

## INDUCTION OF IMMUNITY AND TOLERANCE IN VITRO BY HAPTEN PROTEIN CONJUGATES

### I. THE RELATIONSHIP BETWEEN THE DEGREE OF HAPTEN CONJUGATION AND THE IMMUNOGENICITY OF DINITROPHENYLATED POLYMERIZED FLAGELLIN\*†

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A critical event in the immune response is the interaction of antigen with the surface receptors of immunocompetent cells. This interaction may result in two different consequences: an immune response or immunological tolerance.

Little is known at the single-cell level about the way in which antigen can dictate such contrasting events. Experiments performed *in vivo* are of limited usefulness in analysis of molecular mechanisms, where because of the antigen's inhomogeneous distribution and catabolism the relation between the amount of antigen injected and its concentration accessible to lymphocyte receptors is unknown (1). The mechanism by which antigen interacts with lymphocyte surfaces may be more directly studied using dissociated lymphoid cells *in vitro* (2, 3), where immunological tolerance may be rapidly induced with high concentrations of the antigen, polymeric flagellin of *Salmonella adelaide* (POL),<sup>1</sup> whereas lower concentrations of the same antigen induce an antibody response (4). This form of tolerance was termed high zone tolerance. Feldmann and Diener found that the induction of tolerance with antigen *in vitro* may be greatly facilitated by the presence of small concentrations of added antibody (5-8). The amount of antigen needed to induce tolerance could be reduced 10<sup>6</sup>-fold in the presence of antibody (7). This form of tolerance, necessitating the presence of both antigen and antibody, was termed "antibody-mediated tolerance," and in appropriate

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<sup>1</sup> *Abbreviations used in this paper:* AFC, antibody-forming cells; ATC, activated thymus cells; B cell, bone marrow- or bursal equivalent-derived lymphocytes; BSA, bovine serum albumin; dansyl, 5-(dimethylamino)-1-naphthyl determinant; DNP, 2,4-dinitrophenyl determinant; DRC, donkey red cells; FCS, fetal calf serum; F $\gamma$ G, fowl gamma globulin; Fla. flagella; H $\gamma$ G, human gamma globulin; KLH, keyhole limpet hemocyanin; MON, monomeric form of flagellin (in this communication the oxidized form, incapable of repolymerization, was used); POL, polymerized flagellin, unless otherwise specified, of *Salmonella adelaide*; SRC, sheep red cells; T cells, thymus-derived lymphocytes.

circumstances is equivalent, in antigen concentration terms, to "low zone tolerance" *in vitro* (7-9).

These findings have posed a major dilemma. How does an immunocompetent cell determine whether the amount of cell surface antigen is such as to cause immunity, or tolerance (10)? What is the mechanism of this antigen-induced immunological discrimination between immunity and tolerance? To probe further into the mechanism by which an immunocompetent cell distinguishes whether antigen is to act as a signal for immunity or for tolerance, it was necessary to use a more precise *in vitro* system. Instead of natural antigens in which the number and configuration of antigenic determinants is not known, chemically defined antigens were used (11). This communication presents the details of an antigenic system which is well suited to studying the molecular mechanism of immunity and tolerance induction, since the antibody response to the antigen used, dinitrophenylated polymeric flagellin (DNP-POL) does not require the presence of the known cellular helper mechanisms. An optimal response occurs in the absence of thymus-derived cells (T cells) (12), carrier reactive cells,<sup>2</sup> and phagocytic cells (13).<sup>2</sup> Thus the reaction of antigen with only one cell type, the bone marrow-derived antibody-forming cell precursors (B cells), initiates the antibody response. The system permits the reaction of antigen with surface receptors on B cells to be studied, and interpretations are not complicated by the necessity of considering helper mechanisms.

By varying the degree of conjugation of the hapten DNP onto the carrier POL, it was found that the immunogenic properties of the complex varied greatly depending on the degree of hapten conjugation. DNP-POL preparations with relatively few DNP groups were capable of inducing antibody responses to the DNP determinant, but even at very high doses could not induce tolerance. DNP-POL preparations with many DNP groups failed to induce immunity, even when tested over a very wide range of concentrations, but were tolerogenic. Thus by changing the degree of hapten conjugation, a complete reversal of immunological properties was achieved. The consequences of these findings on the mechanism of discrimination between immunity and tolerance will be discussed.

#### *Materials and Methods*

*Animals.*—Mice of the highly inbred CBA/H/Wehi strain were used at 70-110 days (14).

*Tissue Culture.*—The method of Feldmann and Diener (7) was used, employing Eagle's medium from Grand Island Biological Co., Grand Island, N. Y., supplemented with 5% fetal calf serum (FCS). Most experiments were assayed after 4 days to ensure that a maximal response was obtained.

*Cell Suspensions.*—Spleen and thymus suspensions were prepared and counted as described previously (5). Activated thymus cells (ATC) were prepared (14) and washed three times through FCS before use in tissue culture. Cell viability was assessed by eosin exclusion (15).

*Antigens.*—Bacterial flagella (Fla) and polymeric flagellin (POL) were prepared by the method of Ada et al. (16), monomeric flagellin by acid dissociation of POL, and oxidized by the method of Parish (17). Crystalline bovine serum albumin (BSA) was obtained from Armour Pharmaceutical Co., Chicago, Ill., fowl gamma globulin (F $\gamma$ G) was prepared (18),

<sup>2</sup> Feldmann, M. 1972. Induction of immunity and tolerance *in vitro* by hapten protein conjugates. II. Carrier independence of dinitrophenylated polymerized flagellin. *Eur. J. Immunol.* In press.

and human gamma globulin was obtained from Commonwealth Serum Laboratories (Melbourne, Australia). Human immunoglobulin G(H $\gamma$ G) was isolated by batch extraction on diethylaminoethyl-(DEAE-)cellulose (19).

Dinitrophenylated (DNP) antigens apart from flagellin were prepared by the method of Eisen (20).

Dinitrophenylated flagella (DNP-Fla), dinitrophenylated polymeric flagellin (DNP-POL), or dinitrophenylated monomeric flagellin (DNP-MON) were prepared as described previously (11).

"Dansyl" chloride [5-(dimethylamino)-1-naphthalenesulfonyl chloride] was obtained from Calbiochem (Los Angeles, Calif.). Dansyl proteins were prepared by the method of Paul et al. (21).

Donkey red cells (DRC) were obtained from Commonwealth Serum Laboratories. Sheep red cells (SRC) and DRC were stored for a week in Alsever's solution, and washed three times in saline before use.

