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24 November 1972

Hepatitis B Virus Antigen: Validation and Immunologic Characterization of Low-Titer Serums with ^{125}I-Antibody

Abstract. *Radioimmunoassay was used to determine the serologic subspecificities of 85 blood donor serums positive for hepatitis B virus–associated antigen. There was complete agreement with results obtained by immunoprecipitation of 43 serums. The remaining 42 serums were negative by immunoprecipitation but followed type-specific immunology by radioimmunoassay, and this served as a validation for authentic hepatitis B virus antigen.*

The use of highly sensitive testing procedures, such as radioimmunoassay, has established that the hepatitis B virus antigen occurs much more frequently among blood donors and hepatitis patients than has been found by less sensitive immunologic methods (*1*, *2*). Study of antigenic subspecificities as epidemiological markers is limited because most of the low-titered serums cannot be analyzed by immunoprecipitation in agar gels. Some difficulty is also encountered in assessing a serum that is positive by radioimmunoassay but cannot be confirmed by any other method. Both of these difficulties can be minimized if radioimmunoassay procedures are used to determine antigen subtype specificities. We now report that immunologic specificities established by immunoprecipitation were correctly identified by radioimmunoassay. In addition, many serums which were negative by immunoprecipitation were successfully subtyped by radioimmunoassay.

Soon after the discovery of Australia antigen and its association with hepatitis type B, Levine and Blumberg (*3*) showed evidence of the possible serologic heterogeneity of this antigen. Later, the nature of the heterogeneity was clarified by LeBouvier (*4*, *5*) and by Kim and Tilles (*6*). LeBouvier's characterizations suggest that three antigenic determinants are carried on antigen particles. One of these, designated a, is group-specific and is common to all antigenic types. Two others, designated d and y, are type-specific and are mutually exclusive. Although it was not well characterized, LeBouvier also indicated that a fourth determinant, x, was unequally shared by the antigenic subsets. Further classification of the determinants has been provided by Bancroft *et al.* (*7*), with the discovery of two new mutually exclusive determinants, w and r. Thus, three patterns emerge, ayw, adr, and adw. A putative ayr was not discovered by Bancroft, but has since been reported by Holland *et al.* and LeBouvier *et al.* (*8*).

The above immunologic characterizations were performed by immunoprecipitation with the micro-Ouchterlony technique. We have shown that ^{125}I-labeled specific immunoglobulin can be used in a highly specific and highly sensitive radioimmune procedure (*1*). The test takes advantage of the multiple combining sites on each viruslike particle. Briefly, a mixture of antibodies specific for a, d, and y determinants was fixed to a solid surface, usually polypropylene or polystyrene (test tubes). A test serum (0.1 ml) was pipetted into the tube and incubated at room temperature for 16 hours. During this period, antigen in the serum was fixed to the solid-phase antibody at the liquid-surface interface. The contents of the tube were then aspirated, the tube was washed, and ^{125}I-labeled antibody was then added. During the subsequent 90-minute incubation period at room temperature the purified radioactive antibodies were fixed at the available free combining sites on the antigen particles. The unreacted radioactive antibody was removed, and radioactivity in the tubes was counted. The count rate was in direct proportion to the amount of antigen in the original sample. For general detection of the antigen in an unknown sample by radioimmunoassay we used a mixture of antibodies to the known antigenic determinants. The antibodies generally were in unequal proportion, and the test showed a slightly greater sensitivity for ad subtypes as compared to the ay subtype. Ginsberg *et al.* (*9*) have used this differential feature of the test to correctly identify a number of antigen subtypes in serums previously characterized by agar gel diffusion. It is obvious that great specificity can be obtained if the labeled antibody consists of only a single serologic type, antibody to a, d, or y.

