

## Tolerance Induction with Aggregated Bovine Gamma Globulin in C57Bl/6 Mice<sup>1</sup> (37594)

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In 1961, Dresser (1) described preparation of an antigen, bovine  $\gamma$ -globulin (BGG), which would not induce immunity when injected into CBA mice while the original material would. The so-called adjuvanticity of the original material was thought to reside in aggregated or particulate matter which could be removed by high speed centrifugation. The soluble component left behind would induce immunological paralysis when injected into the same strain mice, but could induce immunity if given with an adjuvant.

Essentially similar findings were reported for the antigen human  $\gamma$ -globulin (HGG) by Golub and Weigle (2), who, in addition, showed important strain differences in respect to ability of centrifuged HGG to induce tolerance. Strain C57Bl/6 mice, for example, could be easily paralyzed by as little as 50  $\mu$ g of HGG which had been spun at 30,000g for 30 min, but Balb/C mice were not paralyzed by even 10 mg. From these data, it was postulated that Balb/C mice were considerably more efficient in processing the trace amounts of aggregated  $\gamma$ -globulin to give an immune response while C57Bl/6 mice were paralyzed by the major toleragenic component.

Further confirmation of this postulate was seen in the ability to remove the component responsible for immunization of the Balb/C mice by  $\text{Na}_2\text{SO}_4$  fractionation and by biologic filtration through normal mice, procedures considered suitable for removal of aggregated materials. Some doubt, however, was cast on this interpretation by the observation that even centrifugation for 1 hr at 105,000g was

unable to remove the component producing immunity in the Balb/C mice (2).

Similar findings (3), which we have confirmed, concerned the use of BGG in Balb/C mice, namely, that ultracentrifugation at 100,000g for 0.5 hr does not remove the component immunizing this strain but biologic filtration through this strain does. Because these data suggested the possibility that a property other than aggregation was being screened, we decided to investigate directly the effect of aggregation of BGG and see whether a totally aggregated preparation could still produce tolerance in a susceptible strain.

*Materials and Methods.* Adult strain C57Bl/6 mice of both sexes which are readily paralyzed by 0.2 mg of centrifuged BGG were used throughout. They were routinely injected ip with the test dose of BGG in saline. One week later they were immunized with 100  $\mu$ g of BGG in complete Freund's adjuvant and 1 wk after that, put on iodized water and challenged ip with a test dose of about 3  $\mu$ g <sup>131</sup>I labeled BGG. Trace labeled BGG was prepared fresh each time by the chloramine-T procedure (4). Whole body counting of the mice was performed daily by placing them in a plastic container into a large crystal gamma counter. Counting was conducted for at least 5 days or until background levels were obtained.

Since heat aggregation of BGG gave a product which was unstable on standing, it was felt that a more suitable preparation could be made for testing by use of the bi-functional coupling reagent bis-diazotized benzidine (BDB) (5). To 20 ml of a 1% solution of BGG in barbital buffer (pH 8.5) was added 0.1 ml of a 1% solution of BDB

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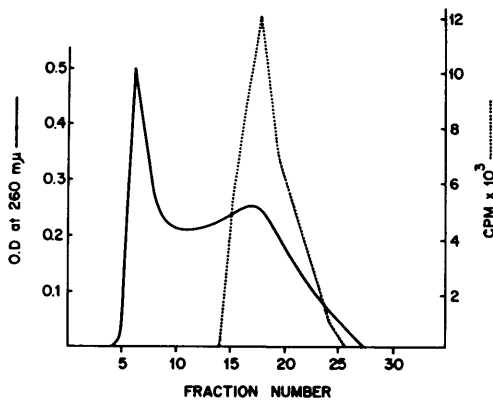


FIG. 1. Fractionation of BDB-aggregated BGG with added  $^{131}\text{I}$  labeled BGG on a Bio-Gel A, 5 M column. (—) Protein concentration on left ordinate; (····) gamma radiation on right ordinate.

giving a molar ratio of 2 BDB/1BGG. The solution was stirred at room temperature for 30 min and then dialyzed extensively against buffer with glycine added.

A portion of this material was mixed with a small amount of  $^{131}\text{I}$  labeled BGG and put on a Bio-Gel A, 5 M column. Elution with 0.05 M borate buffer (pH 8.5) yielded two main fractions as shown in Fig. 1. The first, appearing just after the void volume, contained aggregates of molecular weight about  $10^7$ ; the last, a broad shoulder containing all the radioactivity, probably contained smaller aggregates and unaggregated BGG.

To purify the larger aggregates for use in tolerance induction, they were first concentrated from the original reaction mixture and freed of unaggregated BGG by precipitation  $3\times$  from 0.6 M  $\text{Na}_2\text{SO}_4$  and re-solution in saline. After the last precipitation, the aggregates were dissolved in 0.05 M borate buffer (pH 8.5) and run on a Bio-Gel A, 5 M column with the same buffer. The fractions comprising just the sharp peak, appearing immediately after the void volume, were collected, pooled and analyzed for protein content. When this fraction was subjected to centrifugation at 100,000g for 30 min, about 40% of the total protein could be accounted for in the pellet. Essentially, all the protein was precipitable by 0.6 M  $\text{Na}_2\text{SO}_4$ .

