Immunology, 1975, **28**, 885.

Antibody Formation in the Mouse Induced by Hapten–Carrier Complexes

H. SNIPPE, W. G. GRAVEN AND P. J. WILLEMS

Department of Immunology, Laboratory of Microbiology, State University Utrecht, The Netherlands

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

Summary. The influence of hapten, carrier and their ratio in a complex on T-cell helper stimulation and antibody formation against the dinitrophenyl (DNP) hapten was studied. Complexes of DNP with bovine serum albumin (BSA), bovine gamma-globulin (BGG), isologous mouse immunoglobulin (MIG) and polyvinylpyrrolidone (PVP) as carriers were used. Optimal antibody formation against DNP was obtained with complexes with an intermediate hapten:carrier ratio (DNP_{16}–BSA, DNP_{43}–BGG and DNP_{48}–MIG). DNP–PVP complexes were not active either in the primary or in the secondary response. The anti-BSA titre was independent of the number of DNP groups on the complex used for immunization. Inhibition of DNP-plaque formation by spleen cells of immunized mice shows an increase of the inhibitory capacity of the complex with the increase of the hapten–carrier ratio. DNP_{16}–PVP was the only PVP complex which was inhibitory. These results suggest that helper cells involved in the antibody formation against BSA and DNP are reactive with different parts of the complex. Priming of mice with carrier or complex after cyclophosphamide (Cy) treatment followed by a secondary injection with complex 10 days later gave strong indications that there is a greater involvement in stimulation of helper T cells by determinants of the complex (new antigenic determinants (NAD) or NAD–DNP groups) or DNP, than by true BSA determinants. This holds for both the IgM and IgG responses.

INTRODUCTION

Humoral antibody response to hapten–carrier conjugates requires co-operation between thymus-dependent lymphocytes (T cells) and bone marrow-derived thymus-independent lymphocytes (B cells) (Mitchison, 1971). Although both cell types are involved in the specificity of the antibody response, the B lymphocytes are precursors of the antibody-forming cells, while the exact role of T lymphocytes in this co-operation remains unclear (Miller and Mitchell, 1968; Munro and Hunter, 1970). It is postulated that for optimal co-operation to take place at least two different antigenic determinants on the same molecule are required: one able to react with and possibly activate T cells, the other recognizing specific receptors on the antibody-producing B cells (Rajewsky, Schirrmacher, Nase and Jerne, 1969).

Correspondence: Dr H. Snippe, Department of Immunology, Laboratory of Microbiology, State University Utrecht, Catharijnesingel 59, Utrecht, The Netherlands.

Using the *in vitro* incorporation of tritiated thymidine into the DNA of dividing lymphoid cells as a measure of activity, Snippe, Nab and van Eyk (1974) showed that carrier alone does not prime for a response against hapten–carrier complex, but that all complexes independent of the hapten:carrier ratio or kind of carrier were able to prime. In contrast the response in the subsequent *in vitro* stimulation depended to a large extent on the hapten:carrier ratio. The target cells of the *in vitro* anti-hapten–carrier response were chiefly T cells, with some contribution to the response by B cells (Snippe and van Eyk, 1974).

As the number of hapten groups for priming was not critical, only for *in vitro* stimulation, it seemed worthwhile to investigate the influence of the number of hapten groups on antibody formation. The minor importance of the carrier in priming for *in vitro* tests prompted us to investigate the role of the hapten:carrier ratio in antibody induction against the carrier.

Kerckhaert, van den Berg and Willers (1974) showed that treatment with cyclophosphamide (Cy) before priming caused enhancement of delayed hypersensitivity (DH), when the interval between priming and elicitation was at least 10 days. They presumed that this enhancement was due to T-cell proliferation in the presence of antigen (education of T cells). Application of this technique to priming with carrier and hapten–carrier complexes would result in educated T cells reacting against hapten (T_h) or carrier. A study of antibody formation against carrier or hapten might give information on the determinants to which the T cells had responded.

MATERIALS AND METHODS

Animals and immunization

Inbred BALB/c mice were bred and maintained in the Laboratory of Microbiology, State University, Utrecht, The Netherlands. Twelve-week-old male mice divided into groups containing four to five mice were injected intraperitoneally (i.p.) with different amounts of DNP–protein dissolved in 0·2 ml of saline, emulsified in 0·2 ml of Freund's complete adjuvant (FCA), which contains killed *Mycobacterium* H37 Ra (Difco Laboratories, Detroit, Michigan). For intracutaneous (i.c.) immunization DNP–protein was dissolved in 0·1 ml of saline, emulsified in 0·1 ml of FCA.

