EXPERIMENTAL STUDIES ON THE BRIDGING HYPOTHESIS OF ANAPHYLAXIS

Haptenic Determinants Required to Elicit Immediate-Type Reactions in Calf Skin by Separate or Concurrent Sensitization with Reagins of Different Specificity

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Recent evidence has been provided that IgE represents a distinct class of immunoglobulins that initiate immediate-type hypersensitivity reactions in man and also in other species (1). Using a priming regimen to elicit a good reaginic antibody response, we have been able to demonstrate that antibodies of the IgE class are capable of eliciting tissue sensitization in the bovine species (2) as well. The molecular basis for the mediator role of IgE involves the linking of the Fc part to membrane receptors on basophilic granulocytes and mast cells (3, 4). The current general opinion holds that for eliciting of an optimal anaphylactic response the allergen must carry at least two haptenic determinants per molecule within a relatively short distance (5, 6) and reactions occurring consequent to monovalent interactions are considered rather exceptional (7, 8).

The apparent requirement for bivalency of the hapten was interpreted as indicating that the bridging of two combining sites on adjacent cell-bound antibody molecules is the prerequisite for the release of pharmacologically active substances (9). The possibility that bridging of two cell-bound reaginic antibody molecules is the initial step of the reaction was also suggested by the fact that anti-IgE or even the F(ab')2 fragment thereof provides histamine release from basophils, whereas the Fab fragment failed to do so (10). So far, however, there is no final proof that the formation of a complex made up of two adjacent membrane-fixed antibody molecules linked together by a bivalent hapten is a critical requirement for the elicitation of immediate-type reactions. In view of these considerations, we compared several bivalent and multivalent haptens (DNP determinant) differing in molecular size and in the distance of haptenic groups quantitatively with regard to their capacity to evoke skin responses in calves passively sensitized with anti-DNP antibodies.

The present studies were mainly designed to provide direct evidence for the bridging hypothesis by concurrent sensitization of skin sites with antibodies of
different specificities permitting a bivalent hapten carrying noncross-reactive antigenic determinants to be effective in eliciting anaphylactic reactions. The results provide compelling evidence that cross-linking of two adjacent membrane-fixed antibody molecules by a bivalent hapten may be at least one, if not the only pathway for effective elicitation of immediate-type reactions.

Materials and Methods

Proteins and Chemical Reagents. Bovine serum albumin (BSA) was prepared according to the procedure of Michael (11). Edestin was obtained from Serva, Feinbiochemica, Heidelberg, West Germany, and 2,4-dinitrobenzenesulphonate (DNBS) from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y. Gramicidin-J was supplied from Sigma Chemical Co., St. Louis, Mo. and p-arsanilic acid from Fluka A. G., Buchs, Switzerland. \( \varepsilon \)-DNP-lysine was prepared and characterized according to a previously described method (12).

Hapten-Substituted Proteins. p-azobenzenearsonate BSA (ABA-BSA) containing 33 ABA groups/molecule was prepared according to standard procedure (13). The preparation of ABA\( _{11} \)-edestin (Ed) has been described in detail (13). DNP\( _{11} \)-Ed and DNP\( _{14} \)-BSA were prepared by reaction of DNBS with the appropriate proteins as previously outlined (14). The numerical subscripts refer to the average number of moles of haptenic groups per mole of carrier.

Monovalent Haptens. \( \varepsilon \)-DNP-aminocaproic acid (DNP-cap) was prepared and characterized following the method of Carsten and Eisen (15) and (4-hydroxy-3-azobenzenearsonic acid)-phenylacetic acid (ABA-HPA) was synthesized as previously described (16).

Bivalent Haptens. \( \varepsilon \)-N-(\( \varepsilon \)-N-DNP-aminocaproyl)-\( \varepsilon \)-N-DNP-L-lysine dicyclohexylammonium salt (DNP-DNP) was prepared by dissolving \( \varepsilon \)-N-DNP-lysine hydrochloride hydrate (367 mg, 1 mmol) partially in dimethylformamide (15 ml) containing triethylamine (280 \( \mu l \)). To this was added the \( N \)-hydroxysuccinimide ester of DNP-cap (394 mg, 1 mmol) dissolved in dimethylformamide (4.0 ml) by vigorous mixing. Stirring of the reaction mixture was continued for an additional 20 h at room temperature. At the end of this period a small amount of a yellow precipitate was removed by filtration and the solution was evaporated in vacuo at 55°C. The residue was taken up in 0.2 N HCl and extracted with ethyl acetate. The ethyl acetate extract was dried over anhydrous Na\( _2 \)SO\( _4 \) and finally evaporated in vacuo to dryness. The compound was crystallized by addition of ether and dicyclohexylamine (200 \( \mu l \)) and recrystallized from hot ethanol. The yield (520 mg) was 67% of theoretical (mp, 168 - 175°C).

