Brief Definitive Report

ASSOCIATION BETWEEN MITOGENICITY AND IMMUNOGENICITY OF 4-HYDROXY-3,5-DINITROPHENACETYL-LIPOPOLYSACCHARIDE, A T-INDEPENDENT ANTIGEN*

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Thymus-independent (TI) induction of antibody production is the simplest model for the study of B-cell triggering mechanisms (1) and the direct B-cell responses to many bacterial products appear to be of major importance in the economy of the immune system (2). One common characteristic of TI antigens is their ability to induce polyclonal antibody synthesis in B lymphocytes when added in high concentrations (1) but specific antibody synthesis when given in 10^{-6} or lower concentrations (1). Another common property, which is not shared by all TI antigens, is their structure of repeating antigenic determinants. These characteristics constitute the basis for two current competing "one signal" concepts of B-cell activation. Thus, it has been postulated that B-cell triggering is the result of one specific signal generated by the interaction of suitably presented antigenic determinants with the surface Ig receptors, or alternatively, that B cells are activated by one nonspecific signal delivered by the nonspecific polyclonal B-cell activating (PBA) properties of the antigen (for review see 3). The latter model ascribes to the surface Ig receptors on B cells a passive function of focusing nonspecific triggering signals onto the cell membrane.

To distinguish between these two alternatives, we have made use of the antibiotic polymyxin B (PB), which is competent to inactivate the nonspecific activating properties of a well known TI antigen and a PBA, namely lipopolysaccharide (LPS), from gram-negative bacteria (4).

Materials and Methods

Mice. B10.5M ($H\text{-}2^b$) and C57BL ($H\text{-}2^b$) mice of both sexes, 6–8 wk of age from our own colony, were used in all the experiments.

Culture Conditions. The method for the study of polyclonal proliferative responses to LPS and LPS-PB conjugates, as well as for the induction of specific TI anti-4-hydroxy-3,5-dinitrophenacetyl (NNP) antibody responses by NNP-LPS, has been described earlier (5, 6).

LPS. LPS from *Escherichia coli* O55:B5 obtained by phenol-water extraction (7) was used throughout these experiments. The hapten NNP was conjugated to LPS as previously described (6), and the biological characterization of the conjugate used in the present experiments has been presented before (8). The final conjugation ratio was 10^{-5} M NNP/mg LPS, and the amount of LPS

* This work was supported by the Ollie and Elof Ericsson Foundation, the Eva and Oscar Ahreń Foundation, and the Anders Otto Svärd Foundation.

in the stock solution was approximately 10 mg/ml. Serial fourfold dilutions were prepared for testing and are recorded in the tables and figures as Log_4 dilutions. Thus, 5 Log_4 dilution = 1:1,024.

Polymyxin B Sulphate. PB (batch 14124) was a gift from 3 M Riker Laboratories, Great Britain.

Detection of Antibody-Producing Cells. The number of antibody-producing cells (plaque-forming cell, PFC) to the hapten NNP was determined by the use of a modification of the hemolytic plaque assay in agar (9) for detecting low-affinity (polyclonal) or high-affinity (specific) antihapten PFC.

Inhibition of Rosette-Forming Cells. NNP-LPS was mixed with different amounts of PB and allowed to interact for 15 min at room temperature. Anti-NNP spleen cells from hyperimmunized mice were admixed and left for additional 15 min at room temperature. Without subsequent washing NNP-coated SRBC were added and the suspensions centrifuged for 10 min at 4°C to allow formation of rosette-forming cell (RFC). Details of this method have been published elsewhere (10).

Dialysates. Different concentrations of LPS and PB dissolved in balanced salt solution (BSS) were mixed at room temperature and allowed to react for 30 min. The mixtures were thereafter transferred to dialysis bags (Union Carbide Corp., New York) and extensively dialyzed against BSS to remove unreacted PB.

Results

PB Inhibits Mitogenicity of LPS. LPS was pretreated with PB, followed by dialysis to remove excess PB. The treated LPS was subsequently added to spleen cells. Results of such an experiment are shown in Fig. 1. As can be seen, untreated LPS dialyzed against BSS induced a proliferative response in spleen B cells exhibiting a typical dose response profile. PB-treated LPS preparations, however, were significantly less mitogenic. The degree of inhibition of mitogenicity increased with the PB concentrations used for inactivation. PB treatment of LPS did not completely abolish mitogenicity, and the preparations were competent to induce a proliferative response, but much higher LPS concentrations were required to achieve the same level of responses.

