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Freedom from Transmission of Hepatitis-B of Gamma-Globulin and Heat-Inactivated Plasma Protein Fraction Prepared from Contaminated Human Plasma by Fractionation with Solid-Phase Polyelectrolytes[1]

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Abstract. Plasma contaminated with hepatitis B surface antigen (HBsAg) and shown by others to be infectious when injected in a dilution of 1:1,000,000 in chimpanzees, was fractionated by a solid-phase polyelectrolyte (PE) procedure for its content of plasma protein fraction (PPF) and γ-globulin (immune serum globulin; ISG). Quantitative Ausria II radioimmunoassays showed that nearly half the HBsAg was bound by the PE and could be eluted at low pH, while the rest was found in the heat-inactivated PPF. When the ISG was concentrated to 16%, the 13 mg/kg (comparable to a human dose) was injected intramuscularly in 6 chimpanzees, or when the PPF was heated at 60°C for 10 h and injected intravenously in 2 chimpanzees, there was no clinical or laboratory evidence of hepatitis B infection after 12 months, although 1 chimp of 2 who received the same material showed a borderline positive anti-HBsAg antibody result on one of 52 weekly serum samples. Since the new PE fractionation method is essentially nondenaturing, and simpler than the classical ethanol procedures, it was important to establish the noninfectivity of the final products.

The first practical procedures for the large-scale fractionation of human plasma, utilizing ethanol, were reported by *Cohn et al.* [5] in 1946 (method VI) and *Oncley et al.* [17] (method IX). These methods led to the precipitation and purification of many clinically valuable plasma proteins and have been used widely since, starting with large pools of plasma for maximum efficiency. Indeed, for the preparation of immune serum globulin (ISG) the Bureau of Biologics (BOB), of the Food and Drug Administration (FDA) requires a minimum of 1,000 donors per batch to ensure an adequate mixture of clinically important antibodies [4].

Although the BOB requires that individual blood donor units be tested for hepatitis

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B surface antigen (HBsAg) by 'third generation' procedures, usually radioimmunoassay (RIA), these methods are not sufficiently sensitive to detect in various plasma fractions the minimum number of virus particles causing infectivity in man [20] or chimpanzees [2, 4, 14]. Studies with different HBsAg-rich sera have shown that the incubation period before onset of hepatitis B tends to vary inversely with the size of the antigen dose [2, 10], suggesting a relatively constant proportion of intact infectious virions (Dane particles) in these sera [8], but the correlation between the antigen titer and the infective potential in any given sample is virtually impossible to predict.

If the plasma from only 1 of every 100 blood units is contaminated with 10^8 particles/ml of HBsAg, a concentration considered equivocal or negative by RIA [8, 14], the entire pool will be contaminated to the extent of 10^6 particles/ml, an antigen concentration which might be found in infectious sera [14]. Several early studies [9–11, 22] demonstrated HBsAg reactivity in antihemophilic factor (AHF/factor VIII), Cohn fraction I (fibrinogen), fraction III containing coagulation factors II, VII, IX and X, fractions IV and V containing plasma protein fraction (PPF), and normal serum albumin (NSA). The safety of PPF and NSA is ensured by pasteurization at 60 °C for 10 h [10, 11] but hepatitis from administration of fractions that cannot be heat treated is well documented [7, 19, 21], including ISG made by zinc precipitation as in Cohn method XII [18], DEAE-chromatography [12], ether fractionation [3], ammonium sulfate precipitation [1] or, on occasion, even Cohn method VI and IX [6, 11]. Under these conditions, Cohn fraction II (ISG) rarely contains HBsAg, however, and the material is generally not infectious [11].

In a recent report, we described a nondenaturing fractionation method for nearly all the clinically useful plasma proteins, using solid-phase polyelectrolytes (PEs), which is much simpler and usually gives a higher yield than the Cohn methods for comparative fractions. For instance, using PEs, albumin can be produced directly from plasma in a three-step procedure [13].

To make certain that PE-produced ISG was antigen free, we used the method to fractionate gamma globulin from purposely contaminated plasma and remove the contained antigen to less than 5×10^4 HBsAg particles/ml. This is approximately 10,000 times below the sensitivity of the Ausria II RIA [8, 14]. PPF produced from the same contaminated plasma was heat-inactivated for 10 h at 60 °C. Both materials were injected in chimpanzees without evidence of any clinical toxicity.

Materials and Methods

Polyelectrolyte Resin

The PE resin for these experiments (designated E-100) was supplied as a dry powder by the Monsanto Company, St. Louis, Mo. It was stored under nitrogen in a desiccator until used. E-100 is an insoluble cross-linked copolymer of ethylene/maleic anhydride which is substituted with dimethylaminopropylimide functional groups [13] (fig. 1). Prior to use, the E-100 was equilibrated at pH 4.0 in 0.15 *M* NaCl (to protonate the resin). It was then washed 3 times in pH 4.0 saline and resuspended in fresh saline, pH 7.0. There are no known E-100 breakdown products and the PE is almost completely removed by centrifugation at each fractionation step. The few residual 'fines' remaining are removed by filtration through a 0.22 μM sterilization filter.

