

## MECHANISM OF THYMUS-INDEPENDENT IMMUNOCYTE TRIGGERING

### MITOGENIC ACTIVATION OF B CELLS RESULTS IN SPECIFIC IMMUNE RESPONSES

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We have suggested before that thymus-independent immune responses to certain antigens are caused by special properties of the antigens concerned, namely that they are B-cell mitogens (1). Actually all thymus-independent antigens (TI)<sup>1</sup> we have tested so far were found to be competent to activate DNA and polyclonal antibody synthesis in nonprimed B cells cultured in vitro (2). Since the TI antigens can directly induce division and differentiation in B cells, it is evident that T-B cells cooperation would not be required for the induction of specific immune responses. However, there is an apparent paradox between the ability of a mitogen to induce an antigen-specific immune response to its own antigenic determinants, and its ability to activate polyclonal antibody synthesis in all B cells, irrespective of their specificities. The paradox can be easily explained by assuming two fundamentally different types of antigen binding to the cell surfaces. At low (immunogenic) concentrations, TI antigens would be preferentially bound by Ig receptors on specific antigen-binding B cells, whereas at high (mitogenic) concentrations also, other B cells (lacking specific receptors for the antigenic determinants of these molecules) would bind a sufficient number of molecules to become activated. Once triggering concentrations were reached on the cell surface, the cell would be activated and secrete the immunoglobulin for which it is genetically programmed. In terms of this argument, T-cell independence could be considered the most sensitive test for B-cell mitogenicity, since it is the "physiological" expression of direct B-cell activation.

The present experiments were designed to test this hypothesis critically, by use of the hapten 4-hydroxy-3,5-dinitrophenyl (NNP) coupled to the B-cell mitogen lipopolysaccharide (LPS) (3). The purpose of this work was to investi-

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<sup>1</sup> *Abbreviations used in this paper:* BSS, balanced salt solution; FCS, fetal calf serum; HRBC, horse red blood cells; LPS, lipopolysaccharide from *E. coli* 055:B5; NIP, 4-hydroxy-3-iodo-5-nitrophenyl; NNP, 4-hydroxy-3,5-dinitrophenyl; NNP-cap, NNP- $\epsilon$ -aminocaproic acid; PFC, plaque-forming cells; RFC, rosette-forming cells; SRBC, sheep red blood cells; TD, thymus-dependent antigen; TI, thymus-independent antigen.

gate whether NNP-LPS molecules would preferentially bind to NNP-specific cells and activate them to specific anti-NNP antibody synthesis at such low mitogen concentrations that fail to activate polyclonal antibody synthesis. It will be shown that optimal doses of NNP-LPS for mitogenic activation of high-affinity NNP-specific B cells are several orders of magnitude lower than optimal doses required for polyclonal activation. At the latter low concentrations of the hapten-mitogen conjugate, only hapten-specific cells are activated. Thus, this activation gives rise to a specific, thymus-independent immune response, with the typical kinetics of an antigen response.

### *Materials and Methods*

*Mice.*—Mice of the inbred strains A, B10.5M, and F<sub>1</sub> hybrids between these strains were used in different experiments. The mice were used at 4–12 wk of age. Congenitally athymic (nude) mice on BALB/c background were obtained from Bomholtgaard, Ry, Denmark.

*Mitogens.*—Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 was obtained by phenol water extraction according to the method of Westphal et al. (4). For the coupling of NNP to LPS, 40 mg of NNP-azide was added to 100 mg of LPS in 6 ml of carbonate-bicarbonate buffer (pH 9.2) and mixed at room temperature for 1 h. The reaction was halted by adding 50 mg of glycyl-glycine, and the mixture was separated on a Sephadex G-50 column using 1 M potassium buffer for elution. The first peak collected was passed again in the same column after adjusting the pH to 7. The collected material was then dialyzed against distilled water. The content of LPS was estimated from the dry weight of the sample, and the NNP content was determined by spectrophotometry. The final conjugation ratio was 10<sup>-5</sup> M NNP/mg LPS. LPS or NNP-LPS were dissolved in balanced salt solution (BSS) to the desired concentration.

*Antigens and Immunization.*—Sheep red blood cells (SRBC), always obtained from the same sheep donor, were stored in sterile Alsever's solution. The SRBC were washed twice in BSS before use. An analogous procedure was followed when horse red cells (HRBC) were used.

Haptenated sheep or horse red cells were prepared as described by Pasanen and Mäkelä (5). NNP-azide 1.0 or 0.01 mg/ml final concentration, respectively, or 4-hydroxy-3-iodo-5-nitrophenyl (NIP)-azide (1 mg/ml final concentration) was mixed with washed sheep red cells in a carbonate-bicarbonate buffer (pH 9.2) at room temperature for 45 min. Haptenated red cells were then extensively washed in BSS. The NNP haptenation procedure yielded red cells with different epitope density referred to as NNP nos. 1, 2, and 3, respectively. For immunization *in vitro*, approximately 10<sup>7</sup> red cells were added to each culture dish.

*Preparation of Lymphocytes.*—For studies on antibody synthesis, spleens were removed and teased in ice-cold BSS. The cells were washed three times in 50 ml of cold BSS and subsequently resuspended in tissue culture medium. For induction of DNA synthesis, spleen and lymph nodes were cut in pieces and pressed through a 60 mesh stainless steel screen into BSS. The cells were washed three times in 50 ml of BSS (400 g for 10 min) and thereafter resuspended in culture medium to the desired cell concentration. Cellular and viability counts were performed in a Burker hemacytometer after staining the damaged cells with 0.02% trypan blue.

