Immunology, 1975, **28**, 897.

Delayed Hypersensitivity in the Mouse Induced by Hapten–Carrier Complexes

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(*Received 5th July* 1974; *accepted for publication 11th November* 1974)

Summary. Delayed hypersensitivity (DH) in the mouse was studied with complexes of dinitrophenyl (DNP) as hapten and bovine serum albumin (BSA), mouse immunoglobulin (MIg) and polyvinylpyrrolidone (PVP) as carrier. Priming with BSA induced strong DH against this carrier, but DH of decreasing strength against complexes with increasing DNP:carrier ratio. Priming with DNP–BSA complexes never resulted in a DH against BSA or DNP_3–BSA. Injections of DNP_{16}–BSA and DNP_{28}–BSA induced positive DH which increased with the hapten:carrier ratio of the eliciting antigen.

The complexes with an isologous carrier DNP_{48}–MIg or DNP_{90}–MIg induced positive reactions against both complexes but not against the weakly substituted DNP_{11}–MIg. The latter only primed for itself. The importance of the DNP groups as determinant in these DH reactions is stressed by the cross-reactions between DNP–BSA and DNP–MIg complexes and by the induction by DNP_{16}–PVP of positive DH against DNP_{28}–BSA. Cyclophosphamide (Cy) treatment before priming with complexes induced enhanced DH against complexes with sufficient hapten: carrier ratio. Priming with carrier under Cy treatment induced no DH against complexes. All these results indicate that carrier determinants are not involved in the DH against complexes. After priming with complexes with a low hapten: carrier ratio the DH is directed against new antigenic determinants (NAD) groups. After priming with complexes with high ratios DH is directed against DNP groups. With adoptive local transfer of spleen cells of primed animals and pretreatment of these cells with anti-thymocyte serum or anti-plasma cell serum and complement it was possible to demonstrate that the T cell was responsible for the DH reactions.

The involvement of different determinant groups on the hapten–carrier complexes in immune reactions is discussed.

INTRODUCTION

Immunization with the low molecular weight azobenzenearsonate (ABA) conjugate of *N*-acetyltyrosine produces a state of relatively pure delayed hypersensitivity (DH), that was shown to be specific for this hapten (Leskowitz, Jones and Zak, 1966). Hanna and Leskowitz (1973) demonstrated that the amino acid portion of the molecule is of paramount importance in determining the immunogenic properties of the conjugate in

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DH reactions *in vivo* and *in vitro*. Using complexes of dinitrophenyl groups (DNP) as hapten and serum protein as carrier Snippe, Nab and van Eyk (1974) demonstrated that in the *in vitro* lymphocyte stimulation test DNP groups or DNP groups combined with part of the carrier were more reactive than the carrier. The target cells of this response were mainly T cells (Snippe and van Eyk, 1974). Antibody formation was studied with the use of complexes with different hapten:carrier ratios and after education of T cells in the presence of carrier or hapten–carrier complexes (Snippe, Graven and Willems, 1975). They found strong indications that T helper cells are reactive with DNP groups or DNP–new antigenic determinant (NAD) groups, and probably to a lesser degree with true carrier determinants. While in the *in vitro* stimulation test the complexes with the highest DNP:carrier ratio were the most active ones (Snippe *et al.*, 1974), the complexes with an intermediate hapten:carrier ratio gave better anti-DNP antibody formation (Mosier, Johnson, Paul and McMaster, 1974).

In this paper the role of hapten, carrier and NAD groups in the delayed hypersensitivity *in vivo* was studied.

MATERIALS AND METHODS

Animals and immunization

Inbred female BALB/c mice were raised in the Laboratory of Microbiology, State University, Utrecht, The Netherlands. The animals were used at an age of about 12 weeks. For each experiment groups containing five to six mice were used. Different amounts of DNP–protein dissolved in 0·1 ml of saline, emulsified in 0·1 ml of Freund's complete adjuvant (FCA) which contained killed *Mycobacterium* H_{37} Ra (Difco Laboratories, Detroit, Michigan) were injected intracutaneously (i.c.) on the abdomen, divided over four sites.

