Immunogenic Properties of Human Gamma Globulin Free of Endotoxin Contamination (35242)

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The immunological responsiveness of an animal to foreign serum proteins depends upon the physical state of the administered material. The supernatant after ultracentrifugation of soluble bovine γ globulin (BGG) is capable of inducing immunologic tolerance in mice while injection of the sedimented material results in an antibody response (1). It has also been shown that soluble, nonaggregated human γ globulin (HGG) is tolerogenic while heat-aggregated HGG is immunogenic in adult rabbits and mice (2). When phagocytizable material from commercial bovine serum albumin (BSA) was removed by "biological filtration" in the rabbit, the remaining fraction became nonimmunogenic and in some cases tolerogenic when infused into other rabbits (3). In a subsequent series of experiments (4) BSA was separated into tolerogenic and immunogenic fractions by gel filtration. Since immunogenicity can be restored to ultracentrifuged serum proteins by the addition of the aggregated fraction, it was proposed that the denatured fraction behaved as an adjuvant (5). This interpretation was strengthened by the observation that "biologically filtered" BSA becomes immunogenic when injected together with bacterial endotoxin, a known adjuvant (4). Similarly, tolerogenic supernatant BGG behaves as an immunogen when injected with bacterial endotoxin into adult mice (5). Since commercial BSA is usually contaminated with bacterial endotoxin (6) it has been suggested that contaminating endotoxin may be responsible for the immunogenicity of this material in adult rabbits (7). The possibility must be considered that the immunogenic material which is removed by biologic filtration, ultracentrifugation, and gel filtration is endotoxin which when present acts as a nonspecific adjuvant and is responsible for the immunogenicity of soluble serum proteins. The fact that endotoxins are adjuvants (8) supports such an hypothesis.

The present studies were designed to answer the question of whether contaminating endotoxin was responsible for the immunogenicity of the aggregated fraction of serum proteins. The results show these materials can be immunogenic in the absence of contaminating endotoxin.

Materials and Methods. Preparation of antigen. Human γ globulin (HGG) fraction II (Pentex) was used for all immunizations as well as for the quantitative determination of precipitating anti-HGG antibody. An HGG solution in a concentration of 20 mg/ml in phosphate buffered saline 0.15 *M*, pH 7.4 (PBS) was centrifuged at 105,000*g* at 20° for 90 min, using a Spinco rotor J65. The top two-thirds of the supernate was divided into equal volumes designated Fractions A and B, aggregate free but aggregable material. The remainder of the supernate was discarded. The sedimented material was redissolved in one-third the original volume of PBS and designated Fraction C, which contained aggregated material present in the original preparation as well as any other material that would sediment at 105,000*g*. Fraction B was heated at 63° for 20 min in a water bath. The materials were adjusted in PBS to the appropriate concentration for later immunizations by optical density determinations at 278 nm. Thus, Fraction A contained unheated, unaggregated material; Fraction B contained heated aggregated material; Fraction C contained unheated, sedimented material.

Immunization of rabbits. Twenty-four New Zealand albino rabbits of either sex, weighing approximately 2.5 kg, were used throughout. Groups of eight animals each were the recipients of the three different fractions. At the time of immunization, the materials were tested for pyrogenicity as described below. The eight animals in each group were given 5 mg iv of the appropriate HGG fraction (either A, B, or C) in 1 ml of PBS. Two weeks later, all animals received 2 mg of unaltered (unheated, unsedimented) HGG iv in 1 ml of PBS. Two weeks following the second injection, four animals from each of the three groups were exsanguinated and the sera were stored at —20°. The remaining animals were secondarily challenged with 5 mg iv of Fraction B, HGG (aggregated) and 2 weeks later were injected with 2 mg iv of unaltered HGG. These latter animals were exsanguinaged 2 weeks following the last injection and all of the sera were then assayed for anti-HGG antibody levels.

Pyrogen test. Pyrogen testing was carried out as previously described (9). Temperatures (°) were monitored with rectal thermistors[1] connected to a recording telethermometer.[1] On the day prior to the initial immunization, rabbits were trained for 6 to 8 hr. On the day of immunization, temperatures were monitored for at least 1 hr to insure a steady base line and animals were used only if the base line temperature was between 38.5 and 39.9°.

Determination of antibody response. Quantitative determination of precipitating anti-HGG antibody was performed with ^{125}I HGG according to Katz *et al.* (10) as follows: 0.2 ml of antiserum was reacted with 0.1 ml of ^{125}I HGG in varying concentrations from 10 to 3200 μg/ml. The mixtures were incubated at 37° for 1 hr and then at 4° for 24 hr. The samples were centrifuged at 2500 rpm for 30 min at 4° and the radioactivity of an aliquot of each supernatant was measured in a NaI crystal scintillation counter. Curves were constructed by plotting percentage of antigen precipitated against antigen concentration. From these curves, the antigen concentration at which 50% of added antigen was precipitated was determined (P_{50}). P_{50} is expressed as micrograms of HGG added per milliliter of antiserum.

[1] Yellow Springs Instrument Company, Yellow Springs Ohio.

TABLE I. Quantitative Determination of Precipitating Anti-HGG Antibody with ^{125}I-HGG.