The purity of the product was ascertained by thin-layer chromatography on silica gel using a solvent system of benzene-ethyl acetate-acetic acid in a vol ratio of 70:20:10. Elemental analysis showed the following results:

\[
\begin{align*}
C_{12}H_{16}N_5O_11 & \\
C & 55.95 & 6.78 & 14.50 \\
N & 55.26 & 6.65 & 14.50
\end{align*}
\]

\( \varepsilon \)-N-DNP-\( \varepsilon \),N-[(4-hydroxy-3-azobenzenearsonic acid)-phenacetyl]-L-lysine (DNP-ABA): the dicyclohexylammonium salt of \( \varepsilon \),N-(4-hydroxyphenacetyl)-\( \varepsilon \),N-DNP-L-lysine (50 mg, 0.08 mmol) prepared as described previously (17) was treated with 1 ml of 0.2 N HCl and extracted with ethyl acetate to remove dicyclohexylamine. The ethyl acetate extract was evaporated to dryness and the residue was dissolved in borate-buffered NaCl (0.14 N NaCl, 0.02 M borate buffer pH 9.0).

Abbreviations used in this paper: ABA, p-azobenzenearsonate; ABA-HPA, (4-hydroxy-3-azobenzenearsonic acid)-phenylacetic acid; BPO, D-benzylpenicilloic acid; BSA, bovine serum albumin; DNBS, 2,4-dinitrobenzenesulphonate; DNP-ABA, \( \varepsilon \),N-DNP-\( \varepsilon \),N-[(4-hydroxy-3-azobenzenearsonic acid)-phenacetyl]-L-lysine; DNP-cap, \( \varepsilon \)-DNP-aminocaproic acid; (DNP)\( _2 \)-Gram (DNP)\( _2 \)-gramicidin-J; DNP-DNP, \( \varepsilon \),N-(\( \varepsilon \),N-DNP-aminocaproyl)-\( \varepsilon \),N-DNP-L-lysine dicyclohexylammonium salt; DST, direct skin test; Ed, edestin; P-K, Prausnitz-Küstner; TAS, Tris-albumin NaCl.
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The diazonium salt was prepared by dissolving arsanilic acid (108.5 mg, 0.5 mmol) in 4.7 ml of 0.6 N HCl. After cooling to 0°C, 0.55 mmol of NaN₃ (1.0 ml) was added and stirred for 30 min in an ice bath. After this, 1.2 ml of the diazonium salt solution (0.105 mmol) was added dropwise to 2 ml DNP-lysine-HPA and the mixture was stirred for 2 h with pH maintained at 9.0 by addition of NaOH. The reaction product was precipitated by acidification to pH 2.5 with 2 N HCl, centrifuged, dissolved in H₂O at pH 8.5 by addition of NaOH, and then reprecipitated at pH 2.5. The precipitate was redissolved in borate-buffered NaCl and passed twice through a 2.8 × 100-cm column of Sephadex G-15 to accomplish separation of monoazo from bisazo derivatives.

The monosubstituted material was further purified by repeated precipitation at pH 2.5. The yield (25 mg) of the noncrystallized preparation was 50% of theoretical and the elemental analysis showed the following results:

\[
\text{C}_{26}\text{H}_{31}\text{N}_{11}\text{As}
\]

\[
\begin{array}{ccc}
\text{Calculated:} & 46.30 & 4.04 & 12.46 \\
\text{Found:} & 48.59 & 4.24 & 11.41 \\
\end{array}
\]

As shown by paper chromatography, using a solvent system of butanol-pyridine-water in a vol ratio of 6:4:3, the monoazo derivative was free of disubstituted material (the migration ratio of monosubstituted to disubstituted material was 1.22:1). In addition, contamination of the monoazo derivative with the disubstituted compound was ruled out by its ineffectiveness in eliciting skin reactions in sites singly sensitized with anti-ABA antibody.

\[(\text{DNP})_{2}\text{-Gramicidin-J}\equiv(\text{DNP})_{2}\text{-Gram}\]\n
Gramicidin-J was suspended in 2% (wt/vol) K₂CO₃ in water and subsequently reacted with 2,4-dinitrofluorobenzene according to the method of Montgomery et al. (18). The purified dinitrophenylated gramicidin-J was shown to be homogenous by thin-layer chromatography on polyamide sheets (F 1700 Micropolyamid, Schleicher and Schüll, Dassel, West Germany) using benzene-glacial acetic acid (80:20 vol/vol) in the first and formic acid (90%) and water (50:50 vol/vol) in the second dimension (19). The extent of dinitrophenylation was determined by amino acid analysis (6 N HCl, 72 h, 110°C); 0.016 mol of residual ornithine/2 mol of proline were found.

