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In vitro Stimulation of Spleen Cells of the Mouse by DNP–Carrier Complexes

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Summary. Hapten–carrier complexes were prepared with the 2,4-dinitrophenyl group (DNP) as a hapten and bovine serum albumin (BSA), bovine gamma-globulin (BGG) (heterologous), mouse immunoglobulin (MIg) (isologous) and polyvinyl pyrrolidone (PVP) (thymus-independent) as carriers. All complexes could be used for priming, independent of the type of carrier or the number of DNP groups. No *in vitro* response, however, could be obtained with any of the DNP–PVP complexes or with the complexes with a low hapten/carrier ratio (about 3). Priming with carriers alone resulted in some *in vitro* activity on challenge with the homologous DNP–carrier complexes, but this response was less than after priming with one of the homologous DNP–carrier complexes. Cross-reactions between DNP–MIg, DNP–BGG and DNP–BSA complexes could be obtained. In almost all instances *in vitro* challenge with BSA with the highest number of DNP groups (DNP_{28}–BSA) resulted in the highest activity. Inhibition of the *in vitro* reaction could to a certain extent be obtained with ε-DNP–L-lysine. These experiments suggest that: (1) for priming, the hapten/carrier ratio is of no importance and the influence of the type of carrier is low; (2) *in vitro* stimulation can only be obtained with complexes with a high hapten–carrier ratio; when this ratio approaches a maximum (DNP_{28}–BSA) the type of the carrier used by priming seems to be irrelevant; (3) the data from these experiments, from cross-reactions and from inhibition reactions suggest that the stimulating activity of DNP_{28}–BSA is due to the DNP groups, but the activity of complexes such as DNP_{16}–BSA, DNP–BGG and DNP–MIg is at least partly due to the 'new antigenic determinant' (NAD) or DNP–NAD groups.

INTRODUCTION

The immune response requires the participation of thymus-derived (T) and bone marrow-derived (B) cells. Both cell types recognize antigens; T cells appear to be involved in cell-mediated immune reactions while the antibody response to many antigens involves the co-operation of both B and T cells (Claman and Chaperon, 1969). Although the mechanism underlying the dependence on T cells of activation of B cells in the im

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mune response is not fully understood (Dukor and Hartmann, 1973), the suggestion of Mitchison, Rajewsky and Taylor (1970) that determinants on the carrier react with T cells and haptenic determinants with B cells is widely accepted.

Mitogens are able to induce non-specific DNA synthesis, PHA, Con A and allogeneic cells activating exclusively T cells (Doenhoff, Davis, Leuchars and Wallis, 1970), endotoxin and dextran sulphate activating B cells (Greaves and Roitt, 1968; Diamantstein, Rühl, Vogt and Bochert, 1973), while pokeweed mitogen is able to activate both cell types (Stockman, Gallagher, Heim, South and Trentin, 1971). Specifically induced DNA synthesis is considered a valid correlate of cell-mediated immunity *in vitro* (Bloom, 1971). The contribution of the carrier and haptenic determinants in this process is under discussion (Schlossman, Herman and Yaron, 1969; Osborne and Katz, 1973). We have performed a series of experiments utilizing 2,4-dinitrophenyl hapten–carrier conjugates as stimulating antigens of *in vitro* cultures of normal and primed mouse lymphoid cells. The use of such conjugates permits observation of DNA synthesis, reflecting stimulation by either the hapten or the carrier component of the molecule, and thus allows further definition of the specificity of antigen-induced DNA synthesis. In addition, the influence of the type of carrier and of the number of haptenic groups per carrier molecule on the stimulation of DNA synthesis was investigated.

MATERIALS AND METHODS

Mice

Male BALB/c mice were raised in the Laboratory of Microbiology, State University, Utrecht, the Netherlands. The animals were used at an age of about 10 weeks.

Preparation of cell suspensions

Cell suspensions from the lymphoid organs of the mice were prepared as described by Kerckhaert, Benner and Willers (1973).