*Medium.*—For induction of DNA synthesis the medium used was Eagle's minimal essential medium in Earle's solution obtained from Flow Laboratories, Irvine, Scotland, containing glutamine (2 mM), 100 IU of penicillin, and 100 µg streptomycin/ml. In all experiments, 10 mM of *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) buffer (Flow Laboratories) was present in the medium and the pH adjusted to 7.0–7.2. For induction of immuno-

globulin synthesis the same medium supplemented with nonessential amino acids and pyruvate was used as described by Mishell and Dutton (6).

*Sera.*—Most experiments were carried out in serum-free medium, but in certain experiments 10% fetal calf serum (Rehatuin batch no. 268; Armour Pharmaceutical Co., Chicago, Ill.) was used to the medium.

*Experimental Procedure.*—Activation of DNA synthesis was performed in plastic tissue culture tubes (no. 2054; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and 1 ml of lymphocyte suspension containing  $2 \times 10^6$  cells/ml was added to each tube. Thereafter 0.1 ml of the mitogens used were admixed. Triplicate cultures were incubated in plastic boxes filled with a mixture of 10% CO<sub>2</sub>, 83% N<sub>2</sub>, and 7% O<sub>2</sub>. At various time intervals 2  $\mu$ Ci/ml in a volume of 0.1 ml of [<sup>3</sup>H]thymidine (The Radiochemical Centre, Amersham, England; sp act of 5 Ci/mM) was added to each tube. 24 h later the cultures were harvested by pouring them onto Sartorius membranes (0.8  $\mu$ m pore size) (Membrane Filter GmbH, Göttingen, West Germany) in a Millipore multimanifold sample collector (Millipore Corp., Bedford, Mass.). The cells were successively washed in 15 ml of 0.9% saline and 4 ml of ice-cold 5% trichloroacetic acid. The filters were placed in scintillation vials and left to dry overnight. 5 ml of scintillation fluid was added to each vial and the samples were counted in a scintillation spectrometer (Tri-Carb; Packard Instrument Co., Inc., Downers Grove, Ill.). The data are presented as counts per minute per culture.

Induction of antibody synthesis was performed in plastic Petri dishes (no. 3001; Falcon Plastics) 1-ml cultures containing  $10^7$  cells were set up in triplicate and incubated at 37°C as described above, rocking on a platform at 8 oscillations per min. The cultures were fed daily with a nutritional cocktail, as described earlier (7). At various times after onset, the cells were harvested and washed three times in BSS before the plaque assay. The cultures were assayed individually (indicated by standard errors in tables and figures).

*The hemolytic plaque assay* used was modified according to Bullock and Möller (8). In short the following procedure was followed: 0.6 ml of 0.5% agar (Bacto Agar; Difco Laboratories, Inc., Detroit, Mich.) in BSS with 0.05% DEAE-dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) was kept at 46°C. 0.05 ml of red cells or haptened red cells diluted 1:8 in BSS was added to the agar together with 0.2 ml of washed lymphocytes and 0.05 ml of guinea pig serum (diluted 1:4 in BSS). The mixture was plated in 0.2-ml spread spots on plastic Petri plates and incubated for 3 h at 37°C. Plaque-forming cells (PFC) were counted using indirect light. Total number of cells were counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.) and the viability was assayed by the trypan blue exclusion test.

*The rosette inhibition assay* was performed as described by Möller et al. (9). Briefly, washed spleen cells were mixed *in vitro* with various concentrations of either LPS or NNP-LPS and incubated at room temperature for 30 min. Thereafter, NNP-SRBC target cells were added and the cell suspension was immediately centrifuged for rosette-forming cells (RFC) formation. The cell suspensions were kept at 4°C until the enumeration of RFC had been completed.

## RESULTS

*Haptentation Does Not Interfere with the Mitogenic Properties of LPS.*—The purpose of this work was to investigate the activation induced by a strong B-cell mitogen on cells that can specifically bind the antigenic determinants present on the mitogen molecules. We selected the determinant NNP because the detection method (hemolytic plaque assay) is convenient, relatively high numbers of PFC can be detected in nonprimed animals against this determinant (8), and the affinity distributions among the specific cells can be easily determined (5, 10). However, the NNP-LPS preparation would be suitable for this purpose only if LPS retained full mitogenic activity even after haptentation. In order to study

this, a wide range of concentrations of NNP-LPS was tested in parallel with a nonhaptened preparation of LPS, for the ability to activate DNA synthesis in normal spleen cells. Results of one experiment are shown in Fig. 1. Normal spleen cells were cultured together with either preparation of the mitogen, and activation of DNA synthesis was measured after 2 days in culture, when peak activity occurs (7). The dose-response curves were similar for both forms of the mitogen, indicating that they did not differ with regard to nonspecific mitogenic activation of B cells.

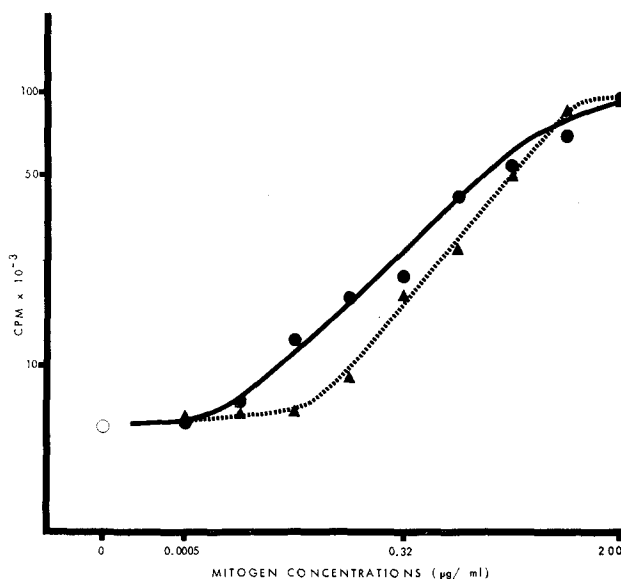


FIG. 1. Incorporation of [<sup>3</sup>H]thymidine (means of triplicate cultures, the SE being smaller than the symbols) into normal B10.5M spleen cells cultured for 2 days in serum-free medium ( $2 \times 10^6$  cells/ml per culture) with various concentrations of LPS (▲---▲) or NNP-LPS (●—●). Background incorporation in nonstimulated cultures is also indicated (○).