**Immunization.** Antisera against the ABA group (anti-ABA antibody) as well as against the DNP determinant (anti-DNP antibody) were prepared by immunizing pregnant Simmental cows, 2–3 yr of age (450–500 kg), with either 120 mg of ABA-Ed, DNP-Ed or (DNP)₂-Gram, respectively, emulsified in an adjuvant composed of aluminium hydroxide gel, paraffin oil, and sorbitan trioleate (20). At appropriate times after priming (8 wk) a secondary challenge was performed after the immunization schedule as outlined previously (21). Antisera were obtained 10 days (early primary antibody) and 56 days (late primary antibody) after priming as well as 7 days after the second injection (secondary antibody). The collection and preparation of the colostrum has been described previously (22).

**Anti-IgE Antibody.** IgE-containing fractions were obtained from colostrum by ion exchange chromatography and gel filtration (2). Rabbits were immunized with this fraction included in complete Freund’s adjuvant (Difco Laboratories, Detroit, Mich.). The antisera was rendered monospecific for IgE by absorption with IgG, IgA, IgM, and fetal calf serum. Because of the lack of purified bovine IgE the amount of antibody present in the anti-IgE antiserum could not be quantitated.

**Direct Skin Test (DST).** Skin tests were performed in calves 2 wk after feeding colostrum of the dam immunized with (DNP)₂-Gram. The hair along the flank of both sides was clipped and 20.0 ml Tris-albumin NaCl(TAS:0.01 M Tris, 0.14 NaCl 0.03% BSA pH 7.3) containing 2.5% Evans blue was given by intravenous injection.

Haptens were diluted in TAS and reagent vol of 0.05 ml each were carefully injected intradermally using disposable syringes. Each test was done in duplicate and reactions appearing 30 min after the injection were read and recorded as the highest dilution of the hapten evoking threshold skin reactivity (5-mm diameter). Two kinds of controls were included: (a) specific inhibition of the reaction by monovalent haptenes and (b) injection of noncross-reactive haptens.

Anti-IgE was used to study the presence of cell-bound IgE molecules on mast cells in skin sites of either newborn colostrum-deprived calves or of those 4 wk of age. The animals received Evans blue
intravenously followed by intracutaneous administration of appropriate dilutions of anti-IgE. The diameter of skin reactions was measured 30-60 min after injection of the antibodies.

Passive Transfer Test. Bovine reaginic antibody was assayed by the Prausnitz-Küstner (P-K) reaction in either newborn colostrum-deprived calves or in 4-wk old calves (30-80 kg of body weight). Dermal sites were sensitized with 0.15-ml samples of reaginic antiserum or colostral whey serially diluted in TAS. After a latent period of 72 h, calves received Evans blue intravenously. Immediately thereafter, passively sensitized test sites and nonsensitized control sites were challenged by injection of 0.05 ml of hapten and the skin scored as outlined above. The reciprocal of the highest dilution of serum or colostrum evoking skin reactions larger than 5 mm in diameter was taken as threshold reactivity. For each experiment at least two calves were used and the order of intradermal injection was varied. Appropriate controls of the specificity of the reactions were included by using noncross-reactive haptens. Two points of importance should be emphasized about the reaginic antibody of the IgE class. Firstly, this antibody loses skin-sensitizing ability by heating at 56°C and is optimally detected 72 h after injection into skin sites. These are characteristic properties of IgE class antibodies and rule out any contribution of skin-sensitizing antibodies of the IgG subclasses (2).

Inhibition Studies. Inhibition experiments were performed by mixing monovalent haptens dissolved in TAS with bivalent or multivalent haptens at different molar ratios. The mixture (0.05 ml) was tested for its ability to elicit P-K reactions in calves sensitized with reaginic antiserum or colostral whey. The specificity of inhibition by monovalent haptens was documented from their failure to influence skin reactions to noncross-reactive bivalent or multivalent haptens.

Results

The structural formulas of the univalent and bivalent haptens used in the present study are shown in Fig. 1.

