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Monophosphoryl Lipid A Behaves as a T-Cell-Independent Type 1 Carrier for Hapten-Specific Antibody Responses in Mice

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It is known that the lipopolysaccharide (LPS) of gram-negative bacteria, in addition to being a potent adjuvant, is an effective carrier for covalently associated haptens. However, the toxic nature of most forms of LPS precludes their use as adjuvants or carriers for human vaccines. 4′-Monophosphoryl lipid A (MLA), a derivative of LPS with attenuated toxicity, is currently being tested in humans as an immunological adjuvant. In this study, MLA was tested for its ability to function as a carrier for a small hapten, the trinitrophenyl group (TNP). MLA was first modified by addition of 6-aminocaproic acid to the 6′ position of the disaccharide backbone (Cap-MLA). TNP was then attached to Cap-MLA via the free amino group, yielding TNP-Cap-MLA. Immunization of normal mice with TNP-Cap-MLA resulted in high-titer anti-TNP responses of immunoglobulin M and all immunoglobulin G subclasses. Furthermore MLA, like other T-cell-independent type 1 (TI-1) carriers, induced responses in athymic and X-linked immunodeficient mice. In all cases, immunization with either MLA alone or TNP-Cap plus MLA failed to induce measurable anti-TNP antibodies of any isotype, indicating that covalent association of MLA and hapten was necessary for MLA's carrier activity to be manifested. These properties of MLA make it a potential candidate as a carrier for vaccine subunit components, such as small peptides, especially for situations in which T-cell help is impaired, as occurs following human immunodeficiency virus type 1 infection.

There is increasing interest in the development and use of vaccines that contain the minimal antigenic structures necessary for generation of protective immunity (7). This trend has been motivated primarily by the expectation that such (subunit) vaccines will be safer than conventional vaccines. A difficulty encountered with this approach, however, is that the immunogenicity inherent in complex antigens, such as whole bacteria, is lost with simpler structures of lower molecular weight, such as peptides or proteins. Consequently, considerable attention has been focused on new methods of enhancing the immunogenicity of these simpler antigens without introduction of significant chemical complexity (1). One such approach has been to covalently link the antigen directly to a suitable immunopotentiating entity (carrier), so that all of the components necessary to achieve the desired immune response are contained in the same molecular complex. Examples of carriers that have been used with peptide or protein antigens include muramyl dipeptide, the N-terminal region of Braun's lipoprotein, antibodies directed against antigen-presenting cells, cytokines, peptide sequences from cytokines, and peptides corresponding to epitopes recognized by T-helper cells (3, 5, 15, 18, 27, 34).

In general, antigens and carriers can be classified according to the nature and intensity of the antibody responses that they elicit in immunodeficient and neonatal mice (Table 1 and reference 11). Proteins are generally classified as T-cell-dependent (TD) antigens, because they elicit much lower antibody responses in athymic (e.g., *nu*/*nu*) mice than in normal mice. In contrast to TD antigens, T-cell-independent (TI) antigens are immunogenic in both normal and athymic mice. Polysaccharides and lipopolysaccharides (LPS) represent the main types of TI antigens. TI antigens can be further classified on the basis of their immunogenicity in mouse strains possessing an X-linked immunodeficiency (Xid) that renders them unresponsive to polysaccharide antigens (e.g., CBA/N mice [33]). On this basis, LPS is classified as a TI type 1 (TI-1) antigen (active in CBA/N mice), and polysaccharides are TI-2 antigens (not active in CBA/N mice). TI-1 antigens induce antibodies of all isotypes, such that generally immunoglobulin G2a (IgG2a) is more abundant than IgG1, while TI-2 antigens elicit primarily IgM and IgG3, with IgM greater than IgG3 (13, 35).

LPS is a potent adjuvant when administered in combination with protein antigens (22). Furthermore, LPS itself acts as a TI-1 antigen, and this antigenic character is imparted to covalently attached haptens. Thus, the antitrinitrophenyl (anti-TNP) responses elicited by TNP conjugated to LPS from *Escherichia coli* (TNP-LPS EC [35]) or LPS from *Brucella abortus* (TNP-LPS BA [4]) are characteristic of TI-1 antigens. TI-1 antigens have been proposed as candidates for vaccines in immunosuppressed populations, such as human immunodeficiency virus-infected individuals, because of their ability to elicit a protective antibody response in the absence of functional helper T-cell populations (12). Unfortunately, the practical use of LPS as an immunological carrier has been precluded by its highly toxic nature. This has led to a search for naturally occurring or modified forms of LPS with reduced endotoxicity but with strong adjuvant activity (14, 32).

