# Altered Immunogenicity Produced by Change in Mode of Linkage of Hapten to Carrier (36400)

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The protein carrier in a hapten–protein conjugate has a profound effect on the size of the antihapten immune response. In general, the more immunogenic the carrier, the larger the response to the hapten (1). Production of tolerance to the carrier in animals immunized with hapten–carrier conjugates causes a decrease in the antihapten response (2). The nature of the hapten, also seems to affect the size of the immune response. Conjugates with dinitrophenyl (DNP) or picryl (TNP) haptens usually elicit more antihapten antibody than conjugates with azobenzoate or azobenzenearsonate (3).

Despite such findings a meaningful comparison of the immunogenic potency of haptens is made difficult by the many variables associated with the production of conjugates, such as degree of coupling, the type of bond formed, the site of attachment and the nature of the carrier. However, it has been suggested (4) that haptens coupled to carrier proteins by azo linkages give only modest antibody levels. This was in contrast to amide coupled haptens which under similar conditions, provoked considerably more hapten specific antibody.

In order to determine the effect of the mode of coupling of a hapten on the immune response to a hapten–carrier conjugate, the response to one hapten (DNP) coupled in different ways to one carrier, bovine serum albumin (BSA), was studied.

*Materials and Methods.* DNP-conjugates were prepared by reaction of proteins, recrystallized 2,4-dinitrobenzenesulfonic acid and $K_2CO_3$ in aqueous solution (5). Extent of conjugation was calculated by optical density measurements at 360 m$\mu$, using 17,530 as the molar extinction coefficient for DNP–lysyl residues. Two conjugates were prepared, $DNP_{29}$–BSA and $DNP_{10}$–BSA.

As an alternate method of conjugation, 2, 4-dinitrophenyldiazonium fluoroborate was first prepared from 2,4-dinitroaniline by a modification of the procedure of Metzgar, Wofsy, and Singer (6). This was coupled to protein carriers by reaction in cold acetate buffer at pH 5.0. Degree of conjugation was estimated according to Eisen, Carsten, and Belman (7) using a molar extinction coefficient at 360 m$\mu$ of 7980 for DNP-azo-*p*-cresol. It was estimated that about 23 and 9 DNP-azo groups/molecule of BSA were coupled in the two preparations used ($DNP_{23}$-azo–BSA and $DNP_9$-azo–BSA).

A third method for conjugation utilized a sulfur-mustard-DNP compound kindly provided by Dr. A. Soloway. Twenty milligrams of DNP-mustard dissolved in 2 ml of dioxane was added dropwise during 8 hr to 50 mg of BSA in 50 ml of distilled water. The reaction mixture was held at room temperature with continuous stirring and adjusting the pH to 7.5 with 0.01 *N* NaOH solution. The reaction was allowed to proceed for another 24 hr at room temperature with stirring. The number of DNP groups coupled to the BSA was estimated, using the DNP–lysine extinction coefficient at 360 m$\mu$ of 17,350 at pH 7.4 and found to be 11 DNP groups/molecule of BSA ($DNP_{11}$-mustard–BSA). All conjugates used were purified first by precipitation with trichloracetic acid, then they were redissolved in borate buffer (pH 8.2), and run through a column of Sephadex G-25.

Outbred, white male guinea pigs were immunized with 100 $\mu$g of the different DNP–BSA conjugates incorporated in complete Freund's adjuvant (CFA) injected into the 4 foot pads. Three weeks later they were

TABLE I. Production of Hemolytic ($\gamma_2$) Antibodies to DNP–Hapten and BSA Carrier in Guinea Pigs Immunized with DNP-BSA, DNP-azo-BSA and DNP-mustard-BSA.

| | Antibody response to | | | |
|---|---|---|---|---|
| | DNP | | BSA | |
| Antigen used for immunization | No. of animals | Av. titer | No. of animals | Av. titer |
| $DNP_{29}$-BSA | 8/8[a] | 1/5120 | 0/8 | 0 |
| $DNP_{29}$-azo-BSA | 8/8 | 1/320 | 8/8 | 1/1280 |
| $DNP_{10}$-BSA | 8/8 | 1/2560 | 3/8 | 1/20 |
| $DNP_9$-azo-BSA | 8/8 | 1/160 | 8/8 | 1/5120 |
| $DNP_{11}$-mustard-BSA | 8/8 | 1/2560 | 5/8 | 1/40 |