![Structural formulas of DNP and ABA haptens.](image-url)

**Fig. 1.** Structural formulas of DNP and ABA haptens.
Induction of Reaginic (IgE) Antibody Responses in Cattle. Groups of four and two pregnant cows, respectively, were immunized with either DNP-Ed or ABA-Ed (120 mg each). All animals received a second injection of the antigen (same dose) 8 wk after primary immunization. Cattle were bled 10 and 56 days after priming and again 7 days after the secondary challenge. The reaginic activity of their antisera was analyzed by P-K reactions in nonsensitive calves.

The reagin synthesis to either DNP-Ed or ABA-Ed is illustrated in Fig. 2. A rather low reaginic response (P-K titer 5) was elicited by day 10 (early primary antibody) after priming with either hapten. When tested 8 wk after primary immunization (late primary antibody), cows injected with DNP-Ed showed a striking increase in reaginic antibodies yielding average P-K titers of 1,600. By contrast, ABA-Ed-primed animals consistently failed to elicit a similar increase in reaginic response late after priming (P-K titer 20). Cows of both groups responded to a booster injection on day 56 with a substantial reaginic antibody response, which, however, never exceeded that measured 8 wk after priming.

The level of DNP-specific IgE antibodies present in the colostral whey was very similar to that obtained in the serum after secondary challenge. On the other hand, in the colostrum of cows secondarily challenged with ABA-Ed, levels of reaginic antibodies (P-K titer 200) were detected up to 10 times the serum concentration.

Effect of Antibody Affinity on Efficacy of DNP-Substituted Haptens as Elicitors of Skin Reactions. In previous experiments (23) differences were noted in the skin test responses to DNP conjugates, varying in the degree of substitution, which appear to be dependent upon antibody affinity. These
findings prompted us to explore the effectiveness of multivalent and bivalent haptens in eliciting P-K reactions in skin sites sensitized with reaginic antisera obtained at day 10 (early primary) and 56 (late primary) as well as 7 days after secondary challenge of a cow immunized with DNP-Ed.

As shown in Table I, skin sites prepared with early primary antibody gave positive P-K reactions only with DNP-BSA in doses of \(6 \times 10^{-3}\) μmol of the haptenic group, whereas an equimolar solution of DNP-DNP was incapable of evoking a comparable response. In contrast, DNP-DNP and DNP-BSA elicit equally intense P-K reactions in sites sensitized with either late primary or secondary antibody when used at hapten doses of \(6 \times 10^{-4}-6 \times 10^{-3}\) μmol. The monovalent DNP-cap (not shown in table) was consistently incapable of eliciting P-K reactions in calves sensitized either with primary or secondary anti-DNP reaginic antibody.

Effectiveness of Bivalent and Multivalent Haptens in Eliciting Skin Reactions.

Two cows immunized twice with 120 mg (DNP)_2-Gram 8 wk apart synthesized and secreted DNP-specific IgE antibody into colostrum after secondary challenge as indicated by P-K reactions in passively sensitized newborn calves. When viewed in the light of previous findings (18) it was reasonable, however, to assume a rather low concentration of reaginic anti-(DNP)_2-Gram antibody in the colostrum. Therefore, we have examined the question if an increase of the capacity of mast cells to combine with IgE molecules would favor an effective sensitization of skin sites. It should, however, be recognized that incidental IgE molecules bound to the mast cell surface interfere competitively with passive sensitization. This problem was resolved by the unique finding that skin mast cells of the newborn colostrum-deprived calf completely lacked IgE molecules on their surface. This conclusion was deduced from the finding that the injection of anti-IgE into skin sites of newborns failed to elicit any reaction. On the other hand, anti-IgE even at a dilution of \(10^{-8}\) resulted in induction of reversed type P-K reactions in calves after uptake of colostrum.

DST were performed on 2-wk old calves sensitized by intestinal uptake of reaginic anti-DNP antibodies via colostrum. The effectiveness of two bivalent haptens, differing in structural rigidity, as well as of a multivalent hapten in eliciting P-K reactions is summarized in Table II. Sensitized calves were found to be strongly reactive to DNP-DNP and DNP-BSA, whereas (DNP)_2-Gram failed to elicit P-K reactions when tested at comparable doses of the hapten (\(1 \times 10^{-4}-6 \times 10^{-4}\) μmol). DNP-DNP and DNP-BSA were still found to be equally effective in eliciting skin reactions when used at a 1,000-fold lower hapten dose. From the experimental details and the results given in Table II it is also apparent that skin sites of colostrum-deprived newborns prepared by injection of anti-(DNP)_2-Gram reaginic colostrum were sensitized less efficiently. This is implied by the observation that DNP-DNP and DNP-BSA both were only effective at the highest hapten dose (\(6 \times 10^{-4}\) μmol) and failed to elicit any skin reaction in calves after feeding colostrum.