Antigens

The preparation of DNP–BSA (2,4-dinitrophenylated bovine serum albumin), DNP–MIg (isologous mouse immunoglobulin) and DNP–PVP (polyvinylpyrrolidone, molecular weight 360,000) complexes was described earlier by Snippe *et al.* (1974). Complexes with the following composition were prepared: DNP_3–BSA; DNP_{16}–BSA; DNP_{28}–BSA; DNP_{11}–MIg; DNP_{48}–MIg; DNP_{90}–MIg, and analysed according to the method of Eisen, Carsten and Belman (1954). DNP_{16}–PVP, DNP_{35}–PVP and DNP_{91}–PVP (PVP, molecular weight 360,000) were prepared and analysed according to Snippe *et al.* (1974).

Assay for delayed hypersensitivity (DH)

The DH reactions were determined by measuring the increase in footpad-thickness (footpad swell test) as described by Kerckhaert, van den Berg and Willers (1974). In all experiments the test dose was given by an injection into the left footpad of 25 μg of antigen suspended in saline to a total volume of 0·05 ml. Siliconed needles (size $0{\cdot}5 \times 16$ mm) were used in this assay. A footpad swelling of 0·25 mm is regarded as positive. The results were expressed as the increment of the footpad-thickness in 1/10 mm $\pm$ standard error of the mean of six to eight mice.

Cyclophosphamide treatment

Cyclophosphamide (Cy) was obtained from Koch-Light Laboratories (Colnbrook,

Bucks). The mice received an intraperitoneal (i.p.) injection of Cy (300 mg/kg) in 0·5 ml of saline, 8 hours before an i.c. immunization with antigen.

Cell transfer

Mice were immunized i.c. with 50 μg of hapten–carrier complex in FCA. At day five, 2×10^7 nucleated spleen cells (untreated or treated with antisera) and 25 μg of antigen in Eagle's basal medium (MEM) to a total volume of 0·05 ml were transferred in the left footpad of recipients. Footpad thickness was measured at different hours after the transfer.

Anti-thymocyte sera (ATS) and anti-plasma cell sera (APCS) were prepared as described by Snippe and van Eyk (1974). ATS was absorbed with IgG2B tumour cells and APCS with thymus cells. The C source was guinea-pig serum previously absorbed with thymus or IgG2B tumour cells. Cells (2×10^8/ml) were treated with an equal volume of ATS (diluted 1:3) or APCS (diluted 1:6) for 30 minutes at 37°, followed by a treatment with C (diluted 1:6) for 30 minutes at 37° and washed twice with MEM. For transfer experiments the equivalent of 2×10^7 spleen cells as starting material were used.

Histology

Histology on the footpads and popliteal lymph nodes was performed according to the methods of Keuning, van der Meer, Nieuwenhuis and Oudendijk (1963) by Dr A. van den Broek, Department of Histology, State University of Groningen, The Netherlands.

TABLE 1

THE EFFECT OF INTERVAL BETWEEN SENSITIZATION AND CHALLENGE

Interval (days)	Footpad thickness ($\times 10^{-1}$ mm ± s.e.)	Interval	Footpad thickness ($\times 10^{-1}$ mm ± s.e.)
3	0·8 ± 0·15	7	4·3 ± 0·6
4	2·4 ± 0·5	8	4·6 ± 0·4
5	4·1 ± 0·5	10	5·2 ± 0·6
6	3·9 ± 0·7	14	6·0 ± 0·6

Groups of six mice were immunized i.c. with 50 μg of DNP_{16}–BSA in FCA. At different intervals a challenge was given with 25 μg of DNP_{28}–BSA into the left footpad. The increase in footpad thickness was measured at 24 hours. s.e. = Standard error of the mean.

RESULTS

THE EFFECT OF AN INTERVAL BETWEEN SENSITIZATION AND CHALLENGE ON DH

Mice were immunized with 50 μg of DNP_{16}–BSA. At different intervals after immunization, groups of six mice were tested with 25 μg of DNP_{28}–BSA and the footpad swelling was measured 24 hours later. DH was positive on day 4 and increased steadily (Table 1). Similar results were obtained with DNP–MIg complexes. For practical reasons an interval of 11 days between immunization and challenge was chosen for further experiments.

THE EFFECT ON DH OF THE ELICITING DOSE OF DNP–CARRIER COMPLEX

In preliminary experiments doses of antigen ranging from 10 to 100 μg were used for

sensitization. For the different antigens used 50 μg gave the most consistent results. In further experiments this amount of antigen was chosen for sensitization. The dose of antigen necessary for testing was further investigated.