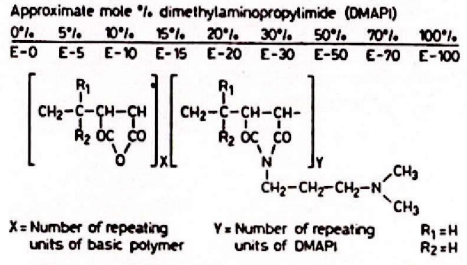

Fig. 1. Approximate structure of ethylene maleic anhydride (EMA) polyelectrolytes (5% cross-linked methylaminobispropylamine).

Polyethylene Glycol (PEG)

PEG of 4,000 mol.wt. (approved by the FDA for pharmaceutical use) was obtained from the Chemical Division of Union Carbide Corp., Tarrytown, N.Y.

All other materials were reagent grade or better.

HBsAg Plasma

HBsAg-rich plasma, subtype adr, was obtained from Dr. *Jay H. Hoofnagle* at the Bureau of Biologics.[2] The plasma was diluted 1:10 in phosphate-buffered saline and stored in 1.0 ml aliquots at –60 °C until needed. The antigen concentration in the diluted stock material was 2.5×10^{11} particles/ml, as quantitated by our modification of the Ausria II RIA [8, 14]. *Barker et al.* [2] showed that this HBsAg-rich plasma caused hepatitis in chimpanzees when injected in dilutions up to 1:1,000,000. Subtype adr was chosen because it is rather rare in this country and would permit epidemiologic studies of any intercurrent infection that might develop.

Animals

Chimpanzees imported from Africa were caged individually, and quarantined for at least 12 months prior to injection, while serologic and tuberculin tests were carried out. The 8 chimpanzees selected for this study were RIA-negative for hepatitis B antigen and antibody and nonreactive for tuberculosis. They were housed and maintained at the New York University Laboratory for Experimental Medicine and Surgery in Primates at Sterling Forest, N.Y.

Before each animal was injected, it was tested biweekly for at least 9 months for HBsAg and anti-HBsAg by solid-phase radioimmunoassay (Ausria II and Ausab, respectively, Abbott Laboratories, Chicago, Ill.). Levels of SGOT and SGPT were determined by the Karmen-LaDue method [23] and results were expressed in Karmen units. After each animal was injected, its serum was tested for these enzymes and for HBsAg at weekly intervals for 9 months. All of these animals were shown to be susceptible to hepatitis B infection when they were used at a later date for different experiments.

Removal of HBsAg from Fractions with E-100

The HBsAg-rich plasma, subtype adr, was diluted in whole plasma negative for antigen by the Ausria II test to a concentration of 2.0×10^8 particles/ml. The plasma used for dilution was also negative for antibody by the Ausab RIA. This plasma (1.25 liters) was diluted 1:8 with pyrogen-free distilled water to give 10 liters of starting material, and 200 g of E-100 was mixed with the dilute plasma (20 mg/ml) in a batch procedure at pH 7.0 for 120 min at room temperature (fig. 2). The dilution has since been changed to 1:4 and the mixing time shortened to 30 min with the same results [13]. Careful attention was given to efficient mixing and all glassware, mixing vessels, lyophilizing vials, etc., were steam sterilized prior to use. The experiments were done either at 4 °C or room temperature under aseptic conditions.

The PE was removed from the plasma by centrifugation; and 5,000 ml of the γ-globulin-rich supernatant (S-1) was concentrated to 1.0 ml by our pepsin-PEG method [8] to permit quantitative assay of the antigen. 10 mg of fresh E-100/ml was added to the remaining S-1 and the pH adjusted to 8.0 with NaOH to further purify the ISG and remove the HBsAg. This second supernatant (S-2) was processed twice more with 10 mg of the fresh PE/ml; samples of the S-2, S-3 and S-4 supernatants were taken for characterization of the protein and for quantitative antigen assay. ISG from these supernatants was concentrated to 16% pro-

[2] Now at the Veterans Administration Hospital, Washington, D.C.

Table I. Protocol for injection of E-100 prepared γ-globulin (ISG) and PPF into chimpanzees

Chimp No.	Material[a]	Dose/kg[b]	HBsAg particles/ml
90/ 99	ISG (2nd adsorption)	13 mg/kg – 1.0 ml	$<10^4$
103/107	ISG (3rd adsorption)	13 mg/kg – 1.0 ml	$<10^4$
92/101	ISG (4th adsorption)	13 mg/kg – 1.0 ml	$<10^4$
111/113	PPF	360 mg/kg – 57.0 ml	1.3×10^8
Controls[c]	HBsAg-adr antigen	1.0 ml	$10^8 - 10^{12}$

[a] The PPF and γ-globulin fractions were concentrated to 87 and 160 mg/ml protein, respectively, with PEG. The PPF was heat treated for 10 h at 60 °C prior to injection.

