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Synthetic Toll-Like Receptor 4 Agonists Stimulate Innate Resistance to Infectious Challenge

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A compound family of synthetic lipid A mimetics (termed the aminoalkyl glucosaminide phosphates [AGPs]) was evaluated in murine infectious disease models of protection against challenge with *Listeria monocytogenes* and influenza virus. For the *Listeria* model, intravenous administration of AGPs was followed by intravenous bacterial challenge 24 h later. Spleens were harvested 2 days postchallenge for the enumeration of CFU. For the influenza virus model, mice were challenged with virus via the intranasal/intrapulmonary route 48 h after intranasal/intrapulmonary administration of AGPs. The severity of disease was assessed daily for 3 weeks following challenge. Several types of AGPs provided strong protection against influenza virus or *Listeria* challenge in wild-type mice, but they were inactive in the C3H/HeJ mouse, demonstrating the dependence of the AGPs on toll-like receptor 4 (TLR4) signaling for the protective effect. Structure-activity relationship studies showed that the activation of innate immune effectors by AGPs depends primarily on the lengths of the secondary acyl chains within the three acyl-oxy-acyl residues and also on the nature of the functional group attached to the aglycon component. We conclude that the administration of synthetic TLR4 agonists provides rapid pharmacologic induction of innate resistance to infectious challenge by two different pathogen classes, that this effect is mediated via TLR4, and that structural differences between AGPs can have dramatic effects on agonist activity in vivo.

The toll-like receptors (TLRs) comprise an evolutionarily conserved receptor family that is capable of detecting and responding to microbial challenge (1). The TLRs recognize a variety of pathogen-specific components, including lipopolysaccharide (LPS), CpG DNA, and microbial membrane and cell wall components (20). Toll-like receptor 4 (TLR4) is critical for the recognition of LPS (30, 31), and considerable progress has recently been made in understanding the interaction of TLR4 with critical accessory molecules implicated in LPS recognition (8, 9, 17, 21, 22, 32, 40, 41). In this regard, it appears that LPS binding protein (LBP) promotes the binding of LPS to CD14, which in turn facilitates the association of the lipid A component of LPS with MD-2 to form a soluble complex that serves as an activating ligand for TLR4 on the cell surface. Binding of the MD-2/LPS complex to TLR4 results in the aggregation of TLR4 into lipid rafts and the activation of several distinct intracellular signaling pathways that results in increased transcription of many genes encoding cytokines, defensins, chemokines, and alpha/beta interferons (28, 38). These effector molecules determine a wide range of biological activities, including further production of cytokines, enhancement of microbicidal activity of phagocytic cells, and migration/maturation of dendritic cells (5, 23, 36).

Before the discovery that LPS interacts with TLR4, evidence demonstrating the importance of innate immune activation in controlling infection with gram-negative bacteria was obtained in studies of C3H/HeJ mice. These mice harbor a signaling defect in TLR4 that renders them LPS hyporesponsive (30, 31). *Salmonella enterica* serovar Typhimurium-induced morbidity and mortality occurs at much lower doses for C3H/HeJ mice than wild-type mice (27), presumably because C3H/HeJ mice are unable to mount innate immune responses early enough to control the infectious burden in the period prior to the development of adaptive responses (33). Similar results have been obtained when C3H/HeJ mice were evaluated in models of infection with *E. coli* (10), *Neisseria meningitidis* (42), and *Francisella tularensis* (18).

Administration of purified LPS has been found to confer prophylactic protection from subsequent bacterial or viral challenge in various animal models (4, 25), presumably via stimulation of innate immunity. Recently, the intrauterine administration of LPS in cattle was shown to facilitate clearance of chronic intrauterine infections associated with infertility (34). However, despite these potentially beneficial effects, the pharmacologic use of purified LPS (or lipid A) is precluded by its extreme toxicity; LPS is highly pyrogenic and promotes systemic inflammatory response syndrome (12). In an effort to uncouple the immunomodulatory effects of lipid A from its toxicity, Masihi et al. (19) developed monophosphoryl lipid A (MLA); MLA comprises the lipid A portion of LPS from which the (*R*)-3-hydroxytetradecanoyl group and the 1-phosphate have been removed (24) by successive acid and base hydrolysis. LPS and MLA induce similar cytokine profiles, but MLA is at least 100-fold less toxic (24, 39). MLA, the active ingredient of

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MPL adjuvant, has been administered in over 273,000 doses in human clinical studies of the next generation of vaccine adjuvants (6). MLA alone or in combination with other molecules elicits protective responses when administered prior to challenge with pathogenic viruses (influenza virus), bacteria (*Listeria monocytogenes* and *Salmonella enterica* serovar Enteritidis), or parasites (*Toxoplasma gondii*) (19, 29, 39). MLA is a natural biological product composed of multiple structurally distinct pharmacophores. The intrinsic heterogeneity of MLA hinders analysis of the structural features responsible for induction of innate immune responses.