Mice were immunized with 50 μg of DNP_{16}–BSA and at 11 days tested with graded amounts of DNP_{28}–BSA. After 24 hours the footpad swelling was measured (Fig. 1). Optimal responses were obtained with eliciting doses of 10–40 μg. Based on these and corresponding experiments with DNP–MIg complexes it was decided to use 25 μg of DNP–carrier complex as test dose in the following experiments.

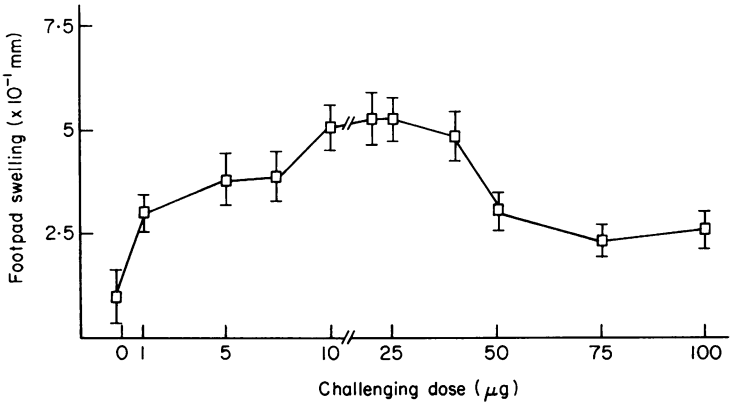

FIG. 1. The challenging dose for DH with DNP–carrier complexes. Groups of six mice were immunized with 50 μg of DNP_{16}–BSA. At day 11 varying amounts of DNP_{28}–BSA were injected in the left footpad. The increase in footpad thickness was measured at 24 hours. Vertical bars indicate standard errors of the mean.

HOMOLOGOUS AND HETEROLOGOUS REACTIONS WITH CARRIER AND HAPTEN–CARRIER COMPLEXES IN DH

To investigate the role of the carrier in DNP–carrier complexes on DH, groups of mice were primed with 50 μg of BSA or DNP–BSA complex, followed 11 days later by a challenge with BSA or DNP–BSA complex.

Strong positive DH was observed when mice primed with BSA were challenged with BSA or DNP_3–BSA (Fig. 2a). The amount of footpad swelling decreased when the hapten: carrier ratio of the complex used for challenge increased.

When DNP–BSA complexes were used for priming, no positive reaction was obtained after challenge with BSA or DNP_3–BSA (Fig. 2b, c and d). The low responses were not due to the dose used for challenge. Either increase or decrease of the dose did not alter the result. Clear-cut positive reactions were obtained when the challenge was given with DNP_{16}–BSA or DNP_{28}–BSA, the reaction in the latter case being higher than with DNP_{16}–BSA.

DH AGAINST HAPTEN (ISOLOGOUS) CARRIER COMPLEXES

In reactions with DNP–MIg complexes the carrier does not play a role and only DNP and NAD groups are reactive. DH was studied with these complexes. Groups of mice were immunized with 50 μg of the following complexes: DNP_{11}–MIg; DNP_{48}–MIg; DNP_{90}–MIg. Each group was challenged with 25 μg of DNP_{11}–MIg, DNP_{48}–MIg and DNP_{90}–MIg and the DH was measured at day 11. It was observed that sensitization of mice with

