitis was the diagnosis established for 43 biopsies. These biopsies were taken from 34 patients (30 males, 4 females), ranging in age from 19 to 74 years, mean 33 years (table V.6).

Thirty biopsies with chronic active hepatitis exhibited only mild activity. In 4 cases, differentiation from chronic persistent hepatitis was difficult. Most of the 30 biopsies exhibited mild periportal fibrosis. However, in 7 biopsies the fibrosis was marked with extensive porto-portal septum formation. In 2 biopsies the extensive fibrosis was accompanied by nodular regeneration. When cirrhosis could not be excluded, the liver changes in these cases were classified as "probable" cirrhosis. As a result of pronounced lobular changes with necrosis of hepatocytes in 2 of the 30 biopsies, problems in differentiation between prolonged acute hepatitis and exacerbation of chronic active hepatitis existed.

Table V.6 *Age distribution of patients with chronic active hepatitis.*



In 12 biopsies the activity of the chronic hepatitis was moderate. In general the fibrosis in these biopsies was more marked; however none of the biopsies exhibited sufficient signs to be classified as cirrhosis. In 2 of the smaller biopsies

however cirrhosis could not be ruled out. As a result of a coexisting florid lobular hepatitis component in 5 of these 12 biopsies, acute exacerbation of the chronic active hepatitis was considered. In these cases prolonged acute hepatitis with transition to chronic hepatitis was the differential diagnosis. One biopsy showed a severe chronic active hepatitis with extensive active septum formation.

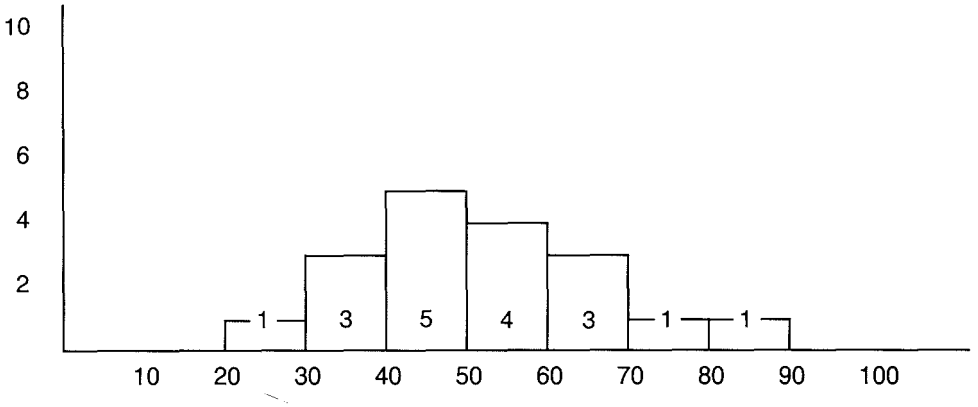
Table V.7 *Relation between the severity of chronic active hepatitis and the degree of fibrosis and lobular changes.*

	n	fibrosis mild	fibrosis marked	probably cirrhosis	lobular changes
CAH (mild)	30	23	5	2	2
CAH (moderate)	12	3	7	2	5
CAH (severe)	1	0	1	0	0

V.3.1.4 Cirrhosis

Cirrhosis was the diagnosis established for 20 biopsies taken from 18 patients (17 males, 1 female). The age of these patients ranged from 26 to 81 years with a mean of 51 years (table V.8). All patients had clinically established portal hypertension with variable degrees of splenomegaly, esophageal varices and, in some cases, ascites.

Table V.8 *Age distribution of patients with cirrhosis.*



In only 3 cases was the cirrhosis not accompanied by inflammatory infiltrates in the fibrotic septa; as a result the cirrhosis was classified as "inactive" in these cases. In one of the cases of "inactive" cirrhosis, the cirrhosis was of the "incomplete septal" type; this was the youngest patient in this group (26 years). The other 2 inactive cirrhosis were micronodular in one and mixed micro-macro nodular in the other. In 17 of the 20 biopsies the cirrhosis occurred together with inflammatory infiltrates in the fibrotic septa, thus resembling chronic persistent hepatitis or chronic active cirrhosis. In 6 of these 17 biopsies the chronic active hepatitis was even the more striking phenomenon.

In most biopsies exhibiting active cirrhosis the hepatocytes in the cirrhotic nodules showed more of less severe cytoplasmic and nuclear anaplasia; these atypical hepatocytes usually contained abundant cytoplasm with a finely granular eosinophilic aspect (oncocytic change). Nuclei were prominent and hyperchromatic with prominent amphophilic nucleoli. The degree of atypia correlated with the severity of the accompanying chronic active hepatitis component.

Follow-up

During 15 years of follow-up 4 patients died due to concomitant diseases. One patient was no longer HBsAg positive 7 years after the biopsy which reveiled cirrhotic transformation. Three patients developed a hepatocellular carcinoma 4, 5 and 15 years, respectively, after the biopsy which established the existence of a cirrhosis (in the mean time two died).

Table V.9 *Characteristics of patients and biopsies with cirrhosis.*

type of cirrhosis	n	hepato-cellular atypia	died from concomitant disease	loss of HBsAg	hepato-cellular carcinoma
incomplete septal	1	-	-	-	-
inactive cirrhosis	2	-	-	-	-
cirrhosis with CPH	2	+	-	-	-
cirrhosis with CAH	15	+/+++	4	1	3

+ = slight atypia ++ = moderate atypia +++ = severe atypia

V.3.1.5 **Minimal lesions**

Minimal histological changes were seen in 14 of the 165 biopsies. These biopsies were taken from 14 HBsAg seropositive patients (5 females and 9 males) ranging in age from 26 to 55 years (average: 33 years). Nine of the 14 patients were blood donors, accidentally found to be HBsAg-positive during routine blood screening. In some of these biopsies some scattered lymphocytes were found in a minority of the portal triads. On the basis of the presence of these lymphocytic infiltrates in one biopsy, the diagnosis of an aspecific reactive hepatitis was considered. The liver architecture in all biopsies was completely intact. No appreciable fibrosis was found in any of the biopsies.

In most biopsies the hepatocytes showed mild cellular and nuclear anisomorphism, and occasionally a slight disarray of hepatocyte plates. The most dominant feature in all biopsies was the presence of large irregular clusters of hepatocytes with the ground-glass appearance (see chapter VII).

In one of the biopsies a mild coarse vacuolar steatosis and in another a small amount of hemosiderin pigment in Kupffer's cells were seen.

V.3.1.6 **Aspecific reactive hepatitis**

Aspecific reactive hepatitis was the diagnosis established for 11 of the 165 biopsies. These biopsies were taken from 11 individuals (1 female and 10 males) ranging in age from 19 to 50 years (average: 30 years). Seven of these 11 patients belonged to the group of blood donors who were found by chance to have HBsAg.

As the diagnosis indicates, the generally mild changes were aspecific and did not fit under the heading of one of the more definite types of hepatitis. The overall picture was that of a "restless liver". In most biopsies mild inflammatory infiltrates, composed mainly of lymphocytes, were found scattered throughout the portal tracts and in some places in the liver parenchyma. Throughout the liver parenchyma Kupffer's cells were slightly swollen and contained small amounts of ceroid pigment. Also some ceroid-loaded macrophages were seen in the portal tracts. The histological architecture of the liver was intact. Slight irregular fibrosis was seen in 5 of the 11 biopsies. In 2 cases the residual phase of acute hepatitis was included in the differential diagnosis. In 1 biopsy mild and in another more pronounced deposits of hemosiderin pigment were seen, mainly in the cytoplasm of Kupffer's cells. In this group of 11 biopsies larger clusters of ground-glass hepatocytes were also an important feature.

V.3.2 ASYMPTOMATIC HBSAG-POSITIVE SUBJECTS (GROUP A)

This group of HBsAg seropositive individuals was composed chiefly of voluntary blood donors (41), together with:

1. asymptomatic contacts of HBsAg-seropositive individuals (2).
2. individuals who were found by chance to be HBsAg-positive during routine medical examination (2).
3. patients found to be HBsAg-positive at routine screening by a clinic for venereal diseases (6).

V.3.2.1 **Blood donors**

For the group of 41 voluntary blood donors (8 females and 33 males) the results of the histological classification are given in table V.10.

Table V.10 *Histological liver changes, age and sex of 41 voluntary blood donors.*

	n	males	females	age range	age mean
acute hepatitis	9 (22%)	9	0	22-50y	34y
- self-limiting	3	3	0	22-43y	32y
- transition into chronic	6	6	0	29-50y	36y
chronic persistent hepatitis	10 (24½%)	8	2	22-49y	29y
chronic active hepatitis	5 (12%)	4	1	21-32y	25y
active cirrhosis	0 (0%)	0	0	-	-
inactive cirrhosis	1 (2½%)	1	0	26y	26y
minimal lesions	9 (22%)	5	4	26-55y	33y
aspecific reactive hepatitis	7 (17%)	6	1	23-50y	29y
total	41 (100%)	33	8		

During clinical follow-up of the hepatitis B infection, one or more biopsies were taken from 8 blood donors in the 5 years time period of this study. Between the first and second biopsies the time interval ranged from 5 to 13 months. On long-term follow-up, after the 5 years time period of this study, several additional biopsies were taken. The histological diagnose established for the initial and follow-up biopsies are given in table V.11. Clinical follow-up of the voluntary blood donors ranged from 8 months to 15 years.

Table V.11 *Histological diagnosis and degree of fibrosis for initial and follow-up biopsies from 8 seropositive blood donors*

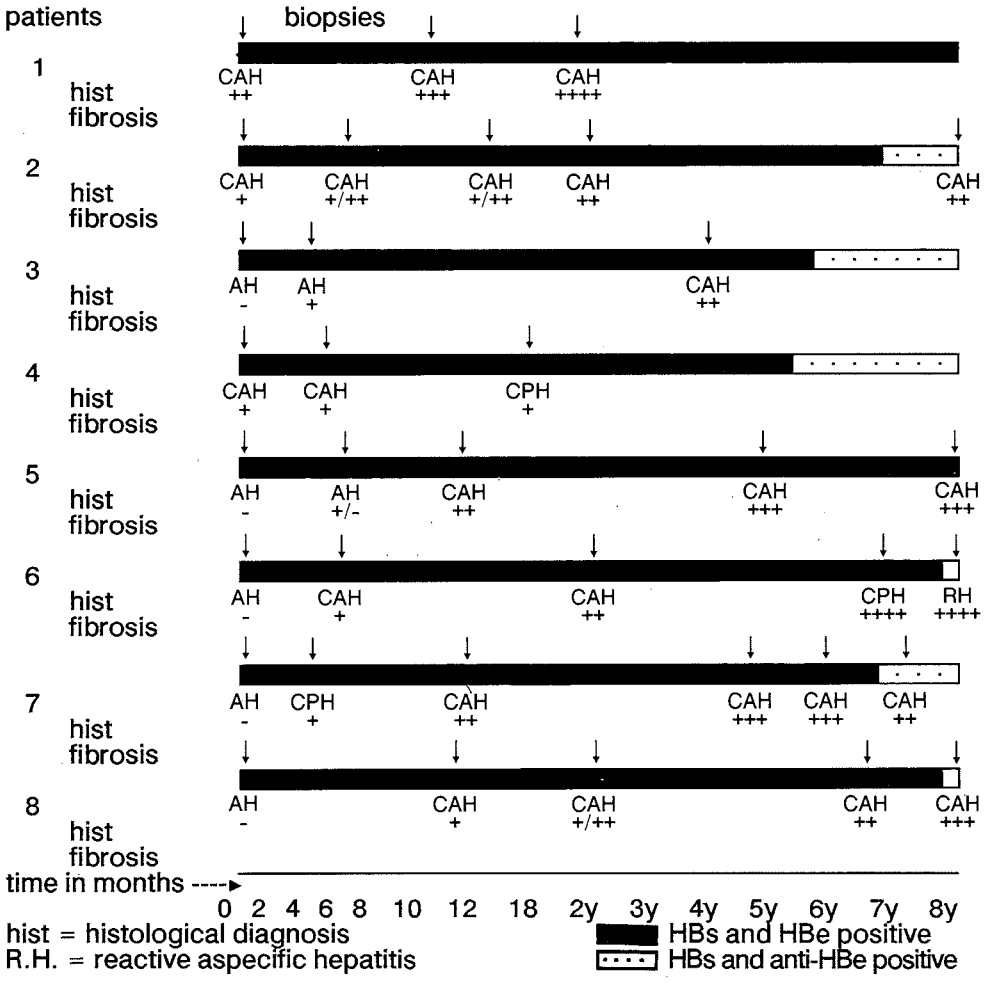


Table V.11 shows the initial and follow-up biopsies, the respective histological diagnose and the degree of fibrosis for 8 blood donors, followed because of the

Table V.10 shows the initial and follow-up biopsies, the respective histological diagnose and the degree of fibrosis for 8 blood donors, followed because of the finding and persistance of a hepatitis B viral infection. The ordinate represents the months after the first biopsy. A.H.: acute hepatitis; C.A.H.: chronic active hepatitis; C.P.H.:chronic persistent hepatitis.

Eight biopsies were taken as part of a series of follow-up biopsies. In a series of 4 biopsies from 1 patient 2 biopsies showed chronic persistent hepatitis, while the other 2 showed chronic active hepatitis.

V.3.2.1.1 Acute (lobular) hepatitis

Acute (lobular) hepatitis was the diagnosis established for the initial biopsy from 9 blood donors. In this group 3 individuals had self-limiting hepatitis, i.e. HBsAg had disappeared from the blood within 6 months of the accidental discovery of HBsAg. The other 6 remained HBsAg-positive for at least 6 years.

The 3 blood donors with self-limiting acute hepatitis exhibited the histological picture of classical fully developed acute hepatitis. In these biopsies marked ballooning degeneration and focal, mainly centrilobular, necrosis of hepatocytes were present; no bridging or confluent necrosis was seen. The 3 patients developed jaundice 50, 40 and 14 days, respectively, after the discovery of HBsAg in the serum by the blood transfusion services. Obviously the HBV infection in these individuals was detected during the incubation period. In these cases the liver biopsy was performed 8 days before and 8 and 9 days after the onset of jaundice. Before the jaundice they had complained of fatigue and general distress; one had arthralgia and a dermal rash. The patient with acute hepatitis who developed jaundice 8 days after the liver biopsy, had complained of fatigue and distress in the right upper abdominal region four weeks before the liver biopsy. At the time of the liver biopsy SGPT was elevated 100-fold and SGOT 50-fold. In contrast bilirubin was only mildly elevated as was alkaline phosphatase. Cholestasis was not seen in the biopsy of this patient, in contrast to the marked cholestasis with bile plugs in bile canaliculi in the other 2 cases. The portal tracts in all 3 patients exhibited edema and mild inflammatory infiltrates, composed of polymorphonuclear leukocytes, lymphocytes and plasma cells. In addition some portal tracts showed mild bile duct proliferation. In 1 patient an epithelioid cell granuloma was present in one of the portal tracts. In all 3 cases the serum was free of HBsAg 1 to 3 months after jaundice started. The peak of the SGPT values during the disease in these patients was 1740 IU/L on the average; the average value of SGOT on the day of the biopsy was 950 IU/L.

The other 6 individuals with lobular hepatitis exhibited in general the histological picture of a more advanced stage of acute hepatitis. Swelling and

proliferation of ceroid-loaded macrophages and Kupffer's cells were found in all biopsies, mainly centrilobular. In some biopsies numerous macrophages were also present in the portal areas. In contrast to the extensive mesenchymal reaction the number of Councilman bodies was small. Ballooning degeneration and mitotic activity was less evident than in the first 3 patients. In only 1 of the biopsies was mild cholestasis seen. None of the 6 blood donors had ever had jaundice and during follow-up jaundice also did not develop. Five of the 6 had complained 1 week to 3 months before the biopsy of general distress and/or arthralgia. One donor with severe arthralgia was positive for both HBsAg and anti-HBs. Only 1 of the 6 had no special complaints. Three of the 6 had donated blood about 6 months before the time HBsAg was found accidentally but had not been HBsAg-positive at that time. The other 3 subjects were donating blood for the first time. During clinical follow-up the course of the disease in 5 of the 6 donors was also evaluated histologically. The follow-up biopsies from these donors showed the histological features of a chronic hepatitis (table V.10).

The liver tissue from 8 of the 9 donors with the histological picture of acute hepatitis did not contain hemosiderin or copper deposits. In 1 case the biopsy revealed a minimal amount of hemosiderin pigment in the Kupffer's cells. All biopsies contained small (6) or moderate (3) quantities of eosinophilic leukocytes, mainly in the portal tracts.

V.3.2.1.2 Chronic persistent hepatitis

Chronic persistent hepatitis was the histological diagnosis established for the initial biopsy from 10 volunteer blood donors (8 males, 2 females ranging in age from 22 to 49 years). All biopsies showed prominent round-cell infiltrates, consisting mainly of lymphocytes and some plasma cells, in more than one of the portal tracts. Only 1 biopsy revealed "spill-over" of the infiltrate. In the other biopsies the infiltrates were restricted to the portal tracts. There was no evidence of piecemeal necrosis in any of the biopsies. In 2 biopsies most of the portal tracts contained a mild infiltrate; these cases provided diagnostic difficulties; differential diagnosis included aspecific reactive hepatitis and residual phase of acute hepatitis.

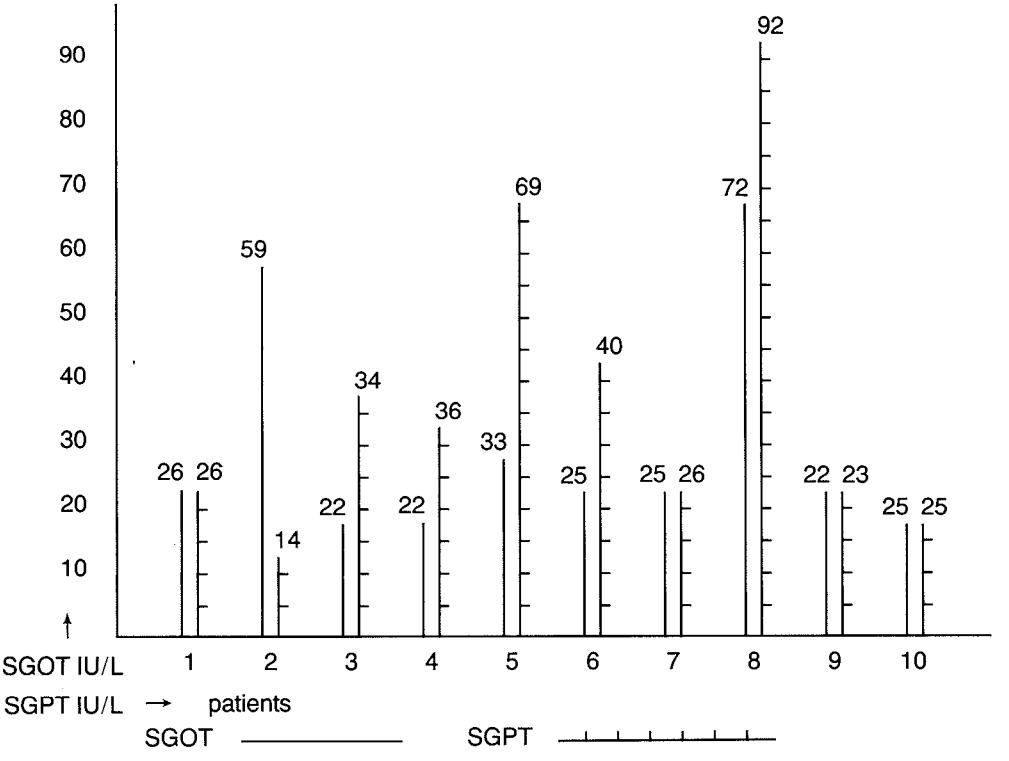
Lobular changes in all biopsies were restricted to scattered small foci of proliferated macrophages and/or Kupffer's cells mixed with some lymphocytes. In these foci degenerating hepatocytes, sometimes with the features of a Councilman body, were seen sporadically. All biopsies contained groups of hepatocytes with little or moderate anisocytosis and anisokaryosis. In 4 of the 10 biopsies marked fibrosis was present, sometimes with porto-portal septum formation which caused focal parcelling of the liver parenchyma. These fibrotic

septa contained no or only minimal inflammatory infiltrates. In 4 biopsies foci of hepatocytes contained intracytoplasmic fat vacuoles. Hemosiderin pigment, in both Kupffer's cells and hepatocytes, was seen only in one biopsy.

The serum SGOT values, see fig.V.3, ranged from 22 to 72 IU/l with an average of 33 at the time of the biopsy. With respect to the serum SGOT levels, the serum SGPT levels, ranging from 14 to 92 IU/L with an average of 38, were higher in 6, similar in 2 and lower in 2 cases.

In all 10 cases the serum bilirubin levels were within the normal limits. None of the patients had a history of jaundice. Five individuals had complained of general distress and nausea 4 to 12 months before the finding of HBsAg; the other 5 never experienced signs or symptoms suggesting the presence of a subicteric acute hepatitis.

Fig.V.3 *Serum transaminase levels measured in sera from 10 blood donors taken at the time of the liver biopsy, which was indicative for chronic persistent hepatitis.*



All donors were followed clinically and biochemically. During follow-up the course of the liver enzyme tests justified histological follow-up in only 1 case (patient 8, fig.V.3). The biopsy was taken five years after the initial biopsy and showed chronic active hepatitis with marked fibrosis.

V.3.2.1.3 Chronic active hepatitis

The initial biopsy from 5 volunteer blood donors (4 males, 1 female) showed the histological picture of chronic active hepatitis. In all biopsies all portal tracts contained marked round-cell infiltrates, consisting of lymphocytes and plasma cells. In general the infiltrates were concentrated on the interface between the portal tracts and the liver parenchyma. In several places the parenchyma was invaded by the inflammatory infiltrate with destruction of the limiting plate. In these areas degenerating hepatocytes, sometimes with the aspect of Councilman bodies, were present. In 2 of the biopsies the inflammatory infiltrates were intermingled with a marked quantity of eosinophilic leukocytes. The activity of the chronic hepatitis varied from mild (3) to moderate (2). In all biopsies periportal fibrosis was present, together with the formation of some porto-portal septa in 1 biopsy, and with extensive porto-portal septa and nodular regeneration of the liver parenchyma in another.

In 2 of the 5 biopsies a small amount of hemosiderin pigment was present. The serum SGOT values for this group ranged from 32 to 86 I.U./L with an average of 56. The SGPT values, which with one exception were higher than the SGOT values, ranged from 30 to 120 I.U./L with an average of 70 (fig.V.4).

Follow-up

During clinical and biochemical follow-up, repeat biopsies were taken from 3 of the 5 patients during the five year study time: 3 from 1 patient, and 2 from 2 patients. On the long-term follow-up biopsies were taken from all 5 patients. Four of the 5 donors reveiled HBe seroconversion after 3, 6, 6 and 10 years, respectively. Subsequently 2 of these 4 also became HBsAg seronegative.

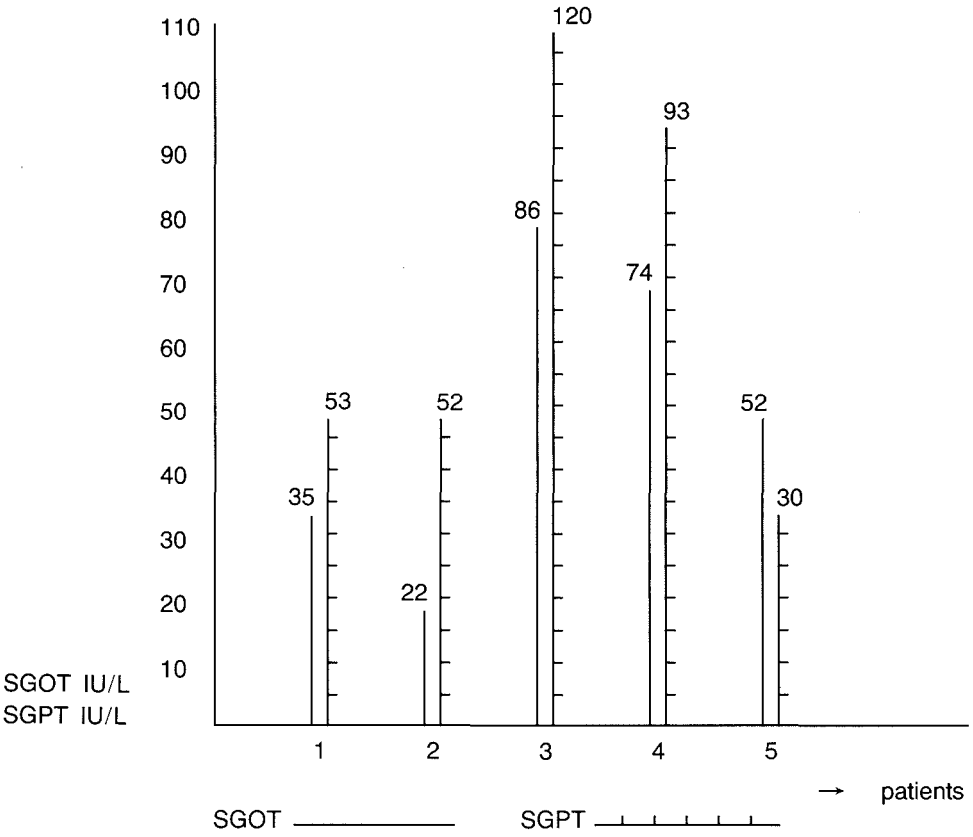
V.3.2.1.4 Cirrhosis

Cirrhosis was found in one volunteer blood donor. The cirrhosis was from the incomplete septal type. There were only minimal inflammatory infiltrates in the gracile fibrous septa.

V.3.2.1.5 Minimal lesions

Minimal lesions (9 donors: 5 males, 4 females) or an aspecific hepatitis (7 donors: 6 males, 1 female) were the diagnosis established for 16 of the 42 volunteer blood donors.

Fig.V.4 *Serum transaminase levels measured on the day of biopsy in 5 blood donors with chronic active liver disease.*



The changes in the biopsies from blood donors with aspecific hepatitis comprised focal anisocytosis and anisokaryosis of the hepatocytes, numerous binucleated hepatocytes, activation of scattered Kupffer's cells which contained

some ceroid pigment and inconspicuous inflammatory infiltrates in the portal tracts: the latter were too small for a diagnosis of chronic persistent hepatitis. Differentiation form chronic persistent hepatitis was difficult in 1 of the 7 cases. End-stage of acute hepatitis was included in the differential diagnosis for another biopsy, which showed fibrotic septa and many swollen macrophages loaded with ceroid pigment. Two biopsies exhibited mild fibrosis together with scattered hepatocytes with intracytoplasmic fat vacuoles (steatosis). Iron pigment was not found in any of the 7 biopsies.

Periodic acid-Schiff-positive globules in the cytoplasm of hepatocytes, characteristic of α_1-antitrypsin, were seen in 1 biopsy. Proliferation of bile ductules was evident in another biopsy.

For the group of subjects with aspecific reactive hepatitis the SGOT levels in sera taken at the time of the biopsy ranged from 16 to 26 with a mean value of 21. The SGPT levels ranged from 18 to 25 (mean value 22). Six of 7 donors with an aspecific hepatitis were found to be HBsAg-positive at the time of their first visit to the blood transfusion services. However, 1 patient had been HBsAg-negative at the time of a blood donation 1 year before.

The 9 biopsies with histological changes scored as minimal lesions showed no inflammatory infiltrates. In 1 biopsy moderate and in another biopsy minimal steatosis was present. Fibrosis was not seen in any of these biopsies. No iron and copper pigment could be demonstrated.

Both the liver biopsies with minimal lesions and those with aspecific hepatitis contained large numbers of "ground-glass" cells (see chapter VII).

At the time of the biopsy the serum SGOT levels for the minimal lesion group ranged from 14 to 33 (mean: 22); the serum SGPT levels ranged from 12 to 32 (mean: 28). The bilirubin levels were all within the normal limits.

Seven of the 9 aspirant donors were found to be HBsAg-positive during their first medical examination which included routine screening of the blood for the presence of HBsAg; 2 of the 9, however, had been active donors, for 10 and 2 years respectively. Their blood had been HBsAg-negative at the times of 8 and 3 previous donations, respectively, the last being only 6 months earlier.

V.3.2.2 **Asymptomatic hepatitis B positive subjects, except blood donors**

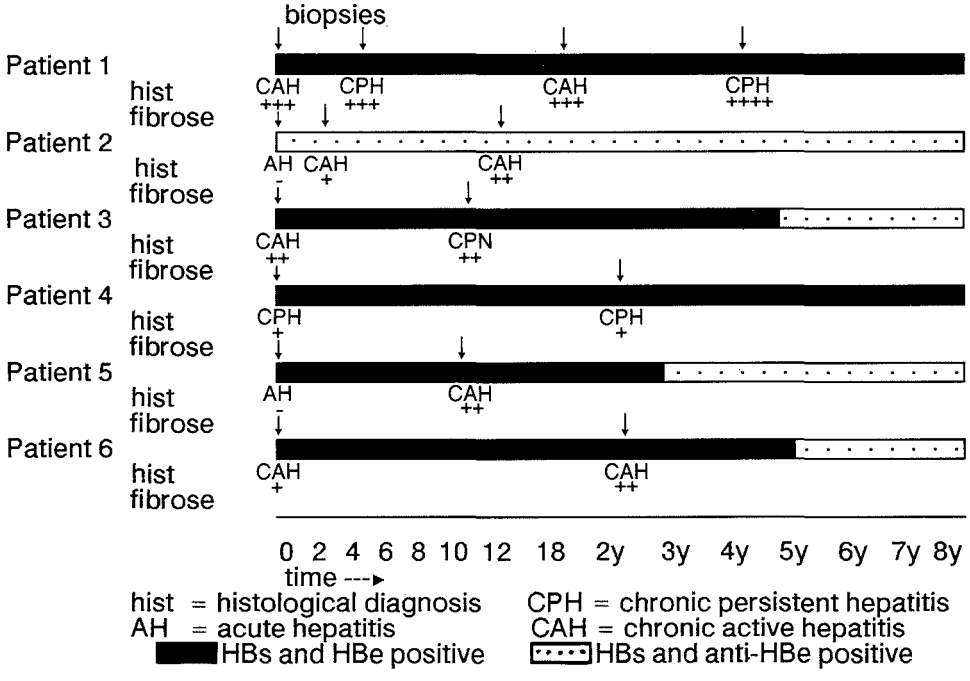
The histological diagnosis for the 6 patients, found to be HBsAg positive by chance, during a visit to an outpatient clinic for venereal disease, are:
chronic persistent hepatitis (2 patients); chronic active hepatitis (2 patients); minimal lesions (1 patient); aspecific reactive hepatitis (1 patient).

From the two subjects found HBsAg-positive by screening of family contacts of known HBsAg carriers one had chronic persistent hepatitis, the other had

aspecific reactive hepatitis. The 2 patients found HBsAg seropositive on routine medical examination reveiled chronic active hepatitis in one and acute hepatitis in the other.

From 6 out of the 10 cases follow-up biopsies were available. The results are given in table V.11.

Table V.11 *Histological diagnosis and degree of fibrosis for initial and follow-up biopsies from 6 asymptomatic HBsAg-seropositive subjects*



V.3.3 SYMPTOMATIC HBSAG SEROPOSITIVE PATIENTS (GROUP B)

The classification of the histological changes in the biopsies from 88 symptomatic HBsAg seropositive patients are given in table V.12.

Most of all acute hepatitis cases in this study, belonged to the group of symptomatic hepatitis B infections (33 of 43 cases). From the 33 acute hepatitis cases, 31 were accompanied by jaundice. Transformation into chronic hepatitis occurred in four, including the two anicteric cases. From the symptomatic hepatitis B patients ± 13 per cent had chronic persistent hepatitis, compared to ± 25 per cent in the asymptomatic hepatitis B subjects.

As could be expected, most of the patients with chronic active hepatitis and cirrhosis had symptomatic liver diseases, i.e. 21 of 29 and 17 of 18, respectively.

On the other hand hepatitis B infection with minimal histological changes and aspecific hepatitis were relatively more frequent in the asymptomatic hepatitis B carriers.

Table V.12 *Histological classification of liver changes for 88 symptomatic HBsAg carriers*

acute hepatitis	33
chronic persistent hepatitis	11
chronic active hepatitis	21
cirrhosis active	15
cirrhosis inactive	2
minimal lesions	3
reactive hepatitis	3

V.4 DISCUSSION

A wide range of liver changes was found in the liver biopsies from the 139 HBsAg-seropositive patients. The histological changes in all biopsies fitted one of the histological categories of the hepatitis B reaction pattern. However, despite exact definition of the histological criteria for the various categories of hepatitis, problems in classifying the liver changes were encountered in a large number of cases. These problems are inherent to all classification systems, where a continue spectrum of a disease is splitted artificially in categories.

As far as the group with acute hepatitis is concerned the most serious problems occurred when there were prominent portal infiltrates, which sometimes invaded the surrounding liver parenchyma. When there were signs of a subsiding lobular hepatitis component, as seen in late-phase acute hepatitis, these prominent portal infiltrates suggested in all cases a transition from acute hepatitis to chronic hepatitis. In this study an acute hepatitis with a prominent portal infiltrate was seen in 21 of the 47 biopsies (48 per cent); on the basis of this histological aspect, acute hepatitis, suggestive for transition to chronic hepatitis, was the diagnosis in these cases (table V.13). Three of these 21 biopsies revealed a fully

developed acute (lobular) hepatitis and 18 a late-phase acute hepatitis. Thirteen of these biopsies were taken from 9 patients, who ultimately developed a histologically proven chronic hepatitis, while 8 biopsies came from 8 patients with a proven self-limiting acute hepatitis. Of the last group of 8 patients, 5 were (however) drug-addicts and the prominent portal infiltrates in these drugaddicts may be explained to be caused by other pathogenetic factors than the hepatitis B infection. In contrast only 3 out of 26 patients with an acute hepatitis without prominent portal inflammatory infiltrates developed chronic hepatitis. So, a prominent portal infiltrate, sometimes with the features of piecemeal necrosis had some but slight prognostic value, in predicting the transition of acute hepatitis to chronic hepatitis. All other light microscopic aspects seen in acute hepatitis are shown to have less prognostic value.

Histological stages of acute hepatitis (fully developed, late and residual stages), correlated well with clinical and biochemical findings. Biopsies with the histological picture of an acute fully developed hepatitis were found to be taken meanly 12 days after jaundice started, whereas the biopsies with the histological picture of a late stage of acute hepatitis were taken 23 days after jaundice started and were accompanied by significant lower transaminase levels as compared with fully developed acute hepatitis. Most of the acute hepatitis cases which transformed in chronic hepatitis were the late-stage types. Probably this is the result of low rate of liver cell necrose on a protracted time-scale which will influence in the liver tissue the relation between necrotic hepatocytes and ceroid macrophages in favor of the ceroid macrophages.

Presence of iron-pigment in the biopsy series of acute hepatitis was inconspicuous. In only 12 out of 47 cases (± 21 per cent) iron pigment was detected. This is in contrast to the studies of Hengeveld (1979) who found in 12 out of 20 patients (60 per cent) iron pigment, partly in macrophages, partly in sinusoidal lining cells and in all biopsies (10), taken more than 2 weeks after jaundice started, he detected iron pigment. Eosinophilic granulocytes were found in 27 of the 47 biopsies with acute hepatitis, in large numbers in 10 cases. From this finding it is shown that eosinophilic leukocytes may not be used to discreminate between an acute viral hepatitis and an acute drug-induced hepatitis.

In this study the differentation between CPH and CAH was not only justified by the differences in transamines in both groups but also by differences in architectural disturbances of the liver parenchyma and prognosis. Nevertheless pronounced fibrosis was found in some biopsies with the histological picture of a chronic persistent hepatitis. Obviously in these cases the CPH must be considered as a temporaly state of a basically more severe liver disease or as a state after a more severe liver disease e.g. status after severe acute hepatitis or after HBcAg seroconversion. The relatively good correlation between disturbances in

liver enzymes and histological liver changes are in contrast with the study of Seef 1975, who found a poor correlation between clinical and histological diagnosis.

Ages of patients with acute hepatitis, chronic persistent hepatitis and chronic active hepatitis differed not significantly, whereas cirrhosis was found in older age groups. This finding can be explained by the time period, necessary for the evolution of cirrhosis from a chronic (active) hepatitis. However a more agressive course of a hepatitis B infection in older age individuals cannot be excluded in this study.

Of the 43 patients with a histologically classified acute hepatitis, 11 developed a chronic hepatitis. In 9 of these 11 patients, the acute hepatitis ran an anicteric course, which fits with the finding of Sherlock (1975) who stated that most cases of chronic hepatitis start as a grumbling subclinical acute hepatitis and that icteric acute hepatitis rarely transforms into a chronic liver disease. Moreover most of the patients with chronic hepatitis in this study do not reveal an anamnestic period of jaundice.

Table V.13 *Clinical and histological aspects associated with the course of an acute hepatitis with prominent portal infiltrates.*

course of hepatitis	biopsies n	patients n	jaundice +	jaundice -	fully developed hepatitis	late phase type of hepatitis
self-limiting	8	8	8	0	3	5
transition into chronic	13	9	1	12	0	13

From this study it is concluded that the presence of a prominent portal infiltrate, sometimes with the characteristics of piecemeal necrosis is not always a sign for transition to chronic hepatitis. For asymptomatic HBsAg seropositive subjects the wide spectrum of histological changes in liver biopsies is described by several authors (Klinge, 1973; Feinaro, 1974; Woolf, 1974; Villeneuve, 1976; Lok, 1988). However, there are discrepancies between these reports as far as the incidence, nature and severity of the liver abnormalities are concerned. These discrepancies could be explained by differences in the populations studied by the various authors (Feinman, 1975). In the studies on asymptomatic blood donors, some investigators excluded asymptomatic HBsAg-positive donors with abnormal liver function tests (Reinicke, 1972; Shrago, 1977), while others analysed all HBsAg

blood donors, irrespective of their liver function tests (Griffin, 1973; Villeneuve, 1976).