We report here the results of in vivo structure-function studies of a family of synthetic lipid A mimetics, the aminoalkyl glucosaminide phosphates (AGPs) (13, 14). Intravenous or intranasal/intrapulmonary administration of AGPs was evaluated in standardized murine models of nonspecific resistance to bacterial and viral challenges. Our findings indicate that the ability of the AGPs to induce protective innate immune responses is TLR4 dependent and that the biological activity of these molecules is dependent on specific structural features.

MATERIALS AND METHODS

Mice. Female BALB/c, C3H/HeOuJ, and C3H/HeJ mice were obtained from Jackson Laboratories, Bar Harbor, Maine. Mice were 7 to 9 weeks of age when the experiments were initiated. Mice were anesthetized for treatment or challenge by intraperitoneal administration of a preparation containing ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg). All animals were used in accordance with guidelines established by the Public Health Service and the Institutional Animal Care and Use Committee at Corixa Corporation, Hamilton, Mont.

AGPs. All AGPs were synthesized at Corixa Corp., Hamilton, Mont., as previously described (13). Stock solutions (1 mg/ml) of AGPs (presented as CRX compounds) were made in 0.2% triethanolamine (TEOA; Sigma) (pH 7). Dilutions were made in sterile phosphate-buffered saline (PBS) for influenza studies or TEOA for *Listeria* studies. For intravenous administration, AGPs were injected into the lateral tail vein (BALB/c mice) or retro-orbital venous plexus (C3H mice) in a volume of 100 μl. For intranasal/intrapulmonary administration, a manual pipettor was used to place 20 μl of AGP preparations up the noses (10 μl per nostril) of anesthetized mice lying on their backs.

***Listeria monocytogenes* challenge model.** An aliquot of *Listeria monocytogenes* (serotype 10403; originally obtained from M. L. Gray, Montana State University) containing $\sim 5 \times 10^9$ bacteria (stored at −70°C) was added to 24 ml of sterile brain heart infusion broth (Gibco) and incubated in a shaking water bath at 37°C until mid-log growth phase (approximately 3 h to achieve an optical density of 0.3 at 550 nM, equivalent to $\sim 10^9$ bacteria/ml). Serial dilutions (10-fold) of the *L. monocytogenes* culture were made in sterile PBS. One hundred microliters of the 10^3/ml dilution was spread on each of three tryptic soy agar plates to confirm bacterial concentration and lack of contamination from other organisms. For intravenous challenge, 100 μl of the 10^6/ml dilution was administered via the tail vein (BALB/c mice) or retro-orbital plexus (C3H mice) to provide a challenge dose of $\sim 10^5$ *L. monocytogenes* bacteria.

Listeria-infected mice were euthanized by CO_2 overdose 2 days after challenge, and spleens were harvested and placed in 4 ml of sterile PBS (16- by 100-mm glass tubes). Spleens were homogenized for 10 s using an Ultra-Turrax T25 probe homogenizer (Junkel & Kunkel). Five 10-fold serial dilutions of splenic homogenates were generated, and 100 μl of each dilution was spread on a 10-cm-diameter tryptic soy agar plate (Remel Cat 01917). The plates were incubated at room temperature, and *Listeria* CFU were counted 72 h later. Protection was defined as the difference between the mean $\log_{10}$ number of bacteria per spleen in the treatment groups and the mean $\log_{10}$ number of bacteria per spleen in the control group treated with vehicle alone.

Influenza virus challenge model. Stock influenza virus A/HK/68 (H3N2), originally obtained from Philip Wyde (Baylor College of Medicine, Houston, TX), was prepared by passage through mice and stored as 1-ml aliquots at −70°C. For intranasal delivery of AGPs or influenza virus, BALB/c mice were anesthetized and 20-μl volumes containing five 50% lethal doses of influenza virus were administered (10 μl/nostril) using a manual pipettor. Mice were monitored for mortality, clinical signs of disease, and weight change for 21 days following viral challenge.

The clinical symptoms observed for disease severity were recorded as disease index scores and included ruffled fur, labored breathing, and hunched posture. An arbitrary scale of 0 to 3 was used to score severity of disease. The disease index presented in the figures is an average value for all mice within a group for the 21-day period following viral challenge. The weight of the mice is a cumulative value for all the mice in a group on each day the weights were determined. Survival rates are presented as the percentages of mice in each group that were alive at the end of the 21-day period following viral challenge. Mice alive at the end of the test period were considered to be fully recovered.

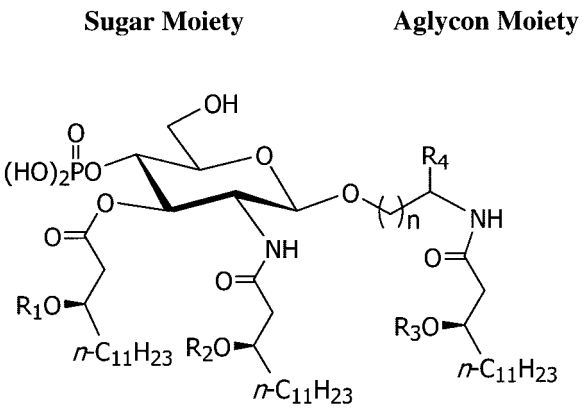

FIG. 1. General structure of AGPs.