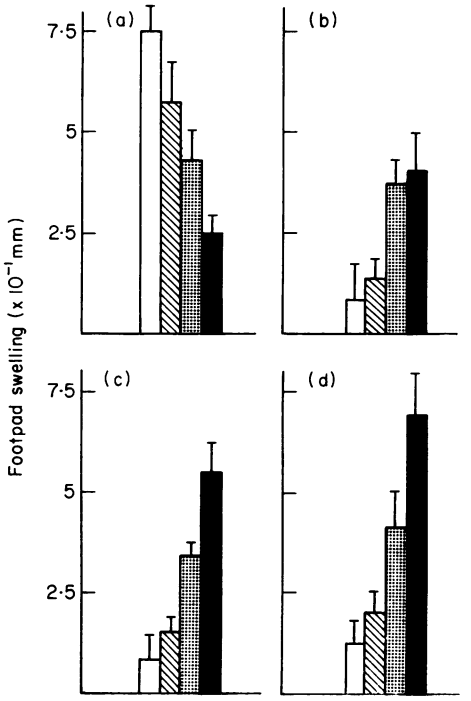

FIG. 2. Homologous and heterologous DH reactions with carrier and hapten–carrier complexes. Groups of twenty mice were immunized i.c. with 50 μg of (a) BSA, (b) DNP_3–BSA, (c) DNP_{16}–BSA or (d) DNP_{28}–BSA. At day 11 each group of five mice were challenged in the left footpad with 25 μg of BSA (blank columns), DNP_3–BSA (hatched columns), DNP_{16}–BSA (stippled columns) or DNP_{28}–BSA (solid columns). The increase in footpad thickness was measured at 24 hours. Vertical bars indicate standard errors of the mean.

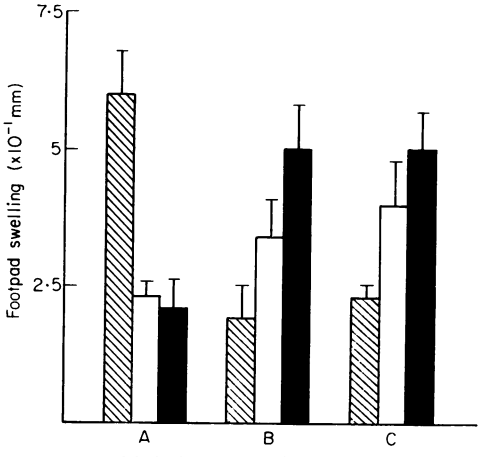

FIG. 3. DH against DNP complexes with isologous carrier. Groups of eighteen mice were immunized i.c. with either 50 μg of (A) DNP_{11}–MIg, (B) DNP_{48}–MIg or (C) DNP_{90}–MIg. At day 11 six mice of each group were challenged with either 25 μg of DNP_{11}–MIg (hatched columns), DNP_{48}–MIg (blank columns) or DNP_{90}–MIg (solid columns) in the left footpad. The increase in footpad thickness was measured at 24 hours. Vertical bars indicate standard errors of the mean.

DNP_{11}–MIg resulted only in a footpad swelling after challenge with the same complex, indicating DH on the NAD or NAD–DNP groups rather than on the DNP groups alone (Fig. 3). Sensitization with DNP_{48}–MIg or DNP_{90}–MIg gives only a DH when challenged with a complex with a high DNP:carrier ratio. The isologous carrier alone never sensitized for DH.

CROSS-REACTIONS IN DH OF COMPLEXES OF DNP WITH DIFFERENT CARRIERS

As the experiments with complexes with high DNP:carrier ratio suggest a DH reaction against the DNP groups, cross-reactions between complexes, differing in the carrier should be possible.

Groups of mice were immunized with 50 μg of DNP_{16}–BSA, DNP_{48}–MIg or DNP_{90}–MIg. At day 11 the challenge was given with DNP_{48}–MIg, DNP_{90}–MIg or DNP_{28}–BSA. If mice were sensitized with DNP_{16}–BSA, DH was only observed after challenge with a complex with a high DNP:carrier ratio (DNP_{90}–MIg) (Table 2). Sensitization with DNP–MIg complexes followed by a challenge with DNP_{28}–BSA only yielded a positive reaction when DNP_{90}–MIg was used for sensitization.

TABLE 2

CROSS-REACTIONS BETWEEN DNP–BSA AND DNP–MIg COMPLEXES IN DH

Sensitization	Challenge	Footpad swelling ($\times 10^{-1}$ mm ± s.e.)
DNP_{16}–BSA	DNP_{48}–MIg	1·6 ± 0·3
DNP_{16}–BSA	DNP_{90}–MIg	3·9 ± 0·2
DNP_{48}–MIg	DNP_{28}–BSA	1·8 ± 0·1
DNP_{90}–MIg	DNP_{28}–BSA	3·8 ± 0·2
DNP_{16}–PVP (10 μg)	DNP_{28}–BSA	3·8 ± 0·6
DNP_{16}–PVP (30 μg)	DNP_{28}–BSA	3·2 ± 0·5

Groups of six mice were immunized i.c. with 50 μg of DNP_{16}–BSA, DNP_{48}–MIg, DNP_{90}–MIg or 10 or 30 μg of DNP_{16}–PVP. At day 11 a challenge with either 25 μg of DNP_{48}–MIg, DNP_{90}–MIg or DNP_{28}–BSA was given into the left footpad. The increase in footpad thickness was measured at 24 hours. s.e. = Standard error of the mean.

