Immunofluorescence detection of new antigen-antibody system (δ/anti-δ) associated to hepatitis B virus in liver and in serum of HBsAg carriers

M. RIZZETTO,[1] M. G. CANESE, S. ARICÒ, O. CRIVELLI, C. TREPO, F. BONINO, AND G. VERME

From the Department of Gastroenterology, Ospedale Mauriziano Umberto I, Turin, Italy, the Electron Microscopy Centre of the Faculty of Medicine, University of Turin, Italy, and INSERM U45, and Laboratory of Hygiene, University Claude Bernard, Lyon, France

SUMMARY   A new antigen-antibody system associated with the hepatitis B virus and immunologically distinct from the HB surface, core, and *e* systems is reported. The new antigen, termed δ, was detected by direct immunofluorescence only in the liver cell nuclei of patients with HBsAg positive chronic liver disease. At present, the intrahepatic expression of HBcAg and δ antigen appears to be mutually exclusive. No ultrastructural aspect corresponding to the δ antigen could be identified under the electron microscope. δ antibody was found in the serum of chronic HBsAg carriers, with a higher prevalence in patients with liver damage. The nuclear fluorescence patterns of HBcAg and δ antigen were similar; it is only possible to discriminate between the two antigens by using the respective specific antisera.

While studying liver biopsies from patients who were seropositive for the hepatitis B surface antigen (HBsAg) in direct immunofluorescence, it was noted that an antiserum against the hepatitis B core antigen (HBcAg), as well as staining specimens in which core particles could be demonstrated by the electron microscope (EM), also reacted with additional biopsies which did not contain core particles (at electron microscopy) and were negative with other reference antisera against HBcAg.

When the EM core positive and core negative specimens were tested with several HBsAg positive sera, it soon became apparent that some sera reacted with either one or the other liver substrate; this suggested that there were two distinct nuclear antigenic specificities.

The identification of this new antigen and of its antibody as an immunological system independent of other known reactions associated with the HB virus is reported in this communication. Provisionally, we propose that it should be called δ.

[1]Address for correspondence: Dr M. Rizzetto, Department of Gastroenterology, Ospedale Mauriziano Umberto I, Cs. Turati 46, 10128 Turin, Italy.

Methods

PREPARATION OF STANDARD FLUORESCENT ANTISERA AGAINST δ ANTIGEN (δ ANTISERUM), AGAINST HBCAG (HBC ANTISERUM), AGAINST HBSAg (HBS ANTISERUM), AND AGAINST *e* ANTIGENS (e_α + e_β ANTISERUM), STANDARD δ ANTIGEN (δ) AND HBCAG POSITIVE LIVER SUBSTRATES

A fluorescein isothyocianate (FITC) conjugated antiserum against HBsAg was prepared from Beheringwerke rabbit precipitating serum RBBO4 (Rizzetto *et al.*, 1976b). A FITC conjugated antiserum against *e* antigens (e_α + e_β) was prepared from a human serum as previously described (Trepo *et al.*, 1976).

A FITC antiserum monospecific against HBcAg and one monospecific against δ were prepared from the blood of two apparently healthy HBsAg carriers; both sera were negative when tested by the Reuma and Waaler-Rose techniques. The gamma globulin fractions, isolated after precipitation with $(NH_4)_2SO_4$, did not contain autoantibodies (in indirect Immunofluorescence (IFL)), antibodies against HBsAg, *e* antigens, or *e* antibodies.

After conjugation with FITC, the HBc antiserum

reacted at a titre of 1 in 320 with substrates in which core particles had been detected by the electron microscope and with a reference core positive liver specimen (kindly provided by Dr K. Krawczynsky, The National Institute of Hygiene, Warsaw, Poland).

FITC conjugated δ antiserum did not react with the reference HBcAg positive substrate but gave an identical nuclear fluorescence pattern on livers from HBsAg carriers which did not contain core particles under the electron microscope. Such livers were considered as δ specific substrates.