Effectiveness of a Bivalent Hapten Carrying Noncross-Reactive Determinants as Elicitor of Skin Reactions in Doubly Sensitized Calves. The current opinion holds that the effective initiation of immediate-type reactions requires the bridging of adjacent cell-bound antibody molecules by haptens carrying at least
# Table 1

**Effect of IgE Antibody Affinity on Capacity of Bivalent and Multivalent DNP-Haptens to Elicit P-K Reactions in Calves**

| DNP-specific IgE antibody used to prepare skin sites* | Hapten injected (μmol) | Diameter of skin reactions (mm)‡ |
|------------------------------------------------------|------------------------|----------------------------------|
|                                                      |                        | $6 \times 10^{-4}$       | $6 \times 10^{-4}$       | $6 \times 10^{-5}$       | $6 \times 10^{-6}$       |
|                                                      |                        | DNP-BSA | DNP-DNP | DNP-BSA | DNP-DNP | DNP-BSA | DNP-DNP | DNP-BSA | DNP-DNP |
| Early primary (2)§                                   | 8                      | 0       | 0       | 0       | 0       | 0       | 0       | 0       | 0       |
| Late primary (200)                                   | 20                     | 23      | 20      | 21      | 18      | 20      | 16      | 14      |        |
| Secondary (200)                                      | 17                     | 17      | 13      | 13      | 11      | 13      | 10      | 11      |        |

* Sensitized with 0.15 ml reaginic antiserum obtained at day 10 (1° early) and 56 (1° late) after priming as well as 7 days after secondary challenge (2°) of a cow immunized with DNP-Ed (see Materials and Methods).

† Each reaction diameter represents means of duplicates recorded in two calves.

§ Dilution of reaginic antiserum.
two antigenic determinants (5). The present experiment was designed to gain more conclusive evidence in support of the bridging hypothesis. Thus, skin sites were prepared by separate or concurrent sensitization with anti-DNP reaginic serum (diluted 1:100) and anti-ABA colostrum (diluted 1:25) both obtained from cows after secondary challenge. As shown in Table III, skin sites singly sensitized with either DNP- or ABA-specific IgE antibodies respond as expected to DNP-DNP and ABA-BSA, respectively, whereas DNP-ABA consistently failed to provoke P-K reactions when tested at comparable hapten doses (6 × 10⁻³ - 2 × 10⁻⁴ μmol). The most notable feature, however, is the fact that DNP-ABA employed as a bivalent hapten with noncross-reactive haptenic determinants, as well as DNP-DNP and ABA-BSA behaved comparatively effective in eliciting P-K reactions in doubly sensitized calves, if equimolar hapten solutions were

**Table II**

Comparative Effectiveness of Bivalent and Multivalent Haptens in Eliciting P-K Reactions in Skin Sites of Calves Passively Sensitized by IgE Class Antibody from Cows Immunized with (DNP)₂-Gram

| Hapten injected (μmol) | Diameter* of P-K reactions (mm) |
|-----------------------|---------------------------------|
|                       | Direct skin tests§ | Passive transfer tests§ |
|                       | DNP-DNP | (DNP)₂-Gram | DNP-BSA | DNP-DNP | (DNP)₂-Gram | DNP-BSA |
| 6 × 10⁻⁴              | 19      | 0           | 20      | 11      | 0           | 12      |
| 1 × 10⁻⁴              | 17      | 0           | 16      | 0       | 0           | 0       |
| 2 × 10⁻⁴              | 16      | ND          | 15      | 0       | ND          | 0       |
| 4 × 10⁻⁴              | 13      | ND          | 10      | 0       | ND          | 0       |
| 8 × 10⁻⁴              | 9       | ND          | 9       | 0       | ND          | 0       |

* Average diameter of duplicate tests in two calves.
† Test substances were injected into skin sites of calves passively sensitized by feeding colostrum from cows after secondary challenge with (DNP)₂-Gram.
§ Skin sites of nonsensitized calves prepared by injection of anti-DNP reaginic colostrum.

