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Comparison of *in vitro* immunogenicity, tolerogenicity and mitogenicity of dinitrophenyl–levan conjugates with varying epitope density

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Summary. The mitogenicity, immunogenicity and tolerogenicity of various DNP–levan (DNP–LE) conjugates were compared using *in vitro* methods. Anti-DNP antibody synthesis induced by DNP–LE conjugates was related to the epitope density of DNP, but was not affected by macrophage depletion. However, polyclonal stimulation by the same DNP–LE conjugates was macrophage dependent and was not influenced by the degree of hapten conjugation. These results imply that mitogenicity of an antigen is not necessarily related to the specific triggering of B cells.

INTRODUCTION

The mechanisms by which antigens induce responses by lymphocytes are still the subject of controversy. Direct triggering of B cells by T-independent antigens represents the simplest model for studying B-cell stimulation (Feldmann, 1974). Although some of these antigens have been reported to be polyclonal B-cell mitogens (Coutinho and Möller, 1973) the question is open whether the induction of the polyclonal mitotic response and specific antibody response are causally connected (the latter being a consequence of the former), or whether the two effects are unrelated.

The aim of this investigation was to compare the mitogenicity, immunogenicity and tolerogenicity of various DNP–levan conjugates by *in vitro* methods. Specific anti-DNP responses induced by these conjugates were correlated with the epitope density of the DNP determinant and the intensity of mitogenicity was assessed using various concentrations of carrier (levan). Further, a comparison was made of the macrophage dependence of specific antibody activation and polyclonal B-lymphocyte stimulation by these haptenated LE conjugates.

MATERIALS AND METHODS

Animals

Male CBA/T6T6 mice, bred at Wellcome Research Laboratories, were used at 10–12 weeks of age.

Mitogens and antigens

Lipopolysaccharide (LPS) from *Veillonella alcalescens* was prepared as described previously (Ivanyi, Lehner and Burry, 1973).

Phytohaemagglutinin A (PHA) was obtained from Wellcome Reagents Ltd (Beckenham, Kent).

Native levan (LE) was prepared from *Corynebacterium levaniformis* (Miranda, 1972).

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DNP–LE conjugates were prepared according to the technique of Axen, Porath and Ernback (1967). LE was activated by cyanogen bromide at respectively pH 9, 11, 11·5 for $DNP_{0.7}LE$, $DNP_{1.2}LE$ and $DNP_{2.5}LE$ conjugates. Activated LE was incubated for 24 h with DNP–lysine in 0·5 M $NaHCO_3$ as described previously (Desaymard and Feldmann, 1975a).

The degree of substitution of LE, which is a polydisperse polymer of mean molecular weight 4×10^7 Daltons, was expressed for 40,000 molecular weight, the size of monomeric flagellin, to facilitate comparison with earlier work on epitope density, which was performed with conjugates with polymerized flagellin (DNP–POL), where the substitution was expressed per 40,000 Daltons.

The concentration of LE was measured by the cysteine technique of Diesche (1949). The molarity of DNP was calculated from the extinction coefficient at 360 nm ($E^{1M}/1$ cm 17,400).

Cell suspensions

Spleens were removed aseptically and cell suspensions prepared by using a loose fitting glass homogenizer. The cells were washed twice through a layer of FCS and then resuspended in RPMI 1640 medium (Grand Island Biological, Grand Island, New York) enriched with added L-glutamine (2 mM/ml), penicillin (100 u/ml), streptomycin (100 μg/ml) and 5 per cent foetal calf serum (FCS) (Biocult). Cell counts and viability were assessed by the trypan blue dye exclusion test (Fallon, Frei, Davidson, Trier and Burk, 1962).