Each antiserum was absorbed with rat, pig, and human HBsAg negative livers, with immuno-globulins from an HBsAg negative serum and with blood cells from individuals of group AB, and tested in direct IFL against rat liver, kidney, and stomach and against human surgical and post mortem specimens of pancreas, thyroid, stomach, colon, adrenal, ovary, testis, and brain.

To perform IFL screening for HBc and δ antibodies, a standard HBcAg and a standard δ positive liver were obtained at necropsy from two HBsAg positive patients who died from cirrhosis; several blocks of each liver were immediately frozen. The standard HBcAg and δ substrates were tested in direct immunofluorescence (DIFL) with HBs, $e_\alpha + e_\beta$ and δ antisera and with a reference HBc antiserum (kindly provided by Dr K. Krawczynsky), and in indirect IFL with a HBc chimpanzee antiserum (kindly provided by the NIH, Bethesda, USA).

DEMONSTRATION OF SPECIFICITY OF δ FLUORESCENCE

To exclude the possibility of anti-IgG reactivity, the standard antiserum was tested by DIFL with kidney glomeruli and HBcAg positive liver nuclei containing bound IgG, demonstrated by mono-specific FITC anti-human IgG rabbit antisera. Immunofluorescence, blocking, and absorption experiments were carried out to assess the specificity of the δ reaction. δ antiserum was tested in DIFL on substrates containing cytoplasmic HBsAg, cytoplasmic e_β and nuclear e_α antigens (Trepo *et al.*, 1976).

In the blocking tests, the standard δ positive substrate was covered for 30 minutes with high titre human and chimpanzee HBc antisera, with human and rabbit HBs antisera, and with human sera strongly reacting against the e_α and the e_β antigens by immunodiffusion (ID), and subsequently tested in DIFL with the standard δ antiserum.

Absorption experiments were carried out using (1) an HB core rich fraction prepared according to the method of Huang (Huang and Groh, 1973) by pronase digestion of an HBcAg positive liver well

characterised by IFL and electron microscopy studies; (2) e_α and e_β antigen enriched fractions obtained from human sera as previously reported (Trepo *et al.*, 1976); (3) anti-human IgG (Behering-werke TNYO5). These three preparations were able to abolish (1) HBcAg, (2) e_α and e_β, and (3) any fluorescence when incubated for one hour at 37° and overnight at 4° with the respective antisera. The standard δ antiserum, titred to end point, was incubated (1/5 VV) for one hour at 37° and over-night at 4° with the above preparations and sub-sequently tested by DIFL on the standard δ substrate.

BIOPSY SCREENING FOR HBcAg, HBsAg, IgG DEPOSITS AND IN VITRO COMPLEMENT (C) FIXATION CAPACITY (VCF) BY IMMUNO-FLUORESCENCE (IFL)

One hundred and thirty-seven liver biopsies were retrospectively studied: 54 from HBsAg sero-negative patients with a variety of liver disorders, and 83 from HBsAg sero-positive patients whose histological diagnosis is reported in Table 1.

Table 1 *Incidence of δ in liver of 83 HBsAg sero-positive individuals with and without liver disease, divided in disease categories compared with incidence of HBcAg in same groups*

Histological diagnosis	Number of patients examined	δ positive	HBcAg positive	δ + HBcAg positive
Asymptomatic chronic carriers with normal liver histology	18	—	—	—
Acute hepatitis	20	—	1	—
Chronic persistent hepatitis	13	6	2	—
Chronic active liver disease	23	5	10	—
Inactive cirrhosis	9	2	4	—
Total	83	13	17	0

Gastric and rectal biopsies from two patients with a positive δ reaction in their livers were also available for IFL studies.

Each specimen was tested in direct immuno-fluorescence for the presence of the δ antigen, HBcAg, and HBsAg with the respective mono-specific fluorescent antisera, for the presence of immunoglobulins deposits with antihuman IgG, A, M commercial antisera (Behering-Hoechst), and for the capacity to fix C *in vitro*, as previously described (Rizzetto *et al.*, 1976a).