[b] Normal human dose: γ-globulin 7.0–10.0 mg/kg; albumin 160–400 mg/kg.

[c] Control animals were injected with adr-HBsAg in dilution range of 1:1 to 1:1,000,000 by *Barker et al.* [2].

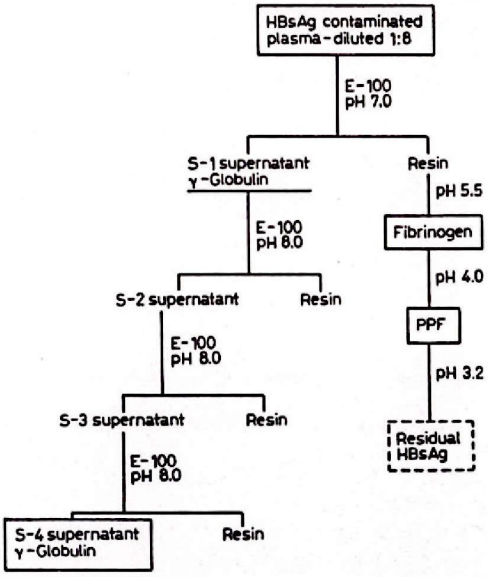

Fig. 2. Preparation of γ-globulin and PPF from HBsAg-contaminated human plasma with E-100. Supernatant 1 (S-1) was cycled 3 more times to remove possible impurities. Fibrinogen, PPF and the residual HB_sAg were eluted from the resin after the first adsorption.

tein by 20% (w/v) PEG, pH 6.5, filtered through sterile 0.22 μm filters (Millipore Corp., Bedford, Mass.) and injected intramuscularly into chimpanzees according to the protocol in table I. The PEG concentration in the final ISG and PPF products was less than 0.1% as determined by assay [15].

Most of the fibrinogen was eluted from the PE resin used for the first adsorption with 0.15 *M* NaCl at pH 5.5, and the PPF (97% albumin) was eluted at pH 4.0 with 2,500 ml of distilled water at a protein concentration of 10 mg/ml (fig. 2). The HBsAg in the fibrinogen and PPF fractions (500 ml each) was concentrated 500 times by the pepsin-PEG procedure and assayed by the quantitative RIA.

The PPF was sterile filtered, mixed with 0.02 *M* sodium caprylate-0.02 *M* tryptophanate to stabilize the protein, and heated for 10 h at 60 °C to ensure noninfectivity. The preparation was concentrated to 87 mg protein/ml by precipitation with 20% (w/v) PEG, pH 4.8. Since commercial PPF solutions are typically 5% (w/v), we felt an 8.7% solution would be adequate for these experiments. We have subsequently prepared PPF and albumin as 25% solutions. Two chimpanzees were injected intravenously with a solution containing 6 g (360 mg/kg) – equivalent to a human dose (table I).

The HBsAg adsorbed on the PE was eluted with 0.05 *M* lysine, pH 3.2, and concentrated 625 times for quantitative RIA.

Quantitative HBsAg

The number of HBsAg particles/ml at each fractionation stage was determined by the modified Ausria II RIA [8, 14] which has a standard error of less than 20%.

Protein Assay

The concentration of total protein was measured by the biuret method [16] using commercial ISG or PPF as a standard. Purity was assessed by electrophoresis on preformed agarose gel plates (ACl Corp., Palo Alto, Calif.). Electrophoresis was performed in 0.05 *M* barbital buffer, pH 8.6, and the gels were stained with Coomassie brilliant blue for 1 h. Gels were scanned in a Schoeffel Corp. scanning spectrodensitometer, and the scan was integrated by an on-line Digital Equipment PDP 11/10B minicomputer.

Results

Removal of HBsAg

Although the HBsAg was adsorbed from contaminated plasma at pH 7.0 and the γ-globulin was processed at pH 8.0 in these studies, our present data show that adsorption of HBsAg on E-100 appeared to be optimal at pH 6.5–7.0 and the purity of ISG prepared with E-100 showed a similar pH dependence (fig. 3).