Culture medium

Eagle's basal medium (EBM) was used as previously described by Snippe, Bruynzeel and Willers (1973). 2-Mercaptoethanol (3×10^{-5} M) was added before use (Heber-Katz and Glick, 1972; Broome and Jeng, 1973).

Antigen

Mouse immunoglobulin (MIg) was prepared according to Walters, Moorhead and Claman (1972) using BALB/c mice. Bovine serum albumin (BSA) was purchased from Poviet, Amsterdam, Netherlands. Bovine gamma-globulin (BGG) was purchased from Sigma Chemical Company, Missouri.

The 2,4-dinitrophenyl group (DNP) was used as hapten. DNP–protein coupling was carried out and analysed according to the method of Eisen, Carsten and Belman (1954) with 2,4-dinitrobenzene sulphonic acid sodium salt (Eastman Kodak, New York) and yielded products with the following ratios: DNP_7–MIg, DNP_{32}–MIg, DNP_3–BSA, DNP_{16}–BSA, DNP_{28}–BSA, DNP_3–BGG and DNP_{14}–BGG.

Preparation of DNP–PVP complexes

PVP (polyvinyl pyrrolidone, molecular weight 360,000, K 90, Fluka, Switzerland)

was dissolved in 0·01 N NaOH. Varying amounts of 10^{-5} M PVP in 0·01 MNaOH were added to a solution of 10^{-3} M dinitrophenyl hydrazine in the same solvent. The mixture was incubated at 37° for 72 hours in the dark, dialysed against distilled water and lyophilized. For the determination of the number of DNP groups on a PVP molecule it was necessary to prepare a standard. For this purpose acetone–DNP was prepared in the same manner. At 430 nm this compound has a molecular extinction coefficient of 1250 specific for the (C=N–NH–DNP) group(s). As PVP itself has no extinction at 430 nm only the C=N–NH–DNP groups in the PVP molecule are measured. On a basis of weight of starting material the DNP/PVP ratio was calculated. The following conjugates were obtained: DNP_8–PVP, DNP_{35}–PVP and DNP_{92}–PVP.

In vivo *priming of mice*

Mice were immunized with 0, 10 or 100 μg of DNP–protein dissolved in 0·1 ml of saline, emulsified in 0·1 ml of Freund's complete adjuvant (FCA) containing killed *Mycobacterium* $H_{37}R_a$ (Difco laboratories, Detroit, Michigan). The antigen was injected intracutaneously in the abdomen, divided over four sites.

In vitro *stimulation and* [^3H]*thymidine incorporation assay*

The stimulation of DNA synthesis by antigen was studied in a manner similar to that of Snippe *et al.* (1973). Eleven days after priming 15×10^6 spleen cells from immunized or control mice (which had received only FCA) were cultured in 3 ml of EBM with different concentrations of antigen (10, 30, 100 or 500 μg per tube) for 64 hours. One microcurie of [^{3}H]thymidine (Radiochemical Centre, Amersham) (1·0 Ci/ml, specific activity 23 Ci/mmol) was added to each tube for the final 24 hours of culture, after which the cultures were terminated. The ratio of incorporation (R value) was calculated by dividing the number of disintegrations per minute (d.p.m.) of the stimulated culture by the number of d.p.m. of the unstimulated culture.

RESULTS

INFLUENCE OF THE NUMBER OF DNP GROUPS ON THE BSA MOLECULE ON THE *in vitro* STIMULATION OF MOUSE SPLEEN CELLS

A number of BALB/c mice were immunized with 10, 100 or 500 μg of DNP–BSA. The spleen cells of these animals and unimmunized controls were challenged *in vitro* with 10, 30 or 100 μg of the same DNP–BSA complex. The amount of [^{3}H]thymidine incorporated was measured. Fig. 1 shows that there was no difference in activity between spleen cells from unsensitized animals and animals sensitized with DNP_3–BSA. However, when DNP_{16}–BSA was used (Fig. 1b) a challenge with DNP_{16}–BSA produced an amount of [^{3}H]thymidine incorporation which increased with the challenging dose. With DNP_{28}–BSA an even more pronounced effect could be observed, especially for the higher challenging doses (Fig. 1c). The influence of the immunizing dose was less apparent.