4′-Monophosphoryl lipid A (MLA; Fig. 1) is an attenuated derivative of LPS that lacks many of the endotoxic properties of the parent molecule and yet retains potent adjuvant and immunostimulating activities (36, 38). MLA, marketed as MPL

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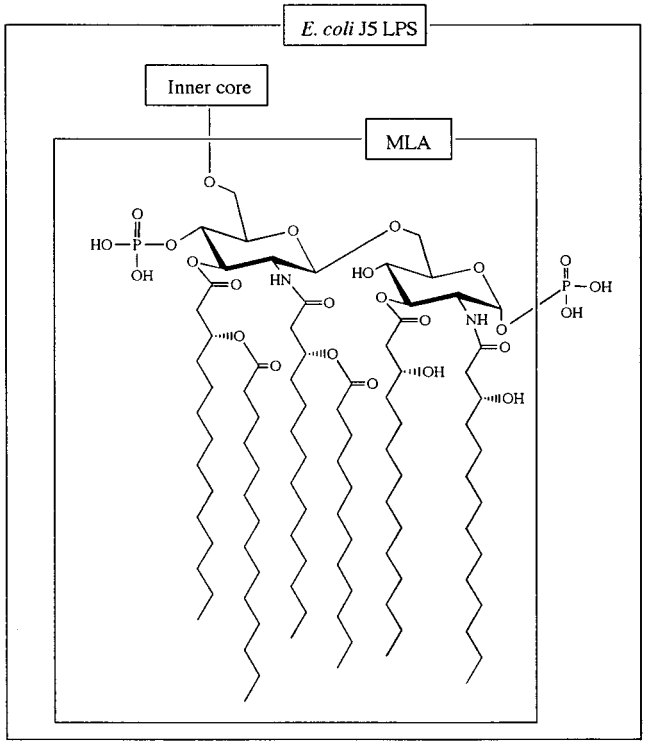

FIG. 1. Relationship of MLA to LPS. Treatment of LPS with refluxing 0.1 N HCl causes loss of the glycosidic phosphate at the 1 position and the core residues attached to the 6′ position.

immunostimulant, has been tested in several clinical trials involving a variety of indications, formulations, and routes of administration (10, 23, 24, 28, 30, 31, 37, 39, 40). Overall, these studies have shown that MLA is safe in humans at doses that are active with respect to a number of immunological markers, including induction of cytokines, activation of cytotoxic T cells, and protection against a subsequent endotoxin challenge. The utility of MLA as an adjuvant for human vaccines is supported by several of these clinical studies (10, 23, 28, 37, 39). For example, in a recent series of studies, it was found that addition of MLA to recombinant hepatitis B and herpes simplex vaccines resulted in higher seroconversion rates and larger antibody titers, without any increase in the reactogenicity of the vaccines (23, 37, 39).

In view of the encouraging clinical results obtained to date with MLA, it was of interest to determine whether MLA retained the ability of LPS to act as a TI-1 carrier for a covalently attached hapten. To this end, a novel conjugate of TNP and MLA was prepared and evaluated with respect to its ability to induce anti-TNP antibody responses in normal, athymic (*nu/nu*), and Xid (CBA/N) mouse strains.

TABLE 1. Classification of antigens or carriers on the basis of antibody responses in various mouse strains

Classification	Typical structure	Antibody response		
		Athymic[a]	Xid[a]	Normal[b]
TD	Protein	−	++	IgG1 > IgG2a
TI-1	LPS	+	++	IgG2a > IgG1
TI-2	Polysaccharide	+	−	IgM > IgG3

[a] Relative overall antibody response.
[b] Relative isotype response.