*NNP-LPS Is Preferentially Bound by NNP-Specific Cells.*—The experimental design requires that the NNP determinants on the LPS molecule can be specifically bound by anti-NNP receptors on lymphocytes. To study whether this was the case with the preparation of NNP-LPS used, we employed the technique of inhibition of hapten-specific rosette-forming cells by soluble hapten, as described by Möller and Mäkelä (11). In this technique, lymphocytes are allowed to react with soluble hapten for 30 min at room temperature, after which indicator erythrocytes (NNP-coated SRBC) are added and the rosettes formed at 4°C. If soluble hapten is present during the incubation period it will combine with specific cell receptors and will prevent further antigen binding when given on the erythrocytes. Therefore, rosette formation is inhibited and the phenome-

non has been shown to depend on the concentration of the hapten and on the affinity of the receptors on the specific cell population (8). If NNP determinants on the LPS molecule were available for the binding by NNP-specific cells, the NNP-LPS preparation would also efficiently inhibit NNP-specific rosette-forming cells. And as can be seen in Fig. 2, a typical inhibition curve for hapten conjugates was found with NNP-LPS, whereas LPS did not inhibit rosette formation.

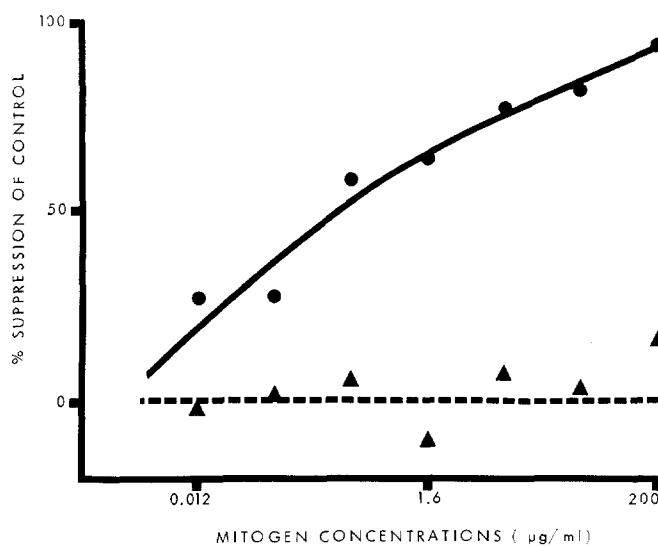


FIG. 2. Inhibition of NNP-RFC by NNP-LPS and LPS. Spleen cells from Ax5M mice primed 6 days before with NNP-HRBC were incubated with various concentrations of NNP-LPS (●—●), LPS (▲—▲), or with BSS (control), and the rosettes formed with NNP-SRBC no. 3. The percent suppression of control was calculated by the formula:

$$100 \times \frac{\text{no. of RFC in control} - \text{no. of RFC in experimental group}}{\text{no. of RFC in control}}$$

*Differential Dose Requirements for Activation of NNP-Specific Cells and for Polyclonal Activation.*—Once it had been established that a mitogen molecule containing NNP determinants was preferentially bound to NNP-specific cells, the hypothesis outlined in the introduction was tested. It is well known that B-cell mitogens activate immunoglobulin synthesis and secretion in nonprimed B-cell populations, irrespective of their specificities (3). Therefore, this preparation of hapten-mitogen should also be able to activate polyclonal antibody synthesis. However, if our hypothesis is correct, activation of antibody synthesis in NNP-specific B cells would occur at lower concentrations of NNP-LPS than activation of non-antigen-specific cells, since the mitogen would be preferentially bound to NNP-specific cells and triggering concentrations would be reached on the surface of these cells at lower concentrations of the mitogen in the medium.

Therefore, a wide range of NNP-LPS concentrations was added to normal spleen cells in culture, and the mitogen-induced PFC response was measured after 2 days in culture (7). The activation of antibody production against an unrelated antigen (SRBC) was determined as a measure of polyclonal activation, whereas the stimulation of specific cells was tested by enumerating anti-NNP PFC. Moreover, it is known that the avidity of the anti-NNP PFC can be studied by using target red cells coated with different epitope densities in the hemolytic plaque assay (5, 10). Therefore, we could study the avidity distributions of the anti-NNP PFC activated by different concentrations of NNP-LPS. Fig. 3 shows the results of such an experiment. The dose-response profile for polyclonal activation, as measured by anti-SRBC PFC, was the same as reported elsewhere for LPS preparations (3): increased numbers of PFC starting to appear at LPS concentrations about  $0.1 \mu\text{g/ml}$ , the response gradually increasing with higher concentrations, until a plateau was reached with concentrations of the mitogen of about  $10 \mu\text{g/ml}$ . This shows that the hapten-mitogen

