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An enzyme-linked immunosorbent assay (ELISA) for the detection of IgG and IgM anti-idiotypes directed against anti-HBs molecules

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A simple and specific enzyme-linked immunosorbent assay (ELISA) has been developed to detect circulating IgG and IgM anti-idiotypic antibodies directed against anti-HBs molecules using 96-well polyvinyl microtitre plates as the solid phase and HRPO-labelled goat anti-HBs as conjugate. Anti-idiotype reactions were observed in the supernatant portion after precipitation of immune complexes from sera with polyethylene glycol 6000 (PEG). Both IgG and IgM with anti-idiotype activity were detected concurrently in HBsAg-positive sera from HBV-infected patients and asymptomatic HBV carriers. Anti-idiotype activity was absent in HBsAg-negative sera from healthy persons, and in patients with non-A, non-B hepatitis and viral hepatitis A. However, such antibodies could be demonstrated in the sera of two out of eight HBsAg vaccine recipients negative for anti-HBs but in none of 11 recipients positive for anti-HBs after receiving a booster immunising dose of HBsAg vaccine. Those sera showing positive anti-idiotype reactions were free from rheumatoid factor and HBsAg/IgM or HBsAg/IgG complex activity.

An analysis of anti-idiotype positive sera for anti-HBs, HBeAg and HBV-specific DNA-polymerase activity demonstrated these markers in 20%, 30% and 60% of cases, respectively. The presence of anti-idiotypic antibodies was presumed to permit a more active multiplication of hepatitis B virus.

Key words: ELISA; Anti-idiotype, IgG; Anti-idiotype, IgM; Hepatitis B virus; Anti-HBs, goat

Introduction

The regulation of host immune responses by idiotypic-anti-idiotypic (Id-anti-Id) interactions has been documented in experimental animals (Eichmann and Rajewsky, 1974; Robertson, 1977; Woodland and Cantor, 1978; Bona and Paul, 1979). Although little is known about the occurrence of auto-anti-Id antibodies in the course of the normal human immune response, the presence of these antibodies in man has been shown in association with certain diseases, such as, systemic lupus erythematosus (Abdou et al., 1981), in patients with mixed cryoglobulins (Geltner et al., 1980) and in IgA-deficient individuals (Cunningham, 1981). More recently, Troisi and Hollinger (1985) have demonstrated anti-idiotypic antibodies directed against anti-HBs during viral hepatitis B infection. This is probably the first report of an anti-Id response during a natural viral infection and may be very helpful in understand-

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Abbreviations: HBV, hepatitis B virus; anti-Id, anti-idiotypic antibodies; HRPO, horseradish peroxidase enzyme; anti-HBs, antibody against HBsAg.

ing the mechanism of liver damage during active disease and the basis of regular viral multiplication in the carrier state. A detailed study of anti-Id in sera from different stages of HBV infection is still awaited.

The aim of the present study was to develop an enzyme-linked immunosorbent assay (ELISA) to detect anti-Id antibodies to anti-HBs in the serum samples from hepatitis B patients with the long term objective of understanding the regulatory role of these antibodies in the immunopathogenesis of hepatitis B virus (HBV) disease.

Materials and methods

Sera

HBsAg-positive sera were obtained from patients with acute viral hepatitis (40), fulminant hepatitis (40), chronic active hepatitis (8), subacute viral hepatitis (10) and asymptomatic HBsAg carriers (52). HBsAg-negative sera used in the present study were from non-A, non-B hepatitis (26), viral hepatitis A (15) and healthy persons (57) negative for all hepatitis A and B markers. In addition, 19 HBsAg vaccine recipients who were negative for HBsAg, were also bled immediately after a booster immunization dose of vaccine and sera obtained for anti-Id analysis. The diagnosis of hepatitis A was based on IgM anti-HAV positivity in the sera. The patients with acute viral hepatitis, fulminant hepatic failure, subacute hepatitis and chronic active hepatitis were diagnosed using accepted clinical, biochemical and, occasionally, histological criteria (Trey and Davidson, 1970; Krishnamurthy et al., 1976; Tandon et al., 1982, 1983) (Table I). The presence of HBV infection was established by the detection of anti-HBc IgM and absence of IgM anti-HAV in the sera of patients with acute diseases such as AVH, FHF and SAFH and the presence of HBsAg and HBV-specific DNA polymerase in the sera of patients with chronic active hepatitis. All cases of chronic active hepatitis were biopsy-proven. The diagnosis of non-A, non-B hepatitis was established by exclusion criteria. Rabbit anti-human IgG (γ-chain specific; 4 mg/ml) and anti-human IgM (μ-chain specific; 4 mg/ml) were purchased from Dakopatts, Denmark.