To study the relationship between δ or HBcAg positive hepatocytes and those positive for IgG or VCF, serial sections were stained alternatively with the δ or HBc FITC conjugated antisera and with rodamine conjugated antihuman IgG or C_3 antisera,

as previously described (Rizzetto et al., 1976a).

For additional experiments, sections were also fixed with absolute methanol, ethanol, and acetone.

SEROLOGICAL SCREENING FOR HBSAg AND *e* ANTIGEN (*e*) AND FOR ANTIBODIES AGAINST HBSAg (HBSAb), AGAINST δ (ANTI-δ), AGAINST HBCAg (HBCAb) AND AGAINST *e* (ANTI-*e*)

HBsAg and HBsAb were detected in radioimmunoassay (by courtesy of Dr Peyretti, The Blood Bank, Turin).

As circulating δ was never found by ID in the serum of patients with and without δ in their livers, δ antibody had to be detected by IFL; because of intrinsic IgG background fluorescence in the standard substrate, only direct immunofluorescence could be used. With this method the frequency of anti-δ was assessed in the sera of 60 patients, whose immunological and clinical features are reported in Table 2.

Table 2 *Incidence of anti-δ in sera of 60 patients with and without serological evidence of exposure to HB virus, divided in disease categories and incidence of HBc antibodies in same groups*

Serological status		Number of patients examined	Diagnosis	Anti-δ positive sera	HBcAb positive sera	Anti-δ + HBcAb positive sera
HBsAg	HBsAb					
—	—	11	Normal controls	—	—	—
—	+	12	Chronic liver disease	—	—	—
+	—	17	Asymptomatic carriers with normal livers	2	9	1
+	—	8	Acute hepatitis	—	2	—
+	—	12	Chronic liver disease	6	3	—

Aliquots of the gamma globulin fractions of the sera under study were left overnight dialysing in a bath containing FITC 0·1 mg/ml in carbonate bicarbonate buffer pH 9, dialysed thereafter against phosphate buffered saline for 48 hours, absorbed with rat and human HBsAg negative liver powders, and tested in direct IFL for anti-δ and HBcAb on the positive standard substrates. Parallel indirect IFL tests for HBcAb were carried out with 1/10 diluted sera on the reference HBcAg positive substrate, which did not exhibit any background intrinsic IgG fluorescence.

e and anti-*e* were detected in double immuno-

diffusion in agarose (Magnius and Espmark, 1972) against standard anti-*e* and *e*.

ELECTRON MICROSCOPY

The standard HBcAg and δ positive livers and four biopsies positive for δ in IFL were studied with the electron microscope.

Some samples from each biopsy were fixed for two hours in 3% buffered glutaraldehyde, postfixed for two hours in 1% osmium tetroxide and then dehydrated with ethanol and embedded in Araldite (Durcupan ACM Fluka); other samples were fixed for one hour in 4% phosphate buffered paraformaldehyde, prepared according to Karnowsky (Karnowsky, 1965), dehydrated with acetone, and embedded in Araldite or Epon-Araldite. Postfixation with osmium was omitted after paraformaldehyde fixation. Ultra-thin sections from the fixed blocks were stained with uranil acetate and lead citrate.

To localise δ positive nuclei, electron microscopy was carried out on ultra-thin sections serial to semi-thin ones stained in IFL with δ anti-serum (Canese and Bussolati, 1974).

Results

SPECIFICITY OF δ ANTIGEN-ANTIBODY SYSTEM

δ antiserum stained only liver biopsies from HBsAg seropositive patients (Table 1); 54 liver specimens from HBsAg seronegative subjects, rectal and gastric biopsies from two patients with a positive δ reaction in their livers, and all the animal and human substrates that were tested were negative.

Fluorescence was always limited to the nuclei of the hepatocytes, the proportion of positive cells varying from a few to over 80% in the section. The staining usually appeared to be diffuse and reticular, and was only occasionally granular with brighter areas at the periphery of the nuclei; nucleoli remained unstained (Figure). Liver cell cytoplasms and Kuppfer cells never stained.