**Table III**

Comparative Effectiveness of DNP-DNP, ABA-BSA, and DNP-ABA in Eliciting P-K Reactions in Calves Using Separate or Concurrent Sensitization of Skin Sites with Two Noncross-Reacting Reaginic Antibodies

| Skin sites prepared with antibody to* | Challenging hapten injected (μmol) | Diameter of skin reactions (mm)‡ |
|--------------------------------------|-----------------------------------|---------------------------------|
|                                      | DNP-DNP | ABA-BSA | DNP-ABA |
|                                      | 6 × 10⁻³ | 1 × 10⁻³ | 2 × 10⁻⁴ |
|                                      | 6 × 10⁻⁴ | 1 × 10⁻³ | 2 × 10⁻⁴ |
|                                      | 6 × 10⁻⁴ | 1 × 10⁻³ | 2 × 10⁻⁴ |
| DNP§                                 | 22      | 22      | 20      |
| ABA§                                 | 0       | 0       | 0       |
| DNP§ + ABA||| 20      | 18      | 17      |

* Sensitized with 0.15 ml reaginic antibody.
‡ Means of duplicate tests.
§ Anti-DNP reaginic serum diluted 1:100.
|| Anti-ABA reaginic colostrum diluted 1:25.
used. By contrast both bivalent and multivalent haptens up to $6 \times 10^{-2} \mu\text{mol}$ were found to be incapable of evoking immediate type reactions when injected into nonsensitized skin sites.

**Inhibitory Effect of Univalent Haptens on P-K Reactions Elicited by Bivalent and Multivalent Conjugates.** As shown in preceding experiments, in contrast to the capacity of bivalent and multivalent DNP haptens in eliciting immediate type reactions, the monovalent DNP-cap was found not to be effective. It is apparent from the results in Table IV, however, that monovalent haptens can, in fact, specifically inhibit P-K reactions. Skin sites were prepared by separate or concurrent sensitization with anti-DNP reaginic serum and anti-ABA colostrum.

![Table IV](#)

| Skin sites prepared with antibody to | Challenging hapten§ (a) | Inhibitor (b) | Molar ratio (a)/(b) | Skin reaction§ |
|------------------------------------|------------------------|---------------|--------------------|----------------|
| DNP                               | DNP-DNP                | —             | —                  | 18             |
|                                   | DNP-DNP DNP-cap        | 5             | 0                  |
|                                   | DNP-BSA DNP-cap        | 10            | 0                  |
| ABA                               | ABA-BSA                | —             | —                  | 20             |
|                                   | ABA-BSA ABA-HPA        | 6             | 0                  |
| DNP + ABA                         | DNP-ABA                | —             | —                  | 15             |
|                                   | DNP-ABA DNP-cap        | 1             | 0                  |
|                                   | DNP-ABA ABA-HPA        | 1             | 0                  |

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* A mixture of univalent and challenging hapten was injected into each site.

§ $6 \times 10^{-2} \mu\text{mol}$ of challenging haptens were injected.

§ Means of duplicate tests recorded in two calves.

|| Anti-DNP reaginic serum diluted 1:50. Anti-ABA reaginic colostrum diluted 1:12.5.

as outlined in the preceding experiment. Thus, skin reactions elicited by either DNP-DNP or DNP-BSA were completely inhibited by the monovalent DNP-cap, when used at a 5 to 10 times molar excess. Analogously, the activity of ABA-HPA could be demonstrated by inhibition of the response to ABA-BSA. As expected, monovalent haptens equally effectively inhibited the response to DNP-ABA in skin sites of doubly sensitized calves even at equimolar hapten doses. The specificity of inhibition by monovalent haptens was documented from the absence of inhibiting noncross-reactive haptens as elicitors of P-K reactions.

**Discussion**

The present study has established that cows suitably primed with relatively low doses of hapten-protein conjugates developed hapten-specific responses of the IgE antibody class. Although the immunization procedure consistently induced IgE antihapten antibody, there is some evidence of an haptenic preference. Thus,
a striking increase and persistence of reaginic antibody synthesis was induced by a single injection of DNP-Ed as reflected by significantly lower P-K titers at 10 days (5) than at 56 days (1,600) after priming. The present results are in accord with the intense and persistent reagin synthesis obtained in certain mouse strains with minute doses of antigen (24), but clearly different from the early transient pattern of reaginic antibody response in rats (25). However, ABA coupled to the same carrier molecule as that used for preparation of DNP-conjugates failed to elicit more than a low reaginic response. The finding that cows vary widely in the IgE antibody synthesis to distinct haptens on the same carrier protein may be indicative for the involvement of regulatory mechanisms such as (a) genetic control of the immune response and (b) the chemical nature of the haptenic determinant. Challenge with the hapten-protein conjugate used for priming again elicited a marked reaginic response and the amount of IgE antihapten antibody secreted into colostrum was occasionally found to be up to 10 times the serum concentration, which is consistent with the observation made in nonallergic women (1).