Removal of phagocytic cells

Columns of Sephadex G-10 were equilibrated with RPMI 1640 supplemented with 10 per cent FCS. Cells at a concentration of 2×10^8/ml were passed rapidly through the columns using a ratio of 2×10^8 cells per 10 ml of packed Sephadex G-10. Cells were eluted with twice the column bed volume of culture medium (M. Hoffmann, personal communication). The percentages of phagocytes, monocytes and B lymphocytes in untreated and effluent suspensions were determined by labelling with fluorescent *Corynebacterium parvum* particles (Bell and Shand, 1972), by staining for nucleoli with toluidine blue at pH 5·1 (Smetana, 1961) and by membrane fluorescence with antimouse immunoglobulin (Wellcome Reagents) (Froland and Natvig, 1972) respectively. Seventy per cent of cells were recovered without significant alteration in the proportion of Ig-bearing cells. Effluent suspensions contained less than 0·5 per cent actively phagocytic and 3 per cent monocytic cells according to morphological criteria.

Peritoneal macrophages

Six millilitres of saline were injected into the peritoneal cavity and withdrawn with a syringe. Cells were washed twice through a layer of FCS and enumerated using a trypan blue dye exclusion test.

In vitro *antibody synthesis*

Mouse spleen cells were cultured in a modified Marbrook–Diener system (Marbrook, 1967; Diener and Armstrong, 1967). Cells (15×10^6) in Eagle's minimum essential medium (MEM) supplemented with 5 per cent FCS were incubated for 4 days at 37° in a humid atmosphere containing 10 per cent CO_2 in air.

In vitro *tolerance induction*

Various concentrations of antigen were added to plastic Petri dishes which contained 6×10^7 mouse spleen cells in 4 ml Eagle's MEM supplemented with 5 per cent FCS. Petri dishes were incubated for 4 h at 37° in a 10 per cent CO_2 humidified incubator. After harvesting, cells were washed twice in Earle's saline through a layer of FCS and cultured for 4 days with either 0·5 μg of $DNP_{2.5}LE$ or 0·1 μg of $DNP_{2.8}$ dextran.

Anti-DNP–antibody-forming cells (AFC)

These were enumerated using the technique of Cunningham and Szenberg (1968). Sheep red blood cells (SRC) were sensitized with rabbit anti-SRC Fab′ conjugated with DNP (DNP–SRC). Specific AFC were calculated by subtracting the number of plaques obtained with SRC from that obtained with DNP–SRC. Results were expressed as arithmetic means ± standard errors.

DNA synthesis

Cultures containing 4×10^6 spleen cells in 1 ml of medium RPMI 1640 were prepared, harvested and assayed as described in detail previously (Ivanyi and Lehner, 1970). The results of lymphocyte stimulation are presented both as stimulation indices (SI) representing the ratio of [^{14}C]thymidine uptake between antigen and saline-stimulated cultures and as c.p.m. per 4×10^6 viable lymphocytes.

RESULTS

Stimulation of DNA synthesis by LPS, levan and haptenated levan

The effect of 5 to 500 μg/ml of LPS, levan and DNP–LE on the DNA synthetic activity of normal spleen cells were assessed (Table 1). Increased DNA synthesis was induced by all three compounds under the culture conditions used. The optimal concentration of LPS was 10 μg/ml, while a plateau level of stimulation was produced by DNP–LE and LE over a wide dose range (10–500 μg/ml). The mitogenic potential of LE and of DNP–LE with conjugation ratios ranging from 0·7 to 2·5 DNP groups/4×10^4 molecular weight, was similar, even though the latter varied greatly in the immunogenicity of their DNP epitopes (Fig. 1).

The effect of depletion of adherent cells on stimulation of DNA synthesis induced by LPS, levan or DNP–LE

Passage of spleen cells through a Sephadex G-10 column markedly diminished the increased DNA synthesis induced by LE and DNP–LE-stimulation indices were diminished by 84–99·7 per cent (Table 2). In contrast, the SI of LPS was diminished by only 45 per cent. Cellular analysis of the filtrate indicated that a 90–95 per cent loss of macrophages had been achieved, as judged by uptake of fluorescent *C. parvum*, without measurable Ig-bearing cell depletion. Again the loss of mitogenicity observed with LE or DNP–LE was comparable in extent and was not influenced by the degree of DNP conjugation.

