A SYNTHETIC GLYCOLIPID WITH B-CELL MITOGENIC ACTIVITY

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Endotoxic lipopolysaccharide (LPS) molecules from Gram-negative bacteria consist of a polysaccharide and lipid moiety. This lipid has been found to be responsible for most of the endotoxic reactions, including B-cell mitogenesis (1–3).

The chemical structure of the lipid moiety was studied by analyzing "Lipid A" preparations obtained from LPS (1, 4), followed by structural investigation of the endotoxic glycolipid of *Salmonella minnesota* R595 which consists of lipid and 2-keto-3-deoxyoctonate (KDO) only, without the polysaccharide. These studies led to the discovery of a new class of glycolipids in which the long-chain carboxylic acids are esterified or amide-bound to D-glucosamine phosphates (4). The findings have been confirmed by several laboratories (5, 6).

Accordingly, two of us (Asselineau and Nowotny) synthesized model compounds similar in structure to some components of Lipid A, by esterifying various amino sugars with long-chain carboxylic acids, or by substituting one H of the primary amino group with various fatty acids in an amide-type linkage. In this report we present evidence that a simple synthetic glycolipid *N*-palmitoyl D-glucosamine (NPG) is mitogenic for mouse B lymphocytes.

Materials and Methods

Animals. Normal 6–8-wk old female C57Bl/6J (Jackson Laboratories, Bar Harbor, Maine) or C_3H/HEN, or nu/nu mice (Division of Research Services, NIH) were used for the mitogen experiments. Cells from these three strains respond well to endotoxin in vitro.

Reagents and Media. RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) was supplemented with glutamine (2 mM/ml), penicillin (100 μg/ml), and streptomycin (100 μg/ml). Hepes buffer was titrated to pH 7.2 with NaOH and added to the media to produce a final concentration of 15 mM. Concanavalin A (Con A) (2 × crystallized) (Nutritional Biochemical Corp., Cleveland, Ohio) was used at a final concentration of 1 μg/ml in culture and phytohemagglutinin (Wellcome Research Laboratories, Beckenham, England) at 2 μg/ml. LPS from *S. minnesota* and the natural glycolipid from the rough mutant of *S. minnesota* R595 were obtained and prepared as previously described (7).

Synthesis of NPG. *N*-palmitoyl-D-glucosamine (NPG) was synthesized by the procedure of Fieser and associates (8). The NPG was recrystallized twice from petroleum ether-benzene = 1:1, m.p. 198–202°C. The product was homogeneous in thin-layer chromatography using chloroform: methanol:water = 140:35:4.

Solubilization for Biological Assays. Both synthetic NPG and the natural glycolipid R595 were insoluble in water. Therefore, in order to use them in culture they were first suspended in pyrogen-free distilled water at a concentration of 1 mg/ml and then sonicated for 1 min at 1.7 amp. (Branson Instruments Co., Stamford, Conn.). They were then sterilized by boiling for 30 min. After heating, the synthetic glycolipid formed a precipitate, therefore it was redispersed by sonication for 1 min, and made isotonic with appropriate amounts of sterile, pyrogen-free 9% NaCl. The natural glycolipid was treated in a similar fashion, but without heating. Two fatty acids, palmitic and myristic acid (Applied Science Laboratories, State College, Pa.), and glucosamine·HCl (Sigma Chemical Co., St. Louis, Mo.) were treated in exactly the same fashion as the synthetic glycolipid and used as controls.

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Cell Collection and Tissue Culture. Spleens were removed aseptically and single cell suspensions were prepared by rinsing the cells from the splenic capsule with medium from a needle and a syringe. The spleen cells were washed twice and suspended at a concentration of 2×10^6 cells per ml in medium supplemented with 2% FCS (Industrial Biological Laboratories, Rockville, Md.). Triplicate 0.2-ml samples were cultured along with the test materials in round bottom, plastic microtrays (Cooke Engineering Co., Alexandria, Va.) for 72 h at 37°C, in a humidified atmosphere of 5% CO_2 in air and then pulsed with 0.5 μCi of tritiated thymidine ([^{3}H]Tdr, 6.0 Ci/mM, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.) for the last 4 h of culture. Cultures were harvested using a multiple automated harvesting apparatus (Mash II, Microbiological Associates, Inc., Bethesda, Md.) and counted in a Packard Tri-Carb Scintillation Counter (Packard Instrument Co., Downers Grove, Ill.). The geometric mean of the counts per minute (cpm) of triplicate samples was determined and the results expressed as the stimulation ratio (E/C), where E/C is defined as the ratio of mean cpm in the experimental cultures divided by the mean cpm in the control (unstimulated) cultures.

Limulus Lysate Clotting Assay. The *Limulus* assay was carried out by the procedure of Levin et al. under pyrogen-free conditions (9). All glass or metal instruments were heated at 180°C for 3 h before the experiments. The mitogenic NPG preparation never caused gel formation but at a concentration of 100 μg/ml did produce an incomplete (viscous) reaction. In contrast, the LPS preparation produced a viscous change at 0.001 μg/ml.