Table V.14 *Results of histological analysis of liver biopsies from healthy asymptomatic HBsAg-seropositive subjects in several studies.*

Series		Total	Normal	Abnormal			
		n	or Asp.	CPH	CAH	Cirrh.	AH
Prince	1969:bl	1	0	1	0	0	0
Klinge	1971:bl	21	10	11	0	0	0
Singleton	1971:bl	10	2	3	2	1	2
Iworson	1972:bl	21	10	11	0	0	0
Reinicke	1972:bl	24	6	17	0	1	0
Griffin	1973:bl	12	5	7	0	0	0
Simon	1974:bl	9	6	0	2	0	1
Ichida	1975:bl	20	19	0	0	0	1
Feinman	1975:bl	29	17	10	0	1	0
Woolf	1975:bl	36	19	11	5	1	0
Villeneuve	1976:bl	31	5	24	0	2	0
Shrago	1977:bl	26	11	13	0	0	0
Wesdorp	1979:bl	12	4	1	4	3	0
Dormeyer	1981:bl	88	78	6	4	0	0
Feinman	1982:bl	36	21	15	0	0	0
This serie	1989:bl	41	16	10	5	1	9

bl = blooddonors

Table V.14 shows the results of the histological evaluation of biopsies, taken from asymptomatic HBsAg seropositive individuals in several studies. The spectrum of lesions reported by the different investigators, varied from predominantly "normal liver histology" to serious lesions such as cirrhosis or chronic active hepatitis. However, in most studies the majority of liver biopsies revealed no or slight changes; chronic active hepatitis and cirrhosis were rare findings. As in the present study the more severe liver changes were always accompanied by abnormal liver function tests. However, subjects with chronic persistent hepatitis may reveal normal or partly abnormal liver function tests. The relatively severe liver changes found in blood donors in the study of Wesdorp (1979) can be explained by abnormal liver enzymes as the selection criterium for the performance of a

liver biopsy. Also Singleton (1971) found in 9 out of 10 asymptomatic blood donors proportionally many severe liver changes: hepatitis, with chronic active hepatitis in 1, acute hepatitis in 2 and active cirrhosis in 1 and inactive cirrhosis in another case. However in contrast with the studies of Reinicke and Iworson, which reported on volunteer blood donors, the study of Singleton concerned a group of payed donors, with a high incidence of intraveneus drug abusers.

In contrast to the findings of others this present study of volunteer blood donors showed a striking incidence of lobular (acute) hepatitis. In three blood donors HBsAg was detected in the preicteric period and acute hepatitis was self-limiting. Six patients however were detected with an anicteric acute hepatitis which transformed into a chronic type of hepatitis. Within ten years four of these blood donors showed HBe seroconversion and one lost HBsAg subsequently.

As to be expected liver biopsies with minimal lesions or compatible with the picture of "aspecific reactive hepatitis" are encountered more often in the group of asymptomatic HBsAg seropositive subjects (19 of the 51: 39 per cent) than the group of symptomatic HBsAg seropositive patients (6 of the 88: 6,5 per cent). Among the symptomatic HBsAg seropositive patients acute hepatitis, including acute hepatitis that is likely to transform into chronic hepatitis, and active cirrhosis were the most common diagnosis (41 and 20 of the 89 patients, respectively).

CHAPTER VI

DETECTION AND LOCALIZATION OF HEPATITIS B AND DELTA ANTIGENS AND IMMUNOGLOBULIN IN LIVER TISSUE

An immunochemical study

CHAPTER VI

DETECTION AND LOCALIZATION OF HEPATITIS B AND DELTA ANTIGENS AND IMMUNOGLOBULIN IN LIVER TISSUE

An immunochemical study

VI.1. SUMMARY

The presence and localization of hepatitis B and D antigens were investigated in 165 biopsies from 139 HBsAg-positive patients.

The mutual relationships between these antigens and the concomitant histological changes in the liver parenchyma were studied.

In fresh frozen liver tissue three distinct patterns, based mainly on the distribution of HBsAg in the liver tissue, were distinguished. These patterns correlate well with, and provide additional diagnostic and prognostic parameters for, the light microscopical diagnosis:

a. solitary pattern: expression of HBsAg in only an occasional macrophage and degenerating hepatocyte. This pattern correlates with acute self-limiting hepatitis.
b. diffuse or honeycomb pattern: all hepatocytes exhibit linear to finely granular deposits along the cell membranes, particularly on the outer surface. The pattern may be combined with the presence of scattered hepatocytes with intracytoplasmic HBsAg (ground-glass hepatocytes). Moreover, HBcAg and/or HDV may be present. On the basis of the presence and localization of HBcAg the pattern is subdivided:
    1. no HBcAg or HBcAg confined to the nuclei.
    2. combined nuclear and cytoplasmic HBcAg localization.

    The diffuse HBsAg pattern correlates with chronic persistent and chronic active hepatitis while progression of chronic hepatitis is determined by the presence and localization of HBcAg.
c. focal pattern: large groups of hepatocytes contain abundant intracytoplasmic HBsAg. These hepatocytes correspond in general with the ground-glass cells seen in light microscopy. HBcAg is not present. Light microscopy shows minimal changes or slight aspecific hepatitis.

The results suggest that a combination of immune mechanisms, determined by

HBsAg and possible HBcAg localization along hepatocytic membranes and direct cytopathogenic effects related to cytoplasmic HBcAg localization, plays a role in the pathogenesis of hepatitis B. Ground-glass cells are supposed to be a sign of integration of HBV-DNA in the host cell genome. The presence of immunoglobulins in the nucleus and the cytoplasm of hepatocytes correlated with the presence and localization of HBcAg and/or HDV and is the result of adherance of intrinsic anti-HBc and/or anti-delta antibodies present in serum to HBcAg and/or HDV during the immuno-histochemical procedures. This phenomenon provides the means for finding other (viral) antigen/antibody systems in (liver) tissues.

VI.2 INTRODUCTION

After the discovery of hepatitis B antigen (HBAg), then known as Blumberg's Australia antigen (1965), several immunofluorescence studies on the demonstration and localization of HBV- and later HDV- in liver tissue were published.

In the early investigations various types of hepatitis were studied by means of the direct or indirect fluorescence technique, using both heterologous and homologous antisera to HBAg but the exact localization of HBAg in hepatocytes remained controversial. Some authors found HBAg chiefly in the nucleus (Millman, 1969; Coyne, 1970; Nielsen, 1970; Nielsen, 1971), others only in the cytoplasm (Edgington, 1971; Hadziyannis, 1972), while still others found HBAg in both the nucleus and the cytoplasm in variable patterns and quantities (Nowoslawski, 1970; Kater, 1972; Gerber, 1972; Krawczynski, 1972; Hadziyannis, 1973).

Most of the controversy was resolved when it became clear that HBAg is composed of at least two distinct components with well-defined morphological and antigenic correlates (Almeida, 1971; Brzosko, 1973; Huang, 1973). These components are hepatitis B core (HBcAg) and hepatitis B surface (HBsAg) antigen, both defined by their own antibody system (anti HBsAg and anti HBcAg, respectively). In a patient's serum these antigens can appear together in one particle (Dane particle) as a complete virion, but in infected liver cells the two antigens have distinct localizations.

Immunofluorescence studies, using specific antisera against core antigen, have shown that HBcAg is found mainly in the nuclei of hepatocytes and that the expression of this antigen correlates microscopically with the presence of 21-25 nm particles (Huang, 1971). HBsAg could only be demonstrated in the cytoplasm and/or along the cell membranes of hepatocytes (Edgington, 1971; ten Kate, 1974; Roos, 1975; Gudat, 1976). In ultrathin sections intracytoplasmic HBsAg was associated with 20 nm spherical or tubular particles located predominantly in the rough and smooth endoplasmic reticulum (Stein, 1971).

As a rule HB antisera obtained from hyperimmunized animals appear to contain high levels of antibodies against the surface antigen whereas those obtained from HBsAg seropositive patients contain antibodies directed exclusively against the core antigen and those from convalescent hepatitis B patients contain variable amounts of HBc and HBs antibodies. The differences in the expression of HBsAg in the cytoplasm and HBcAg in the nuclei of hepatocytes led to the suggestion by Gudat et al. (1975) that these expression patterns could be related to the immune responsiveness of the patient and could therefore have diagnostic and prognostic implications. Indeed morphological and immuno-fluorescence studies by ten Kate (1974), Akeyama (1974) and Ray (1976) suggested that HBc, and especially HBs, fluorescence patterns correlate with stage and prognosis of the liver disease.

Several authors compared the sensitivity of HBsAg serology with that of HBsAg fluorescence in liver tissue. Edgington (1971), Krawczynski (1972) and Ray et al. (1974, 1976) demonstrated positive fluorescence of HBAg antigens in liver tissue from HBsAg-negative patients. Krawczynski, however, compared the immuno-fluorescence patterns found for liver tissue obtained at autopsy with the findings of the HBsAg assays performed with sera and pericardial fluids, using the insensitive immunodiffusion technique. The studies of Ray et al. (1974, 1976), who used a modified fluorescence method, also demonstrated positive HBsAg fluorescence in livers from HBsAg-seronegative patients. Serological detection in these studies was performed with radioimmunoassays. This latter study suggested that immunofluorescence studies of liver tissue are a more sensitive technique than radioimmunoassays of serum for the detection of hepatitis B antigens.

In the liver HBAg has been detected not only in hepatocytes but also in Kupffer's cells and endothelial lining cells as well as macrophages in the portal tracts (Coyne, 1970; Edgington, 1971; Hadziyannis, 1974). Outside the liver, e.g. in bone marrow, testis, spleen or mesentery, deposition of HBAg was rare (Coyne, 1970). The presence of HBAg in vessel walls in HBsAg seropositive patients with polyarthritis nodosa (Prince and Trepo, 1971; Cocke, 1971; Nowoslawski, 1972; Brzosko, 1974) and in glomeruli (de Man, 1989) is highly suggestive of immune complex deposits (Steffelaar, 1977).

Immmunoglobulins have been demonstrated in the liver along sinusoids (Kater, 1972) and on surface membranes as well as in the nuclei of liver cells (Gerber, 1972; Hadziyannis, 1973; ten Kate, 1974; Roos, 1975). The deposits of immuno-globulin along liver cell surface membranes are thought to be caused by viral-induced changes in the composition of the cell membranes which are responsible for destruction of the hepatocytes. The localization of immunoglobulins in hepatocytic nuclei is a subject of controversy.

In this chapter the localization of HB antigens (HBsAg, HBcAg and HBeAg), Delta antigen and immunoglobulins in liver tissue and liver cell suspensions is described. The variation in the appearance of HBc in the nuclei and HBsAg in the cytoplasm led to the recognition of several viral expression patterns; these patterns could be correlated with light microscopical findings (see chapter IV). Most of the patients were followed clinically, biochemically and in some cases morphologically in order to study the course of the disease. The significance of the various fluorescence patterns as a tool for histological staging and determination of the prognosis of the disease is discussed.

VI.3 RESULTS

VI.3.1 HBAG IN LIVER TISSUE AND ISOLATED LIVER CELLS

Of 252 liver biopsies subjected to immunofluorescence to detect the presence of HBAg, 165 were obtained from HBsAg-seropositive patients and 87 from HBsAg-seronegative patients.

Except in one case, none of the 87 liver biopsies from seronegative patients showed specific fluorescence with either anti-HBsAg and anti-HBcAg antisera. The one positive case in the group of HBsAg seronegative patients exhibited HBc fluorescence but no HBsAg fluorescence.

In total 161 of the 165 liver biopsies obtained from HBsAg seropositive patients showed positive HBsAg fluorescence. HBsAg was found chiefly in hepatocytes, but sometimes also in the cytoplasm of Kupffer's cells and endothelial lining cells as well as the walls of vessels in the portal tracts. Of the 161 biopsies with a specific HBsAg fluorescence, 43 also showed positive HBcAg fluorescence (table VI.1).

Table VI.1 *Results of HBsAg and HBcAg fluorescence studies of 252 biopsies from HBsAg-seropositive and HBsAg-seronegative patients.*

	n	HBsAg + n	HBcAg + n
liver tissue from HBsAg seronegative patients	87	0	1
liver tissue from HBsAg seropositive patients	165	161	43

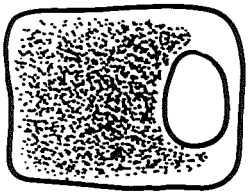
The results of the HBs and HBc fluorescence studies of liver tissue specimens were compared with the results found for isolated hepatocytes in suspension and/or sedimentation preparations made from the liver biopsies of 45 patients

VI.3.2 LOCALIZATION OF HBSAG IN LIVER CELLS

Immunofluorescence studies, using monospecific HBsAg antisera, revealed that HBsAg appears within the hepatocytes only in the cytoplasm or along the cell surface. Nuclear localization of HBsAg was never seen.

Within the cytoplasm of the hepatocytes some distinctive HBsAg distribution patterns were identified:

1. hepatocytes with a diffuse granular, almost homogeneous, bright HBsAg fluorescence in the cytoplasm, leaving the nucleus and a small part of the biliary pool of the cytoplasm free of fluorescence (homogeneous fluorescence hepatocytes-HFH)



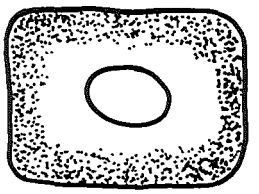
= HB_SAg

Fig.VI.1
homogeneous
fluorescence
hepatocyte (HFH)

These HFH appeared in liver biopsies in large clusters or as solitary cells scattered throughout the liver tissue. In sedimentation preparations of isolated hepatocytes these cells were also detected, but the granular fluorescence then overlapped the nucleus. Suspensions of isolated hepatocytes contained only a sporadic HFH. These HFH corresponded with the so-called ground-glass hepatocytes in light microscopy (Hadziyannis, 1973) (see chapter VII). This was confirmed by subsequent histochemical staining of slides containing HFH cells with hematoxylin azophloxin or aldehyde thionin (fig.VII.6).

2. hepatocytes with a varying bright rim of granular fluorescence along the periphery of the cytoplasm, leaving the nucleus and the perinuclear parts of the cytoplasm free of HBsAg fluorescence (buttonhole fluorescence hepatocytes-BFH). These cells were almost always found in small or large

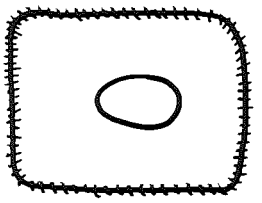
clusters. In light microscopy these cells were sometimes faintly recognizable on hematoxylin azophloxin-stained slides; depending on the quantity of HBsAg they were visualized fairly easily with the aldehyde thionin and Shikata stains.



= HB_SAg

Fig.VI.2
buttonhole
hepatocyte (BFH)

3. hepatocytes which exhibit a fine granular almost linear fluorescence along the cell surface, not only along the sinusoidal parts but also along the intercellular parts of the cell membrane (cell surface fluorescence hepatocytes-SFH). Cells with this type of fluorescence were encountered as a rule



= HB_SAg

Fig.VI.3
hepatocyte with
cell surface
fluorescence (SFH)

throughout the entire liver biopsy in all liver lobuli. Although the intensity of the fluorescence of these SFH did vary only slightly within a biopsy, the difference between various biopsies may be marked. In sedimentation preparations of isolated hepatocytes these cells showed a fine granular fluorescence which also covered the nucleus (fig.VI.4).
Similarly, suspensions of isolated hepatocytes exhibited a fine granular fluorescence along the cell surface, indicating that the HBsAg had been exposed to the outer surface of the liver cells. The fluorescence granules usually were not distributed as uniformly over the cell surface as in frozen sections, since they tended to form aggregates on the former (fig.VI.5). Sometimes a "capping" phenomenon was seen. Light microscopically no features corresponding to these SFH were seen.

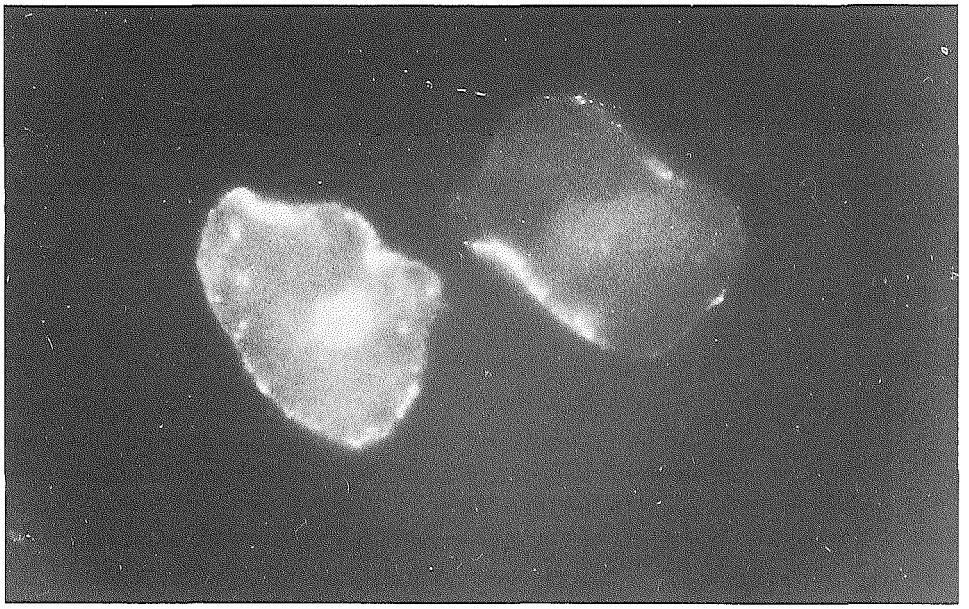
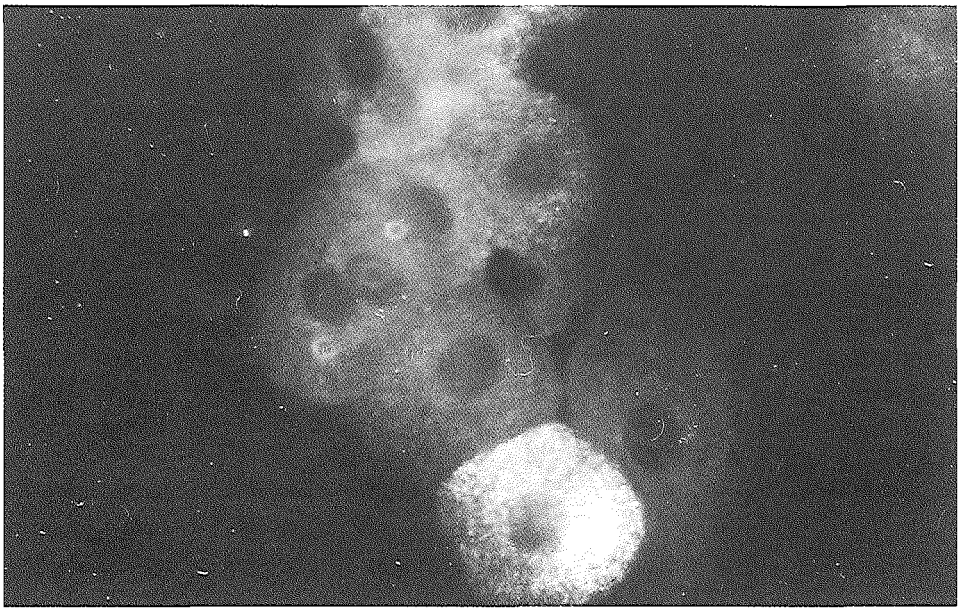


Fig.VI.4 *Suspension preparation of two hepatocytes with HBsAg located on the cell surface (direct immunofluorescence method, anti-HBsAg 600x).*

Fig.VI.5 *Sedimentation preparation of hepatocytes showing HBsAg deposits, irregular distributed on the outer surface (direct immunofluorescence method, anti-HBsAg 600x).*



VI.3.3 Influence of fixatives and fluorescence techniques on HBsAg fluorescence

The HBsAg-positive hepatocytes with bright homogeneous fluorescence or the buttonhole aspect could also be detected in sections of paraffin-embedded liver tissue by means of immunofluorescence and immunoperoxidase methods. However, the hepatocytes with cell membrane-bound fluorescence could generally not be demonstrated in formalin-fixed paraffin-embedded material. HBsAg fluorescence staining of frozen sections was only slightly affected by prior fixation with 2 per cent paraformaldehyde, alcohol or acetone as well as by heating during incubation with antisera. On the other hand prior fixation with 4 per cent formaldehyde or 1 per cent glutaraldehyde induced a strong background fluorescence, which masked the specific fluorescence.

Incubation of sections with anti HBsAg antisera for less than one hour diminished the intensity of fluorescence (table VI.2). Using microwave irradiation during incubation period within 5 minutes optimal fluorescence results were obtained.

Table VI.2 *Effects of varying treatments of frozen liver tissue sections on HBsAg fluorescence results*

slide pretreatment	aHBs incubation	HBsAg fluorescence results cytoplasmic	membrane	background
dried unfixed	1 hour 20°C	+ +/+ + +	+ +/+ + +	+/-
dried unfixed	½ hour 20°C	+	+	+/-
dried unfixed	1 hour 40°C	+ + +	+ + +	+
dried unfixed	½ hour 40°C	+ +	+ +	+/-
PBS 15 min	1 hour	+ +/+ + +	+ +	+ +
acetone 10 min	1 hour	+ + +	+ +/+ + +	+/-
ethanol 15 min	1 hour	+/+ +		
formaldehyde 15 min	1 hour	+	+/+ +	+ +
paraformaldehyde 2 % 15 min	1 hour	+ + +	+ + +	+ +
glutaraldehyde 1 % 15 min	1 hour	+ +	+/+ +	+ +
sublimate 15 min	1 hour	+/-	+/-	+ +
aceton 10 min	5 min micro	+ + +	+ + +	+/-

- = negative + = slight + + = moderate
+ + + = clear/severe micro = microwave irradiation

VI.3.4 LOCALIZATION OF HBcAG IN LIVER CELLS

With the fluorescence technique, using a homologous antiserum, HBcAg was found to lie in most cases almost exclusively in the nuclei of hepatocytes. However in some cases nuclear localization occurred together with localization in the cytoplasm: in particular patients with active liver disease and cirrhosis and patients undergoing immunosuppressive therapy sometimes exhibited large amounts of HBcAg in the cytoplasm (chapter XI). HBcAg could be detected in varying percentages of the nuclei, ranging from a sporadic nucleus to nearly all nuclei. No specific localization of HBcAg-positive hepatocytes was found within the hepatic lobuli. Within the nuclei HBcAg caused a fine granular pattern of variable intensity. The granules were usually scattered diffusely throughout the nucleus. Sometimes, however, these granules had accumulated near the nuclear membrane. The often pronounced nucleoli did not seem to contain HBcAg. Cytoplasmic HBsAg fluorescence in general occurred together with nuclear HBcAg, localized in several scattered or small groups of hepatocytes.

Light microscopy did not reveal nuclear features consistent with the presence of HBcAg. With the double labelling immunofluorescence technique using FITC and TRITC-labelled antisera against HBsAg and HBcAg, it was shown that HBcAg is nearly always present in hepatocytes exhibiting cell membrane-bound HBsAg fluorescence (fig.VI.6).

In sedimentation preparations of isolated hepatocytes HBcAg was also found in the nuclei of the hepatocytes. In suspensions HBcAg fluorescence could not be detected. Hepatocytes with bright homogeneous HBsAg cytoplasmic fluorescence, indicating impressive quantities of HBsAg, rarely contained HBcAg (fig.VI.6).

VI.3.5 LOCALIZATION OF eAG

The localization of eAg in general was identical to that of HBcAg (chapter IX).

VI.3.6 LOCALIZATION OF DELTA ANTIGEN

Only one of the 165 liver biopsies revealed delta antigen. Delta antigen was found in the nucleus of a small percentage of hepatocytes, together with HBsAg along the cell membranes. No HBcAg was found. The nuclear localization of Delta antigen in a small percentage of hepatocytes was also found in biopsies from later dates.

In liver grafts, transplanted in HBV-positive, HDV-positive patients, HDV-antigen

was detected together with HBcAg, both in the nucleus and the cytoplasm of hepatocytes (see chapter XI).

VI.3.7 HBsAg and HBcAg immunofluorescence patterns in liver tissue

On the basis of the distribution of HBsAg and HBcAg in liver tissue, distinct patterns can be distinguished:

1. **the solitary pattern**. In this pattern only a few scattered cellular elements in the liver tissue are positive for HBsAg. HBcAg is rarely found in these cases. The HBsAg-positive cells in the liver tissue are usually Kupffer's cells or macrophages with a coarse granular cytoplasmic fluorescence. These cells are situated within the sinusoidal spaces or lie within the portal tracts. Sporadically, a HBsAg-positive degenerating hepatocyte (Councilman body) may be found, but no vital HBsAg-positive hepatocytes are seen.

2. **the diffuse pattern**. Classically this pattern is characterized by a fine cell membrane-bound fluorescence of all hepatocytes in the liver tissue, yielding a honeycomb-like fluorescence aspect (fig.VI.6, page 145). In addition to this cell membrane-bound fluorescence pattern, scattered hepatocytes exhibit a bright homogeneous fluorescence. The number of these latter hepatocytes differs from one patient to the next and probably to some extent within one and the same liver. Sometimes small groups of buttonhole fluorescence hepatocytes are present. In liver tissue exhibiting this diffuse HBsAg pattern, HBcAg is frequently found in the nuclei of the hepatocytes, sometimes in combination with cytoplasmic localization. The number of HBcAg-positive cells can range from sporadic to almost all hepatocytes. High percentages of HBcAg-positive hepatocytes are found especially in livers from patients on immunosuppressive therapy. The HBcAg-positive hepatocytes are distributed in foci. This focal distribution is not related to a specific area within the lobulus. The localization of HBcAg is nearly always restricted to hepatocytes with cell membrane-bound fluorescence, while hepatocytes with intracytoplasmic HBsAg fluorescence are often negative for HBcAg (fig.VII.9, page 160).

3. **the focal pattern**. This pattern is characterized by coherent groups of hepatocytes with a bright intracytoplasmic HBsAg fluorescence (fig.VI.7, page 145). In addition, groups of hepatocytes with buttonhole fluorescence and/or cell membrane-bound fluorescence may also be found. The groups of HBsAg-positive hepatocytes have no specific location within the hepatic

lobulus and can cover more than one lobulus. This HBsAg pattern never included HBcAg. The inhomogeneous distribution of HBsAg-positive hepatocytes may cause a false-negative fluorescence finding.

According to the above-mentioned criteria most of the biopsies can be classified under one of the three fluorescence patterns. Sometimes, however, a pattern may be found with intermediate features. Cirrhotic livers in particular may exhibit different patterns in different nodules.

The incidence and distribution of the solitary, diffuse and focal patterns over the 165 liver biopsies are given in table VI.3.

Table VI.3 Incidence of HBcAg in the different HBsAg fluorescence patterns

			with HBcAg	without HBcAg
		n	n	n
solitary	pattern	28	0	28
diffuse	pattern	100	36	64
focal	pattern	22	0	22
negative		4	1	3
intermediate	pattern	11	6	5
total		165	43	122

None of the biopsies with the solitary or focal pattern contained HBcAg. HBcAg fluorescence was seen in 36 of the 100 biopsies (36 per cent) with a diffuse pattern. The number of nuclei positive for HBcAg ranged from sporadic to more than 80 per cent (table VI.4).

Eleven biopsies exhibited a fluorescence that did not fit one of the three fluorescence patterns. In 6 of these 11 biopsies cell membrane-bound fluorescence occurred only within groups of hepatocytes (not diffuse); 3 of these 6 biopsies were also HBcAg-positive. The other 5 biopsies were characterized by foci of bright homogeneous cytoplasmic HBsAg fluorescence together with hepatocytes that produced a cell membrane-bound HBsAg fluorescence. Three of these biopsies exhibited HBcAg fluorescence. Four out of the 165 biopsies were negative for HBsAg, one of which contained HBcAg.

Table VI.4 *Estimated percentage of HBcAg-positive hepatocytes in biopsies with a diffuse HBsAg fluorescence pattern*

	n
no fluorescence	63
sporadic	5
between 5 and 20 per cent	20
between 20 and 50 per cent	6
50 per cent and higher	6
total	100

VI.3.8 IMMUNOGLOBULIN AND COMPLEMENT DEPOSITS IN LIVER TISSUE AND ISOLATED LIVER CELLS FROM HBSAG-POSITIVE PATIENTS (table VI.5)

The direct or indirect immunofluorescence technique for frozen sections revealed that all biopsies from HBsAg-positive as well as HBsAg-negative patients contained diffuse deposits of immunoglobulin and complement in varying quantities in the sinusoidal spaces. These deposits were localized in particular in Disse's spaces (fig.VI.8). Biopsies from patients with inflammatory liver disease generally produced a more intense fluorescence than those from patients without inflam-matory liver changes. The immunoglobulin isotypes found were IgG and, to a lesser extent, IgM and IgA. Moreover the titer of complement factors in the sinusoidal spaces was low.

Deposits of IgG or IgM were detected along the cell membrane of hepatocytes in 98 of the 165 liver biopsies from HBsAg seropositive patients and in 43 of the 87 liver biopsies from HBsAg seronegative patients. This IgG and IgM fluorescence pattern was identical to the honeycomb-like fluorescence pattern found for HBsAg. Deposits of complement were never found in this honeycomb-like cell membrane-bound fluorescence pattern.

Immunoglobulin deposits were detected in the nuclei of hepatocytes in 42 of the 165 liver biopsies from HBsAg seropositive patients and 1 of the 87 biopsies from HBsAg seronegative patients. In the biopsies from HBsAg seropositive patients nuclear immunoglobulin fluorescence was always localized in groups of scattered hepatocytes (fig.VI.9). In the same biopsies HBcAg fluorescence exhibited an identical distribution pattern. Only 1 biopsy with HBcAg fluorescence lacked immunoglobulins; this biopsy came from a renal transplant recipient on high-dose immunosuppressive therapy. This was also the only one of the 43 patients with HBcAg fluorescence who exhibited minimal anti-core activity in the serum.

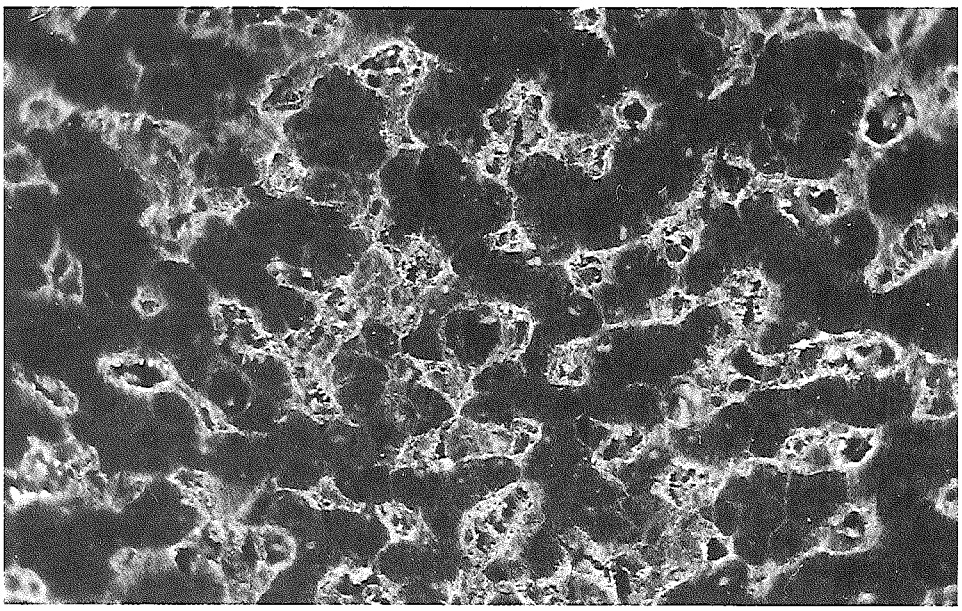
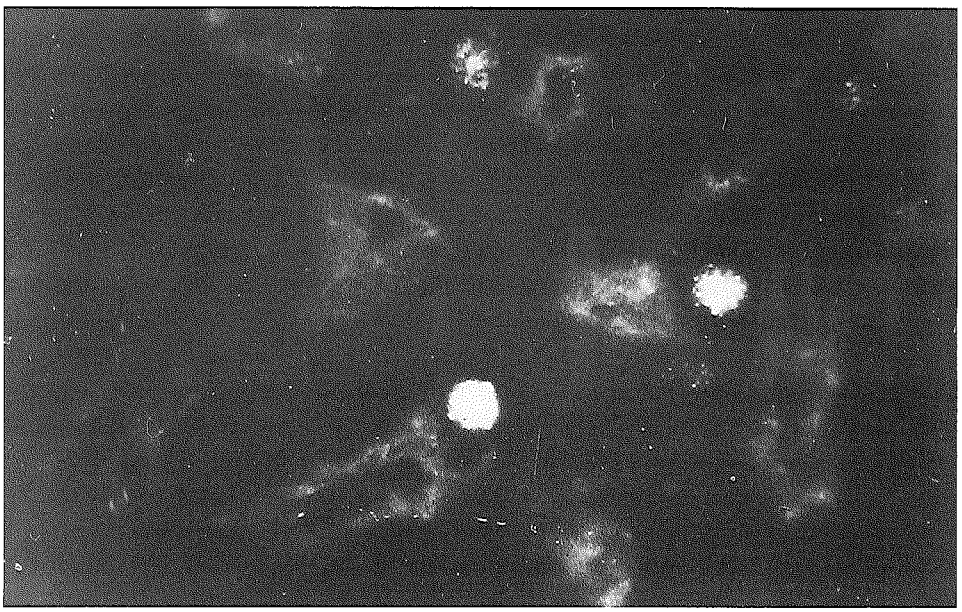


Fig.VI.8 *Normal IgG sinusoïdal fluorescence pattern in a section of frozen liver tissue (320x).*

Fig.VI.9 *Perisinusoïdal IgG deposits together with bright fluorescence of IgG deposits in a nucleus indicating the presence of HBcAg (anti-IgG, direct one-step fluorescence method, 600x).*



Electron microscopy showed core particles in the nuclei only in those cases in which nuclear immunoglobulin and/or HBcAg fluorescence staining had been demonstrated (see chapter X). All biopsies with nuclear deposits of immunoglobulin also exhibited deposition of immunoglobulin along the cell membrane of the hepatocytes.

Table VI.5 *Deposits of immunoglobulins in liver tissue*

	n	sinusoidal	cell membrane bound IgG	cell membrane bound IgM	nuclear IgG	nuclear IgM
liver tissue from HBsAg-positive patients	165	165	98	39	42	2
liver tissue from HBsAg-negative patients	87	87	43	29	1	0

In 40 of the 42 biopsies the nuclear immunoglobulin deposits were of the IgG class; the other two contained both IgG and IgM deposits. None of the biopsies showed deposition of IgA or complement in the nuclei. The biopsy with nuclear deposits of immunoglobulin from the patient without HBsAg-positive serology showed granular IgG fluorescence of all hepatocytic nuclei. The biopsies with nuclear immunoglobulin deposits from HBsAg-positive patients as well as the above-mentioned HBsAg-negative patient acquired nuclear complement fixation after incubation of the slides with fresh human serum.

Eluates of the liver tissue showed anti-core activity in biopsies from HBsAg seropositive patients. Eluate of the liver tissue from the HBsAg seronegative patient showed antinuclear activity. The latter patient was known to have lupus erythematosus.

VI.3.9 Relation between (HBsAg and HBcAg) fluorescence patterns and liver histology

Table VI.6 gives the relation between the HBsAg fluorescence patterns described above and the histological changes in the liver biopsies from HBsAg-seropositive patients.

Table VI.6 *Relation between HBsAg fluorescence pattern and histological diagnosis*

histological diagnosis	n	HBsAg fluorescence patterns sol	diff	focal	interm	neg
acute hepatitis	47	26	15	0	5	1
chronic persistent hepatitis	30	0	28	2	0	0
chronic active hepatitis	43	1	38	0	3	1
cirrhosis active	17	1	12	1	3	0
cirrhosis inactive	3	0	1	2	0	0
minimal lesions	14	0	3	11	0	0
aspecific reactive hepatitis	11	0	3	6	0	2
total	165	28	100	22	11	4

sol = solitary diff = diffuse interm = intermediate neg = negative

More than 50 per cent of the liver biopsies graded histologically as acute hepatitis yielded a solitary pattern, a pattern that is seen only sporadically in the other histopathological categories of liver changes for the group of HBsAg seropositive patients. Approximately 30 per cent of the biopsies of acute hepatitis exhibited a diffuse fluorescence pattern, while an intermediate fluorescence pattern was seen in 5 patients (± 10 per cent). Three of the latter showed cell membrane-bound HBsAg not as a diffuse pattern but in groups of hepatocytes. The liver biopsies from the other two patients yielded a fluorescence pattern intermediate between the diffuse and focal HBsAg fluorescence patterns. Biopsies graded histologically as chronic persistent hepatitis gave a diffuse fluorescence pattern in all but two cases. The two exceptions exhibited a focal fluorescence pattern.

Table VI.7 *Relation of HBcAg with histological diagnosis and HBsAg fluorescence patterns.*

histological diagnosis	HBcAg n	HBsAg diffuse	HBsAg intermediate
acute hepatitis	6	4	2
chronic persistent hepatitis	9	9	
chronic active hepatitis	22	20	2
cirrhosis	5	3	2
minimal lesions	1	1	
aspecific changes	0	0	
total	43	37	6

In chronic active hepatitis too the diffuse fluorescence pattern prevailed (88 per cent); in only one case was a solitary and in three cases an intermediate fluorescence pattern seen, while no fluorescence could be detected in one biopsy. In 2 out of the 3 cases with an intermediate fluorescence pattern only small groups of hepatocytes showed cell-membrane bound fluorescence; in the other one, the fluorescence pattern was intermediate between the focal and diffuse patterns. One of the patients with chronic active hepatitis and cell membrane-bound HBsAg fluorescence in some groups of hepatocytes was found to exhibit extensive HBcAg fluorescence.

Liver biopsies with cirrhosis gave a wider variation in the fluoresence pattern; in most cases the diffuse pattern predominated.

About 70 per cent of the liver biopsies showing minimal or aspecific liver changes exhibited the focal pattern. A relatively large percentage (20 per cent) of biopsies with aspecific liver changes was negative for HBsAg.

HBcAg was found exclusively in biopsies with a diffuse or intermediate fluorescence pattern and was associated with nearly all types of histological changes (table VI.7).

VI.3.10 FOLLOW-UP STUDIES OF PATIENTS WITH HISTOLOGICALLY GRADED ACUTE HEPATITIS

The 43 patients graded histologically as acute hepatitis were followed clinically. In 31 of these 43 patients HBsAg was cleared from the serum and the elevated levels of transaminase normalized within eight months. No histological follow-up of these patients is available.

Biopsies of the 31 patients with self-limiting acute hepatitis gave a solitary fluorescence pattern in 26 cases, a diffuse fluorescence pattern in 1 and a partial cell membrane-bound fluorescence of groups of hepatocytes in 3 cases. One biopsy was completely negative for HBsAg and HBcAg.

