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Role of the Carrier Protein in the Antibody Elicited to DNP Hapten.* (31892)

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The anti-hapten response of an animal immunized with a hapten-carrier conjugate is, to a large extent, influenced by the carrier molecule(1-4). It has been shown that the carrier not only influences the amount of anti-hapten antibody but the type and avidity of the immunoglobulin as well(5). The degree of conjugation of the hapten to the carrier and the genetic constitution of the immunized animal also markedly affect the anti-hapten response (6-8).

The present studies were designed to examine the relationship between the antigenicity of a carrier molecule and its effectiveness as a carrier. In order to keep variables such as the genetic constitution, sex, age, previous immunologic history of the immunized animals and the size and composition of the hapten-carrier complex as constant as possible, the following criteria were adopted: (a) The animals were to be highly inbred, of the same age, sex, and reared as identically as possible; (b) The carriers were to be proteins of the same size and function isolated from various species of animals including the animals to be immunized; and (c) The chemical structure of the hapten and number of haptenic groups attached onto each carrier were to be kept constant.

Reported below are studies of antibody elicited to the dinitrophenol (DNP) hapten in inbred mice immunized with different DNP-γ 2 globulin conjugates. The γ 2 globulin carriers were isolated from bovine, rabbit, rat and isologous mouse sera and the number of DNP groups per carrier molecule was the same for each conjugate.

Methods and materials. Preparation of carriers and hapten-carrier conjugates. Purified γ 2 globulin preparations of bovine (BGG), rabbit (RGG), and rat (RtGG) were obtained by DEAE-cellulose chromatography of commercially available fractions.‡ Purified mouse γ 2 globulin (MGG) was prepared by the method of Fahey and Horbett(9) using

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‡ BGG (Pentex lot #18); RGG (Pentex lot #36); and RtGG (Pentex lot #1064).

sera from donor C_3H/HeJ mice not used in immunization experiments. The purity of each γ 2 globulin preparation was established by immunoelectrophoresis using antisera prepared against whole bovine, rat, rabbit, and C_3H/HeJ sera. Each of the antisera showed at least 12 precipitation arcs when whole sera were analyzed, including those corresponding to the major immunoglobulin classes. Purified γ 2 globulin preparations showed a single arc by immunoelectrophoresis corresponding to the mobility of γ 2 globulin.

The dinitrophenyl derivatives of the purified proteins were prepared by a modification of the method of Eisen *et al*(10). Equal concentrations (12.5 mg/ml) of the protein, 2, 4 dinitrobenzensulfonate ($DNBSO_3$),§ and K_2CO_3 in water were incubated at 37°. Periodically, small aliquots of the reaction mixture were withdrawn and rapidly passed through a Sephadex-G-25 column to separate the DNP-protein conjugate from unreacted $DNBSO_3$. The conjugate was analyzed for DNP/protein ratio as described by Carsten and Eisen(11). When the mole ratio of DNP/protein was approximately 30, the reaction mixture was immediately passed through a Sephadex-G-25 column. By this method all conjugates prepared contained 30 $\pm$ 2 DNP groups per protein molecule.

Immunization. Fourteen-week-old male C_3H/HeJ‖ mice were used in these experiments. This strain was chosen because preliminary experiments, performed on 4 inbred strains (A/J; AKR/J; AKD_2F_1 and C_3H/HeJ), indicated that the C_3H/HeJ strain was the best anti-DNP responder. Groups of 40 mice were given 3 courses of DNP-protein conjugate (1 mg of conjugate per course in Difco complete Freund's adjuvant) subcutaneously on days 1, 28, and 35. Twenty animals from each group were bled *via* the retro-orbital plexus every other day from days 2-16, and days 36-50. The sera obtained were individually stored at —45°.

