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ENZYME-IMMUNOASSAY IN THE DIAGNOSIS OF HUMAN HEPATITIS A: SPECIFIC AND NON-SPECIFIC REACTIONS

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Summary. — Solid-phase enzyme-immunoassay (EIA) used for the detection of hepatitis A virus (HAV) often reveals a non-specific activity which can be reduced or fully eliminated in the presence of normal serum. The factor responsible for this activity appeared to be a non-viral EIA-active material (NVEAM) that non-specifically reacted with normal serum of some mammalian species (human, monkey, rabbit, cattle). The HAV and NVEAM have been separated by CsCl gradient centrifugation, where the HAV banded in a narrow zone at 1.34 g/cm^3, whereas the NVEAM could be found in a wider zone with an average density of 1.31 g/cm^3. Non-immunological character of the non-specific activity was demonstrated by the inhibitory effect of weak non-ionic detergents (0.05% Tween 20 or bile, 1 : 500). The conditions for preferential binding of the HAV by immune sera and elimination of non-specific reaction have been determined.

Key words: *hepatitis A virus*; *enzyme-immunoassay*; *non-specific reactions*; *separation in CsCl density gradient*

Introduction

Solid-phase radio- and enzyme immunoassays (RIA and EIA) are widely used in the research of human hepatitis type A (HA) as well as for diagnostic purposes (for review see Zuckerman and Howard, 1979; Frösner *et al.*, 1980). The both were proved to be highly sensitive and specific tests suitable for detection of hepatitis A virus (HAV) antigen and corresponding (anti-HAV) antibody. One of the main requirements for their specificity was the elimination of false-positive reactions occurring between the anti-HAV sera and an EIA-active non-viral material often present in the stool extracts collected from HA patients. This material was revealed by its characteristic binding ability with normal non-immune serum (Purcell *et al.*, 1976; Hall *et al.*, 1977; Mathiesen *et al.*, 1978; Coursaget *et al.*, 1980).

Since in our routine diagnostic surveys on HAV excretion employing the EIA such non-specific material was found quite regularly in the stool

Table 1. Blocking of HAV and NVEAM in the stool extracts with normal and anti-HAV sera

Stool extract	Blocking with						Interpretation[1])	Results of immune electron microscopy[2])
	PBS		Normal serum (No. 6)		anti-HAV serum (No. 5)			
	OD_{492}	P/N	OD_{492}	P/N	OD_{492}	P/N		
122-1	1.836	29.6	1.953	32.0	0.123	2.1	+	+
176-1	0.524	8.5	0.525	8.6	0.085	1.7	+	+
122-2	0.525	8.5	0.265	4.3	0.058	1.0	+	+
1118-1	0.435	7.0	0.260	4.3	0.048	1.0	+	+
114-1	1.996	32.2	1.625	26.3	0.235	4.7	+	+
166-1	0.567	9.1	0.214	3.3	0.051	1.0	+	+
PV	0.573	9.2	0.430	5.6	0.044	1.0	+	+
627-2	0.445	7.2	0.161	2.6	n.t.	n.t.		—
515-1	0.436	7.0	0.118	1.9	0.091	1.5	—	—
200	0.237	3.8	0.089	1.6	0.059	1.0	—	—
248	0.266	4.3	0.031	0.5	0.037	0.6	—	—
NS*)	0.062	1.0	0.061	1.0	0.050	0.8	—	—

*) Negative sample: pool of 20 stool extracts from healthy children; n.t. — not tested.
[1]) + = HAV antigen present; — = HAV antigen absent.
[2]) + = HAV particles present; — = HAV particles absent.

extracts with or without the HAV, it seemed expedient to study non-specific interaction between normal sera and the non-viral EIA-active material (NVEAM) as well as some properties of NVEAM.

Materials and Methods

Stool specimens. Eleven stool specimens in the form of 17% aqueous extracts have been selected. The extracts were prepared according to the method described by Brandley *et al.* (1976) and were found to differ in the content of HAV and NVEAM. Specimens 122-1, 122-2, 1118-1, 114-1 and 627-2 (Table 1) were collected from icteric HA cases between the second and the fourth day after the onset of jaundice; specimens 176-1, 166-1, 515-1 and 200 were taken from unicteric patients with HA 3-11 days after the first aminotransferase abnormalities; specimen 248 was taken from a convalescent case. In addition, and HAV-containing stool extract (PV) prepared and tested in Phoenix Laboratories Division, CDC (Phoenix, U.S.A.) has been included into the study.