Antigens

The preparation of DNP–BSA (2,4-dinitrophenylated bovine serum albumin), DNP–BGG (bovine gamma-globulin), DNP–MIg (isologous mouse immunoglobulin) and DNP–PVP (polyvinylpyrrolidone, molecular weight 360,000) was described earlier by Snippe, Nab and van Eyk (1974). Complexes with the following composition were prepared: DNP_3–BSA; DNP_{16}–BSA; DNP_{28}–BSA; DNP_3–BGG; DNP_{10}–BGG; DNP_{33}–BGG; DNP_{43}–BGG; DNP_{11}–MIg; DNP_{48}–MIg; DNP_{90}–MIg and analysed according to the method of Eisen, Carsten and Belman (1954). DNP_7–PVP, DNP_{16}–PVP, DNP_{35}–PVP and DNP_{91}–PVP were prepared and analysed according to Snippe, Nab and van Eyk (1974).

Cyclophosphamide treatment

Cyclophosphamide (Cy) was obtained from Koch-Light Laboratories (Colnbrook, Bucks). In one group of experiments the mice received 8 hours before i.c. immunization

an i.p. injection of 300 mg of Cy/kg in 0·5 ml of saline. In another experiment the immunization was given i.p. on different days after Cy treatment.

Determination of the number of plaque-forming cells (PFC)

The number of PFC against DNP was determined according to the technique of Jerne and Nordin (1963) using trinitrophenylated sheep red blood cells (TNP–SRBC) prepared according to Rittenberg and Pratt (1969). The technique of Dresser and Wortis (1965) was used to determine the number of indirect (IgG) PFC. Anti-IgG was prepared according to Zaalberg, van der Meul and van Twisk (1968) and used in a dilution of 1:40.

Passive haemagglutination test (HA)

The antibody titre against BSA was determined with the passive HA test. BSA–SRBC were prepared according to Onkelinx, Meuldermans, Jonian and Lontie (1968) with glutaraldehyde as a coupling reagent. Two-fold dilutions of antiserum in PBS (phosphate-buffered saline with 1 per cent normal rabbit serum) were prepared. 0·03 ml of a suspension of 2 per cent BSA–SRBC was added to 0·1 ml of the diluted antiserum. Haemagglutination was determined after 4 hours. The reciprocal value of the end-point of haemagglutination was taken as the titre.

RESULTS

INFLUENCE OF THE NUMBER OF DNP GROUPS ON HOMOLOGOUS AND HETEROLOGOUS CARRIERS ON ANTIBODY FORMATION *in vivo*

To study the influence on the number of hapten groups on the antibody-inducing capacity of DNP–carrier complexes, groups of five mice were i.p. immunized with 10, 100 or 1000 μg of DNP_3–BSA, DNP_{16}–BSA or DNP_{28}–BSA. Starting on day 4 after immunization, mice were killed daily and the number of PFC in the spleen was determined. When immunized with DNP_3–BSA, only a low response with 100 μg could be obtained (Fig. 1a). Anti-DNP responses were obtained with DNP_{16}–BSA and DNP_{28}–BSA, although the time of optimal response differed with the dose used (Fig. 1b and c). The responses to 10 μg of DNP_{16}–BSA and DNP_{16}–BSA were significantly lower than with higher doses. Corresponding, but lower, responses were obtained with DNP–BGG and DNP–MIg complexes. The response to DNP_3–BGG was negative (Fig. 1d). Positive responses were obtained with higher DNP:BGG ratios (Fig. 1e and f). With the DNP–MIg complex reactions were already positive on day 5 (Fig. 1h and i). Again the response to the complex with a low hapten:carrier ratio (Fig. 1g) was weak.

No antibody formation could be obtained with DNP_7–PVP, DNP_{35}–PVP and DNP_{90}–PVP neither in primary nor in secondary responses. Priming with DNP_{35}–PVP or DNP_{90}–PVP did not result in a secondary response when boosted with DNP_{28}–BSA.

INHIBITION OF ANTI-DNP–PFC BY DNP–CARRIER COMPLEXES

The above results show that complexes with BSA are superior for antibody induction to complexes with BGG and MIg. It is also clear that DNP_{16}–BSA and DNP_{48}–MIg are superior to complexes of the same carrier with higher hapten:carrier ratios. On the other hand, DNP–PVP complexes did not induce antibody formation, although these