In homologous reactions with complexes of DNP–PVP no positive DH could be obtained. As DNP–PVP complexes were able to prime for *in vitro* stimulation with DNP_{28}–BSA (Snippe *et al.*, 1974), graded amounts of DNP–PVP complexes were used for priming. At day 11 a challenge was given with DNP_{28}–BSA. Only priming with 10 or 30 μg of DNP_{16}–PVP yielded a positive reaction when tested with DNP_{28}–BSA (Table 2). Other doses or other DNP–PVP complexes used gave negative results.

THE EFFECT OF Cy TREATMENT ON THE DH WITH HAPTEN–CARRIER COMPLEXES

Kerckhaert *et al.* (1974) described enhanced DH on sheep red blood cells when Cy injection preceded the sensitization, and the eliciting injection was given 10 days later. This experiment was repeated with DNP–BSA complex. Groups of mice received an i.p. injection of Cy (300 mg/kg) and 8 hours later 50 μg of DNP_{16}–BSA. At different times after immunization the mice were tested with 25 μg of DNP_{28}–BSA. Fig. 4 shows that DH is impaired till 5 days after immunization and strongly enhanced between days 8 and 11.

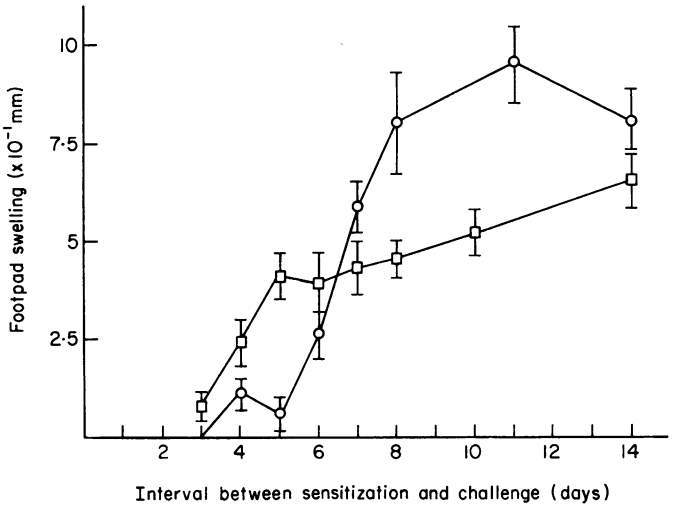

FIG. 4. The effect of Cy treatment before priming on DH. Groups of six mice were immunized i.c. with 50 μg of DNP_{16}–BSA in FCA 8 hours after an i.p. injection of saline (□) or Cy (○). At varying days after immunization 25 μg of DNP_{28}–BSA was injected into the left footpad. The increase in footpad thickness was measured at 24 hours. Vertical bars indicate standard errors of the mean.

To investigate if this response is due to recognition by T cells of the carrier or the DNP group, mice were treated with Cy, immunized with BSA or DNP–BSA complexes and challenged with DNP_3–BSA, DNP_{16}–BSA or DNP_{28}–BSA. Treatment with Cy before sensitization with BSA did not influence DH upon challenge with complexes. After Cy treatment and priming with DNP_3–BSA, DH against DNP_{16}–BSA and DNP_{28}–BSA was enhanced (Fig. 5b, column A), both to an equal level. DNP_3–BSA gave no increased response. Priming with DNP_{16}–BSA resulted also in an enhanced DH against DNP_{16}–BSA and DNP_{28}–BSA (Fig. 5, column B). After priming with DNP_{28}–BSA (Fig. 5, column C) the effect was less pronounced. These results indicate that the T cells educated during Cy treatment recognize DNP or NAD groups but not carrier.