PB Inhibits the Capacity of LPS to Induce Polyclonal Antibody Synthesis. Similar experiments were performed to evaluate the capacity of PB-treated LPS to induce polyclonal antibody synthesis in resting B cells. This was done by determining numbers of high rate antibody secreting cells (PFC) against sheep red blood cells (SRBC) coupled with a high density of haptenic determinants. As can be seen in Table I, PB-treated LPS was significantly inhibited in the ability to induce polyclonal antibody synthesis. The inhibition was not complete, and higher concentrations of PB-treated LPS could induce polyclonal antibody synthesis.

PB Inhibits the Specific TI Immune Response to a Hapten LPS Conjugate. Having established that PB is competent to inhibit the nonspecific B-cell-activating properties of LPS, we investigated its ability to interfere with the specific TI immune response to a hapten-LPS conjugate. If B-cell triggering in specific responses is also the result of nonspecific signals delivered by PBA properties of LPS, the responses should be expected to be inhibited. Alternatively, if specific B cells were triggered by one specific signal delivered by the "pattern of antigen presentation," no effect should occur after PB treatment. Since the amount of LPS in the conjugate concentrations, which are required to induce optimal in vitro responses to the hapten, is extremely low (ranging from 10 pg/ml to 10 ng/ml), low nontoxic concentrations of PB, directly added to the

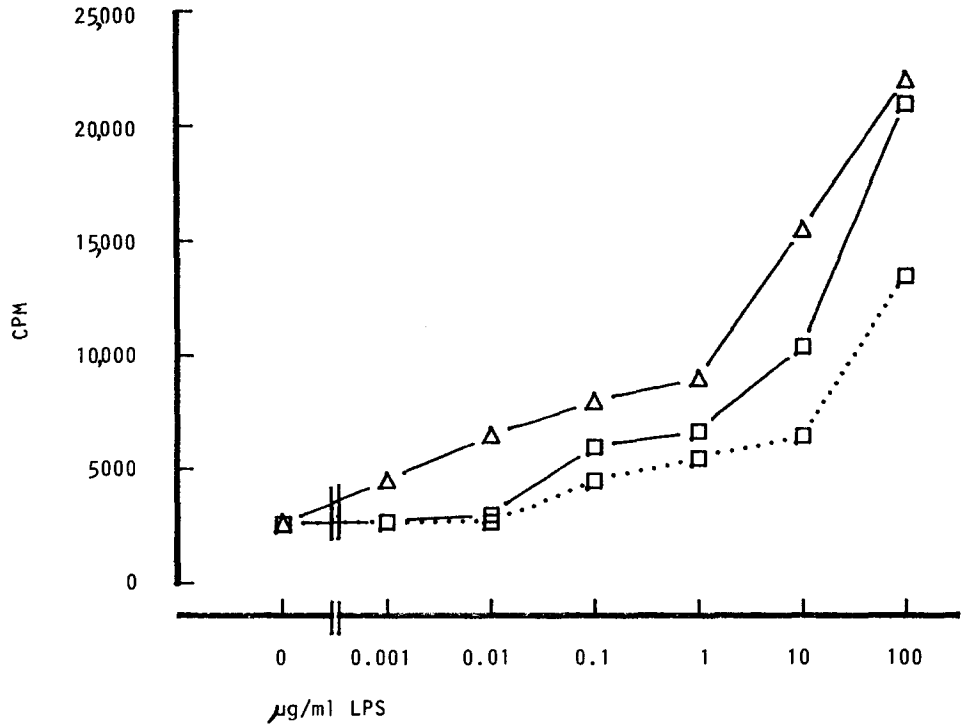

FIG. 1. Induction of DNA synthesis by LPS pretreated with 0 (△), 20 (— □), or 200 (····· □) μg/ml of PB.

TABLE I

Inhibition of Polyclonal Response to LPS by Pretreatment with PB in Percent of Normal PFC Response

	LPS			
	100 μg/ml	10 μg/ml	1 μg/ml	0.1 μg/ml
	μg	μg	μg	
200 μg/ml PB	2	28	44	45
20 μg/ml PB	0	19	28	31

cultures, should be sufficient to inactivate the mitogen. As shown in Fig. 2, normal spleen cells given NNP-LPS mount a good high-avidity antihapten PFC response in vitro. As for all other direct B-cell responses, the dose-response profile is bell shaped, superoptimal concentrations of the antigen resulting in paralysis of the hapten-specific cells.

Addition of PB results in a shift of the whole dose-response curve to the right, indicating that a loss of immunogencity took place in the presence of the inhibitor. The enhanced PFC response seen with high amounts of PB could reflect a decreased toxicity of LPS. Therefore we conclude that PB, which is competent to inhibit the nonspecific B-cell-activating properties of LPS, is also competent to inhibit the specific antihapten response to a hapten-LPS conjugate, strongly suggesting that the nonspecific activating properties of the antigen are strictly required for the induction of the specific responses.