Eleven of the 43 patients remained HBsAg seropositive for at least 4 years. One patient died 5 months after the biopsy was taken with continually positive HBsAg serum tests. For 9 of the patients with persisting HBsAg-positive serum the histological changes in follow-up biopsies could be studied. In 4 cases the second biopsy, taken approximately six months after the first, still showed the histological pattern of acute hepatitis. However, one year after the first biopsy, all 9 follow-up biopsies indicated chronic hepatitis (some chronic persistent, some chronic active).

A diffuse fluorescence pattern was seen in the first biopsy of 9 and a mixture of the diffuse and focal fluorescence patterns in 2 of the 11 patients whose liver disease progressed from acute to chronic hepatitis. All but 2 of these patients were clinically anicteric. Of the 31 patients with acute self-limiting hepatitis 29 were icteric, while the other two experienced a period of doubtful icterus.

The next table (VI.8) gives the combined results of the histological and fluorescence studies of the follow-up study. From these data it is clear that the fluorescence pattern remained constant, despite the fact that the histology of the liver disease changed in sequential biopsies from these patients.

Of the 13 cases graded histologically as acute hepatitis and a diffuse fluorescence pattern only three included a sporadic hepatocyte with cytoplasmic HBsAg fluorescence. HBcAg could only be demonstrated in 2 biopsies.

VI.3.11 FOLLOW-UP STUDY OF PATIENTS WITH HISTOLOGICALLY GRADED CHRONIC HEPATITIS

Chronic persistent or chronic active hepatitis was diagnosed in 30 and 43 of the 165 liver biopsies, respectively. These biopsies were obtained from 60 patients. All patients remained HBsAg-positive during a follow-up period of at least four years. Twelve follow-up biopsies were obtained from 7 patients. The fluorescence pattern for the first biopsy was of the diffuse type in all cases and

Table VI.8 *Histological and fluorescence results for the first and follow-up biopsies from 9 HBsAg-positive patients. Note: constant HBsAg expression, changing histological diagnosis*

Patients		1e biopsies									
A	hist.	AH						CPH			
	Fl.	diff						diff			
	GG.	-						+			
	HBc	-						+			
B	hist.	AH	CPH								
	Fl.	diff	diff								
	GG.	-	+								
	HBc	-	+								
C	hist.	AH		AH			CAH				
	Fl.	diff		diff			diff				
	GG.	-		+			+				
	HBc	-		+			-				
D	hist.	AH	AH				CAH				
	Fl.	diff	diff				diff				
	GG.	-	-				+				
	HBc	-	+				+				
E	hist.	AH				AH					
	Fl.	diff				diff					
	GG.	-				-					
	HBc	-				+					
F	hist.	AH				CAH					
	Fl.	diff				diff					
	GG.	-				+					
	HBc	-				+					
G	hist.	AH				AH					
	Fl.	diff				diff					
	GG.	-				+					
	HBc	-				+					
H	hist.	AH				CAH					
	Fl.	diff				diff					
	GG.	-				+					
	HBc	-				+					
I	hist.	AH				CAH(mild)				CAH(mod)	
	Fl.	diff				diff				diff	
	GG.	-				+				+	
	HBc	+				+				+	
months ----▸		0	2	4	6	8	10	12	16	20	24

hist. = histology Fl. = fluorescence pattern diff = diffuse fluorescence pattern

this pattern remained diffuse later. However, the histological diagnosis changed during follow-up in 4 cases from CAH to CPH or from CPH to CAH.

If HBcAg was found in one biopsy, HBcAg could nearly always be detected in subsequent biopsies from the same patient. Ground-glass cells were found scattered throughout the liver tissue in 14 of the 19 biopsies.

One patient with CAH who exhibited a diffuse fluorescence pattern subsequently developed cirrhosis, similarly associated with a diffuse fluorescence pattern. One year later the patient died as a result of a hepatocellular carcinoma. The follow-up biopsies never revealed a change from one type of fluorescence pattern to another.

VI.4 DISCUSSION

HBsAg could be demonstrated in 161 of the liver biopsies from HBsAg seropositive patients and none of those from HBsAg seronegative patients. We could not confirm the observation of Ray et al. (1974, 1976) that the immunofluorescence technique for liver tissue is more sensitive for detection of HBsAg than radioimmunoassay of serum. HBcAg was, however, found in 1 biopsy from a HBsAg seronegative patient. Also immunofluorescence may be enhance the detection of HBV infections slightly. The liver biopsies from four HBsAg seropositive patients in our study gave negative HBsAg fluorescence. One of the 4 liver biopsies from HBsAg seropositive patients with negative HBsAg fluorescence was characterized by late-phase acute hepatitis. In this patient HBsAg cleared from the serum a few weeks after the biopsy was taken. Thus, in this case the negative HBsAg immunofluorescence should not be considered as a false negative result but as a predictor of the benign course of the HBV infection. Therefore, as seen in the liver biopsies from all patients with an acute self-limiting hepatitis, HBV is as a rule cleared from the liver long before the serum becomes negative. The second case with negative immunofluorescence was a patient with chronic active hepatitis. HBsAg was detected in one serum sample but all other sera were negative (a false-positive serological result?). The sera of the third and fourth patients with negative HBsAg immunofluorescence remained HBsAg-positive during follow-up. The biopsies from both patients showed only limited aspecific histological changes. The absence of HBsAg in liver tissue is in these cases unlikely: sampling errors due to the focal distribution of HBsAg (which is consistent with this type of histological change) seems to be a more probable explanation of the negative results.

HBsAg was found in the cytoplasm of hepatocytes, particularly ground-glass hepatocytes, but also along the cell membranes of hepatocytes, in Kupffer's cells

and sometimes in the walls of vessels in the portal tracts. HBsAg was never found in hepatocytic nuclei. The most sensitive method for detection of HBsAg in liver tissue is the fluorescence method using air-dried or acetone-fixed frozen sections. Sublimate-fixed, trypsin-treated frozen sections yield better morphology with retention of the specific fluorescence. For rapid detection of HBsAg in liver tissue microwave stimulated incubation can be used, which yields good fluorescence results within 5 minutes.

Immunofluorescence studies of paraffin-embedded formalin-fixed material show intracytoplasmic HBsAg in ground-glass and buttonhole cells. In contrast cell membrane-bound HBsAg fluorescence of hepatocytes was detected only sporadically: with this method we would have missed 34 patients with pure cell membrane-bound fluorescence staining of the liver biopsy. The peroxidase-labelled antibody method for formalin-fixed paraffin-embedded liver tissue allows detection of intracytoplasmic HBsAg and also, according to some authors, membrane-bound HBsAg in cell membranes of hepatocytes (Busachi, 1978).

Only 43 of the 161 patients with HBsAg fluorescence exhibited core antigens (± 27 per cent). In our study core antigens always existed, except in one patient, in combination with cell membrane-bound HBsAg. Also the study of Gudat (1977) revealed that HBcAg may in rare cases occur without detectable HBsAg. In hepatocytes with a marked accumulation of HBsAg, as seen in HFH or ground-glass hepatocytes, HBcAg could be demonstrated only sporadically.

Probably these ground-glass cells harbor the genetic material of the virus in a hidden form, possibly incorporated in the genome of the host hepatocyte. The incorporation of viral DNA in the cellular genome differs from patient to patient and is possibly dependent on the rate at which the infected hepatocyte is destroyed. In general the destruction of hepatocytes cannot be attributed to a direct toxic effect of the virus itself, because there is no direct relation between the concentration of HBcAg and liver cell damage. Sometimes, especially in immunosuppressed patients, enormous accumulations of HBcAg are found without mentionable liver cell necrosis.

Accumulations of HBsAg in the hepatocytes too cannot be the cause of liver cell damage; on the contrary a reverse relation between the levels of HBsAg in the liver and liver cell necrosis seems to exist, as suggested by the findings for liver tissue from HBsAg carriers. The data seem to indicate that damage of the liver tissue is related instead to host factors. But also location of HBcAg in the hepatocyte may be an important factor for liver cell necrosis, as shown for recurrence of hepatitis B in liver grafts (see chapter XI). Dysfunction of the immune reaction of the patient, in particular, which causes infected hepatocytes to be eliminated, seems to lead to liver cell necrosis. An essential factor in this process appears to be the incorporation of HBsAg in the liver cell membrane. All of our

material from patients with chronic hepatitis contained hepatocytes with cell membrane-bound fluorescence. Moreover very early stages of acute hepatitis are characterized by small groups of hepatocytes with cell surface fluorescence. Obviously these smaller groups of hepatocytes can disappear, presumably via the host's immune reaction, as was the case in the 3 patients with early acute hepatitis combined with a cell membrane-bound fluorescence pattern.

When acute hepatitis was associated with the presence of HBsAg on the cell membranes of all hepatocytes, causing a diffuse honeycomb-like fluorescence pattern, the acute hepatitis always changed into a chronic type of hepatitis. Thus, all 15 patients with acute hepatitis and a diffuse fluorescence pattern in the liver biopsy developed chronic hepatitis, as seen in the follow-up biopsies. HBsAg immunofluorescence is therefore an important tool for prediction of the outcome of an acute hepatitis.

Chronic active or chronic persistent hepatitis was the diagnosis for 66 of the 73 patients with a diffuse membrane-bound HBsAg fluorescence pattern. This pattern is characterized by scattered hepatocytes with intracytoplasmic fluorescence (ground-glass cells). However, the HFH only appeared in the diffuse fluorescence pattern after the infection had been present for some time. The shortest time interval between onset of hepatitis B and detection of HFH was three months. Since HFH seem to appear some time after acquisition of hepatitis B infection, HFH or ground-glass cells can only be used sporadically to predict transition of acute hepatitis to chronic hepatitis. A diffuse fluorescence pattern with some scattered HFH can persist for many years, independent of therapeutic intervention.

As already mentioned, these scattered HFH exhibited HBcAg only sporadically and probably generally incorporated in the genome of HFH. Because of the solitary localization of these HFH cells they obviously are not capable of extending this genetic constitution by replication.

In contrast in patients with minimal or aspecific hepatic lesions and a focal fluorescence pattern showing compact fields of HFH, this transfer seems to occur by clonal proliferations. In such cases HBcAg might be incorporated in the genome of the host hepatocyte with preservation of the potency of the hepatocytes to replicate. In this focal pattern the virus appears to be tolerated not only by the immune system but also by the hepatocytes of the patient. One might speculate that changes in hepatocyte DNA induced by incorporation of viral DNA may be the cause of lupus erythematosus in association with persistent hepatitis B (Looi, 1982).

In our material two patients graded histologically as chronic persistent hepatitis exhibited the focal fluorescence pattern.

Based on this focal HBsAg fluorescence pattern chronic persistent hepatitis

as seen in these two cases should be differentiated from chronic persistent hepatitis associated with a diffuse HBsAg fluorescence pattern, as observed in the other 28 patients with chronic persistent hepatitis. This diffuse HBsAg pattern has been shown to be closely associated with chronic active hepatitis.

In view of these differences in viral expression pattern within the histological group of patients with chronic persistent hepatitis, there is evidence that chronic persistent hepatitis is an non-entity and only a histological receptacle. On the same grounds the 6 patients with a histological diagnosis of minimal or aspecific lesions but a diffuse fluorescence pattern have to be considered as a separate group with the viral expression pattern of chronic active hepatitis; 5 of these 6 patients were on immunosuppressive therapy and 2 developed chronic hepatitis after withdrawal of these drugs. The patterns of viral expression in the liver, which are similar for chronic persistent and chronic active hepatitis, suggest that the two types of hepatitis are basically the same.

Deposition of immunoglobulins along the cell membranes of hepatocytes in liver tissue sections and on hepatocytes in suspensions with the same pattern as HBsAg suggests that not only cellular but also humoral immune mechanisms play a role in the destruction of hepatocytes. The cellular immune reaction is reflected histologically in piecemeal necrosis in chronic active hepatitis.

There is strong evdence that immunoglobulin deposits in the nuclei of hepatocytes in HBsAg seropositive subjects represent an in vitro induced phenomenon. Deposits of immunoglobulins were only found in biopsies in which core particles were revealed by ultrastructural examination and HBcAg in the nuclei were demonstrated by immuno-histochemical examination. No nuclear immunoglobulin deposits were found in HBcAg-negative biopsies. Nuclear immunoglobulin deposits were never accompanied by deposits of complement in the nuclei.

However, incubation of the HBcAg-positive slides with fresh human serum produced complement fixation on the same place as the immunoglobulin deposits which suggests the formation of antigen antibody complexes in these places.

The nuclear immunoglobulins could be removed from the nuclei by elution with KSCN (4M) and citrate acid (pH 3.2). The eluates revealed anticore activity, as demonstrated by the core-positive liver tissue from a patient with minimal anticore serum antibody titers.

The results of these experiments indicate that anti-HBc antibodies diffuse from the sinusoidal blood spaces during the incubation period and bind in the second instance to nuclear core antigens that happen to be present (fig.VI.9).

Immunoglobulin deposits were found by the same in vitro mechanism in liver biopsies which revealed nuclear Delta antigen in patients with HDV superinfec-

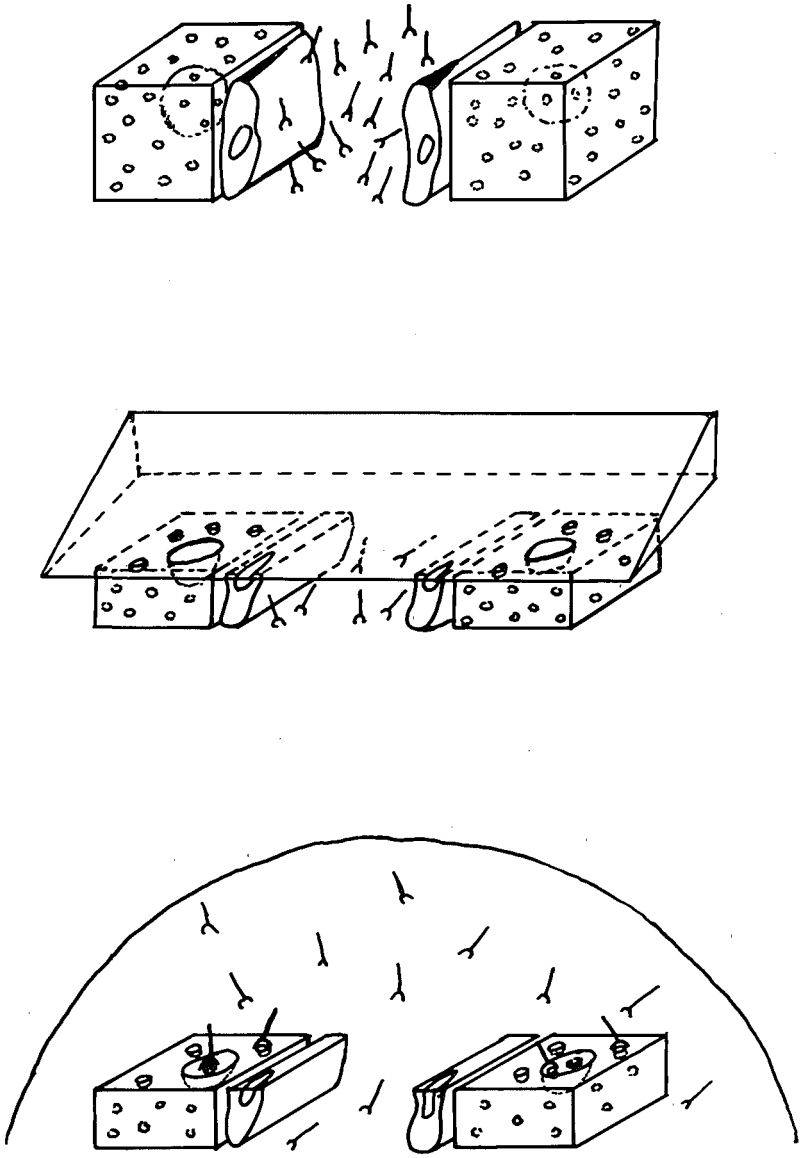


Fig.VI.9 *Schematic drawing showing the mechanism of immune complex formation in frozen tissue sections during the immunohistochemical incubation procedure. Circulating antibodies present in the sinusoidal spaces (e.g. aHBcAg or aHDAg):*

1. *dissolve in the incubation droplet during the incubation procedure.*
2. *bind to the corresponding antigens (e.g. HBcAg or HDAg) which are hidden in the tissue cells before tissue cutting. The mechanism explains the simultaneous detection of various known and unknown antigens of viral or tissue origin and the correspondent circulating antibody.*

tion. In these cases serum anti-delta-antibodies binds to the Delta antigen present in the nuclei of hepatocytes.

The same phenomenon can be demonstrated in tissues from patients with lupus erythematosus and a high titer of circulating antinuclear antibodies. These biopsies exhibit nuclear fluorescence by binding of anti-nuclear antibodies to the DNA present in the nuclei of the hepatocytes; a granular fluorescence is present in all nuclei in these cases in contrast to the nuclear fluorescence in scattered groups of hepatocytes observed in HBV-infected livers.

The fine granular cytoplasmic IgG and/or IgM fluorescence of hepatocytes in liver tissue from patients with primary biliary cirrhosis (ten Kate, 1981) can also be explained by this in vitro mechanism. In these liver biopsies antimitochondrial antibodies from serum in the sinusoidal spaces diffuse into incubation fluids and bind to the cytoplasmic mitochondria.

In HBsAg seropositive patients this phenomenon reveals not only the presence of HBcAg in liver biopsies but also the presence of circulating anticore antibodies. In our series there was only one case out of the 43 patients with detectable HBcAg in the liver without detectable IgG fluorescence. This was a renal transplant recipient on high-dose immunosuppressive therapy with very low anticore antibody titer.

By using antibodies against the various immunoglobulin classes, we could also determine the immunoglobulin class of the anticore antibodies. In 42 cases these antibodies were of the IgG class; only 2 were of the IgG and IgM classes.

In fact, this phenomenon enhances the possibility of discovering new antigen antibody systems, e.g. in the case of persistent non-A, non-B hepatitis.

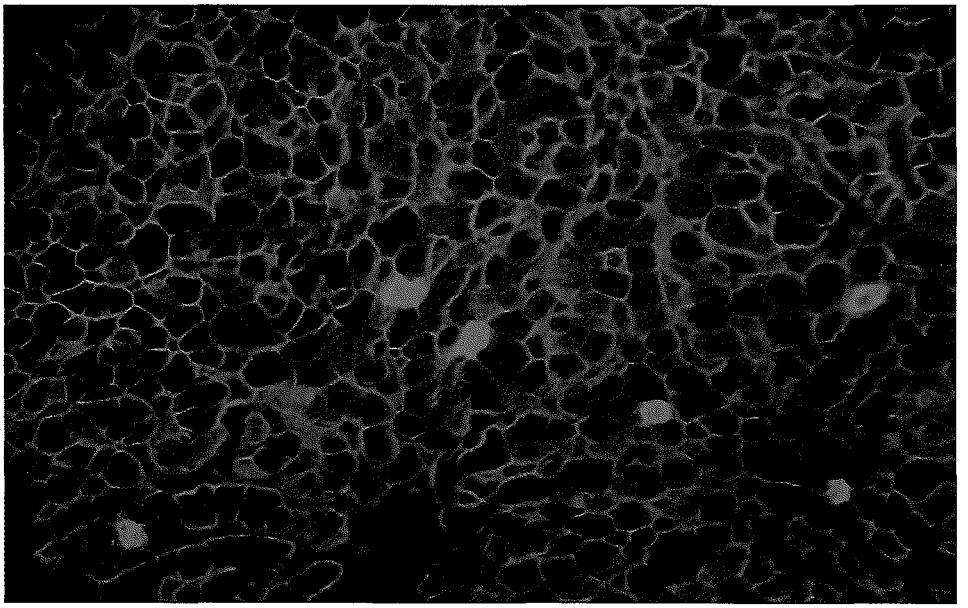
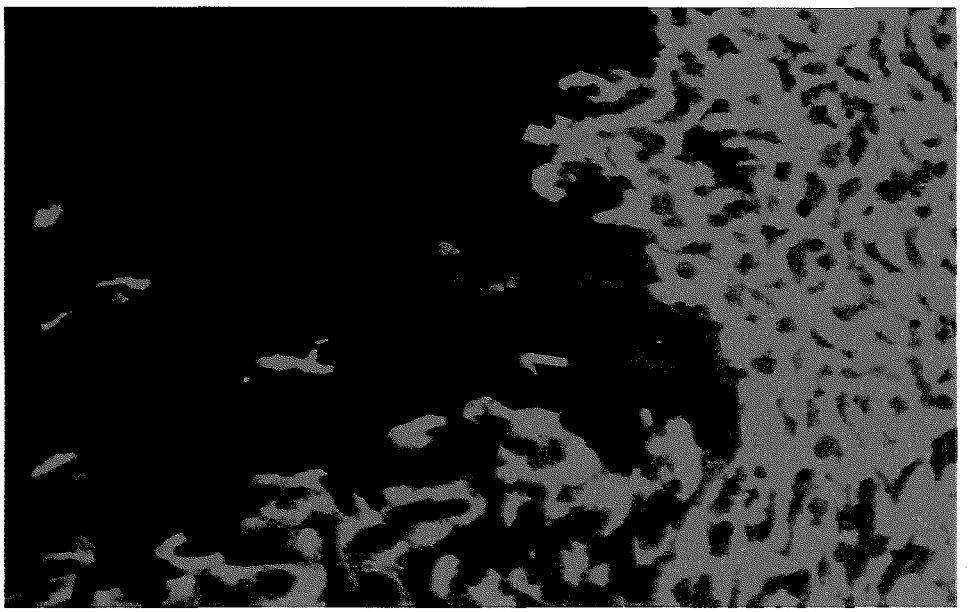


Fig VI.6 Diffuse HBsAg fluorescence pattern (direct one-step immunofluorescence, (150x).

Fig VI.7 Focal HBsAg fluorescence pattern (direkt one-step immunofluorescence, (150x).



CHAPTER VII

THE GROUND-GLASS HEPATOCYTE

Its value in light microscopical diagnosis of hepatitis B infection

CHAPTER VII

THE GROUND-GLASS HEPATOCYTE

Its value in light microscopical diagnosis of hepatitis B infection

VII.1 SUMMARY

The presence of ground-glass hepatocytes (GGH) was studied in routinely stained hematoxylin-azophloxin (HA) sections of 1850 consecutive paraffin-embedded liver biopsies.

To determine the staining characteristics of GGH as well as the value of special staining methods for the detection of hepatitis B surface antigen (HBsAg) in liver tissue, additional sections of all liver biopsies consecutive to the HA sections were also stained with aldehyde thionin and in most cases orcein. The specificity of each positive staining result for the presence of HBsAg was determined by immunohistochemical detection techniques for both frozen and paraffin-embedded sections. Hepatocytes with a ground-glass appearance were seen in 150 HA stained sections of liver biopsy specimens obtained from both HBsAg seropositive and HBsAg seronegative patients with various liver diseases.

However GGH positive for aldehyde thionin and orcein were only present in liver biopsy specimens from patients with HB antigenemia of more than three months duration. Aldehyde thionin and orcein-positive GGH were never found in liver tissue from either HBsAg seronegative patients or patients with acute self-limiting hepatitis B infection. The number and distribution of GGH varied with the clinicopathological type of the HBV liver disease. In order to be able to judge those liver changes caused by hepatitis B virus in biopsies from patients with liver disease caused by more than one etiological factor, knowledge of the presence and distribution of GGH is important. A large percentage (93 per cent) of the biopsies from HBsAg seropositive patients with minimal liver changes were positive for GGH; in these biopsies the GGH were distributed in large clusters (focal pattern). Of the biopsies from patients with chronic persistent, chronic active and aspecific reactive hepatitis, nearly two-thirds contained GGH. In chronic persistent and chronic active hepatitis the number of GGH was low; the GGH appeared in liver tissue as isolated cells surrounded by normal hepatocytes (solitary pattern). Weak positive staining of hepatocytes by aldehyde thionin and orcein was sometimes also seen in biopsies from HBsAg seronegative patients, without however concomitant ground-glass appearance on routine HA-stained slides.

Therefore not only a positive staining of the hepatocytes by aldehyde thionin or orcein but also ground-glass appearance on HA-stained slides are mandatory for a histological diagnosis of HBV infection.

It is concluded that GGH that are positive to the aldehyde thionin and/or orcein stain are specific for the presence of a persistent HBV infection. As confirmed by follow-up biopsies, their number and distribution during the natural course of the liver disease remain fairly constant over many years. On the other hand their number and distribution can be an important tool to the histological diagnosis and classification of hepatitis B liver disease.

VII.2 INTRODUCTION

Hepatocytes are known as hepatocytes with a ground-glass appearance, i.e. ground-glass hepatocytes, if the cytoplasm in hematoxylin and azophloxin (eosin) stained sections contains a very finely granular, weakly eosinophilic material, usually as a cytoplasmic inclusion body. GGH were noted by many pathologists (Desmet, Scheuer, Popper) before their nature, pathogenesis and significance were known.

Hadziyannis et al. (1973) discovered that hepatocytes with a cytoplasm that was proven to contain abundant HBsAg by immuno-histochemical methods exhibit the ground-glass aspect in routinely stained liver sections.

In (immuno)electron microscopy studies these hepatocytes showed proliferation of the endoplasmic reticulum with dilated cisternae, which contained tubular and circular structures that were identical to the HBsAg containing particles found in the serum of HBsAg seropositive subjects (Ahamed, 1971; Stein, 1972; Gerber, 1974; Huang, 1974; Trump, 1976).

The material in these GGH has special histochemical staining characteristics and can easily be identified by the Gomori's aldehyde fuchsin, aldehyde thionin (ten Kate, 1974), orcein (Shikata, 1974; Kostich, 1977) and trichome (Gubetta, 1974) stains.

The results for sections of paraffin-embedded liver tissue stained with aldehyde fuchsin, aldehyde thionin and orcein appear to correlate well with specific immunofluorescence and immuno-peroxidase stains for HBsAg (Nayak, 1975; Afroudakis, 1976).

Furthermore hepatocytes with the ground-glass aspect are in themselves neither specific nor pathognomonic for the presence of HBsAg since they have also been found in liver tissue from HBsAg seronegative patients with drug-induced and other liver diseases (Harriman, 1955; Klinge, 1968; Gerber, 1981; Callea, 1986). However these latter GGH do not appear to have the same histo-

chemical staining characteristics as the ground-glass hepatocytes found in HBsAg-positive biopsies. The number and distribution of GGH in liver tissue obtained from HBsAg seropositive patients appear to vary with the histopathological category of the hepatitis.

In biopsies from patients with HBV infections different authors have found varying percentages of biopsies positive for GGH (Shikata, 100 per cent; Deodhar, 55 per cent; Gerber 27 per cent). To determine the number of biopsies with GGH in various liver diseases and to appraise the diagnostic significance of these cells, 1850 consecutive liver biopsies from HBsAg seropositive and seronegative patients were screened for the presence of GGH.

Moreover, to evaluate the value of special histochemical stains for the detection of specific hepatitis B infections, adjacent sections of all biopsies were stained with aldehyde thionin and, in most cases, orcein. The findings were compared with the results of immunohistochemical staining methods, using immunofluorescence and immunoperoxidase techniques.

VII.3 MATERIAL AND METHODS

The material consisted of 1850 consecutive formalin-fixed paraffin-embedded liver biopsies received for evaluation during the period 1973-1977. Forty biopsies, were too small (less than 0.5 cm) or not properly fixed and were excluded from the study.

The remaining biopsies (1810) came from patients with all kinds of liver disease. The age of the patients ranged from 2 months to 97 years. Sera from 750 patients were tested for the presence of HBsAg, including the sera from all 150 patients with GGH in their liver biopsies. The remaining patients exhibited no clinical or morphological evidence of an HBV infection, therefore there was no reason for testing the serum for the presence of HBsAg.

Serial sections of all liver biopsy specimens were stained with hematoxylin azophloxin, hematoxylin azophloxin saffron, periodic acid Schiff, periodic acid Schiff after diastase digestion, Gomori's reticulin, Azan, and, in most cases, orcein (see chapter III). Moreover all biopsies from HBsAg seropositive patients were tested for the presence of HBsAg by immunofluorescence and immunoperoxidase techniques (see chapter III).

Moreover a fresh sample from 252 of the 1810 liver biopsy specimens was separated before formalin fixation and snap-frozen in liquid nitrogen. A small piece of the same 252 biopsies was also prepared for electron microscopy studies. Sections of the snap-frozen as well as formalin-fixed material were investigated for the presence of HBsAg by immunofluorescence and immunoper-

oxidase techniques (see chapter III). Sera from all 252 patients, were examined for the presence of HBsAg. The HA-stained sections were screened for the presence of hepatocytes with the ground-glass aspect. The number of GGH in the liver biopsies was scored semi-quantitatively as +, ++, +++: + less than 1 ground-glass hepatocyte per mm^2; ++ 1-10 ground-glass cells per mm^2; and +++ more than 10 ground-glass cells per mm^2.

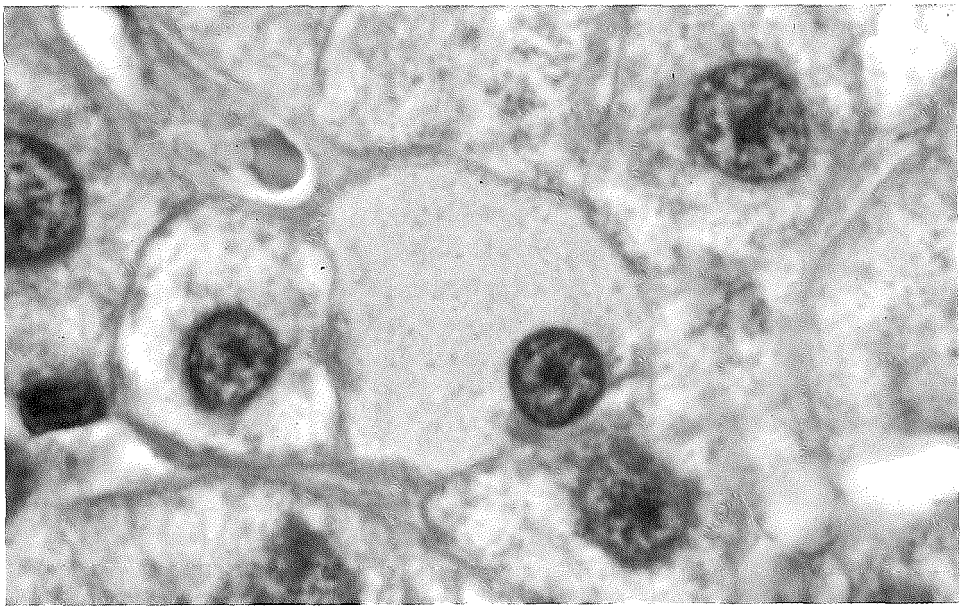
Determination of the range of the scores was based on the experience about the range of GGH in liver tissue. Ground-glass hepatocytes were defined as normal-sized hepatocytes with a very finely granular, almost homogeneous, weakly eosinophilic aspect in all or part of the cytoplasm. This aspect of the hepatocytes contrasts with the irregular coarsely granular cytoplasm of normal hepatocytes. Hepatocytes with oncocytic changes in the cytoplasm were excluded (diffuse granular deeply eosinophilic).

VII.4 RESULTS

VII.4.1 PREVALENCE

Ground-glass hepatocytes were detected in 150 (±8 per cent) of the 1810 liver biopsies.

Fig.VII.1 *Ground-glass hepatocyte in liver tissue from a HBsAg seropositive patient (H and A, 500x)*



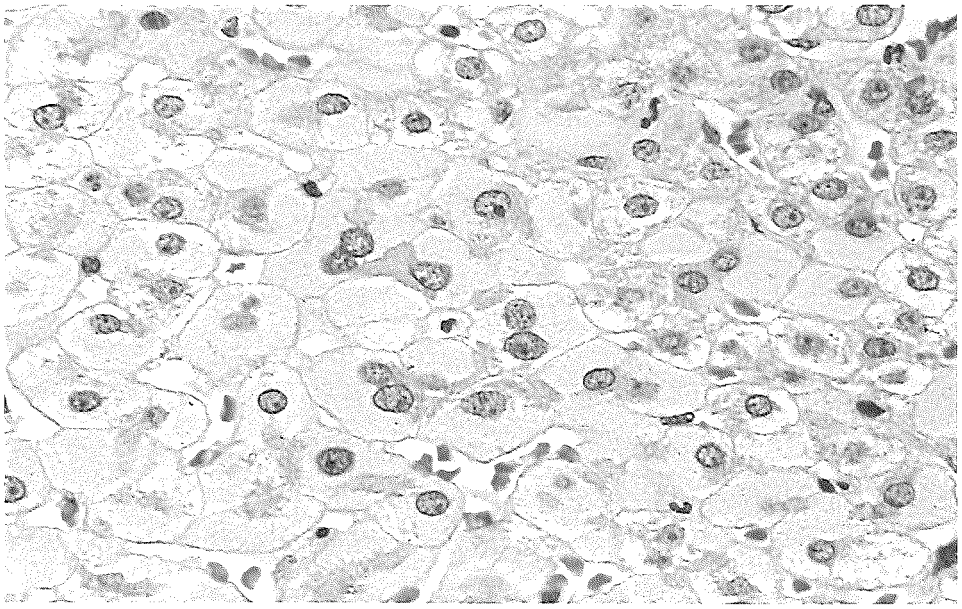
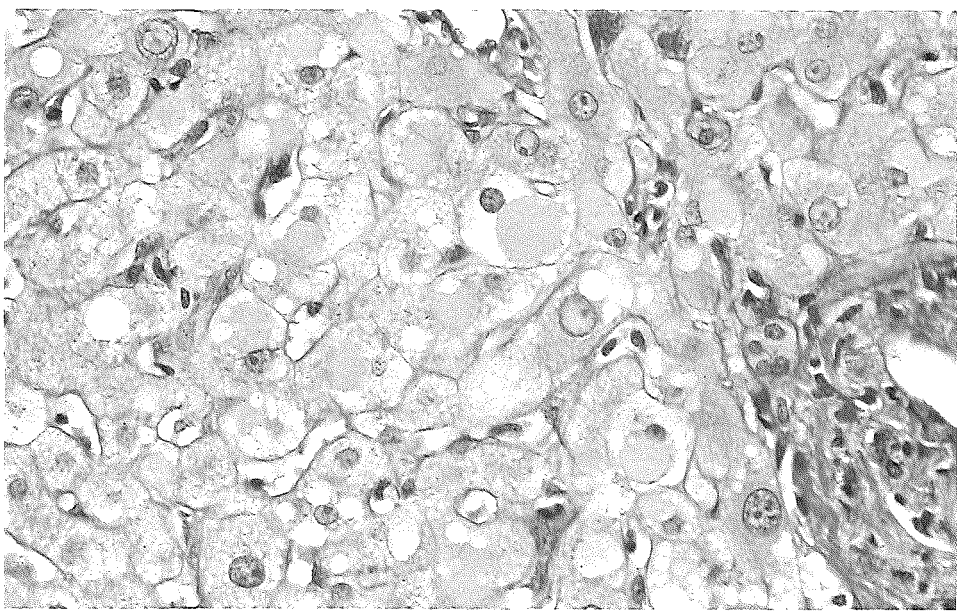


Fig.VII.2 *Large group of ground-glass hepatocytes in liver tissue of a HBsAg seropositive subject. Note the accumulation of preexistent cytoplasmic organelles on the biliary pole and the binucleation (H and A, 360x).*

Fig.VII.3 *Ground-glass hepatocytes in a liver biopsy from a HBsAg seronegative patient (H and A, 360x).*



Out of these 150 biopsies 103 were obtained from patients who were HBsAg seropositive; the other 47 biopsies came from HBsAg seronegative patients.

In biopsies from HBsAg seropositive patients the GGH appeared as smaller and larger groups (focal pattern) or isolated cells distributed irregularly throughout the liver parenchyma (solitary pattern).

Table VII.1: *Distribution pattern and number of ground-glass cells in liver tissue from HBsAg seropositive and seronegative patients.*

	biopsies n	ground-glass cells focal			solitary		
		+	++	+++	+	++	+++
HBsAg seropositive	103	-	-	35	31	31	6
HBsAg seronegative	47	-	-	45	-	2	-

+ = < 1, ++ = 1-10, +++ = > 10 ground-glass cell per mm^2.

In the focal pattern the groups of GGH were not restricted to a special area of the hepatic lobulus. The number of GGH in the focal pattern was almost always scored as +++. In the solitary pattern the GGH were found in varying numbers (table VII.I). In some biopsies only an occasional ground-glass cell could be detected. In biopsies from HBsAg seronegative patients the number of GGH was in general larger and they were nearly always grouped in large clusters. Solitary GGH were rarely seen in biopsies from HBsAg seronegative patients (table VII.1).

VII.4.2. Morphological aspects:

Cytologically the typical ground-glass cell in biopsies from HBsAg seropositive patients was characterized by a circumscribed mass of very finely granular, nearly homogeneous, eosinophilic material, lying as an inclusion body in the cytoplasm and separated from the cell membrane by a halo of optically clear material (fig.VII.1 and fig.VII.2). Sometimes fine vacuolization was seen in the center of the homogeneous material. The nucleus was displaced toward the periphery of the cytoplasm, usually to the biliary pole. Moreover, in the periphery of the cell and close to the eccentric nucleus the preexisting coarse granular cytoplasmic organelles had clumped together in small areas (fig.VII.1 and 2). Frequently the GGH were binucleate. Mitotic figures were never seen in GGH.

The cytological aspect of the GGH in biopsies from HBsAg seronegative patients varied. In most cases the cytoplasm showed a more diffuse eosinophilic change without displacement of the nucleus. This eosinophilic change was mostly pale. GGH were found in liver biopsies from patients with all kinds of liver disease, predominantly however in those with cirrhosis. An explanation for the ground-glass change was not clear in most cases. Sometimes the patient was known to have used a drug that has been reported to cause ground-glass transformation of hepatocytes, e.g. chlorpromazine and diphenylhydantoin (Jezequel, 1972; Mullick, 1972; Ishak, 1982). Only 4 biopsies from HBsAg seronegative patients contained GGH that were nearly identical cytologically to those found in liver biopsies from HBsAg seropositive patients. In some of these cases the eosinophilic material appeared as a sharply circumscribed mass in the cytoplasm of the hepatocyte (fig.VII.3).