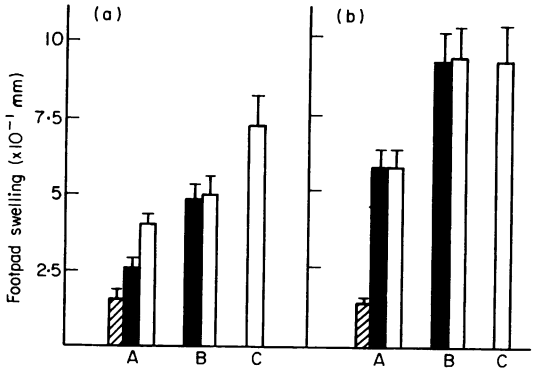

FIG. 5. The effect of Cy treatment on DH against hapten–carrier complexes. Groups of mice were immunized i.c. with 50 μg of (A) DNP_3–BSA, (B) DNP_{16}–BSA or (C) DNP_{28}–BSA 8 hours after treatment with (a) saline or (b) Cy. At 11 days after immunization groups of six mice were challenged with either 25 μg of DNP_3–BSA (hatched columns), DNP_{16}–BSA (solid columns) or DNP_{28}–BSA (blank columns) in the left footpad. The increase in footpad thickness was measured at 24 hours. Vertical bars indicate standard errors of the mean.

ADOPTIVE TRANSFER OF DH

In order to confirm the role of T lymphocytes in the reactions on DNP–carrier complexes, adoptive transfer experiments were performed. No footpad swelling was detected when spleen or lymph node cells of primed animals were injected i.v. into normal or sublethally irradiated recipients followed by a challenge. Blazkovec, Sorkin and Turk (1965) were able to transfer locally passive delayed hypersensitivity to tuberculin in inbred guinea-pigs. This method was applied in our system. Spleen cells of mice primed with 50 μg of DNP_{16}–BSA were injected together with the eliciting antigen (25 μg of DNP_{28}–BSA in 0·05 ml of MEM) into the footpad of normal mice. A footpad swelling could be measured between 2 and 9 hours after the transfer (Fig. 6). Recipients of cells of non-primed animals and antigen were taken as control animals. The peak response was found at 3 hours after the transfer with some reactivity in the control mice. These background reactions were weaker at 6 hours. In further experiments the period of 6 hours between transfer and reading of the reaction was used.

Preliminary histological examination after the local passive transfer gave no indica-

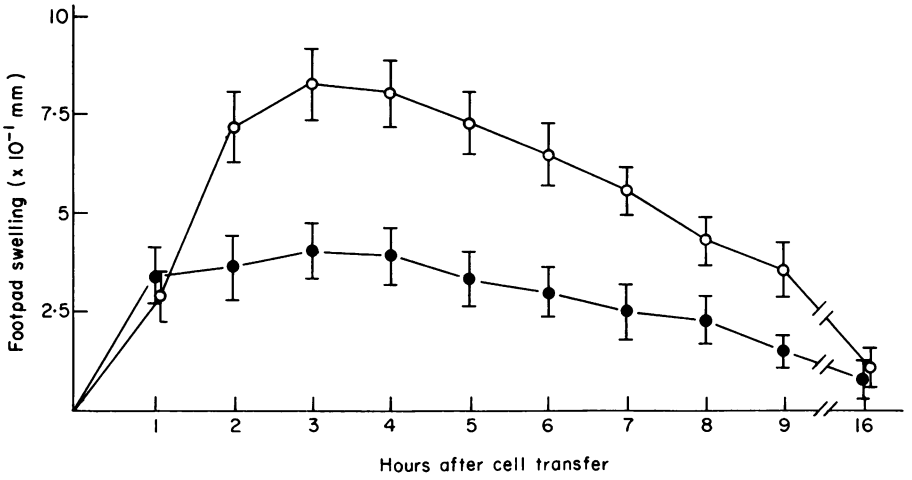

FIG. 6. Time course of the footpad swelling after the adoptive transfer of cells of primed mice. Mice were immunized with 50 μg of DNP_{16}–BSA. At day 5, 2×10^7 nucleated spleen cells of these mice (○) or control mice (●) were mixed with 25 μg of DNP_{28}–BSA in a total volume of 0·05 ml MEM and injected in the left footpads of two groups of fourteen recipients. The footpad swelling was measured at different hours after the transfer. Vertical bars indicate standard errors of the mean.

tions of activity in the draining lymph nodes at the time of the reaction (6 hours). In the footpads of the test and control mice the histological appearance was overall similar. The main difference was the intensity of the reaction observed. The cells injected formed a dense mass in the dermis, consisting of mononuclear and polymorphonuclear cells. The test footpads showed a progressing inflammation recognized by infiltration of mononuclear cells around and in the blood vessels, while in the connective tissue many mononuclear cells were observed. In the control footpads inflammation could hardly be seen. The number of pyroninophilic cells was not significantly increased. Macroscopically oedematous fluid was present mainly in the test footpads.