THE ROLE OF THE CARRIER ON THE *in vitro* STIMULATION BY CARRIER–DNP COMPLEXES

In order to investigate the role of the carrier, hapten–carrier complexes containing the heterologous proteins BSA and BGG, the isologous protein MIg and the thymus–independent antigen PVP were used. Mice were primed with 10 and 100 μg of hapten–carrier combinations. The spleen cells of these mice and of unstimulated animals were

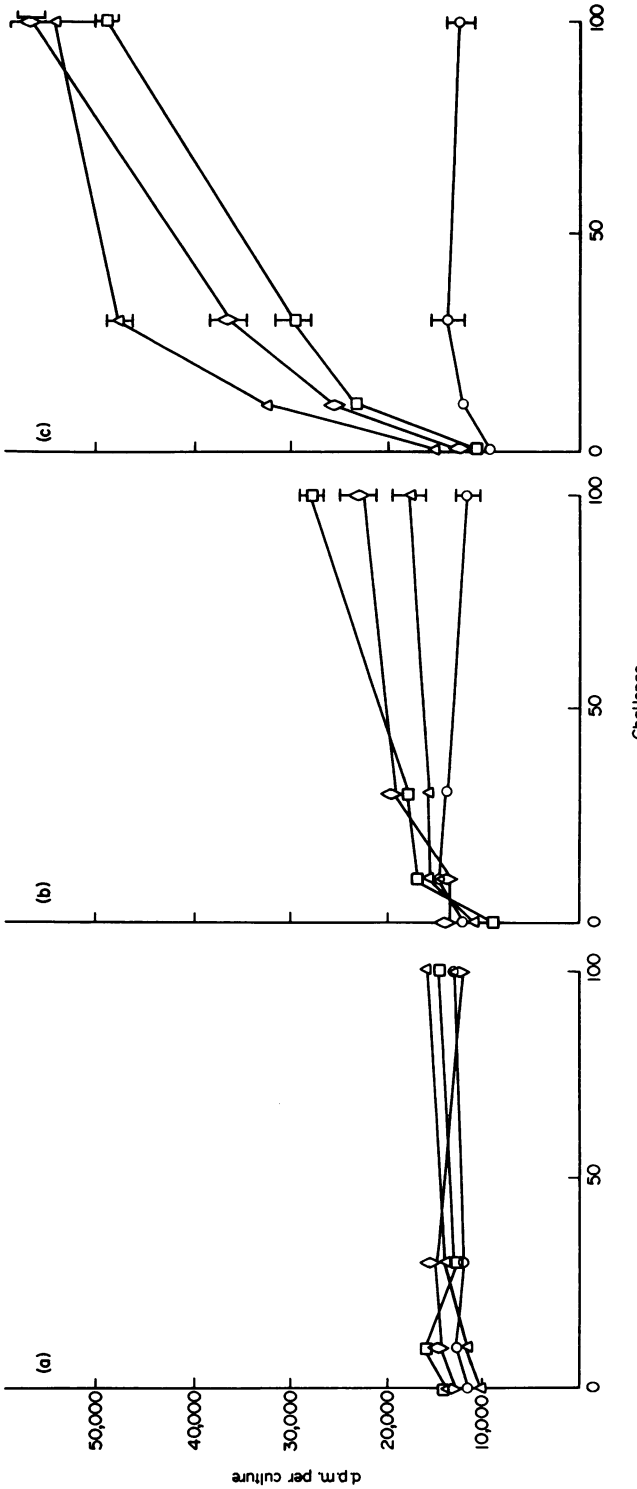

FIG. 1. Influence of the DNP/BSA ratio on the *in vitro* stimulation of mouse spleen cells. Groups of four mice were immunized with 10 (□), 100 (△) or 500 (◇) μg of DNP–BSA complex. The pooled spleen cells of each group and those of unimmunized controls (○) were cultured in triplicate with 10, 30 or 100 μg of the same DNP–BSA complex. The amount of [^{3}H]thymidine incorporated was measured. The results are given for (a) DNP_3–BSA, (b) DNP_{16}–BSA and (c) DNP_{28}–BSA. Vertical bars indicate standard errors of the mean.