VII.4.3 Ultrastructural aspects of ground-glass cells

In some of the biopsies the GGH could be detected as a result of their homogeneous structureless cytoplasm. Ultrastructurally liver tissue from both HBsAg seropositive and HBsAg seronegative patients contained hepatocytes with an excessive increase in smooth endoplasmic reticulum. In proven HBsAg-positive liver tissue typical circular and tubular structures were found in this endoplasmic reticulum (fig.X.1).

These structures were not detected in the GGH present in liver biopsies from HBsAg seronegative patients.

VII.4.4 Special staining characteristics of ground-glass cells stained with aldehyde thionin or orcein

Only GGH in biopsies from HBsAg seropositive patients reacted positively to the aldehyde thionin and orcein stains, whereas GGH in biopsies from HBsAg seronegative patients were consistently negative (fig.VII.4 and 5).

With the aldehyde thionin or orcein staining methods, GGH in liver tissue from HBsAg seropositive patients were detected much more easily than on HA-stained slides, especially when only a sporadic cell was present (fig.VII.6). With these special staining methods, positive cells were found in 7 additional biopsies from HBsAg seropositive patients. Three of these 7 biopsies contained only a sporadic ground-glass cell, which was missed in the HA-stained sections; in the other 4 biopsies some scattered hepatocytes or groups of hepatocytes gave a positive orcein and aldehyde thionin reaction only in the periphery of the cytoplasm. Although typical GGH were not detected in HA-stained sections, the presence of

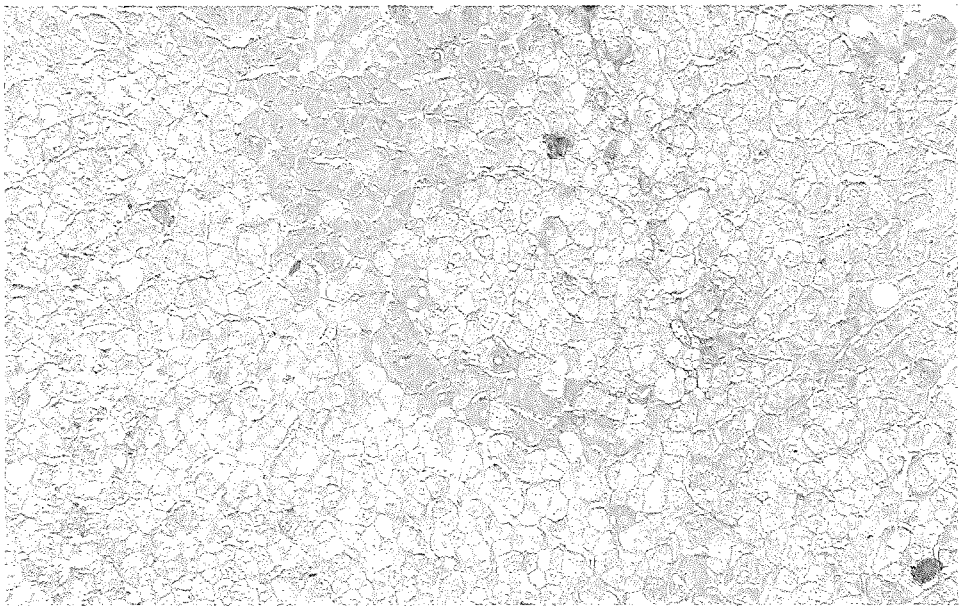
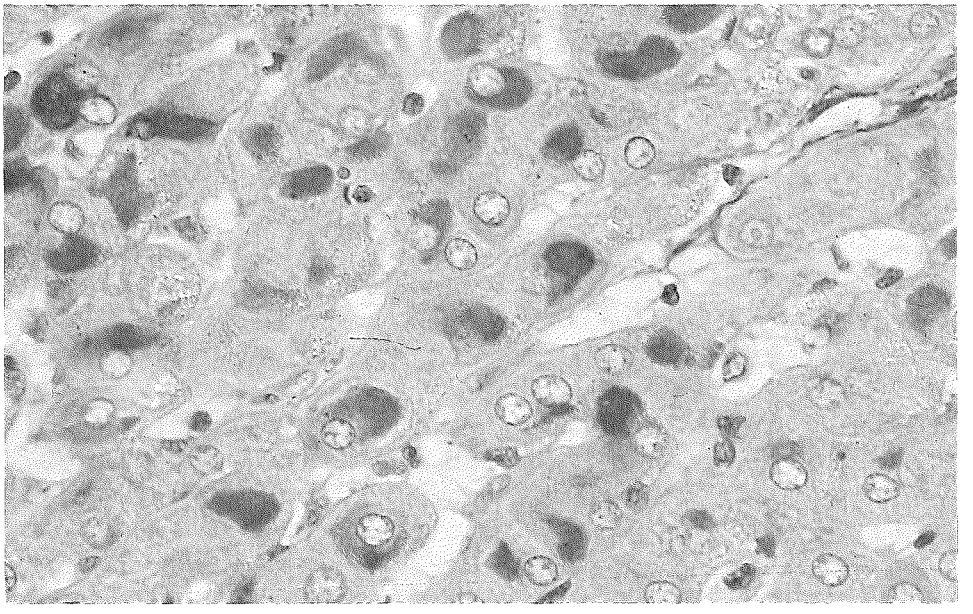


Fig.VII.4 *Large group of aldehyde thionine positive ground-glass hepatocytes in otherwise normal liver tissue (120x).*

Fig.VII.5 *Large group of ground-glass hepatocytes, positive with orcein staining method (360x).*



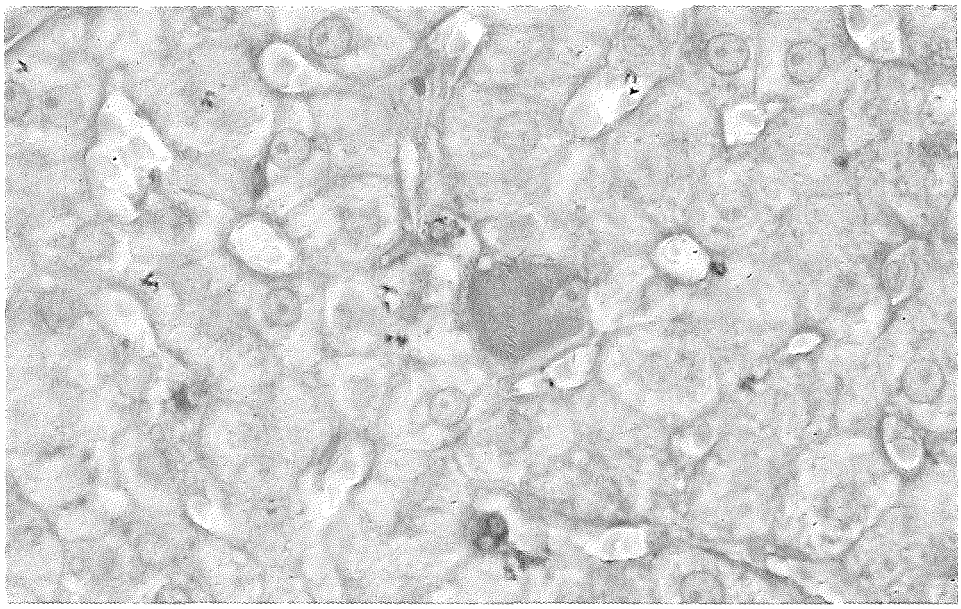
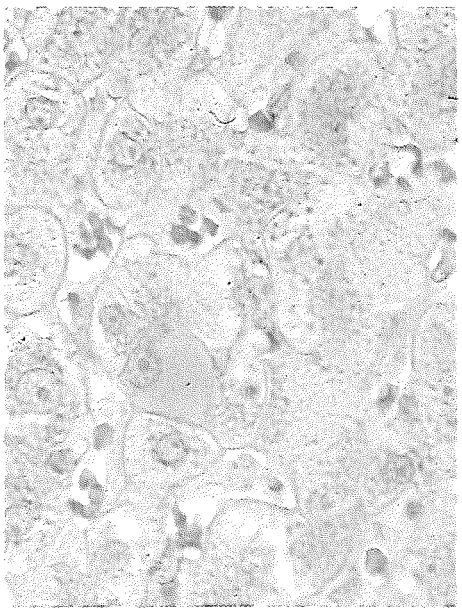
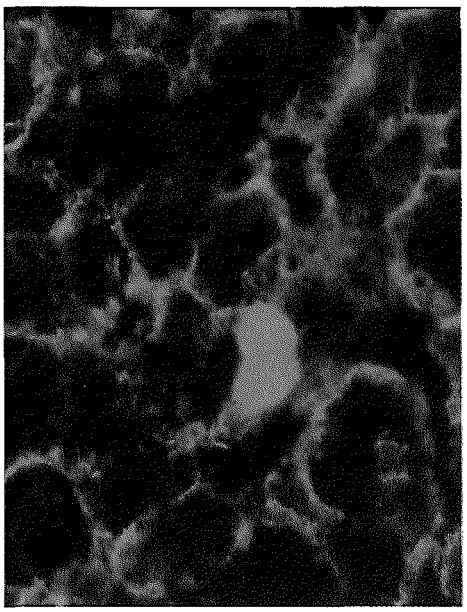


Fig.VII.6 *Liver tissue from a HBs-seropositive patient with an isolated ground-glass hepatocyte, easely detected by the positive reaction for aldehyde thionin (380x).*

Fig.VII.7 *Ground-glass hepatocyte in consecutive sections of frozen liver tissue, positive for both aldehyde thionin (right) and monospecific anti-HBsAg antibodies, using immunofluorescent method (left) (300x).*





a finely granular eosinophilic change in the periphery of the cytoplasm of these hepatocytes was scored as "possibly present". Aldehyde thionin and orcein stain not only the ground-glass material in HBsAg-positive hepatocytes but also other structures, especially elastin, lipofuchsin and copper-binding protein. Weakly positive staining was sometimes seen in hepatocytes which did not exhibit the ground-glass aspect in HA-stained sections. This weak staining, which was encountered in biopsies from both seronegative and seropositive patients, was localized especially along the margins of the biopsy specimens.

A disadvantage of both the aldehyde thionin and orcein stains was the inconsistency of staining results, that varied with the brand and lot of the dye as well as the maturity of the solutions. In addition to the aldehyde thionin and orcein staining procedures, GGH were also easily recognized in Azan-stained sections; in contrast these cells were not recognized, or only with great difficulty, in PAS, PAS after diastase digestion, Gomori's, EG and Fe-stained sections.

Table VII.2 *Distribution pattern and number of ground-glass cells in different types of hepatitis B liver disease*

	n	ground-glass hepatocytes					
		solitary pattern			focal pattern		
		+	++	+++	+	++	+++
acute hepatitis that has transformed into chronic hepatitis	6	2	3	0	0	0	1
chronic persistent hepatitis	24	12	7	1	0	0	4
chronic active hepatitis (mild)	25	13	11	0	0	0	1
chronic active hepatitis (moderate)	11	2	6	0	0	0	3
cirrhosis	14	2	1	4	0	0	7
aspecific reactive hepatitis	9	0	1	1	0	0	7
minimal lesions	14	0	2	0	0	0	12
total	103	31	31	6	0	0	35

+ = < 1, ++ = 1-10, +++ = > 10 ground-glass cells per mm^2.

VII.4.5 GROUND-GLASS HEPATOCYTES AND THE DIFFERENT CATEGORIES OF HBV HEPATITIS

Ground-glass cells were found in liver biopsies from patients with chronic hepatitis, cirrhosis, minimal lesions and aspecific reactive inflammatory changes and sometimes (prolonged) acute hepatitis that progressed to chronic hepatitis. In chronic persistent, chronic active and prolonged acute hepatitis the GGH occurred mainly as solitary cells and very rarely exhibited the focal pattern, whereas liver biopsies with minimal lesions or aspecific reactive hepatitis, were characterized by GGH in the focal pattern (fig.VII.2 and fig.VII.4). In cirrhosis both patterns were observed (table VII.2). In cirrhotic livers the number of GGH and sometimes the distribution often varied from nodule to nodule.

VII.4.6 DETECTION OF HBSAG BY THE IMMUNOFLUORESCENCE AND IMMUNOPEROXIDASE METHODS

The presence of HBsAg in GGH in the biopsies from HBsAg seropositive patients could be confirmed by immunofluorescence and immunoperoxidase techniques, using serial sections of the paraffin-embedded and frozen liver tissue (fig.VII.7 and 8). On the other hand HBsAg could not be demonstrated by immunofluorescence and immunoperoxidase methods in the GGH present in biopsies from HBsAg seronegative patients.

In the biopsies from HBsAg seropositive patients the number of hepatocytes positive for HBsAg according to the immunological methods was in general slightly higher than the number of hepatocytes positive for aldehyde thionin and orcein. The increase in the number of positive hepatocytes obtained by immunological techniques could be explained by the presence of hepatocytes with faint HBsAg cytoplasmic staining but without an evident ground-glass aspect in the HA-stained sections. The HBsAg-positive material was localized in hepatocytes mainly in the periphery of the cytoplasm.

The specificities of the immunofluorescence and immunoperoxidase methods for detection of HBsAg were nearly the same, being strongly dependent on sufficient blocking of non-immunological binding of antibodies to the tissue sections.

When sections of the 252 snap-frozen liver biopsy samples were examined for the presence of HBsAg by the immunofluorescence technique, more biopsies were found to be HBsAg-positive than when the formalin-fixed embedded parts of these biopsies were tested.

The more sensitive detection of HBsAg in frozen liver tissue can be attributed to the detection of cell membrane HBsAg localization, which could detected

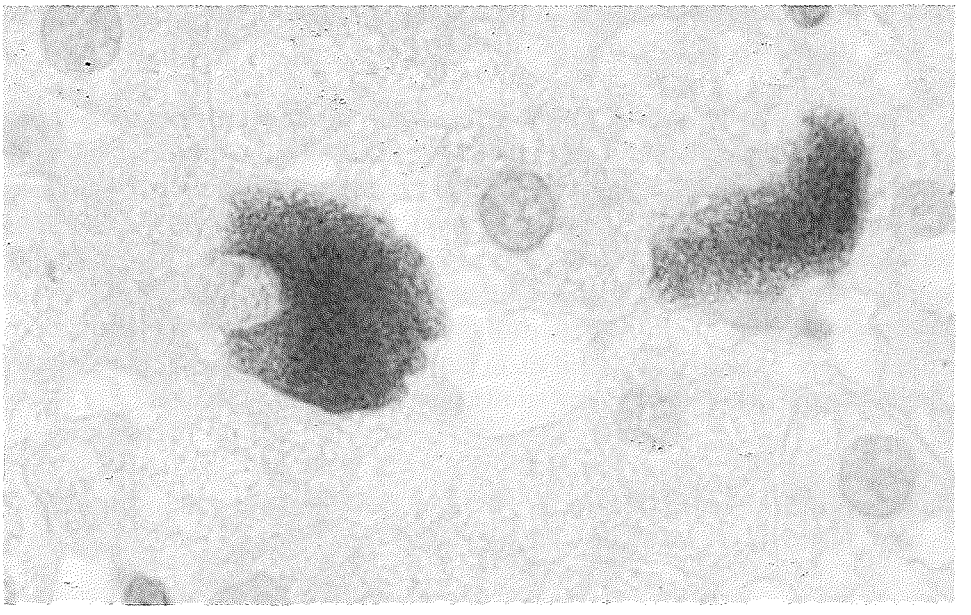
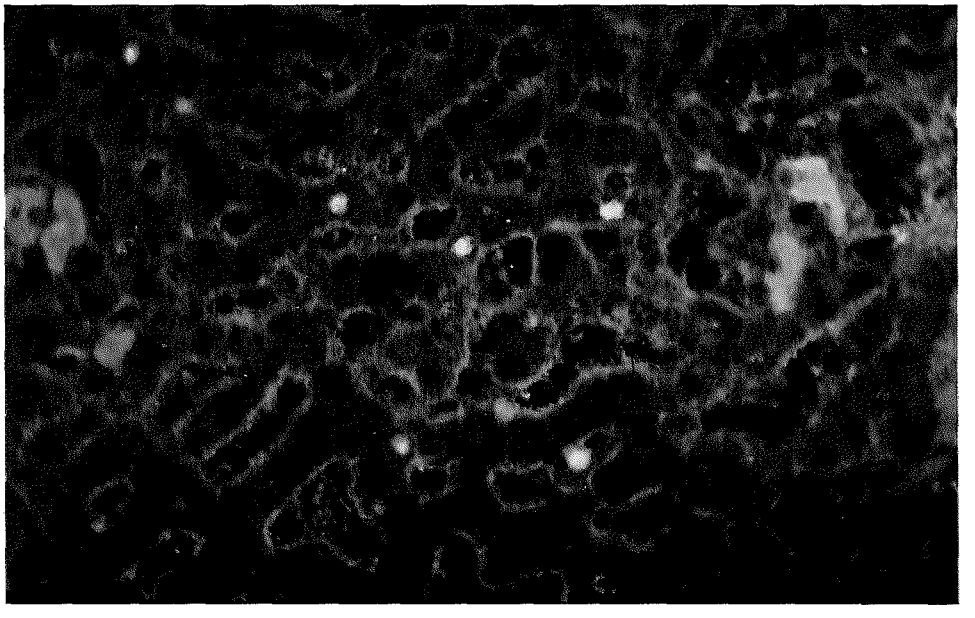


Fig.VII.8 *Liver tissue with two hepatocytes with extensive accumulation of HBsAg, demonstrated by anti-HBsAg antibodies using indirect immunoperoxidase method (500x).*

Fig.VII.9 *Presence of HBsAg and HBcAg in different hepatocytes, using FITC-labeled (HBcAg) and TRITC-labeled (HBsAg) antibodies (200x).*



easily in frozen liver tissue and only sporadically in liver tissue fixed in formalin and embedded in paraffin (see chapter VI).

VII.4.7 PREVALENCE OF GROUND-GLASS HEPATOCYTES IN THE VARIOUS MORPHOLOGICAL CATEGORIES OF HEPATITIS B INFECTION

The clinical evaluation of 750 patients with liver disease included serological tests for the presence of HBsAg. Because there was no clinical or morphological evidence of hepatitis B infection in the remaining cases, serological testing was not carried out.

Out of the above-mentioned 750 patients, 145 had a positive serum reaction to HBsAg; 189 biopsies were obtained from these 145 patients.

In 103 of these biopsies (55 per cent) GGH were found in the HA-stained sections while 110 biopsies (59 per cent) reacted positively to the aldehyde thionin and orcein stains.

Table VII.3 *Prevalence of ground-glass cells in different types of hepatitis B liver disease*

	biopsies n	ground-glass hepatocytes negative	positive
acute hepatitis	48	42	6
self-limiting	32	32	0
transition into chronic	16	10	6
chronic persistent hepatitis	35	11	24
chronic active hepatitis	56	20	36
minimal lesions	15	1	14
aspecific hepatitis	13	4	9
cirrhosis	22	8	14

None of the patients with an acute self-limiting hepatitis exhibited GGH. However 6 of the 16 biopsies from patients with an acute hepatitis that progressed to chronic hepatitis contained GGH usually only a few scattered throughout the biopsy.

The percentage of biopsies positive for GGH was the highest for the group with minimal lesions, namely 93 per cent (13 out of 14). In the other groups, i.e. a-pecific reactive hepatitis, chronic persistent hepatitis, chronic active hepatitis and cirrhosis, the percentages of biopsies that stained positive for GGH were approximately the same, ranging from 65.5 per cent for the group with chronic active hepatitis to 70.5 per cent for the group with chronic persistent hepatitis (fig.VII.10).

However in these last groups the distribution pattern of the GGH varied (table VII.3). In the group with aspecific reactive hepatitis the focal pattern with clusters of positive cells predominated, while in the group with chronic persistent and chronic active hepatitis the solitary pattern was usually encountered.

VII.4.8 Ground-glass hepatocytes in serial biopsies from HBsAg seropositive patients

More than 1 biopsy were obtained from 30 patients during the period of the study (1973-1977): 2 biopsies from 19 patients, 3 biopsies from 8 patients and 4 biopsies from 3 patients. The histological diagnosis and the presence or absence of ground-glass hepatocytes in these serial biopsies are shown in fig.VII.11. GGH could not be detected in either the initial or the follow-up biopsies from 7 patients (1, 3, 9, 14, 24, 34, 41). One of these patients (3) had a severe acute self-limiting hepatitis with bridging necrosis. The first biopsy was taken during the acute phase of the hepatitis, the second biopsy six months later when the liver function tests had nearly normalized and HBsAg had been cleared from the serum.

In two cases (1, 9) the first biopsy revealed an acute hepatitis. In the next biopsy from one of these patients (9) the acute hepatitis had changed to a histological picture of a chronic persistent hepatitis; however at the time of this second biopsy the serum had just converted from HBsAg positive to anti-HBs (self-limiting hepatitis). In the other case (1) the histological findings for the next biopsy remained the same; however according to biopsies taken after the end of the study period transition of the acute hepatitis to chronic active hepatitis occurred. Both the initial and the follow-up biopsy (biopsies) showed chronic active hepatitis in two cases (14, 24), chronic persistent hepatitis in one (41) and active cirrhosis in the other case (34).

The two serial biopsies from one patient (20) contained doubtful ground-glass cells in HA-stained sections, but small numbers of positive cells were evident in both the orcein and aldehyde thionin-stained sections. A chronic active hepatitis was demonstrated in the first biopsy and an active cirrhosis in the second.

Both the first and subsequent biopsies from 11 patients contained GGH (2

Fig.VII.10 *Schematic representation of the percentages of biopsies positive for ground-glass hepatocytes in the different morphological categories of hepatitis B*

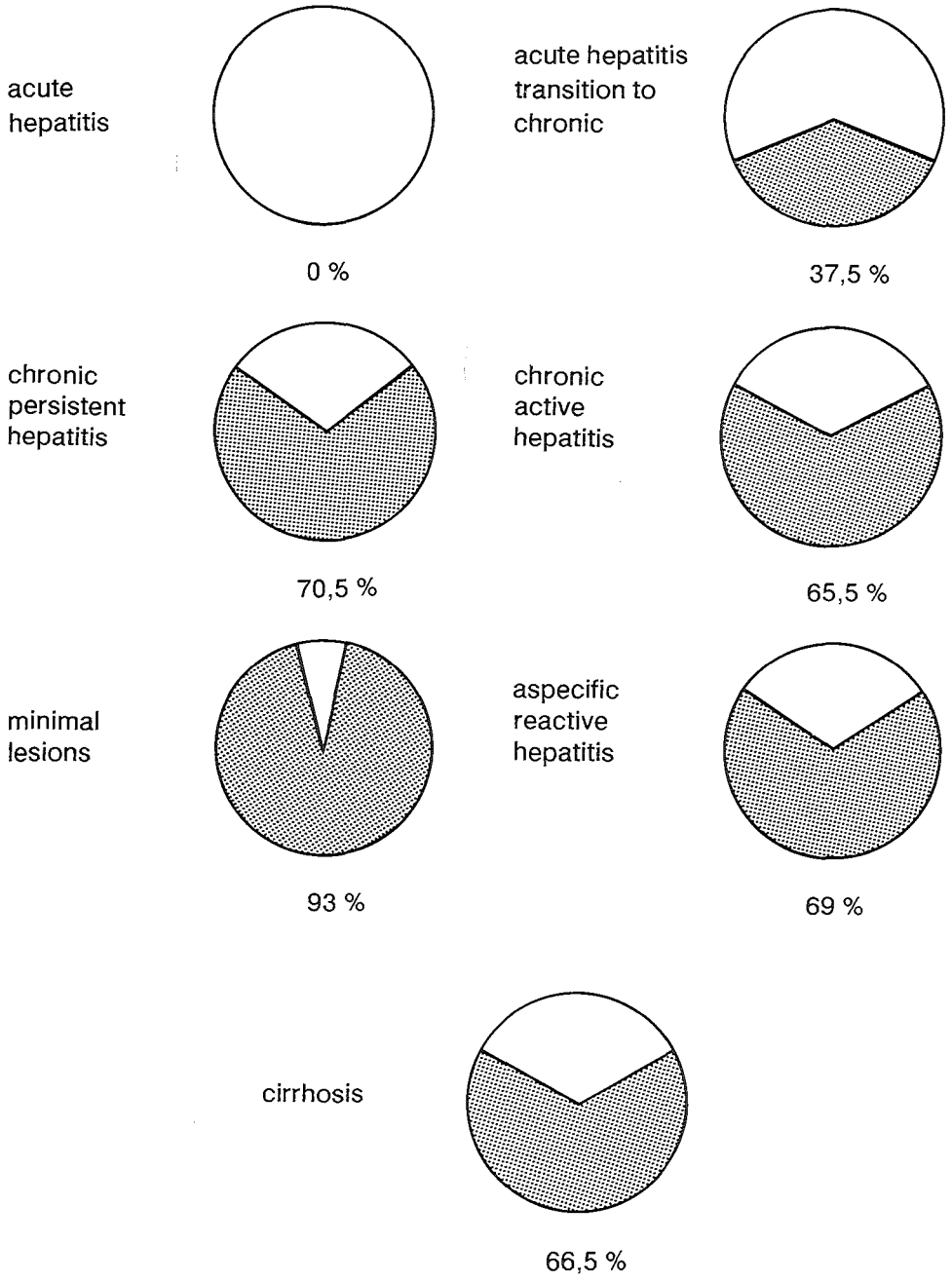
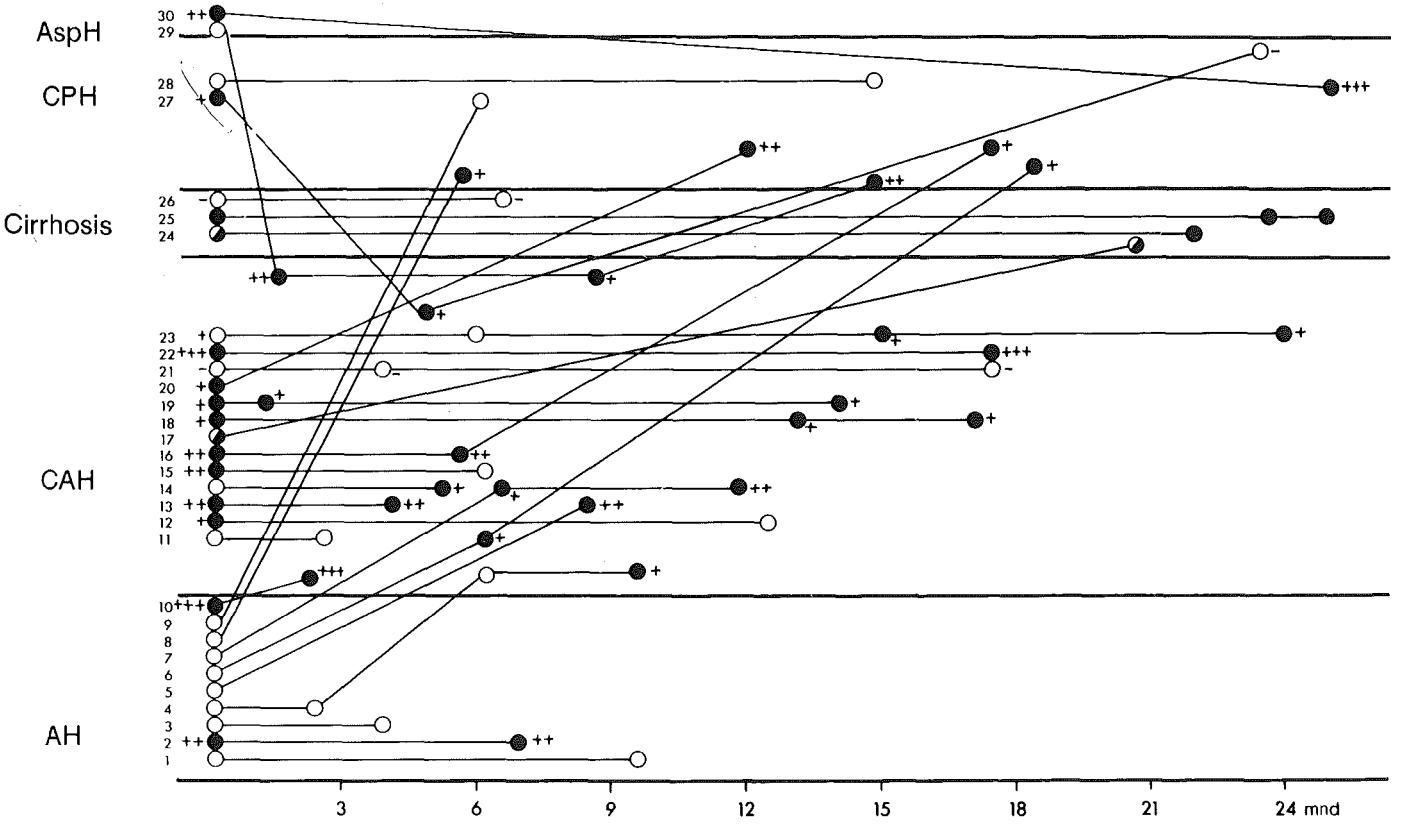


Fig.VII.5 *HBsAg positive patients (n = 30 vertical axis) subdivided according to histological diagnosis for the first biopsy. White dots: biopsies without ground-glass cells, dark dots: biopsies with ground-glass cells. Follow-up biopsies are shown in relation to time and histological changes (reaction pattern)*



+ = < 1, + + = 1-5, + + + = > 5 ground-glass cells per mm^2

biopsies each from 7 patients and 3 biopsies each from 4 patients). For two of these 11 patients (2, 10) the initial diagnosis was an acute hepatitis while the subsequent biopsy was indicative of an acute hepatitis for the second time in one case (2) and a chronic active hepatitis in the other (10). For four of these 11 patients (16, 21, 22, 25) chronic active hepatitis was diagnosed on the basis of the initial biopsy as well as the subsequent biopsy (biopsies) while two patients (19, 23) exhibited chronic active hepatitis in the first and a chronic persistent hepatitis in one of the follow-up biopsies. The first and the subsequent biopsies from two patients (32, 33) reveiled active cirrhosis. In one case (45) the first biopsy exhibit the picture of aspecific reactive hepatitis while a chronic persistent hepatitis was seen in the second biopsy.

In another 11 cases one or more biopsies contained no GGH, while GGH could be detected in the rest of the serial biopsies. For 5 of these 11 patients acute hepatitis without GGH was seen in the first biopsy (biopsies); the acute hepatitis changed in one case (8) into a chronic persistent and in 4 cases (4, 5, 6, 7) to a chronic active hepatitis in the follow-up biopsies. One or all of these follow-up biopsies contained GGH.

In 4 patients (15, 17, 18. 26) the diagnosis for the first biopsy was chronic active hepatitis, a picture that did not change in the follow-up biopsies. In one of the serial biopsies from these 4 patients no GGH could be detected, while the others contained small numbers of GGH. For one patient (40) the first biopsy yielded a diagnosis of chronic persistent hepatitis, the second biopsy showed the histologic picture of a chronic active hepatitis and the third biopsy was again indicative of a chronic persistent hepatitis. In the last case (44) the first biopsy showed an aspecific hepatitis without GGH, while the histological picture of the second biopsy had changed to chronic active hepatitis with many scattered GGH, a picture that persisted in the third biopsy. The fourth biopsy, however, was indicative of chronic persistent hepatitis.

VII.5 DISCUSSION

For many years ground-glass hepatocytes in H and A/E stained sections have been related to the presence of a hepatitis B viral infection (Hadziyannis, 1974).

However GGH with a nearly similar cytological aspect have been demonstrated in livers from HBsAg seronegative individuals. In most cases this phenomenon could be linked to the use of a pharmaceutical drug, such as Antabuse (Bruquera, 1986).

This ground-glass appearance is characterized by finely granular to homogeneous, weak eosinophilic changes in the liver cell cytoplasm. This cytoplasmic as-

pect contrasts with that of the surrounding hepatocytes on routine hematoxylin, eosin or azophloxin-stained slides. These GGH must be distinguished from oxyphilic granular hepatocytes (Lefkowitch, 1980) or hepatic oncocytes (Gerber, 1981). The oxyphilic granular or oncocytic aspect is caused by an extraordinary increase in cytoplasmic mitochondria, while the ground-glass aspect is caused by a proliferation of smooth endoplasmic reticulum (Klinge, 1968; Hadziyannis, 1973).

An oncocytic change in the hepatocytes is also encountered in HBsAg seropositive patients with chronic active hepatitis and cirrhosis (Lefkowitz, 1980) and in patients with cirrhosis of all etiologies (Gerber, 1981).

GGH were detected in 150 of the 1810 liver biopsies in this study. The largest proportion (103) of these biopsies were obtained from HBsAg seropositive patients, the others (47) from HBsAg seronegative patients. GGH in biopsies from HBsAg seropositive patients usually differed morphologically from the GGH in biopsies from HBsAg seronegative patients.

The morphological aspect of the former is typified by a eosinophilic change in a circumscribed part of the hepatocytic cytoplasm and the eccentric position of the nucleus, together with the presence of pre-existing cytoplasmic organelles in the hepatocyte (fig.VII.1).

In addition GGH in liver tissue from HBsAg seropositive patients could be distinguished from GGH in liver biopsies from HBsAg seronegative patients by their specific staining reactions to orcein (Shikata, 1974) and aldehyde thionin (Klinge, 1973; ten Kate, 1973). Moreover all biopsies with GGH that were positive after orcein and aldehyde thionin staining showed accumulation of cytoplasmic HBsAg, as demonstrated by immunochemical detection methods using serial sections, whereas orcein and aldehyde thionin-negative GGH were HBsAg-negative. These observations, which are in agreement with the results of the studies of Shikata (1974) and Deodhar (1975), demonstrated that the presence of orcein and/or aldehyde thionin-positive hepatocytes indicates the existence of an HBV infection. These staining characteristics seem to be based on the binding of the dyes by disulfide bands, present in the excessive accumulated HBsAg (Vyas, 1972; Sukena, 1972; Shikata, 1974), in cisternae of smooth endoplasmic reticulum.

The detection of GGH in liver tissue from HBsAg seropositive patients was easier with the earlier mentioned special stains as compared with the HA stain. When only a few randomly distributed ground-glass cells are present, they will easily be missed in HA-stained sections.

In our material, GGH were detected by orcein and aldehyde thionin staining in only three additional cases. In these cases the GGH were missed in HA stained slides even after careful screening. In all three cases the liver tissue contained

only a sporadic ground-glass cell. Moreover, by using these special stains, hepatocytes with insufficient HBsAg in the cytoplasm to cause the typical ground-glass aspect in HA-stained sections could also be demonstrated. In this study 7 (3.5 per cent) of the 189 biopsies from HBsAg seropositive patients were found to contain HBsAg only after orcein and aldehyde thionin staining. In 4 cases the positive hepatocytes did not exhibit the typical ground-glass aspect in the HA-stained sections.

In most biopsies from HBsAg-positive patients the number of aldehyde thionin or orcein-positive cells was greater than the number of ground-glass hepatocytes in the HA-stained sections. This increase in number can be explained partly by the presence of insufficient quantities of HBsAg in the cytoplasm to induce the typical ground-glass aspect. From this observation it is concluded that only when HBsAg has accumulated in bulk in the endoplasmic reticulum the antigen will cause the ground-glass appearance. The small difference in number between ground-glass cells in HA-stained sections and in orcein-stained sections in this study is in sharp contrast with the findings of Gubetta (1977). He found that of his orcein-positive cases, less then one-half exhibited ground-glass hepatocytes in HA-stained sections.

Although these special staining procedures offer the advantage of detecting small quantities of ground-glass hepatocytes, both orcein and aldehyde thionin-stained slides are difficult to interpret in many cases. There are several explanations for these difficulties. Firstly, the orcein and aldehyde thionin stains are not specific for HBsAg but also react with other structures in the liver biopsy, e.g. elastin, lipofuchsin and copper-binding protein. Secondly, both stains produce aspecific reactions, especially along the margins of the biopsies and in particular when fixation is not optimal. Thirdly, both orcein and aldehyde thionin vary considerably in staining properties from lot to lot, which makes it difficult to obtain consistent staining results. For these reasons many variations in the orcein staining procedure have been suggested (Masachika, 1982) and alternative staining procedures have been proposed (Gubetta, 1977). For these reasons, it is essential for the diagnosis of a HBV infection that hepatocytes not only give a positive reaction with orcein and aldehyde thionin stains but also show in HA-stained sections a typical ground-glass aspect. Despite the greater specificity, the sensitivity of the immunofluorescence and immunoperoxidase staining methods for detection of HBsAg on sections of paraffin-embedded liver tissue was only slightly better than that for the histochemical aldehyde thionin and orcein stains, confirming the results of the studies of Gerber (1975) and Afroudakis (1976). We did not see an increase in the number of positive biopsies, only that the number of hepatocytes detected with the immunological method was greater than the number of aldehyde thionin and orcein-positive hepatocytes. The immunofluores-

cence and immunoperoxidase methods for paraffin-embedded liver tissue were nearly similar in results and sensitivity. The staining results were highly dependent on effective blocking of aspecific binding of the antisera on the slides. However with respect to the specificity of the immuno-peroxidase method for detecting HBsAg, the study of Omata (1980) showed that positive staining of HBsAg is not only caused by specific binding of the HBs antibodies to HBsAg but also by non-immunological binding of horse-rabbit peroxidase to HBsAg.

In this particular case this phenomenon has no implications for the detection of HBsAg, only for the detection of other antigens in HBsAg-positive liver tissue by means of the immunoperoxidase technique. Moreover, in addition to the affinity of horse-rabbit peroxidase for HBsAg, other investigators have shown a non-immunological binding of horse-rabbit peroxidase to alcoholic hyalin (Sim and French, 1976) and lymphocyte cell membrane (Molin, 1978).

Ground-glass hepatocytes were detected in all types of hepatitis B liver disease, except acute self-limiting hepatitis, however the quantity and distribution pattern of the ground-glass hepatocytes differ from one type of liver disease to the other. The focal pattern, with many ground-glass cells arranged in clusters, is typical for HBV carriers with minimal histological changes, indicating that the presence of virus surface protein is not necessarily hepatotoxic.

As already mentioned in chapter V, HBcAg cannot be demonstrated in these cases, despite the abundant production of HBsAg; the viral genome seems to be incorporated in the DNA of the host, as shown by Shafritz (1981) for HBV liver disease and hepatocellular carcinoma. During this integration specific HBcAg expression is deleted. The focal distribution pattern suggests a clonal proliferation of GGH in these cases.