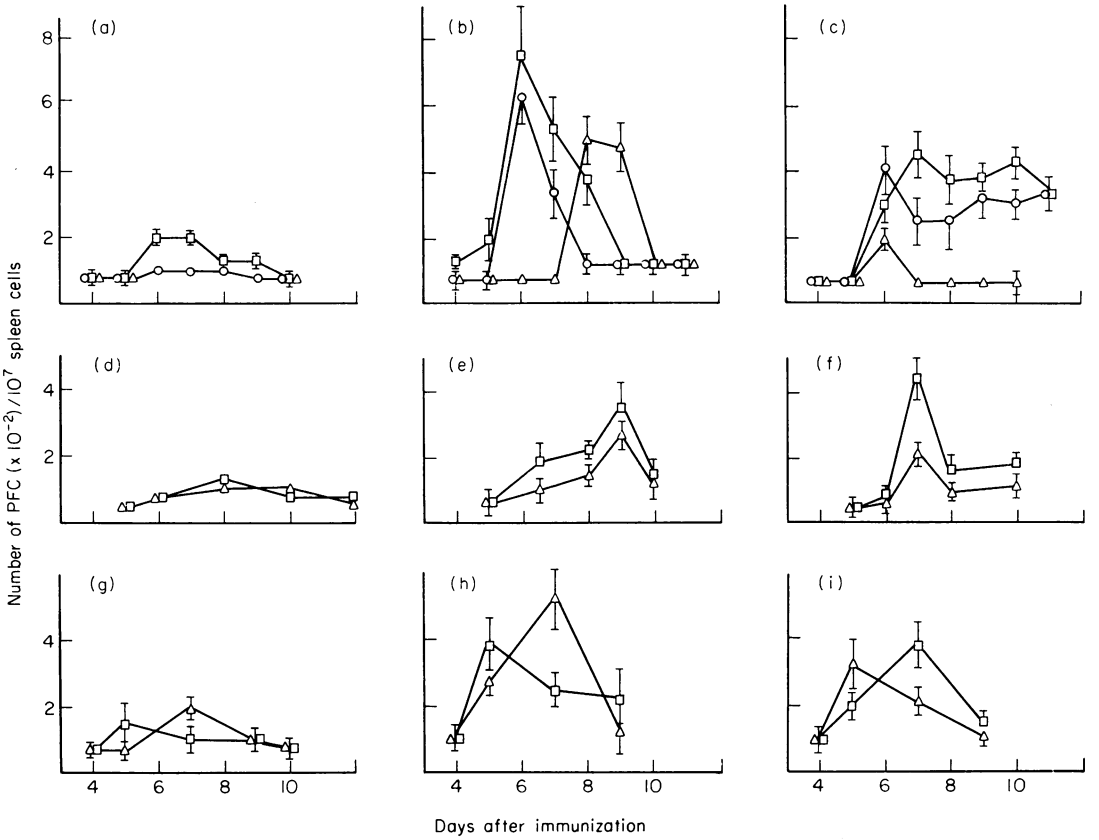

FIG. 1. Influence of the DNP: carrier ratio and amount of complex injected on the number of direct PFC in the mouse spleen. Groups of five mice were immunized with 10 (△), 100 (□) or 1000 (○) μg of DNP–carrier complex in FCA. The number of PFC in 10^7 spleen cells were measured. Vertical bars indicate standard errors of the mean. The results are given for (a) DNP_3–BSA, (b) DNP_{16}–BSA, (c) DNP_{28}–BSA, (d) DNP_3–BGG, (e) DNP_{33}–BGG, (f) DNP_{43}–BGG, (g) DNP_{11}–MIg, (h) DNP_{48}–MIg and (i) DNP_{90}–MIg.

TABLE 1

INHIBITION OF DIRECT PFC AGAINST TNP–SRBC BY DNP–CARRIER COMPLEXES

Complex	Amount giving 50 per cent inhibition (nM)	Complex	Amount giving 50 per cent inhibition (nM)
DNP_3–BSA*	1685	DNP_3–BGG	623
DNP_{16}–BSA*	16	DNP_{10}–BGG	18
DNP_{28}–BSA	5	DNP_{33}–BGG	2
DNP_{11}–MIg	19	DNP_{48}–BGG	2
DNP_{27}–MIg	6	DNP_{16}–PVP	82
DNP_{90}–MIg	4	ε-DNP–L-lysine	90,000

Groups of three mice were immunized with 100 μg of DNP_{16}–BSA or * 100 μg of DNP_{48}–MIg in FCA. At day 6 the number of direct PFC against TNP–SRBC in spleen cells was measured in the presence or absence of different amounts of DNP–carrier complex. The reduction in the numbers of PFC was determined. From these figures the numbers of nM (nanomoles) of complex giving 50 per cent inhibition were calculated by interpolation.

complexes were able to sensitize cells for *in vitro* and *in vivo* stimulation with DNP_{28}–BSA (Snippe *et al.*, 1974; Snippe, Willems, Graven and Kamp, 1975). To investigate whether these differences could be explained by a different behaviour of DNP groups on various carriers in their reaction with cell receptors or antibody molecules, inhibition experiments were performed.