Table 1. Stimulation of spleen cells by various doses of mitogens

Mitogen	Stimulation index of concentrations (μg/ml)					
	5	10	20	50	100	500
PHA	96	115	—	—	—	—
LPS	56	76	—	52	—	—
LE	35	46	—	—	54	48
$DNP_{0.7}LE$	44	47	57	—	61	—
$DNP_{1.2}LE$	34	44	—	—	59	51
$DNP_{2.5}LE$	41	45	—	—	51	43

— = Not tested.
Mean values from 1–5 experiments.
c.p.m. (mean value) of control cultures (saline) = 7·36.

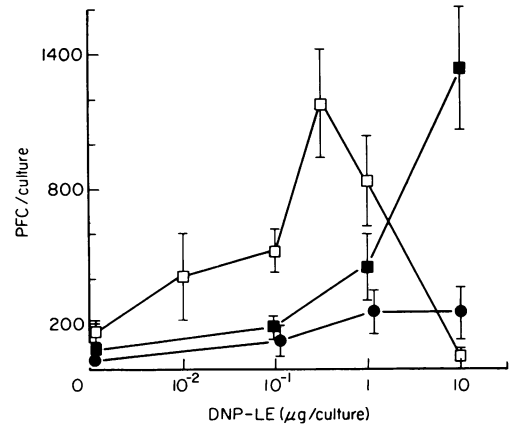

Figure 1. The effect of epitope density on anti-DNP response. Primary response to DNP–LE conjugates: (●) $DNP_{0.7}LE$; (■) $DNP_{1.2}LE$; (□) $DNP_{2.5}LE$. Arithmetic means±s.e. (n = 9).

Effect of adding peritoneal cells on the DNA synthesis of column-purified spleen cells

In order to confirm that inhibition of the mitogenic responses following passage of spleen cells through a Sephadex G-10 column was attributable to macrophage depletion, the effect of reconstitution with peritoneal cells was investigated. The effect of adding 1, 5 or 10 per cent peritoneal cells to Sephadex G-10 column-fractionated spleen cells is shown in Table 3. The response to all the mitogenic compounds was adequately restored by addition of 1 per cent cells, but less so when a greater number (5 or 10 per cent) was used.

Immunogenicity and tolerogenicity of DNP–LE conjugates

The immunogenicity and tolerogenicity for Bμ cells of DNP conjugates with various TI antigens depends on the conjugation ratio or epitope density (Desaymard and Feldmann, 1975b; reviewed by Feldmann, 1974). The conjugates used in the present study were chosen so as to represent different immunological potentials. $DNP_{0.7}LE$ produced no significant *in vitro* anti-DNP response, even with concentrations up to 100 μg/ml (Fig. 1 and unpublished data). $DNP_{1.2}LE$ was

Table 2. The effect of macrophage removal on lymphocyte stimulation

Mitogen	Culture (μg/ml)	[^{14}C]thymidine incorporation: c.p.m.* N	c.p.m.* G-10	SI N	SI G-10	Percentage of suppression
PHA	10	87,280	69,375	217·6	206·4	5·2
LPS	10	40,050	18,156	99·8	54·0	45·9
Levan	5	16,531	260	41·2	0·8	98·0
	10	16,987	489	42·3	1·4	96·7
	100	19,460	600	48·5	1·8	96·3
$DNP_{0.7}LE$	5	14,265	342	35·6	0·1	99·7
	10	18,724	388	46·7	1·1	97·7
	20	19,365	448	48·3	1·3	97·3
$DNP_{1.2}LE$	5	17,111	374	42·6	1·1	97·4
	10	18,231	246	45·5	0·7	84·6
	100	18,581	730	46·3	2·2	95·2
$DNP_{2.5}LE$	5	13,569	534	33·8	1·6	95·3
	10	14,180	347	35·4	1·0	97·2
	100	14,465	823	36·0	2·4	93·3
Saline		401	336			

Percentage of cells	N	G-10
Monocytes	9	2·8
Macrophages	5	0·5
Ig-bearing lymphocytes	38	37

* N = untreated lymphoid suspension; G-10 = lymphoid suspension passed through Sephadex G-10 column.