*Enumeration of Antibody-Forming Cells.*—Antibody-forming cells (AFC) to SRC and DRC were detected by Cunningham and Szenberg's modification of the hemolytic plaque technique (22). The number of AFC to DNP was enumerated by separate assays of 0.1-ml aliquots of the cultured cell suspension with SRC and with SRC coated with rabbit anti-SRC Fab' conjugated with DNP (23), and subtracting the number of AFC obtained with unconjugated SRC from those obtained with DNP-SRC. Cells forming antibody to POL were assayed by the bacterial immunocytotoxicity method, as described by Diener (24).

*Irradiation.*—Irradiation was performed as described in reference 5.

*Electron Microscopy.*—One drop of a solution of dilute flagella or POL in 1% phosphotungstic acid solution (pH 7.4) was deposited onto a carbon-coated support grid. Excess fluid was removed with the edge of a piece of filter paper, and then allowed to dry. Specimens were scanned in a Philips EM 300 electron microscope (Philips Electronic Instruments, Australia) at magnifications ranging from 20,000 to 180,000. These electron microscopic examinations of DNP-Fla and DNP-POL preparations were kindly performed by Mr. Andrew Abbot.

## RESULTS

*Induction of a Primary Anti-DNP Response In Vitro.*—The kinetics of the development of the primary anti-DNP response to 100 ng/ml of DNP<sub>1.5</sub>-POL<sup>3</sup> in vitro was determined (Fig. 1). There was no response to DNP in the absence of added antigen, in contrast to other reports (25, 26). For comparison a secondary response, 8 wk after priming with 50  $\mu$ g of DNP-Fla,<sup>4</sup> is also shown. Both primary and secondary responses occur rapidly, with peak responses usually occurring by day 3.

DNP proteins apart from DNP-POL could not elicit antibody responses with unprimed cells in vitro, even when a wide range of antigen doses and conjugation ratios were tested. Shown in Table I is the lack of immunogenicity of dinitrophenylated fowl gamma globulin (DNP-F $\gamma$ G), DNP-H $\gamma$ G, dinitrophenylated keyhole limpet hemocyanin (DNP-KLH), DNP-BSA, and DNP-MON of *Salmonella adelaide* or *S. typhimurium* to normal CBA spleen cells in

<sup>3</sup> DNP<sub>1.5</sub>-POL indicates a preparation with an average of 1.5 DNP groups per monomeric flagellin unit of DNP-POL.

<sup>4</sup> Since POL and Fla have very similar physical and immunological properties, DNP-Fla is included in the generic term "DNP-POL."

vitro. Of special importance is the lack of immunogenicity of DNP-MON (made with oxidized monomer), compared with DNP-POL.

*Secondary Response to the DNP Determinant In Vitro.*—Attempts were made to elicit anti-DNP responses in spleen cells from mice immunized with different DNP conjugates. Antibody responses were obtained using cells from mice primed in vivo, and secondarily stimulated in vitro with DNP-F $\gamma$ G, DNP-H $\gamma$ G, DNP-MON, and DNP-KLH. The anti-DNP response obtained using spleen cells of mice primed with 100  $\mu$ g of DNP-F $\gamma$ G in Freund's com-

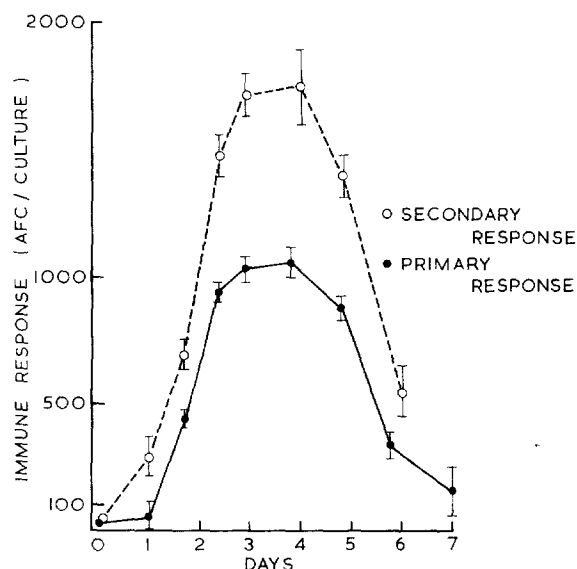


FIG. 1. Kinetics of the primary and secondary anti-DNP responses in vitro. Each point represents the arithmetic mean of four to eight cultures. The error bars in this and all other graphs in this paper indicate one standard error of the mean. In other experiments the secondary anti-DNP response varied from 2-10 times the magnitude of the primary response depending on the time and antigen dose used for priming.

plete adjuvant 2 months previously is illustrated in Table II. 1000-2000 AFC/culture occurred at day 4 with 0.1-10  $\mu$ g/ml of DNP-F $\gamma$ G. Antigens incapable of eliciting primary responses could thus cause secondary anti-DNP responses.

*Stimulation of Unprimed Spleen Cells to Respond to Hapten Protein Conjugates by the Addition of Activated Thymus Cells.*—Since it has been shown that the in vitro IgM response to POL (or DNP-POL) is thymus independent, whereas that to MON requires thymus-derived (T) cells (12), it is possible that the reason for the inability of DNP-MON to immunize unprimed spleen cells in vitro is a deficiency in flagellin-reactive T cells. This possibility was directly tested by adding "activated" thymus cells (ATC) to normal spleen

TABLE I  
Capacity of Hapten Protein Conjugates to Immunize Normal CBA Mouse Spleen Cells

Antigen	Specific anti-DNP response to various antigen concentrations (g/ml)							
	10 <sup>-9</sup>	10 <sup>-8</sup>	10 <sup>-7</sup>	10 <sup>-6</sup>	10 <sup>-5</sup>	10 <sup>-4</sup>	10 <sup>-3</sup>	10 <sup>-2</sup>
	<i>AFC/culture</i>							
DNP <sub>15</sub> -FγG	0	0	0	0	40	60	0	0
DNP <sub>4</sub> -FγG	0	0	0	0	0	0	0	0
DNP <sub>1</sub> -POL	100	320	600	900	720	820	840	—
DNP <sub>2.5</sub> -POL	80	420	720	510	100	—	0	—
DNP <sub>1.5</sub> -POL ( <i>S. typhimurium</i> )	40	1170	1280	1470	1200	1080	—	—
DNP <sub>1</sub> -MON	0	0	0	40	80	0	0	0
DNP <sub>3</sub> -MON	0	0	0	0	0	0	0	0
DNP <sub>4.5</sub> -MON	0	0	0	0	0	0	0	0
DNP <sub>3</sub> -MON ( <i>S. typhimurium</i> )	0	0	0	80	0	0	0	0
DNP <sub>12</sub> -HγG	0	0	0	0	0	0	0	0
DNP <sub>700</sub> -KLH	0	0	0	0	0	0	0	0
DNP <sub>1800</sub> -KLH	0	0	0	0	0	0	0	0
DNP <sub>12</sub> -BSA	0	0	0	0	0	0	0	0