Sera. The anti-HAV positive serum samples used in the experiments were as follows: (i) two serum samples from early convalescents taken at the day 30 (No. 1) and 40 (No. 2) after the onset of the HA, the diagnosis having been confirmed by determination of specific IgM antibodies in both cases; (ii) a late HA convalescent serum independently tested for anti-HAV in three different laboratories (No. 3); (iii) anti-HAV human serum (Burton) employed in CDC, Phoenix as an internal control (No. 4) and (iv) a serum sample from a chimpanzee experimentally infected with HAV (No. 5). As negative (control) serum samples sera taken from healthy children were used (the absence of anti-HAV has been previously established) as well as sera from non-immune rabbits, rhesus monkeys and a chimpanzee serum specimen sampled before the experimental HAV infection (No. 6). Chimpanzee sera No. 5 and No. 6 were received from the Research Resources Branch, NIAID, Bethesda, U.S.A. Anti-human IgG serum has been purchased from ICN Pharmaceuticals, Inc.

EIA procedure — determination of HAV antigen. The EIA test was performed mainly according to Duermeyer (1980) in four steps using, as the solid phase, the Linbro (Flow Laboratories Ltd.) flexible vinyl microtitration plates (Cat. No. 76-364-05): (i) coating the wells with 100 µl of serum

No. 1 (1:25.000) followed by 18—20 hr incubation at 22 °C; (ii) washing the wells with phosphate buffered saline (PBS; pH 7.2) supplemented with 0.05% Tween-20 and adding 50 μl of antigen followed by additional incubation for 18—20 hr at 22 °C; (iii) washing and adding 50 μl of immunoglobulin labelled with peroxidase, incubation for 2 hr at 22 °C:, (iv) washing and adding 100 μl of enzyme substrate (ortho-phenylene-diamine plus hydrogen perixode), incubation in the dark at 22 °C for 40 min. The reaction was stopped by adding 50 μl of 2 M sulphuric acid. The

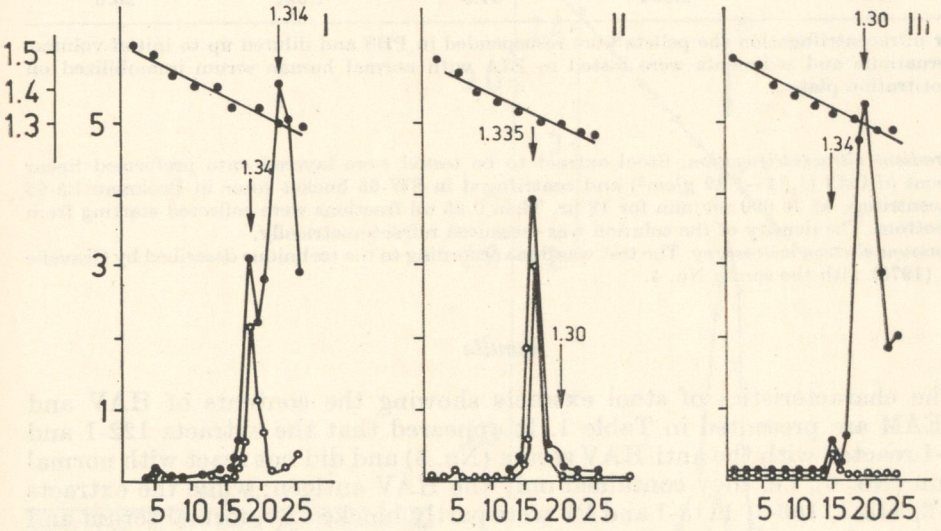

Fig. 1.

Banding of HAV and NVEAM in CsCl gradient

After adding 0.2 ml of 17% stool extract on the top of the gradient it was centrifuged in SW-65 rotor for 18 hr at 36,000 rev/min. The fractions were tested in EIA: to detect the HAV (empty circles) 2.5% human serum containing no anti-HAV antibodies was used for blocking. The detection of NVEAM and NVEAM + HAV was performed in the presence of foetal calf serum (black circles). I — stool sample No. 114, II — stool sample No. 122-1, III — stool sample No. 122-2.