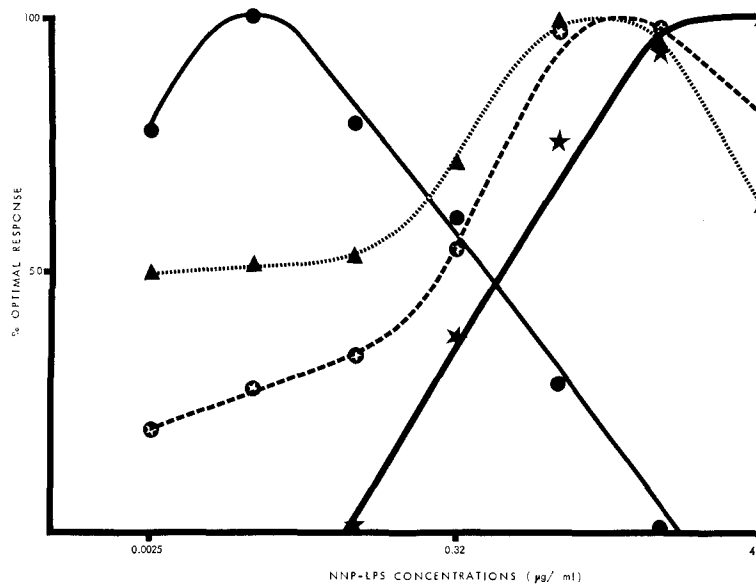


FIG. 3. Normal B10.5M spleen cells were incubated in triplicate cultures with different concentrations of NNP-LPS in serum-free medium ( $10 \times 10^6$  cells/ml per culture) and the number of PFC was determined after 2 days against: NNP-SRBC no. 3, no. 2, and no. 1, and SRBC. Numbers of anti-SRBC PFC were subtracted from the numbers of anti-NNP SRBC of different epitope densities, for each culture, to obtain absolute numbers of anti-NNP PFC of each avidity group. Background numbers of PFC in nonstimulated cultures were subtracted and the percent of optimal response of each avidity distribution was calculated. Anti-SRBC PFC  $\star-\star$ ; high-avidity anti-NNP PFC  $\bullet-\bullet$ ; low-avidity anti-NNP PFC  $\odot-\odot$ ; intermediate-avidity anti-NNP PFC  $\blacktriangle-\dots-\blacktriangle$ .

conjugate has an unimpaired ability to activate polyclonal antibody synthesis. However, the anti-NNP response induced by the haptenated mitogen showed a completely different dose-response curve. Optimal concentrations for the activation of high-affinity NNP-specific B cells (0.00256–0.0128  $\mu\text{g}/\text{ml}$ ) failed to activate polyclonal antibody (Fig. 3) and DNA (Fig. 1) synthesis. Furthermore, the anti-NNP-specific cells were inactivated by concentrations of NNP-LPS that were optimal for polyclonal activation, presumably by the same type of mechanism that accounts for the inactivation of all B cells by supraoptimal concentrations of any B-cell mitogen. However, this high dose inactivation proceeded faster (as a function of NNP-LPS concentration) for specific cells, and it follows that the dose-response profile is not only shifted to the left for specific cells, but also was more narrow than for polyclonal activation. In Fig. 3 is also shown the response induced by NNP-LPS in NNP-specific B cells of lower average affinity for the hapten, detected as PFC using a higher epitope density on the target red cells. Cells of very low affinity showed a dose-response curve more similar to the polyclonal curve, and cells of median affinity had dose requirements in between the high- and low-affinity cells. With the highest epitope coat it was indeed possible to detect very high numbers of PFC (12) that could be shown to be of very low affinity for the hapten, most likely representing many different specificities. When responses to a cross-reacting antigen (NIP) were measured (results not shown), optimal activating concentrations were higher than for low-affinity NNP-specific cells, but lower than for polyclonal activation. However, a significant response was still obtained with very low concentrations that failed to activate polyclonal antibody synthesis. This later finding can be explained in terms of higher cross-reactivity displayed by high-affinity antibodies (13). The same type of findings were obtained by using a different preparation of LPS that had been coupled to fluorescein isothiocyanate (G. Möller and A. Coutinho, unpublished observations).

*Inhibition of Specific Binding Prevents Preferential Activation of Specific Cells.*—If the results described above were due to preferential binding of the hapten-mitogen conjugate to specific hapten-binding B cells, it would be possible to prevent the preferential (“specific”) activation of NNP-binding cells, by inhibiting the specific binding of the mitogen to these cells. It has been shown before (14) that the immune response of mouse spleen cells *in vitro* to NNP-red cell conjugates could be inhibited by adding free hapten (NNP- $\epsilon$ -aminocaproic acid [cap]) to the cultures, presumably by inhibiting binding of the immunogenic material. We have used the same molar concentrations of NNP-cap that had been used for the inhibition of primary immune responses in the Mishell-Dutton system, for studying the inhibition of mitogenic stimulation induced by NNP-LPS. Normal spleen cells were cultured with doses of NNP-LPS optimal for activation of high-affinity NNP-specific cells, or alternatively, with optimal concentrations for polyclonal activation that completely suppressed the specific anti-NNP response. In the same experiments, equal concentrations of

nonhaptenated LPS were tested in parallel. As shown in Fig. 4, optimal concentrations of NNP-LPS were completely nonactivating when NNP-cap was present in the cultures, the numbers of PFC being the same as the background in nonstimulated cultures and in cultures treated with the same submitogenic dose of nonhaptenated LPS. On the other hand, the inactivation induced by high doses of NNP-LPS was prevented by free hapten, and the anti-NNP response

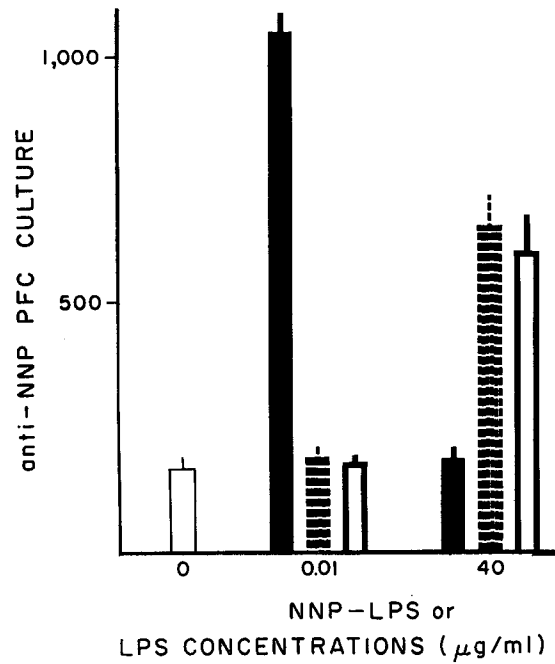


FIG. 4. Normal B10.5M spleen cells were incubated in triplicate cultures in serum-free medium with the indicated concentrations of NNP-LPS (filled bars), NNP-LPS in presence of  $2 \times 10^{-4}$  M NNP-cap (hatched bars), or LPS (open bars), and the PFC response was measured after 2 days against NNP-SRBC no. 3, and SRBC. Values are given as means and SE of anti-NNP PFC (SRBC PFC subtracted for each culture) in triplicate cultures. Background numbers of PFC in nonstimulated cultures are also indicated.