Hepatitis B assays

HBsAg was detected in sera by the micro-ELISA technique of Gandhi and Tandon (1984). HBeAg, anti-HBe and anti-HBs were detected using commercial EIA kits from Abbot Lab., U.S.A. Hepatitis B virus (HBV)-specific DNA-

TABLE I

BIOCHEMICAL, SEROLOGICAL AND HISTOLOGICAL STATUS OF DIFFERENT HBV-INFECTED PATIENTS

Group	Number	SGOT	SGPT	HBsAg positive	IgM anti-HBc positive	IgM anti-HAV positive	Histology positive
(1) Acute viral hepatitis	40	10 N [a]	20 N	40	40	0	20
(2) Fulminant hepatitis	40	15 N	30 N	40	40	0	31 (Post-mortem)
(3) Chronic active hepatitis	8	3 N	5 N	8	3	0	8
(4) Subacute viral hepatitis	10	4.5 N	8 N	10	10	0	6 (Post-mortem)
(5) Healthy HBV carriers	52	1 N	1 N	52	ND [b]	ND	ND

[a] Normal value for transaminases.
[b] Not determined.

polymerase activity was assayed according to the method of Fang et al. (1981).

Precipitation of immune complexes

The removal of HBsAg/IgM or HBsAg/IgG immune complexes before the analysis of sera for anti-Id was carried out by polyethylene glycol precipitation of sera according to the procedure of Anh-Tuan and Novak (1980). In brief, the serum sample was diluted 1 : 10 in 0.1 M boric acid containing 0.025 M borax and 0.075 M NaCl (pH 8.4, BBS). To 1 vol. of diluted serum, 1 vol. of 3.5% polyethylene glycol 6000 (PEG) in BBS was added. The mixture was incubated for 5 h at 20°C followed by 13 h at 4°C and then the precipitate was pelleted at 1600 × *g* for 60 min at 5°C. The clear supernatant was decanted into the clean tubes for anti-Id testing and the pellet washed twice with BBS containing 1.75% PEG before it was solubilised in BBS. In order to confirm the removal of complex by PEG treatment a separate experiment was designed. HBsAg/IgM or HBsAg/IgG complex activity in serum samples was determined by the ELISA procedure of Gandhi et al. (1986). Two HBsAg/IgM complex positive sera (with OD ratio values of 16.5 and 18.5, where the OD ratio represents the ratio of OD of test serum and negative control serum) were first treated with 3.5% PEG as described above and then complex activity was determined in both supernatant and precipitate after dissolving the latter in an equivalent volume of PBS. The complex activity in the supernatants, as determined by ELISA (OD ratios: 0.98 and 0.86, respectively) was found to be nil. However, the resolubilised precipitates contained significant amounts of complex activity (OD ratio: 15.8 and 17.8, respectively). Identical results were obtained with HBsAg/IgG positive sera suggesting that PEG treatment completely removes immune complexes from serum.

ELISA procedure

A solid-phase ELISA method was developed to detect circulating IgG and IgM anti-idiotype antibody against anti-HBs molecules. The details of the assay system were as follows: 96-well polyvinyl microtitre plates from Nunc were activated by exposure to UV light for 3 h before the assay. After activation, 50 μl of rabbit anti-human IgG or IgM diluted 1 : 5000 in 0.1 M carbonate buffer, pH 9.6, were added to each well to coat the plates. Plates were incubated for 20 h at 25°C and then washed three times (for 5 min) with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T).