For inhibition with DNP–BSA complexes mice were immunized with DNP_{48}–MIg. For the other inhibition experiments DNP_{16}–BSA was used for immunization. Groups of three mice were immunized i.p. with 100 μg of the antigen in FCA. After 6–7 days the mice were killed and their spleens removed. Graded amounts of the DNP–carrier complexes or ε-DNP–L-lysine were added to the spleen cells and the number of PFC was determined. The results were expressed as percentage inhibition, compared to control systems without inhibitor. Table 1 shows that the inhibitory capacity of the DNP–BSA complexes increased with the number of hapten groups of the complex. The same was true for DNP–MIg and DNP–BGG complexes (Table 1). DNP_3–BSA and DNP_3–BGG were the weakest inhibitors, but were still 50 and 150 times, respectively, as active as ε-DNP–L-lysine. With increasing hapten:carrier ratios the concentration causing 50 per cent inhibition decreased to a level of 2–5 nanomol (nM). The results with DNP_{33}–BGG and DNP_{48}–BGG show that a further increase of hapten groups per carrier molecule had no further effect on inhibition. DNP–PVP complexes were only weakly inhibitory, except for the DNP_{16}–PVP complex. The carriers BSA, MIg, BGG or PVP did not give any inhibition.

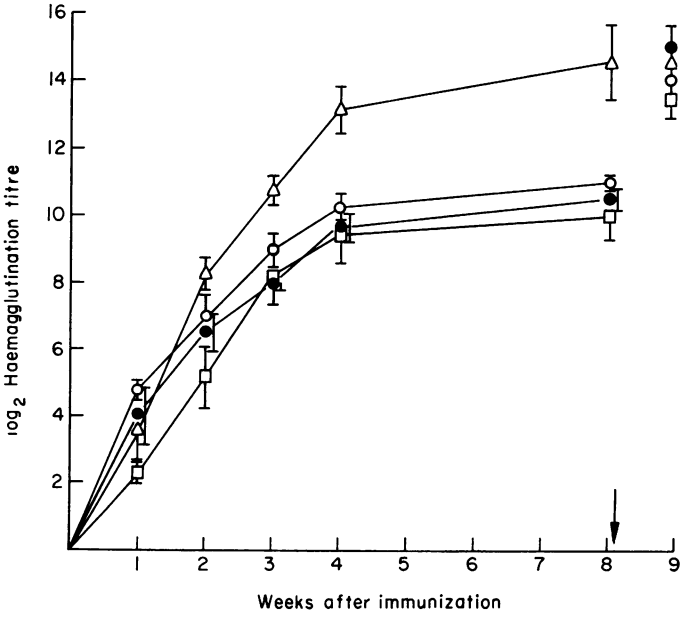

FIG. 2. The effect of the DNP/BSA ratio on the antibody response against the carrier. Groups of six mice were i.p. immunized with 100 μg of BSA (△), 100 μg of DNP_3–BSA (○), 100 μg of DNP_{16}–BSA (□), 100 μg of DNP_{28}–BSA (●) or PBS (■) in FCA. At 8 weeks after the primary injection all animals received a booster injection of 100 μg of BSA (↓). The haemagglutination titre of individual mice against BSA–SRBC was determined at varying periods after primary immunization and 8 days after the secondary injection. Vertical bars indicate standard errors of the mean.

EFFECT OF THE DNP:BSA RATIO ON THE ANTIBODY RESPONSE AGAINST THE CARRIER

The former experiments suggest that the DNP groups play a major role in antibody induction against hapten. Earlier work (Snippe *et al.*, 1974) suggested that complexes with high DNP:carrier ratios behave as if they consist only of DNP groups. In order to test the ability of the DNP–BSA complexes to induce antibody formation against the carrier, groups of six mice were i.p. immunized with 100 μg of BSA or DNP–BSA complexes. Blood was collected weekly up to 8 weeks after the immunization and the haemagglutination titre against BSA-coated SRBC was determined for individual mice. From Fig. 2 it is clear that the response induced with BSA alone was higher than that induced with the DNP–BSA complexes. The different complexes induced antibody formation to a same level. Moreover, boosting with BSA after 8 weeks resulted in responses which were identical, irrespective of the antigen (BSA or complexes) used for priming.

THE SECONDARY IMMUNE RESPONSE AGAINST DNP–BSA AFTER Cy TREATMENT OF MICE

Kerckhaert *et al.* (1974) used Cy treatment followed by an antigen injection as a means of suppressing antibody formation and obtaining educated T cells. Maximal T-cell stimulation was obtained at 10 days after Cy treatment and antigenic stimulation. This method could be useful in investigating the role of the carrier in the stimulation of T_h cells if antibody formation against DNP after a booster was taken as a parameter. The duration of the suppression of antibody formation by Cy treatment was determined by the following experiment. Mice were injected with Cy (300 mg/kg). Starting on day 5 till day 25 after