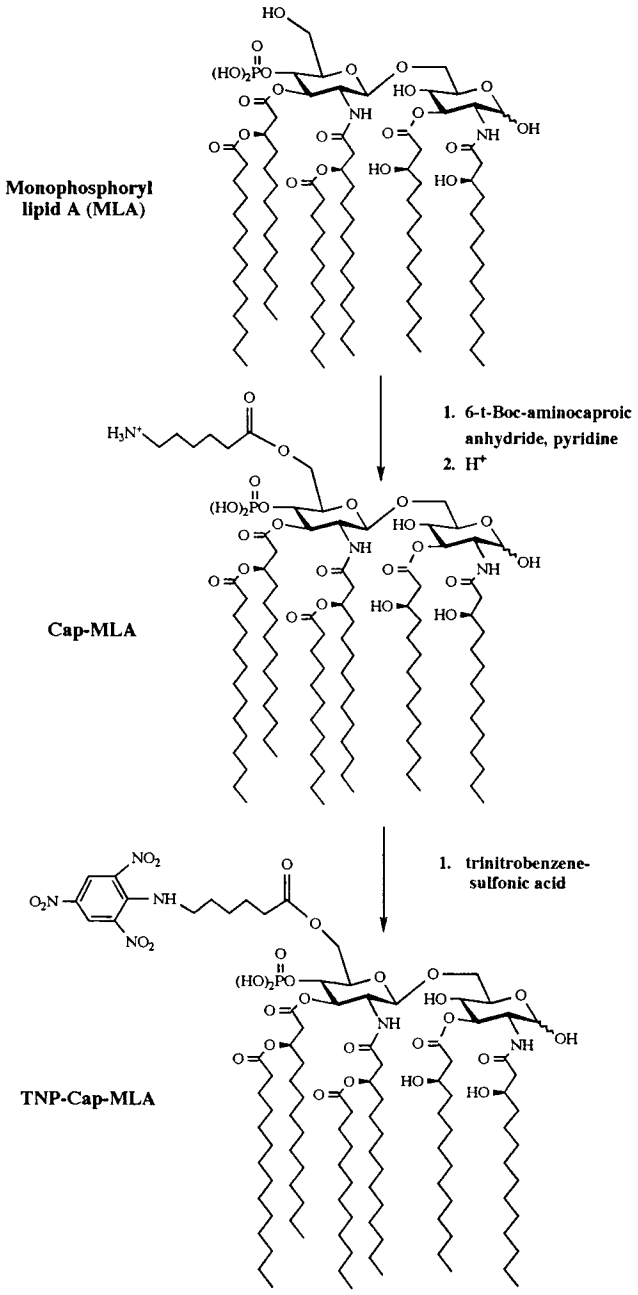

FIG. 2. Preparation of TNP-Cap-MLA. Reaction of MLA with the protected anhydride occurs primarily at the primary hydroxyl at the 6′ position (25). Following removal of the protecting group, Cap-MLA can be reacted with trinitrobenzenesulfonic acid as described in the text.

MATERIALS AND METHODS

Immunogens. (i) TNP-Cap-MLA. The procedure used to prepare TNP-Cap-MLA (see below) is summarized in Fig. 2. All manipulations involving trinitrophenylated materials were done in reduced light. Cap-MLA was prepared from *E. coli* J5 MLA as described by Myers et al. (25). Cap-MLA was trinitrophenylated by reaction with picrylsulfonic acid (trinitrobenzenesulfonic acid) in aqueous solution. Cap-MLA (10.9 mg) was first dispersed in 8.0 ml of water containing 0.125% (vol/vol) triethylamine, using sonication at 45°C to promote solubilization. To this solution were then added 1.95 ml of 0.5 M triethylammo-

