Preparation and Immunological Properties of a Brilliant Blue-Protein Conjugate

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ABSTRACT

Brilliant Blue F.C.F. (BB), after purification to remove high molecular weight, blue-colored contaminants present in commercial preparations, was conjugated to bovine serum albumin (BSA). Phosphorus oxychloride was used to transform the sulfonate groups of BB to sulfonyl chloride groups; the product of this reaction was then allowed to react further with the protein. The conjugate thus prepared was purified by Sephadex G-25 column chromatography and found to contain 7 moles of BB per mole of BSA. Immunological studies in the rat indicated that neither IgE nor IgG a antibodies to BB could be detected in the sera of immunized animals. This would tend to suggest that BB possesses a low degree of allergenicity. However, the possibility that some individuals under appropriate circumstances could respond with allergic manifestations to BB cannot be dismissed.

Penicillin and other anti-infective agents are widely used in the treatment of mastitis in dairy cows by intramammary infusion. The subsequent appearance of residues in milk could constitute a hazard to public health (3). Several preventive measures have been suggested to curtail this problem, among which is the incorporation of tracer dyes in intramammary preparations. This would provide a simple means for eliminating the use of milk from treated cows during and shortly after treatment.

Brilliant Blue (BB) can be successfully used in intramammary creams given to the lactating cow (1), as well as in the cow which has been dried off (4), without detrimental effects. The methodology for detection of the tracer dye was reviewed and further improvements of the ion-exchange method were described in a paper by Macintosh and Vilim (13). The small column method (13) was used successfully in a field trial comparing depletion of tracer dye and penicillin following intramammary infusion with dye-marked mastitis cream to healthy cows (21). Suggestions have been made, however, that low concentrations of the dye, below the detection level, may contaminate the milk supply. Because of this possibility, the safety of BB is of paramount importance.

Some of the FD&C-approved synthetic colors in foods and drugs have been implicated in allergic reactions (12,14) that range from urticaria to severe asthma. Most reported cases of allergy involve tartrazine, but sporadic cases involving other dyes can be found in the medical literature. Similarly, food colors have been implicated in hyperactivity in children (5). However, the association of hyperactivity with color additives has not been validated in controlled, double-blind experiments (6).

Recently, we have used an animal model to study the allergenic and immunogenic properties of drugs (9-11). We have been able to correlate anaphylactic antibody production (IgE, IgG a) in Wistar rats to drug determinants with a rather high risk of allergy in man to these drugs. The procedure consists of (a) preparing conjugates of the low molecular weight compound (hapten) with proteins, (b) immunizing groups of Albino Wistar rats with the conjugate and (c) analyzing the sera from immunized animals for presence of antibodies, and determining their specificity and type, using the conjugate in combination with the hapten. To obtain information regarding the allergenic potential of BB, it was decided to carry out a similar study with the dye.

MATERIALS AND METHODS

Materials

BB, C.I. No. 42090, was obtained from Matheson, Coleman and Bell Manufacturing Chemists (Norwood, Ohio, U.S.A.). pertussis vaccine
Preparation of BB sulfonyl chloride and BB-protein conjugate

BB sulfonyl chloride groups to react with BSA; the remaining groups would be hydrolyzed back to the original sulfonate groups. Spectroscopy of BB-BSA conjugate in the ultraviolet region revealed substantial changes when compared with similar spectra of BSA and BB, indicating that conjugation was effected. In the visible region, the spectra of BB and BB-BSA conjugate overlapped, both showing a sharp peak at 630 nm. The optical density at 630 nm was used to determine the BB content of the conjugate. The conjugate thus prepared, containing 7 moles of BB per mole of protein, was used to immunize Albino Wistar rats under various schedules of immunization for anaphylactic antibody production. Analysis of sera from animals immunized with BB7-BSA indicated that the conjugate failed to induce production of detectable levels, by the methods employed, of (a) IgE antibodies to BB in animals immunized with the conjugate and either B. pertussis or alum as adjuvants and (b) of IgG2 antibodies in animals immunized with the conjugate and Freund’s adjuvant. Emphasis was placed on anaphylactic antibodies as these antibodies participate directly in the induction and development of allergic reactions. As anaphylactic antibodies could not be detected in the sera of immunized animals, this would tend to indicate a low degree of allergenicity for this dye.

**RESULTS AND DISCUSSION**

As BB is a hapten and thus non-immunogenic in conventional methods of antibody induction, attempts were made to conjugate the dye to a macromolecular carrier before undertaking immunological studies.

Figure 1 shows the structure of BB, the conversion of its sulfonate groups to sulfonyl chloride groups and the further reaction with the amino groups of BSA. Even though during the first reaction more than one sulfonate may react with phosphorus oxychloride, steric hindrance would make it improbable for more than one of the sulfonyl chloride groups to react with BSA; the remaining groups would be hydrolyzed back to the original sulfonate groups. Spectroscopy of BB-BSA conjugate in the ultraviolet region revealed substantial changes when compared with similar spectra of BSA and BB, indicating that conjugation was effected. In the visible region, the spectra of BB and BB-BSA conjugate overlapped, both showing a sharp peak at 630 nm. The optical density at 630 nm was used to determine the BB content of the conjugate. The conjugate thus prepared, containing 7 moles of BB per mole of protein, was used to immunize Albino Wistar rats under various schedules of immunization for anaphylactic antibody production. Analysis of sera from animals immunized with BB7-BSA indicated that the conjugate failed to induce production of detectable levels, by the methods employed, of (a) IgE antibodies to BB in animals immunized with the conjugate and either B. pertussis or alum as adjuvants and (b) of IgG2 antibodies in animals immunized with the conjugate and Freund’s adjuvant. Emphasis was placed on anaphylactic antibodies as these antibodies participate directly in the induction and development of allergic reactions. As anaphylactic antibodies could not be detected in the sera of immunized animals, this would tend to indicate a low degree of allergenicity for this dye.

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The results of this study were obtained using a BB-protein conjugate. The possibility remains that other types of conjugates or other forms of the dye (metabolic or degradation products) could play a more important role in allergy. Animal experimentation has shown that BB is excreted almost completely as such after oral administration (7,19), absorption from the gastrointestinal tract is minimal (7,19) and metabolism plays, if any, an insignificant role during oral feeding although the liver is capable of metabolizing the compound to unidentified products (2). However, a number of factors can modify metabolic rates, metabolic pathways and absorption of various compounds. Thus it is conceivable that certain individuals on occasion could handle the dye differently.

BB in a mixture with FD&C yellow No. 5 (tartrazine) and FD&C red No. 2 (amaranth) was used by Hosen (8)
for provocative tests. Using the sublingual technique 15% of 500 patients reacted to the color mixture. How many, if any, of these reactions were due to BB was not indicated. Although findings in our study suggest a low degree of allergenicity for BB, this does not imply that individuals possessing a low constitutional degree of allergy. Thus these results might indicate that the risk of allergy for this dye is low. However, the possibility remains that some individuals with a low "threshold of allergy" under appropriate conditions could develop allergic manifestations when exposed to the dye.

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REFERENCES