TABLE 1

STIMULATION OF PRIMED MOUSE SPLEEN CELLS BY HOMOLOGOUS DNP–CARRIER COMPLEXES

Antigen	Amount per culture (μg)	d.p.m. per culture ± s.d.* after immunization with: 0 μg	10 μg	100 μg
DNP_{28}–BSA	0	9854 ± 1207	14632 ± 689	11880 ± 920
	10	11249 ± 1294	32471 ± 1297	24623 ± 761
	100	12103 ± 1816	54450 ± 5308	56133 ± 861
DNP_{32}–MIg	0	16425 ± 653	16443 ± 1115	14649 ± 1172
	10	17843 ± 1138	17698 ± 1462	33522 ± 2957
	100	21042 ± 1392	31905 ± 1407	50637 ± 1018
DNP_{14}–BGG	0	8120 ± 612	10604 ± 1473	8167 ± 598
	10	8452 ± 532	10515 ± 1301	12764 ± 673
	100	8542 ± 428	14669 ± 958	22430 ± 1812
DNP_{35}–PVP	0	10332 ± 726	10159 ± 609	10594 ± 737
	10	11606 ± 901	10788 ± 721	10175 ± 1247
	100	11558 ± 214	10914 ± 714	10841 ± 1247

Groups of six mice were immunized with 0, 10 or 100 μg of either DNP_{28}–BSA, DNP_{32}–MIg, DNP_{14}–BGG or DNP_{35}–PVP. Pooled spleen cells (15×10^6) of each group were cultured in triplicate with the same antigens in different concentrations for 64 hours and the last 24 hours of incubation pulsed with 1 μCi of [^{3}H]thymidine. Results are given as the mean of three cultures.

* s.d. = Standard deviation.

TABLE 2

STIMULATION OF PRIMED MOUSE SPLEEN CELLS BY CARRIER AND DNP–CARRIER COMPLEX

Antigen (100 μg per culture)	d.p.m. per culture ± s.d.* after immunization with: None	100 μg of carrier	100 μg of DNP–carrier
None	14681 ± 497	16129 ± 583	19962 ± 473
BSA	15681 ± 1050	20278 ± 1511	22711 ± 1828
DNP_{28}–BSA	17159 ± 583	38018 ± 1130	94501 ± 3443
None	14681 ± 497	12514 ± 679	19003 ± 724
MIg	14436 ± 1651	14163 ± 767	21532 ± 823
DNP_{32}–MIg	15326 ± 876	15037 ± 616	56294 ± 1364
None	14681 ± 497	15865 ± 591	20465 ± 1764
BGG	15190 ± 746	17264 ± 914	27162 ± 1681
DNP_{14}–BGG	14960 ± 810	27723 ± 1086	69120 ± 4768
None	14681 ± 497	15402 ± 1103	12545 ± 960
PVP	13598 ± 1063	17605 ± 1210	13622 ± 306
DNP_{92}–PVP	14378 ± 80	15925 ± 2440	12954 ± 328

Groups of four mice were immunized with 0 and 100 μg of either carrier or DNP–carrier. Pooled spleen cells (15×10^6) of each group were cultured in triplicate with either 100 μg of the same carrier or DNP–carrier complex for 64 hours and the last 24 hours of incubation pulsed with 1 μCi of [^{3}H]thymidine. Results are given as the mean of three cultures.