Treatment of the donor cells with ATS and complement completely abolished the activity of the transferred cells (Table 3). No impairment of activity was found upon

TABLE 3

THE EFFECT OF ANTISERUM AND COMPLEMENT ON THE ADOPTIVE TRANSFER OF DH IN A LOCAL SYSTEM

Treatment of cells	Antigen	Footpad thickness ($\times 10^{-1}$ mm $\pm$ s.e.)
None	No	1·4 ± 0·3
None	Yes	4·9 ± 0·2
ATS + C*	No	0·8 ± 0·2
ATS + C*	Yes	1·2 ± 0·4
C*	Yes	4·5 ± 0·7
APCS + C†	No	1·7 ± 0·2
APCS + C†	Yes	4·5 ± 0·3
C†	Yes	4·4 ± 0·4

Mice were immunized with 50 μg of DNP_{16}–BSA. At day 5, 2×10^7 nucleated spleen cells of these mice were treated with antiserum or MEM and complement. To the cells a solution of 25 μg of DNP_{28}–BSA or MEM was added to a total volume of 0·05 ml and this mixture was injected in the left footpad of the recipients. The footpad swelling was measured at 6 hours after transfer and calculated as the mean value of six to eight mice. s.e. = Standard error of the mean.

* Fresh guinea-pig serum absorbed with plasma-cell tumour IgG2B.
† Fresh guinea-pig serum absorbed with thymus cells.

treatment of the cells with an anti-B-cell serum (APCS) and C. This suggests that mainly T cells are involved in the reactions studied.

DISCUSSION

A hapten–carrier complex represents three kinds of antigenic determinants: (1) hapten; (2) native carrier determinants; (3) new antigenic determinants (NAD) introduced through the hapten–carrier coupling reaction (Landsteiner, 1962; Rubin, 1972). When a hapten is coupled to an isologous protein, the hapten–carrier complex lacks foreign native carrier determinants. In a previous paper (Snippe *et al.*, 1974) it was suggested that for lymphocyte proliferation *in vitro* the stimulating activity is due to DNP groups in complexes with high hapten:carrier ratios (DNP_{28}–BSA). Complexes with low hapten:carrier ratios (DNP_3–BSA) were not active, while the activity of complexes with intermediate hapten:carrier ratios was at least partly due to NAD or DNP–NAD groups. The target cell for these activities was mainly the T cell, with some contribution of the B cell (Snippe and van Eyk, 1974). Both lymphocyte proliferation *in vitro* and *in vivo* are characterized by a high degree of specificity. With hapten–carrier systems DH reactions are elicited most readily by the immunizing conjugate (Dutton and Bullman, 1964).

To investigate the role of the different parts of hapten:carrier complexes in the cellular response *in vivo*, DH reactions with footpad swelling as parameter were performed. Priming with BSA induced a good response against the carrier but decreasing responses for complexes with increasing number of hapten groups (Fig. 2a). This suggests DH against the carrier. Conversely, after priming with complexes, induced DH increased with increasing hapten:carrier ratios. The hapten:carrier ratio used for testing is even more important. The response increased from nil for BSA alone to a high response against DNP_{28}–BSA. In this case DH is obviously mainly directed against the complexes and

not against the carrier. These results are in accordance with those found in the *in vitro* responses, where DNP_{28}–BSA behaved as a molecule containing mainly DNP determinants, while the complexes with lower hapten:carrier ratio reacted also with NAD or DNP–NAD determinants.