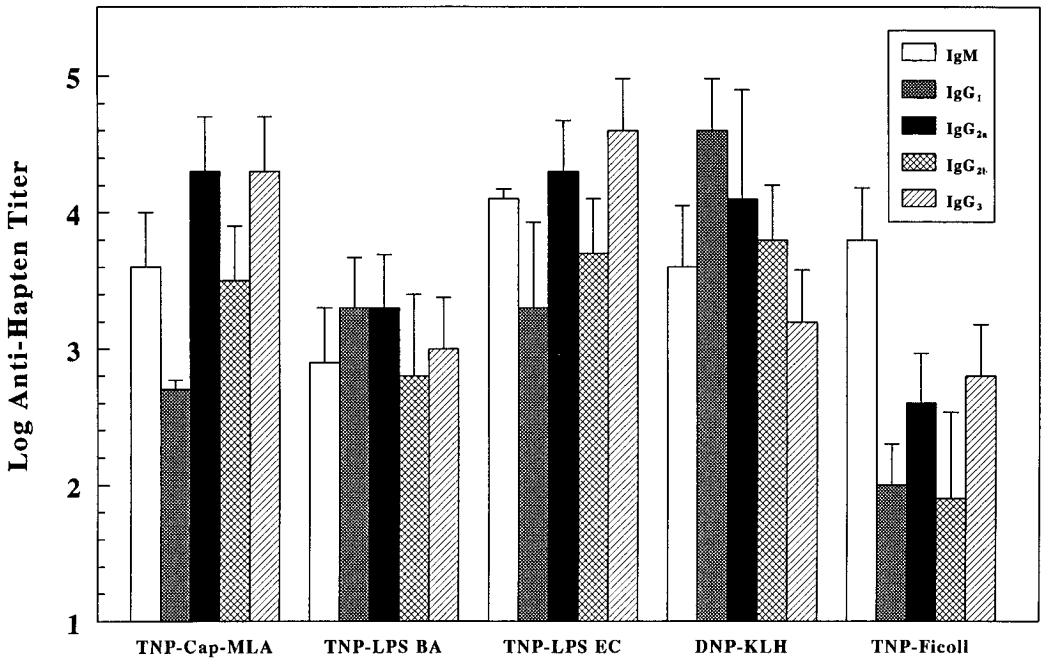

FIG. 3. Anti-TNP antibody responses in BALB/c mice. Mice were immunized three times at 2-week intervals and bled 5 days after the last immunization. The sera were tested for anti-TNP antibodies by ELISA. Results are expressed as the $\log_{10}$ of the ELISA titers.

nium bicarbonate (pH 7.7) and 0.975 ml picrylsulfonic acid (5% [wt/vol] aqueous solution; Sigma). The resulting solution was stirred at 37°C for 8 h, dialyzed (molecular weight cutoff of 1,000) against distilled water at 4°C, and lyophilized. The resulting residue was dissolved in 5.0 ml of chloroform-methanol (2:1 [vol/vol]) and washed with 2.0 ml of water, and the organic fraction was evaporated to dryness. The crude TNP-Cap-MLA was purified by preparative thin-layer chromatography on silica gel (Empore soft thin-layer chromatography plates; Analytichem), using chloroform-methanol-water-ammonium hydroxide (50:31:6:2 [by volume]) as the developing solvent. Sections of the plates containing the desired product were excised, and product was eluted with chloroform-methanol-water (65:25:4 [by volume]) followed by chloroform-methanol (2:1 [vol/vol]). The resulting solution was washed once with 0.1 N HCl followed by water and was then evaporated to dryness. The final yield was 9.9 mg of TNP-Cap-MLA. TNP content in the resulting TNP-Cap-MLA was determined from the A_{350}, using a molar extinction coefficient of 15,400 (29), and phosphorus was determined by colorimetric analysis (2). On the basis of these assays, the molar ratio of TNP to phosphorus in this preparation of TNP-Cap-MLA was 1.1 (expected value of 1.0). The preparation contained one TNP group per 4.4 kDa of molecular mass, based on both phosphorus and UV absorbance. (Compare with values for TNP-LPS conjugates given below.)

(ii) TNP-LPS EC. TNP-LPS derived from *E. coli* O55:B5 was purchased from List Biological Laboratories (Campbell, Calif.) and contained of 0.34 TNP group per 5 kDa of LPS by spectrophotometry.

(iii) TNP-LPS BA. LPS was obtained from cells of *B. abortus* 1119.3, which were graciously supplied by the U.S. Department of Agriculture (Ames, Iowa), by extraction with *n*-butanol–water followed by treatment with proteinase K (26). LPS BA was trinitrophenylated as described by Betts et al. (4) and contained 0.63 TNP group per 5 kDa of LPS by spectrophotometry.

(iv) DNP-KLH. *N*-2,4-Dinitrophenyl-β-alanylglycylglycyl-substituted keyhole limpet hemocyanin (DNP-KLH) was prepared as described previously (19–21).

(v) TNP-Ficoll. TNP-Ficoll was prepared from aminoethyl-carbamethyl-Ficoll as described by Betts et al. (4).

(vi) TNP-Cap. *N*-(2,4,6-trinitrophenyl)-6-aminohexanoic acid (TNP-Cap) was prepared by reaction of picrylsulfonic acid with 6-aminohexanoic acid in phosphate-buffered solution at pH 7. The reaction was allowed to proceed for 60 min, then the solution was acidified and extracted with chloroform-methanol (2:1 [vol/vol]), and the organic phase was evaporated to yield TNP-Cap.