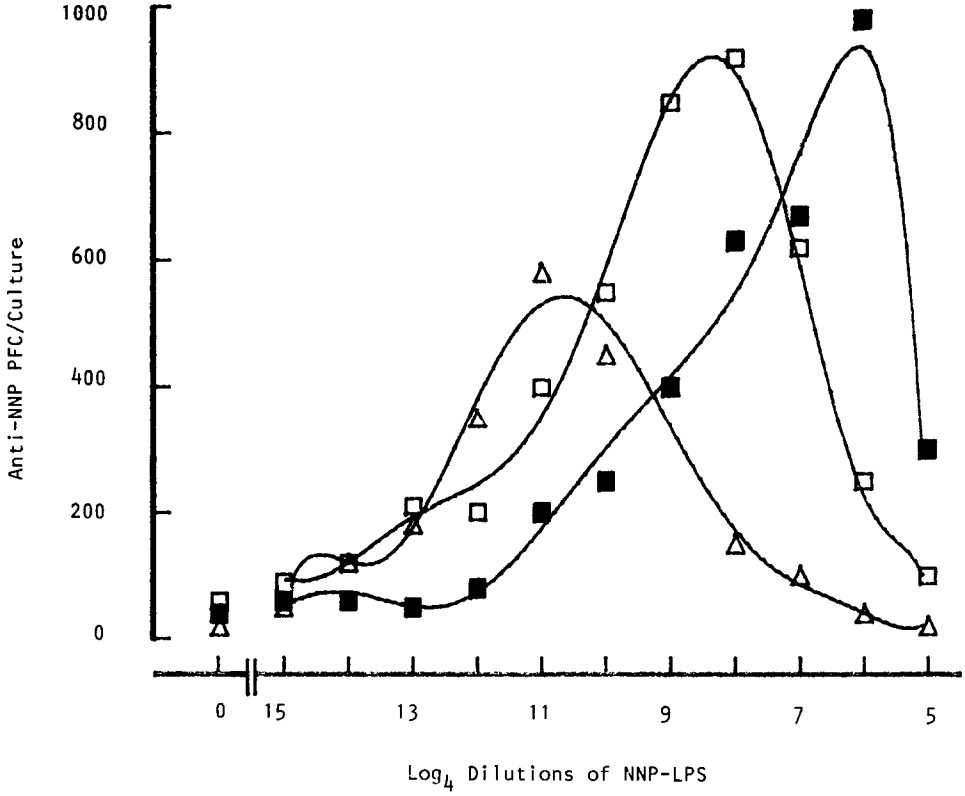

FIG. 2. Induction of specific anti-NNP PFC with NNP-LPS conjugates after addition of 0 (△), 1 (□), or 10 (■) μg/ml PB.

PB Does Not Show Immunological Cross-Reactivity with the Hapten NNP. To conclude that the PB-induced inhibition is nonspecific, it should be demonstrated that PB does not cross-react with the antigen. PB was incorporated into the agar used for the assay of PFC with cells that had been activated by NNP-LPS in vitro. As can be seen in Table II, no inhibition was detected. Thus, we conclude, that the inhibition of the TI immune response to NNP-LPS by PB is not due to cross-reactivity of PB with the hapten NNP.

PB Does Not Interfere with the Expression of NNP Determinants on the Immunogenic Conjugate. It seems possible that PB could specifically inhibit the response to the conjugate by interacting with the conjugate in such a way that the availability of the haptenic groups would be changed. To test this possibility, we used a rosette inhibition assay. This is a good test for functional availability of haptenic groups, and the amount of inhibition depends on the amount of hapten in the particular conjugate that is to be studied. As can be seen in Fig. 3, the NNP-LPS conjugate was competent to inhibit hapten-specific RFC, demonstrating that haptenic groups were available for the inhibition. Furthermore, the conjugate, pretreated with inhibitory concentrations of PB, was as inhibitory for RFC formation. Inhibition was specific since the SRBC-RFC response was unaffected by NNP-LPS conjugates.