Antigen concentrations are expressed as fractions of g/ml: 10<sup>-9</sup> g/ml = 1 ng/ml, 10<sup>-6</sup> g/ml = 1 μg/ml, etc. Each value represents the arithmetic mean of 8-24 cultures, in at least two experiments. The value of 0 represents a value of less than 20% of the SRC background response which was the limit of detection of specific DNP plaques. Thus values of less than about 20 AFC/culture would be taken as 0. The minimal responses to DNP-FγG, DNP-MON shown in this table were not reproducible; in other experiments there was no detectable response. DNP-MON was made using oxidized MON, incapable of repolymerizing

TABLE II  
Secondary Response to DNP-FγG and DNP-MON in Vitro

Priming in vivo	Antigen in vitro	Anti-DNP response
	μg/ml	<i>AFC/culture ± SE</i>
	DNP <sub>18</sub> -FγG	
100 μg DNP <sub>8</sub> -FγG i.p.	100	80 ± 40
8 wk before	10	160 ± 70
" " "	10	320 ± 90
" " "	0.1	100 ± 40
	DNP <sub>8</sub> -FγG	
" " "	100	160 ± 30
" " "	10	1800 ± 280
" " "	1	1400 ± 300
" " "	0.1	980 ± 160
" " "	0.01	300 ± 100
	DNP <sub>2</sub> MON	
50 μg DNP <sub>2</sub> -POL i.p.	10	680 ± 160
4 wk before	1	980 ± 200
" " "	0.1	240 ± 140
" " "	0.01	60 ± 40

Each value is the arithmetic mean of four cultures ± the standard error of the mean of a single comparative experiment. Further experiments confirmed these results and are published elsewhere (see footnote 2).

cells cultured with antigens such as DNP-F $\gamma$ G or DNP-MON. The effect of the addition of ATC to flagellin on the anti-DNP response of normal spleen to DNP-MON is shown in Fig. 2. The DNP response was stimulated by these carrier-primed T cells, to the same level as caused by DNP-POL.

*The Effect of the Frequency of Hapten Substitution on the Immune Response to DNP-POL In Vitro.*—Preliminary experiments using DNP-POL revealed that not all batches were equally immunogenic. The response to 0.1–100  $\mu$ g/ml of three batches of different hapten conjugation rates is shown in Fig. 3. Marked differences between the batches were noted. The least conjugated preparation, with 0.7 DNP groups per monomeric unit in POL (DNP<sub>0.7</sub>-POL)

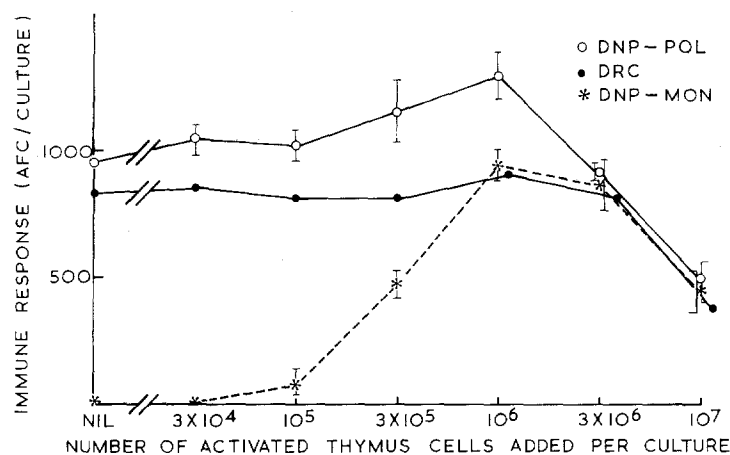


FIG. 2. Stimulation of the capacity of normal spleen cells to respond to DNP-MON by thymus cells activated to monomeric flagellin. The data shown were pooled from two experiments, and each point is the arithmetic mean of four to eight cultures  $\pm$  the standard error of the mean. There was no significant enhancement of the response elicited by DNP-POL.

was immunogenic even at doses up to 3  $\mu$ g. With a higher degree of hapten substitution, 2.7 groups per monomeric unit of POL (DNP<sub>2.7</sub>-POL), a somewhat different immunogenic behavior emerged. The anti-DNP response occurred at the same antigenic threshold, and peak immunogenicity occurred at about 100 ng/ml. At higher concentrations, 3  $\mu$ g/ml or more, the immune response was diminished.

Also shown on the same graph is the lack of an immune response at any of the doses tested to DNP<sub>3.7</sub>-POL.

There was thus a marked change, depending on the degree of conjugation, in the capacity of DNP-POL to induce anti-DNP responses. The immunogenic behavior of low conjugates differed from that of native POL, which is not immunogenic at high doses, as did that of high conjugates. The antibody responses to DNP and POL elicited by various DNP-POL preparations were

thus compared. The capacity to induce immunity or tolerance to POL and DNP did not change in parallel (Table III).

The correlation between hapten substitution and immune responsiveness was therefore studied in more detail. 39 batches of DNP-POL of two different bacterial strains were prepared to encompass the possible substitution rates. The immunogenic properties of each batch were tested over a dose range of 1 ng–10  $\mu$ g/ml. Each experiment included at least two doses of an optimally immunogenic preparation and each batch was tested at least twice. The data