We have now purified and radiolabeled the group-specific antibody to a and the two type-specific antibodies to d and y. When these were individually used in our direct radioimmunoassay (as in Table 1), antigen-positive serums fell into the y or d subsets. This provided a method for the detection and immunologic characterization of antigen samples not amenable to characterization by immunoprecipitation; also it served to validate samples found

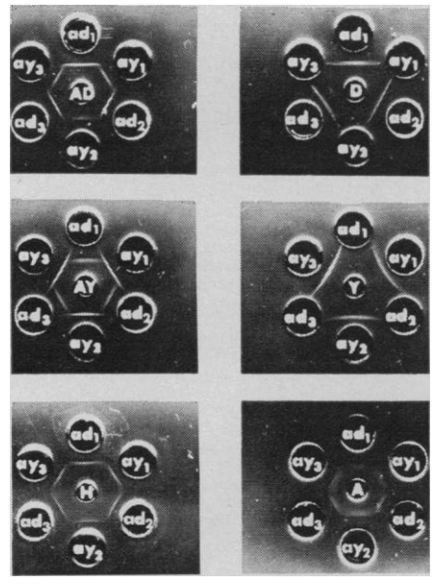

Fig. 1. Immunodiffusion specificity of hepatitis B antigen–positive serums. Three ad-type and three ay-type serums were placed alternatively in the peripheral wells of micro-Ouchterlony plates, as indicated. Antiserums of different origin were placed in the center wells. AD is serum from guinea pigs immunized with an ad antigen; D is the antiserum to ad after absorption with an ay antigen; AY is serum from guinea pigs immunized with an ay antigen; Y is the antiserum to ay after absorption with an ad antigen. A is purified antibody obtained from the complex formed by antiserum to ay with an ad antigen; H is antiserum of human origin showing wide spectrum and does not discriminate among antigen types.

positive by radioimmunoassay but not confirmable by other immunologic methods.

Immunoprecipitation was carried out by the micro-Ouchterlony technique (*4*) with antiserum produced in guinea pigs with purified antigen (*1*) or a specimen of human antiserum showing broad-spectrum characteristics. Purified antibody for radioimmunoassay was prepared from guinea pig antiserum by absorption with specific antigens (*1*). For example, serum from animals immunized with an ad-type antigen was absorbed with an ay-type antigen and the resulting complex (ay antigen plus antibody to a) was separated by low-speed centrifugation. Dissociation and separation of the complex yielded purified antibody globulin to a. This was used for immunoprecipitation analyses, and, after labeling with ^{125}I, for radioimmunoassay. The supernatant from the absorption, containing antibody to d, was used as a type-specific serum for immunoprecipitation. A portion of it was absorbed with an ad-type antigen, giving a complex of ad antigen with antibody to d. Dissociation and separation of this complex, as above, gave purified antibody globulin to d. Subsequent iodination gave ^{125}I-labeled antibody to d for radioimmunoassay. We followed similar procedures, beginning with an ay antigen as immunogen and obtained antiserum to y for immunoprecipitation, and ^{125}I-labeled antibody to a and to y for radioimmunoassay. Figure 1 shows typical immunoprecipitation patterns with three ad-type and three ay-type antigens. The absorbed antiserums appeared to be highly specific for the d and y determinants, respectively. The unabsorbed antiserums gave spur formations related to the subdeterminants. In contrast, the purified antibody to a and the broad-spectrum human antibody was nondiscriminative and gave lines of identity with antigen subtypes.

A group of 85 serums, positive by radioimmunoassay, was selected for serotyping by immunoprecipitation. These fell into four groups. Twenty-nine serums showed immunoprecipitin bands with the human antiserum and with the guinea pig antiserum to d. Another 14 serums interacted with human antiserum and the guinea pig antiserum to y only. Slight immunoprecipitin lines were observed with another 18 samples when the human antiserum or unabsorbed guinea pig serums were used, but the precipitin lines were too faint to observe spurring in the Ouchterlony plates, and no bands were visible with the type specific antiserum to d or to y. Twenty-four of the samples, positive by radioimmunoassay, gave no observable precipitin lines with any of the antibodies. They could not be found positive by any other immunological test, including complement fixation and counterelectrophoresis.