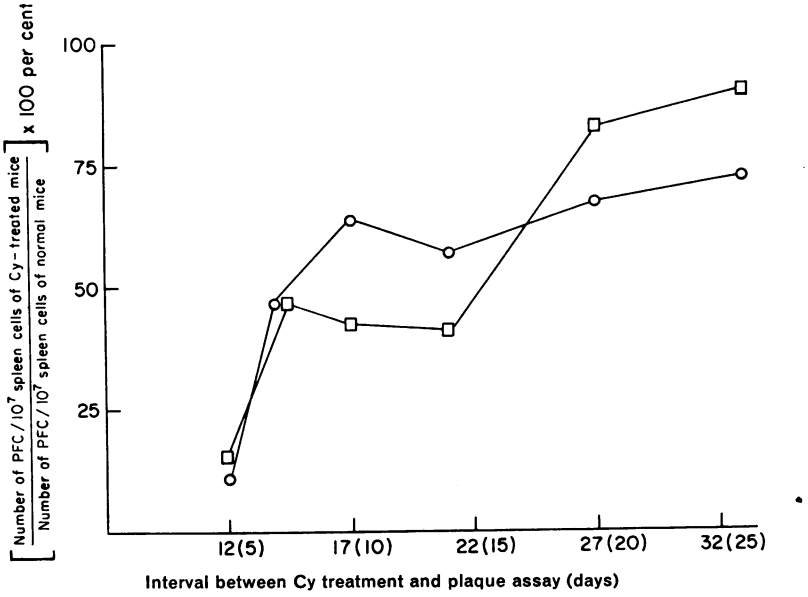

FIG. 3. The effect of variation in the interval between Cy treatment and immunization on the antibody response against DNP–BSA complex. Mice were i.p. injected with Cy (300 mg/kg). After 5, 7, 10, 15, 20 and 25 days, shown in parentheses, groups of five Cy-treated and five non-treated controls were injected with 100 μg of DNP_{16}–BSA (○) or DNP_{28}–BSA (□) in FCA. The numbers of anti-DNP–PFC were determined 7 days after immunization. The response of the Cy-treated animals is expressed as percentage of the response of the non-Cy-treated controls.

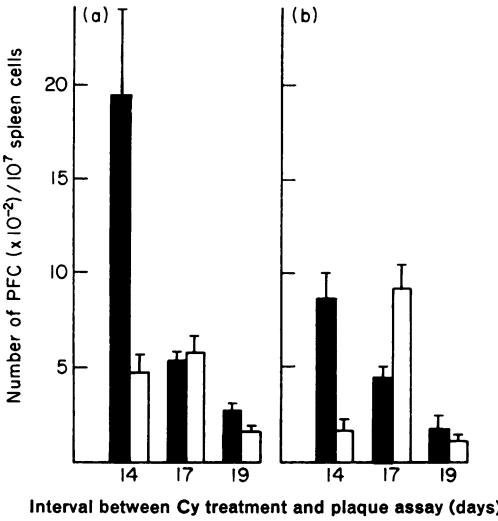

FIG. 4. The immune response against DNP–BSA in mice primed with BSA after Cy treatment. Groups of five mice were injected with Cy (□) or saline (■) and after 8 hours they were i.c. primed with (a) 25 μg or (b) 100 μg of BSA. On day 10 an injection with 100 μg of DNP_{16}–BSA was given i.p. The number of direct PFC in 10^7 spleen cells was determined on days 14, 17 and 19 after the first immunization. Vertical bars indicate standard errors of the mean.

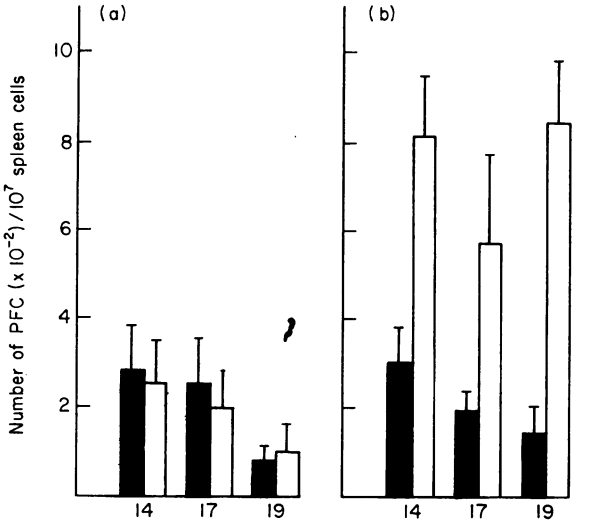

FIG. 5. The secondary immune response against DNP_{28}–BSA in mice primed with this complex after Cy treatment. Groups of five mice were injected with Cy (□) or saline (■) and after 8 hours they were i.c. primed with 100 μg of DNP_{28}–BSA. On day 10 a secondary injection with (a) 25 μg or (b) 100 μg DNP_{28}–BSA was given i.p. The number of direct PFC in 10^7 spleen cells was determined on days 14, 17 and 19 after the first immunization. Vertical bars indicate standard errors of the mean.