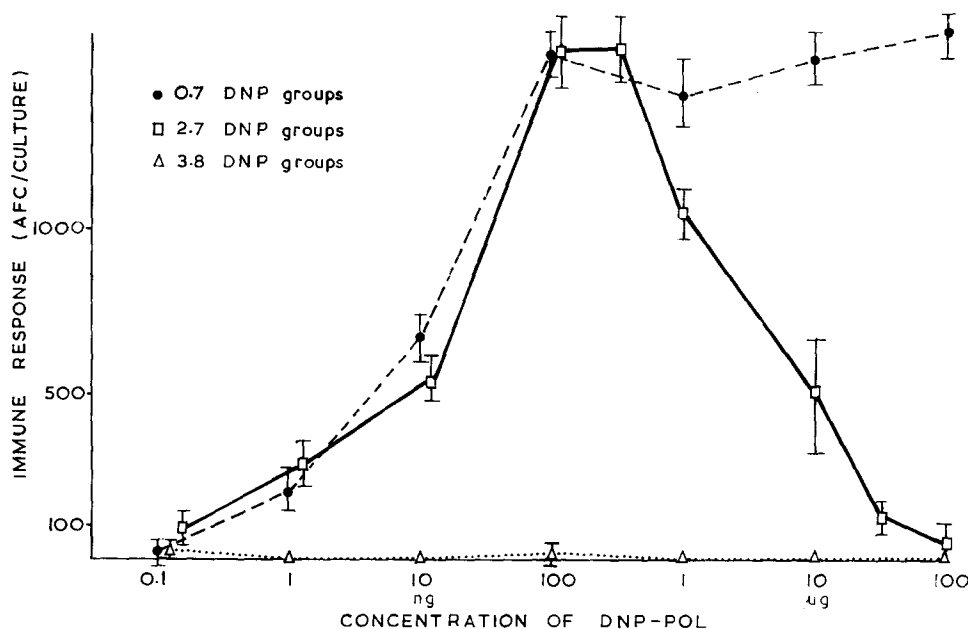


FIG. 3. The effect of altering the number of DNP groups conjugated to POL on the primary anti-DNP response elicited by DNP-POL in vitro. A typical experiment is shown, with four cultures per point. Similar results were always obtained with these substitution ratios.

obtained (22 experiments) were pooled, and expressed as percentages of the response of the reference batch. Fig. 4 shows the average (as a percentage) of the optimal response obtained with each preparation in various experiments. The lowering in immunogenicity with preparations with more than 2–3 DNP/MON unit was reproducible. The substitution curve does not extend beyond about 5–6 DNP/MON unit because of depolymerization of POL.

*Lack of Immunogenicity of Highly Conjugated DNP-POL.*—Possible reasons for the lack of immunogenicity were tested:

(a) *Depolymerization:* Since DNP-MON does not elicit primary anti-DNP responses in vitro, the lack of immunogenicity of highly conjugated DNP-POL

may have been due to depolymerization. Since POL is a much larger molecule than MON (mol wt MON = 40,000, mol wt POL = 40,000 × hundreds), it is easy to sediment the POL. All preparations used were polymeric. The physical state of the DNP-POL preparations was also checked by electron microscopy using phosphotungstic acid as a negative stain (Materials and Methods). Rods of POL or flagella can be clearly seen whereas MON cannot. Fig. 5 illustrates unconjugated POL, and an immunogenic and a tolerogenic batch of DNP-POL. With the batches of *S. adelaide* POL, and with the method of con-

TABLE III  
*Comparison of the Antibody Response to DNP and to POL Elicited by Various DNP-POL Conjugates*

Antigen	Antibody response	
	Anti-DNP	Anti-POL
$\mu\text{g}$	<i>AFC/culture</i> $\pm$ <i>SE</i>	<i>AFC/culture</i> $\pm$ <i>SE</i>
POL		
0.1	<20	840 $\pm$ 180
100	<20	<20
DNP <sub>0.7</sub> -POL		
0.1	1080 $\pm$ 120	680 $\pm$ 160
100	840 $\pm$ 240	<20
DNP <sub>1.9</sub> -POL		
0.1	1140 $\pm$ 60	580 $\pm$ 210
100	120 $\pm$ 40	<20
DNP <sub>3.8</sub> -POL		
0.1	<20	<20
100	<20	<20

Normal spleen cells were cultured for 3.7 days with the above antigens. Responses to DNP and POL were assayed. Each value is the arithmetic mean of four cultures  $\pm$  the standard error of the mean.

jugation used, depolymerization did not begin to be an appreciable problem until there were 4–5 DNP groups/MON of POL, and was usually complete by 6–8 DNP/MON. For this reason the data shown in Fig. 4 are only plotted up to DNP<sub>6</sub>-POL.

Electron microscopy did not reveal any alteration in the usual heterogeneity in the length of the rods of DNP-POL or DNP-Fla as compared with the unconjugated preparations. Since DNP-MON does not inhibit the response elicited by DNP-POL unless it is present in 100-fold excess, very small degrees of depolymerization would not influence the response.

(b) *Changes in the tertiary structure of the monomeric units of DNP-POL:*



The conjugation of a bulky aromatic group such as DNP onto lysine (or methyl-lysine) residues could alter the tertiary structure of the flagellin molecule. This potential change in tertiary structure should also be caused by the conjugation of other similar groups onto lysine residues. The dansyl group was used to investigate whether the effect of a high degree of DNP conjugation was immunologically specific, or was due to changes in the flagellin making it less immunogenic. Dansyl<sub>4</sub>-POL was prepared and conjugated with DNP. The immunogenicity of DNP<sub>1</sub>-dansyl<sub>4</sub>-POL, DNP<sub>1.9</sub>-dansyl<sub>4</sub>-POL was compared with that

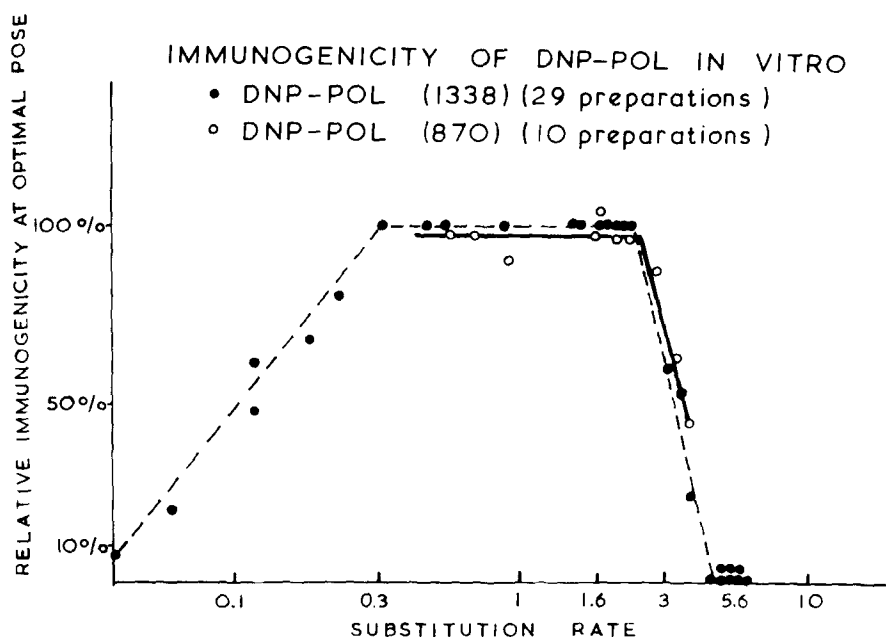


FIG. 4. The effect of DNP substitution on the anti-DNP response elicited by DNP-POL in vitro. Summary of data with all the batches of DNP-POL prepared. DNP flagella (14 batches) behave in a similar fashion. The data are only plotted until six groups per mole because of depolymerization.

