reproducible end point. Figure 3 shows the relation between ART titres and the percentage deflections from the baseline produced by the neat sera. The levelling off at higher titres is probably due to the total removal of reactive particles.

The results show that the modified ART produces more positive results than the RPR card test (Fig. 2). Of 49 sera positive by both methods, 18 (39%) showed higher titres with the modified ART. This may be due to the use of serum as a diluent for the modified ART or the increased sensitivity of the detection system.

Particle counting has demonstrated reaginic flocculation reactions in a continuous flow system,

and we have also carried out virus haemagglutination tests using similar apparatus. Further developments suggest that specific treponemal haemagglutination tests may be performed using this detector system.

References

- McGrew, B. E., DuCros, M. J. F., Stout, G. W., and Falcone, V. H. (1968). Automation of a flocculation test for syphilis. *American Journal of Clinical Pathology*, **50**, 52-59.
- Portnoy, J., Brewer, J. H., and Harris, A. (1962). Rapid plasma reagin card test for syphilis and other treponematoses. *Public Health Reports*, **77**, 645-652.

A direct immunofluorescence method for the detection of hepatitis B core antigen in formalin-fixed and gelatin-embedded liver specimens

H. YOSHIKAWA, Y. ITOH, Y. AKAHANE, F. TSUDA, Y. MIYAKAWA, and M. MAYUMI

From the Hepatitis Division, Tokyo Metropolitan Institute of Medical Science, and the Third Department of Internal Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Infection with hepatitis B virus (HBV) and the responses of the host to it are much more complicated than were previously thought. To date, at least three distinct antigen-antibody systems, that is, hepatitis B surface antigen (HB_sAg)—anti-HB_s, hepatitis B core antigen (HB_cAg)—anti-HB_c, and e antigen—anti-e, have been identified in the serum of individuals infected with HBV. The diagnostic and prognostic values of determining these markers in the serum have already been established (Almeida *et al.*, 1971; Magnus and Espmark, 1972; Hoofnagle *et al.*, 1973).

HB_sAg was demonstrated in the cytoplasm, and HB_cAg in the nucleus, of hepatocytes by immunofluorescence in unfixed frozen sections (Brzosko *et al.*, 1973). The immunofluorescence method has been successfully extended to formalin-fixed liver specimens for the demonstration of HB_sAg, and this has opened the way for retrospective studies of necropsy material (Huang *et al.*, 1976). We now report a simple method for detecting HB_cAg in formalin-

fixed liver specimens by a direct immunofluorescence method.

Material and methods

Liver tissue obtained at necropsy from patients with various hepatic disease and which had been fixed and stored in 10% formalin was used. Three blocks of about 1 cm³ size were cut, one from the left lobe, another from the right lobe, and the third from the centre of the liver. They were washed free of formalin in tap water for 12 hours. The specimens were then incubated in distilled water containing 0.01% (w/v) NaN₃, and 5% (w/v) gelatin (Difco Laboratories, Detroit, Mich.) at 37°C for 12 hours, and successively in water supplemented with 10% gelatin at 37°C for 4 hours. The specimens were transferred onto a Petri dish filled with 10% gelatin at 37°C, and the dish was allowed to consolidate at room temperature. The liver specimens were then cut from the gelatin plate and snap-frozen in n-hexane cooled in a dry ice/acetone bath.

Anti-HB_c was obtained from the serum samples of asymptomatic carriers of HBV containing a high titre of anti-HB_c (immune adherence haemagglutination titre of 2¹⁶ or higher). The gamma-globulin fraction was prepared by precipitation in 33%—saturated (NH₄)₂SO₄ solution followed by chromatography on DEAE cellulose. Anti-HB_c γ-globulin was then labelled with fluorescein isothiocyanate (FITC). Similarly, γ-globulin fractions of rabbit and human anti-HB_s antisera (passive haemagglutination titre of 1:20000 or higher) were isolated and labelled with FITC, and fluoresceinated anti-HB_s reagents were obtained.

Cryostat sections were cut at 4 μ and mounted on a slide glass with egg-white/glycerol (1:1). They were fixed by immersion in acetone for 1 minute at

Fig. 1 Staining of HB_eAg in a cryostat section of formalin-fixed and gelatin-embedded liver of a patient with cirrhosis by the direct immunofluorescence method. Positive immunofluorescence is observed exclusively in the nucleus of hepatocytes ($\times 450$)

Fig. 2 Staining of HB_sAg in a cryostat section of formalin-fixed and gelatin-embedded liver of the same patient as in Fig. 1 by the direct immunofluorescence method. Fluorescence is observed in the cytoplasm of hepatocytes. No nuclear fluorescence is seen ($\times 450$).