Cy treatment, groups of five Cy-treated and five non-treated mice were injected i.p. with 100 μg of DNP_{16}–BSA or DNP_{28}–BSA in FCA. The numbers of anti-DNP–PFC were determined 7 days after immunization. The response of Cy-treated animals was

calculated as percentage of the response of the non-treated controls (Fig. 3). At 7 days after Cy treatment values reached were about 50 per cent of the normal responses and remained at this level for at least 7 more days. Even at 25 days after Cy treatment the response was below normal. This period of impaired responsiveness permits a study of the effect on the anti-DNP response of education of T_h on either the carrier alone or on one of the complexes. Groups of mice were treated with Cy or saline, followed 8 hours later by an i.c. injection of 25 or 100 μg of BSA in FCA. After 10 days all mice received an i.p. injection of 100 μg of DNP_{16}–BSA in saline. Four, 7 or 9 days later mice of the Cy and saline groups were killed and the numbers of PFC against DNP were determined in the spleens. Fig. 4 shows that after Cy treatment and BSA injection antibody formation against DNP is impaired on day 14. The suppressive effect of Cy was eliminated by day 17 (in contrast to Fig. 3), while education with 100 μg of BSA resulted in an enhanced

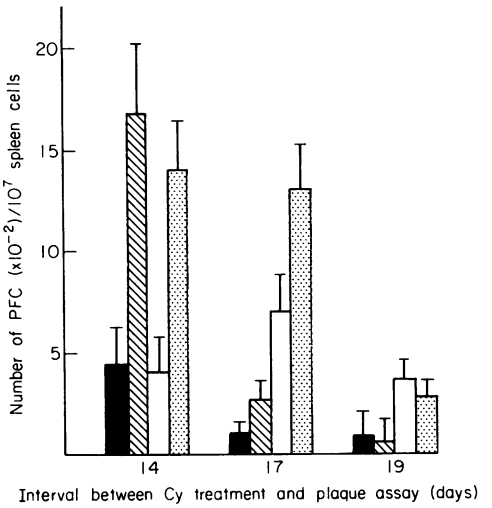

FIG. 6. The secondary immune response against DNP_{16}–BSA in mice primed with this complex after Cy treatment. Groups of five mice were injected with Cy or saline and after 8 hours they were i.c. primed with 100 μg of DNP_{16}–BSA. On day 10 a secondary injection with 25 μg of DNP_{16}–BSA was given i.p. The number of direct (blank columns) and indirect (stippled columns) PFC in the Cy group and the direct (solid columns) and indirect (hatched columns) PFC in the control group were determined on days 14, 17 and 19 after the first immunization. Vertical bars indicate standard errors of the mean.

response on day 17. The response was almost entirely an IgM response. The IgG response was very weak and is not given in the figures. When mice were immunized with 100 μg of DNP_{28}–BSA and boosted with 25 or 100 μg of DNP_{28}–BSA, almost identical responses were obtained for both doses (Fig. 5). When, however, the mice were pretreated with Cy followed by a primary injection with 100 μg of DNP_{28}–BSA, the booster with 100 μg of DNP_{28}–BSA resulted in a considerably enhanced IgM response on all 3 days tested. No such enhancement was found after boosting with 25 μg of DNP_{28}–BSA (Fig. 5). The difference in numbers of direct (IgM) PFC which depended on the challenge suggested an investigation of the IgG responses, especially after challenge with 25 μg of antigen. As Mäkelä (1970) has indicated, with a decrease in the hapten : carrier ratio, there is a relative increase in the amount of IgG produced, so the IgG and IgM responses were studied with DNP_{16}–BSA (Fig. 6). Mice were primed with 100 μg of DNP_{16}–BSA with or without preceding Cy treatment. At day 10 all mice were challenged with 25 μg of DNP_{16}–BSA.

Again 4, 7 and 9 days later the number of direct (IgM) and indirect (IgG) PFC were determined. Without Cy treatment a high IgG response was found on day 14, while both the IgM and IgG responses faded on days 17 and 19. At day 14 the responses of the Cy-treated animals were almost identical to those of the non-Cy-treated. However, at days 17 and 19 there were strongly enhanced IgM and even more enhanced IgG responses.

DISCUSSION

It is postulated that in order to obtain optimal co-operation between T and B cells at least two different antigenic determinants on the same molecule are required (Rajewsky *et al.*, 1969). According to this postulate, it seems fundamental to assume that any antigenic group, irrespective of molecular size and complexity, which is able to react with and activate T cells, should be able to function as a carrier for other antigenic determinants linked to it. Indeed Hanna and Leskowitz (1973) demonstrated that azobenzenearsonate (ABA) groups function as a carrier co-operating in the production of antibody to bovine serum albumin (BSA) as determinant. Taylor and Iverson (1971) have shown that dinitrophenyl (DNP) and 4-hydroxy-3-iodo-5-nitrophenyl acetic acid haptens can function as carriers in stimulating antibody production for other haptenic groups or protein antigens. Janeway and Paul (1972), however, claimed that the DNP helper function detected by Taylor and Iverson (1971) is mediated by humoral antibodies, since it could be transferred passively by serum. Hanna and Leskowitz (1973) were able to exclude a helper function of antibodies against ABA in their experiments.