Table 3. Restoration or suppression of the mitotic response by adding macrophages to G-10-depleted spleen cells

G-10 depletion of spleen cells	Addition of peritoneal cells (per cent)*	Saline (c.p.m.)	LE c.p.m.	LE SI	$DNP_{0.7}LE$ c.p.m.	$DNP_{0.7}LE$ SI	$DNP_{2.5}LE$ c.p.m.	$DNP_{2.5}LE$ SI	LPS c.p.m.	LPS SI
−	None	785	36,124	46·0	31,146	39·6	27,630	35·2	50,057	63·7
+	None	828	5954	7·2	4350	5·2	4592	5·5	16,099	19·3
+	1	743	35906	48·3	29,092	39·1	26,226	35·3	49,915	67·1
+	5	1279	27,141	21·2	22,242	17·4	24,154	18·9	32,394	25·3
+	10	1091	15,800	14·5	13,594	12·5	14,686	13·5	26,522	24·3

All mitogens were used at 10 μg/ml of culture.

* Seventy per cent macrophages.

immunogenic and showed dose related augmentation of response, but was not tolerogenic at any level. $DNP_{2.5}LE$ was immunogenic at 0·3 μg/ml, less so at 1 μg/ml and fully tolerogenic at 10 μg/ml. The stability and validity of tolerance induced by $DNP_{2.5}LE$ was controlled by transfer experiments (Table 4). Preincubation of cells with 20 μg of LE or up to 100 μg of $DNP_{1.2}LE$ did not impair the anti-DNP response induced by challenge with an immunogenic dose of $DNP_{2.5}LE$ or In contrast, preincubation of cells with 10 μg of $DNP_{2.5}LE$ before challenge reduced the anti-DNP response by

Table 4. Induction of tolerance with LE and DNP–LE conjugates *in vitro*

Induction* (μg/ml)	Challenge (μg/ml)	IgM anti-DNP response (PFC/culture)†
Nil	Nil	30±3
Nil	$DNP_{2\ 5}$LE 0·5	1689±264
LE 20	$DNP_{2\cdot5}$LE 0·5	1430±216
LE 0·5	$DNP_{2\cdot5}$LE 0·5	1367±343
$DNP_{1\cdot2}$LE 100	$DNP_{2\cdot5}$LE 0·5	1676±857
$DNP_{1\cdot2}$LE 100	$DNP_{2\cdot5}$LE 0·5	1583±428
$DNP_{2\cdot5}$LE 10	$DNP_{2\cdot5}$LE 0·5	160±47
$DNP_{2\cdot5}$LE 1	$DNP_{2\cdot5}$LE 0·5	1240±319
$DNP_{2\cdot5}$LE 0·1	$DNP_{2\cdot5}$LE 0·5	1583±307

* Four hours pre-incubation of normal CBA mouse spleen with LE or DNP–LE prior to challenge.

† Arithmetic mean of three cultures±s.e. (experiment reproduced three times).

90 per cent. The tolerogenic effect of $DNP_{2\cdot5}$LE was related directly to the amount of conjugate which was added during preincubation. This inhibition was specific for DNP as the response to SRC was normal.

The anti-DNP response could be induced by DNP–LE conjugates in spleen cell suspensions depleted of macrophages. The effect on three different batches of DNP–LE used at their respective optimum doses is shown in Table 5. Similarly, no significant effect resulted from macrophage depletion (*P* value greater than 0·2), followed by restoration with 10^5 peritoneal cells. This is in total contrast with the effect produced on the polyclonal proliferative response (Table 2).

Table 5. Macrophage independence of the anti-DNP response to DNP–LE conjugates

Source of cells	Antigen (μg/culture)	PFC/culture* Anti-DNP	PFC/culture* Anti-SRC
Normal spleen cells	$DNP_{2\cdot5}$LE 0·3	610±170	n.d.
	$DNP_{1\cdot7}$LE 0·5	783±56	n.d.
	$DNP_{1\cdot2}$LE 10	520±128	196±30
	LPS 30	328±79	216±17
	Nil	87±6	40±11
Macrophage-depleted spleen cells†	$DNP_{2\cdot5}$LE 0·3	380±129‡	n.d.
	$DNP_{1\cdot7}$LE 0·5	590±125§	n.d.
	$DNP_{1\cdot2}$LE 10	410±170¶	32±9
	LPS 30	296±14	191±15
	Nil	50±15	12±5

n.d. = Not determined.