Measurement of antibody titer. Anti-DNP titers were measured by micro passive hemagglutination using DNP-human erythrocyte preparations. 1, 3 difluoro-4, 6 dinitrobenzene (FDNFB)¶ was coupled to human type AB red cells by the method of Bullock and Kantor(12). Microtitrations were performed with the apparatus and disposable microtiter plates supplied by the Cook Engineering Co., Alexandria, Va. Serial 2-fold dilutions of mouse sera in pH 7.2 buffered saline were prepared in 0.025 ml volume. To these samples were added 0.025 ml of DNP-red cell preparation and the titers read from the settling patterns. To measure the levels of antibody directed against the carrier proteins, a similar titration was performed using formalinized, tanned erythrocytes coated with the appropriate carrier protein. Carrier sensitized red cells were prepared by the method of Daniel *et al*(13), and only those preparations which gave high titers with known antisera directed against the particular carrier were used.

Results. Fig. 1 shows the anti-DNP responses of the 4 groups of mice after primary stimulation. Each point represents the average of 20 animals. In all groups detectable levels of antibody were first noted on day 4 and reached peak titer on day 10. The levels of anti-DNP, after primary immunization, were 13 times as high with bovine carrier protein as with mouse carrier protein while the RGG and RtGG were about 2.8-3.4 times as effective in causing a DNP response as the isologous mouse protein. Essentially the same behavior was seen after the third stimulation, the relative peak anti-DNP titers for the bovine, rabbit and rat carrier proteins being 9.2, 2.0, and 2.2 times greater respectively than the response with the isologous mouse carrier protein.

Fig. 2 shows the average antibody titers to the carrier proteins in the same groups of animals. The greatest response was shown to the bovine carrier and no response to mouse protein.

Discussion. The above results suggest a direct relationship between the antigenicity of a protein molecule and its effectiveness as a hapten carrier. The carriers used in this study were proteins with similar molecular weights. They also presumably had similar confor-

§ Eastman Organic Chemical, Rochester, N. Y.

‖ Purchased from Jackson Memorial Laboratories, Baltimore, Md.

¶ Aldrich Chemical Co., Inc., Milwaukee, Wisc.

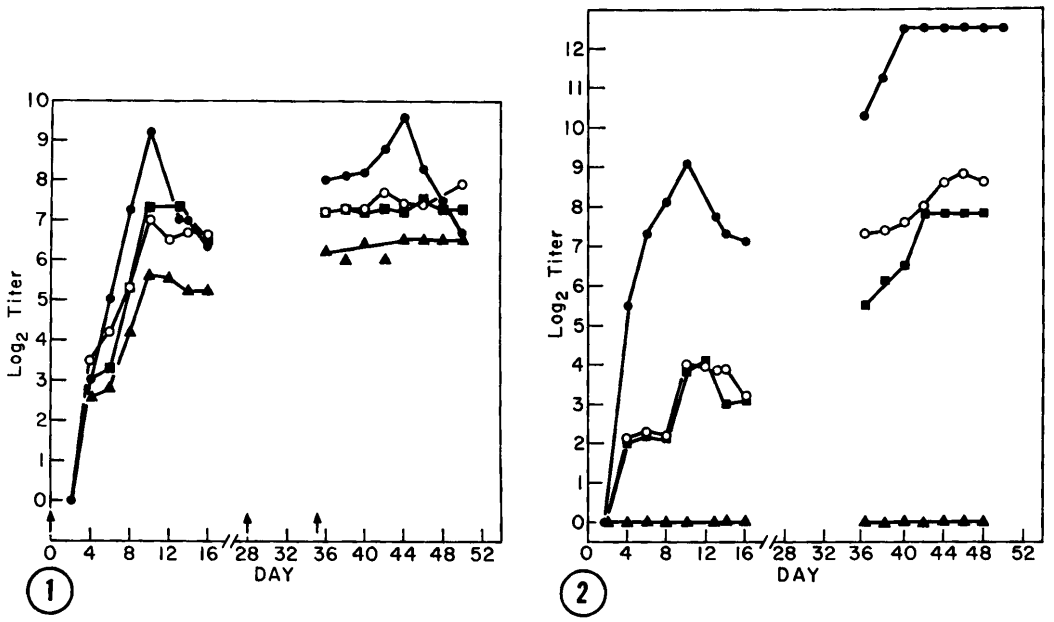

Fig. 1. Anti-DNP responses in mice injected with DNP-γ 2 globulin conjugates. Log_2 titer *vs.* time after primary injection. Carrier proteins: ● bovine, ○ rabbit, □ rat and △ mouse.
Fig. 2. Anti-carrier responses in mice injected with DNP-γ 2 globulin conjugates. Log_2 titer *vs.* time after primary injection. Carrier proteins: ● bovine, ○ rabbit, □ rat and △ mouse.