Although this issue is not yet resolved, the persistent and boosterable reaginic response indicates that there is no suppressor mechanism in the cow capable of terminating pre-existing IgE synthesis. This interpretation does not concur with observations in the rat system in which regulation of the reaginic response either by IgG antibody (25) or by carrier-specific T cells (26) has been documented.

Only a small amount of data is available at present suggesting that with time after immunization there is a progressive increase in the average affinity of IgE antibody for a haptenic determinant (27). The findings obtained in the present studies permit the conclusion that cows exhibited a marked increase in affinity of DNP-specific IgE antibody between 10 days (early 1° antibody) and 8 wk (late 1° antibody) after priming. This interpretation is strongly suggested by the observation that the bivalent DNP-DNP is virtually equally effective as the multivalent DNP-BSA in eliciting P-K reactions in skin sites sensitized with either late 1° or 2° IgE antibody, but failed to induce a comparable reaction in sites pretreated with early 1° antibody. These findings confirm previous reports on the development of immediate-type reactions in human subjects (23) and guinea pigs (28) in which bivalent haptens were shown to be regularly effective in eliciting skin reactions mediated by high affinity IgG antibody, but failed to evoke a response in skin sites prepared with low affinity antibody. Optimal distances of haptenic groups seem to be a prerequisite for effective elicitation of P-K reactions. This conclusion was based on the finding that α,εN-bis-DNP-lysine in which haptenic groups are closely spaced unlike DNP-DNP failed to evoke skin reactions. The present data confirm earlier observations that optimally spaced haptenic groups were also maximally effective in eliciting wheal-and-flare reactions in sensitized man (29). These results are of general significance in demonstrating (a) the importance of antibody affinity and (b) the requirement of at least a bivalent hapten whose determinant groups are separated by optimal distances for effective elicitation of P-K reactions.

The present study clearly demonstrates that cows synthesize and secrete

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3 Mossmann, H., and D. K. Hammer. Unpublished results.
reaginic IgE antibody into colostrum after secondary challenge with the bivalent (DNP)_2-Gram. The successful transfer of reaginic DNP-specific antibodies was demonstrated by direct skin tests in newborn calves after feeding colostrum. Most notably the bivalent DNP-DNP and the multivalent DNP-BSA were equally effective in evoking skin reactions in sensitized calves, whereas (DNP)_5-Gram consistently failed to do so when equimolar hapten solutions were used. This was also true for colostrum-deprived newborns, whose skin sites were prepared by injection with colostral whey. The comparative ineffectiveness of (DNP)_2-Gram may be ascribed to the rigid, cyclic structure of the molecule, which displays a two-fold axis of symmetry. This interpretation is consistent with the observation that rigid bivalent haptens were considerably less effective than the more flexible aliphatic molecules in eliciting immediate type reactions in humans (29).

Of particular importance in the present study was the finding that newborn colostrum-deprived calves obviously lack IgE molecules on the mast cell surface. This conclusion was based on the observation that anti-IgE injected intradermally consistently failed to elicit a definite skin reaction. By comparison, anti-IgE even at a dilution of 10^{-8} was sufficient for induction of a reversed type P-K reaction in calves after uptake of colostrum. Thus, newborn colostrum-deprived calves whose mast cell receptors are completely devoid of IgE molecules may provide a unique model to investigate the molecular mechanisms involved in immediate-type reactions more precisely.

An additional implication arising from the results is the fact that the newborn calf efficiently can develop IgE-induced immediate-type reactions, whereas newborn rats (30) and rabbits (31) were found to be refractory. These results may be interpreted to indicate that all of the factors responsible for the release of and/or reactivity to mediators capable of inducing capillary permeability are present in the newborn calf. Most notably, concurrent sensitization of skin sites with IgE antibodies of different specificity has enabled us to provide direct proof that cross-linking of two adjacent reaginic molecules fixed to the mast cell surface by a bivalent hapten is required for effective elicitation of immediate type reactions. Thus, the bivalent DNP-ABA carrying two noncross-reactive haptenic groups was consistently effective in evoking P-K reactions in double sensitized skin sites. This hapten, however, failed to induce skin reactions in singly sensitized calves when injected at hapten doses up to 6 \times 10^{-3} \mu mol. Principal among the implications arising from the results presented here is the clear impression that DNP-ABA does not act as a monovalent molecule with intrinsic toxicity (5) but, indeed, functions as a bivalent hapten in doubly sensitized skin sites.