in this case was comparable to that induced by the same concentration of nonhaptenated LPS. It seems likely, therefore, that the preferential mitogenic activation induced in hapten-specific cells by submitogenic concentrations of NNP-LPS was due to the fact that only specific cells bound a sufficient number of mitogen molecules to become activated. Free hapten did not prevent activation of specific cells when high NNP-LPS concentrations were used, although the hapten prevents specific binding of NNP-LPS to NNP-reactive cells. In the presence of free hapten specific cells can only bind the conjugate nonspecifically as any other B cell. The fact that these specific cells could still be activated,



even when the Ig receptors were presumably occupied by the free hapten, strongly indicates that B-cell activation does not take place via these surface receptors.

*NNP-LPS as an Antigen.*—

*Specificity:* In the preceding experiments, the activation of NNP-specific cells was measured by PFC formation after 2 days of in vitro incubation with the mitogen conjugate. Though the responses were mitogen induced, they were strictly specific for the hapten at certain concentrations of the hapten-mitogen conjugate. As shown above, optimal concentrations of NNP-LPS for induction of high-affinity NNP-specific cells did not induce increased DNA or polyclonal antibody synthesis, nor could the same concentrations of nonhaptened LPS activate polyclonal or NNP-specific responses. Moreover, it could be shown that these low concentrations of NNP-LPS activate exclusively, or mostly, high-affinity anti-NNP cells, since small or no increases in PFC over background were found in the same cultures, when tested against target cells coated with a higher epitope density (Table I). On the other hand, the absolute numbers of high-affinity anti-NNP PFC induced by these concentrations of NNP-LPS were always as high as, and usually higher than, the numbers of anti-NNP PFC of the same affinity distribution induced by optimal concentrations of nonhaptened LPS, when measured after 2 days of culture, at the peak of polyclonal

TABLE I  
*Avidity Distribution of the Cells Responding to NNP-LPS or LPS*

Mitogen in culture	Anti-NNP PFC/culture detected with			Ratio low:high avidity PFC
	Low epitope density (high-avidity cells)	High epitope density (high-and low- avidity cells)	(low-avidity cells)	
NNP-LPS 0.0025 $\mu\text{g}/\text{ml}$				
Exp. 1*	978 $\pm$ 71	1,057 $\pm$ 38	79	0.08
2‡	1,087 $\pm$ 158	1,410 $\pm$ 111	323	0.29
3‡	1,643 $\pm$ 78	2,085 $\pm$ 92	442	0.27
LPS 100 $\mu\text{g}/\text{ml}$				
Exp. 4*	318 $\pm$ 70	3,702 $\pm$ 31	3,383	10.61
5*	577 $\pm$ 27	4,700 $\pm$ 104	4,123	7.15
6*	568 $\pm$ 28	5,172 $\pm$ 321	4,604	8.11

Normal B10.5M spleen cells were stimulated in triplicate cultures in serum-free medium by NNP-LPS or LPS at the indicated concentrations. The PFC response was determined against NNP-coated SRBC with a low (for high-affinity cells) or with a high (for low-affinity cells) epitope density. Numbers of anti-SRBC PFC were subtracted for each culture, as well as the anti-NNP PFC of the corresponding avidity group detected in background non-stimulated cultures. Since all PFC of high avidity are also visualized with a higher epitope density, whereas the reverse does not occur, absolute numbers of low-avidity PFC were obtained by subtracting numbers of high-avidity PFC from detected numbers of low-avidity PFC for each culture.

\* Harvested after 2 days in culture.

‡ Harvested after 3 days in culture.

activation. These findings indicate that all the high-affinity anti-NNP-specific cells, and only those, are activated by these submitogenic concentrations of the hapten-mitogen conjugate.

*Kinetics:* The present results suggest that mitogenic activation of normal spleen cells *in vitro* can give rise to specific immune responses. However, it has been previously reported that the kinetics of the *in vitro* antibody responses is markedly different between mitogenic and antigenic responses. Thus, the mitogen-induced responses are characterized by a sharp peak on day 2 of culture, and background levels are reached after 4 or 5 days (7). Contrariwise, antigenic responses appear after the 1st days of culture and reach a maximum on day 4 or 5 (6). We followed cultures stimulated with various concentrations of NNP-LPS or LPS for up to 5 days and measured the PFC responses against NNP and SRBC. Some of the results are summarized in Fig. 5 and Table II. Mitogenic activation by optimal doses of LPS followed the expected kinetics, for both SRBC and NNP PFC, with a sharp peak on day 2 of culture. However, the response to optimally stimulating doses of NNP-LPS for high-affinity NNP-specific cells increased throughout the whole culture period.