Due to the presence of overwhelming numbers of ground-glass cells, HBV carriers with minimal lesions are seldom missed, as shown in this study and the studies of Gerber (1975) and Deodhar (1975). If sufficient biopsy material is present (>0.5 cm), sampling errors, whereby GGH can be missed, are not likely because of the homogeneous distribution of the ground-glass cells throughout the whole liver, as indicated by the study of Nakaso (1982).

The solitary distribution of GGH is a pattern seen specifically in biopsies of chronic hepatitis (both chronic persistent and chronic active) or prolonged acute hepatitis that progresses to chronic hepatitis. This similarity in the number and distribution of HBsAg among these different morphological categories of hepatitis suggests that these types of hepatitis basically belong to one group, and that the histological differences are an expression of small differences in immune reactivity of the host against viral antigens. In biopsies with the solitary pattern of GGH, variable amounts of HBcAg could often be detected in the nuclei of varying numbers of hepatocytes (see chapter V)(fig.VII.9). However, HBcAg could be

detected in only a minority of GGH and the solitary distribution suggests an inability of these cells to proliferate in these cases. In the solitary pattern the scattered ground-glass cells are surrounded by normal-looking hepatocytes. The number of GGH varies in most cases from + to + +, with a tendency toward increasing numbers as the activity of the chronic liver disease increases. This observation contrasts with the observations of Gubetta (1977) and Paradinas (1981). Paradinas found more ground-glass hepatocytes in inactive liver disease than in active liver disease. However these differences, which were not significant, can be explained by the fact that the group of inactive liver diseases was composed of both minimal lesions and chronic persistent hepatitis. In most of their cases of chronic persistent hepatitis, Paradinas found also only a few GGH and the high mean value can be attributed to the large number of GGH in biopsies, showing minimal lesions.

In this study the percentages of biopsies with GGH in the chronic active and chronic persistent hepatitis groups were nearly identical, 65.5 per cent and 70.5 per cent, respectively. These percentages are in agreement with the findings of Deodhar (1975), who used orcein stain. He found GGH in 7 out of 11 biopsies with chronic persistent hepatitis and in 27 out of 40 biopsies with chronic active hepatitis or with cirrhosis. The study of Gubetta (1977), who used orcein as well as trichrome, also showed that 70 per cent of the biopsies with chronic persistent hepatitis contained GGH. However he found GGH in only 22 per cent of the liver biopsies with chronic active hepatitis.

With the exception of the group of patients with acute self-limiting hepatitis, who exhibited in the liver tissue no ground-glass cells at all, GGH were detected in all histological categories. The lowest percentage of GGH was found for the group that underwent transition from (prolonged) acute hepatitis, to chronic hepatitis (37.5 per cent). However during histological follow-up of all cases of acute hepatitis that progressed to chronic active hepatitis, GGH could always be detected in one of the later biopsies. From these findings it is concluded that in acute hepatitis the detection of GGH with special affinity for aldehyde thionin or orcein forecasts transition of this acute hepatitis to a chronic hepatitis. The development of GGH (incorporation of viral DNA in the hepatocyte genome) takes some time; in our series the shortest time within which GGH were detected was nearly 3 months after the onset of acute hepatitis. However the assessment of the exact date of the onset of the acute hepatitis was very difficult, because the liver disease in nearly all patients with the histological features of acute hepatitis that progresses to chronic hepatitis is, according to the findings of Sherlock (1976), clinically anicteric and in some cases subicteric. Reinfected liver grafts for which the exact date of infection was known, exhibited ground-glass cells six months later (see chapter XI).

As shown in chapter V immunofluorescence studies using of frozen liver tissue, provide in a much earlier stage of the acute hepatitis an indication of an unfavorable course by the presence of a cell membrane-bound HBsAg fluorescence pattern of all hepatocytes in these cases. This cell membrane-bound fluorescence pattern is lost in most cases after formalin fixation, as was also reported by Gubetta (1977).

From follow-up biopsies there is some evidence that the number and distribution of GGH in one patient remain quite constant, at least for some years. In many cases the number and especially the distribution pattern of GGH are more constant than the histological diagnosis of the liver disease. We could not demonstrate an increase in the number of GGH during the course of the disease.

Although screening of liver tissue for the presence of ground-glass hepatocytes is not a sensitive method for detection of hepatitis B infection, the presence of these cells has some prognostic value in acute hepatitis and is an essential tool for the grading hepatitis B liver disease. This is especially important when more than one etiological factor seems to be responsible for the changes in the liver.

In conclusion, as far as the assessment of an HBV infection by various stainings on formalin fixed liver biopsy material is concerned, it can be stated that:

1. No histochemical and immunochemical reactions diagnostic for an HBV infection are found in acute self-limiting HBV hepatitis.
2. In chronic HBsAg-positive patients precise light microscopic diagnosis of an HBV infection is possible of a maximum of ± 90 per cent in the category of minimal histological changes. In the other clinico-pathological categories lower percentages of biopsies, in which a diagnosis of an HBV infection can be established, were found.
3. Most of these diagnoses can be established on the basis of routinely stained hematoxylin azophloxin (eosin) slides.
4. Specific immunohistochemical detection methods do not lead to an increase in the number of diagnosis, but are indispensable for specification of the definitive diagnosis.
5. There are few arguments that support the routine use of histochemical staining methods to detect an HBV infection.

CHAPTER VIII

EXPRESSION OF THE DETERMINANTS OF HEPATITIS B SURFACE SUBTYPES IN LIVER TISSUE

CHAPTER VIII

EXPRESSION OF THE DETERMINANTS OF HEPATITIS B SURFACE SUBTYPES IN LIVER TISSUE

VIII.1 SUMMARY

The presence of different HBsAg determinants in HBV-infected liver tissue was demonstrated by means of the immunofluorescence technique using monospecific antisera against the "a", "d" and "y" determinants. These experiments proved that liver tissue from HBsAg ("ad") seropositive patients contains the "a" determinant as well as the "d" determinant. Similarly liver tissue from HBsAg ("ay")-seropositive patients contained the "a" and the "y" determinant.

By using the double labelling immunofluorescence technique it was shown that the different determinants have the same localization in liver tissue. It is concluded that these determinants are formed by the same mechanism and that none of the determinants are liver cell-derived.

VIII.2 INTRODUCTION

The determinants of hepatitis B surface antigen (HBsAg) have been investigated by several authors (Le Bouvier, 1971; Couroucé, 1974). Studies on HBsAg-positive serum have resulted in the detection of several antigenic determinants, indicated by the letters "a", "d", "y", "w" and "r". In this system the "a" determinant has been found to be the obligatory group-specific determinant, which in turn can be classified according to the presence of four subdeterminants (Couroucé, 1974). The "d" and "y" as well as the "w" and "r" determinants re-present two pairs of mutually exclusive determinants, giving rise to the following hepatitis B surface subtypes: "adr", "adw", "ayw", "ayr" (Le Bouvier, 1971; Bancroft, 1972).

The presence of the different determinants in liver tissue has barely been documented. Most HBsAg studies were performed with antisera against HBsAg "ad" or "ay". Houthoff (1976) suggested that the "a" determinant is not present in liver tissue. Our studies on the localization of HBsAg in liver tissue, reported in the previous chapters, were based on the use of an antiserum against HBsAg subtype "ad" that contained antibodies against both the "a" determinant (high titers) and the "d" determinant (low titers).

In this chapter the results of a study on the specific localizations of the "a", "y" and "d" determinants in liver tissue from both HBsAg (subtype "ad" and "ay") seropositive and HBsAg seronegative patients are given; antisera against the "a", the "y" and the "d" determinants, respectively, were used (chapter III).

VIII.3 MATERIAL AND METHODS

Liver biopsy tissue from 20 HBsAg seropositive and 8 HBsAg seronegative patients was tested with monospecific antisera against "ad", "a", "d" and "y" determinants of the hepatitis B surface antigen, using direct and indirect immunofluorescence techniques (see chapter III).
Experiments were completed by blocking and double staining procedures.

VIII.4 RESULTS

Anti "ad"

In 19 of the 20 liver biopsies from HBsAg seropositive patients one of the previously described fluorescence patterns (focal or diffuse) was observed. The solitary pattern was not seen. None of the liver biopsies from the 8 HBsAg seronegative patients were positive for the "ad" determinants.

Table VIII.1: *Localization of HBsAg "a", "d" and "y" determinants in liver tissue from 20 HBsAg "ad" and "ay" seropositive patients.*

serology		immunofluorescence		
"ad"	"ay"	"a"	"d"	"y"
15	-	14/15	12/15	0/15
-	5	5/5	0/5	3/5

Anti "a"

The immunofluorescence results with antiserum against the "a" determinant of HBsAg were identical to those found with the anti "ad" antiserum. Reactivity was completely abolished by preincubation of the anti "a" antiserum with HBsAg ("adr"), HBsAg ("adw") or HBsAg ("ayw").

Anti "d"

Of the liver biopsies from HBsAg ("ad") and HBsAg ("ay") seropositive patients tested with anti "d" antiserum only those from HBsAg ("ad") seropositive patients exhibited (in most cases) fluorescence. The fluorescence pattern was the same as that found with HBsAg ("ad") and HBsAg ("a") antisera. However, two HBsAg ("ad") seropositive patients reacted positively to anti "ad" and anti "a" antisera but negatively to anti "d" antiserum. Moreover in a few cases there was some difference in the intensity of fluorescence obtained with anti "ad" antiserum, on one hand, and anti "d" antiserum, on the other. By double staining of the liver slides with rabbit anti "a" antiserum and guinea pig anti "d" antiserum, using TRITC and FITC-labelled anti-rabbit and anti-guinea pig antisera, respectively, as second layer, the localizations of the "a" and "d" determinants were shown to be exactly the same. Both were found in the cytoplasm as well as along the cell membranes of the hepatocytes. No fluorescence was found after preincubation of the anti "d" antiserum with HBsAg ("ad")-positive serum. Preincubation of the anti "d" antiserum with HBsAg ("ay") sera did not influence the outcome of the fluorescence studies.

Anti "y"

Anti-HBsAg ("y") antiserum only stained liver tissue from HBsAg ("ay")-seropositive patients. However, liver biopsies from 2 of the 5 HBsAg ("ay")-seropositive patients reacted negatively to the anti "y" antiserum, although both biopsies exhibited fluorescence with anti "ad" and anti "a" antisera. In the other biopsies the localization of the "y" determinant was identical to that of the "a" determinant. Both could be demonstrated along the cellular membrane and in the cytoplasm of hepatocytes. Preincubation of the anti "y" antiserum with HBsAg ("ay") serum abolished the reactivity, whereas preincubation with HBsAg ("ad") serum did not alter the results of the fluorescence studies.

VIII.5 DISCUSSION

The results of these fluorescence experiments demonstrate that both the common group-specific "a" determinant as well as the "y" and "d" determinants of HBsAg are present in liver tissue from HBsAg seropositive patients. This conflicts with the results of Houthoff and Houwen (1976) who could not find the "a" determinant in liver tissue from either "ad" or "ay" seropositive patients and suggested that proteins derived from liver cell membranes participate in the

formation of HBsAg. This suggestion seems unlikely in view of the fact that our rabbit anti "a" antiserum, which contained high titers against the "a" determinant and low titers against the "d" determinant, yielded positive fluorescence in liver tissue from both HBsAg "ay" and "ad" seropositive subjects. The "a", "d" and "y" determinants were found both in the cytoplasm of hepatocytes and along the cell membranes. Double staining techniques revealed the identical localizations of these determinants. Although the "a", "d" and "y" determinants were found at the same localization in the liver tissue, the quantity of the "a" determinant, on the one hand, and the "d" and "y" determinants, on the other, varied, as estimated from the intensity of the fluorescence. In two HBsAg ("ad") and two HBsAg ("ay") seropositive patients the "d" and "y" determinants could not even be detected in the liver tissue, while these tissues were positive for the HBsAg ("a") determinant. This is in accordance with the studies of Van Elven (unpublished), which showed a variation in the ratio of "a" to "d" antigens in HBsAg "ad" positive sera and of "a" to "y" antigens in HBsAg "ay" positive sera. Although the common "a" determinant is always combined with the "d" or the "y" determinant in HBsAg, the free capsid material of HBV does not seem to have a fixed "a", "d" and "y" composition. When using immunological techniques for the detection of HBsAg in liver tissue it is recommended that an antiserum with a high anti "a" activity be used because antisera with anti "d" and "y" activity tend to produce false-negative results.

CHAPTER IX

HBeAg AND anti-HBe

Localization of HBeAg in liver tissue; relation between HBeAg and anti-HBe in serum and hepatitis B antigens in liver tissue. A light microscopical and immunohistochemical study.

CHAPTER IX

HBeAg AND anti-HBe

Localization of HBeAg in liver tissue; relation between HBeAg and anti-HBe in serum and hepatitis B antigens in liver tissue. A light microscopical and immunohistochemical study.

IX.1 SUMMARY

Sixty-three serum samples from 62 patients with histologically graded hepatitis B liver disease were examined for the presence of HBe-antigen (HBeAg) and HBe-antibody (anti-HBe). The incidence of HBeAg and anti-HBe in serum was estimated for the various histological types of liver disease. The presence and localization of HBeAg, HBsAg, HBcAg and immunoglobulins in liver tissue were studied by immunofluorescence and compared with the presence of HBeAg and anti-HBe in serum.

HBeAg was found mainly in chronic hepatitis B infection. A relation existed between the activity of chronic hepatitis B and the presence of HBeAg in serum and in liver tissue. Of the patients with cirrhotic liver disease, however, only 3 out of 10 were HBeAg-seropositive. The only patient with minimal histological changes and HBeAg-positive serum was on immunosuppressive therapy, indicating that the presence of HBeAg in itself does not cause active liver disease. Sera from 3 out of 13 patients with acute self-limiting hepatitis exhibited HBeAg. However, HBeAg was found in 5 out of 6 patients with an acute hepatitis that progressed to chronic liver disease.

All but four HBeAg seropositive patients exhibited HBcAg in the nuclei of hepatocytes versus one out of 7 patients with anti-HBe-positive serum and 2 out of 21 patients with neither HBeAg nor anti-HBe in the serum.

Immunofluorescence studies of liver tissue showed identical nuclear localizations for HBeAg and HBcAg. In all HBeAg seropositive patients HBsAg fluorescence was found along the cell surface of the hepatocytes.

From this it was concluded that:

- a high level of HBeAg in the serum is an indicator of the presence of chronic hepatitis B infection; an early preicteric stage of acute self-limiting hepatitis B infection has to be excluded.

- HBsAg-positive patients with cirrhosis are relatively infrequent HBeAg positive. This low prevalence of eAg in cirrhosis is probably due to e-conversion, which may occur after long-term HBsAg carriership.
- the presence of HBeAg is related to the severity of chronic hepatitis.
- there is a close relation between HBeAg and HBcAg in the liver as far as both incidence and localization are concerned.
- there is no direct relation between the numbers of HBeAg and HBcAg-positive hepatocytes and the level of HBeAg in serum.
- twenty-three out of twenty-six (88 per cent) patients with HBcAg and HBeAg in the liver tissue were found to have HBeAg in the serum.

IX.2 INTRODUCTION

In 1972 Magnius and Espmark described a new antigen-antibody system, designated HBe antigen (HBeAg) and HBe antibody (anti-HBe), in hepatitis B surface antigen (HBsAg) positive sera. They found HBeAg in sera from both persistent carriers of HBsAg and patients in the incubation period of a hepatitis B infection (Magnius, 1975). HBeAg was found exclusively in HBsAg-positive sera and is therefore apparently linked to the HBV. HBeAg did not appear to be a subtype of the hepatitis B surface antigen from which it differed both immunologically and biochemically (Magnius, 1975).

Not only the observation that the presence of HBeAg in patient sera seemed to be closely associated with the occurrence of Dane particles and DNA polymerase activity, but also the finding that HBeAg in serum was associated with hepatitis B core antigen (HBcAg), strongly suggests that HBeAg is a component of the Dane or core particle. In their study, Takahashi et al. (1979) confirmed this hypothesis by demonstrating the masked presence of HBeAg in the cores of the Dane particles. Since the Dane particle is known to represent the complete virion of HBV, the presence of HBeAg in serum is considered to be an important marker of the infectivity of HBsAg-positive serum.

The exact nature of HBeAg, which seems to exhibit antigenic polymorphism (Budkowska, 1979), was however far from clear (McAuliffe, 1976). Studies of Neurath et al. (1977) showed that HBeAg has the physicochemical and immunological properties of an immunoglobulin, predominantly of the IgG4 subclass. Specific antigenic sites, designated as HBe determinants, do however differentiate HBeAg from other immunoglobulins.

Clinical studies (Nielson, 1974; Trepo, 1976; Smith, 1976; Eleftheriou, 1976) indicate that the presence of HBeAg is associated with the activity of hepatitis, and that HBeAg may be used as a prognostic marker for acute hepatitis (Thamer,

1976). In accordance with the findings of these studies, several other studies have indicated that chronic active hepatitis B may improve after seroconversion from HBeAg to anti-HBe and is then usually associated with a favorable outcome of the disease, as shown by a sustained biochemical and histological remission (Realdi, 1980; Fattovich, 1986). In contrast Fattovich (1988) has shown that patients with anti-HBe positive chronic hepatitis B infection may have continuing inflammatory activity and a poor prognosis. This continuing inflammatory activity in anti-HBe positive patients seems to be caused by low level HBV replication (Lok, 1984).

As far as the localization of HBeAg in liver tissue is concerned, two completely conflicting reports exist. Trepo (1976) demonstrated HBeAg in the cytoplasm of hepatocytes, whereas Arnold (1977) localized the HBeAg exclusively in hepatic nuclei. Therefore the presence of HBeAg and anti HBe was studied in a series of HBsAg seropositive patients with various histopathological types of liver disease. In addition the localization of HBeAg in liver tissue and the relation of HBeAg in serum with the localization of HBcAg, HBsAg and immunoglobulins in liver tissue were investigated.

IX.3 MATERIAL AND METHODS

IX.3.1 LIVER TISSUE

Sixty-three liver biopsies were obtained from 62 HBsAg seropositive patients with a True-cut needle (Travenol^{R}). All liver biopsies were prepared as described in chapter III and graded histologically as described in chapter IV.

IX.3.2 SERA

Serum samples from all 62 patients were acquired on the same day as the liver biopsy specimen and tested for the presence of HBsAg by radioimmunoassay (Abbott Laboratories, IL, USA). The presence of HBeAg and anti-HBe antibodies was determined by both by a double immunodiffusion method and radioimmunoassay (Abbott Laboratories, IL, USA).

IX.3.3 ANTISERA

Slides of the snap-frozen part of the liver biopsies were tested with antibodies against HBsAg, HBcAg and HBeAg and with HBe-positive sera (see chapter III).

IX.4 RESULTS

HBeAg and anti-HBe in sera from patients with different histological types of hepatitis B, measured by the immunodiffusion method

HBeAg was detected in the sera of 5 out of the 19 patients with histologically graded acute hepatitis (26 per cent), 2 out of 9 patients with chronic persistent hepatitis (22 per cent) and 9 out of 16 patients with chronic active hepatitis without cirrhotic changes (56 per cent). One out of 10 patients with cirrhotic liver changes and 1 out of 9 subjects with minimal or aspecific reactive liver changes had HBeAg in their serum.

Anti-HBe was found in the serum of only 1 of the 19 patients (5 per cent) with acute hepatitis, 2 of the 9 patients (22 per cent) with chronic persistent hepatitis, 2 of the 16 patients (13 per cent) with chronic active hepatitis, 3 of the 9 subjects (33 per cent) with minimal or aspecific liver disease and 2 of the 10 patients (20 per cent) with cirrhosis.

HBeAg and anti-HBe in sera from patients with different histological types of hepatitis B, measured by radioimmunoassay

Three sera from patients with histologically graded acute hepatitis, one serum from a patient with chronic persistent hepatitis, three sera from patients with chronic active hepatitis and one serum from a patient with cirrhosis, all negative for both HBeAg and anti-HBe according to the immunodiffusion technique, were HBeAg-positive according to the radioimmunoassay.

In addition to the anti-HBe positive cases found by the immunodiffusion technique, anti-HBe could be detected by radioimmunoassay in sera from four patients with acute hepatitis and two patients with minimal or aspecific liver disease (table IX.1).

Of the 19 patients with histologically graded acute hepatitis, 13 normalized biochemically and serologically. In contrast 6 patients developed a chronic liver disease. Initially five of these 6 patients were serologically positive for HBeAg, while only three of the 13 patients with acute self-limiting hepatitis were positive for HBeAg. Two of these three patients were blood donors who were found to be HBsAg-positive by chance in the pre-icteric phase of an acute hepatitis B; in one patient a liver biopsy and a serum sample were taken during this period whereas the others were biopsied in the early icteric phase. One of these patients had both HBeAg and anti-HBe in the serum.

Table IX.1 *Presence of HBeAg and anti-HBe in sera from patients with different histological types of hepatitis B, as assessed by the immunodiffusion technique and radioimmunoassay (in parentheses)*

histological type	n	HBeAg n	HBeAg %	anti-HBe n	anti-HBe %
acute hepatitis	19	5 (8)	26(42)	1(5)	5(26)
chronic persistent hepatitis	9	2 (3)	22(33)	2(2)	22(22)
chronic active hepatitis	16	9(12)	56(75)	2(2)	13(13)
cirrhosis	10	1 (3)	10(30)	2(2)	20(20)
minimal or aspecific liver changes	9	1 (1)	11(11)	3(5)	33(55)

The patients in the acute hepatitis group with anti-HBe positive-serum proved to have a self-limiting acute icteric hepatitis. At the time of the liver biopsy these patients had had jaundice for more than 10 days.

Table IX.2 *HBeAg and anti-HBe in histologically graded acute hepatitis B*

	n	HBeAg	anti-HBe
self-limiting hepatitis	13	3	5
acute hepatitis with transition to chronic hepatitis	6	5	0

Out of the group of 16 patients with chronic active liver disease, 7 patients had a mild degree of chronic active hepatitis with transaminase levels no more than 3 times the normal value. Four of these patients were serologically positive for HBeAg. Nine patients had moderate or severe chronic active hepatitis. For 8 of these 9 patients HBeAg could be demonstrated in the serum.

Table IX.3 *HBeAg and anti-HBe in serum related to the various grades of chronic active hepatitis*

chronic active hepatitis	n	HBeAg n	%	anti-HBe n	%
mild	7	4	57	2	28
moderate/severe	9	8	90	0	0
total	16	12	75	2	13

The only patient with minimal liver changes and HBeAg in his serum was a renal transplant recipient receiving high doses of immunosuppressive agents.

In the group of 10 patients with cirrhosis of the liver, cirrhosis was combined with a chronic active hepatitis in 8 cases (active cirrhosis). The three patients in this group of 10 patients with HBeAg detectable in the serum had cirrhosis with a mild degree of chronic active hepatitis in two cases and with a severe degree of CAH in the third. One of the patients with mild CAH was on immunosuppressive therapy. The two patients with positive anti-HBe serum exhibited a moderate and severe chronic active hepatitis component, respectively. The two patients with inactive cirrhosis had neither HBeAg nor anti-HBe.

Correlation of HBeAg and anti-HBe in serum with HBsAg distribution in liver tissue

This study of 63 biopsies revealed that HBsAg occurred in three distinct patterns (see also chapter IV).

a. **A solitary pattern**, in which only a few scattered Kupffer's cells and portal macrophages are positive for HBsAg, as indicated by a granular cytoplasmic fluorescence; this pattern was seen in 12 of the 63 biopsies.
b. **A diffuse pattern**, in which all hepatocytes show cell membrane-bound linear fluorescence. In this pattern varying numbers of scattered hepatocytes also exhibit intracytoplasmic fluorescence; this pattern was seen in 34 of the 63 biopsies.
c. **A focal pattern**, in which clusters of hepatocytes show bright intracytoplasmic HBsAg fluorescence. The remaining hepatocytes in the liver tissue either exhibit faint cell membrane-bound HBsAg fluorescence or are negative for HBsAg.

HBeAg could be detected in 27 of 63 serum samples tested from HBsAg-positive patients. In 25 of the 27 cases HBeAg in serum was associated with a diffuse HBsAg pattern in the liver tissue. These 25 liver biopsies represented all histological types of hepatitis B liver disease. The other two cases, both patients with acute self-limiting hepatitis, exhibited a solitary pattern.

Anti-HBe was found together with the solitary pattern in 5 cases, the diffuse pattern in 5 cases and the focal pattern in 6 cases.

Table IX.4 *Correlation of HBsAg fluorescence pattern in liver tissue and HBeAg or anti-HBe in serum.*

	n	focal pattern	diffuse pattern	solitary pattern
HBeAg	27	-	25	2
anti-HBe	16	6	5	5

Correlation of HBeAg and anti HBe in serum with the presence of HBcAg in liver tissue

Liver biopsy specimens from all patients were tested for the presence of HBcAg by the immunofluorescence technique and, in a few cases, by electron microscopy. HBeAg was found in 27 of the 63 samples tested. In 23 of these cases the corresponding liver biopsies revealed HBcAg. In four cases HBeAg was found in the serum without HBcAg in the corresponding liver biopsy. Two of these four HBcAg-tissue-negative, HBe-seropositive cases were patients with an acute self-limiting hepatitis.

Table IX.5 *Correlation of HBeAg and anti-HBe in serum with the presence of HBcAg in liver biopsies.*

HBeAg/anti-HBe	HBcAg n	HBcAg pos.	neg.
HBeAg positive	27	23	4
HBeAg negative/anti-HBe negative	21	2	19
anti-HBe positive	16	1	15

The sera from 21 of the 62 patients contained neither HBeAg nor anti-HBe while core particles could be detected in the corresponding liver biopsies from 2 of these 21 patients. In one case anti-HBe in serum was associated with the presence of HBcAg in the liver tissue. Table IX.6 shows the relation between HBeAg and anti-HBe in serum and the presence of HBcAg in liver tissue for the various histological groups of hepatitis B.

Table IX.6 *Correlation between HBeAg and anti-HBe in serum and HBcAg in liver tissue for various histological types of hepatitis.*

		HBeAg pos		HBeAg neg anti-HBe neg		anti-HBe pos	
	n	core +	core -	core +	core -	core +	core-
acute hepatitis self limiting	13	1	2	-	6	-	5
acute hepatitis with transition into CH	6	5	-	-	1	-	-
chronic persistent hepatitis	9	3	-	-	4	-	2
chronic active hepatitis	16	10	2	1	1	-	2
minimal lesions	4	1	-	-	1	-	2
reactive hepatitis	5	-	-	-	2	-	3
cirrhosis	10	3	-	1	4	1	1
total	63	23	4	2	19	1	15

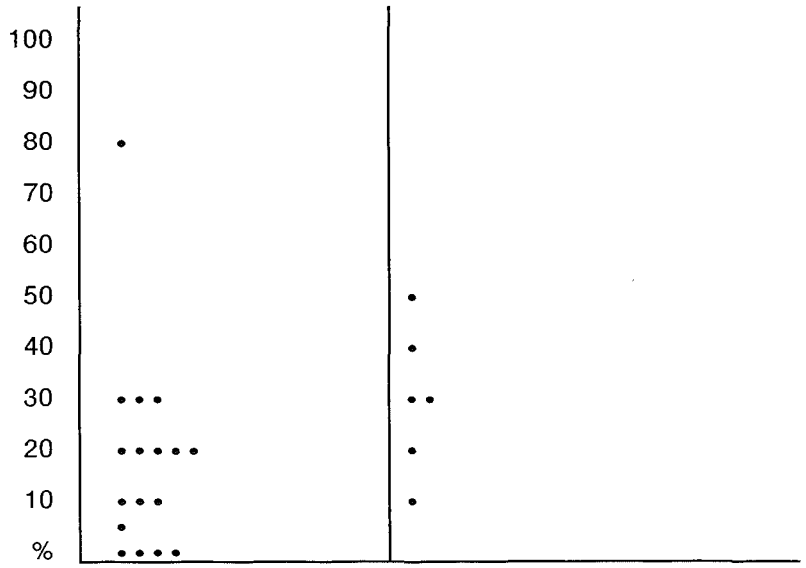
Of the group of patients with acute self-limiting hepatitis three had HBeAg-positive serum; only one of these three patients was also positive for HBcAg in the liver biopsy. Of the 6 cases of acute hepatitis that progressed to chronic hepatitis only one was negative for both HBcAg in liver tissue and HBeAg in serum.

In the histological group of chronic persistent hepatitis, 3 of the 9 patients were positive for both HBeAg in serum and HBcAg in liver tissue. Two of the nine patients had anti-HBe in serum and no detectable HBcAg in the liver tissue.

In the group with chronic active hepatitis 10 of the 16 patients were positive for both HBeAg in serum and HBcAg in the liver. In 2 cases the serum showed anti-HBe without HBcAg in the liver tissue. Of the 2 patients negative for HBeAg and anti-HBe, one, however, showed HBcAg in the liver tissue.

In the group of 9 patients with minimal lesions or aspecific reactive hepatitis one patient, a renal transplant recipient on high-dose immunosuppressive therapy, exhibited HBeAg in the serum as well as HBcAg in the liver specimen. Five of the 9 patients were anti-HBe seropositive without detectable HBcAg in the liver, while 3 patients were negative both for HBeAg and anti-HBe in serum and HBcAg in the liver tissue.

Table IX.7 *Relation between the percentage of HBcAg-positive hepatocytic nuclei and HBeAg in serum as determined by immunodiffusion and radioimmunoassay.*



For 3 of the 10 patients with cirrhosis HBeAg could be detected in the serum, while 4 patients were positive for HBcAg. Two patients showed anti-HBe, 1 with and 1 without HBcAg in the liver tissue. Four patients were negative for HBeAg and anti-HBe in the serum as well as for HBcAg in the liver tissue.

The percentage nuclei positive for HBcAg in the liver biopsy ranged from sporadic to 80 per cent, with a mean value of ± 20 per cent, for the group of

patients with HBeAg-positive serum as measured by the immunodiffusion technique and from 5 per cent to 60 per cent, with a mean value of 30 per cent, for the group of patients who were HBeAg-positive only with the sensitive radioimmunoassay (table IX.7).

Localization of HBeAg in liver tissue

All 63 liver biopsy specimens were tested with HBeAg and anti-HBe-positive sera using the indirect and direct immunofluorescence technique. In most cases HBeAg was only detected in the nuclei of hepatocytes; in some cases, however, nuclear HBeAg localization was combined with localization in the cytoplasm of hepatocytes. All 26 biopsies positive for HBc antigen also exhibited positive fluorescence with anti-HBe antisera and HBeAg-positive sera; the latter yielded a distribution identical to that seen for HBcAg. Preincubation of the slides with a monoclonal mouse anti-HBcAg antiserum before incubation of the slides with the human HBcAg antiserum resulted into complete blocking of the specific HBcAg fluorescence. On the other hand incubation of slides with anti-HBe antisera after preincubation with monoclonal mouse HBcAg-antiserum yielded nuclear fluorescence.

IX.5 DISCUSSION

Sixty-three HBsAg-positive sera from 62 patients with various types of liver disease were tested for the presence of HBeAg and anti-HBe. The results of the serological tests were correlated with the results of immunofluorescence studies to demonstrate the presence of HBcAg in the liver biopsies. These biopsies were taken on the day the sera were sampled.

Twenty-seven of the tested sera were positive for HBeAg and 16 for anti-HBe. Twenty-one (33 per cent) of the sera, however, were negative for both HBeAg and anti-HBe. When the immunodiffusion technique was used 35 of 63 sera were negative; this number dropped to 21 with the radioimmunoassay. The relatively low percentage of cases positive for anti-HBe or HBeAg (68 per cent) is in contrast to the results reported by Mushawar, 1978, Tabor, 1980, Aldersville, 1980 and Mushawar, 1981, who detected either HBeAg or anti-HBe in about 90 per cent of the HBsAg-seropositive serum samples. The low numbers of either HBeAg or anti-HBe may partly be explained by the relatively large group of patients with acute self-limiting hepatitis.

In our material there was a close positive correlation between the presence of HBeAg in the serum and HBcAg in the nuclei of hepatocytes in the liver biopsy,

a correlation that was also found by Murphy (1976) and Trepo (1976). In only four cases was a patient positive for HBeAg in the serum and negative for HBcAg in the liver tissue. Two of these patients had a self-limiting acute hepatitis. Two patients, however, had a chronic active hepatitis. In a follow-up biopsy obtained two years later from one of these patients HBcAg could be detected. Because HBcAg is found mostly in foci of hepatocytes, the absence of HBcAg in the first biopsy probably represents a sampling error. Despite the close correlation between the presence of HBcAg in liver tissue and HBeAg in serum, remarkable differences in HBeAg titers and the number of HBcAg-positive hepatocytic nuclei were found. Although this discrepancy can be partly attributed to sampling of the liver tissue, because the positive nuclei are irregularly distributed, the differences in HBeAg serum levels and the number of HBcAg-positive nuclei are too frequent and too striking for this to be the only explanation. In one case more than 80 per cent of the nuclei in the biopsy were positive for HBcAg whereas the HBeAg serum titer was very low. On the other hand 3 patients with sparse HBcAg-positive nuclei had a high HBeAg titer. Moreover, for 2 patients without detectable HBeAg in the serum, the number of HBcAg-positive nuclei ranged from 5 to 60 per cent. The mean number of HBcAg-positive nuclei for the group with HBeAg detected by the immunodiffusion technique was lower than that for patients with HBeAg detected only by radioimmunoassay. The discrepancy between the quantity of HBeAg and HBcAg in tissue and serum may be explained by differences in the excretion of core particles from the hepatocytes or by differences in the degree of lysis of hepatocytes loaded with viral particles. However in this study no direct relation was found between hepatocyte necrosis and HBeAg titer.

Although the core particle contains the genetic information and the potency of the HBV, our observations on the close correlation between the presence of HBeAg in serum and core particles in liver tissue confirm the value of the demonstration of HBeAg in serum as a sign of infectious potency and viral replication. In addition, however, our data also indicate that demonstration of HBeAg and HBcAg in liver tissue is supplementary to the detection of HBeAg in serum for the assessment of infectivity and viral replication activity; anti-HBe in serum does not exclude the possibility of an infectious type of hepatitis B liver disease. For example in one patient with active cirrhosis, HBcAg in the liver biopsy was accompanied by the presence of anti HBe in serum. Follow-up biopsies from this patient confirmed this simultaneous presence of HBcAg in liver tissue and anti HBe in serum, thus excluding the possibility that the initial samples were taken during conversion of HBeAg to anti-HBe with the subsequent disappearance of HBcAg. In contrast to the study of Murphy (1976) our results demonstrate that a strong correlation exists between HBcAg in liver tissue and the presence of HBeAg in serum.

The studies of Okada (1976), Beasly (1977) and Mushahwar (1981) on the vertical transmission of hepatitis B from mothers to their children and our own observation of infection of a liver graft by the hepatitis B virus in an anti-HBe seropositive patient prove that not only HBeAg-positive subjects but also anti-HBe-positive subjects have to be considered as potentially infectious. Only patients with a focal HBsAg fluorescence pattern have been shown to be consistently negative for HBcAg and HBeAg in both serum and liver tissue.

Of the patients with histologically graded acute hepatitis and HBeAg in the serum, 5 out of 8 underwent transition to chronic active hepatitis. Only 1 of the 6 patients with acute hepatitis that progressed to chronic hepatitis was negative for HBeAg in the serum. In contrast all but three patients with acute self-limiting hepatitis were negative for HBeAg. This confirms the predictive significance of HBeAg in late stage acute hepatitis for the natural course of the disease, as already mentioned by Nielsen (1974). Moreover, in cases of acute hepatitis persistent high serum titers of HBeAg are shown to be highly predictive of transition to chronic hepatitis (van Hattum, 1986).

In our study a strong correlation was found between the severity of chronic hepatitis B infection and the presence of HBeAg. HBeAg-positive serum was found for 3 of the 9 patients with chronic persistent hepatitis, 4 of the 7 patients with mild chronic active hepatitis and 8 of the 9 patients with severe chronic active hepatitis. Only in liver cirrhosis was there a lack of correlation between activity of the inflammation and the presence of HBeAg. Eight of the 10 patients with cirrhosis revealed a mild or more severe chronic active hepatitis component, while only 3 patients were positive for HBeAg. One of these two patients was also on immunosuppressive therapy. Another patient with cirrhosis and chronic active hepatitis was positive for anti HBe, a serum profile that was also found in 1 case by Mushahwar (1981) and for several patients in the study of Fattovich (1988). These results suggest that long-standing hepatitis B has a tendency to correlate with declining HBeAg titers and sero-conversion from HBeAg to anti HBe.

Minimal and aspecific lesions in liver tissue were accompanied by the presence of HBeAg in only 1 of the 9 patients. This patient was the only one in this group on immunosuppressive therapy, indicating that positive HBeAg and HBcAg titers are not in themselves related to inflammatory infiltrates.

Striking was the close relation between the cell membrane bound HBsAg pattern and the presence of HBeAg in serum and HBcAg and HBeAg in liver tissue and the severity of the liver disease. On the other hand a focal HBsAg pattern was found for patients without evidence of liver cell necrosis, a HBeAg-negative serology and a HBcAg-negative liver biopsy.

This study on the localization of HBeAg in liver tissue demonstrates the close relation between HBeAg and HBcAg on the cellular level. HBeAg was only found

in nuclei and cytoplasm from liver tissue specimens which also contained HBcAg. In most HBcAg-positive liver biopsies, the numbers of HBcAg and HBe-containing hepatocytes were equal. These results are in agreement with those of Arnold (1977) who found, using specific FITC and rodamine-labelled antisera against HBcAg and HBeAg, a nuclear localization for both HBc and HBeAg. In our studies it was impossible to purify human anti-HBe antisera of anti-HBc antibodies using an immunoabsorbent technique. This phenomenon is also mentioned by Neurath (1977), which leads to the supposition that HBeAg itself is an immunoglobulin.

Immunofluorescence experiments using HBeAg-positive sera yielded the same results as those obtained with anti-HBe and HBcAg antisera. However, this cannot result from immunoglobulin activity of HBe alone although it may point to the presence of anti-HBc antibodies in the test sera. A special difficulty, not mentioned by Arnold (1977), is the presence of anti-HBc antibodies in the serum, and thus in the biopsy itself, which could also bind to the core particles in hepatocytes during the immunofluorescence procedure. During the testing of biopsies for the presence of HBeAg and HBcAg by means of a double immunofluorescence method, some of the core particles could be masked by intrinsic anti-HBc. Therefore the conclusion that HBeAg and HBcAg are not always present simultaneously in the nuclei of hepatocytes is not proven.