Taken collectively, these results are not directly comparable to the recent findings of Magro and Alexander (32) who demonstrated effective histamine release by blood leukocytes from doubly immunized rabbits elicited by a bivalent hapten carrying two noncross-reactive determinant groups. Considering their system, however, two points of importance should be emphasized: (a) it is uncertain whether IgE or IgG class antibodies or both contribute to sensitization of leukocytes and (b) there is no experimental evidence that appropriate doses of the bivalent octamethylene-diamine derivative containing DNP and D-benzylpenicilloic acid (BPO) groups on opposite sites were incapable of eliciting
histamine release from leukocytes of rabbits, singly immunized to either DNP or BPO. Thus, a monovalent interaction between antibody and haptenic molecule which may suffice to elicit an immediate type reaction (5) cannot be ruled out.

We have presented detailed evidence for the failure of monovalent haptens to elicit immediate type reactions in sensitized calf skin. It is apparent from the results, however, that monovalent haptens can, in fact, specifically inhibit the response to bivalent and multivalent haptens in either singly or doubly sensitized skin sites. It is clear from the data that reactions elicited by DNP-ABA in doubly sensitized skin sites were completely inhibited by equimolar concentrations of either DNP-cap or ABA-HPA. As expected, in singly sensitized skin sites a 5 to 10 times molar excess of the monovalent hapten was needed for effective inhibition of immediate type responses. The data obtained are at variance with the recent findings of Magro and Alexander (32) who showed that more than a 1,000-fold molar excess of the monovalent hapten is required for complete inhibition of the histamine release from rabbit leukocytes. However, their results cannot be easily explained and it seems necessary to recall that virtually all DNP substituents combined with the ε-amino groups of lysine have strong affinity for anti-DNP antibodies (23). A satisfactory reconciliation of the discrepancies will require a more defined reaginic system in which purified and characterized reactants are used.

Summary

The present study has established, that cows suitably immunized with either DNP-edestin (DNP-Ed), di-DNP-gramicidin-J [(DNP)₂-Gram], respectively, or p-azobenzene arsonate-Ed (ABA-Ed) synthesized and secreted reaginic antibodies (IgE) into colostrum. Whereas ABA-Ed failed to elicit more than a low response, there was however a persistent and increased antibody synthesis between 10 and 56 days after priming with DNP-Ed.

Bivalent and multivalent DNP haptens differing in molecular size, degree of substitution, and rigidity were compared for their effectiveness in eliciting Prausnitz-Küstner (P-K) reactions in either newborn colostrum-deprived calves or in those 4 wk of age. The sensitization with reaginic anti-DNP antibody has been accomplished either by feeding colostrum of the immunized dam or by intradermal injection of reaginic serum or colostral whey. It could be demonstrated that equimolar doses of the bivalent α,N-(ε,N-DNP-aminocaproyl)-ε,N-DNP-L-lysine and the multivalent dinitrophenylated bovine serum albumin were equally effective in eliciting reactions in skin sites provided that a high affinity antibody was used for sensitization. By contrast, the comparatively rigid, bivalent hapten, (DNP)₂-Gram consistently failed to induce comparable reactions. Furthermore, it was clearly shown that optimal distances of determinant groups on the haptenic molecule are a prerequisite for positive P-K reactions, since α,ε,N-bis-DNP-lysine failed to induce comparable reactions.

Concurrent sensitization of skin sites with reaginic anti-DNP and anti-ABA antibodies provides the final proof that cross-linking of two adjacent reaginic molecules on the mast cell surface by a bivalent hapten is required for effective
elicitation of immediate-type reactions. This has been accomplished by utilizing the bivalent \( \epsilon,N\text{-DNP-} \alpha,N\text{-[(4-hydroxy-3-azobenzenearsonic acid)-phenacetyl]} \text{-L-lysine (DNP-ABA)} \) carrying noncross-reactive haptenic groups, which was consistently effective in eliciting P-K reactions in doubly but never in singly sensitized skin sites. It is apparent from the results that equimolar doses of monovalent haptens could completely inhibit the response to DNP-ABA. The present studies finally establish that mast cells of newborn colostrum-deprived calves lack IgE molecules on their surface. Thus, mast cells of newborn calves may be unique, to investigate the molecular mechanisms involved in immediate-type reactions more precisely.

We are indebted to Dr. U. Schmitz-Hübner for helpful discussions and to Mr. F. Bühler for skillful technical assistance. The authors wish to express their appreciation to the Landwirtschaftsschule Hochburg for making their dairy herd available.

Received for publication 30 July 1974.

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