4°C. The fluoresceinated reagents were flooded onto the sections which were incubated at 37°C for 30 minutes. They were then rinsed in a phosphate buffer (0.01 M, pH 7.2) containing 0.15 M NaCl for two 5-minute periods. They were examined with a Leitz fluorescence microscope (Orthoplan, West Germany) operating at incident light excitation. The specificity of the staining was confirmed by blocking test, in which the slide was pretreated with corresponding, unlabelled antibody.

Results and comments

Figure 1 shows the staining of a formalin-fixed and gelatin-embedded liver specimen, obtained at necropsy from a case of liver cirrhosis, for HB_eAg by the direct immunofluorescence method. The fluorescence is exclusively localised in the nucleus. About 80% of the hepatocytes are positive with a clear bright fluorescence and virtually no background staining. In Fig. 2 the staining of HB_sAg in the liver specimen of the same patient is seen. It is localised in the cytoplasm and no nuclear fluorescence is observed. The liver biopsy specimens were divided into two parts, one of which was sectioned after fixation in formalin and gelatin embedding, and the other was sectioned unfixed but gelatin embedded; the detectability of both HB_eAg and HB_sAg by the direct immunofluorescence method was similar in both specimens.

It may be helpful to determine the presence and distribution of hepatitis B antigens *in situ*, since variable morphological manifestations of type B hepatitis are thought to be induced by immune responses of the host to them (Gudat *et al.*, 1975). Both HB_sAg and HB_eAg have been successfully demonstrated by direct and indirect immunofluorescence methods in unfixed liver biopsy specimens. Attempts have been made to demonstrate them in formalin-fixed, paraffin-embedded liver specimens which had been stored for an indefinite period. Huang (1975) described a method to detect HB_sAg in formalin-fixed paraffin sections of the liver. The remarkable stability of HB_sAg, which is reported to be of a lipoprotein nature, is apparently responsible for his success. He also tried to demonstrate HB_eAg in paraffin sections of the liver and he noted that HB_eAg staining by direct immunofluorescence was much less sensitive than that by the indirect method. The practical usefulness of his indirect method seems to be restricted, however, by non-specific staining and by false positive results due to immunoglobulins in hepatocytes. To cope with these difficulties, the pretreatment of paraffin sections by trypsin digestion has recently been introduced (Huang *et al.*, 1976).

The basis for the success of a simple, one-step staining for HB_eAg in formalin-fixed liver specimens reported here is apparently the embedding process for which we used gelatin, avoiding the dehydration process for embedding in paraffin with subsequent deparaffinisation and rehydration of paraffin sections, all of which involve treatment with organic solvents such as ethanol, chloroform, and xylene. Our direct immunofluorescence method performed on formalin-fixed and gelatin-embedded liver specimens, for both HB_eAg and HB_sAg, is at least as sensitive as that performed on unfixed, frozen sections. In addition, when formalin-fixed gelatin sections are stained by haematoxylin and eosin for routine morphological observations, a much better resolution is obtained than when the same staining is done on unfixed, frozen sections, making the comparison and integration of the results by fluorescent and routine stainings on serial sections of the liver much easier. We hope the present method will find a number of applications in attempts to elucidate the pathogenesis of hepatic diseases induced by HBV infections.

This work was supported in part by the grants from Tokyo Metropolitan Government and the Japanese Ministry of Health and Welfare.

References

- Almeida, J. D., Rubenstein, D., and Stott, E. J. (1971). New antigen-antibody system in Australia-antigen-positive hepatitis. *Lancet*, **2**, 1225-1227.
- Brzosko, W. J., Madalinski, K., Krawczynski, K., and Nowoslawski, A. (1973). Duality of hepatitis B antigen and its antibody. I. Immunofluorescence studies. *Journal of Infectious Diseases*, **127**, 424-428.
- Gudat, F., Bianchi, L., Sonnabend, W., Thiel, G., Aenishaenslin, W., and Stalder, G. A. (1975). Pattern of core and surface expression in liver tissue reflects state of specific immune response in hepatitis B. *Laboratory Investigation*, **32**, 1-9.
- Hoofnagle, J. G., Gerety, R. J., and Barker, L. F. (1973). Antibody to hepatitis-B-virus core in man. *Lancet*, **2**, 869-873.
- Huang, S.-N. (1975). Immunohistochemical demonstration of hepatitis B core and surface antigen in paraffin sections. *Laboratory Investigation*, **33**, 88-95.
- Huang, S.-N., Minassian, H., and More, J. D. (1976). Application of immunofluorescent staining on paraffin sections improved by trypsin digestion. *Laboratory Investigation*, **35**, 383-390.
- Magnius, L. O., and Espmark, J. A. (1972). New specificities in Australia antigen positive sera distinct from le Bouvier determinants. *Journal of Immunology*, **109**, 1017-1021.