Monospecific human and animal antisera against HBsAg, HBcAg, and *e* (e_α + e_β) did not stain the nuclei of any δ positive specimen, while anti-δ never stained HBcAg positive biopsies, either one or the other determinant being expressed in the liver.

Fluorescence was not blocked by previous incubation of the δ positive substrate with HBs, HBc, e_α + e_β anti-sera, or abolished after absorption of δ antiserum with fractions containing e_α, e_β and core particles in high concentrations; conversely, it was completely abolished after absorption with antihuman IgG serum.

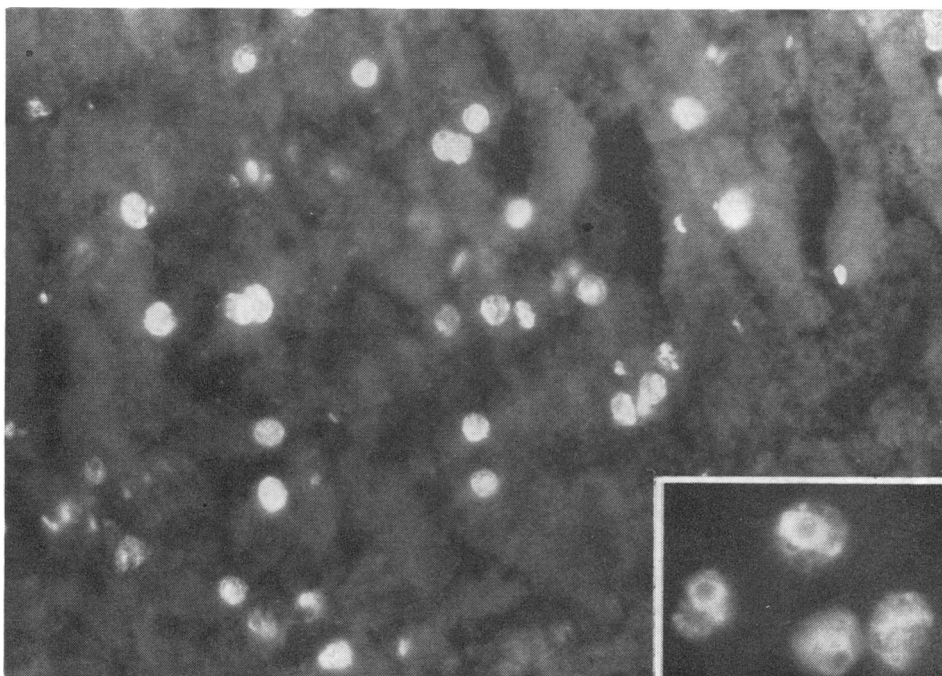Most of the δ positive specimens reacted also

Figure   δ nuclear fluorescence pattern (× 250) in the post-mortem liver from a patient with active cirrhosis. Inset: same case, nuclear fluorescence, more evident in the perinucleolar membrane.

with human IgG antisera and all of them exhibited a strong in vitro C fixing capacity (Table 3); both reactions were localised in the same nuclei containing the δ determinant, as demonstrated by double staining experiment with FITC and RITC labelled conjugates.

Slide fixation with absolute methanol denatured the antigen and the IgG reacting site to a variable degree, fixation with acetone or ethanol for up to 30 minutes did not affect either of them.

ELECTRON MICROSCOPY
The nuclei of the standard HBcAg positive liver, fixed with glutaraldehyde and formaldehyde, were

Table 3   Clinical, histological, and immunological features in 13 patients with δ positive, HBcAg negative reactions in their livers