It could be argued that low concentrations of nonhaptened LPS would have

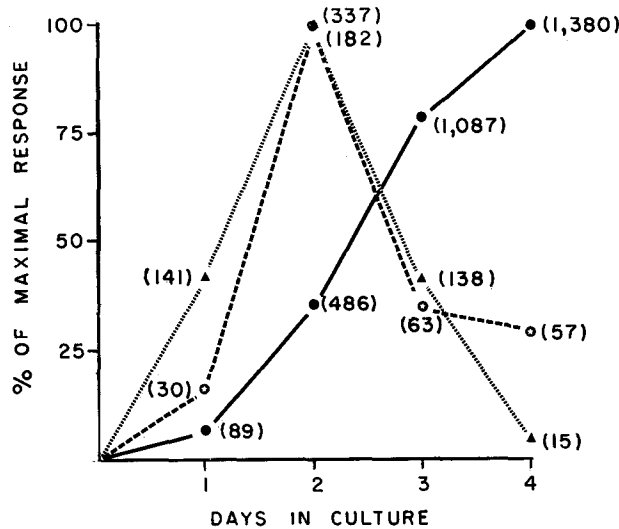


FIG. 5. Normal B10.5M spleen cells were cultured in serum-free medium ( $10 \times 10^6$  cells/ml per culture) in the presence of  $100 \mu\text{g}$  of LPS or  $0.0025 \mu\text{g}$  of NNP-LPS in triplicate cultures. At different days the PFC response was detected against NNP or SRBC. Background numbers of PFC in nonstimulated cultures were subtracted and the percent of maximal response per culture was calculated each day. ▲.....▲ anti-NNP PFC with  $100 \mu\text{g}$  LPS; ⊙.....⊙ anti-SRBC PFC with  $100 \mu\text{g}$  LPS; ●—● anti-NNP PFC with  $0.0025 \mu\text{g}$  NNP-LPS (no anti-SRBC PFC above background were detected with  $0.0025 \mu\text{g}$  NNP-LPS). The mean of absolute numbers of PFC above backgrounds are indicated in parentheses.

TABLE II  
*Kinetics and Specificity of the "Late" PFC Response of Normal Spleen Cells to NNP-LPS or LPS*

Mitogen in culture	PFC/culture against			
	Day 3		Day 4	
	SRBC	NNP	SRBC	NNP
—	15 ± 8	27 ± 12	15 ± 7	18 ± 11
LPS 0.0025 µg/ml	15 ± 0	35 ± 15	13 ± 2	15 ± 7
NNP-LPS 0.0025 µg/ml	15 ± 7	1,670 ± 78	10 ± 2	1,876 ± 83

For culture and plaque assay conditions, see footnote of Table I. Anti-NNP PFC detected with a low epitope density on the target red cells.

the same effect and cause some PFC response to appear later in the culture. But, as shown in Table II, this is not the case. When the same low concentration of LPS and NNP-LPS was tested in parallel, LPS did not induce any stimulation of PFC against NNP or SRBC, whereas NNP-LPS induced a specific anti-NNP response, which peaked on day 4 of culture.

Several reasons may be responsible for the deterioration of the polyclonal response after the 1st 2 days, such as direct cell damage by high doses of mitogen, deterioration of the culture conditions as a result of the intense metabolic activity in these cultures, because a large proportion of the cells are activated, shortage of nutrients and/or mitogen molecules, and the release of toxic materials by activated and damaged cells. Whatever the reason, it is clear that when only a small number of lymphocytes are activated, as in the case of the specific responses induced by NNP-LPS, the response follows the kinetics of antigen responses, even though the stimulus was mitogenic.

*The responses to NNP-LPS are thymus independent:* The response to the NNP determinant on the hapten-mitogen conjugate appears to fit the generally accepted definition for antigenic responses, since it is specific and exhibits the kinetics of this type of responses. However, these experiments also strongly suggest that this response is the result of the mitogenic stimulation of specific NNP-binding cells confronted with the mitogenic properties of the LPS molecule. If this were the case, the anti-NNP response should be expected to be independent of helper cells, such as T cells. The fact that the response can be induced in serum-free media (all the experiments above) by itself suggests thymus independence, since it was shown before that thymus-dependent (TD) antigens do not induce primary *in vitro* responses in this type of system, unless further nonspecific B-cell stimuli are provided (15).

We tested the anti-NNP responses in spleen cells from congenitally athymic mice, and compared them with the responses induced in normal mouse spleen cells, after stimulation with both NNP-LPS and NNP-HRBC. The later antigen was chosen because it lacks mitogenic activity and is, therefore, highly TD (2). Thus, the NNP determinants were presented to the cells on a mitogenic and

on a nonmitogenic carrier, the hapten density presumably being much higher on the non mitogenic carrier. According to our hypothesis, different requirements for helper cell activity should be expected for each of the carriers.

As shown in Table III, NNP-LPS induced comparable anti-NNP responses in normal or T-deficient (nude) spleen cells, whereas NNP-HRBC, which was a good immunogen when normal spleen cells were used, did not induce any response above background values in nude spleen cells. It can also be seen that the numbers of PFC induced by NNP-HRBC in normal spleen cells were considerably higher than the numbers induced by NNP-LPS. This could be explained by amplification mechanisms involved in T-cell helper activity, which do not participate in the response to NNP-LPS, or by the presence of fetal calf serum (FCS) in NNP-HRBC cultures, since FCS is required to support that type of responses (15).

TABLE III  
*Thymus Independence of the In Vitro Antibody Response to NNP when Coupled to LPS*

Antigen in culture	Spleen cells	Anti-NNP PFC/10 <sup>6</sup> recovered viable cells	
		Day 3	Day 4
NNP-HRBC*	Normal	713 ± 126	3,304 ± 431
	"Nude"	n.t.‡	15 + 2
NNP-LPS§	Normal	750 ± 13	960 ± 128
	"Nude"	844 ± 40	1,012 ± 50

Since induction of in vitro responses by NNP-HRBC requires the presence of FCS (15), these cultures received serum supplementation as described under Materials and Methods. The PFC numbers shown are the differences in numbers of PFC in antigen-stimulated cultures above the numbers of PFC detected in background, nonstimulated cultures. For other culture and plaque assay conditions see footnote of Table I. Anti-NNP PFC detected with a low epitope density on the target red cells.