Abscissae: fraction number (0.25 ml each); ordinates: optical density at 492 nm and density constant ρ

resulted optical density of the solutions was measured in Perkin-Elmer, model 402 spectrophotometer at $\lambda = 492$ nm. The content of HAV was expressed as P/N ratio, i.e. the ratio of OD_{492} of specimen tested to OD_{492} of control specimen, the latter being a pool of 20 normal stool speciments free from HAV and NVEAM. The P/N = 2.1 was taken as threshold. The globulin fraction from serum No. 4 has been used for conjugation with horse-radish peroxidase (HRPO, type VI, RZ = 3.0, Sigma Biochemical Corp.) according to the procedure described by Nakano and Kawaoi (1974). The conjugate was calibrated in „chessboard" (conjugate versus antigen) titration to determine the working dilution, which was 1:200 throughout the experiments.

Determination of NVEAM. For quantitative determination of NVEAM, normal anti-HAV antibodies-free human serum has been used at 1:1,000 dilution for coating the wells of microtitration plates. Later on the test was performed as mentioned above. The NVEAM content was presented as P/N, where P = OD_{492} of tested specimen and N = OD_{492} of control. The control was the same as for HAV determination. In an alternative variant the NVEAM was determined by blocking effect of normal serum introduced between the steps (ii) and (iii) for 2 hr at 37 °C.

Table 2. Distribution of NVEAM after differential ultracentrifugation

Stool extracts	Supernatant		Sediment	
	OD_{492}	P/N	OD_{492}	P/N
627-2	4.285	61.2	3.384	48.2
122-2	5.430	77.6	2.124	30.3
114-1	2.394	47.9	1.331	26.6

After ultracentrifugation the pellets were resuspended in PBS and diluted up to initial volume. Supernatants and sediments were tested in EIA with normal human serum immobilized on microtitration plates.

Gradient ultracentrifugation. Stool extract to be tested were layered onto preformed linear gradient of CsCl (1.24—1.49 g/cm^3) and centrifuged in SW-65 bucket rotor in Beckman L5-65 ultracentrifuge at 36,000 rev/min for 18 hr. Then 0.25 ml fractions were collected starting from the bottom. The density of the solution was measured refractometrically.

Immune electron microscopy. The test was done according to the technique described by Gravelle *et al.* (1975) with the serum No. 4.

Results

The characteristics of stool extracts showing the contents of HAV and NVEAM are presented in Table 1. It appeared that the extracts 122-1 and 176-1 reacted with the anti-HAV serum (No. 5) and did not react with normal serum (No. 6), i.e. they contained only the HAV antigen; while the extracts 122-2, 114-1, 166-1, 1118-1 and PV were partly blocked by normal serum and further on by specific anti-HAV serum. Seemingly the HAV and the NVEAM were simultaneously present in these extracts. The extracts 515-1, 200, 248 and perhaps 627-2 were fully blocked by any serum, regardless whether it did or did not contain anti-HAV antibodies, i.e. whether NVEAM was found in the extracts only.

The NVEAM reacted in a similar way with several normal human sera as well as with the sera of non-immune rabbits, rhesus monkeys and the lots of commercially available bovine serum. IgG fraction extracted from

Table 3. Effect of detergents on the adsorption of NVEAM and HAV onto immobilized serum

Medium for adsorption	NVEAM[1]		HAV	
	OD_{492}	P/N	OD_{492}	P/N
PBS	2.073	40.6	0.546	19.0
+ 0.05% Tween-20	0.513	10.1	0.632	20.1
+ bile, 1 : 500	0.183	3.6	0.563	19.6
+ 0.01% Sodium desoxycholate	1.308	25.6		
+ bile, 1 : 500 (preincubation)[2])	1.605	31.1		

[1]) NVEAM from CsCl gradient band with buoyant density of 1.29 g/cm^3 (see Fig. 2).
[2]) Material was incubated in bile for 24 hr at 37 °C and diluted 1 : 10 before testing.