CHAPTER X

HEPATITIS B

An electron microscopical study

CHAPTER X

HEPATITIS B

An electron microscopical study

X.1 SUMMARY

Twenty-five liver biopsies randomly selected from a series of 165 liver biopsies from HBsAg seropositive patients were investigated immunohistochemically for the presence of hepatitis B antigens and ultrastructurally for the presence of viral particles. In all biopsies in which HBcAg was demonstrated by immunochemical methods, 23-27 nm particles could be detected in the hepatocytes. These particles were found mainly in the nuclei in large clusters scattered among the nuclear chromatin. In contrast, all HBcAg-negative biopsies lacked 23-27 nm viral particles. In some biopsies scattered hepatocytes were found with extensive accumulations of round and filamentous particles in widened cisternae of the endoplasmic reticulum. The presence of these particles in hepatocytes correlated with the presence of HBsAg-positive ground-glass hepatocytes seen in light microscopy. Ultrastructurally no viral structures could be found that correlated with the HBsAg detected immunohistochemically along the cell membranes of the hepatocytes.

X.2 INTRODUCTION

Ultrastructural studies of liver tissue obtained from HBsAg-seropositive patients have disclosed hepatitis B viral structures and a variety of cytoplasmic and nuclear alterations in hepatocytes and sinusoidal lining cells (Schaffner, 1966; Lapis and Schaff 1979; Yamada, 1982; Phillips, 1981).

The core of the Dane particle, the complete HB virus, was identified ultrastructurally as an uncoated 21 - 25 nm particle, distributed mostly within the nucleus in a diffuse pattern or in large or small aggregations (Huang, 1971; Gerber 1974). Sometimes core particles were found in the cytoplasma of hepatocytes (Huang, 1974). The structural substrate of HBsAg could be identified as characteristic filamentous structures lying within widened cisternae of the proliferating smooth endoplasmatic reticulum (Stein, 1972). In transverse sections the intracisternal filaments appeared as small ring-like structures with a diameter of 35 nm. Sometimes these filamentous structures were found to be

continuous with the outer covering of the coated particles, like the Dane particle in serum (Huang, 1974).

In acute hepatitis B the ultrastructural aspects essentially do not differ from those seen in other types of acute viral hepatitis. Generally the hepatocytes show degenerative-necrotic and regenerative changes simultaneously. Rounded necrotic dark cells with barely identifiable organelles, sometimes with nuclear fragments (Councilman bodies) (Klion and Schaffner, 1966), are seen together with swollen hepatocytes characterized by marked distension of the endoplasmatic reticulum (balloon cells), swollen mitochondria and aspecific degenerative changes in the nucleus (Schaffner, 1966; Ruebner, 1968). Lytic necrosis of ballooning hepatocytes is often preceeded by marked swelling of the vascular pole and bleb formation. Sinusoidal lining cells (Kupffer's cells and endothelial cells) are enlarged and contain lipofuscin, ceroid hemosiderin, bile and necrotic hepatocytic debris. Only in early acute HBV infection can nuclear core particles be found ultrastructurally. The presence of core particles in later stages of acute hepatitis is an indication of on-going HBV infection (transition to chronic hepatitis).

In chronic hepatitis B both intracisternal filamentous HBsAg and nuclear core particles were found, whereas asymptomatic hepatitis B carriers usually exhibited only filamentous HBsAg (Sun, 1974). Structural substrates of membrane-bound HBsAg have not been described.

In this study the presence of viral particles was investigated in 25 of the 165 liver biopsies and compared with the presence of viral antigens detected by means of immunochemical methods.

X.3 RESULTS

The results of the ultrastructural study of the presence of viral particles in liver biopsy material from 25 HBsAg seropositive patients are summarized in table X.1

Table X.1 *Presence of viral particles detected by electron microscopy in 25 liver biopsies from HBsAg-positive patients*

23-27 nm particles (nuclear)	10/25
filamentous/round cytoplasmic particles	8/25
negative	11/25

In 8 of the 25 liver biopsies the cytoplasm of a few scattered hepatocytes contained accumulations of filamentous and round structures in widened cisternae of the smooth endoplasmatic reticulum (fig.X.1). These hepatocytes could be already detected easily in the semi-thin sections by their typical cytoplasmic aspect in the toluidin blue stain (fig.X.2). In all 8 cases that part of the tissue used for light microscopical evaluation showed HBsAg-positive ground-glass hepatocytes. No cytoplasmic viral structures were found in 17 biopsies; in 8 of these 17 cases HBsAg-positive ground-glass cells were found in the part prepared for light microscopy (table X.2).

Table X.2 *Correlation between groundglass cells and filamentous and circular viral structures in the cytoplasm of hepatocytes*

Ground-glass hepatocytes	filamentous/circular particles present	absent
present	8	8
absent	0	9

In 10 of the 25 biopsies large and small clusters of 23-27 nm particles were detected. These particles were localized mainly in the nuclei, between the nuclear chromatin (fig.X.3 and X.4). In all 10 cases HBcAg (and IgG in the same location) were detected immunohistochemically in frozen and formalin-fixed sections of the biopsies. In contrast neither HBcAg nor 23-27 nm particles were detected in the 15 other biopsies.

Table X.3 *Correlation between 23-27 nm nuclear particles and HBcAg in nuclei of hepatocytes*

HBcAg	± 27 nm particles present	absent
present	10	0
absent	0	15

In only 4 biopsies could filamentous structures be found in the cytoplasm together with ± 23-27 nm particles in the nuclei. In only one case were 23-27 nm particles detected in the nucleus of a hepatocyte with filamentous particles in the cytoplasm.

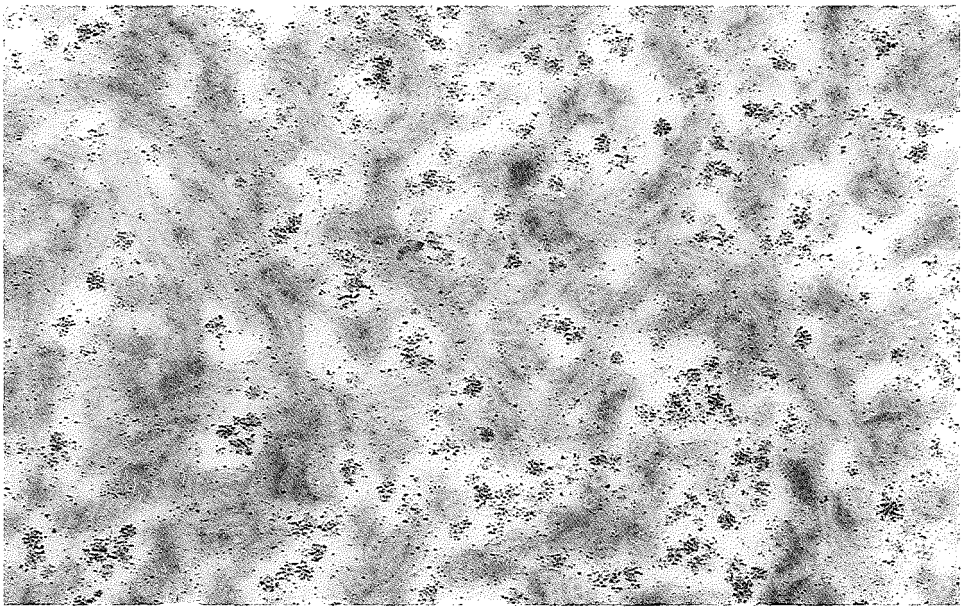
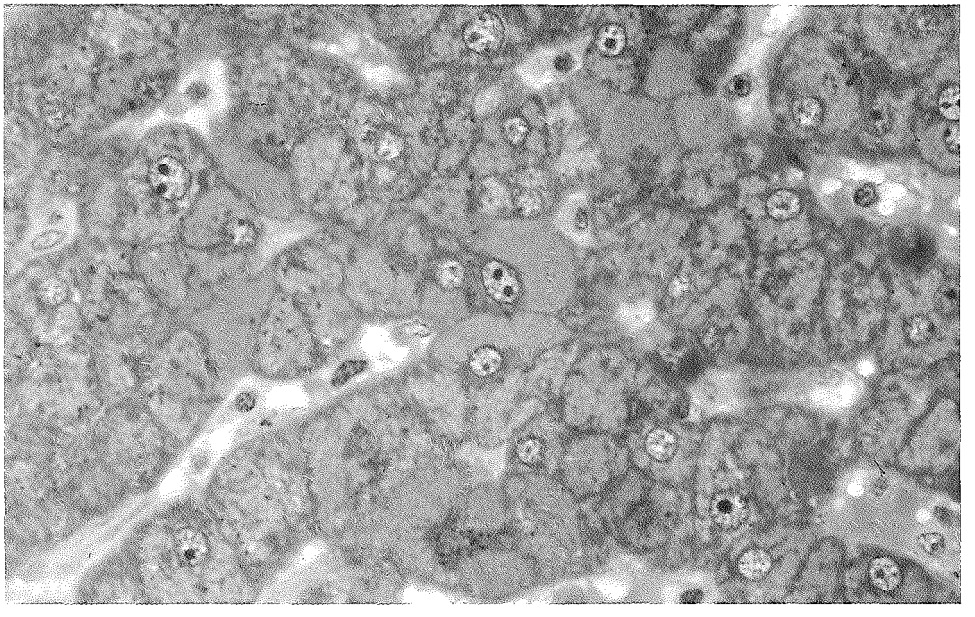


Fig.X.1 *Hepatocyte with many filamentous structures within dilated cisternae of proliferated smooth endoplasmic reticulum. In cross sections the endoplasmic reticulum is seen to contain one or more of these filaments (52.000x)*

Fig.X.2 *Semi-thin section of epon-embedded liver tissue, showing many hepatocytes with faint staining homogeneous cytoplasm, corresponding to the accumulation of HBsAg-related filamentous structures (toluïdin blue, 150x)*



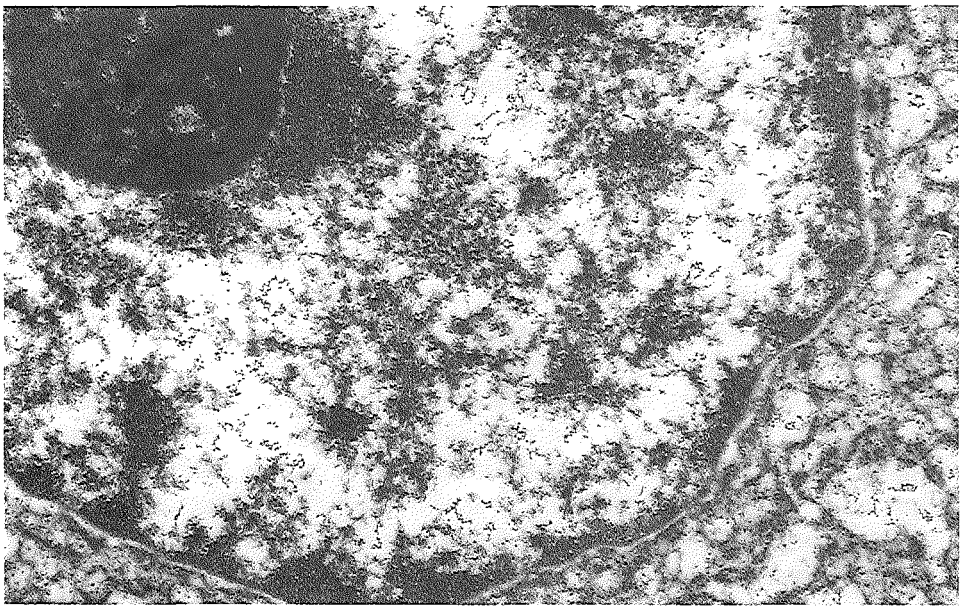
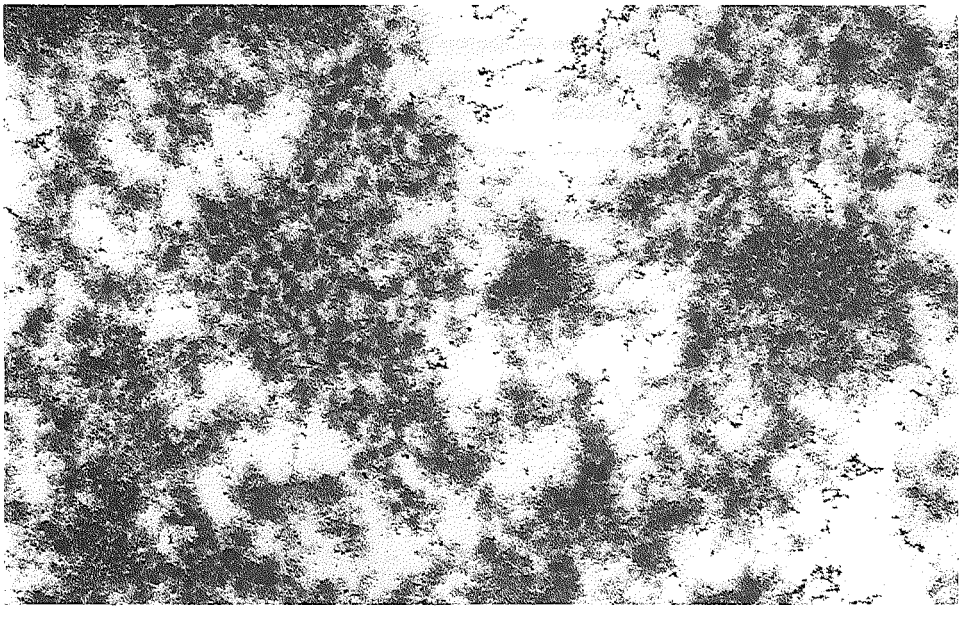


Fig.X.3 *Electron micrograph of the nucleus of a hepatocyte, showing clusters of loosely arranged uncoated 18-27 nm core-particles (22.000x)*

Fig.X.4 *Electron micrograph of clusters of core particles within the nucleus of a hepatocyte (52.000x)*



X.4 DISCUSSION

As described by several authors (Stein, 1972; Huang, 1974; Yamada, 1977; Lapis, 1979), viral particles can be found in liver cells from HBsAg seropositive patients in the nucleus, as ± 23 nm particles, as well as the cytoplasm. Most cytoplasmic viral structures are detected in widened cisternae of the rough and smooth endoplasmic reticulum. These particles are round or filamentous, the length of which can vary, and structurally are comparable to the round and filamentous particles found in the sera of HBsAg seropositive patients. Only HBsAg antigenic determinants, representing non-infectious surplus viral coat material, are present on these particles.

The results of this study are in accordance with these earlier studies. In contrast to some other studies 23-27 nm particles were not seen in the cytoplasm, probably as a result of selection of the investigated material. The main purpose of this study was to determine the sensitivity of the ultrastructural detection method for hepatitis B viral infections. Because no structural substance is known for cell membrane-bound HBsAg, which is the most sensitive immunochemical sign of a chronic hepatitis B infection (see chapter VI), only 14 of the 25 biopsies exhibited ultrastructural signs of hepatitis B viral infection. The ultrastructural method for detection of core particles was not better and much more complicated than immunohistochemical methods for detection of HBcAg antigen. Moreover, by immunoelectron microscopy, HBcAg determinants were displayed not only by the core particles but also in nuclear and cytoplasmic ground substance (Yamada, 1982). In only half of the biopsies was there a correlation between the presence of intracytoplasmic viral structures as demonstrated by electron microscopy, and intracytoplasmic HBsAg as demonstrated by immunohistochemical methods. The discrepancy could be explained by tissue sampling. It is concluded that for the detection and diagnosis of hepatitis B infections, ultrastructural studies of liver tissue are not a valuable tool.

CHAPTER XI

COURSE OF HEPATITIS B AND DELTA INFECTION IN LIVERS, AUXILIARY TRANSPLANTED IN HEPATITIS B POSITIVE PATIENTS

A light microscopic and immunochemical study

CHAPTER XI

COURSE OF HEPATITIS B AND DELTA INFECTION IN LIVERS, AUXILIARY TRANSPLANTED IN HEPATITIS B POSITIVE PATIENTS

A light microscopic and immunochemical study

XI.1 SUMMARY

Four patients who received a heterotopic auxiliary liver graft for decompensated liver cirrhosis due to hepatitis B, associated in two cases with hepatitis delta virus(HDV) superinfection, were studied. The consecutive appearance of hepatitis B and D antigens in the grafts was investigated in serial liver biopsies by immuno-histochemical methods and compared with the viral antigenic profiles in the host livers. The histological changes in the livers were studied in relation to the viral expression patterns.

One week after transplantation HBsAg could already be detected in two grafts; this was accompanied by a minimal amount of HBcAg in the graft of the only patient with HBcAg in the host liver. HDAg was detectable in both patients with HDV cirrhosis; in one case HDAg was present in the absence of HBsAg. In the three-week graft biopsy HBsAg was observed in a linear pattern along the cell membranes of all hepatocytes in all patients. This HBsAg pattern was associated with nuclear and especially cytoplasmic localization of HBcAg in two patients and nuclear and to a lesser extent cytoplasmic localization of delta antigen in the other two. At three months, widespread delta expression with extensive coexpression of HBcAg was observed in the grafts of the two HDV positive patients.

All patients developed a mild lobular hepatitis, histologically demonstrated between the 47th and 107th posttransplantation day. In the two HBV-positive, HDV-negative patients the hepatitis B infection induced cirrhotic transformation in the graft within one year. In the HDV-positive patients the hepatitis B and D infection persisted, with transition of the acute hepatitis to a mild chronic active hepatitis with slight or moderate fibrosis after one year.

It is concluded that HBV and HDV infection in livers, auxiliary transplanted in HBV or HDV positive patients is demonstrable by immunological methods within 1-3 weeks after transplantation; HBV infection in liver grafts appears to be a rapidly progressive disease; coinfection with HDV does not aggravate the acute hepatitis and may even suppress the aggressiveness of the hepatitis B infection.

There is evidence that liver cell damage may be due to direct cytopathic effects of HBV while cytopathic effects of HDV plays a minor role. It should be emphasized, however, that these observations are made in effectively immunosuppressed patients.

XI.2 INTRODUCTION

Liver transplantation has become a feasible form of treatment for patients with acute and chronic liver failure of various etiologies.

Cirrhosis due to hepatitis B is one of the generic indications for liver transplantation (Corman, 1970; Maddrey, 1988). A complication of liver transplantation for chronic hepatitis B is the frequent recurrence of hepatitis B or D in the transplanted liver, despite passive immunization or antiviral therapy (Demetris, 1986; Portman, 1986; Lauchart, 1987; van Thiel, 1987; Rizzetto, 1987; Samuel, 1988). The clinical implications of recurrence of the hepatitis B or D infection are not well known since preliminary reports range from excellent tolerance (Lauchart, 1987; Samuel, 1988) to an aggressive disease in which histological cirrhosis develops within less than a year (van Thiel, 1987).

A program for auxiliary partial liver transplantation was started in our hospital in October, 1986 (Terpstra et al). Five chronic hepatitis B patients with decompensated cirrhosis received a heterotopic liver graft, four of whom survived beyond 1 month. This transplantation technique is such that the host liver and thus the source of the hepatitis B infection remains in situ; therefore serological viral parameters cannot be used to monitor viral events in the grafted livers.

In this prospective study, we used serial liver biopsies to assess the evolution of hepatitis B infection in the auxiliary transplanted livers. For this purpose the various markers of hepatitis B and D infection as well as the accompanying histological changes in the liver tissue were investigated. The mechanisms of liver damage due to the viral infection will be discussed.

XI.3 PATIENTS AND METHODS

XI.3.1 PATIENTS

Pretransplant demografic data, liver disease and viral HBV and HDV markers are given in table XI.1. Two patients (II and III) had an HDV superinfection. One of the other two showed signs of active viral replication (IV), whereas the other (I) did not.

Table XI.1 *Pretransplant demographic data, liver disease and viral HBV and HDV markers*

Patient	age/ sex	pretransplant liver disease	viral markers liver HBs	HBc	HDAg	serum HBs	HBe	DNAp	aHD
I	36/M	cirrhosis (micro) + CAH moderate hepato-cellular atypia	+	-	-	+	-	-	-
II	41/M	cirrhosis (macro) + CAH severe hepato-cellular atypia	+	-	+	+	-	-	+
III	32/M	cirrhosis (macro) + CAH moderate hepato-cellular atypia	+	-	+	+	-	-	+
IV	51/M	cirrhosis (macro) + CAH moderate hepato-cellular atypia	+	+	-	+	+	+	-

DNAp = DNA-polymerase CAH = chronic active hepatitis

Patient I

A 35 year old, HBs- and HBe-seropositive male with a liver cirrhosis was admitted twice for hepatic coma and intractable ascites in 1985. One year before transplantation HBeAg and HBV-DNA polymerase became negative. The pre-operative liver biopsy then revealed a macronodular cirrhosis with signs of a mild chronic active hepatitis component and moderate hepatocellular atypia. HBsAg was found in a linear pattern along hepatocyte cell membranes; HBcAg, HBeAg and HDAg could not be detected.

Patient II

A 40 year old male suffered from a decompensated liver cirrhosis due to a chronic hepatitis B and D infection. Intractable ascites and muscle wasting were the

indication for liver transplantation. The serum was and remained positive for HBsAg, anti-HBe and anti-HD; HBeAg, and HBV-DNA polymerase could not be detected. A preoperative liver biopsy showed a cirrhosis with a moderate chronic active hepatitis component. Focally moderate atypia of hepatocytes was seen. HBsAg was present in a diffuse pattern along the cell membranes of the hepatocytes. HDAg was found in about 5 per cent of the hepatocytes; HBcAg and HBeAg could not be detected.

Patient III

A 32 year old male was admitted for ascites. Liver cirrhosis due to chronic hepatitis B with D superinfection was diagnosed. Persistent ascites associated with continued inflammation was the indication for liver transplantation. Serum was positive for HBsAg, anti-HBe and anti-HD, negative for HBeAg and HBV-DNA polymerase. Liver biopsy showed macronodulair cirrhosis with moderate atypia of hepatocytes and a mild chronic active hepatitis. HBsAg was detected both along cell membranes and in the cytoplasm of the hepatocytes. In a sporadical hepatocyte delta antigen was detected in the nucleus.

Patient IV

A 52 year old male, with intractable ascites was found to be HBsAg positive 15 months prior to transplantation. Additional serum tests were positive for HBeAg, HBV-DNA polymerase and HBV-DNA, and remained so till the day of transplantation. A liver biopsy showed a macronodular cirrhosis with a moderate chronic active hepatitis. Focally the liver parenchyma showed a moderate hepatocellular atypia. HBsAg was found in a linear pattern along the cell membranes of all hepatocytes in all nodules and in the cytoplasma of a few hepatocytes. HBcAg was found in more than 50 per cent of the hepatocytes in the nuclei and focally also in the cytoplasm; HDAg was not detectable.

XI.3.2 Liver transplantation

All four patients received a heterotopic auxiliary partial liver transplant, as described by Terpstra (1988). The liver grafts were reduced in size by segment resection of the left lobe. Transplantation was performed with both arterial and portal inflow and venous drainage throughout the suprahepatic vena cava of the graft into the recipient vena cava as craniad as possible. Biliary drainage was by choledochojejunostomy with a Roux-and-Y loop.

XI.3.3 IMMUNOSUPPRESSIVE REGIMEN

Prednisolone (25 mg) was administered intravenously prior to surgery and every 6 hours during the first 24 hours; it was then tapered off to a maintenance dose of 15 mg orally on the 14th postoperative day. Equine antilymphocyte globulin (Institute Mérieux, Lyon, France; 425 lymphocytotoxic units/kg/day) and azathioprine (1 mg/kg i.v.) were given untill the 7th postoperative day. Cyclosporine A was started on the 6th postoperative day in a dosage of 3 mg/kg/24 h via a continuous intravenous infusion and adjusted to plasma levels of 200 ng/l (conventional radioimmunoassay, Sandoz, Basle). After withdrawal of the biliary drainage catheter on the 10th postoperative day, cyclosporine A was administered orally in a dosage of 4 times the daily intravenous dose, divided into 2 daily portions and adjusted to trough plasma levels of 100-200 ng/l.

XI.3.4 LIVER BIOPSIES

Needle (Tru-Cut R) biopsies of the transplanted livers were taken according to protocol, 30 minutes after implantation of the graft and one week, three weeks, three months, six months and one year after transplantation. At the time of transplantation a needle biopsy of the recipient liver was also taken. Additional biopsies were only obtained in the event of clinical or biochemical signs of graft dysfunction. Light microscopical and immunohistochemical procedures are carried out as described in chapter III. An overview of the immunohistochemical techniques and the antibodies used, are given in table XI.2.

Table XI.2 *Antibodies and techniques used for the immunohistochemical studies on liver biopsies*

Antigens	Method	Antibody	Source
HBsAg	dIF	Pab	C.L.B.
HBsAg	iIF/iIP	Mab	Organon
HBcAg	iIF/iIP	Mab	Organon
HBeAg	iIF/iIP	Mab	Thomas
HDV	dIF/dIP	Mab	Rizzetto
IgG	dIF	Mab	Kallestad

dIF: direct fluorescence method
iIF: indirect fluorescence method
Pab: polyclonal antibody
dIP: direct immunoperoxidase method
iIP: indirect immunoperoxidase method
Mab: monoclonal antibody

HBsAg antibodies were tested against positive and negative controls. The monoclonal anti-HBe antibody was negative for anti-HBc activity in the standard radioimmunoassay (Corab, Abbott, U.S.A.). Similarly the monoclonal anti-HBc antibody was negative in the radioimmunoassay for anti-HBe activity (Abbott, U.S.A.). Non-immune serum was substituted for the primary antibody to obtain a negative control for each staining procedure; no staining occurred.

Liver tissue from seropositive patients with chronic hepatitis B and D was used as the positive control.

XI.4 RESULTS

XI.4.1 RECIPIENT LIVER BIOPSIES

Biopsies taken from the recipient's liver during the auxiliary liver transplantation procedure showed a mixed micro/macro-nodular cirrhosis combined with a chronic active hepatitis component in all cases. In all four livers moderate to severe atypia was seen in the hepatocytes in some parenchymal nodules.

Immunohistochemical studies on frozen liver tissue revealed HBsAg expression along the membranes of nearly all hepatocytes in the liver tissue of all four biopsies; in three of the four patients (patients I, II, IV) the liver biopsies revealed also intracytoplasmic HBsAg expression in some nodules.

HDAg could be detected in about 5 per cent of the hepatocytic nuclei in the liver tissue of two patients (patients II, III). Neither HBcAg nor HBeAg could be demonstrated in either of these biopsies. One biopsy (patient IV) contained HBcAg and HBeAg, mainly in the cytoplasm but also in the nuclei of the hepatocytes. HBcAg, HBeAg and HDAg were not detected in the biopsy from patient I.

XI.4.2 30-MINUTE BIOPSIES

The biopsies taken from the liver graft 30 minutes after recirculation showed only minor light microscopic changes. HBsAg, HBcAg, HBeAg and HDAg could not be detected in any of these biopsies.

XI.4.3 ONE-WEEK BIOPSIES

The one-week protocolled biopsies were actually taken on the fifth, seventh, eight and ninth days after transplantation.

Light microscopically all biopsies showed an acute cholangiolitis with moderate centrilobular hepatocellular and canalicular cholestasis in all cases. Only mild

liver cell necrosis with scattered acidophil bodies was present; characteristic signs of acute rejection (Snover 1984) were not found.

Hepatitis B surface antigens could be detected in two of the four biopsies (patients III and IV, fig.XI.1). Only patient I, who exhibited no signs of active viral replication before transplantation, did not test positive for any of the hepatitis B or hepatitis D antigens.

In patients II and III, who were positive for HDAg prior to transplantation, HDAg was already present in 50 and 1 per cent of the hepatocytic nuclei, respectively, one week after transplantation; HBcAg was not detected in either biopsy. In the biopsy from patient IV, who exhibited active hepatitis B replication prior to transplantation, a weak nuclear HBcAg fluorescence was detected in a sporadic hepatocyte.

IgG deposits were detected in the biopsy, which revealed HDV in 50 per cent of the nuclei, in an identical pattern and localization of the HDV antigen as result of binding of intrinsic HDV antibodies to HDV antigen in the liver tissue.

XI.4.4 THREE-WEEK BIOPSIES

The three-week biopsy of the protocol was taken on days 20, 20, 21 and 24, respectively.

In all cases a mild acute cholangiolitis was still present, together with a mild lobular cholestasis. In one biopsy round cell infiltration of some bile ductules was consistent with the development of grade I graft rejection (patient II).

All biopsies revealed viral antigens. HBsAg was detected in a linear pattern along the cell membranes of all hepatocytes in all four liver grafts. In patients II and III this HBsAg pattern was combined with nuclear accumulation of delta antigen; HBcAg could not be detected in these cases. In the other two cases (patients I and IV) HBsAg occurred in combination with HBcAg and HBeAg, localized in the nucleus of a sporadic hepatocyte in patient I and in the cytoplasm of about 40 per cent of the hepatocytes in patient IV (fig.XI.1).

XI.4.5 THREE-MONTH BIOPSIES

Histologically three out of four biopsies were characterized by a lobular (acute) hepatitis with necrosis of many scattered mainly centrilobular hepatocytes. In one biopsy (patient I) only minimal changes were observed. None of the biopsies, however, contained ground-glass hepatocytes. In all four biopsies a bright linear cell membrane-bound HBsAg fluorescence was seen (fig.VI.6 page 145), accompanied in two cases (patients I and IV) by mild cytoplasmic staining (fig.XI.1).

In the two HDV-positive patients HDAg was detected in about 50 per cent of

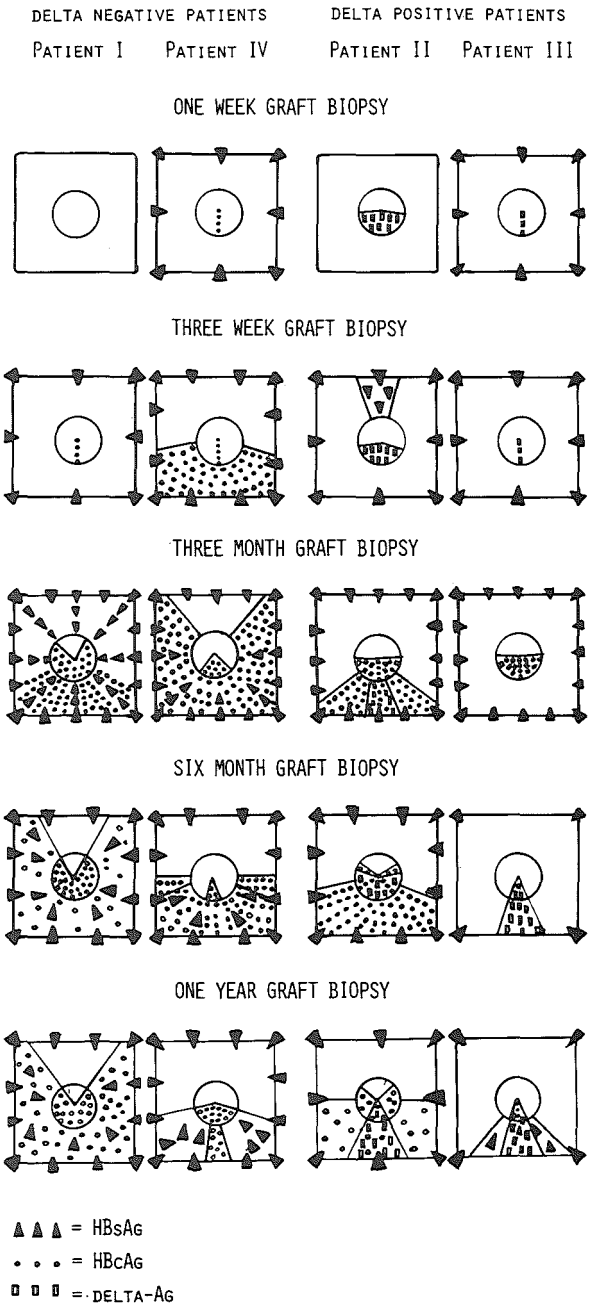


Fig.XI.1 *Schematic drawing of HBV and HDV expression patterns in serial liver biopsies from livers, transplanted in two HBV-positive (I and IV) and two HDV-positive (II and III) patients.*

the hepatocytes, mainly in the nuclei. HDAg expression was combined with HBcAg expression in both the nucleus and the cytoplasm in patient II and only in the nucleus in patient III (fig.XI.1 and XI.2).

The two HBV-positive, HDV-negative patients showed HBcAg and HBeAg expression in more than half of the hepatocytes, partly in the nucleus and partly in the cytoplasm.

XI.4.6 Six-month biopsies

The biopsies of the HBV-positive, HDV-negative patients showed an ongoing acute hepatitis, with focal and extensive centrilobular (patient I) hepatocyte necrosis (fig.XI.3). In patient IV this acute (lobular) hepatitis was combined with periportal hepatitis, indicating a transition to chronic hepatitis. Biopsies from the HDV-positive patients revealed a transition from acute hepatitis to mild chronic active hepatitis in patient II and a mild cholangiolitis in patient III. The biopsies of the HBV-positive, HDV-negative patients exhibited both mild and severe fibrotic changes, whereas the HDV-positive patients showed only minimal or mild fibrosis (table XI.3).

In all biopsies linear HBsAg deposits were found along the cell membranes of all hepatocytes. In the biopsies of patient I and IV, this pattern was combined with intracytoplasmic HBsAg expression. In the HBV-positive, HDV-negative patients HBcAg and HBeAg were detected in more than 50 per cent of the hepatocytes, in the nucleus but also in the cytoplasm. The biopsies from the HDV-positive patients revealed HDAg together with nuclear and cytoplasmic (patient II) HBcAg and HBeAg. HDAg was present in 50 per cent of the hepatocytes but restricted to the nucleus in patient II; in patient III HDAg expression was found in only a small percentage of the hepatocytes, both in nucleus and cytoplasm (fig.XI.4).

IgG was demonstrated in sections of the frozen liver tissue in a distribution pattern identical to HBcAg and/or HDAg, and due to in vitro binding of intrinsic anti-HBc and anti-HDV antibodies to their complementory antigens.

XI.4.7 One-year biopsies

The biopsies from both HBV-positive, HDV-negative patients (I and IV) showed severe fibrosis with cirrhotic transformation of the hepatic parenchyma (table XI.3. and fig.XI.5 and XI.6). The fibrous septa contained only an inconspicuous aspecific inflammatory infiltrate. In the parenchymal nodules most hepatocytes were enlarged due to the abundant finely granular cytoplasm, that reacted faintly positively to HBsAg antibodies. HBsAg-positive "ground-glass" hepatocytes were present sporadically in patient I and in abundance in patient IV. There was exten

sive HBcAg and HBeAg expression located in the nucleus as well as the cytoplasm of about 80 per cent of the hepatocytes in patient I and mainly in the nucleus of about 40 per cent of the hepatocytes in patient IV. This nuclear localization of HBcAg and HBeAg in patient IV is in contrast to the mainly cytoplasmic localization in the six-month biopsy (fig.XI.1).

In contrast to the cirrhotic transformation found for both HBV-positive, HDV-negative patients, only a minimal or mild fibrosis was seen in the HDV-positive patients (fig.XI.7 and XI.8). Both HDV-positive patients exhibited signs of a mild chronic active hepatitis (fig.XI.9). Ground-glass hepatocytes could not be detected. HBsAg was found along the cell membrane of the hepatocytes and not in the cytoplasm.

For patient III the numbers of HBeAg- and HDAg-positive cells and the antigen distribution patterns were essentially the same as in the six-month biopsy. In patient II the number of HDAg-positive hepatocytes had clearly diminished, while the HBcAg and HBeAg expression remained constant (fig.XI.1).