Experimental protocol. BALB/c, BALB/c *nu/nu*, and (CBA/N × DBA/2) F_1 mice were purchased from Charles River Laboratories (Wilmington, Mass.). All mice were female and were 6 to 8 weeks old. LPS immunogens were first dispersed in water or 0.1% aqueous triethylamine with sonication at 40 to 45°C and then diluted with saline to the desired concentrations. Groups of five mice were immunized intraperitoneally with 50 μg of immunogen contained in 0.2 ml of saline. Mice were immunized three times at 2-week intervals and were bled via the tail vein on day 5 after each immunization. Control groups of each strain were injected with the separate conjugate components (i.e., MLA, LPS EC, LPS BA, MLA plus TNP-Cap, or TNP-Cap) and treated identically to the test groups. Each experiment was performed three times with similar results. A representative experiment is shown for each mouse strain.

Measurement of antibody titers. Enzyme-linked immunosorbent assays (ELISAs) were performed by using TNP-bovine serum albumin-coated plates as described by Betts et al. (4). Sera were added at different dilutions and detected by isotype-specific goat anti-mouse antibodies (anti-μ, anti-γ_1, anti-γ_{2a}, anti-γ_{2b}, and anti-γ_3) obtained from Southern Biotechnology Associates (Birmingham, Ala.). Log titers were obtained from the $\log_{10}$ of the endpoint titration, using serial five-fold or twofold dilutions of the sera. The endpoint titers represent the intercept of the linear part of the titration curve and the *x* axis. Serum samples from each mouse were assayed individually, and the results were averaged for each group. In all cases, the titers that are reported were obtained following the third injection.

Statistical analyses. All data are reported as the means ± standard deviations of the titers obtained in individual mice. Statistical significance was evaluated by using either the Wilcoxon signed rank (one-tailed) test or the Wilcoxon rank sum (two-tailed) test (8).

RESULTS AND DISCUSSION

TNP-Cap-MLA elicits anti-TNP antibody responses in BALB/c mice. It is well known that both LPS EC and LPS BA are effective carriers for the TNP hapten, in that both TNP-LPS EC and TNP-LPS BA are able to elicit strong anti-TNP antibody responses of all isotypes in normal mice (4, 35). However, these two types of LPS differ dramatically with respect to their endotoxic nature; LPS BA was found to be 300 to 10,000 times less potent than LPS EC in a variety of assays of endotoxic activity (14). This finding indicated that the ability of LPS to act as a carrier is not linked inseparably to its toxic activity. Like LPS BA, MLA is much less toxic than LPS EC (36, 38). It was therefore of interest to determine if MLA prepared from LPS EC would retain the ability of LPS to act as a carrier for the TNP hapten. To this end, an amino-containing derivative of MLA, Cap-MLA (25), was trinitrophenylated, and the resulting TNP-Cap-MLA (Fig. 2) was tested in parallel with TNP-LPS EC, TNP-LPS BA, DNP-KLH, and TNP-Ficoll in BALB/c mice. All of these haptenated materials elicited sig-