Each of the 85 serums was then subjected to three radioimmunoassay procedures. ^{125}I-Labeled antibody to a, to d, and to y were independently used in the labeling step of each test. On the basis of count rate results and immunoprecipitation, the samples were grouped into five categories (Table 1). Groups 1 and 2 were the serums characterized as ad or ay by agar gel diffusion. In these cases it was obvious that the ratio of counts with radioactive antibody to d and antibody to y could be used to assess correctly the immunologic specificities. The mean ratios (count/min) of antibody to d to antibody to y (anti-d/anti-y) were 9.03 for ad subtypes and 0.16 for ay subtypes. The results for the 18 samples that were positive in agar gel diffusion, but not identifiable as to antigenic subtype are shown as groups 3a and 3b. The immunologic specificities were clearly indicated by the radioimmunoassay results. Eight of the samples were ad type, with a ratio of 7.8, and ten of the samples were ay type, showing a ratio of 0.14. The fourth group, positive by radioimmunoassay but negative by agar gel diffusion, counterimmunoelectrophoresis, and complement fixation, showed a very similar distribution of anti-d/anti-y ratios. Thirteen sam-

Table 1. Summary of radioimmunoassay (RIA) determination of immunologic specificities of hepatitis type B antigen. Group 1 was immunodiffusion-positive with wide spectrum human antiserum and guinea pig antiserum to d. Group 2 was immunodiffusion-positive with wide spectrum human antiserum and guinea pig antiserum to y. Group 3 was immunodiffusion-positive with wide spectrum human antiserum only. Group 4 was immunodiffusion-negative with all antiserums. Group 5 represents four samples showing atypical immunologic specificities. Ten negative (Neg) control replicate samples were processed to establish normal serum count rates and method variations.

Group	Number of serums	Subtype by immunodiffusion	^{125}I-labeled antibody to (mean count/min)					RIA specificity
			a	d	y	d/y		
						Mean	Range	
1	29	ad	2040	2446	271	9.0	(4.6–11.4)	ad
2	13	ay	3094	382	2321	0.16	(0.11–0.19)	ay
3a	8	Neg	1839	1862	239	7.8	(5.1–9.9)	ad
3b	10	Neg	2742	321	2334	0.14	(0.11–0.16)	ay
4a	10	Neg	681	880	186	4.7	(2.7–7.6)	ad
4b	11	Neg	1052	172	1003	0.17	(0.08–0.25)	ay
5	28*	*ay* (*d*)	2696	844†	1762	0.48		ay (d)
	49512*	Neg	148	612	166	3.7		d
	50275*	Neg	281	777	226	3.4		d
	49673*	Neg	591	1720	485†	3.5		ad (y)
Negative control	10	Neg	162	191	138			
S.D.			± 13	± 39	± 26			

* Serum identification number. † The number of counts per minute was appreciably higher than in normal serum control.

ples showed d-type specificities (ratio 4.7), and 11 samples showed y-type specificities (ratio 0.17).

Of the total 85 serums, four samples showed interesting variations from the simple ad and ay subsets (Table 1, group 5). Sample No. 28 showed clearly an ay subtype by immunoprecipitation with antiserum to y, and, in addition, a faint precipitin line was observed with antiserum to d. The radioimmunoassay showed a variation in the count rate ratio with antibodies to d and to y: 0.48 compared to a mean value of the ay group of 0.16. As will be obvious from the experiments on specificity, this increased ratio was due to an elevated count rate with the labeled antibody to d—844, in replicate analyses, compared to a mean value of 382 for the group. Thus, a logical interpretation was that this serum sample contained not only predominantly a and y specificities, but also contained d specificity to a limited extent. This result suggested that this serum contained a large number of virus particles that were carrying ay determinants and a smaller number carrying the d determinant. It is also possible that a laboratory contaminant was introduced, but we feel this was unlikely. An alternate possibility is that the serum contained an antigen other than y that interacted with the antiserum to y. We are attempting to trace the source of the serum to verify the result with another sample from the donor.