of DNP<sub>3</sub>-POL (Fig. 6). DNP<sub>1</sub>-dansyl<sub>4</sub>-POL, and DNP<sub>1.9</sub>-dansyl<sub>4</sub>-POL in the appropriate dose elicited good anti-DNP responses in the appropriate dose. Thus the lack of a response to DNP<sub>3</sub>-POL was directly due to the number of DNP groups present, and not to some conjugation-induced change in the structure of flagellin.

(c) *Tolerance*: Highly substituted DNP-POL could be very tolerogenic. This possibility was tested by preincubating normal spleen cells with various concentrations of DNP<sub>3.8</sub>-POL, a nonimmunogenic preparation. Cells were thus incubated with 100, 30, 10, 3, 1, or 0.3  $\mu\text{g/ml}$  of DNP<sub>3.8</sub>-POL for 6 hr at 37°C

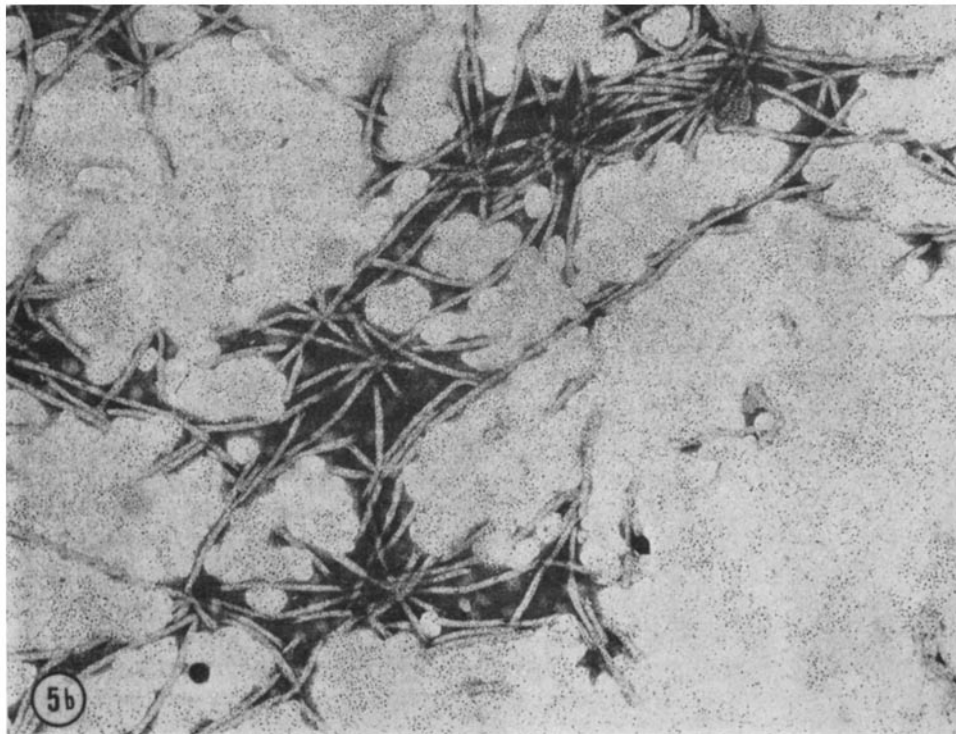
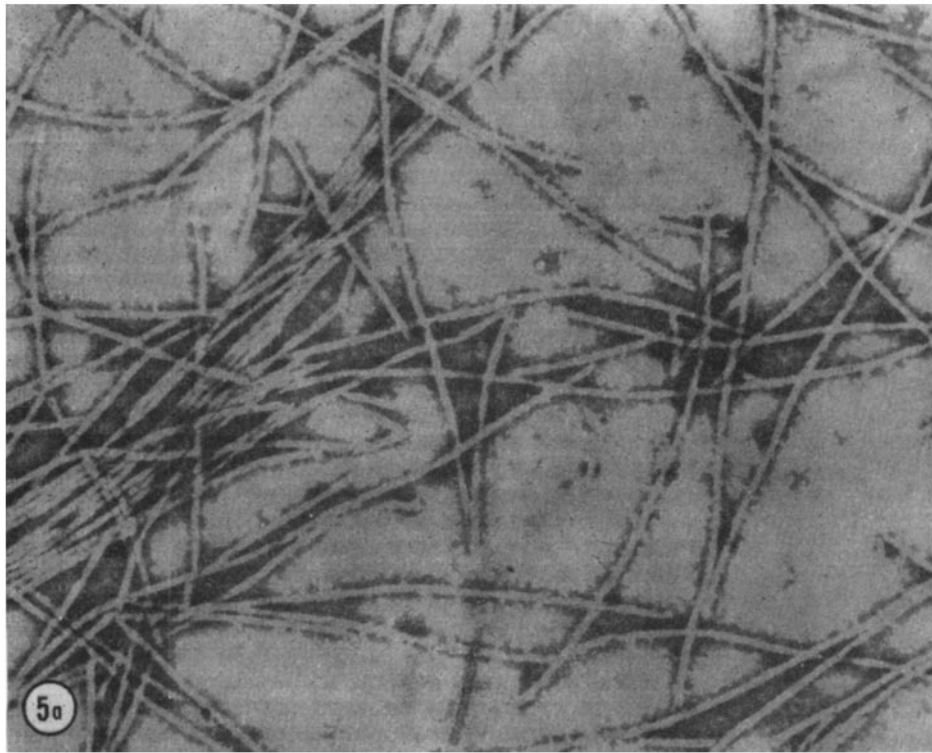




FIG. 5. Comparison of the structure of POL and DNP-POL using phototungstic acid negative stain ( $\times 65,250$ ). (a) POL, (b)  $\text{DNP}_1$ -POL, an obligatory immunogen, (c)  $\text{DNP}_{3.8}$ -POL, an obligatory tolerogen.