Additional binding sites were saturated by adding 200 μl of 0.5% gelatin in PBS to each well and incubating plates for 2 h at 37°C followed by 20 h at 4°C. The plates were washed 2–3 times with PBS-T and then 50 μl of the supernatant obtained after PEG precipitation of the serum was added to each well for anti-idiotype testing. An identical volume of 3.5% PEG in BBS (as a negative control) was also added simultaneously. Incubation was carried out at 37°C for 4 h followed by washing as above. Finally, 50 μl of HRPO-labelled goat anti-HBs (Abbots, U.S.A.) were added to each well, incubation was continued for 2 h at 37°C and the wash cycle repeated. After washing, enzyme activity was measured by adding 50 μl of freshly prepared substrate solution (0.4 mg of *o*-phenylenediamine per ml of 0.1 M phosphate citrate buffer, pH 5.0 containing 0.06% H_2O_2) to each well and incubating the plate in the dark at 20°C for 10 min. The enzyme reaction was stopped by adding 50 μl of 4 N H_2SO_4 and absorbance read at 492 nm. Each sample was tested in duplicate. Based on statistical analyses of presumably negative samples, a cutoff point equivalent to mean plus two standard deviations of such absorbance values was calculated. Any test sample giving an absorbance value greater than the cutoff point was considered positive.

Inhibition assay

The specificity of the present ELISA method was evaluated in an inhibition assay. Sera positive for IgG and IgM anti-idiotype activity against anti-HBs were treated with PEG, as above, and the supernatants re-evaluated in an inhibition assay in which various concentrations of horse anti-HBs (Wellcome) were added to the test wells 3 h before incubation at 37°C with HRPO-anti-HBs as conjugate. Non-immune serum from the same species was used as a negative control reagent and the percent inhibition calculated using the absorbance measured in wells without horse anti-HBs as 100%.

Other assays

HBsAg/IgM or HBsAg/IgG complexes were detected by the ELISA technique of Gandhi et al. (1986) after dissolving pellets in 0.1 M PBS, pH 7.4. Rheumatoid factor activity was evaluated in supernatants as described by Palla et al. (1983).

Results

A total of 150 HBsAg-positive and 111 HBsAg-negative sera were analysed for IgG- and IgM-type anti-idiotypic antibodies directed against anti-HBs. Anti-idiotypic antibodies were detected in only HBsAg-positive sera. The prevalence of such anti-Id antibodies in HBsAg-positive sera from patients with different HBV infections and asymptomatic carriers is shown in Table II. Both IgG and IgM anti-Id antibodies were detected simultaneously except for two sera in which only IgG was present. Anti-Id antibodies were found in higher proportions in sera from healthy HBsAg carriers and in patients with chronic active hepatitis than in other groups. None of the sera from patients with fulminant hepatitis contained anti-idiotype activity. Nor were anti-idiotype antibodies detected in the sera from 26 patients with non-A, non-B hepatitis and 15 patients with viral hepatitis A. Of 19 HBsAg-vaccine recipients, 11 developed anti-HBs after a booster dose of vaccine whereas eight persons did not produce detectable levels of anti-HBs. Only IgG-type anti-idiotypic antibody was detected in the sera from two out of eight anti-HBs-negative and none of the anti-HBs-positive recipients.

The present ELISA method was highly specific.

TABLE III

EFFECT OF HORSE ANTI-HBs ON THE BINDING BETWEEN ANTI-IDIOTYPIC ANTI-HBs AND HRPO-LABELLED GOAT ANTI-HBs (CONJUGATE)

The horse anti-HBs (Wellcome, 4 mg/ml) was diluted in PBS and preincubated before adding HRPO goat anti-HBs in the ELISA assay. Identical assays were run with a plate coated with anti-human IgM.

Horse anti-HBs (dilutions)	% Inhibition of binding activity	
	IgG anti-idiotype (%)	IgM anti-idiotype (%)
1 : 200	25.8	20.9
1 : 100	40.5	42.3
1 : 50	62.4	58.5
1 : 20	73.2	69.2
1 : 10	85.4	77.4

This was revealed by the inhibition assay in which increasing concentrations of unlabelled anti-HBs (horse) added before incubation with conjugate in the ELISA assay, depressed the binding between anti-Id and conjugate (Table III). Furthermore, use of HRPO-anti-HBc and HRPO-anti-HBe as conjugates instead of HRPO-anti-HBs produced no binding. This suggests that the anti-Id antibodies were specifically directed against anti-HBs and not against anti-HBc or anti-HBe.