In the low-titered samples containing the d specificities, there were three samples that did not fit the normal pattern. Serums No. 49512 and 50275 showed abnormally low count rates with iodinated antibody to a—148 and 281 which are in the range of normal serums. Thus these two samples showed a relative "absence" of the group-specific determinant a. One possible explanation for the low reactivity is that the antigens could be complexed with antibody to a in the original serum. In the same group, the radioimmunoassay specificities for sample 49673 indicated the presence of a, d, and y specificities, similar to serum No. 28. Unlike sample 28, the specificities could not be confirmed by immunoprecipitation. These four samples require additional study to ascertain whether they represent variants of the hepatitis B antigen.

In this group of 85 serums from commercial blood donors, 50 were of the d type and 35 were of the y type, as shown by radioimmunoassay. Of the 43 samples that could be typed by immunoprecipitation, 29 were d and 14 were y. In the radioimmunoassay tests the specific activities of the labeled antibodies to a, d, and y were roughly similar (20 μc/μg). In each subset, the count rate observed with antibody to a and the type-specific antibody were grossly similar. For example, in group 1, 2040 count/min were found with antibody to a and 2446 with antibody to d. In group 2, 3094 and 2341 count/min were found with antibody to a and antibody to y, respectively. This suggested that the relative availability of the group-specific determinant a and of the type-specific determinant d or y was quantitatively similar.

The results confirmed that a majority of the samples found positive by our radioimmunoassays procedure were truly hepatitis B virus–associated antigen, since they followed the identical subtyping specificities expected for this antigen. The studies also indicated that there were no substantial differences between low-titer serums and high-titer serums, regarding the antigenic subsets. The relationships of the recently characterized w and r determinants were not dealt with in this study. The guinea pig antiserums were produced with adw and ayw antigens. Therefore, in the cross absorptions to prepare type-specific antiserums, the w antibodies, if present, were probably included in antibody to a, but absent from antibody to d and antibody to y. We have in progress studies utilizing antiserum produced with adr antigens; these studies should give information relating to the contribution of the w and r determinants to the immunologic specificities.

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17 November 1972

Unilateral Cortical Activity in Newborn Humans: An Early Index of Cerebral Dominance?

Abstract. *Spectral analyses show unilateral photic driving in newborn human infants to bilateral repetitive visual stimulation. Results are interpreted as evidence of dominance in the right hemisphere for rhythmic visual stimuli and lack of interhemispheric integration.*

Analyses (*1*) of electrical activity in brains of human newborns provide new evidence on cerebral hemispheric development. Neonatal electroencephalograms (EEG's) from homologous areas of both hemispheres show that few babies have bilateral EEG responses of driving to bilateral photic stimulation. In some newborns only a unilateral response is present and in others no response can be detected. Since a bilateral EEG response normally occurs in adults (*2*), our results with infants may be attributed to a lack of maturation. In light of this, a developmental sequence is postulated, beginning first with no driving, then right unilateral followed by bilateral photic driving. We interpret our results as an early manifestation of cerebral dominance in the ontogeny of interhemispheric integration.

The EEG's (*3*) of two samples (groups 1 and 2) of clinically normal, full-term newborn babies were recorded from right and left occipital areas referenced to the right ear, EEG 1 (O_2-A_2) and EEG 2 (O_1-A_2). Electrooculograms (EOG's) on two additional channels detected eye movement. Infants were stimulated by repetitive light flashes of 3 hertz (three flashes per second) for 4 seconds with a Grass PS-2 photostimulator, intensity setting 8, at a distance of 45.7 cm from the eyes, directly in the line of vision (*4*). After screening for artifacts (*5*), the data obtained from 97 babies (*6*) were analyzed.

The EEG's for each baby were ex-