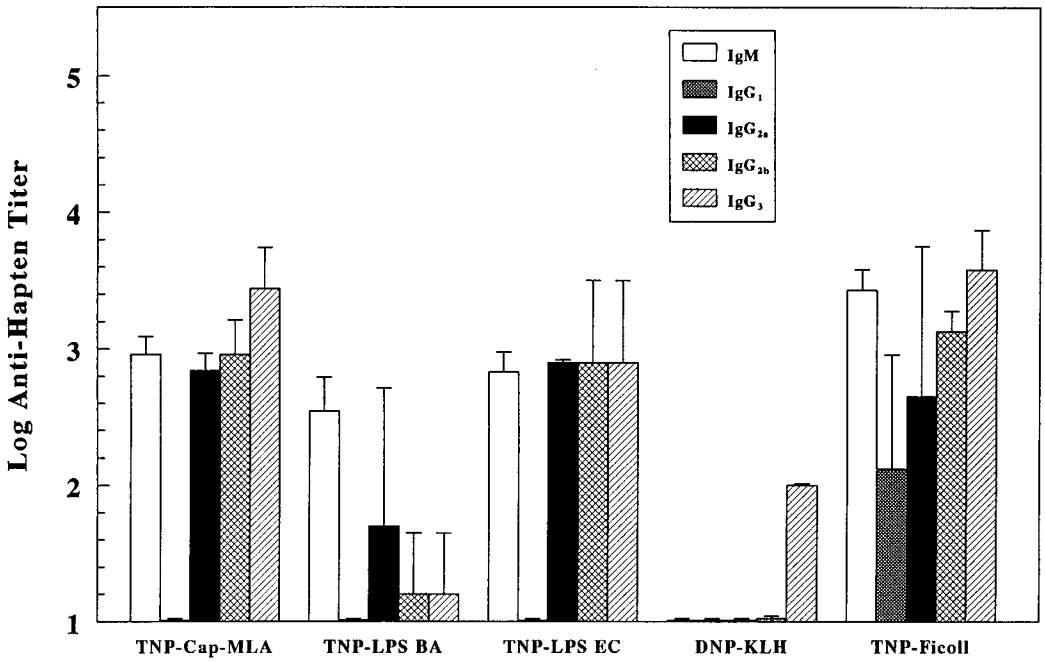

FIG. 4. Anti-TNP antibody responses in athymic (BALB/c *nu/nu*) mice. Mice were immunized three times at 2-week intervals and bled 5 days after the last immunization. The sera were tested for anti-TNP antibodies by ELISA. Results are expressed as the $\log_{10}$ of the ELISA titers.

nificant IgG and IgM anti-TNP responses (Fig. 3). The mean titer of IgG anti-TNP increased after each injection of TNP-Cap-MLA, from 800 to 6,400 to 23,400, suggesting that the number of responding precursor B cells was expanded in an exponential manner to form a memory pool. The profile of isotype titers induced by TNP-Cap-MLA closely resembled that obtained with TNP-LPS EC, suggesting that MLA does retain the ability of LPS EC to act as a carrier for TNP. Both conjugates induced higher levels of IgG2a than of IgG1, consistent with TI-1 antigen behavior ($P < 0.05$ for both conjugates). TNP-LPS BA also elicited significant anti-TNP responses, similar to results of previous experiments (4). As expected, IgM exceeded all IgG isotypes for the TI-2 antigen TNP-Ficoll. Unconjugated MLA, LPS EC, and LPS BA, tested in parallel with the conjugates, were unable to stimulate a detectable anti-TNP response (data not shown), indicating that the observed responses with the conjugates were not simply the result of polyclonal stimulation. Likewise, a mixture of MLA with TNP-Cap did not elicit anti-TNP antibodies (data not shown), indicating that MLA's carrier activity required direct association with TNP in order to be manifested. These results demonstrate that MLA, like LPS EC and LPS BA, is able to provide a strong carrier function for the TNP hapten.

TNP-Cap-MLA induces anti-TNP antibody responses in athymic mice. LPS EC and LPS BA are effective carriers for the TNP hapten in athymic mice, indicating that they are able to act as TI carriers (4, 35). The ability of MLA to act in a T-cell-independent manner was examined by immunizing groups of BALB/c *nu/nu* mice with TNP-Cap-MLA in parallel with the other previously defined immunogens (Fig. 4). DNP-KLH, a TD antigen, failed to induce detectable titers of IgM or of IgG isotypes other than IgG3. A low titer (mean = 100) of anti-TNP IgG3 was induced by this antigen in the experiment shown. In subsequent experiments ($n = 2$), DNP-KLH titers were not increased above those of mice immunized with saline. Thus, the low responsiveness overall against DNP-KLH was consistent with the athymic phenotype for these mice. The polysaccharide antigen TNP-Ficoll generated antibody titers of IgM and all IgG isotypes at levels comparable to those obtained with euthymic BALB/c mice (Fig. 3), as expected for a TI antigen. All of the LPS-derived antigens, including TNP-Cap-MLA, were able to induce measurable IgM and IgG anti-TNP titers in BALB/c *nu/nu* mice. The titers increased to some extent after each immunization. The IgM anti-TNP titer in response to TNP-Cap-MLA increased from 200 to 400 to 950, suggesting some expansion of the responding B-cell pool even in nude mice. The titers induced by TNP-Cap-MLA and TNP-LPS EC were again comparable for all isotypes and were somewhat below those obtained in BALB/c mice. The levels of IgM and IgG2a induced by TNP-LPS BA were not significantly different from those elicited by TNP-Cap-MLA and TNP-LPS EC (TNP-LPS BA versus TNP-Cap-MLA; $P > 0.05$), whereas the levels of IgG2b and IgG3 were lower for TNP-LPS BA (TNP-LPS BA versus TNP-Cap-MLA; IgG2b, $P < 0.01$, and IgG3, $P < 0.005$). As seen in BALB/c mice, no anti-TNP responses were observed in BALB/c *nu/nu* mice injected with MLA, MLA mixed with TNP-Cap, LPS EC, or LPS BA (data not shown).