Table XI.3 *Evolution of liver histology in serial liver biopsies from four liver grafts*

	HBV-positive patients				HDV-positive patients			
	Patient I		Patient IV		Patient II		Patient III	
	fibrosis	hepatitis	fibrosis	hepatitis	fibrosis	hepatitis	fibrosis	hepatitis
one week	-	-	-	-	-	-	-	-
three weeks	-	-	-	-	+	-	-	-
three months	-	AH	+	AH	+	AH	+	-
six months	+	AH	+++	CAH	+	CAH	+/-	CAH
one year	++++	CAH	++++	CAH	+	CAH	+/-	CAH

AH = acute hepatitis
\+ = mild fibrosis
+++ = severe fibrosis
CAH = chronic active hepatitis
++ = moderate fibrosis
++++ = cirrhosis

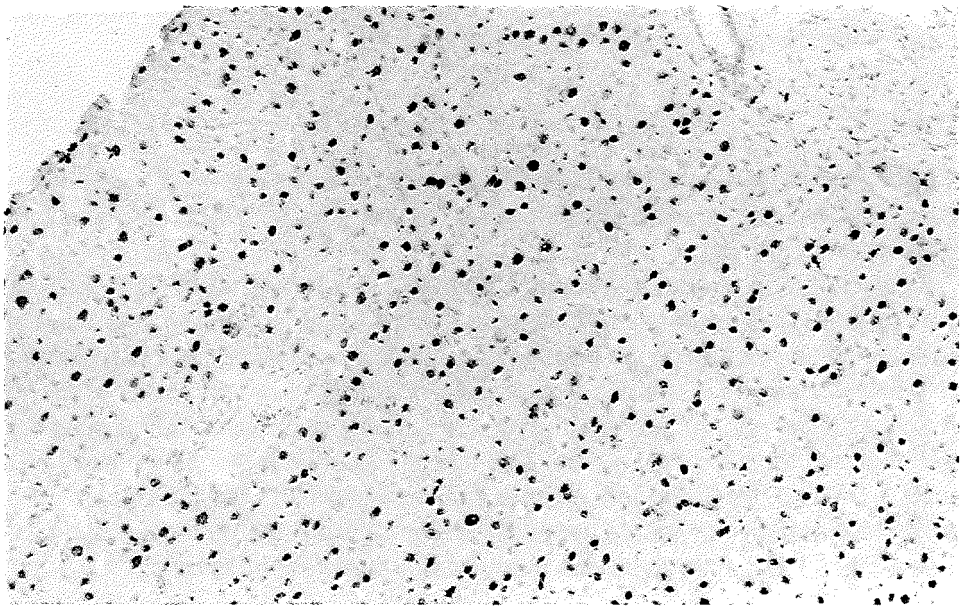
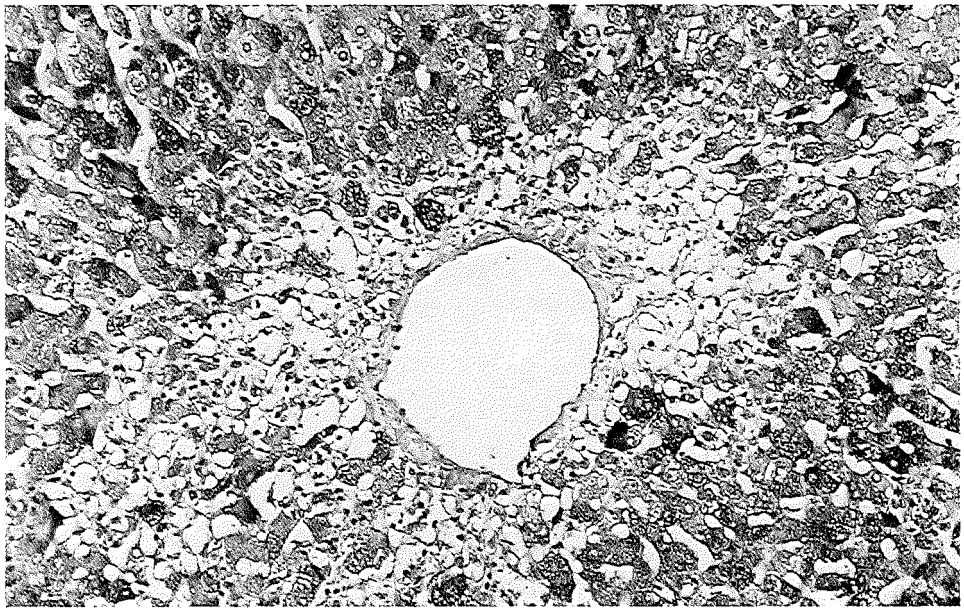


Fig.XI.2 *Three-month biopsy from HDV-positive patient III: more than half of the hepatocytes reveal HDV mainly in the nuclei (anti-HDV; direct peroxidase method, 120x)*

Fig.XI.3 *Six-month biopsy from HBV-positive, HDV-negative patient I. Extensive centrilobular necrosis of hepatocytes. Only slight inflammatory infiltrate present (PAS, 150x.)*



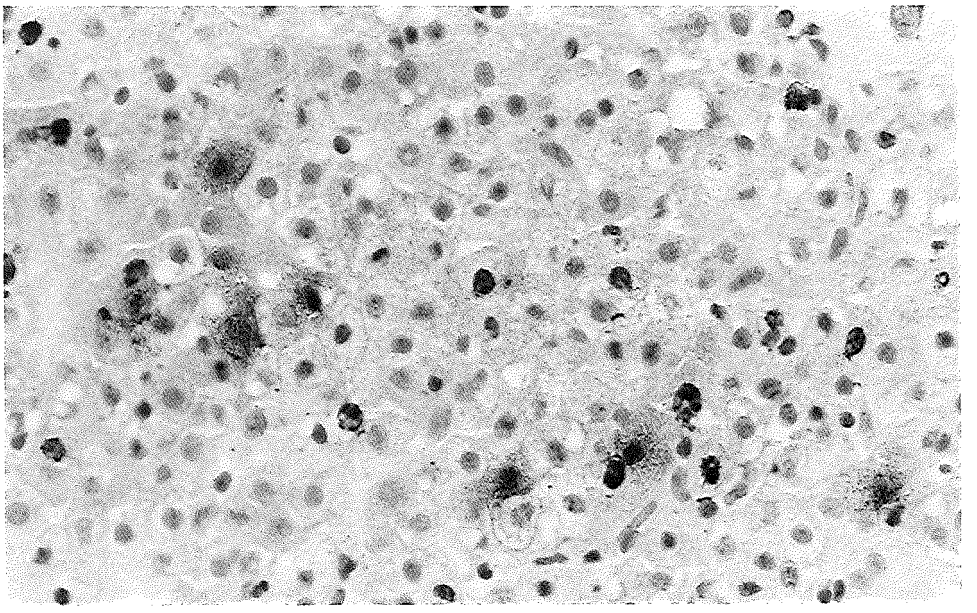
With the same localization and distribution pattern of HBcAg and HDAg, IgG was detected in sections of all frozen liver biopsies.

XI.4.8 INCIDENTAL BIOPSIES

Because of a rise in the bilirubin and/ or serum aminotransferase levels of unknown cause, four non-protocol biopsies were obtained from three of the four patients (II, III and IV).

A 65-day biopsy from HDV-positive patient II showed histological signs of a mild (grade I) rejection based on a mild cholangiolitis combined with venous endothelialitis. In addition the hepatic parenchyma contained several scattered acidophil (Councilman) bodies. Beside HDV-antigen, this biopsy showed HBcAg in about 10 per cent of the hepatocytes, mainly in the nucleus, but sometimes also in the cytoplasm. HDV-positive patient III underwent biopsies on days 51 and 69. On day 51 the mild light microscopic changes in the hepatic lobules were consistent with an early acute hepatitis, whereas on day 69 the picture had progressed to a lobular hepatitis with many scattered acidophil bodies.

Fig.XI.4 *Six-month biopsy from HDV-positive patient III: scattered hepatocytes exhibit HDV-antigen, both in the nucleus and in cytoplasm (anti-HDV antibody; direct immunoperoxidase method, 380x)*



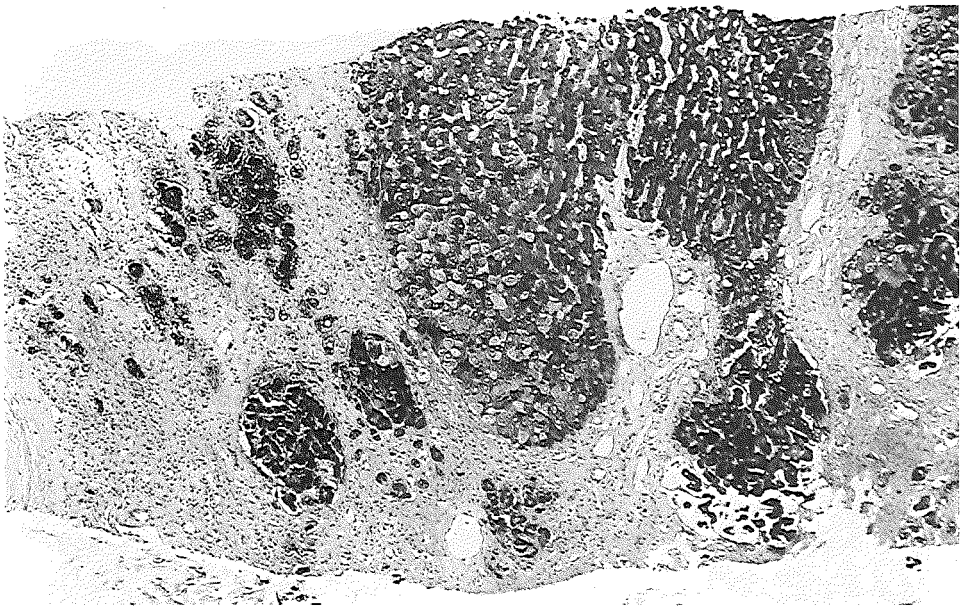
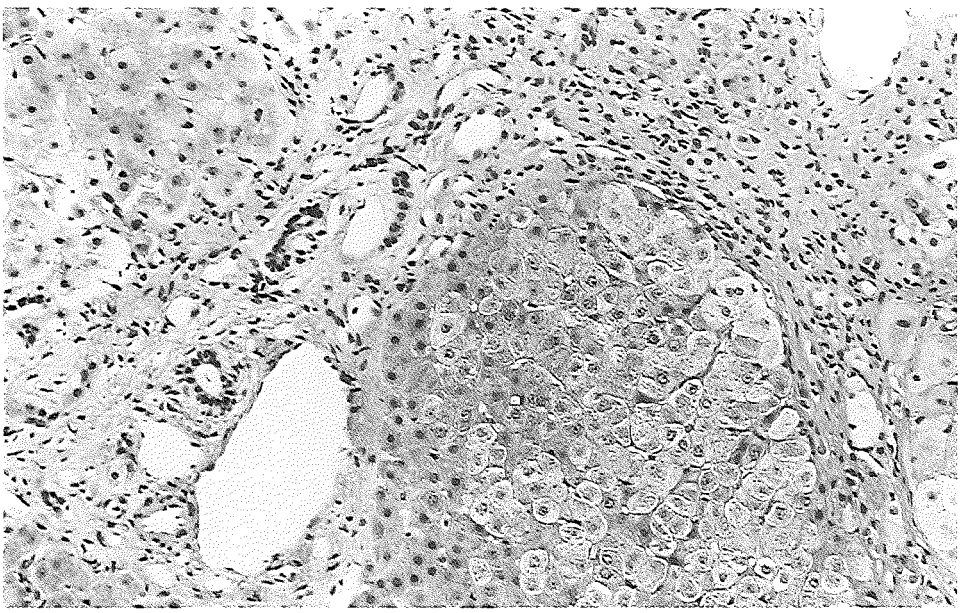


Fig.XI.5 *One-year biopsy from HBV-positive, HDV-negative patient IV. Cirrhotic transformation of the liver parenchyma (PAS 60x).*

Fig.XI.6 *One year biopsy of HBV-positive, HDV-negative patient IV. High power field: only minimal infiltrate in the fibrous septa. In the liver nodule (left) many hepatocytes with ground-glass aspect (HAS, 150x).*



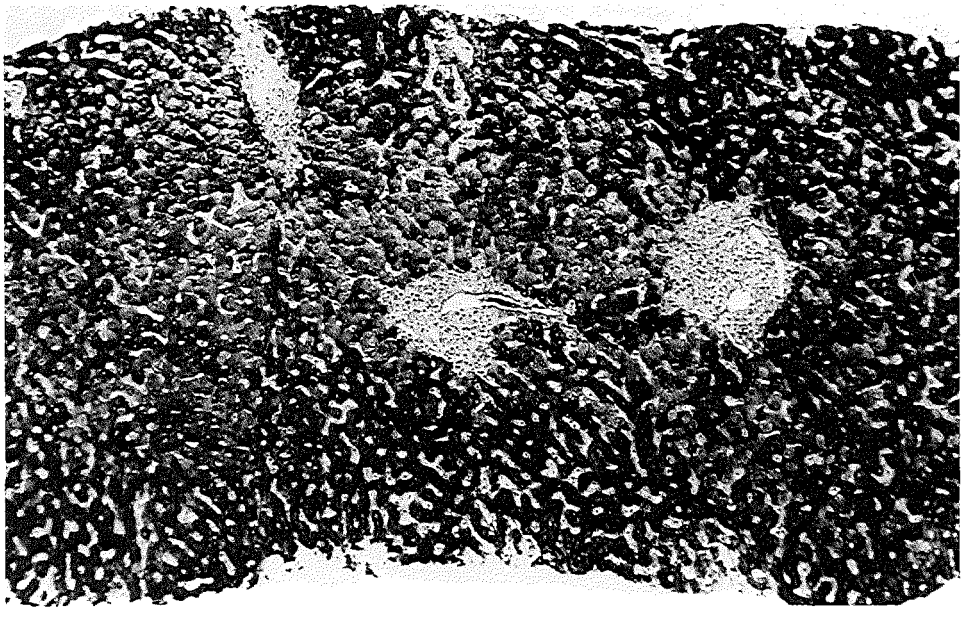
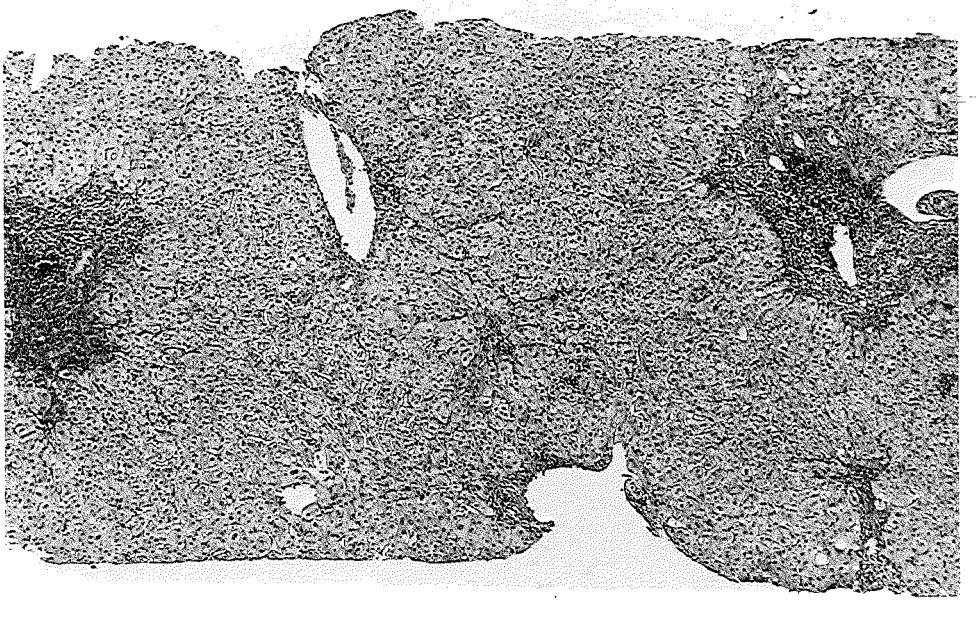


Fig.XI.7 *One year biopsy from HDV-positive patient III. Intact liver architecture. Only slightly enlarged portal tracts (PAS, 60x).*

Fig.XI.8 *One year biopsy from HBV-positive, HDV-positive patient II. Intact architecture; irregular enlarged portal tracts (mild periportal fibrosis) (HA, 60x).*



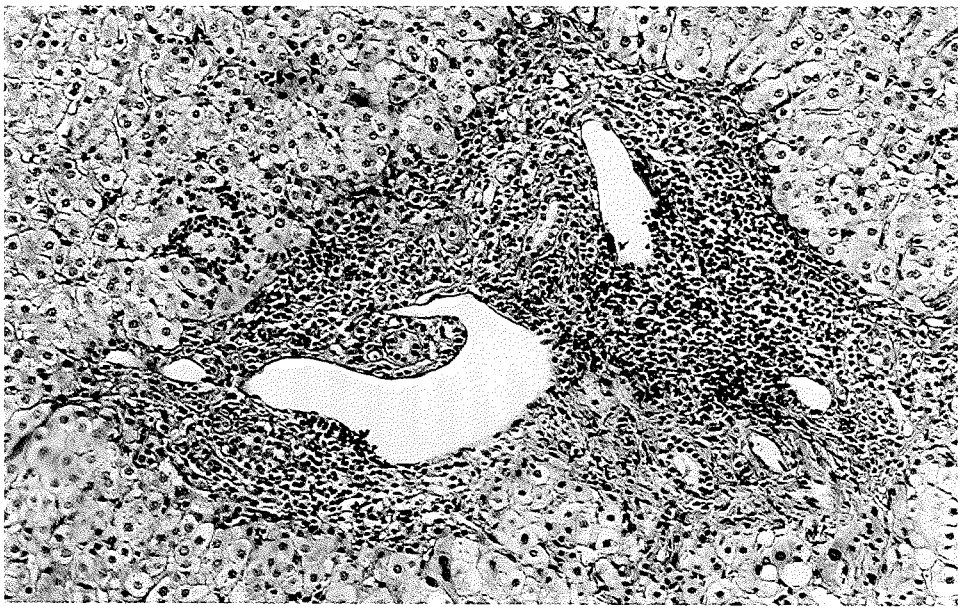


Fig.XI.9 *One year biopsy from HBV-positive, HDV-positive patient II: periportal hepatitis: roundcell infiltrate extending into the liver parenchyma (HA, 150x).*

On day 51 nuclear HDV expression in about 70 per cent of the hepatocytes was combined with nuclear and cytoplasmic localization of HBcAg and HBeAg in about 30 per cent of the hepatocytes. On day 69 HBcAg had increased and was present in the cytoplasm of about 40 per cent and the nucleus of about 70 per cent of the hepatocytes. HDV was slightly diminished, being present in about 50 per cent of the hepatocyte nuclei. A 47-day biopsy from patient IV showed not only cholangiolitis but also a mild lobular hepatitis component. HBsAg appeared as a bright fluorescence pattern along the cell membranes of the hepatocytes and occasionally in the cytoplasm.

XI.4.9 Relation between serum transaminase levels, histology and viral antigens in liver tissue

In fig.XI.10,11,12 and 13 liver transaminase levels in the four patients are given in relation to the time of the post-transplantation biopsies and the presence of viral antigens in the biopsies. Despite the presence of HBsAg along the cell membranes of the hepatocytes and HDV-antigen (patient II and III) in the one-week and three-week biopsies, transaminase levels are not elevated. Simultaneously with the appearance of HBcAg in the liver tissue, transaminase levels are elevated.

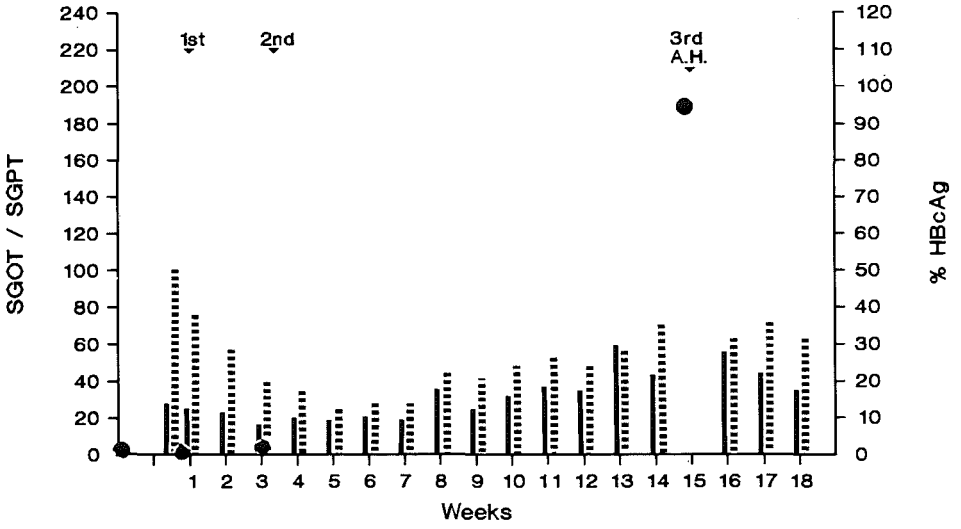
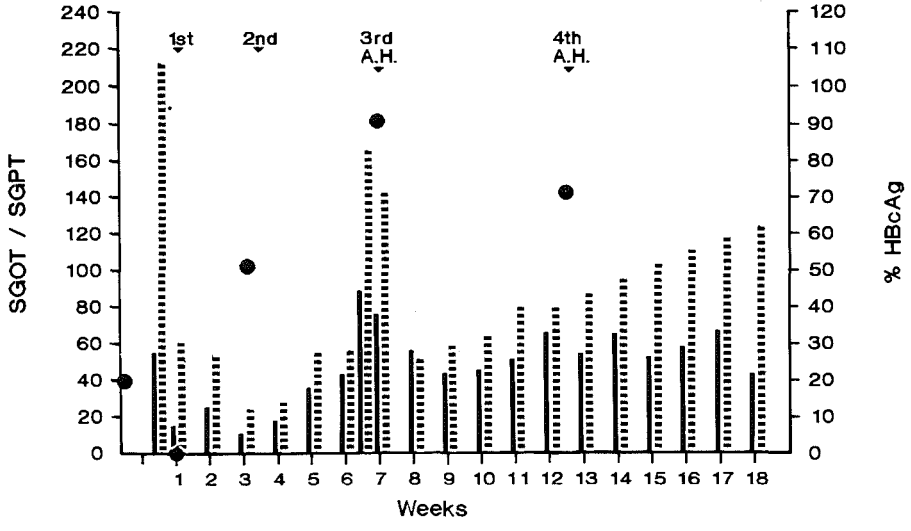


Fig.XI.10 *HBV-positive patient I: posttransplantation levels of SGOT and SGPT, related to the time of the biopsies and the viral antigens, present in the biopsies.* ● = *HBcAg*

Fig.XI.11 *HBV-positive patient IV: posttransplantation levels of SGOT and SGPT, related to the time of the biopsies and the viral antigens, present in the biopsies.* ● = *HBcAg*



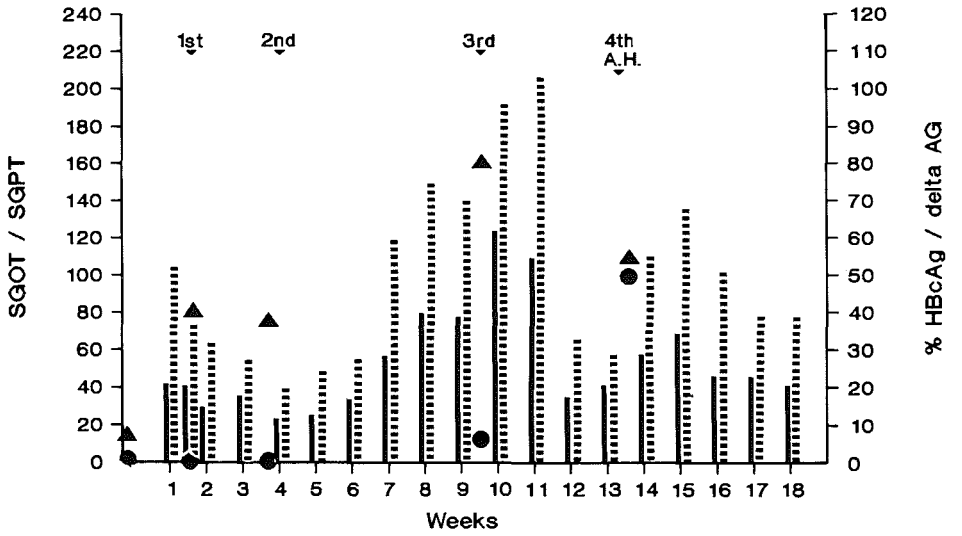
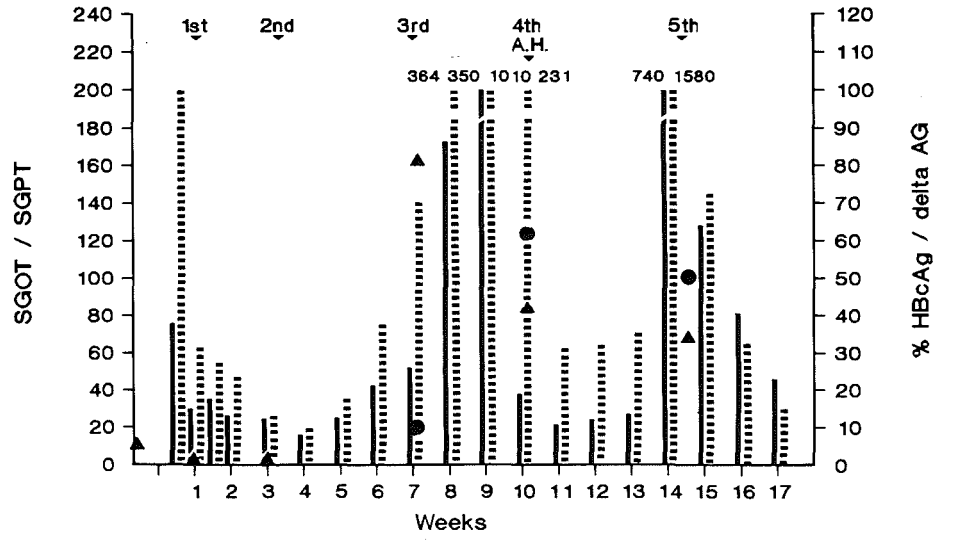


Fig.XI.12 *HDV-positive patient II: posttransplantation serum levels of SGOT and SGPT, related to the time of the biopsies and the viral antigens in these biopsies.* ●= *HBcAg* ▲= *delta antigen*

Fig.XI.13 *HDV-positive patient III: posttransplantation levels of SGOT and SGPT, related to the time of the biopsies and the viral antigens in these biopsies.*
●= *HBcAg* ▲ = *delta antigen*



XI.5 DISCUSSION

Within the framework of an auxiliary partial liver transplant (A.P.L.T.) program five HBsAg-positive patients with decompensated cirrhosis underwent transplantation. Four out of the five survived for more than one month and were followed for at least one year; serial liver biopsies of the grafts were taken and special attention was directed toward appearance and evolution of the viral infection as well as the accompanying histological changes in the grafts.

At the time of transplantation one patient exhibited signs of active HBV replication, as indicated by positive HBeAg and DNA-polymerase activity in the serum. The other three patients showed no signs of active HBV replication, although two had an active HDV superinfection.

After transplantation the liver grafts of all four patients exhibits signs of active HBV infection together with active HDV infection in the two HDV-positive patients. The infection of the graft in the HBV-positive patient with signs of active viral replication could be expected on forehand. The infection of the liver grafts by HBV, however, in the HBeAg-negative patient and by both HBV and HDV in the HDV-positive patients is not self-evident, but consistent with the findings after orthotopic liver transplantation. In these latter patients "dormant" HBV is presumably reactivated. In three of the four patients viral infection of the graft was already apparent in the one-week biopsy. For the HBV-positive, HBeAg-negative patient (case I), however, the graft infection could not be demonstrated until the three-week biopsy. This difference in time point of graft infection may be explained by the time necessary for reactivation of the silent HBV infection. In one of the HDV-positive patients viral graft infection was demonstrated only by the presence of HD viral antigen in 40 per cent of the hepatocytic nuclei without concomitant HBV markers.

One of the early signs of an HBV or HDV infection of the liver grafts comprise localization of HBsAg along the cell membrane of all hepatocytes. This early diffuse HBsAg expression along the cell membrane of the hepatocytes may be explained by different mechanisms. Firstly, HBsAg circulating in the serum of the patient binds immediately after transplantation to specific receptors on the cell surface of the hepatocytes. In support of this possibility specific binding of HBsAg to the liver cell surface has been shown to occur intermediated by poly-albumine and is assumed to be the first step in the penetration and infection of hepatocytes (Thung, 1984; Gerber, 1985; Wright, 1987). This specific binding of viral antigens to antigens on the cell surface is also demonstrated to play a role by other viral infections (Philipson, 1981). The difference in the time of appearance of HBsAg in the HBeAg positive and in the HBe-negative patient might then be explained by higher HBsAg levels in the HBeAg-positive than in the HBeAg-negative patient.

Another more acceptable explanation for the early appearence of the diffuse HBsAg expression is a nearly simultaneous HBsAg production in all hepatocytes following infection of the hepatocytes by the hepatitis B virus. In that case the synthesis of HBsAg proteins by the replicating virus would proceed more rapidly or be more abundant than the synthesis of HBc and HBeAg proteins. In support of the synthesis hypothesis of the cell membrane HBsAg is the fact that HBsAg has been shown to be already detectable on the third day after inoculation of experimentally infected duck siblings (Freiman, 1988).

Moreover Krugman et al, 1979, who studied the natural history of hepatitis B infection in children inoculated with the MS-2 strain hepatitis virus, found an interval of only six days between exposure and the appearance of HBsAg in serum.

In the early stage of infection the hepatocyte cell membrane apparently is a predilection locus for the newly synthesised HBsAg antigens, thus probably changing the antigenic composition of the cell membranes of all hepatocytes. This altered antigenic cell surface composition is suggested in many studies (Gerber, 1985; Alberti, 1984; Mondelli, 1984) as a putative mechanism for cell-mediated immune cell lysis and viral clearing.

The diffuse expression of HBsAg on the hepatocyte cell surface was obviously not accompanied by increased destruction of hepatocytes, as indicated by the absence of hepatitis in these early biopsies.

On the contrary the liver cells exhibited high regenerative activity, as indicated by the numerous mitotic figures in the hepatocytes (Willemse, 1989). However, it has to be stressed that these patients were on effective immunosuppressive therapy, with suppression of immune mechanisms. Although HBsAg was detected mainly along the cell membranes of the hepatocytes in the early infection phase (one-and three-week biopsies), later it also appeared together with HBc in a faintly diffuse pattern in the cytoplasm of the hepatocytes of the HBV-positive, HDV-negative patients. In the six-month biopsies from both HBV-positive, HDV-negative patients typical HBsAg-positive ground-glass cells could be detected. In the two HDV-positive patients, HDAg appeared before or simul-taneously with HBsAg and long before HBcAg; HDAg was already present on the 7th-9th post-transplantation day (fig.XI.1). This observation, also reported by others (Rizzetto, 1987), suggests infection and replication of HDV in liver cells without obvious help from the hepatitis B virus. In one case (patient II), on the 9th posttransplantation day, 40 per cent of the hepatocytes stained positive for HDAg without HBsAg along the cell membrane and without detectable HBcAg in the cytoplasm or nucleus of the hepatocytes.

In the 3 month-biopsies HDAg was detected together with HBcAg. In these patients the time lag between the appearance of HBsAg on one hand and HBcAg

and HBeAg on the other was even more impressive.

The very early appearance of excessive amounts of HDAg in the liver transplants was not associated with major morphological changes. This observation does not support a direct toxic effect of HDV on the hepatocyte.

The frozen but not the paraffin sections of all liver biopsies revealed IgG in the same distribution pattern as HBcAg and HDAg indicating in vitro immune complex for-mation between HDAg and HBcAg antigens in liver tissue and intrinsic anti-HD and anti-HBcAg in sinusoidal serum (Rizzetto, 1981).

All livers revealed an acute hepatitis, demonstrated light microscopically between day 47 and 107, with slight (patient I) to moderate (patient II, III, IV) elevation of SGOT and SGPT ranging from 70 to 1010 i.u./l (fig.XI.10, 11, 12 and 13). The onset of abnormal transaminases, obvious related with the HBV infection of the graft was detected as early as 35 days and as late as 56 days after transplantation. The appearance of these abnormal serum transaminases after HBV exposure differs only slightly from the time of appearance of abnormal transaminases after exposition of HV in the children in the study of Krugman (1979).

In all livers the acute lobular hepatitis coincided with the appearance in the liver tissue of HBcAg located in three of the four biopsies mainly in the cytoplasm and less obviously in the nuclei. This distribution pattern differs from that seen in most livers with untreated chronic persistent and chronic active hepatitis, in which HBcAg occurs predominantly in the hepatocytic nuclei and less in the cytoplasm (see chapter VI).

Possibly of major interest is the observation that the hepatitis was accompanied by an unusually mild inflammatory infiltrate. The concomitant appearance of HBcAg and the lobular cell damage and the virtual absence of an inflammatory infiltrate suggest that a direct toxic effect of cytoplasmic HBcAg rather than immunological events plays a role in the pathogenesis of viral hepatitis B. Cellular immunological mechanisms are also less likely, because rejection phenomena were not prominent in any of the biopsies, indicating adequate suppression of the cellular immune system.

In all patients the acute hepatitis ultimately changed into a mild chronic active or chronic persistent hepatitis although it took 6 months in 3 cases and 12 months in the other for this to happen. Despite the fact that the patients' immunological system had already been sensitized by the hepatitis B virus and despite the use of immunosuppressiva the naturally occurring sequence of lobular hepatitis to portal/periportal hepatitis, as seen by a first infection appeared not to be changed essentially. Also this is not in favor of an immunological mediate disease. Similar to the finding of a progressive hepatitis B infection in many immunosuppressed renal (Parfrey, 1984, 1985) and liver (van Thiel, 1987) transplant recipients, the two HBV-positive, HDV-negative patients in this study

developed liver cirrhosis within one year. It seems likely that enhanced replication of the hepatitis B virus especially with the expression of HBcAg and HBsAg in the cytoplasm of the hepatocytes is an important factor in hepatocyte necrosis and may be due to a direct toxic effect of the virus on the hepatocyte. The importance of cytoplasmic localization of HBcAg in patients with aggressive disease was also stressed in the study of Hsu, 1988.

Since there is strong evidence that, in the natural course of a hepatitis B infection, a T cell-mediated immune mechanism against membrane-bound HBsAg and HBcAg also plays an important role in hepatocyte destruction, we suggest that hepatocyte necrosis might be attribute to two different mechanisms; firstly to an immune mediated mechanism and secondly to a direct cytotoxic effect of HBV.

The mild course of the hepatitis D infection makes a direct toxic effect of HDV improbable. The two HDV-positive patients in this study (especially patient III who showed minimal HBcAg expression) exhibited little architectural liver damage after one year. This relatively favorable course can be explained by suppression of HBV by HDV (Chen, 1988). Nevertheless, the course of the HBV/HDV coinfection in these immunosuppressed patients is in marked contrast to the relatively severe natural course of an HDV infection. Immunological mechanisms may play a role during the HDV infection, and immunosuppression may need to be reevaluated, despite the findings of Rizzetto (1983) who could not demonstrate a beneficial effect of standard immunosuppression in HDV-positive patients.

As complication of the chronic HBV-infection one of the delta negative patients (I), who was HBeAg negative before transplantation, died two years after transplantation of hepatocellular carcinoma which replaced the recipient liver totally and had invaded the liver graft extensively.

GENERAL REMARKS

The studies in this thesis have demonstrated the close relationship between viral expression and pathological changes in liver tissue from HBsAg-positive patients. The patterns of viral expression have been shown to play an important role in the classification of hepatitis B liver disease as well as assessment of prognosis of the disease.
Broadly speaking, three patterns could be distinguished:

A. A pattern, characteristic of (late-phase) acute self-limiting hepatitis with only minimal HBsAg in some scattered macrophages and degenerated hepatocytes. HBcAg is not present (state of viral inacceptance with viral elimination).

B. A pattern characterized by minimal or slight aspecific histological changes and large clusters of hepatocytes with excessive cytoplasmic accumulations of HBsAg; light microscopically these cells are recognized as ground-glass hepatocytes. Despite the presence of excessive HBsAg, no HBcAg can be detected. Incorporation of HDV-DNA in the hepatocyte genome and clonal proliferation of these hepatocytes are apparent in this pattern (state of viral acceptance).

C. A pattern characterized by a diffuse cell membrane-related HBsAg expression (honeycomb pattern), indicative of all types of chronic hepatitis as well as an acute hepatitis which will eventually progress to chronic hepatitis. In many cases HBcAg is present in this pattern. Varying numbers of scattered hepatocytes with intracytoplasmic HBsAg accumulation may be detected; they are the ground-glass cells in light microscopy. Only a few of these ground-glass hepatocytes contain HBcAg. In this pattern there are no signs of clonal proliferation of the ground-glass hepatocytes, nor is there a relation between the number of ground-glass cells and the activity of the chronic hepatitis (state of viral inacceptance without viral elimination).

Viral expression remains constant over prolonged periods, whereas clinical histological activity changes.

The results of the studies demonstrate that it is not the quantity of HBsAg and HBcAg in liver tissue that is related to activity and progression of the liver disease but their localization.

Our findings suggest that HBsAg localization in the cell membrane of hepatocytes causes immune-mediated liver disease, as indicated by portal piecemeal necrosis in light microscopy. The degree of piecemeal necrosis is dependent on host factors. The localization of HBcAg in the cytoplasm of hepatocytes can be related to lobular hepatocyte necrosis.

Studies of reinfected liver grafts show that extensive liver cell necrosis can occur without the presence of lymphocytes. This finding suggests a direct cytopathic effect of HBcAg on the liver cell.

These findings provide evidence that liver cell necrosis is due to both immunological processes, depending upon localization of HBsAg in the cell membrane of hepatocytes, and direct cytopathogenic mechanisms, arising from viral replication and cytoplasmic localization of HBcAg.

The immunological aspect of chronic hepatitis, the severity of which is determined by host factors, presents as periportal hepatitis with piecemeal necrosis. The direct cytopathogenic effect becomes manifest as lobular hepatocytic necrosis and determines to a large extent the prognosis of the chronic hepatitis.

The findings reported in this thesis provide a basis for a strategy for anti-viral treatment of chronic hepatitis B.

Theoretically several approaches to the treatment of a viral infection exist. Because there is strong evidence for a direct relation between viral replication and activity of the liver disease, one therapeutic possibility may be to achieve suppression of viral replication and thus amelioriation of disease activity.

For this purpose drugs that interfere with both viral RNA transcription and the production of viral proteins can be administered in order to suppress replication of the virus.

At present however such drugs are not available. Moreover the infection will not be cured: instead only the effects will mitigated. Such a drug may suppress both the immune mechanism, by diminishing protein (HBsAg) production, and the toxic effects, by reducing proliferation of the virus.

Elimination of the potential of an infected cell to produce a virus is another possibile approach.

To accomplish this aim, drugs can be used that eliminate all infected hepatocytes from the liver. As shown in chapter VI, in chronic hepatitis all hepatocytes appear to be infected by the virus, as demonstrated by the diffuse cell membrane-bound HBsAg localization, so that such a therapy would imply elimination of all hepatocytes.

If this elimination were to occur rapidly, the result would be massive liver cell necrosis and death of the patient.

A more realistic way to eliminate the potential of infected cells to produce virus would be to cure infection of the hepatocyte.

Such a therapy may be achieved by inhibition of viral DNA synthesis. Since viral DNA formation depends on reverse transcription, drugs such as phosphonoformic acid that interfere with reverse transcriptase would prevent the generation of cccDNA. However preliminary results with this type of drug are not hopeful. A decline in viral replication activity is followed by rapid restoration of this activity after withdrawal of the drug.

Finally, another possibility is to cure the chronic infection by creating a situation in which newly formed hepatocytes are protected against reinfection by the virus. In this way, the number of infected hepatocytes will slowly decrease, as a result of regeneration of the liver, and all infected cells will eventually disappear.

SUMMARY

Hepatitis B represents a major public health problem of worldwide concern because of its frequent occurrence and severe complications. Hepatitis B infection is characterized by a wide range of histological changes and various clinical courses, which in general are caused by differences in the relation between the virus and the host.

Since the discovery of the Australia antigen by Blumberg and the subsequent recognition of the Australia antigen as a marker for hepatitis B infection by Prince, significant advances in the fields of basic molecular biology and clinical research have been made.

However our knowledge about the pathobiology of the virus and the molecular and cellular basis for the pathogenesis of the liver changes is still incomplete. In particular mechanisms which lead to:

a. liver cell necrosis.
b. evolution of acute to chronic hepatitis.
c. persistance of a hepatitis B infection.
d. development of hepatocellular carcinoma.

are only partly understood and reports on these subjects are conflicting.

In addition the interactions between the hepatitis B virus and the incomplete hepatitis delta virus as well as the accompanying pathological events are far from clear.

By studying the expression patterns found for the hepatitis B and D virusses in relation to histopathological changes in liver tissue from a series of HBsAg seropositive individuals (both patients and apparently healthy carriers), an attempt has been made to contribute to a better understanding of the pathogenetic mechanisms in viral hepatitis.