Recognition by T cells of DNP groups was demonstrated by Moorhead, Walters and Claman (1973) for hapten groups on an isologous carrier and by Snippe and van Eyk (1974) for haptens on isologous and heterologous carriers.

To investigate the role of the hapten–DNP in antibody formation more extensively, the influence of the number of hapten groups on antibody formation was studied. As also found for the *in vitro* stimulation (Snippe *et al.*, 1974) DNP–BSA complexes were superior in the induction of antibody formation against DNP as compared with DNP–MIg and DNP–BGG complexes. The comparison with the thymidine incorporation assay cannot be drawn further, as the complexes with the highest hapten:carrier ratios (DNP_{28}–BSA and DNP_{90}–MIg) were less active than those with a lower ratio (DNP_{16}–BSA and DNP_{48}–MIg). Complexes with a low ratio (DNP_3–BGG) did not induce antibodies or gave a weak response (DNP_3–BSA and DNP_{11}–MIg). These differences in antibody-inducing capacity could be due to better availability of the DNP groups of the intermediate substituted complexes to the receptors on B cells. If steric hindrance with the individual combining sites of the receptors plays a role in the lower antibody inducing activity of complexes with higher DNP:carrier ratios, this should be reflected in the inhibition of the PFC response by these complexes. With increasing hapten:carrier ratios the concentration of complexes causing 50 per cent inhibition decreased to a level of a few nanomoles. Further increase in substitution ratio (DNP_{33}–BGG:DNP_{48}–BGG or DNP_{27}–MIg:DNP_{90}–MIg) had no further effect on inhibition, i.e. on the reaction with antibodies. This seems to exclude steric hindrance on the individual receptor as a cause of the lower antibody induction by complexes with a high ratio. However, a better fit of the intermediate haptenated complexes on more receptors at a time cannot be excluded.

A second explanation for the difference in the higher antibody-inducing capacities of

DNP_{16}–BSA over DNP_{28}–BSA could be a decrease in thymus dependence with increase in the hapten:carrier ratio (Aird, 1971; Sarvas and Mäkelä, 1974). Responses against a given hapten could be higher when involvement of T helper cells is greater. Snippe and van Eyk (1974) found indications that DNP_{28}–BSA in *in vitro* stimulation of T cells behaved like a molecule exposing only DNP groups, while DNP_{16}–BSA contained, besides DNP hapten, new antigenic determinant (NAD) groups. When, on the other hand, antibody formation against BSA was used as a parameter, all DNP–BSA complexes gave the same response, indicating that T cell stimulation by DNP groups is probably of minor importance in this instance (Fig. 2). After boosting with BSA all responses reached the level obtained with BSA alone, suggesting that the DNP–BSA complexes are able to stimulate T helper cells reactive with unmodified BSA determinants. This does not mean that a reaction between DNP groups and T cells is superfluous in antibody formation against DNP groups. To obtain further information about the determinants on the complex reactive with T cells, education of T cells by carrier or complex in Cy-treated animals was performed. Education of T cells with BSA resulted in an enhanced response against DNP_{16}–BSA on day 17. With DNP_{28}–BSA strongly enhanced antibody formation on this complex was found on all days tested. These results suggest either that education of T cells against determinants other than BSA determinants is more effective or that these T cells are better helper cells in the anti-DNP response. The response after education depended greatly on the dose used for boosting. Reduction of this dose to 25 μg of DNP_{28}–BSA showed elimination of suppression due to Cy treatment (compare Figs 3 and 5a), again on the 3 days tested, but no enhanced response. Extension of this study to IgG responses revealed that when BSA was used for education of T cells almost no indirect anti-DNP–PFC could be demonstrated. Education and boosting with DNP_{16}–BSA resulted for both IgG and IgM responses in elimination of Cy suppression on day 14 and enhanced responses on day 19, indicating identical behaviour of both responses in this instance.

Although *in vitro* stimulation experiments (Snippe and van Eyk, 1974) and delayed hypersensitivity experiments *in vivo* suggest reactions between T cells and DNP groups, no evidence for need of stimulation of T helper cells by DNP groups could be obtained. In conclusion, the experiments presented suggest that: (1) several determinants (or haptens) can stimulate T (helper) cells; (2) in antibody formation against haptens in a complex (DNP groups or BSA haptens), different T helper cells can be involved; (3) in antibody formation against one hapten (DNP) T helper cells reactive with different determinants can be involved.

ACKNOWLEDGMENT

The authors gratefully acknowledge Dr J. M. N. Willers for helpful discussions and preparation of this manuscript.