* s.d. = Standard deviation.

challenged *in vitro* with either none, 10 or 100 μg of the same antigen. Table 1 shows that DNP_{35}–PVP is not able to prepare spleen cells for a challenge with either 10 or 100 μg of the same antigen. DNP_{28}–BSA was more active than either DNP_{14}–BGG or D NP_{32}–MIg.

For the reaction of lymphocytes with hapten–carrier complexes an action of carrier molecules on T cells is suggested. To elucidate a role of carrier molecules in our experiments animals were primed with 100 μg of carrier or DNP–carrier complexes and the

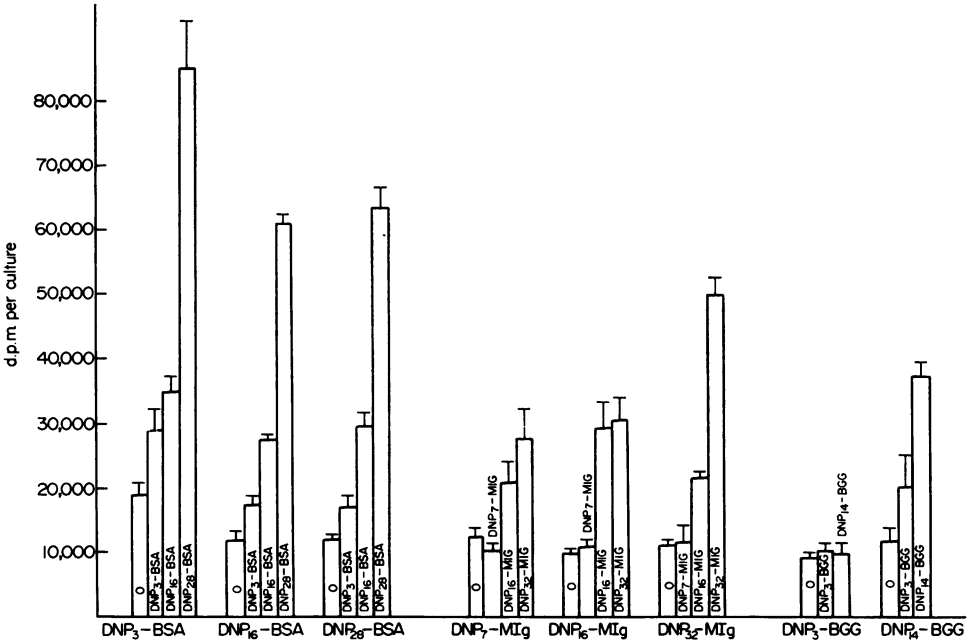

FIG. 2. The role of the hapten on *in vitro* stimulation by DNP–carrier complexes. Groups of four mice were immunized with 100 μg of either DNP_3–BSA, DNP_{16}–BSA, DNP_{28}–BSA, DNP_7–MIg, DNP_{16}–MIg, DNP_{32}–MIg, DNP_3–BGG or DNP_{14}–BGG. Pooled spleen cells of each group were cultured in triplicate with the antigens (indicated in the columns) for 64 hours and 24 hours before the end of incubation were pulsed with 1 μCi of [^{3}H]thymidine.

spleen cells were challenged *in vitro* with 100 μg of corresponding DNP–carrier complexes (Table 2). Priming with carrier alone prepares spleen cells for challenge with hapten–carrier complex in the case of BSA and BGG. However, these reactions are far weaker than when the complexes are used for immunization. Again, PVP and its complex did not show any activity. As expected, injections of the isologous MIg did not result in a priming of the mouse for a challenge with DNP_{32}–MIg.