[a] Number of animals reactive/number of animals tested.

given a booster injection of 100 μg of the homologous conjugate in saline, divided in two intradermal sites. One week later they were bled and the sera were assayed for antihapten and anticarrier $\gamma_2$-hemolytic antibodies (8). For detection of DNP antibodies, tanned sheep red blood cells (SRBC) coated with DNP–human γ-globulin (HGG) or DNP–azo-ribonuclease were used. The presence of anti-BSA was tested using SRBC coated with native BSA.

*Results.* From the results summarized in Table I it is obvious that though all animals immunized with the various BSA conjugates produced specific anti-DNP antibodies, a marked quantitative difference in titer was found among the groups. Animals immunized with $DNP_{29}$–BSA, $DNP_{10}$–BSA and $DNP_{11}$-mustard–BSA responded with high anti-DNP titer, while those immunized with DNP-azo-conjugates produced only low titer antisera to the DNP hapten.

The possibility that the difference in antibody titer obtained is due to differences in specificity of the antibodies elicited by the various DNP-conjugates was ruled out by testing all sera against both DNP–HGG and DNP-azo–RNase. Identical titers of anti-DNP antibodies were obtained using SRBC coated with either one of the two antigens. This finding indicates that the antibodies detected are hapten specific and the difference in titer represents a basic difference in immunogenicity of the DNP–BSA conjugates used.

This dissimilarity in immunogenicity was revealed not only in relation to $\gamma_2$-hemolytic antihapten antibodies but also when $\gamma_1$-skin sensitizing antibodies were tested by passive cutaneous anaphylaxis. None of the animals immunized with $DNP_9$-azo–BSA produced any $\gamma_1$-anti-DNP antibodies, while 5/8 of the animals immunized with $DNP_{10}$–BSA showed an average titer of 1/60.

Another striking difference in immunogenicity between the various DNP–BSA conjugates was found in relation to anticarrier antibodies. Guinea pigs immunized with either DNP-azo–BSA conjugate produced high titers of antibodies to BSA (1/1250–1/5120) while none of the animals immunized with $DNP_{29}$–BSA showed any anti-BSA antibodies, and only some of the animals immunized with $DNP_{10}$–BSA (3/8) and $DPN_{11}$-mustard–BSA (5/8) produced such antibodies in low titers (1/20–1/40).

The ability of the DNP–BSA conjugates to immunize for and elicit delayed-type hypersensitivity was tested by injecting guinea pigs with 10 μg of either DNP–BSA or DNP-azo–BSA incorporated in CFA. Three weeks later, all preparations tested, heavy and light conjugates, produced a strong delayed reaction against the homologous conjugate used for immunization (Table II). The reactions in the DNP-BSA group were somewhat stronger (30 m with 5 μg of test antigen) than that in the azo group (22 mm with 20 μg of test antigen). Delayed reactions to the carrier, however, could not be detected in animals immunized with $DNP_{29}$–BSA, were weak in those immunized with $DNP_{10}$–BSA (10 mm with 20 μg of test antigen) and strong in the azo groups (19 mm with 20 μg of antigen). Decreasing the dose used for immunization to 1 μg of $DNP_{10}$–BSA and $DNP_9$-azo–BSA, showed that of 14 animals immunized with the former antigen all reacted strongly to 5 μg of the homologous conjugate (18 mm induration) but only 3/14 reacted to the carrier BSA (12 mm). In another group of 14 guinea pigs immunized with 1 μg of DNP-azo–BSA, only 2/14 reacted to both BSA and the homologous compound (14 mm). Again, these data show that the DNP–BSA con-

TABLE II. Development of Delayed Sensitivity to BSA and DNP-Conjugates Following Immunization with DNP-BSA and DNP-azo-BSA.