* Arithmetic mean±s.e. (n = six to twelve cultures).

† Depletion of 95 per cent macrophages by passing through Sephadex G-10 column.

‡ $P = 0·3$; § $P = 0·2$; ¶ $P = 0·5$.

Non-specific increase in background AFC levels induced by LPS and LE

LPS has been reported to augment non-specifically background AFC levels in cell cultures (Andersson, Sjöberg and Möller, 1972). In the present experiments, $DNP_{1\cdot2}$LE and LPS raised anti-SRC AFC counts in unfractionated cultured spleen cells by 5–6-fold. Macrophage depletion by either Sephadex G-10 or carbonyl iron powder did not significantly diminish this augmenting effect of LPS, but markedly reduced that of $DNP_{1\cdot2}$LE (Table 5).

Effect of concurrent mitogenic stimulation on the anti-DNP response to DNP–LE

The addition of a highly stimulatory concentration of LE (300 μg/ml) produced a minimal effect on the anti-DNP response elicited by various concentrations of $DNP_{2\cdot5}$LE (Fig. 2). The magnitude of the peak response was not altered, but the optimal stimulating dose was extended beyond 0·3 μg/ml to 1 μg/ml $DNP_{2\cdot5}$LE. No significant impairment of tolerance

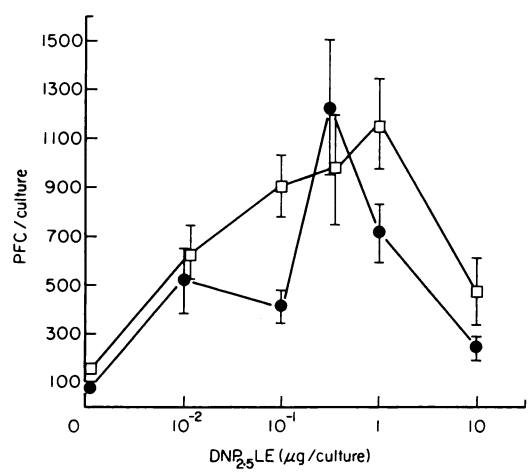

Figure 2. The effect of mitogenic concentration of levan on the anti-DNP response to $DNP_{2\cdot5}$LE. Cultures without (●) or with (□) 300 μg LE per ml. Arithmetic means±s.e. (n = 6).

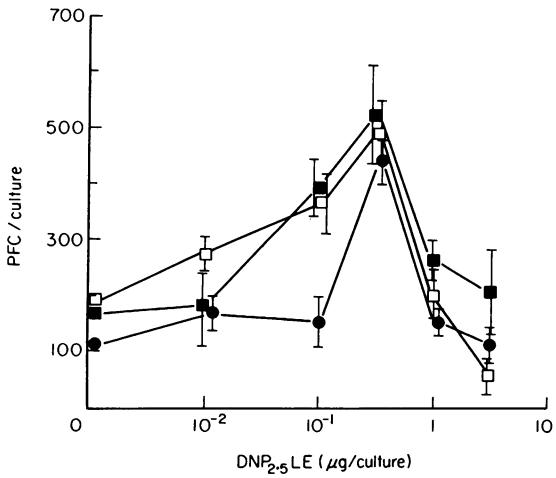

Figure 3. The effect of mitogenic dose of LPS on the anti-DNP response to $DNP_{2\cdot5}LE$. Cultures without (●) or with 3 μg (□) or 30 μg (■) LPS per ml. Arithmetic means ± s.e. (n = 9).

induction was detected. The response to suboptimal concentrations (0·1 ug/ml) of $DNP_{2\cdot5}LE$ was augmented in the presence of 300 μg LE/ml.