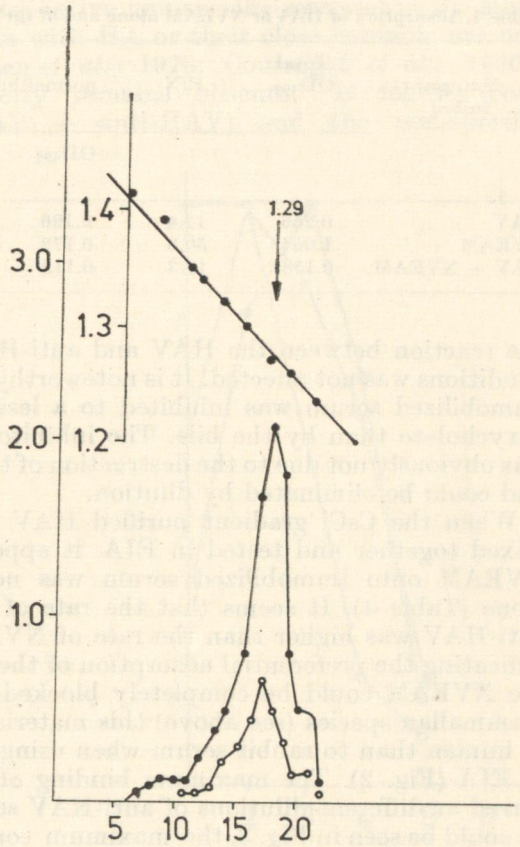

Fig. 2.
Adsorption of NVEAM on immobilized human and rabbit serum
Gradient fractions were tested in EIA using human (●——●) and rabbit (○——○) sera (diluted 1 : 5000 at coating the plates). Abscissa and ordinates as in Fig. 1.

the above sera also appeared to be able to bind the NVEAM. Meanwhile the commercial foetal calf serum (several lots tested) was unable to react with the NVEAM.

The HAV and the NVEAM could be separated by CsCl gradient ultracentrifugation (Fig. 1). The HAV-specific material was banded by a narrow zone buoyant density of 1.34 g/cm³. The HAV particles were visualized by immune electron microscopy in the corresponding fractions. The NVEAM was distributed in a wider zone with mean buoyant density of 1.31 g/cm³. No homogeneous or regular structures were found in these fractions by electron microscopy. The heterogeneity of NVEAM was further confirmed by differential centrifugation (at 130,000 × g for 2 hr) resulting in sedimentation of 28.0 to 44.8% of the NVEAM (Table 2).

Table 3 shows that the weak non-ionic detergents (0.05% Tween-20 and rhesus monkey bile at 1 : 500 dilutions) significantly decreased the binding of NVEAM by the serum immobilized on microtitration plates, whereas

Table 4. Adsorption of HAV or NVEAM alone and of their mixture on immobilized anti-HAV serum

Material tested	Total OD_{492}	P/N	After blocking with normal human serum		After blocking with anti-HAV serum (No. 3)	
			OD_{492}	% of blocking	OD_{492}	% of blocking
HAV	0.256	12.4	0.196	23	0.610	76
NVEAM	1.064	50.8	0.172	84	0.110	90
HAV + NVEAM	0.158	12.3	0.115	55	0.080	69

the reaction between the HAV and anti-HAV serum (No. 1) under similar conditions was not affected. It is noteworthy that adsorption of NVEAM onto immobilized serum was inhibited to a lesser extent by 0.01% sodium desoxycholate than by the bile. The inhibitory effect of non-ionic detergents was obviously not due to the destruction of the NVEAM since it was reversible and could be eliminated by dilution.

When the CsCl gradient purified HAV and NVEAM preparations were mixed together and tested in EIA, it appeared that the adsorption of the NVEAM onto immobilized serum was not as intensive as for NVEAM alone (Table 4). It seems that the rate of reaction between the HAV and anti-HAV was higher than the rate of NVEAM interaction with the serum indicating the preferential adsorption of the HAV from the mixture. Though the NVEAM could be completely blocked by non-immune sera of several mammalian species (see above) this material demonstrated a higher affinity to human than to rabbit serum when using these sera for coating the plates in EIA (Fig. 2). The maximum binding of the HAV and the NVEAM occurred at different dilutions of anti-HAV serum used for coating the plates. As could be seen in Fig. 3, the maximum contentration of HAV was observed at a higher dilution of immobilized anti-HAV sera as compared to the dilution corresponding to the maximum detection of the NVEAM.