REFERENCES

AIRD, J. (1971). 'Thymus dependence of the immune response: Response of the haptenic determinant NIP in mice.' *Immunology*, **20**, 617.

DRESSER, D. W. and WORTIS, H. H. (1965). 'The use of an antiglobulin serum to detect cells producing antibody with low haemolytic efficiency.' *Nature (Lond.)*, **208**, 859.

EISEN, H. N., CARSTEN, S. and BELMAN, S. (1954). 'Studies of hypersensitivity to low molecular weight substances. III. The 2,4 dinitrophenyl groups as a determinant in the precipitin reaction.' *J. Immunol.*, **73**, 296.

HANNA, N. and LESKOWITZ, S. (1973). 'Cooperative effects in antibody formation produced by hapten-specific delayed sensitivity.' *J. Immunol.*, **111,** 410.

JANEWAY, C. A. and PAUL, W. E. (1973). 'Hapten-specific augmentation of the anti-idiotype antibody response to hapten–myeloma protein conjugates in mice.' *Europ. J. Immunol.*, **3,** 340.

JERNE, N. K. and NORDIN, A. A. (1963). 'Plaque formation in agar by single antibody-producing cells.' *Science*, **140,** 450.

KERCKHAERT, J. A. M., BERG, G. J. VAN DEN and WILLERS, J. M. N. (1974). 'Influence of cyclophosphamide on the delayed hypersensitivity of the mouse.' *Ann. Immunol.*, **125C,** 415.

KONTIAINEN, S. (1971). 'Absence of carrier specificity in an adoptive anti-NIP response in the rat.' *Cell Interactions and Receptor Antibodies in Immune Responses* (ed. by O. Mäkelä, A. Cross and T. U. Kosunen), p. 367. Academic Press, London.

MÄKELÄ, O. (1970). 'Analogies between lymphocyte receptors and the resulting humoral antibodies.' *Transplant. Rev.*, **5,** 3.

MILLER, J. F. A. P. and MITCHELL, G. F. (1968). 'Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes.' *J. exp. Med.*, **128,** 801.

MITCHISON, N. A. (1971). 'The carrier effect in the secondary response to hapten–protein conjugates. I. Measurement of the effect with transferred cells and objections to the local environment hypothesis.' *Europ. J. Immunol.*, **1,** 10.

MOORHEAD, J. W., WALTERS, C. S. and CLAMAN, H. N. (1973). 'Immunologic reactions to haptens on autologous carriers. I. Participation of the thymus-derived and bone marrow derived cells in the secondary *in vitro* response.' *J. exp. Med.*, **137,** 411.

MUNRO, A. and HUNTER, P. (1970). '*In vitro* reconstitution of the immune response of thymus-deprived mice to sheep red blood cells.' *Nature* (*Lond.*), **225,** 277.

ONKELINX, E., MEULDERMANS, W., JONIAN, M. and LONTIE, R. (1969). 'Glutaraldehyde as a coupling reagent in passive haemagglutination.' *Immunology*, **16,** 35.

RAJEWSKY, K., SCHIRRMACHER, V., NASE, S. and JERNE, N. K. (1969). 'The requirement of more than one antigenic determinant for immunogenicity.' *J. exp. Med.*, **129,** 1131.

RITTENBERG, M. B. and PRATT, K. L. (1969). 'Anti-trinitrophenyl (TNP) plaque assay. Primary response of Balb/c mice to soluble and particulate immunogen.' *Proc. Soc. exp. Biol.* (*N.Y.*), **132,** 575.

SARVAS, H. and MÄKELÄ, O. (1974). 'Lightly haptenated proteins induce IgM-deficient antibody responses.' *Europ. J. Immunol.*, **4,** 255.

SNIPPE, H., NAB, J. and EYK, R. V. W. VAN (1974). '*In vitro* stimulation of spleen cells of the mouse by DNP–carrier complexes.' *Immunology*, **27,** 761.

SNIPPE, H. and EYK, R. V. W. VAN (1974). 'Cells involved in the *in vitro* stimulation by DNP–carrier complexes of *in vivo* primed mouse spleen cell.' *Immunology*, **27,** 771.

SNIPPE, H., WILLEMS, P. J., GRAVEN, W. G. and KAMP, E. (1975). 'Delayed hypersensitivity in the mouse induced by hapten–carrier complexes.' *Immunology*, **28,** 897.

TAYLOR, R. B. and IVERSON, G. M. (1971). 'Hapten competition and the nature of cell-cooperation in the antibody response.' *Proc. roy. Soc. B*, **176,** 393.

ZAALBERG, O. B., MEUL, V. A. VAN DER and TWISK, M. J. VAN (1968). 'Antibody production by isolated spleen cells. A study of the cluster and the plaque techniques.' *J. Immunol.*, **100,** 451.