Immunizing fraction[a]	Animal no.	Antibody response following primary immunization (P_{50}[b])	Antibody response (P_{50}) following secondary challenge[c]
A	1	0	—
(supernate)	2	0	—
	3	0	—
	4	0	—
	5	0	0
	6	0	0
	7	0	0
	8	0	0
B	9	20	—
(aggregated)	10	10	—
	11	12	—
	12	8	—
	13	37	58
	14	19	41
	15	18	93
	16	30	72
C	17	Died	—
(sediment)	18	9	—
	19	0	—
	20	0	—
	21	51	62
	22	0	0
	23	15	20
	24	25	25

[a] Each animal received 5 mg of either Fraction A, B, or C HGG followed by 2 mg of HGG in 2 weeks. They were bled 2 weeks after last injection.

[b] P_{50} is the antigen concentration (μg/ml of antiserum) at which 50% of added antigen was precipitated. 0 = no specific precipitation was observed when 5 μg of antigen/ml of antiserum was used.

[c] At the time of first bleeding, four animals from each group were challenged with 5 mg of fraction B HGG, then in 2 weeks were rechallenged with 2 mg of HGG and 2 weeks later were exsanguinated.

Results. Pyrogen testing. None of the 24 rabbits tested after the initial injection developed a febrile response, Thus, neither the sedimented, aggregated, nor supernatant HGG contained pyrogenic material.

Antibody responses. As shown in Table I, all eight rabbits that had received HGG Fraction A (supernatant) failed to produce detectable anti-HGG antibody. In contrast, the rabbits given HGG Fraction B (aggregated) produced antibody. Of the eight rabbits which had received Fraction C (unheated sediment) one died before assay and four of seven produced antibody.

To determine if the lack of antibody production in the Fraction A animals was the result of immunolgic tolerance, four of the eight non-responders were challenged with the regimen that had proven immunogenic in all of the Fraction B animals, *i.e.*, 5 mg of aggregated HGG followed in 2 weeks by 2 mg of native HGG. All four of these rabbits proved to be tolerant to HGG by failing to respond to the aggregated material.

In addition, one-half of the Fraction B animals (responders) were challenged with the aggregated material and all manifested a "booster" response to the HGG (Table I). Finally, four of the eight animals of the Fraction C group, three of which were responders were challenged with aggregated HGG. The three responders increased or maintained their antibody levels and the non-responder proved tolerant by failing to make antibody despite the injection of aggregated material (Table I).

Discussion. The present study was undertaken to determine if the immunogenicity of the sedimented or aggregated fraction of a serum protein (HGG) was due to contamination with endotoxin. That such might be the case had been suggested by others (7). It should be noted that in that report (7), BSA was the antigen employed, although it would seem unlikely that the mechanism for development of the immune response to one soluble serum protein would be different from that to another. Therefore it seemed appropriate for us to employ a soluble serum protein in the present studies that was known not be be contaminated with bacterial endotoxin. Different methods have been utilized to separate serum proteins into various fractions: ultracentrifugation (2), biological filtration (3), and gel filtration (4, 6). It is possible that each of these methods could remove contaminating endotoxin from the aggregate-free material but allow it to remain with the aggregated or sedimented material. With the observations of Frei *et al.* (4) using BSA, and Claman (5) using BGG that supernatant, tolerogenic material became immunogenic when injected together with endotoxin, one might speculate that they were merely replacing the endotoxin removed by the separation procedures.

The present study clearly demonstrates that in a system in which a pyrogen-free soluble serum protein (HGG) is separated into tolerogenic and immunogenic fractions by centrifugation and heat aggregation, endotoxin is not a factor in the immunogenicity of the aggregated material. Although pyrogenicity is the only biologic activity of endotoxin examined, this assay is routinely used in our laboratory with sensitivity and reproducibility (9). Since it is well known that the adjuvant activity of endotoxin requires considerably more endotoxin than needed for pyrogenicity, it is unlikely that we did not detect biologically active material. It is probable that the immunogenicity of the aggregated or sedimented material (as compared to the ultracentrifuged HGG) is dependent upon its ability to be taken up by macrophage surface receptors before it is presented to lymphocytes as earlier hypothesized (4). Similarly particulate or aggregated antigen which is concentrated on the surfaces of dendritic macrophages may act as a stimulus for antibody production by predetermined cells. Adjuvant, endotoxin or others, need not be present for this process to occur as shown in this study.

When an antigen is not phagocytizable and potentially tolerogenic, the fact that endotoxin or other adjuvants can render it immunogenic implies that some aspect of the "processing" or "concentrating" step described above is altered by the adjuvant such that the soluble antigens now reach susceptible lymphocytes in a manner which elicits antibody production rather than immunological unresponsiveness.

Summary. Human gamma globulin (HGG) was separated into three fractions following ultracentrifugation: (A) aggregate-free supernatant, (B) heat aggregated supernatant, and (C) sediment. The aggregate-free

material proved tolerogenic in rabbits. The aggregated material was immunogenic in all rabbits tested, and the sedimented material was immunogenic in four of seven rabbits. None of these fractions were pyrogenic and therefore considered to be free of contaminating endotoxins.

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