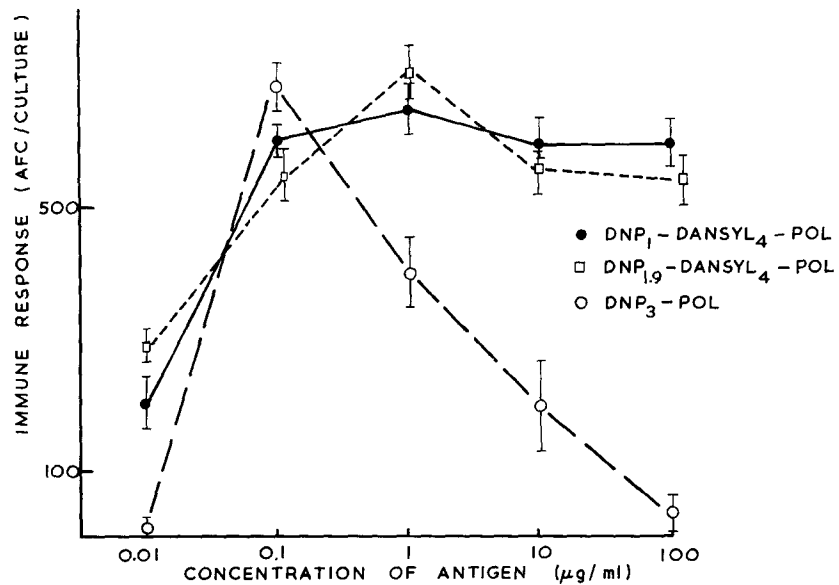


FIG. 6. The immune response to mixed DNP-dansyl-POL conjugates in vitro. This is a typical experiment, in which each point is the arithmetic mean of four cultures. The same result was obtained in two other experiments of this nature using different batches of DNP-dansyl-POL. The mixed conjugates, even with a total of five haptenic groups per MON unit, were fully immunogenic, and were incapable of inducing tolerance.

in vitro, washed thoroughly, and challenged with 100 ng DNP<sub>1.5</sub>-POL (Fig. 7). A state of partial but significant ( $P < 0.05$ ) and specific immunological tolerance was induced, as demonstrated by the normal response to DRC in the same cultures.

*Correlation of the Conjugation Ratio of DNP-POL and the Capacity to Induce Tolerance.*—To correlate DNP substitution with the capacity to induce tolerance, normal spleen cells were incubated for 6 hr at 37°C with 100 μg/ml of various DNP-POL preparations. The cells were washed thoroughly and challenged in vitro with 100 ng of DNP<sub>1.5</sub>-POL. Fig. 8 summarizes the results of 16 experiments of this basic design, and represents the means of the degree of

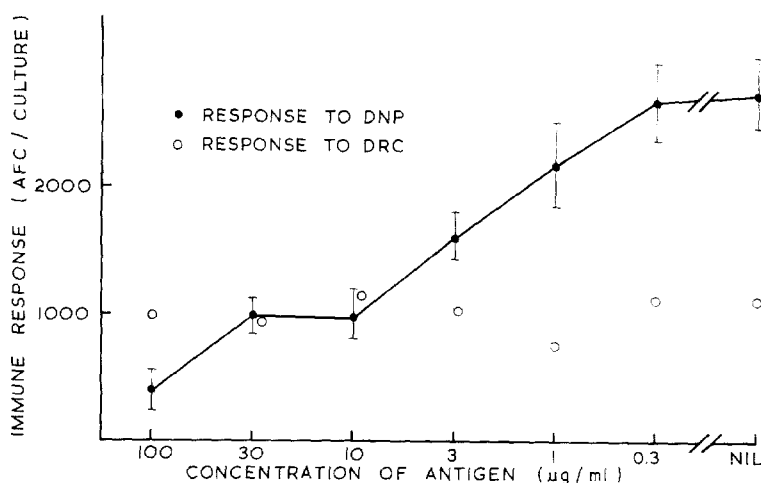


FIG. 7. The capacity of different concentrations of DNP<sub>3</sub>-POL to induce tolerance within 6 hr at 37°C in vitro. The pooled results from two experiments are shown. Significant partial tolerance only occurred with 100, 30, or 10 μg/ml of DNP<sub>3.8</sub>-POL.

tolerance induced. The tolerance induced was compared with cultures preincubated for 6 hr at 37°C without antigen, and washed in parallel. There was a marked relationship between the capacity to induce tolerance and the number of DNP groups per MON unit of DNP-POL. Compounds with less than 1.7 DNP groups did not induce tolerance, even if very high concentrations (3 mg/ml) were used; however, they caused flagellin-specific tolerance. Even when the concentrations of DNP were adjusted to equimolar levels, i.e. much more of a lightly substituted preparation was used than of a highly substituted preparation of DNP-POL, tolerance only occurred with the more highly conjugated compounds (11).

The hapten specificity of the effect of a high degree of hapten conjugation was assessed using mixed DNP-dansyl-POL conjugates. Dansyl [5-(dimethylamino)-1-naphthyl] is an aromatic compound like DNP and thus, if effects

due to the modification of lysine residues were important, it may be expected that dansyl conjugation would reduce the number of DNP groups needed to induce DNP-specific tolerance. It was found that 6-hr pretreatment with

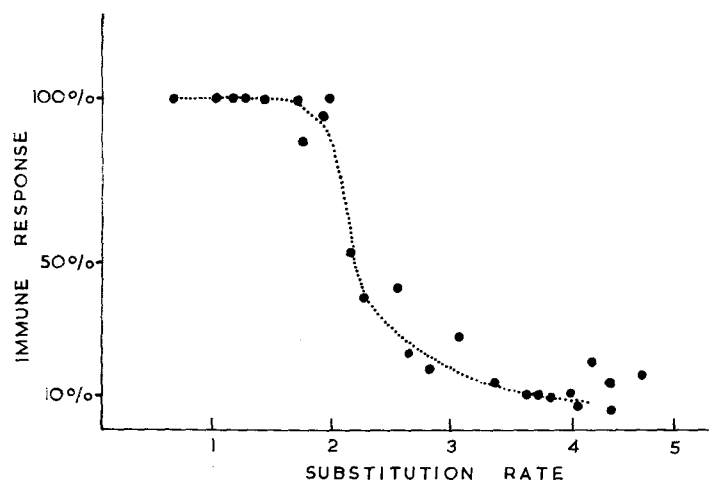


FIG. 8. The pooled results of the effect of DNP conjugation rate on the capacity to induce immunological tolerance in vitro. Each point represents the average residual anti-DNP response obtained in various experiments in which normal spleen cells were treated with 100  $\mu\text{g}/\text{ml}$  of  $\text{DNP}_3\text{-POL}$  in vitro.