| Immunized with 10 μg of | No. tested | Av. delayed reaction (mm) to | | |
|---|---|---|---|---|
| | | $DNP_{10}$-BSA (5 μg) | $DNP_9$-azo-BSA (20 μg) | BSA (20 μg) |
| $DNP_{29}$-BSA | 8 | 30 | ND | 0 |
| $DNP_{10}$-BSA | 8 | 28 | ND | 10 |
| $DNP_{23}$-azo-BSA | 8 | ND | 23 | 18 |
| $DNP_9$-azo-BSA | 8 | ND | 21 | 19 |

jugate is far more immunogenic than its related azo-conjugate in the production of delayed-type immune responses. Differences in the physiochemical properties of the various conjugates used, could be easily predicted assuming that DNP-sulfonate coupled through the ε-amino group of lysine residues, DNP-diazonium fluoroborate through azo bonds to tyrosine and DNP-mustard via ester linkages to –COOH groups. Tests of electrophoretic mobility of the DNP–BSA conjugates showed that $DNP_{29}$–BSA and $DNP_{10}$–BSA have increased anodal mobility of 188% and 125%, respectively, of that of native BSA. DNP-azo–BSA compounds showed no significant change while DNP-mustard–BSA revealed a slight decrease (90% of that of BSA). Whether these differences in the net charge of the DNP–BSA conjugates make any significant contribution to the alteration of immunogenicity of the various conjugates described, could not be determined from the present data.

Another and rather important difference between the various DNP–BSA conjugates used was found to be related to alterations in the antigenicity of the carrier-BSA. Thus, heavily coupled DNP–BSA failed to precipitate rabbit anti-BSA antibodies. It also did not produce delayed hypersensitivity to native BSA when injected into guinea pigs with CFA. On the other hand, heavily coupled DNP-azo–BSA retained 80% of the ability of native BSA to precipitate anti-BSA antibodies as well as to sensitize animals for a delayed-type response to BSA. The lightly coupled conjugates, $DNP_{10}$–BSA and $DNP_9$-azo–BSA, were as efficient in precipitating anti-BSA antibodies as BSA itself but $DNP_{10}$–BSA still showed a diminished ability to sensitize animals for reactivity to BSA.

*Discussion.* These data indicate the critical nature of the mode of coupling a hapten to a protein carrier for eliciting an immune response to either component. With DNP as hapten and BSA as carrier it was seen that conjugation to the ε-amino groups of lysine produced a material that was very immunogenic for the hapten and only poorly so for the carrier. Coupling to tyrosine, on the other hand, resulted in poor immunogenicity for the hapten and good immunogenicity for the carrier. Coupling to carboxyl groups gave a product resembling the lysine coupled material in immunogenicity although the net residual charge effect on the carrier was of opposite magnitude.

Several possible explanations of these data are possible, implicating changes in net charge and a special role for tyrosine in immunogenicity (9). Perhaps the simplest explanation, however, has the virtue of being compatible with present concepts of antibody induction resulting from the binding of antigenic determinants by specific receptors on the surface of immunocompetent cells. This would suggest that the accessibility of various structural groups to coupling by haptens plays the dominant role. Thus, charged and hydrophilic $-NH_2$ and –COOH groups tend to cluster near the surface of macromolecules and their substitution by haptens would place the hapten in a position readily accessible to the receptors of lymphoid cells. At the same time substituents on the surface of the molecule might be expected to interfere sterically with the three-dimensional configuration of the molecular surface leading to a decrease in

native antigenic determinants. Aromatic and hydrophobic groups such as tyrosine, on the other hand, would be more deeply buried in the interior of the molecule and their hapten substituents would, therefore, be much less accessible to cell surface receptors and less likely to alter native determinants.

*Summary.* The immunogenicity of three different conjugates with DNP as hapten and BSA as carrier were compared. Guinea pigs immunized with DNP coupled to amino groups or carboxyl groups responded with high anti-DNP and low anti-BSA antibody production, while those immunized with DNP on tyrosyl residues gave the reverse response. DNP coupled to amino and carboxyl groups produced much more drastic effects on native BSA determinants as measured by precipitin test and delayed sensitivity reactions. It is suggested that haptens coupled to these groups are on the exterior surface of the protein where they are more likely to contact receptors of immunocompetent cells and more likely to alter surface configurations of the native molecule.

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