Results

Effect of the Physical State of NPG on its Mitogenic Activity. In preliminary experiments we found that the synthetic glycolipid was mitogenic only after an initial treatment of heating and resonication. In order to determine the importance of the physical state on mitogenic activity, purified NPG was treated with heating or sonication in a sequential fashion and its mitogenic ability at each state determined. Each preparation was tested over a wide range of concentrations (0.01–100 μg/ml) and the maximum proliferative response induced reported in Table I.

When NPG was sonicated it formed a turbid suspension which, when tested, was only weakly mitogenic (E/C = 3.0). After heating (100°C × 30 min.), the NPG formed dense aggregates that no longer remained in suspension and in this form, it was also a poor mitogen (E/C = 2.4). However, when this preparation was resonicated (1 min, 4°C, 1.7 amp.) it again formed a turbid suspension which was now a potent mitogen (E/C = 26.0). Interestingly, prolonged sonication (5

TABLE I

Effect of Physical State on the Mitogenic Activity of the N-Palmitoyl-D-Glucosamine

Compound	Treatment	Maximum lymphocyte proliferative response (E/C)
NPG	Mild sonication	3.1 ± 0.9
	Heated	2.3 ± 0.6
	Heated and mild resonication	26.0 ± 4.2
	Heated and prolonged sonication	6.7 ± 2.6

min, 25°C, 1.7 amp) reduced rather than increased the mitogenic activity of NPG.

Several different preparations of NPG were tested and they uniformly required heating and sonication to render them mitogenic. The explanation of this phenomenon is presently unclear. However, two points are important. First, the fact that unheated NPG is not mitogenic is evidence that this compound is not contaminated by endotoxin. Secondly, the NPG glycolipid by itself must be in a proper state of dispersion to activate a B lymphocyte.

Mitogenic Activity of Synthetic Glycolipid on Spleen Cells from Normal and nu/nu Mice. Spleen cells from normal mice were cultured in the presence of a broad dose range of the synthetic glycolipid and their maximum mitogenic response determined (Table II). NPG produced approximately one-half as much

TABLE II

Mitogenic Activities of Various Preparations on Spleen Cells from Normal and nu/nu Mice

Stimulant	Maximum lymphocyte proliferative response (E/C)*	
	Normal	nu/nu
Con A	562.9 ± 13.2	2.8 ± 0.4
PHA	147.1 ± 17.7	0.9 ± 0.1
LPS	28.3 ± 4.1	66.7 ± 3.8
Natural Glycolipid from *S. minnesota* R595	42.1 ± 7.7	58.7 ± 3.7
Crude synthetic NPG	6.8 ± 1.2	22.8 ± 1.2
Recrystallized NPG	14.2 ± 4.2	41.0 ± 2.1
Myristic acid	1.0 ± 0.1	1.3 ± 0.2
Palmitic acid	1.2 ± 0.2	1.1 ± 0.2
Glucosamine	1.0 ± 0.05	1.1 ± 0.05

* Results represent the arithmetic means ± SEM of three separate experiments. The mean baseline proliferation of 2×10^5 normal spleen cells was 190.7 cpm and Nu/Nu spleen cells was 104.0 cpm.

stimulation as did LPS and about one-third as much as did the natural glycolipid, R595. Nevertheless, it is clear that the synthetic glycolipid possessed significant mitogenic activity. Furthermore, it is interesting to note that purification of NPG, which removes unreacted glucosamine, palmitic acid, and other contaminating substances, doubled the mitogenic activity of this compound.

In order to determine if this compound was a B-lymphocyte mitogen, it was tested on spleen cells from athymic, nu/nu mice. As expected, nu/nu spleen cells responded poorly or not at all to the T-lymphocyte mitogens, Con A, and PHA but had an enhanced response to the B-cell mitogens, LPS, and *S. minnesota* R595 glycolipid. Likewise, the response to the synthetic glycolipid NPG was also significantly increased. Neither palmitic nor myristic acids, nor glucosamine by themselves were mitogenic under any conditions tested.

The dose response of the NPG preparations in comparison with that of LPS is presented in Fig. 1. Recrystallized NPG was already mitogenic at a concentration of 0.01 μg/ml, and its mitogenic activity increased up to a concentration of 10 μg/ml, but fell off rapidly at a concentration of 100 μg/ml. In contrast, the unpurified (crude) NPG only began to exhibit mitogenic activity at 1 μg/ml and the response continued to increase to a maximum at 100 μg/ml. In comparison, LPS was a better mitogen at every concentration tested but the measured difference between the dose response curves of LPS and NPG was such that there would have had to have been at least a 10% contamination of the purified NPG