The supernatants obtained after PEG precipitation of anti-Id-positive sera were analysed for rheumatoid factor activity. None of them showed the presence of rheumatoid factor. Similarly, the pellets from these positive sera, after dissolving in PBS, were tested for HBsAg/IgG or HBsAg/IgM complex activity. Each one of these samples was

TABLE II

THE PREVALENCE OF IgG AND IgM TYPE ANTI-IDIOTYPIC ANTIBODIES DIRECTED AGAINST ANTI-HBs IN THE SERA OF DIFFERENT HBV-INFECTED PATIENTS AND HEALTHY HBV CARRIERS

Group	Total number	IgG anti-idiotype		IgM anti-idiotype	
		no. positive	%	no. positive	%
(1) Healthy HBV carrier	52	16	30.7	15	28.8
(2) Acute viral hepatitis	40	5	12.5	5	12.5
(3) Fulminant hepatitis	40	Nil	–	1	2.5
(4) Subacute viral hepatitis	10	1	10.0	Nil	–
(5) Chronic active hepatitis	8	4	50.0	3	37.5

found to be negative for complexes. The complexes were also absent in the supernatants obtained after PEG precipitation of complex positive sera, thereby confirming that the complexes were removed by PEG treatment.

20 anti-Id positive sera were also tested for anti-HBs. Only four sera were found to be positive. Of these four sera, three were weakly positive (titre < 1 : 200) whereas one was strongly positive (titre > 1 : 1000). A further analysis of anti-Id-positive sera for HBV-specific DNA polymerase and HBeAg demonstrated DNA-polymerase activity in 12 out of 20 sera and HBeAg in three out of ten serum samples.

Discussion

The present ELISA method is a simple and highly specific assay system for the detection of anti-idiotypic antibodies directed against anti-HBs molecules. There are two main features of this ELISA method. Firstly, anti-Id was detected in the supernatant obtained after PEG precipitation of serum and, secondly, HRPO-labelled goat anti-HBs was used as the conjugate instead of HRPO-human anti-HBs. The advantage of using supernatants for anti-Id analysis is that HBsAg/IgG or HBsAg/IgM complexes which may give identical reactions in the assay are removed in the precipitate. Using sera containing complexes, the efficacy of complex removal by PEG treatment, as originally demonstrated by Troisi and Hollinger (1985), was confirmed. However, anti-Id antibodies remain in the supernatant and are not removed in precipitate. Similarly, the use of HRPO-labelled goat anti-HBs rather than HRPO-human anti-HBs is advantageous because the former is readily available in commercial kits (Abbots, U.S.A.) and is free from contaminating antibodies which cross-react with human serum components. Human anti-HBs is usually affinity purified but may still be contaminated with other antibodies and produce false results. The use of goat anti-HBs instead of human anti-HBs is based on an earlier report (Troisi and Hollinger, 1985) where the binding of goat anti-HBs with anti-Id against anti-HBs from a human source was found to be as efficient as with human anti-HBs. This is also supported by the findings of Kennedy (1985) who reported interspecies Id cross-reactions associated with anti-HBs and the serological resemblance of mouse anti-HBs with human anti-HBs produced by injecting anti-Id alone (Kennedy and Dreesman, 1984). This was further revealed in the inhibition assay where binding between HRPO-goat anti-HBs and anti-Id could be inhibited by the preincubation of anti-Id with unlabelled horse anti-HBs (Table III). This indicates that anti-HBs produced in a wide variety of mammalian species, after coupling with HRPO, may be safely used as conjugate without a significant change in the binding activity.

The supernatant portion obtained after PEG precipitation of serum samples may still have rheumatoid factor activity which would give identical reactions in this assay system. However, rheumatoid factor as a possible cause of this reaction was ruled out by testing the supernatants for rheumatoid factor activity. None of the samples showed such activity. Similarly, the chances of obtaining false positive reactions due to residual HBsAg/IgG or HBsAg/IgM complexes in the supernatant were reduced by testing the pellet portions for complex activity (Gandhi et al., 1986). The absence of immune complex activity in the pellets clearly indicates that the presence of activity in the supernatants cannot be due to such complexes.