A 5,000-fold concentrated sample of the first supernatant (S-1) containing γ-globulin was RIA-negative, indicating that the first E-100 adsorption reduced the antigen concentration from the initial value of approximately 2.0×10^8 particles/ml to less than 5.0×10^4 particles/ml (table II). Since the fractions resulting from subsequent adsorptions of the supernatant must have still fewer than 5.0×10^4 particles/ml, the S-2, S-3 and S-4 supernatants were not concentrated for

Table II. Distribution of HBsAg after fractionation of contaminated human plasma with E-100

Sample	HBsAg particles/ml	Total number of HBsAg particles[a]
HBsAg (adr)-contaminated plasma	2.0×10^8	2.0×10^{13}
γ-Globulin supernatant	$<5.0 \times 10^4$	$<2.8 \times 10^8$
pH 5.5 E-100 eluate (fibrinogen)	$<5.0 \times 10^5$	$<2.5 \times 10^8$
pH 4.0 E-100 eluate (PPF)	1.3×10^8	3.25×10^{11}
pH 3.2 E-100 eluate (HBsAg)	0.3×10^8	1.0×10^{11}
Total recovery	1.6×10^8	0.4×10^{12}

[a] Each value is adjusted to the total volume of the fraction obtained, although only sample aliquots were concentrated for quantitative RIA.

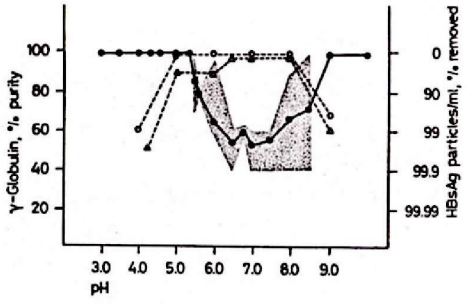

Fig. 3. Effect of pH on purity of γ-globulin and removal of HBsAg during purification with E-100. The purity of γ-globulin is shown after 1st adsorption (S-1; △), and after 2nd adsorption (S-2; ○). The mean (●) and range of data (strippled area) are given for the percent removal of HBsAg particles/ml (5 or more determinations).

assay. The fibrinogen-rich fraction, concentrated 500 times, contained fewer than 5.0×10^5 particles/ml; but the PPF eluate, after 500 times concentration, contained approximately 1.28×10^8 particles/ml. The antigen eluted from the PE and concentrated 625 times contained 0.33×10^8 particles/ml on assay. Nearly all of the HBsAg could be accounted for in the PPF or on the PE (table II).

The 6 chimpanzees injected with PE E-100 γ-globulin and the 2 injected with heat-treated PE E-100 showed no clinical signs or symptoms of viral hepatitis; in no instance were the HBsAg assay results more than 3 SDs from the mean of the negative serum control on any two successive test dates, and only 1 chimp (CH 101-4th adsorption) showed a mildly positive antibody (16 RIA units: $<$ 10 RIA units is negative) on one of 52 weekly serum samples. The SGOT and SGPT levels remained within the normal range (11–34 and 11–44 Karmen units, respectively) established for these animals during the 9 months prior to injection.

Discussion

A new solid-phase polyelectrolyte method devised for the fractionation of PPF (or albumin) and ISG also removes the hepatitis B antigen from the ISG. When the PE ISG was prepared from contaminated plasma and injected in chimpanzees, they failed to develop hepatitis.

The material balance of the HBsAg showed that most of the antigen was either eluted in the PPF fraction or remained on the PE. After heat treatment, the PPF proved noninfective in chimpanzees. The antigen content of ISG and fibrinogen was less than the concentration found in infective sera, as shown by our *in vitro* quantitative procedures and the chimpanzee study.

When other investigators injected chimpanzees with the undiluted adr antigen-positive serum (about 2.2×10^{12} particles/ml) used to contaminate fresh-frozen, antibody-negative plasma for our studies, the animals developed hepatitis within 3 weeks [2]; when a 10^6 dilution (about 2.2×10^6 particles/ml) of the same serum was injected, the incubation period was 13 weeks. As indicated above, antigen levels below 2×10^8 particles/ml are not usually detectable by the Ausria II. These controls were considered adequate because of the mild conditions during PE fractionation.

As expected, the antigen-rich PPF was not infective in chimpanzees after heat treatment by conventional pasteurization techniques [10, 11] now used for PPF and albumin. Pasteurized PPF and albumin frequently contain HBsAg, but have not caused hepatitis in man; to the authors' knowledge no reports have appeared on the effect of injecting pasteurized, antigen-rich PPF in chimpanzees.

The γ-globulin fractionated by the PE procedure in these experiments had a yield of 90% and was more than 99% pure as determined by agarose-gel electrophoresis; it was concentrated to 16% by PEG precipitation. The PPF was obtained in 93% yield, contained approximately 97% albumin, and was concentrated to 87 mg/ml protein. Although Cohn-fractionated ISG and PPF do not usually transmit hepatitis [11], the relatively simple PE fractionation method requires less than 1 day, is easily scaled up from the 10 liters of starting material used in these experiments, and the yield of highly purified albumin is over 90% [13].

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