To eliminate responses against carrier, DNP–MIg complexes were used (Fig. 3). DNP_{11}—MIg induced an appreciable reaction against the homologous complex (Fig. 3, column A) but weak or no reactions against complexes with a higher number of substituents. In this respect it resembled BSA (Fig. 2a). As the reaction cannot be against the carrier, NAD groups seem to be involved. Priming with DNP_{48}–MIg or DNP_{90}–MIg (Fig. 3, columns B and C) induced DH reactions which increase with the hapten:carrier ratio of the eliciting antigen. These are obviously reactions mainly directed against DNP groups. Cross-reactions against DNP_{28}–BSA are only induced by highly substituted DNP–MIg complexes (Table 2). Conversely only DNP_{90}–MIg elicits a cross-reaction after DNP_{28}–BSA priming. This is a strong indication that DNP groups rather than NAD groups are involved in DH against heavily haptenated complexes. Also DH after priming with DNP_{16}–PVP and challenge with DNP_{28}–BSA can only be due to a reaction against DNP groups. It should be emphasized that the use of other DNP–PVP complexes or challenge with DNP_{16}–PVP never resulted in DH. Snippe *et al.* (1974a) demonstrated that all DNP–PVP complexes were able to prime for *in vitro* response on DNP_{28}–BSA. No *in vitro* response against DNP–PVP complexes were possible. No antibody formation could be induced with DNP–PVP complexes. On the other hand DNP_{16}–PVP, but no other DNP–PVP complex, was able to inhibit the development of anti-DNP plaques when mixed with spleen cells of immunized mice (Snippe *et al.*, 1975). It is not clear why only the complexes with a DNP:PVP ratio of 16 are able to prime for reactions *in vivo* and *in vitro* and why challenge is not possible with this complex.

Further evidence for the involvement of NAD or DNP groups is given by the experiments in which Cy treatment followed by priming was used to obtain enhanced DH. No enhanced responses against complexes were found after priming with BSA, but after Cy treatment all DNP–BSA complexes primed for enhanced responses, when tested with DNP_{16}–BSA or DNP_{28}–BSA. This excludes a role of the carrier in these responses.

The adoptive transfer of a systemic DH was not successful, probably due to the small numbers of cells primed for the antigen. Blazkovec *et al.* (1965) performed the local passive transfer of DH to tuberculin in guinea-pigs by simultaneous intradermal injections of cells and antigen. The reaction could be measured at 4 and 24 hours after the transfer. Segre and Sharp (1965) demonstrated in guinea-pigs local passive transfer of DH to diphtheria toxoid if the antigen was injected i.v. 24 hours after the intradermal injection of cells. These reactions were measured after 8 and 24 hours. In our system the local adoptive transfer reactions were positive between 2 and 8 hours. The differences in the time courses in our experiments and those of Blazkovec *et al.* (1965) and Segre and Sharp (1965) might be due to differences in animals, the site of the reaction (footpad versus skin) and the antigen used. Treatment of transferred cells with ATS and APCS showed that T lymphocytes are responsible for the reactivity in these transfer experiments.

The decreasing activity of BSA in inducing DH against complexes with increasing hapten substitution, the activity of complexes of an isologous carrier in inducing DH against itself and against other highly substituted complexes, enhanced DH after priming under Cy treatment are strong indications that DH in these reactions is not directed against the carrier but rather against DNP and NAD groups. From these and earlier

experiments (Snippe *et al.*, 1974, 1975; Snippe and van Eyk, 1974) the following conclusions about the reactivity of hapten–carrier complexes in immune reactions can be drawn. The hapten:carrier ratio is of utmost importance in all immune reactions. In general the response to the complex increases with the increase of the number of hapten groups, with the exception of antibody formation, where complexes with intermediate hapten: carrier ratio are slightly more active. Complexes with a low hapten:carrier ratio and DNP–PVP complexes are able to prime for a subsequent elicitation *in vivo* or *in vitro* with DNP_{28}–BSA, but are not reactive when used for a challenge or in evoking antibody formation. Although antibody formation and DH against BSA determinants is possible after injection of DNP–BSA complexes, these determinants are probably of no importance in the reactions on the DNP groups, e.g. T cells, including T helper cells are reactive on DNP groups, NAD groups and/or DNP–NAD groups. It is suggested that T cells reactive with different epitopes are involved in the reactions with the carrier and complex.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr J. M. N. Willers for helpful discussions and M. J. de Reuver for technical assistance.

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