Sera with anti-idiotypic antibodies were also tested using HRPO-anti-HBc and HRPO-anti-HBe conjugates instead of HRPO-anti-HBs. The absence of any binding activity using these conjugates suggests that the anti-Id antibodies are specifically directed against anti-HBs and not anti-HBc or anti-HBe. Since the host immune response against HBcAg is assumed to play a major role in liver injury during HBV infections (Dienstag, 1984), the presence of anti-HBc was expected in a high proportion of HBV-infected patients. In the sera obtained from HBV patients, anti-HBc was detected in 70% of the cases. Such a high prevalence of anti-HBc and lack of anti-Id against anti-HBc clearly indicates that anti-idiotypes have no role in the regulation of idiotypic anti-HBc production.

Anti-idiotypes were detected in HBsAg positive sera but not in HBsAg-negative sera. The presence

of IgG and IgM anti-idiotypes concurrently suggests that both types of antibodies are produced simultaneously. Jerne (1974) proposed that in general anti-idiotypic antibodies can suppress the synthesis of idiotypic antibodies by their action on a clone of B cells bearing identical idiotypes. Therefore, in the present context, after a critical threshold level is reached, idiotypic anti-HBs would become immunogenic and produce anti-idiotypic antibodies. Such anti-Id would suppress further production of idiotypic anti-HBs. Since in the present study anti-HBs is detectable only in 20% of sera positive for anti-idiotype activity, this implies that persons with anti-idiotype-positive sera have low threshold levels of anti-HBs and thus before anti-HBs reaches detectable levels, it becomes immunogenic and produces anti-idiotypes. These anti-Id against anti-HBs are amongst the major factors regulating viral replication during HBV infection. In acute viral hepatitis, fulminant hepatitis and subacute viral hepatitis, the low prevalence of anti-Id is presumed to be due to the high threshold level of anti-HBs and thus higher production of anti-HBs without any check by anti-Id. Enhanced anti-HBs production results in the elimination of HBV particles by immunological reactions as shown in various other studies (Woolfe et al., 1976; Gimson et al., 1983). Interestingly, however, in the present group of patients with fulminant hepatitis, anti-HBs and DNA polymerase were noted in 14% and 52%, respectively (unpublished data). This is in contrast to previous reports (Woolfe et al., 1976; Gimson et al., 1983) where anti-HBs was detected in a higher percentage of FHF patients. A possible reason for this difference was assumed to be the collection of blood samples in an earlier phase of disease where HBV replication, as shown by DNA-polymerase activity, is continuing without anti-HBs production reaching a detectable level. In asymptomatic carriers low threshold levels of anti-HBs presumably results in earlier production of anti-Id which further reduces the anti-HBs production. Low levels of anti-HBs in these carriers is insufficient to eliminate the HBV particles and viral multiplication is continued for longer periods without any disease symptoms. In chronic active hepatitis, it appears that the threshold level is intermediate and anti-Id formation occurs but not to the extent of stopping anti-HBs production completely. Thus HBV replication is reduced. The data in Table II showing the prevalence of anti-Id in different HBV infections fully support the above hypothesis. The presence of anti-idiotype activity in anti-HBs-negative vaccine recipients and not in anti-HBs-positive recipients also indicates that anti-Id are able to modulate anti-HBs formation. This conclusion is supported by the presence of anti-HBs in only four out of 20 anti-Id-positive cases and suggests that anti-Id regulates anti-HBs formation by a feedback mechanism.

HBeAg was noted in 30% of anti-Id-positive sera whereas DNA-polymerase activity was detected in 60% of such cases. As DNA polymerase is more sensitive and a better marker of HBV replication, it appears that viral replication is quite significant in individuals manifesting anti-Id. Since the elimination of HBV from the circulation depends on both cell-mediated immunity as well as humoral immunity, an impairment of either may result in ensuing viral replication. The presence of anti-idiotype activity permits continuous HBV replication possibly by inhibiting anti-HBs formation.

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