None of the LPS-derived TNP conjugates gave rise to detectable titers of IgG1 in athymic mice (Fig. 4). On the other hand, these antigens were able to induce significant levels of IgG2a in the absence of a functional T-helper cell component. This pattern of isotype responses may reflect the abilities of these antigens to induce various cytokines involved in regulation of immune responses in athymic mice (11). It is known that interleukin-4 and gamma interferon (IFN-γ) have opposing influences on the immune response generated by antigens; interleukin-4 favors humoral responses dominated by IgG1 and IgE, whereas IFN-γ promotes cell-mediated immunity and stimulates a humoral response represented primarily by IgG2a (9). It has also been shown that natural killer cells can function in an antigen-independent manner as a source of IFN-γ (re-

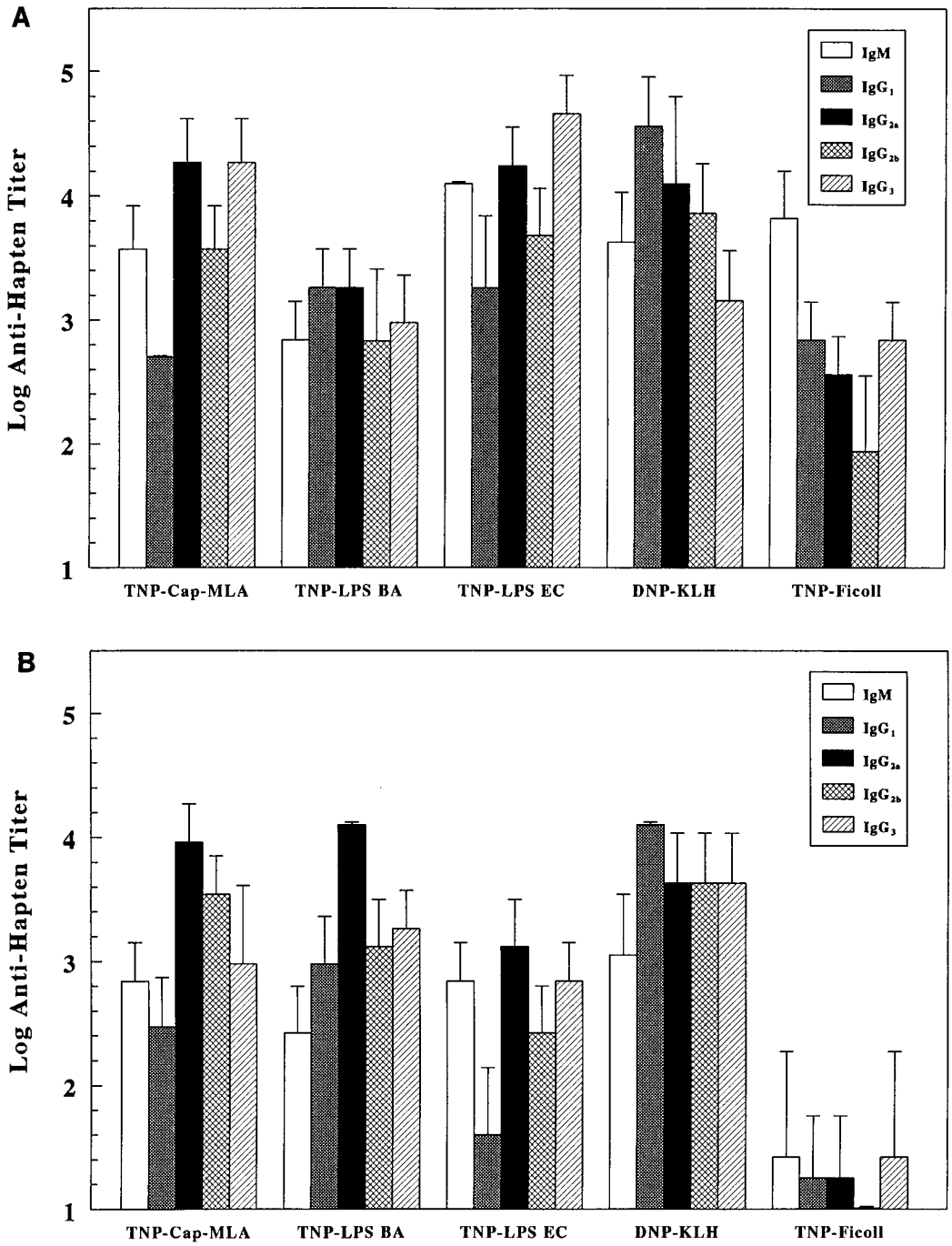

FIG. 5. Anti-TNP antibody responses in (CBA/N × DBA/2)F_1 mice. (A) Responses observed with female mice (normal phenotype); (B) responses observed with male mice (immunodeficient phenotype). Mice were immunized three times at 2-week intervals and bled 5 days after the last immunization. The sera were tested for anti-TNP antibodies by ELISA. Results are expressed as the $\log_{10}$ of the ELISA titers.

viewed in reference 16) and that both LPS (6) and MLA (17) are able to act in concert with tumor necrosis factor alpha to induce release of IFN-γ by natural killer cells. The results obtained for BALB/c *nu/nu* mice with TNP-Cap-MLA, TNP-LPS EC, and TNP-LPS BA are therefore consistent with the known abilities of LPS and MLA to induce IFN-γ from non-T-cell sources and also suggest that these carriers do not induce significant levels of interleukin-4 in athymic mice.

TNP-Cap-MLA stimulates an anti-TNP antibody response in Xid mice. TI antigens can be classified on the basis of their immunogenicity in CBA/N mice. This strain carries an X-linked genetic defect that renders the mice unresponsive to polysaccharide (TI-2) antigens but does not interfere with their response against lipopolysaccharide (TI-1) antigens. LPS EC and LPS BA both function as TI-1 carriers for the TNP hapten, since TNP-LPS EC and TNP-LPS BA are both able to stimulate significant anti-TNP antibody responses in CBA/N mice (4, 35). In this study, the carrier function of MLA was further