Case no.	Age (yr)	Sex	Therapy	Histological diagnosis	Positive nuclei in biopsy (no.)	IFL on liver biopsy			Serum	
						HBsAg	IgG deposits	VCF	e	anti-e
1	43	M	S	CPH	+ + +	−	+	+ + +	+	−
2	35	M	A + S*	CPH	+	−	−	+	−	−
3	23	F	A + S*	CPH	+	−	−	+	+	−
4	35	F	S*	CPH	+ +	−	±	+ +	+	−
5	46	F	−	CPH	+	−	+	+	−	−
6	22	M	−	CPH	+ +	+	±	+ +		
7	42	M	A + S	CALD	+ + +	−	±	+ + +		−
8	64	M	S	CALD	+ + +	−	±	+ + +	−	−
9	53	F	A + S	CALD	+ + +		+	+ + +		
10	61	M	S	CALD	+ + +		+	+ +		−
11	18	F	A + S*	CALD	+ +		−	+ +		−
12	60	F	−	IC	+	−	−	+	−	−
13	52	F	−	IC	+		−	+	−	−

S: steroids. A: azathioprine. *Treatment discontinued one to six months before biopsy.
CPH: chronic persistent hepatitis. CALD: chronic active liver disease. IC: inactive cirrhosis.

filled with several round particles approximately 230 A° in diameter; they were considered to be Dane particle cores.

After extensive ultrastructural search, no core particles could be visualised in the standard δ substrate or in the four δ positive liver biopsies.

PREVALENCE OF δ NUCLEAR FLUORESCENCE IN HBSAg CARRIERS: HISTOLOGICAL AND IMMUNOLOGICAL FEATURES OF PATIENTS WITH δ POSITIVE BIOPSIES

The incidence of δ and HBcAg nuclear fluorescence in 83 HBsAg carriers is reported in Table 1.

δ positive nuclei were observed in 13 patients with chronic liver disease; their histological diagnosis varied from a chronic persistent hepatitis to an inactive cirrhosis (Table 3).

Five patients were being treated with steroids or azathioprine at the time of the biopsy; in four treatment had been discontinued one to six months previously, while four had never received immunosuppressive therapy. The highest number of δ positive nuclei was observed in the immunosuppressed patients, the lowest in those who had never been so treated.

Scattered cytoplasmic HBsAg was observed in four biopsies, e antigen was found in the serum of five out of 11 patients with δ in their liver, anti-e in none of them.

Biopsies were negative from patients with acute type B viral hepatitis or from chronic HBsAg carriers with normal livers.

INCIDENCE OF δ ANTIBODIES IN PATIENTS WITH AND WITHOUT SEROLOGICAL EVIDENCE OF HB VIRUS EXPOSURE

The occurrence of anti-δ and HBcAb in patients with and without serological evidence of contact with the HB virus is reported in Table 2.

Two asymptomatic HBsAg chronic carriers and six HBsAg positive patients with chronic liver disease had circulating anti-δ; three patients in the latter group had δ in their livers (nos. 2, 3, 7, Table 3) and one of the asymptomatic carriers also had HBcAb in his serum.

Discussion

A new antigen-antibody system, distinct from the HBs, HBc, and e systems and called δ, is present in the liver and in the blood of HBsAg carriers. δ antigen was identified by chance, after the observation that an HBcAb positive serum gave a nuclear staining reaction with HBsAg positive specimens in which no core particles could be seen after extensive ultrastructural search.

The new antigen is specific for HB virus infection and was localised in IFL only in the liver cell nuclei of patients with chronic HBsAg seropositive liver disease; patients with HBsAg seronegative disorders, asymptomatic chronic carriers, and those with acute self-limited hepatitis were constantly negative.

Immunofluorescence with antisera against the different HB virus determinants and blocking and absorption experiments demonstrated the specificity and independence of the new antigen. Monospecific HBc and HBs antisera did not stain the nuclei of δ positive substrates, only HBsAg cytoplasmic fluorescence occurring occasionally in these biopsies, while, conversely, δ antiserum never stained HBcAg positive substrates. This suggested mutual exclusion of the intrahepatic expression of the δ and core determinants.

HBsAg positive sera containing precipitating antibodies to e_α and e_β did not stain δ positive substrates, thus demonstrating that the new antigen was also independent of e; this was further confirmed by absorption and blocking experiments with partially purified e_α and e_β antigens and antibodies.