In **chapter I** the history of the discovery of hepatitis B virus and the subsequent advances in hepatitis B research, which is characterized by many serendipities, were reviewed. At the end of this chapter the general aims of the study were described: the central theme is the unravelling of the pathogenetic mechanisms of hepatitis B.

Chapter II is an evaluation of the liver biopsies investigated in this thesis. A series of consecutive liver needle biopsies, received for histological examination by the Department of Clinical Pathology during a five-year period (1973-1977), was studied.

In this period 165 liver needle biopsies from 139 HBsAg seropositive patients (106 males and 33 females) were presented for light microscopical as well as immunohistochemical and electron microscopical examination.

The group of 139 HBsAg seropositive individuals was composed of:

a. apparently healthy asymptomatic HBsAg positive subjects (51)
b. patients with a hepatitis B infection demonstrated during evaluation of a clinically manifest liver disease or other internal diseases partly caused by liver disease (88).

In this chapter demographic data and the possible mode of infection were considered; 41 of the 51 apparently healthy HBsAg positive individuals came from a group of 128 blood donors who were found to be HBsAg positive upon routine screening.

From this finding a prevalence for HBsAg in the Netherlands (Rotterdam) of roughly 0.2 per cent was calculated with a male/female ratio of 4:1. This prevalence for HBsAg is in agreement with prevalences reported for other West European countries and the USA and is far less than the 6-15 per cent found for some Asian and African countries.

All 128 HBsAg-positive blood donors were admitted for clinical evaluation of their HBsAg carriership; a liver biopsy was obtained from 41 (± 25 per cent) blood donors.

In **chapter III**, the methods used for the investigation of the liver biopsy material were described.

Chapter IV focussed on the light microscopical aspects of hepatitis, and in particular hepatitis B. The criteria for and difficulties of the classification of the broad spectrum of microscopical changes were discussed. The relation between light microscopical characteristics and prognosis was considered in detail.

Chapter V dealt with the analysis of liver biopsies from 139 HBsAg positive patients, classified according to the system, described in chapter IV.

The histological findings were compared with clinical and biochemical parameters and the course of the hepatitis B infection. A histological diagnosis of acute hepatitis was established for 43 patients, 10 of whom belonged to the group "apparently healthy individuals". The acute hepatitis was anicteric in 9 cases. All anicteric versus only two cases of icteric acute hepatitis progressed to chronic hepatitis, with prolonged persistence of HBsAg. This provides strong evidence that acute hepatitis with an anicteric course transforms into chronic hepatitis; in contrast of the patients with the clinically more severe icteric acute hepatitis only a few (in this series 6 per cent) underwent progression to chronic hepatitis.

This finding is in agreement with the fact that patients with chronic hepatitis rarely have a history of jaundice.

Light microscopically acute hepatitis could be subdivided roughly into early, fully developed and late phase acute hepatitis, which correlated well with the biochemical and clinical features. No consistent histological features that predict an unfavorable course of acute hepatitis, i.e. transition to chronic hepatitis, were found. Prominent infiltrates in the portal tracts, sometimes with features of piecemeal necrosis, and the absence of cholestasis gave the best correlation with transition to chronic hepatitis.

All cases of acute hepatitis that transformed into chronic hepatitis were of the late-phase type.

In contrast with reports in the literature, biopsies from patients with acute hepatitis contained only minimal iron deposits in this series.

In general asymptomatic individuals with chronic hepatitis exhibited, as was expected, less severe liver changes than the symptomatic ones. On the other hand, the biopsies from 9 asymptomatic blood donors exhibited the pattern of acute hepatitis, 6 of whom ultimately remained anicteric.

Five blood donors had chronic active hepatitis and 1 an incomplete septal cirrhosis.

Seventeen of the 88 symptomatic hepatitis B carriers exhibited severe liver changes with cirrhosis. Cirrhosis developed for the most part in the older age group.

Not only the differentiation of acute self-limiting hepatitis from acute hepatitis which progresses to chronic hepatitis but also the differentiation of chronic persistent hepatitis from chronic active hepatitis, on the one hand, and aspecific reactive hepatitis and the residual phase of acute hepatitis, on the other, was sometimes problematic.

In **chapter VI** the results of immunohistochemical studies for determination of the presence and localization of hepatitis B and D antigens and immunoglobulins were reported.

In the hepatocyte HBsAg could be detected along the cell membrane or in the cytoplasm in a diffuse pattern or as inclusion body. The intracytoplasmic localization of HBsAg correlated in most cases with the presence of ground-glass hepatocytes in H and A-stained formalin-fixed sections of liver tissue. In contrast to the intracytoplasmic HBsAg localization, the membrane localization in general could not be demonstrated in formalin-fixed material.

Using isolated liver cells in suspension it was demonstrated that HBsAg was present along the cell membrane with expression on the outer surface, and that HBsAg was mobile in this cell membrane, as indicated by the capping phenomenon.

HBcAg was demonstrated mainly in the nucleus of the hepatocytes. In some cases this nuclear localization was combined with diffuse localization in the cytoplasm, indicative of active viral replication. HBcAg occurred in nearly all cases in combination with a cell membrane-related HBsAg localization. Sporadically HBcAg was found in hepatocytes with the ground-glass aspect, which correlated with cytoplasmic accumulation of HBsAg.

The localization of HBeAg in liver tissue was identical to that of HBcAg, both being distinct from HBsAg. This finding is also indicative of the close relation, probably involving the same particles, between HBeAg and HBcAg in liver tissue.

Delta antigen was also detected mainly in the nuclei of the hepatocytes, sometimes combined with cytoplasmic localization. In individuals with chronic hepatic disease only a few hepatocytes revealed delta antigen and never in combination with HBcAg.

Concerning the localization of immunoglobulins, the most striking finding for HBsAg-positive patients was the presence of IgG and in some cases IgM deposits in the nuclei of hepatocytes.

Occurrence and localization of these immunoglobulin deposits were identical to those for HBcAg and core particles, as demonstrated by electron microscopy.

The phenomenon was shown to be caused by binding of intrinsic antibodies present in the serum in the sinusoidal spaces to core antigen in the nucleus.

In the same way HBeAg and delta antigen could be demonstrated in eAg or delta antigen-positive liver tissue in patients with anti-HBe and anti-delta activity, respectively, in the serum.

On the basis of the different localizations of the various viral antigens, three viral expression patterns could be distinguished. Localization of HBsAg is the most important for differentiation of the three patterns:

a. solitary pattern: only a few scattered cells (macrophages or degenerated hepatocytes) are present in the liver tissue without concomitant HBcAg. This pattern correlates with and is only present in acute self-limiting hepatitis.
b. focal pattern: in this pattern large clusters of hepatocytes show extensive accumulation of HBsAg in the cytoplasm. No HBcAg can be detected. This pattern correlates with a nearly normal liver tissue with the striking aspect of large groups of ground-glass hepatocytes. This pattern suggests a viral acceptance state with viral DNA incorporated in the viral genoom.
c. diffuse pattern, which correlates with chronic hepatitis. A cell membrane-bound HBsAg localization is seen in all hepatocytes. Some hepatocytes with intracytoplasmic HBsAg (ground-glass cell) can be found dispersed throughout the liver tissue. In this pattern HBcAg can be demonstrated in some hepatocytes (a viral inacceptance state).

On the basis of the presence and especially on the localization of HBcAg, this pattern was subdivided. Cytoplasmic HBcAg localization correlates with rapid progression of the chronic hepatitis.

It is concluded that the localization of viral antigens correlates well with histological patterns and plays an important (prognostic) role in the exact classification of HB disease.

Localization of HBsAg along the cell membrane of hepatocytes is presumed to be an important factor in immune-mediated liver changes, manifested as piecemal necrosis in light microscopy. Active viral replication, with expression of HBcAg in the cytoplasm of hepatocytes, is an important factor in lobular necrosis of hepatocytes and is probably caused by direct hepatotoxic effects.

In **chapter VII** attention was focussed on hepatocytes with the ground-glass aspect in routine HA-stained sections of formalin-fixed paraffin-embedded liver tissue. Ground-glass hepatocytes in routinely stained liver sections are known to occur in association with different liver diseases, hepatitis B viral liver disease being possibly the most important. In these cases the cytoplasm is loaded with hepatitis B surface antigen and shows special affinity for histochemical dyes. The occurrence of ground-glass cells, their staining characteristics, the specificity for a hepatitis B infection and their value for classification of hepatitis B liver disease were investigated.

Ground-glass hepatocytes were found in ± 8% of a series of 1810 liver biopsies. In most cases (103 of the 150) the biopsies came from HBsAg-seropositive individuals. The presence of HBsAg in these ground-glass cells was proven by specific immunohistochemical staining methods. These hepatocytes stained positive with aldehyde thionin and orcein, whereas the hepatocytes in biopsies from HBsAg-negative patients were negative. Except for acute self-limiting hepatitis, ground-glass cells were detected in all variants of HBsAg liver disease in clusters (focal pattern) or scattered throughout the liver tissue (solitary pattern).

The solitary pattern was seen in many cases of prolonged acute hepatitis (37.5 per cent), chronic persistent hepatitis and chronic active hepatitis. The focal pattern was detected in cases of minimal liver changes and in most cases of aspecific reactive hepatitis. These expression patterns were shown to be quite constant in serial liver biopsies. It was concluded that the ground-glass hepatocyte, with its special staining characteristics, forms the hallmark for light microscopical diagnosis of a chronic hepatitis B infection and is an essential aid in grading hepatitis B liver disease, especially when more etiologic factors are responsible for the light microscopical liver changes.

The results of a study on the presence and localization in liver tissue of the determinants characteristic for the different subtypes of HBsAg are reported in **chapter VIII**.

In liver tissue, the "a" as well as the "d" and "y" determinants were demonstrated at the same localization, such that the "a" determinant was always present together with either the "d" or the "y" determinant.

Dissociation of the different determinants of HBsAg was not found in any of the biopsies.

Chapter IX describes the presence of and the relation between HBeAg and anti HBe in serum and HBeAg in liver tissue.

HBeAg was always detected together with and at the same localization as HBcAg. HBeAg and HBcAg was found in liver biopsies from all except four HBeAg-seropositive patients.

In the contrast two patients with HBeAg and anti-HBe-negative serum and one patient with anti-HBe-positive serum revealed HBeAg and HBcAg in the liver tissue.

There was no clear correlation between the titer of HBeAg in serum and the amount of HBcAg and HBeAg in the liver tissue.

It was concluded that an anti-HBe-positive serum may not be used as marker for the absence of infectivity of a patient. This statement was confirmed by the reinfection of an liver graft transplanted into an anti-HBe-positive patient (see chapter XI).

Chapter X presents the results of an electron microscopical study on the presence of viral structures in liver tissue from HBsAg seropositive patients. For all patients with immunohistochemically demonstrated HBcAg, small and large groups of circular structures consistent with core particles were found. No structural counterpart for HBsAg localization along the cell-membrane could be shown.

In **chapter XI** the development and course of hepatitis B and hepatitis delta infections in auxiliary heterotopic liver grafts in patients suffering end-stage (cirrhotic) hepatitis B liver disease are discussed. Serial biopsies from the liver grafts were used to investigate the consecutive appearance of the various hepatitis B and delta antigens and the accompanying histological events in four patients. Prior to transplantation two of these patients were HBV-positive HDV-negative, while the other two had delta superinfection without accompanying signs of active HBV replication. One of the HBV-positive HDV-negative patients exhibited active viral replication, as indicated by HBeAg and HBV-polymerase-positive serum and HBcAg in the liver tissue. The other HBV-positive HDV-negative patient had no signs of active viral replication.

According to the experience in other centers with orthotopic liver grafts in HBsAg-positive patients, the hepatitis B and D virus recurred, in all four liver grafts. The one-week biopsies from the grafts already revealed hepatitis B or D antigens in three out of four patients. Only in the HBV-positive, HDV-negative patient

without signs of active viral replication was recurrence of hepatitis B delayed until the three-week biopsy. This delayed occurrence is probably attributable to the time needed for reactivation of the dormant viral infection. In one of the HDV-positive patients, HDAg was demonstrated as first and only viral antigen in the nucleus of more than 40 percent of the hepatocytes in the one-week biopsy. HBsAg was present in the one-week biopsies from two of the four grafts and in the three-weeks biopsies from all grafts, along the cell membrane of all hepatocytes. Despite immunosuppressive therapy and previous activation of the immune system of the recipient by hepatitis B viral antigens, the sequence of histological events characteristic of a first hepatitis B infection, recurred in all grafts. Acute hepatitis did not occur before 47 days and progressed slowly within six to twelve months to a mild chronic active hepatitis. These observations suggest that the biological processes of the virus in the liver parenchyma determines the course of the disease. Despite a slight inflammatory reaction in the livers, the grafts from both HBV-positive HDV-negative patients exhibited cirrhotic transformation within one year, whereas both HDV-positive patients showed only mild or moderate fibrosis, respectively, after one year.

The appearance of hepatitis in the liver tissue was related to the appearance of HBcAg, especially in the cytoplasm of the hepatocytes, as a sign of marked viral replication. The appearance of HDV in liver tissue and HBsAg on the cell membranes of the hepatocytes could not be correlated with obvious hepatocytic necrosis in these immune-suppressed patients.

SAMENVATTING

Hepatitis B vormt door zijn wereldwijd en frequent voorkomen (± 300 miljoen chronische hepatitis B dragers) en zijn ernstige gevolgen een belangrijk wereldgezondheidsprobleem.

Sinds de ontdekking van het Australië antigeen door Blumberg, Alter en Visnich en vervolgens de onderkenning door Prince, dat de aanwezigheid van dit antigeen een marker van een hepatitis B virus infectie is, zijn er zeer belangrijke vorderingen gemaakt in het Hepatitis B onderzoek, vooral op het gebied van de moleculair biologische eigenschappen van het virus.

Omtrent onze kennis van de pathobiologie van het virus en de moleculaire en cellulaire basis voor de pathogenese van de ziekte blijven daarentegen nog belangrijke gebieden onopgehelderd. Met name de mechanismen welke leiden tot:

a. levercelnecrose.
b. het ontstaan van acute dan wel chronische hepatitis.
c. het persisteren van het hepatitis B virusinfectie.
d. het ontstaan van hepatocellulair carcinoom.

zijn slechts ten dele opgelost en worden als controversieël beschouwd. Ook de interactie van het Hepatitis B virus met het incomplete hepatitis delta virus en de hierbij optredende pathobiologische mechanismen zijn verre van duidelijk.

Door het bestuderen van expressiepatronen van hepatitis B en delta virus, in relatie met histopathologische veranderingen in leverweefsel van een groep hepatitis B surface antigeen positieve patienten, tracht deze studie een bijdrage te leveren tot een beter begrip van pathogenetische mechanismen,welke een rol spelen bij een hepatitis B infectie.

In **hoofdstuk I** wordt een historisch overzicht gegeven van de, door toevallige omstandigheden gekenmerkte, ontdekking van zowel het hepatitis B als het hepatitis Delta virus en de daarop volgende ontwikkelingen. Het hoofdstuk eindigt met de doelstellingen van het onderzoek, waarbij ontrafeling van pathogenetische mechanismen van de door het virus veroorzaakte leverafwijkingen centraal staat.

Hoofdstuk II beschrijft de herkomst van het in deze studie onderzochte levermateriaal. Dit betreft hoofdzakelijk door naaldbiopsie verkregen leverweefsel, ontvangen op de afdeling Klinische Pathologie gedurende de jaren 1973-1977.

In deze periode werden 165 naaldbiopten van 139 HBsAg seropositieve patiënten (106 mannen en 33 vrouwen) aangeboden, die voor lichtmicroscopisch,

immunohistochemisch en electronenmicroscopisch onderzoek konden worden bewerkt.

De groep van 139 patiënten is samengesteld uit twee subgroepen, nl.:

A. "gezonde" asymptomatische hepatitis B dragers (51).
B. patiënten (88) waarbij HBsAg in het serum werd gevonden in het kader van een klinisch manifeste leverziekte, of een andere interne aandoening, die gedeeltelijk het gevolg was van een leverziekte.

Leeftijd en geslachtsverdeling en de mogelijke besmettingswijze worden van alle Hepatitis B dragers gegeven. In de onderzoeksperiode werden op de Bloedtransfusiedienst te Rotterdam 128 donoren HBsAg positief bevonden. Hieruit kon een prevalentie van HBsAg in de Nederlandse (Rotterdamse) bevolking van ± 0.2 worden berekend met een man/vrouw verhouding van ± 4 : 1. Deze prevalentie komt goed overeen met de prevalentiewaarden in andere West-Europese landen en staat in schil contrast met de hoge prevalentiewaarden berekend in delen van Azië en Afrika. Alle 128 positieve donoren werden verwezen voor nader klinisch onderzoek van hun HBsAg dragerschap. In het kader van dit onderzoek werd bij 41 een leverbiopt verricht.

Hoofdstuk III geeft een beschrijving van de bewerking van het leverbiopsiemateriaal voor de verschillende onderzoeksmethoden.

In **hoofdstuk IV** wordt in algemene zin uitgebreid ingegaan op de verschillende lichtmicroscopische reactievormen van de lever op een hepatitis B infectie. De classificatie van deze reactievormen wordt belicht, waarbij ingegaan wordt op de histologische kenmerken van de verschillende groepen en subgroepen van hepatitis, de problemen bij de classificatie en de waarde van verschillende kenmerken voor de prognose van hepatitis.

In **hoofdstuk V** worden de morfologische veranderingen in de 165 leverbiopten van de 139 HBsAg positieve patiënten geanaliseerd en volgens het in hoofdstuk IV beschreven classificatiesysteem ingedeeld.

De histologische bevindingen werden vergeleken met klinische en klinisch-biochemische parameters en met het beloop van de hepatitis B infectie, over een periode van minimaal 2 jaar tot maximaal 15 jaar.

In de gehele groep patiënten werd bij 43 patiënten de diagnose acute hepatitis gesteld, waarvan 9 een anicterisch beloop en 34 een icterisch beloop hadden.

Alle acute leverontstekingen met een anicterisch beloop gingen over in een chronisch hepatitisbeeld, waarbij HBsAg vele jaren positief bleef. Slechts 2 patiënten met een icterische acute hepatitis ontwikkelden een chronische hepatitis. Hieruit wordt geconcludeerd dat chronische hepatitis vrijwel altijd ontstaat via een anicterische (weinig ernstige) acute hepatitis en dat een icterische

hepatitis, welke in de acute phase soms tot ernstig levercelverval aanleiding kan geven, zelden overgaat in een chronische hepatitis. Deze bevinding is in overeenstemming met het feit dat anamnestisch slechts zelden icterus wordt gemeld bij patiënten met een chronische hepatitis. Lichtmicroscopisch konden in de groep van patiënten met acute hepatitis geen, voor het verloop van de virale infectie kenmerkende prognostische aspecten, worden gevonden. Een prominent portaal ontstekingsinfiltraat en de afwezigheid van cholestase tijdens de acute hepatitis zijn het meest significant, doch niet absoluut kenmerkend voor overgang in chronische hepatitis.

In tegenstelling met literatuurgegevens werd in de groep van acute hepatitis patiënten opvallend weinig ijzerpigmentstapeling in het leverweefsel aangetroffen.

Asymptomatische hepatitis B dragers toonden, in de lijn der verwachting, in vergelijking met de symptomatische hepatitis B dragers minder ernstige leverafwijkingen. Toch toonden 9 van de 41 asymptomatische bloeddonoren het histologische beeld van een acute hepatitis, waarvan 6 uiteindelijk anicterisch verliepen. Bij 5 van de 41 donoren werd een chronische actieve hepatitis aangetroffen, welke in alle gevallen gepaard ging met leverenzymstoornissen. In één donor werd een z.g. incomplete septale (inactieve) cirrhose aangetroffen. Geconcludeerd werd dat asymptomatische hepatitis B dragers ernstige leverafwijkingen kunnen tonen, die dan echter altijd met serum transaminase stoornissen gepaard gaan.

Bij de chronische symptomatische hepatitis B dragers werden over het algemeen ernstige afwijkingen aangetroffen; bij 17 van de 88 werd het beeld van cirrhose gezien. De cirrhotische leverafwijkingen werden vooral gezien in de oudere leeftijdsgroep en gingen in de meeste gevallen gepaard met een chronisch actief hepatitisbeeld, welk blijkbaar de oorzaak van de cirrhose was.

Naast de problemen bij de afgrenzing van acute hepatitis en acute hepatitis overgaande in chronische hepatitis, gaf de diagnostiek van chronische persisterende hepatitis vaak moeilijkheden, aan de ene kant met betrekking tot de afgrenzing van niet specifieke hepatitis en hepatitisrestverschijnselen, aan de andere kant met betrekking tot chronische actieve hepatitis.

In **hoofdstuk VI** worden de resultaten van het immunohistochemische onderzoek naar de aanwezigheid en de lokalisatie van de verschillende virale antigenen en immunoglobulinen en complementfactoren in het ingevroren en gefixeerde leverweefsel van de 139 HBsAg positieve patiënten beschreven.

In de hepatocyt kan HBsAg worden aangetroffen langs de celmembraan, diffuus in het cytoplasma en als insluitlichaam focaal in het cytoplasma, nooit in de kern. De intracytoplasmatische HBsAg lokalisatie correleert met het lichtmicroscopisch aangetroffen groundglass aspect van hepatocyten (zie hoofdstuk VII).

In tegenstelling met het cytoplasmatische HBsAg is het celmembraan gebonden HBsAg in gefixeerd leverweefsel veelal niet of moeilijk aantoonbaar.

Bij onderzoek van suspensiepreparaten van HBsAg positief leverweefsel werd HBsAg op het buitenoppervlak van hepatocyten aangetoond en leek op grond van het gevonden "capping" fenomeen mobiel in de celmembraan aanwezig.

HBcAg wordt voornamelijk in de kern aangetroffen en in een klein deel der gevallen, welke hoge virale replicatie activiteit tonen, eveneens en soms alleen in het cytoplasma. HBcAg wordt in de hepatocyt vrijwel altijd gecombineerd aangetroffen met celmembraan gebonden HBsAg en in veel mindere mate met de focale cytoplasmatische lokalisatie van HBsAg. Deze focaal cytoplasmatische lokalisatie van HBsAg lijkt derhalve suggestief voor een zichtbare uiting van de situatie, waarin viraal DNA is ingebouwd in het genoom van de gastheer.

HBeAg lijkt wat lokalisatie betreft niet af te wijken van de lokalisatie van het HBcAg en er werden geen leverbiopten gevonden waarin er een discrepantie bestond tussen aanwezigheid en lokalisatie van HBcAg en HBeAg.

Delta antigeen werd vrijwel alleen in de kernen van de hepatocyten aangetroffen en alleen bij zeer hoge delta virus replicatie ook in het cytoplasma. Over het algemeen sluit de aanwezigheid van delta antigeen de aanwezigheid van HBcAg en HBeAg uit. Bij de HDV positieve transplantatiepatiënten, waar sprake is van een HDV coinfectie, werden echter zowel HBcAg en delta antigeen naast elkaar aangetroffen.

Wat betreft de lokalisatie van immunoglobulinen was de meest opmerkelijke bevinding dat in coupes van ingevroren leverweefsel deposities van immunoglobuline, hoofdzakelijk IgG, werden aangetroffen op dezelfde plaats als core en/of delta antigeen. Dit fenomeen kon worden verklaard door een in vitro binding van intrinsiek in de sinusoidale vaten aanwezige antilichamen tegen core en/of delta antigeen, met het in de hepatocyten aanwezige core en/of delta antigeen. Dit verschijnsel maakt het mogelijk ook andere antigeen/antilichaam systemen op het spoor te komen.

Op grond van de lokalisatie van de verschillende virale antigenen, konden verschillende, voor de classificatie en de prognose kenmerkende patronen worden onderscheiden. De HBsAg lokalisatie speelt in deze patronen de belangrijkste rol:

a. Solitair patroon: hierbij wordt een enkele zwak HBsAg positieve, veelal gedegenereerde hepatocyt of macrofaag gezien, temidden van HBsAg negatief leverweefsel. Dit patroon wordt aangetroffen bij acute hepatitis, welke niet in een chronische hepatitis overgaat.
b. Focaal patroon: hierbij worden naast grote aaneengesloten velden hepatocyten met cytoplasmatische HBsAg lokalisatie (groundglasscellen) kleinere

of grotere velden hepatocyten zonder HBsAg expressie gezien.Dit patroon wordt aangetroffen bij chronische Hepatitis B dragers zonder noemenswaardige lever afwijkingen. HBcAg wordt hierbij niet gedetecteerd.

c. Diffuus patroon: in dit patroon tonen alle hepatocyten een celmembraan gebonden HBsAg lokalisatie (honingraat aspect in lever coupes). Dit patroon wordt gezien bij alle vormen van chronische hepatitis en gaat in een groot deel der gevallen gepaard met HBcAg in de kern of het cytoplasma (actieve virale replicatie).

Geconcludeerd wordt dat de lokalisatie van virale antigenen duidelijk gecorreleerd is met histologische leververanderingen en dat het virale verdelingspatroon bij de classificatie van de leverontstekingen een belangrijke (prognostische) rol speelt.

Pathogenetisch lijkt lokalisatie van HBsAg langs de celmembraan van de hepatocyten belangrijk voor het histologische aspect van zgn. piecemeal necrose bij chronische actieve hepatitis; dit verschijnsel berust op een immunologisch proces. Virale replicatie, vooral met HBcAg expressie in het cytoplasma, is gerelateerd aan de ernst van lobulair levercelverval en bepaald voor een belangrijk deel de snelheid van de progressie van de leverziekte.

In **hoofdstuk VII** wordt ingegaan op de zgn. "groundglass" hepatocyt. Het "groundglass" of matglas aspect van hepatocyten is sterk geassocieërd met de aanwezigheid van een hepatitis B infectie. Het berust op excessieve stapeling van hepatitis B surface antigeen in het cytoplasma van deze hepatocyten. "Groundglass" veranderingen van het cytoplasma van de hepatocyten kunnen echter ook worden aangetroffen bij verschillende andere leveraandoeningen ("drug induced" en cirrhose in het algemeen).

In deze studie werden het vóórkomen, de frequentie, het verdelingspatroon en de histochemische kleuringskarakteristieken van groundglass hepatocyten onderzocht in een serie van 1810 opeenvolgende leverbiopten, die in de periode van dit onderzoek werden ontvangen.

HBsAg positieve groundglass hepatocyten hebben specifieke affiniteit tot bepaalde histochemische kleurstoffen (orceine, aldehyde thionine, aldehyde fuchsine), welke niet wordt aangetroffen bij HBsAg negatieve groundglasscellen.

In 150 van de 1810 leverbiopten (± 8%) werden groundglasscellen aangetroffen. In de meeste gevallen (103 van de 150) ging het hierbij om HBsAg seropositieve patiënten. De aanwezigheid van HBsAg kon in deze groundglasscellen immunohistochemisch worden aangetoond. De groundglasscellen in de biopten van HBsAg seronegatieve patiënten waren HBsAg negatief.

Behalve in de acute genezende HBV infectie worden typische groundglasscellen in meer of mindere mate in alle andere histopathologische vormen van

chronische hepatitis B infectie aangetroffen.

In leverbiopten van patiënten met chronische hepatitis B infectie met minimale of geringe niet specifieke histologische afwijkingen werden in de meeste gevallen (± 90%) groundglasscellen gezien, welke hier in grote aaneengesloten velden gelokaliseerd zijn (focaal verdelingspatroon).

In leverbiopten met chronische persisterende en chronische actieve hepatitis werden in ± 70% van de biopten groundglasscellen gezien, welke solitair, verspreid liggend tussen normale hepatocyten (solitair verdelingspatroon), voorkomen. Dit solitaire verdelingspatroon werd ook gezien in acute hepatitis overgaand in chronische hepatitis (± 47% van de gevallen), waarbij opgemerkt moet worden dat deze cellen niet eerder dan 6 maanden na het begin van de infectie verschijnen.

De leverbiopten van HBsAg positieve patiënten met cirrhose toonden in 66.5% van de gevallen groundglass hepatocyten. Het verdelingspatroon en het aantal groundglasscellen wisselt hier sterk van nodus tot nodus.

In het solitaire verdelingspatroon is het aantal groundglasscellen veel minder groot dan in het focale verdelingspatroon; voor de opsporing is zorgvuldige screening van routinematig gekleurde HA coupes vereist. Voor het opsporen van sporadisch voorkomende groundglasscellen zijn de specifieke histochemische of immunohistochemische kleuringen waardevol. Rekening moet echter worden gehouden met aspecifieke immunohistochemische en histochemische kleurreacties. Voor de diagnose chronische HBV infectie zijn èn een typisch groundglass aspect van het cytoplasma èn een positieve kleurreactie noodzakelijk.

In **hoofdstuk VIII** worden de resultaten van een onderzoek naar de lokalisatie van de determinanten van de verschillende subtypen HBsAg ("a", "d" en "y") gegeven. Zowel de "a", de "d" en "y" determinant wordt in leverweefsel steeds op dezelfde lokalisatie aangetroffen, waarbij de "a" determinant altijd aanwezig is, gepaard gaande met, òf de "y", òf de "d" determinant. Dissociatie in lokalisatie van de verschillende determinanten is hierbij niet aangetroffen. Geconcludeerd wordt derhalve dat alle determinanten op het HBsAg van virale afkomst zijn en direct in een partikel worden geassembleerd.

Hoofdstuk IX gaat in op de lokalisatie van HBeAg in leverweefsel. De relatie van HBeAg in dit leverweefsel met andere virale antigenen (HBcAg, HBsAg) in leverweefsel en met de aanwezigheid van HBeAg en anti HBe in serum, wordt nagegaan. HBeAg komt altijd tesamen en in dezelfde lokalisatie voor als HBcAg. HBeAg en HBcAg worden steeds in combinatie met een diffuus HBsAg fluorescentiepatroon aangetroffen. Er werd sporadisch HBeAg in het serum aangetroffen, wanneer eAg en HBcAg in het leverweefsel niet konden worden aangetroffen.

In een enkel geval werd HBeAg in het leverweefsel aangetroffen in combinatie

met anti HBe in het serum. Geconcludeerd kan worden dat detectie van HBeAg en HBcAg in leverweefsel een gevoelige methode is voor de opsporing van hepatitis B infectie met actieve virale replicatie.

In **hoofdstuk X** worden de bevindingen van het electronenmicroscopisch onderzoek weergegeven. Met name wordt hier de relatie tussen de aanwezigheid van viruspartikels in de hepatocyten en de histochemisch aangetoonde virale antigenen belicht. In alle gevallen, waar immunohistochemisch HBcAg in de hepatocyten werd aangetoond, konden ± 27 nm partikels in de kernen van de hepatocyten worden gezien.

In **hoofdstuk XI** worden de resultaten beschreven van een onderzoek naar de ontwikkeling en het verloop van hepatitis B en delta virus infecties in heterotoop auxillair getransplanteerde levers bij patiënten met een gedecompenseerde HBV of HDV positieve levercirrhose. Met behulp van op bepaalde tijden verrichte leverbiopten kon de volgorde van verschijnen van de verschillende hepatitis B en D antigenen en de daarmee gepaard gaande lichtmicroscopische veranderingen worden nagegaan in vier transplantaten.

Twee van de getransplanteerde patiënten waren vóór de transplantatie HBV positief HDV negatief, terwijl de andere twee HBV negatief, HDV positief waren.

Eén van de HBV positieve patiënten had tekenen van virale replicatie, de andere niet. Een week na transplantatie werden in drie van de vier transplantaten hepatitis B of hepatitis D virus antigenen aangetroffen. Alleen het transplantaat van de HBV positieve, HDV negatieve patiënt zonder virale replicatie toonde pas in het protocollaire 3e week biopt viraal antigeen.

In de twee HBV positieve HDV negatieve patiënten verscheen HBsAg het eerst langs de celmembraan van de hepatocyt, in latere biopten gevolgd door HBcAg. In de HBV negatieve HDV positieve patiënten was HDV het eerst aangetoonde antigeen, in één geval gecombineerd met HBsAg langs de celmembraan. In de HBV negatieve, HDV positieve patiënten verscheen HBcAg later (3e week en 3e maand biopt) tesamen met HDV. HBcAg was gedeeltelijk of grotendeels intracytoplasmatisch gelokaliseerd.

In alle transplantaten ontstond na ± 7 weken het histologische beeld van een acute hepatitis. Het optreden van de acute hepatitis was niet gerelateerd aan het verschijnen van HBsAg en delta antigeen, maar steeds aan dat van HBcAg, vooral gelokaliseerd in het cytoplasma. In beide HBV positieve, HDV negatieve transplantaten ontstond na één jaar een cirrhose, hetgeen een aanmerkelijk sneller beloop is dan bij de meeste natuurlijke hepatitis B infecties. Tegen de verwachting in was de hepatitis in beide HDV positieve patiënten veel minder progressief; na 1 jaar werd slechts geringe fibrose aangetroffen.

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ABBREVIATIONS

AH	acute hepatitis
anti-HBc	antibodies against hepatitis B core antigen
anti-HBe	antibodies against hepatitis B e-antigen
anti-HBs	antibodies against hepatitis B surface antigen
AspH	aspecific hepatitis
Au-Ag	Australia antigen
BHN	bridging hepatic necrosis
CAH	chronic active hepatitis
ccc	covalently closed-circular
CH	chronic hepatitis
CLH	chronic lobular hepatitis
CPH	chronic persistent hepatitis
EG	Lawson-von Gieson's technique for elastin
GGH	ground-glass hepatocyte
HA	hematoxylin and azophloxin
HBAg	hepatitis B antigen
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBF	button-hole hepatocyte
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HDV	hepatitis delta virus
HFH	homogeneous fluorescence hepatocyte
PAS	periodic acid-Schiff
PAS after diastase	periodic acid-Schiff after glycogen digestion by diastases
SFH	cell surface fluorescence hepatocyte
SGOT	serum glutamic oxaloacetic transaminase
SGPT	serum glutamate pyruvate transaminase
SHN	subacute hepatic necrosis

WOORDEN VAN DANK

De tot standkoming van dit proefschrift is niet in de laatste plaats te danken aan en een direct gevolg van de aanwezigheid van een groep zeer enthousiaste hepatologisch geinteresseeerde collega's in de Rotterdamse regio. Een enthousiasme dat tot uitdrukking komt in vele samenwerkingsverbanden op hepatologisch gebied, "de Rotterdamse leverclub" en gezamenlijk onderzoek.

Voor de jarenlange en inspirerende samenwerking dank ik allen. Klinische pathologie floreert niet zonder de aanwezigheid van een geinteresseerde en veeleisende kliniek.

In verband hiermee moeten in de eerste plaats de pioniers van de moderne Rotterdamse hepatologie worden genoemd: prof.dr. M. Frenkel, prof.dr. J.H.P. Wilson, dr. M. de Jong, dr. M. van Blankenstein en mijn collega patholoog-anatomen dr. S. Gratama, A.S.M. Jacobs en dr. A.P.R. Blok, in prettige samenwerking gesteund door de kennis en ervaring van prof.dr. V. Desmet uit Leuven. Aan Roeland Blok ben ik mijn interesse voor de leverpathologie verschuldigd; hij was het ook die mij stimuleerde tot de afronding van dit proefschrift. In deze begin fase werd mijn belangstelling voor hepatitis B gewekt door het klinisch-epidemiologisch onderzoek van "Australië" antigeen seropositieve patiënten van Marck van Blankenstein en dr. F.C.H.A. Kothe, die ik met name dank voor de gastvrijheid op de Rotterdamse bloedbank.

De pioniersfase werd gevolgd door een voortdurende periode van hepatologische bloei op onnavolgbare wijze gestimuleerd door prof.dr. S.W. Schalm samen met prof.dr. O.T. Terpstra en dr. R. Heijtink; voor de plezierige samenwerking en hun stimulerende invloed ben ik hun veel dank verschuldigd.

Vaak denk ik nog terug aan de plezierige samenwerking in de beginfase van het hepatitis onderzoek met dr. Jan Willem Steffelaar, dr. Marius Nap en dr. Ferry van Elven. Ook dank ik allen waarmee ik in de loop der jaren in prettige samenwerking hepatologisch onderzoek heb mogen doen.

Mijn promotor, prof.dr. R.O. van de Heul dank ik voor de vrijheid, die ik kreeg om mijn werkzaamheden en onderzoek naar eigen inzicht te mogen indelen en voor het overdragen van een deel van zijn kennis van de (oncologische) pathologie.

Dr. Th.M. Vroom dank ik speciaal voor de wijze waarop zij mij de voor dit proefschrift belangrijke immunohistochemische technieken bijbracht en voor de daaropvolgende blijvende samenwerking.

Voor de jarenlange samenwerking en vele uren kijken en discussie achter de fluorescentie microscoop dank ik vooral Johan van Lier en de andere analisten,

waarmee ik in de loop der jaren heb samengewerkt. Dr. Vojslav Vuzevski en Piet van der Heul hielpen mij bij het electronenmicroscopisch onderzoek. De foto's werden gemaakt door Paula Delfos. Mevrouw Bominaar dank ik voor de grote vasthoudendheid en nauwkeurigheid bij het uittypen van mijn veelal onleesbare schrijverijen. Dank voorts aan iedereen van de afdeling Pathologische-Anatomie, die op enigerlei wijze heeft bijgedragen aan het tot stand komen van dit proefschrift.

Het proefschrift werd op vakkundige wijze van misstappen in de Engelse taal gezuiverd door mw. G.P. Bieger-Smith. In zeer prettige samenwerking kwam de uiteindelijke vormgeving tot stand met de deskundige hulp van Erik Davids. De omslag van het proefschrift werd getekend door dhr. van Leeuwen.

Tenslotte was dit proefschrift nooit in druk verschenen zonder de inspanningen van Christine, die de P.C. en alle daaraan verbonden programma's feilloos onder de knie kreeg en alle tegenslagen wist op te lossen met hulp van Peter van Vuuren en Sjors Beens.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren in 1942 te Rotterdam. Hij behaalde in 1962 het eindexamen Gymnasium ß aan het Gymnasium Erasmianum te Rotterdam.

De medische studie werd gevolgd in Groningen met co-assistentschappen in Willemstad (Curaçao). Na assistentschappen in Sittard (gynaecologie) en Groningen (intensive care) werd in 1970 het artsexamen afgelegd.

Na als huisarts werkzaam te zijn geweest, werd van 1971-1975 de opleiding tot patholoog-anatoom gevolgd in het Academisch Ziekenhuis Dijkzigt te Rotterdam (hoofd: prof.dr.G. Wielenga)

Sinds 1975 maakt hij deel uit van de staf van de afdeling pathologische anatomie.