**Results.** This soluble, high molecular weight aggregate preparation was tested for ability

to induce tolerance in groups of C57Bl/6 mice. In addition, a highly aggregated, insoluble preparation made by use of a greater BDB/BGG ratio was similarly studied. Figure 2 shows the combined results of 2 experiments comprising 9 mice per group. It may be seen that at a test dose of 2 mg and 0.2 mg, BDB-aggregated but soluble BGG produced a high degree of unresponsiveness comparable to that achieved with 0.2 mg of centrifuged, unaggregated BGG. Even the 0.02 mg dose of aggregated BGG produced partial tolerance while as much as 20 mg of the highly aggregated insoluble BGG left the animals immune.

To follow the fate of these soluble aggregates in normal mice, they were trace labeled with  $^{131}\text{I}$  and approximately 15  $\mu\text{g}$  injected intraperitoneally into groups of mice. Whole body counting revealed that they were cleared at a significantly faster rate than monomeric BGG with 26% remaining at 24 hr and 12% at 48 hr in comparison to 66 and 45%, respectively, for the monomer.

**Discussion.** In summary, the aggregated nature of BDB-treated BGG was shown by molecular filtration on a Bio-Gel column, precipitation with 0.6 M  $\text{Na}_2\text{SO}_4$ , centrifugation at 100,000g and rapid clearance in normal mice. The combination of salt precipitation and gel filtration left no detectable

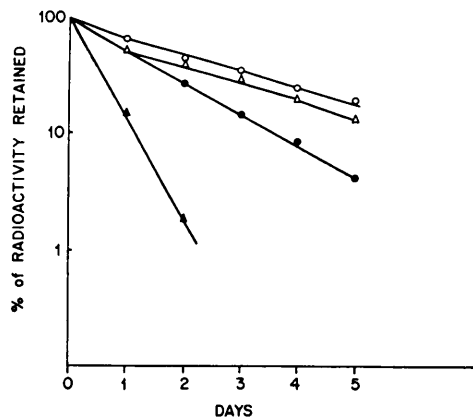


FIG. 2. Clearance of test dose of  $^{131}\text{I}$  labeled BGG from mice pretreated with 0.2 mg BGG (○—), 2 or 0.2 mg of BDB-aggregated BGG (△—), 0.02 mg BDB-aggregated BGG (●—), or saline (immune control) (▲—) and then immunized with 100 mg BGG in complete Freund's adjuvant.

monomeric BGG, yet the material was still capable of eliciting tolerance in comparable doses to monomeric BGG. Insoluble BGG aggregates at even higher doses, however, were not capable of doing so, possibly because of their inability to circulate. These results tend to cast some doubt on the validity of the concept that the "adjuvant-icity" of BGG preparations is dependent solely on the presence of aggregates, at least so far as C57Bl/6 mice are concerned. Unless these aggregates are of the insoluble sort, which can be removed at ordinary centrifugal speeds (10,000g), it would appear that even large molecular weight aggregates (mol wt  $10^7$ ) as long as they remain soluble, are still capable of inducing tolerance in this strain of mice. It is possible that since these soluble aggregates are only partially removed by ultracentrifugation at 100,000g, although completely precipitable by 0.6 M  $\text{Na}_2\text{SO}_4$ , centrifugation leaves sufficient aggregated BGG to immunize the more highly responsive Balb/C strain while salt precipitation and biologic filtration leave only monomeric BGG incapable of immunizing this strain.

Alternatively, it is known that a minor component of BGG produces immunity at the same time the major component elicits tolerance following injection of centrifuged BGG (6). This immunizing fraction is not removable by ultracentrifugation but can be biologically filtered by passage through normal mice presumably because of its shorter half-life *in vivo*. A similar explanation may be offered to explain why Balb/C mice become immune even when given ultracentrifuged BGG or HGG but can be rendered tolerant by biologically filtered material. Rather than soluble aggregates removable only in part by ultracentrifugation, the immunizing capacity may reside in a fraction of globulin that is physicochemically similar to the main component but biologically distinct.

Two observations favor the interpretation that this component might be specific antibody reactive with some constituent antigen of the recipient mouse. One is that biologic filtration as a means of producing toleragenic material seems to be confined to serum

globulin antigens (7); the other is that while normal rabbit  $\gamma$ -globulin induced tolerance in mice, rabbit anti-lymphocyte  $\gamma$ -globulin does not (2).

Regardless of the mechanism involved, it is apparent that a marked difference exists between C57Bl/6 and Balb/C mice. The former strain becomes tolerant even with totally aggregated though soluble BGG while the latter strain is immunized with even trace amounts of material resembling this same aggregate. Some preliminary experiments on biologic filtration suggest that this difference may be reflected in the ability of the respective reticuloendothelial systems to filter out aggregated materials, the Balb/C behaving as though it possessed a highly activated system capable of removing considerably more material per unit of time.

*Summary.* Bovine  $\gamma$ -globulin aggregated by treatment with bis-diazotized benzidine was fractionated by  $\text{Na}_2\text{SO}_4$  precipitation and elution from a Bio-Gel A column to give a highly aggregated though soluble fraction with a molecular weight of about  $10^7$ . This material administered to C57Bl/6 mice still produced tolerance in doses above 0.2 mg comparable to that produced by aggregate-free BGG. By contrast, Balb/C mice require totally monomeric BGG for tolerance induction. The difference in behavior to BGG of these strains is reflected by the differences in ability of their reticuloendothelial systems to filter and clear BGG.

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