characterized by evaluating the anti-TNP response engendered by TNP-Cap-MLA in (CBA/N × DBA/2)F_1 female (normal phenotype) and male (immunodeficient phenotype) mice (Fig. 5). The results obtained with female (CBA/N × DBA/2)F_1 mice were essentially the same as those seen with BALB/c mice (compare Fig. 5A and 3) in that all of the antigens elicited anti-TNP antibodies of all isotypes. The responses observed with TNP-Cap-MLA and TNP-LPS EC were again very similar, with IgG2a more abundant than IgG1 for both antigens ($P < 0.1$ for TNP-LPS EC). As expected, the (CBA/N × DBA/2)F_1 male mice did not respond to the TI-2 antigen TNP-Ficoll (Fig. 5B), confirming that these animals were immunodeficient. The low titers of antibody seen with TNP-Ficoll were not significantly different from those observed with saline-immunized mice (data not shown). TNP-Cap-MLA, as well as the other antigens, induced significant anti-TNP responses in these mice. The LPS- and MLA-derived antigens all stimulated anti-TNP IgG2a more than IgG1, consistent with the expected behavior for TI-1 antigens (TNP-Cap-MLA, $P < 0.05$; TNP-LPS EC, $P < 0.05$). None of the carrier controls elicited detectable anti-TNP responses in either sex of (CBA/N × DBA/2)F_1 mice (data not shown).

In conclusion, the antibody responses induced by TNP-Cap-MLA were similar to those seen with TNP-LPS EC and TNP-LPS BA. All of these conjugates behaved as TI-1 antigens, since they stimulated immune responses in both athymic (BALB/c *nu*/*nu*) and Xid (male [CBA/N × DBA/2]F_1) mice. In contrast, the TD antigen, DNP-KLH, was relatively ineffective in eliciting anti-DNP antibody in athymic mice, and the TI-2 antigen, TNP-Ficoll, did not induce an anti-TNP response in male (CBA/N × DBA/2)F_1 mice. Thus, MLA is able to serve as a carrier for the TNP hapten in the same manner as LPS EC and LPS BA. In future experiments, small peptides will be conjugated to MLA to determine if MLA will provide carrier function even in T-cell-depleted mice. Positive results in such studies would provide a basis for using such peptide-MLA constructs to develop a vaccine for human immunodeficiency virus-infected persons who have impaired $CD4^+$ T-cell function. This approach is supported by the encouraging safety and activity results obtained from preliminary studies with the clinical form of MLA (i.e., MPL immunostimulant) in humans.

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