THE ROLE OF THE HAPTEN ON THE *in vitro* STIMULATION BY CARRIER–DNP COMPLEXES

Groups of mice were immunized with 100 μg of DNP_3–BSA, DNP_{16}–BSA and DNP_{28}–BSA. Each group was challenged with the three complexes. The same experiments were performed for the corresponding hapten–carrier complexes of BGG and MIg. From Fig. 2 it can be seen that priming with DNP_3–BSA and DNP_{16}–BSA prepared just as well as

DNP_{28}–BSA for a challenge with DNP_{28}–BSA. Challenge with either DNP_3–BSA or DNP_{16}–BSA did not result in high [^{3}H]thymidine incorporation after priming with any of the three DNP–BSA complexes.

For the DNP–MIg complexes almost similar results were obtained as for the DNP–BSA complexes. Complexes with high and low numbers of DNP groups prepared the spleen cells equally well for *in vitro* stimulation with DNP_{32}–MIg.

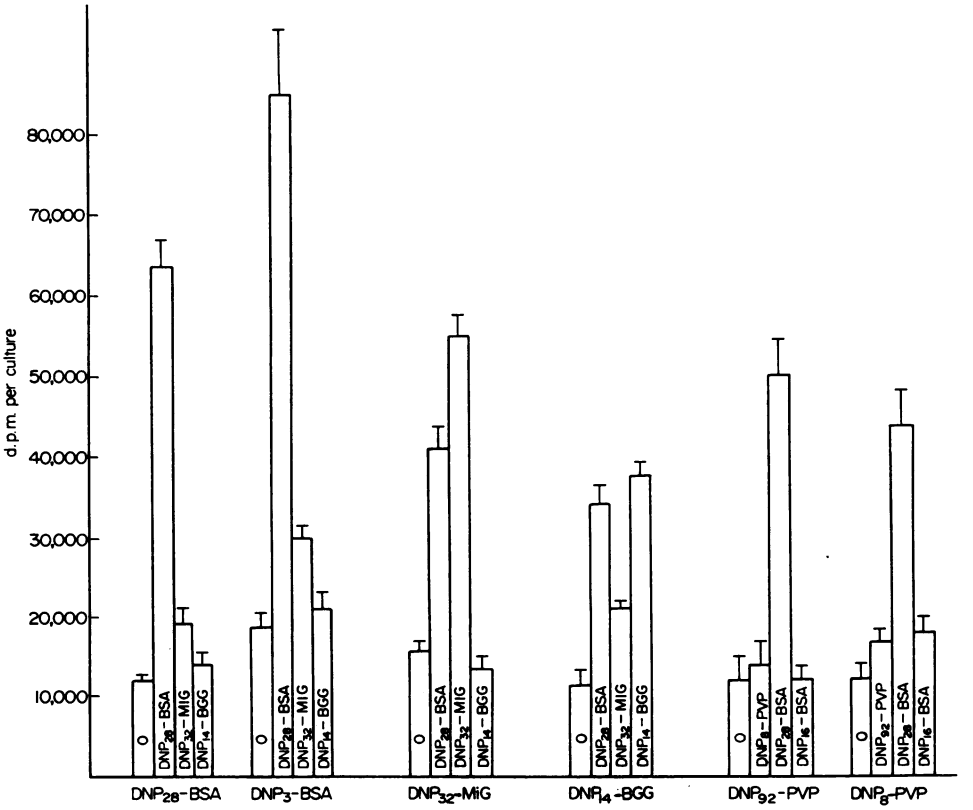

FIG. 3. Cross-reactions in *in vitro* stimulations by DNP complexed to different carriers. Groups of four mice were immunized with 100 μg of either DNP_{28}–BSA, DNP_3–BSA, DNP_{32}–MIg, DNP_{14}–BGG, DNP_{92}–PVP or DNP_8–PVP. Pooled spleen cell of each group were cultured in triplicate with antigens as indicated in the columns for 64 hours and the last 24 hours of incubation pulsed with 1 μCi of [^{3}H]thymidine.

DNP_3–BGG did not prepare for *in vitro* stimulation with DNP_{14}–BGG. On the other hand DNP_{14}–BGG prepared slightly for stimulation with DNP_3–BGG.