TABLE IV

*The Induction of Tolerance with Mixed DNP-Dansyl-POL Conjugates In Vitro*

Antigen used for preincubation	Anti-DNP response
$\mu\text{g}/\text{ml}$	<i>AFC/culture</i> $\pm$ <i>SE</i>
Nil	1340 $\pm$ 180
100, $\text{DNP}_3\text{-POL}$	65 $\pm$ 30*
100, $\text{DNP}_1\text{-dansyl}_4\text{-POL}$	1275 $\pm$ 250
300, $\text{DNP}_1\text{-dansyl}_4\text{-POL}$	1840 $\pm$ 360
100, $\text{DNP}_{1.9}\text{-dansyl}_4\text{-POL}$	840 $\pm$ 280
300, $\text{DNP}_{1.9}\text{-dansyl}_4\text{-POL}$	890 $\pm$ 410
100, $\text{dansyl}_4\text{-POL}$	1475 $\pm$ 310

Normal spleen cells were preincubated with various antigens, washed thoroughly, and challenged with 100 ng of  $\text{DNP}_{1.5}\text{-POL}$  and DRC. The data were pooled from two experiments and each value represents the arithmetic mean of eight cultures  $\pm$  the standard error of the mean. The response to DRC was normal in each group.

\*  $P < 0.01$  compared with all other groups.

either 100 or 300  $\mu\text{g}/\text{ml}$  of  $\text{DNP}_1\text{-dansyl}_4\text{-POL}$  or  $\text{DNP}_{1.9}\text{-dansyl}_4\text{-POL}$  did not cause tolerance, whereas  $\text{DNP}_3\text{-POL}$  induced tolerance (Table IV).

Thus the hapten substitution effect represents a true effect of epitope density on the immunogenic and tolerogenic properties of DNP-POL.

## DISCUSSION

Polymeric flagellin was a good "carrier" molecule *in vitro* since DNP-POL, but not DNP-MON or DNP conjugated to other proteins, elicited a primary anti-DNP response *in vitro* (Table I). Since flagellin polymers of different bacterial strains were suitable carriers, the capacity to immunize must be inherent in the quaternary structure of the protein carrier, and was not a property peculiar to *S. adelaide* POL. Since other DNP proteins, including DNP-MON, were not immunogenic (Table I), the capacity to immunize unprimed spleen cells is related to the repeating antigenic determinants present on polymeric but not on monomeric flagellin.

The antibody response to DNP-POL *in vitro* occurs normally in the absence of T cells (12) but the response to MON treated to prevent its repolymerization requires the presence of T cells (12). Since thymus-independent DNP-POL was immunogenic whereas DNP-MON was not immunogenic, this suggested that the capacity of DNP-POL to immunize unprimed spleen cells was linked to its capacity to immunize B cells directly. *In vitro*, with a restricted number of spleen cells ( $1.5 \times 10^7$ ), there may be too few T cells reactive to carrier protein determinants for effective cell cooperation. T cells are thus rate limiting in normal spleen cultures and the function of T cells determines the magnitude of the response to antigens which need T-B collaboration.<sup>5</sup>

Many proteins can act as carriers for a secondary anti-hapten response *in vitro* (Table II, 27, 28), perhaps because of the presence of more carrier-reactive T cells in primed spleen. This possibility was proven by the capacity of flagellin-reactive ATC to restore the response to DNP-MON, whereas these cells did not enhance the response to DNP-POL (Fig. 2). These results support the concept that DNP-POL elicits a primary response because of its capacity to immunize B cells directly.

Some batches of DNP-POL were much more immunogenic than others. Depending on the degree of DNP conjugation the immunogenicity of DNP-POL varied. Immunogenic preparations were conjugated with relatively few DNP groups, whereas preparations with many DNP groups were much less immunogenic (Figs. 3 and 4). The reasons for the lowered immunogenicity of "highly conjugated" DNP-POL was intriguing as lowered responses occurred with only 3 DNP/MON unit of DNP-POL.

Since DNP-MON did not elicit primary anti-DNP responses *in vitro* depolymerization of DNP-POL as a result of the conjugation is a possible cause of lowered immunogenicity. However, nonimmunogenic batches of DNP-POL were in the polymeric state, as judged by centrifugation and electron microscopic studies (Fig. 5).

<sup>5</sup> Feldmann, M., and A. Basten. 1972. Cell interaction in the immune response *in vitro*. I. Metabolic activities of T cells in a collaborative antibody response. *Eur. J. Immunol.* In press.

To test the possibility that the conjugation of any aromatic group onto POL could reduce the effectiveness of the protein as a carrier for inducing an immune response to the DNP group, the effect on the anti-DNP response of mixed conjugation with DNP and dansyl was ascertained. The additional conjugation of dansyl residues onto immunogenic DNP-POL did not appreciably influence the anti-DNP responses (Fig. 6) unlike the additional conjugation of DNP (Figs. 3 and 4). Conjugation of lysine residues thus does not alter the structure of flagellin in such a way as to reduce its immunogenic capacity as a carrier molecule. Thus the reduced immunogenicity of heavily conjugated DNP-POL implies that antibody-forming cell precursors must be capable of distinguishing the number of DNP groups on the surface of DNP-POL molecules.

Tolerance was the reason for the reduced immunogenicity of highly conjugated DNP-POL (Figs. 7 and 8). Both the degree of substitution, and the antigen dose were important factors in the induction of DNP tolerance (Fig. 8). The degree of DNP conjugation was important, but operated independently of DNP concentration, as  $2 \times 10^{-6}$  M DNP on DNP<sub>1</sub>-POL did not induce tolerance, whereas the same amount as DNP<sub>3</sub>-POL did. Thus the factor of hapten substitution is not merely one of total hapten concentration in solution but also of local concentration or of antigenic determinant density, or "epitope density." Compounds like DNP<sub>0.7</sub>-POL, which did not cause DNP tolerance, caused specific tolerance to POL (Table III). Lowered immunogenicity with highly conjugated hapten protein conjugates has been reported previously (29-31). With hapten protein conjugates which require the participation of carrier-reactive cells, high hapten conjugation may act merely by masking carrier determinants, inhibiting cell cooperation. The results presented above (Figs. 3 and 4) cannot be attributed to this cause, as the antibody primary IgM response to DNP-POL doses not require cell cooperation (12).<sup>2</sup> Reduction in the immunogenicity of highly conjugated DNP flagella has also been noted *in vivo* (R. Huchet and M. Feldmann, to be published). Thus the hapten substitution effect must reflect a basic difference in the reaction pattern of antigenic determinants with B cell surface receptors.