\* Approximately 10<sup>7</sup> NNP-coated HRBC no. 2 were added to each culture dish.

‡ Not tested.

§ 0.0025 µg/ml.

#### DISCUSSION

Since TI antigens can activate B cells directly (in the absence of T-helper cells) during the induction of a primary specific immune response, it has been suggested before that they should also be able to activate all B cells nonspecifically, provided a sufficient number of molecules could interact with the B-cell membranes (1). This was found to be the case. Thus, all TI antigens tested were shown to be nonspecific B-cell mitogens, capable of activating DNA and polyclonal antibody synthesis in nonprimed populations of spleen cells deprived of T cells (2). It should be pointed out that this was found to occur in vitro, as well as in vivo. The ability of TI molecules to trigger B cells directly (B-cell mitogenicity) could explain their thymus independence.

However, it is not immediately apparent how this hypothesis can account for

the specificity of the immune responses induced by TI antigens. In view of the characteristic dose-response curves for polyclonal immunocyte activation exhibited by TI antigens, it seemed likely that immunogenic concentrations of these antigens would be too low to activate nonspecific lymphocytes. However, lymphocytes having specific immunoglobulin receptors for the TI antigen would bind these molecules both "nonspecifically" (as any other B cell) and, in addition, specifically by binding their antigenic determinants. This additional specific binding would result in a larger number of mitogenic molecules being bound to the specific cells, as compared to other B cells. It follows that triggering concentrations of the activating molecules would only be reached selectively on the surface of specific cells. Since B cells can be activated directly by the mitogenic properties of the TI antigen, the response at these concentrations will be detected as a specific and thymus-independent immunocyte activation, even though the basic mechanism of triggering is the same as that activating polyclonal antibody synthesis in other B cells, when higher concentrations of the antigen are used, which allow sufficient nonspecific binding to all B cells for triggering.

This specific preferential binding of the antigenic determinants on TI (mitogen) molecules to specific Ig receptors makes the dose-response profiles markedly different between specific and mitogenic responses. Mitogenic (polyclonal) activation should occur at higher concentrations, whereas induction of specific antigen-binding cells would occur at lower concentrations. However, high concentrations should turn off the specific cells at the same time as nonspecific cells are activated, because these concentrations would be supraoptimal for specific binding lymphocytes resulting in paralysis. This is the case for LPS (3) and all the other TI antigens previously shown to be B-cell mitogens. They all exhibit mitogenicity at concentrations several orders of magnitude higher than the doses reported to be immunogenic *in vitro* or *in vivo* (2, 16-20). TI antigens are also known to be efficient in inducing specific high-zone paralysis and we have shown before that paralytic doses of SIII are mitogenic for other (nonspecific) B cells in the same animals (1).

All these considerations are clearly supported by the experiments described above, using the hapten NNP coupled to the B-cell mitogen LPS. Moreover, it was demonstrated that the differential dose requirements for specific and polyclonal activation, respectively, were dependent upon binding of the NNP determinants (coupled to the LPS molecules) to the NNP-specific cells because: (a) Preferential activation or inactivation of NNP-binding cells by NNP-LPS could be prevented when specific binding was inhibited by free NNP in the cultures; (b) NNP-reactive cells having a lower avidity for the haptenic determinants showed a dose-response profile, which was more similar to nonspecific B cells, than that of high-avidity NNP-reactive cells. The latter result is also predictable from the hypothesis presented above, because low-avidity NNP cells would not bind NNP-LPS as efficiently as high-avidity NNP cells.

These experiments demonstrate that activation of normal spleen cells by a nonspecific B-cell mitogen can result in an exclusively specific immune response to the antigenic determinants present on the mitogen molecule, if suitable concentrations of the mitogen are used. This response is thymus independent, and its specificity is indistinguishable from a thymus-dependent immune response to the same hapten. Even so, it seems highly unlikely that the basic mechanism for triggering induced by NNP-LPS in NNP-specific cells at low concentrations is different from that exerted by the same molecule in nonspecific cells at higher concentrations. The activating (mitogenic) properties of the NNP-LPS molecules are most likely provided by its structure and/or by its mitogenic determinants, but not by the NNP determinants, since NNP determinants on TD carriers do not activate B cells directly. Therefore, the haptenic determinants appear to be exclusively responsible for the specific binding of the nonspecific mitogen. Our experiments clearly suggest that the only specific step in B-cell activation is the binding to the cell membrane, whereas the actual triggering is completely nonspecific.

However, if it is assumed that extensive cross-linking of surface Ig receptors is the fundamental triggering signal (21, 22), it could be argued that such cross-linking would take place in NNP-specific cells, because the NNP determinants were repeated on the LPS molecules. We have pointed out before that cross-linking—if at all necessary for B-cell activation—is not sufficient, because highly hapten-substituted TD proteins do not induce thymus-independent antihapten immune responses (21, 23, 24), whereas low levels of hapten substitution can lead to thymus-independent responses, if the carrier is a TI antigen (B-cell mitogen) (16, 25). Moreover, extensive cross-linking of Ig receptors by bivalent anti-mouse Ig antibodies does not lead to B-cell activation (26). It seems likely, therefore, that presentation of antigen under conditions leading to extensive cross-linking of the antigen-binding receptors cannot be the mechanism of B-cell activation. Rather, triggering by TI antigens appears to be the consequence of the totally nonspecific mitogenic properties of these antigens. Another set of findings is also most likely explained by this hypothesis, namely that TD antigens (SRBC or fowl gamma globulin) can, directly immunize B cells in the presence of TI antigens (B-cell mitogens) like LPS or polymerized flagellin (27–29).