CROSS-REACTIONS IN *in vitro* STIMULATIONS BY DNP COMPLEXED TO DIFFERENT CARRIERS

Mice were primed with 100 μg of one of the following complexes: DNP_3–BSA, DNP_{28}–BSA, DNP_{32}–MIg, DNP_{14}–BGG, DNP_8–PVP and DNP_{92}–PVP. Spleen cells of these mice were stimulated *in vitro* with DNP_{28}–BSA, DNP_{32}–MIg and DNP_{14}–BGG or DNP–

PVP complexes. From Fig. 3 it can be concluded that DNP_{28}–BSA is in all instances able to stimulate spleen cells of DNP-primed mice, even when DNP–PVP was used for priming. When spleen cells of mice primed with DNP_{32}–MIg or DNP_{14}–BGG were used the stimulation with the homologous complex was better or equal to that obtained with DNP_{28}–BSA. Priming with DNP–BSA complexes prepared spleen cells only for stimulation with the homologous antigen.

TABLE 3

HAPTEN INHIBITION BY ε-DNP-L-LYSINE OF *in vitro* STIMULATION BY DNP-CARRIER

Antigen used for senisitzation	Antigen added *in vitro*	d.p.m. per culture ± s.d.* amount of DNP–Lys per culture (μg) 0	200	Percentage inhibition
None	0	14681 ± 447	16873 ± 1041	
DNP_{28}–BSA	0	19962 ± 473	—	
	100	94501 ± 3443	76861 ± 1261	18·7
DNP_{32}–MIg	0	19003 ± 724	—	
	100	56294 ± 1374	46334 ± 4498	17·6
DNP_{14}–BGG	0	20465 ± 1764	—	
	100	69120 ± 4768	49012 ± 3691	29·1

Pooled spleen cells of four mice which had been immunized with 100 μg of either DNP_{28}–BSA or DNP_{32}–MIg or DNP_{14}–BGG were cultured in triplicate for 1 hour with ε-DNP-L-lysine following by 63 hours of incubation with 100 μg of the same antigen. The last 24 hours of incubation the cells were pulsed with 1 μCi of [^{3}H]thymidine. Results are given as the mean of three cultures.
* s.d. = Standard deviation.

INHIBITION OF THE *in vitro* STIMULATION OF SPLEEN CELLS BY LYSINE–DNP

Mice were primed with 100 μg of either DNP_{28}–BSA, DNP_{32}–MIg or DNP_{14}–BGG. Spleen cells of these animals were cultured *in vitro* for 1 hour with or without 200 μg of ε-DNP-L-lysine, followed by 63 hours incubation with 100 μg of the same antigen. Table 3 shows that ε-DNP-L-lysine inhibited the *in vitro* response to all three antigens used to a reasonable extent. In a control experiment no suppression of ε-DNP-L-lysine on the PHA stimulation was found.

DISCUSSION

It is generally accepted that in the immune response B and T cells co-operate. T cells are thought to react with determinants on the carrier, while B cells react with the haptenic groups (Mitchison, Rajewsky and Taylor, 1970). In our experiments the role of the number of haptenic groups on the carrier for priming and for subsequent *in vitro* stimulation was investigated. With a low hapten/carrier ratio (DNP_3–BSA) for both priming and *in vitro* stimulation no response could be obtained even when high doses of complex were used. With increase of the hapten/carrier ratio an increase in response was found. When challenged with DNP_{16}–BSA, however, 100 μg of complex was able to induce a significant response, while for DNP_{28}–BSA 10 μg sufficed for both priming and challenge. This corresponds with the experiments of Schirrmacher and Rajewsky (1970). They found with

inhibition experiments that the number of sulphanilic acid (SULPH) determinants on $SULPH_{31}$–BSA is three times as high as on $SULPH_{13}$–BSA, while the relative number of carrier determinants was three times as high for $SULPH_{13}$–BSA as for $SULPH_{31}$–BSA. When these complexes were used for boosting $SULPH_{31}$–BSA induced an antibody titre which was ten times as high as that obtained with $SULPH_{13}$–BSA. The titre obtained with $SULPH_3$–BSA (the number of SULPH determinants was only 4 per cent of that of $SULPH_{13}$–BSA) was again 1/10 of the titre obtained with $SULPH_{13}$–BSA. This means that the number of determinants and also the effectiveness in antibody stimulation increases disproportionately to the number of hapten groups on the carrier.