How does the hapten density influence the subsequent immunological status of B cells? It is clear from Figs. 3, 4, and 8 that there is a dissociation between the capacity of DNP-POL conjugates to induce immunity and to induce tolerance. There are thus two broad classes of DNP-POL antigens. "Obligatory immunogens" can induce immunity but not tolerance. These compounds may even inhibit the induction of tolerance by an "obligatory tolerogen" a compound whose only demonstrable immunologic behavior is the induction of tolerance.<sup>6</sup> The latter are always heavily substituted. Since electron microscopy demonstrated no difference in the length or in the width of the rods of tolero-

<sup>6</sup> Recently Borel has described the induction of tolerance to DNP using nonimmunogenic dinitrophenylated mouse serum (32).

genic or immunogenic DNP-POL, and since the hapten conjugation effect is strictly antigen specific, the surface receptors of B cells must be capable of distinguishing between an antigen with few or many DNP groups in a given region of its surface.

*Hypothesis: A Mechanism of B Cell Signal Discrimination.*—According to this model the induction of B cell immunity requires the simultaneous interaction of many antigenic determinants with receptor antibody molecules on the surface of B cells. This suggests why antigens with repeating determinants, like DNP-POL, which can make multiple bonds with surface receptors and thus remain firmly cell bound, immunize in the absence of “helper” cells such as T cells and macrophages (12, 13).<sup>7</sup> Antigens without repeating determinants require helper cells to achieve and maintain the necessary simultaneous interaction of antigenic determinants with B cell receptors required for immunization.

The induction of B cell tolerance has some superficial similarities to immune induction. As yet antigens with repeating determinants are the only ones described as capable of causing tolerance in vitro, e.g. POL (4), DNP-POL (11), *Escherichia coli* endotoxin (33). In contrast, antigens without repeating units, such as the monomeric forms of flagellin, fragment A of flagellin, or oxidized monomeric flagellin (6), or serum protein antigens, do not induce B cell tolerance rapidly on present in vitro conditions (8, 34). This fact, together with the need for a high hapten conjugation ratio and concentration for tolerance to occur even with polymeric molecules such as DNP-POL, implicates multiple closely linked reactions of antigenic determinants with receptor antibody molecules, more than are required for the induction of immunity.

Inhibition studies with nonimmunogenic and nontolerogenic DNP conjugates have recently provided direct experimental support for this hypothesis. It was found that the most efficient inhibitors of the anti-DNP response to DNP<sub>1</sub>-POL were multivalent DNP proteins, whereas monosubstituted DNP compounds were much less effective. Similarly the induction of tolerance to DNP with DNP<sub>4</sub>-POL was more readily inhibited by nontolerogenic DNP<sub>1</sub>-POL than by smaller DNP conjugates, DNP<sub>12</sub>-H $\gamma$ G or DNP-lysine.<sup>7</sup> Since DNP-POL makes multiple bonds with the cell surface more readily than the small inhibitors this demonstrates that many very closely spaced reactions of antigenic determinants with B cell receptors are necessary in tolerance induction. A further deduction is that antigen receptors in a given area of the cell membrane must act in unison in order to translate an antigen localization pattern into a meaningful immunological signal.

Experimental data in full agreement with this concept have been acquired using another experimental system. Feldmann and Diener (35) found that the

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<sup>7</sup> Feldmann, M. 1971. Induction of immunity and tolerance in vitro by hapten protein conjugates. III. Elucidation of the mechanism of antigen binding to B cells by the use of hapten inhibition. Manuscript in preparation.



critical function of antibody needed to facilitate tolerance was its capacity to cross-link antigen molecules, since univalent Fab' pepsin fragments did not facilitate antibody-mediated tolerance, whereas  $F(ab')_2$  fragments were as effective as whole IgG. By cross-linking antigen, antibody-antigen complexes may present to B cell receptors a surface of closely spaced antigen receptors which by the hypothesis proposed above is a "tolerogenic signal."

Thus different experimental approaches have yielded results which are consistent with the model of B cell signal discrimination proposed above. Furthermore this model readily explains why polymeric antigens are highly immunogenic, not requiring the helper effect of either T cells or macrophages, yet are also highly tolerogenic in vitro.

#### SUMMARY

Of many dinitrophenylated (DNP) protein conjugates tested, only DNP conjugated to polymerized flagellin (DNP-POL) (or the structurally related bacterial flagella) elicited a primary anti-DNP response in vitro. Other DNP proteins, such as DNP-monomeric flagellin (DNP-MON), were capable of inducing secondary responses in vitro. The capacity of DNP-POL to immunize spleen cell suspensions devoid of thymus-derived cells was the reason for the greater immunogenicity of DNP-POL, since even large numbers of flagellin-reactive activated thymus cells did not increase the anti-DNP response of normal spleen cells immunized with DNP-POL, whereas the thymus-dependent response to DNP-MON was markedly increased.

The capacity of various batches of DNP-POL to immunize normal spleen cells in vitro varied markedly, depending on the number of DNP groups conjugated. DNP-POL with few DNP groups conjugated was immunogenic, but even at very high concentrations did not induce tolerance. In contrast, highly conjugated DNP-POL did not immunize, but readily induced tolerance. DNP-POL with intermediate degrees of conjugation were, like unconjugated polymeric flagellin, capable of inducing both immunity and tolerance. Since DNP-POL immunizes bone marrow-derived lymphocytes (B cells) directly the reduced response was not due to a masking of carrier determinants, necessary for cell collaboration. By using mixed DNP-5-(dimethylamino)-1-naphthalyl (dansyl)-POL conjugates, it was found that the inhibitory effect of a high degree of hapten conjugated was hapten specific. Depolymerization of DNP-POL to DNP-MON, which does not induce primary anti-DNP responses, was excluded by centrifugation analysis and electron microscopy.

The relationship of the degree of hapten conjugation on DNP-POL to the capacity to induce tolerance and immunity in B cells has clarified the mechanism of immunological triggering of these cells. A model of the mechanism of "signal" discrimination between immunity and tolerance in B cells, based on these findings, is proposed.

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