Discussion

In EIA applied to the search of HSV in human excreta, two types of EIA-active material could be demonstrated. One of them - HAV antigen is identified by specific reaction with anti-HAV antibody. The second, in our case called NVEAM, reacts with any serum of certain mammalian species irrespectively of the presence of anti-HAV antibody. Serum component(s) responsible for binding the NVEAM is to be specified. Seemingly the second interaction is non-immunological, i.e. it occurs without involvement of specific antibody. In this aspect the non-specific reaction described and perhaps its substrate — the NVEAM differe from the non-specific EIA--activity due to the presence of coproantibodies to immunoglobulins. This pehnomenon was reported earlier by Yolken and Stopa (1979) in connection with the application of EIA for the detection of human rotavirus in patients,

stools. The findings of RIA- or EIA-active non-specific material in the stool speciments collected from patients with HA or their close contacts are not rare (Purcell *et al.*, 1976: Mathiesen *et al.*, 1978; Coursaget *et al.*, 1980), but the nature of non-specificity remains obscure. It follows from our data that the specific (HAV + anti-HAV) and the non-specific

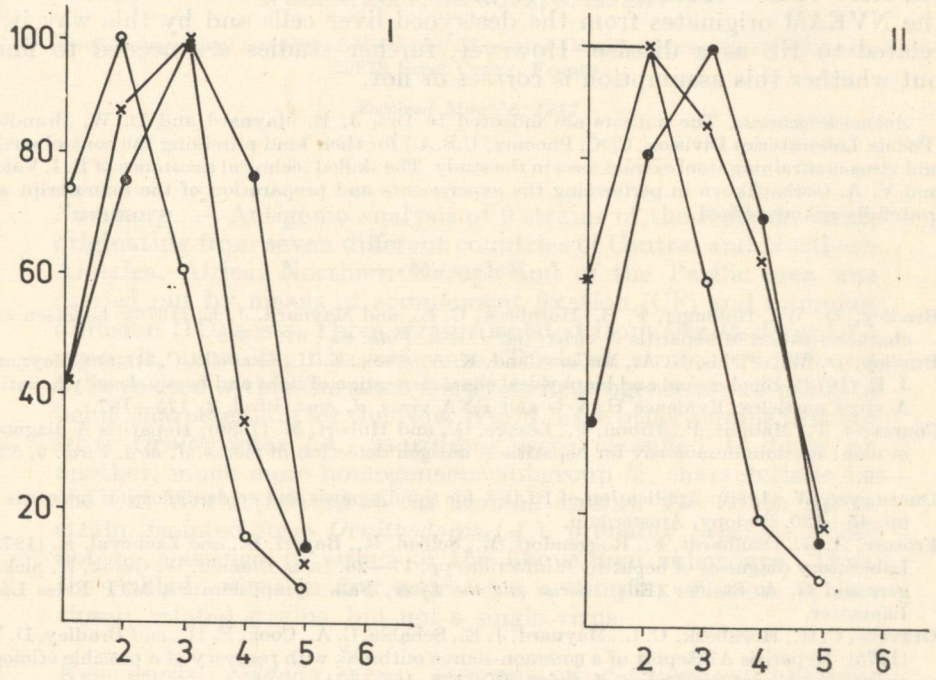

Fig. 3.

Adsorption of HAU and NVEAM on immobilized and-HAV sera

Anti HAV sera at indicated dilutions were used for coating of plates. The amount of immobilized globulin was estimated with peroxidase-labelled anti-human IgG (×——×). The adsorption of HAV (●——●) and of NVEAM (○——○) was determined in the presence of 2.5% normal human serum or foetal calf serum, respectively. I — early convalescent serum No. 1; II — early convalescent serum No. 2. For further details see Materials and Methods.

Abscissa: serum dilutions (dilution reciprocals in $\log_{10}$ values); ordinates: adsorption (%).

(NVEAM + unknown serum component) reactions are different in certain characteristics: (i) the ability of NVEAM to bind the non-immune sera including the sera from animals non-susceptible to human type A hepatitis; (ii) inhibition of non-specific but not specific reaction with weak non-ionic detergents; (iii) the different rate of adsorption of HAV and NVEAM onto immobilized anti-HAV serum; (iv) the different dilutions of immobilized anti-HAV serum that correspond to the maximum binding the HAV and the NVEAM. These characteristics could be taken into account in the routine

RIA and EIA procedures applied to the identification of HAV in human stools.

As to the NVEAM, its composition and origin are now under separate investigation. So far it has been found that this material was not a product of virus degradation since according to the results obtained as well as to the data published by Bradley *et al.* (1978), the proteins released from disintegrated HAV virions could not be detected in EIA or RIA. It is not unlikely that the NVEAM originates from the destryoed liver cells and by this way it is related to HS as a disease. However, further studies are needed to find-out whether this assumption is correct or not.

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