The negative results obtained with DNP_3–BSA do not justify the conclusion that this complex is immunologically inactive, as priming with DNP_3–BSA prepared for a high response with DNP_{28}–BSA. The three different DNP–BSA complexes were equally active when used for priming. This effect was not due to the carrier for the following reasons.

(1) Priming with carrier resulted in a slight response (R = 2) with both protein carriers.

(2) Although DNP–PVP did not prime for homologous stimulation *in vitro*, it did prime for stimulation with DNP_{28}–BSA. The extent of the reaction was of the same level as for priming with one of the DNP–BSA complexes (R = 5).

(3) ε-DNP–L-lysine gave a weak but significant inhibition of the *in vitro* stimulation by DNP–carrier complexes.

These results suggest that the DNP groups play a major role in *in vivo* priming as well as *in vitro* stimulation as measured by the [^{3}H]thymidine incorporation. The priming with DNP–PVP followed by a challenge with DNP_{28}–BSA rules out a possible role for the 'new antigenic determinant' (NAD) (Rubin, 1972) in this case.

For priming, the ratio hapten/carrier and even to a certain extent the type of carrier seems to be of no importance. Probably the *in vivo* conditions are favourable for the presentation of the haptenic moiety of whatever complex to lymphocyte receptors. The conditions for *in vitro* stimulation seem to be quite different as only complexes with a high hapten/carrier ratio were active in this respect.

BGG and the mouse isologous MIg carrier behaved in the same way in the homologous reactions, despite double the number of DNP groups on MIg. When the smaller BSA molecule was linked with about the same number of DNP groups (DNP_{16}–BSA) it was less active as the corresponding BGG complex (DNP_{14}—BGG). Between MIg and BGG complex almost no cross-reactivity could be detected. However, priming with these complexes prepared lymphocytes for challenge with DNP_{28}–BSA. The R values were lower (R = 2) compared with those obtained after priming with BSA or PVP complexes. This suggests that the activity of the DNP–MIg, DNP–BGG and DNP_{16}–BSA in this experiment is due to either NAD or DNP–NAD groups (Rubin, 1972). The reactivity of DNP_{28}–BSA is due to the DNP groups. This molecule seems to contain only DNP determinants, as was found for dinitrophenylated mouse serum albumin (DNP_{32}–MSA) by Rubin (1973).

Evidence for a major role for hapten groups in DNA stimulation was presented by Osborne and Katz (1973). These authors found after priming with DNP–KLH a strong anamnestic response *in vitro*. However, dissociated KLH, the form conjugated to DNP, stimulated only low levels of DNA synthesis in spleen cells of DNP–KLH-primed mice. Moreover, the DNP–KLH induced response could be inhibited more than 50 per cent by the presence of the univalent DNP ligand, ε-DNP–aminocaproic acid.

The results of these authors and our results provide evidence for stimulation of DNA

synthesis in secondary reactions *in vitro* by hapten groups. On the basis of their activities hapten–carrier complexes can be divided into three groups: DNP–PVP complexes and DNP_3–BSA which cause priming only *in vivo*, but are not effective *in vitro*; DNP_{16}–BSA, DNP–MIg and DNP–BGG complexes, which express their reactivity probably through NAD and DNP–NAD groups; and DNP_{28}–BSA, which is suitable for priming and reacts *in vitro* on cells primed with whatever DNP–carrier complex. These reactions are very probably only due to DNP groups.

Experiments on the target cell of this hapten-induced stimulation will be presented in a separate paper (Snippe and Eyk, 1974).

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