No morphological counterpart of the new antigen could be identified by electron microscopy, only granular chromatin clumps being visualised in thin sections serial to others in which corresponding nuclei containing δ could be identified with certainty in IFL.

Material reacting immunologically as human IgG and fixing complement was present in the hepatocytes containing the δ antigen; whether it represents circulating immunoglobulins which entered the cell and formed immune complexes or an intrinsic property of a strong anticomplementary antigen is at present uncertain.

A type of nuclear staining and an IFL distribution pattern similar to the δ and identical IgG and C fixing reactions are known to occur (Arnold *et al.*, 1975; Gerber *et al.*, 1976; Rizzetto *et al.*, 1976a) and were observed in this study in the HBcAg positive biopsies; morphological differentiation of the two antigens is therefore impossible, their recognition being permitted only by the knowledge of the specificity of the challenging antiserum.

As δ antibodies may be present or absent in sera containing HBcAb, screening of liver specimens for the core determinant with HBcAb positive sera has either missed the new reactivity or confused it with HBcAg. We have previously reported strong IgG reactions and C fixing capacity in some biopsies which did not stain with HBc antiserum and explained this as masking of HBc reactive sites by complexed IgG antibodies (Rizzetto *et al.*, 1976a); subsequent testing of these specimens with δ antiserum has shown a positive nuclear reaction,

demonstrating that previous negative results after HBc antiserum represented genuine absence of the core antigen, the IgG and C fixing reactions probably being caused by the hitherto unknown δ antigen.

Recently, the e_α antigen has also been localised in the liver cell nuclei of HBsAg seropositive subjects (Trepo et al., 1976), adding further difficulties in the interpretation of immunofluorescence; HBcAb and anti-e occur often together, and although anti-e was never found together with anti-δ in this study, the limited number of sera tested does not allow us to exclude with certainty the possibility that they may occasionally be associated.

A retrospective survey of 137 biopsies with monospecific antisera revealed an incidence of δ similar to that of HBcAg.

An overlap was also observed between the histological and immunological features of δ positive subjects and those reported in HBcAg positive patients (Gudat et al., 1975; Arnold et al., 1976; Bonino et al., 1976; Ray et al., 1976); a chronic lesion was always observed by histology in their livers, only e, not anti-e, being present in 45% of their sera.

The majority were, or had been, receiving immunosuppressive therapy and fluorescence was strongest in individuals still under treatment; the significance of this association is at present uncertain.

The incidence of anti-δ could be assessed only in a limited number of patients. Indirect IFL proved impossible for the intrinsic background IgG reaction observed in all the positive substrates with enough material available, and attempts to destroy selectively the IgG reaction by fixatives were unsuccessful, the antigen site being also denatured.

Sera were to be tested in direct IFL after conjugation with FITC, a time-consuming procedure which did not allow a large-scale study of the antibody distribution.

Anti-δ was found only in HBsAg positive sera, with a highest incidence in patients with liver disease; an inverse correlation was noted between its titre and that of HBcAb, one antibody usually excluding the other.

Anti-δ was found in several patients with the δ antigen in their liver; in analogy with the HB core system, anti-δ does not imply clearance of the antigen nor resolution of infection and might represent another serological marker of a stage of viral replication different from that identified by HBc antibodies (Hoofnagle et al., 1973).

The nature of the new antigen is obscure; its occurrence in the same type of disorders in which HBcAg is detected and the reputation of the Dane particle as the HB virus unit capable of inducing

the lesion observed in δ positive patients (Nielsen et al., 1973) suggest that the antigen is closely related to the core particle or to the chain of events preceding its assembly or after its clearance. DNA polymerase, circular DNA molecule, and DNA polymerase product are tentative candidates; each of them, however, has been located only inside a mature core envelope (Robinson, 1976), while to be the δ antigen they should exist free and uncovered in the nucleoplasm. Whether this is the case or whether the new antigen is unrelated to any of the known HB virus components is at present under study.

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