These experiments force us to question a generally accepted dogma in immunology, namely that the Ig receptors on the B-cell surface are directly responsible for triggering the cell subsequently to their specific combination with the antigen. Critical evidence to support this dogma does not exist at present. On the other hand, several lines of experimentally supported evidence are accumulating to directly suggest that the actual triggering event of B cells is always basically nonspecific (1, 2, 15, 27, 30–32). In terms of this concept the Ig receptors are only passive focusing devices for mitogens or for T cells—the latter also providing a mitogenic stimulus. The specificity of the responses is determined by the specificity of the antigen binding to Ig receptors, but these re-

ceptors are not involved in the delivery of the activation signal. The actual triggering of the cell is nonspecific and probably carried out via other components of the cell membrane.

Theories maintaining that B-cell activation is caused by specific signals usually explain activation in terms of two signals, one being the specific recognition of the antigen by the Ig receptor, involving an allosteric modification of the receptor, and the other being basically nonspecific such as cross-linking (22, 33) or "pulling" (34) of Ig receptors, binding of associative antibody (35), or nonspecific activating factors generated by helper cells (T cells [36, 37] or macrophages [Schrader, J. W., personal communication]). For this type of hypothesis the failure to activate B cells by bivalent anti-Ig antibodies would be explained by the lack of the first, specific, signal. However, this first signal is not necessary for the nonspecific activation of B cells by mitogens. All these specific hypotheses have in common that the first, specific, signal is necessary but not sufficient for cell activation. Our model states that the antigen binding by Ig receptors does not constitute any signal in the activation process, since the confrontation of B cells with nonmitogenic molecules (TD antigens) does not lead to activation, and because full antibody production can be induced in all B cells without the participation of any specific event, involving combining sites of the surface Ig molecules (e.g. B-cell mitogens). Only one signal seems to be necessary to B-cell activation, and this signal is totally nonspecific, probably mediated by receptors on the cell membrane that are not Ig receptors and that clearly are not the combining sites of Ig receptors. Molecules competent to interact with such receptors are B-cell mitogens, which induce increased antibody production and division in all mature B cells, irrespective of their specificities, and T cell-produced nonspecific factors, which *in vitro* can be shown to induce increased antibody synthesis of all specificities in the absence of antigen (38). Moreover cross-linking does not seem to be necessary for B-cell induction, since molecules of mol wt as low as 2,000 can activate B cells very efficiently,<sup>2</sup> and antibody-producing cells can be activated directly by this type of ligand without the participation of helper cells such as macrophages.<sup>3</sup>

The findings presented in this paper support a "one nonspecific signal" concept of B-cell activation, argue against the active role of antigen-binding Ig receptors for the triggering event, and demonstrate that specific induction of antibody synthesis is not only compatible with mitogenic triggering, but appears to be the only satisfactory way to interpret the findings that TI antigens are also nonspecific B-cell mitogens. The present hypothesis also excludes the possi-

<sup>2</sup> Coutinho, A., G. Möller, and W. Richter. Molecular basis of B cell activation. I. Molecular weight requirements for mitogenicity by modified and native dextrans. Vogt, W., H. Ruh, B. Wagner, and T. Diamanstein. 1973. Stimulation of DNA synthesis in mouse lymphoid cells by polyanions *in vitro*. II. Relationship between adjuvant activity and stimulation of DNA synthesis by polyanions. Manuscripts in preparation.

<sup>3</sup> Coutinho, A., E. Gronowicz, and G. Möller. Triggering of polyclonal antibody synthesis by LPS does not require adherent cells. Manuscript submitted for publication.

bility of tolerance induction by subimmunogenic concentrations of these antigens (low zone) and explains the ease of high-zone tolerance induction (cell paralysis) both in vivo and in vitro by TI antigens. This suggestion has received experimental support from several sources (21, 39-43). However, it should be kept in mind that these findings, and consequently the hypothesis, have been limited to induction of primary IgM responses. Additional requirements that seem to be needed for IgG responses remain to be established.

#### SUMMARY

The present experiments were performed in order to analyze the mechanism by which thymus-independent antigens (nonspecific B-cell mitogens) can induce specific immune responses to antigenic determinants present on the same molecule. The hapten NNP was coupled to the B-cell mitogen, lipopolysaccharide (LPS). The conjugate retained full mitogenic activity and bound specifically to NNP-reactive cells. NNP-LPS activated polyclonal as well as specific anti-NNP antibody synthesis, but the optimal concentrations for induction of specific anti-NNP cells were several orders of magnitude lower than the concentrations required for polyclonal activation. These low concentrations failed to activate nonspecific cells, but they induced specific thymus-independent responses of high-avidity NNP-specific cells with the typical kinetics of antigenic responses in vitro. Furthermore, hapten-specific cells were paralyzed by NNP-LPS concentrations that were optimal for induction of polyclonal activation. Specific activation and paralysis could be abolished by free hapten indicating that selective binding of NNP-LPS to hapten-specific cells was responsible for the specificity of the response. However, the triggering signal lacked specificity, since high-avidity specific anti-NNP cells could still be activated by stimulating concentrations of NNP-LPS in the presence of free hapten, even though the Ig receptor combining sites were presumably occupied by NNP.

The findings show that B cells with specific Ig receptors for the antigenic determinants on mitogen molecules preferentially bind these molecules and become activated at concentrations still insufficient to trigger other B cells that lack specific receptors. It is suggested that activation for primary IgM responses in B cells is the result of "one nonspecific signal." This nonspecific signal is provided by the mitogenic properties of some antigens (highly thymus independent or, alternatively, by nonspecific T-cell factors (for highly T cell-dependent antigens), or both, and the surface structures responsible for triggering are not the Ig receptors. The specific Ig receptors only act as passive focusing devices for nonspecific stimuli, entitling the cell to be selectively activated, even though both the signal and the receptors for the triggering are nonspecific.

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