TABLE II
Effect of Incorporation of Free PB in the Anti-NNP PFC Assay

PB	PFC/culture induced by NNP-LPS 11 Log_4 dilution	Control PFC/culture
μg/ml		
0	520	20
0.001	462	32
0.01	444	18
0.1	556	25
1	476	28

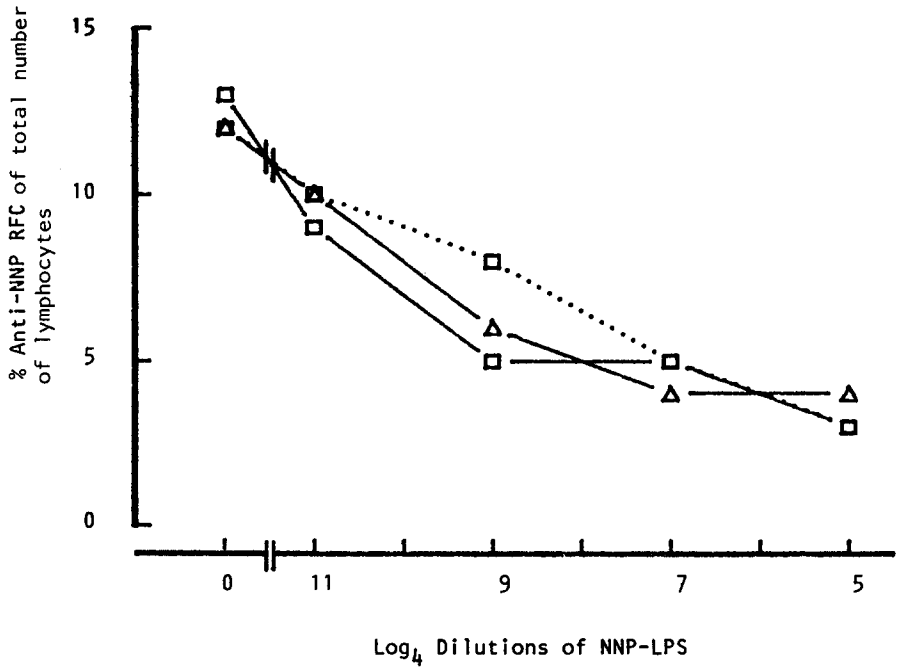

FIG. 3. Rosette inhibition of hyperimmune anti-NNP spleen cells with NNP-LPS conjugates with addition of 0 (△), 1 (—□), or 10 (····□) μg/ml of PB.

Therefore, we conclude that PB does not interfere with the binding of the immunogenic conjugate to the hapten-specific cells. Since RFC inhibition depends on the valency of the conjugates (11), as well as on the total amount of hapten in the reaction, these results indicate that PB does not modify, in any detectable way, the "pattern of determinant presentation" to the specific cells.

Discussion

The present results demonstrate that the immunogenicity of a hapten conjugate is inhibited by inhibiting the nonspecific B-cell activating properties of the carrier. Therefore, it has to be concluded that B-cell activation in specific TI responses does not take place in the absence of a nonspecific (non-Ig-mediated) signal. The results indicate that antigen presentation as such (and therefore the interaction of antigen with Ig receptor) is not disturbed, since the binding of the conjugate to specific cells in the presence of the inhibitor was not affected. These

findings exclude the concept that B-cell triggering is the result of one specific (Ig mediated) signal and strongly argue for a strict requirement for functional "mitogenicity" or PBA activity of the carrier in order to be immunogenic and TI. However, the results do not give information on whether or not an Ig-mediated signal also plays a role in the inductive process, although recent experiments suggest that no signal is generated at the Ig-combining site.

The present experiments should be compared with a recently published report (12) which used the same experimental design, but reached opposite conclusions. Thus, Jacobs and Morrison have shown that PB was competent to inhibit polyclonal (mitogenic) responses induced by LPS. This is in agreement with our findings. However, in their report, PB was found to enhance, rather than to inhibit, the specific antihapten response to a hapten-LPS conjugate. This is in complete contrast to our results. It is unlikely that this phenomenon is specific, since we did not find any cross-reactivity between PB and NNP, the latter being highly cross-reactive with TNP. There may be many explanations to these discrepancies, although the most plausible explanation is the different culture systems used.

PB might prove to be a useful tool for the future investigation of the molecular basis of B-cell activation by LPS, and experiments in this direction are presently being carried out.

Summary

Polymyxin B, which is a basic polypeptide produced by various strains of Bacillus Polymyxa, has previously been shown to prevent the lethal effect of LPS and to neutralize the Schwartzmann reaction. In this study we have investigated the interactions between polymyxin B and lipopolysaccharide (LPS) and hapten LPS conjugates. Polymyxin B was found to suppress mitogenicity of LPS and also to inhibit immunogenicity of the hapten conjugate 4-hydroxy-3,5-dinitrophenacetyl (NNP)-LPS. Inhibition was not due to interference with the expression of NNP determinants nor to cross-reactivity between PB and the hapten. Since mitogenicity and immunogenicity decreased in parallel, we conclude that B-cell activation in specific thymus independent responses does not take place in the absence of a nonspecific (non-Ig-mediated) signal.

The technical assistance of Ms. Yrsa Avellan is gratefully acknowledged. We thank Dr. D. Jacobs for personal communication of her results before their appearance in print.

Received for publication 29 August 1975.

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