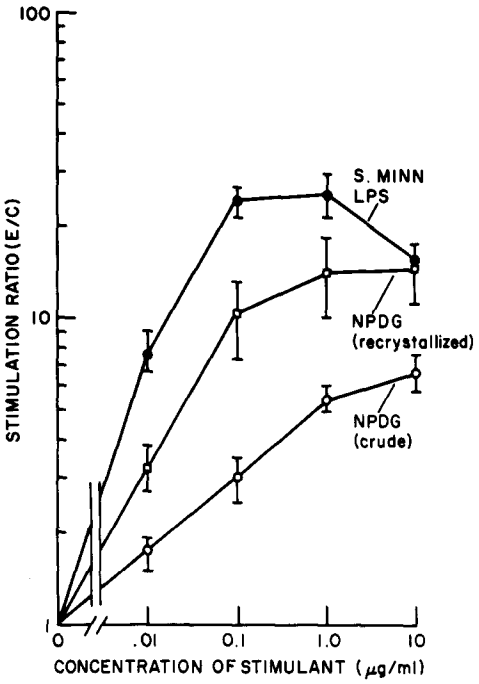

FIG. 1. Mitogenic activity of LPS and synthetic glycolipid on mouse spleen cells. Unpurified (crude) or purified (recrystallized) *N*-palmitoyl-D-glucosamine (NPDG) was assayed for mitogenic activity on mouse spleen cells in comparison to *S. minnesota* LPS. The results are the arithmetic mean of the stimulation ratios of three experiments ± 1 SEM.

with endotoxin to account for the mitogenic activity of this synthetic compound. Since this degree of endotoxin contamination was virtually eliminated by the *Limulus* lysate assay, these data demonstrate that the synthetic NPG itself is mitogenic.

Discussion

A major consideration in any study of this kind is whether the compounds being tested are contaminated with other naturally occurring B-cell mitogens such as endotoxin. It is extremely unlikely that there is any significant endotoxin contamination of our synthetic preparations for several reasons. First, these

preparations by themselves are nonmitogenic unless heated and resonicated, which is not a requirement for endotoxin to be mitogenic. Secondly, purification of these compounds by recrystallization from organic solvents which would remove any endotoxin, if present, increases rather than decreases the mitogenic activity, and furthermore, this preparation of NPG is homogeneous by thin-layer chromatography. Finally, by using the sensitive *Limulus* lysate assay that tests for the presence of endotoxin, we have determined that there could be no greater than 0.001% endotoxin in our glycolipid preparations, which is not enough to account for their mitogenic activity.

According to the reported observations, NPG requires a certain degree of dispersion to be mitogenic. All previously described B-cell mitogens or thymic-independent antigens such as LPS (3), polymerized flagellin (10), and poly I:poly C (11) are high molecular weight, polymeric compounds with multiple repeating determinants. In contrast, NPG is a low molecular weight compound (mol wt, 412), but it does form polymers of different and indeterminate sizes in aqueous solutions. Thus, it is possible that one effect of heating and sonication of NPG is to form micelles with oriented structures, of such a size or orientation that they are in effect polymeric structures with multiple repeating units that thereby act as B-cell mitogens. However, at present we do not know the exact physical state of the NPG in either the mitogenic or nonmitogenic forms. Studies are currently underway in these laboratories to answer this question, including particle size determination of active NPG dispersions by light-scattering photometry and by electron microscopy.

The demonstration of mitogenic activity in a simple, entirely synthetic glycolipid may be of fundamental importance in furthering our understanding of the mechanism of activation by both endotoxin and other B-cell mitogens. There are several tentative conclusions that we can make on the basis of the observation. First, it is clear that only a small component of the endotoxin molecule actually carries the mitogenic signal. However, the remainder of the molecule may be important in both maintaining solubility and possibly orienting the glycolipid or exposing the glycolipid in such a way so that it interacts with the cell membrane. Furthermore, these data suggest that the nature of the mitogenic signal of LPS most probably is a specific physicochemical interaction between the lipophilic moiety of LPS and the lipid fraction of the cell membrane.

Previous findings from our laboratories indicate that not all endotoxic properties are dependent on a single structural property or on one chemical functional group in the endotoxin macromolecule (12). The ability to synthesize compounds with endotoxin-like properties raises the possibility that different compounds can be synthesized with other specific endotoxin-like activities in addition to mitogenicity. Thus, using this approach it may at last be possible to correlate a specific chemical structure with a specific biological activity.

Summary

A synthetic glycolipid, *N*-palmitoyl-D-glucosamine, (NPG) was found to be a potent B-cell mitogen, that stimulated spleen cells from nu/nu as well as from normal mice. The proper dispersion of the water insoluble preparation is critical

for the elicitation of this mitogenic effect. *Limulus* lysate clotting assay indicated that the NPG preparation either contains only 0.001% endotoxin contamination, or that NPG itself is 10^{-5} times less active in this assay than purified endotoxic LPS. Since such low levels of endotoxin concentration are not mitogenic, it is concluded that synthetic *N*-palmitoyl-D-glucosamine, when properly dispersed, is itself a B-cell mitogen.

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