The addition of 3 or 30 μg/ml of LPS did not modify the dose of $DNP_{2\cdot5}LE$ required to give a maximal anti-DNP response (Fig. 3). Tolerance was induced by 1 μg/ml of $DNP_{2\cdot5}LE$, irrespective of the presence of LPS. As with LE, LPS also amplified the anti-hapten response induced by suboptimal doses (0·1 μg/ml) of $DNP_{2\cdot5}LE$. The presence of LE or LPS also invariably increased the level of anti-DNP PFC in the absence of antigen (Figs 2 and 3).

DISCUSSION

We have shown that three DNP–LE conjugates differing substantially in their immunogenic and tolerogenic potencies, as a result of various degrees of hapten conjugations, were as mitogenic as unconjugated levan over a broad dose range. Thus the contrasting immunological properties of the DNP–LE conjugates tested cannot be caused by different degrees of mitogenicity. It was striking to find that depletion of macrophages from spleen cells by passage through a Sephadex G-10 column abolished the polyclonal response induced in them by DNP–LE and LE, yet failed to influence significantly the induction of specific anti-hapten antibody formation. This result both confirms previous observations of the macrophage-independence of *in vitro* responses to polymeric flagellin (POL) (Diener, Shortman and Russell, 1970), DNP–POL (Feldmann, 1972) and DNP–Ficoll (Paul, Karpf and Mosier, 1974), and is complementary to recent demonstrations that some T-independent antigens such as DNP–dextran B512 (molecular weight 2×10^4), DNP–Ficoll or DNP–acrylamide beads (D. Parker and C. Desaymard, unpublished data) are not polyclonal activators. The addition of highly mitogenic doses of LPS or LE into cultures failed to reduce the dose required for tolerization by $DNP_{2\cdot5}LE$ as was proposed by Coutinho and Möller (1975) as a corollary to their 'one non-specific signal theory'. Taken together, the observations presented here and elsewhere (Feldmann, Howard and Desaymard, 1975) are incompatible with the idea that discrimination by B cells between immunity and tolerance with DNP–LE depends on the intensity of mitogenic stimulation to which they are exposed. The results suggest that mitogenic stimulation *per se* is not causally related to the triggering process and reaffirm the importance of epitope density in the discrimination between immunity and tolerance by B cells.

A similar conclusion was arrived at by Klaus, Janossy and Humphrey (1975) by different experimental approaches. They found no correlation between mitogenicity of the carrier and the amplitude of IgM response to the hapten, using several DNP polysaccharide conjugates including our own. They also emphasized the importance of culture conditions for stimulation of DNA synthesis by weak mitogens. Dissociation of immunity and mitogenicity was also observed by Jacobs and Morrison (1975). A TNP–LPS conjugate lost mitogenicity but retained immunogenicity after polymyxin B treatment.

The polyclonal stimulation of [^{14}C]thymidine incorporation by spleen cells in our system depended on the presence of adequate numbers of macrophages. Their depletion by passage through a Sephadex G-10 column suppressed almost completely the LE or DNP–LE-induced response, whereas the SI induced by LPS was only halved. Total restoration of the response was obtained by the addition of 1 per cent peritoneal cells, whether or not they had been treated by anti-θ and anti-B sera in the presence of complement (unpublished data), while higher concentrations of cells were suppressive. These observations confirm that the macrophage-dependence of polyclonal stimulation by the above-mentioned mitogens, which was previously found with human peri-

pheral B lymphocytes (Eisens, Lyle and Parker, 1973; Ivanyi and Lehner, 1974) extends to mouse splenic cells. There is no real discrepancy between our results and previous reports which claim that triggering of B cells by mitogens is direct (Sjöberg, Andersson and Möller, 1972), for the experiments reported in which macrophage depletion was studied involved LPS only. In our experiments, LPS differed from LE and DNP–LE in retaining substantial activity in this circumstance. The mechanism involved in polyclonal B-lymphocyte stimulation is unclear, but one simple possibility is that concentration of many T-independent antigens such as LE by macrophages could cause the release of an endogenous mitogen as a common mediator (Ivanyi and Lehner, 1974).

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