mations, amino acid compositions and numbers of available lysine side chains for attachment of the DNP group. The polypeptide structure of γ 2 globulins from a number of animal species has been shown to be similar. The amino acid compositions of rabbit, horse, and human γ 2 globulins are also similar(14, 15). The number of DNP groups per carrier molecule was carefully controlled and the animals were similar in their physical characteristics and immunologic histories.

The significant differences in the anti-DNP response elicited by the various DNP-γ 2 globulin preparations are therefore probably not ascribable to a difference in the "physical nature" (size and shape) of the various carriers. The similarity of the kinetics of anti-DNP production (appearance of detectable antibody on day 4 after primary stimulation and peak titer on day 10) with the different carriers suggests that there was no basic difference in the overal mechanism of anti-DNP formation for the various immunogens.

There is, however, an obvious parallel between the "foreignness" of the carrier protein and its ability to elicit an anti-hapten response. The isologous mouse carrier protein elicited the poorest response while the carrier protein from that animal most phylogenetically removed from the immunized animal, bovine protein, elicited a 13-fold better response. Those carriers from animals more closely related to the immunized animals gave intermediate responses. The antibody response to the carrier protein provided additional evidence for the parallelism of antigenicity and carrier function for this system.

Although DNP-MGG elicited no detectable antibodies directed against the carrier, significant levels of anti-DNP were observed. Similar observations of an autologous, isologous, or "non-antigenic" carrier eliciting an anti-hapten response have been noted by others(16,17). Such evidence would appear to argue against the necessity of a macromolecule being antigenic in order to function as a carrier. Rüde *et al*(18) have that a conjugate of tetra-O-acetly-β-D-galactopyranose hapten attached to a non-antigenic synthetic copolyaminoacid *via* serine ether linkages was non-antigenic. In contrast, conjugates of a phenyl-β-D-galactopyranose hapten attached to either bovine serum albumin or an antigenic copolyaminoacid *via* tyrosine azo link-

ages were antigenic and elicited antibodies with galactose specificity. Weigle(16) has suggested that the antibody response to homologous protein-hapten conjugates might be directed toward both the hapten and a surrounding surface of the carrier which could have been altered by the process of conjugation. Singer(19) has similarly suggested that, in hapten-protein conjugates, the hapten groups are heterogeneous antigenically and the determinants consist of the hapten plus the immediate environment of the hapten provided by the carrier.

Our data support a previously suggested concept that the mechanism of antibody induction is a multi-stage process involving at least two distinct "recognition" stages: (a) An early stage involving a mechanism which recognizes the antigenicity of the carrier. With a hapten-carrier complex this would involve the recognition that the carrier (or carrier modified by the hapten) is foreign or itself antigenic; (b) A later stage which recognizes the determinant group (or hapten) and results in the synthesis of specific antibodies against the determinant.

Summary. The antibody response to DNP-hapten in inbred mice injected with different carrier proteins was studied under controlled conditions. Use of isogenic serum resulted in the production of antibodies to DNP but none toward the carrier protein. Immunization with DNP conjugates of bovine, rabbit, and rat γ 2 globulins resulted in anti-DNP titers which were 13, 3, and 3 times as high, respectively, as with the isologous DNP-γ 2 globulin conjugate. The responses against carriers paralleled the anti-DNP responses (bovine>rabbit = rat>mouse = 0). The degree of anti-hapten response was directly related to the antigenicity of the carrier molecule. These data support a concept of anti-hapten production involving recognition of the "foreignness" of the carrier or modified carrier.

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