RESULTS

Structural features of AGPs. Previous in vitro studies in our laboratory identified important structural requirements for signaling through TLR4 by AGPs (35). In the current study, the structural correlates of protective activity mediated by AGPs were investigated in infectious challenge models of bacterial (*Listeria monocytogenes*) and viral (influenza virus) agents. The synthesis of the AGPs, all of which are composed of a monosaccharide unit with an N-acylated aminoalkyl aglycon spacer arm, has been described previously (13), and the general structure is shown in Fig. 1. The primary acyl chain length was fixed at 14 carbons for all of the AGPs. The biological effects of variations in secondary acyl chain lengths (R1, R2, and R3) (Fig. 1) and the aglycon component (Fig. 1) were the primary focus of this study.

Requirement of TLR4 for activation of innate immune effectors by AGPs. TLR4 has been identified as an integral component of the LPS receptor complex comprising TLR4, MD-2, and CD14 (1). Mouse strains with mutations in the gene encoding the cytoplasmic signaling domain of TLR4, such as C3H/HeJ, are hyporesponsive to LPS stimulation. This feature was exploited in order to evaluate the requirement for functional TLR4 expression in AGP-mediated enhancement of innate immunity. Groups of C3H/HeJ or wild-type C3H/HeOuH mice were injected via the intravenous route with CRX-524 (see structure [Fig. 1 and Table 1]) in a 0.2% TEOA vehicle or with vehicle alone and challenged intravenously 48 h later with $\sim 10^5$ live *Listeria monocytogenes* bacteria. Two days after challenge, the mice were sacrificed for the determination of splenic CFU. The average number of splenic CFU ($\log_{10}$ value) for CRX-524-treated groups was subtracted from the average

TABLE 1. Specific structures of AGPs

AGP	Chain length (no. of carbon atoms)			R4 functional group	Length of aglycon spacer[a]
	R1	R2	R3		
CRX-526	6	6	6	CO_2H	2
CRX-554	7	7	7	CO_2H	2
CRX-555	8	8	8	CO_2H	2
CRX-537	9	9	9	CO_2H	2
CRX-527	10	10	10	CO_2H	2
CRX-538	11	11	11	CO_2H	2
CRX-560	12	12	12	CO_2H	2
CRX-512	14	14	14	CO_2H	2
CRX-566	6	10	10	CO_2H	2
CRX-565	10	6	10	CO_2H	2
CRX-569	10	10	6	CO_2H	2
CRX-568	6	6	10	CO_2H	2
CRX-567	6	10	6	CO_2H	2
CRX-570	10	6	6	CO_2H	2
CRX-529	14	14	14	H	2
CRX-525	14	14	14	H	3
CRX-557	14	14	14	H	4
CRX-571	14	14	14	H	6
CRX-524	10	10	10	H	2
CRX-522	10	10	10	$CONH_2$	2
CRX-545	10	10	10	CH_2OH	2
CRX-573	10	10	10	$CH_2CH_2CO_2H$	2

[a] Lengths of aglycon spacers are given as numbers of carbon atoms (n + 1).

number obtained for the vehicle-treated control group to determine the magnitude of protection (Fig. 2A).

CRX-524-mediated induction of resistance to *Listeria* challenge was shown to be dependent on the presence of functional TLR4, since this AGP elicited strong protection in wild-type C3H/HeOuJ mice but not in the TLR4 mutant C3H/HeJ mice (Fig. 2A). Similar results were observed for the influenza virus system, in which the intranasal administration of CRX-524 2 days prior to intranasal challenge with a lethal dose of influenza virus induced protection in C3H/HeOuJ mice but not in C3H/HeJ mice (Fig. 2B). We have conducted similar experiments to show that the immunostimulatory activities of other AGPs also depend on the expression of functional TLR4 (data not shown), implicating a general requirement of TLR4 for activation of the innate immune system by this family of lipid A mimetics.

Effects of secondary acyl chain length on induction of innate resistance to infectious challenge. A family of AGP molecules with an L-serine-based aglycon unit (referred to as the L-seryl family) was used to investigate the influence of secondary acyl chain length on the ability of lipid A-related molecules to induce enhanced innate immune function in vivo. As is the case for all AGPs used in the experiments described in this report, each of the three acyl-oxy-acyl residues on the L-seryl AGPs have 14-carbon (tetradecanoyl or myristoyl) acyl chains in the primary positions (Fig. 1). The compounds used in these experiments contained acyl-oxy-acyl residues with 6-, 7-, 8-, 9-, 10-, 11-, 12-, or 14-carbon acyl chains at all three (R1 through R3) secondary positions (Table 1). In the *Listeria* system, L-seryl AGPs in 0.2% TEOA and TEOA alone (vehicle control) were tested for their abilities to induce protection from intravenous challenge 24 h after intravenous administration as described above. In the influenza virus system, L-seryl AGPs were administered to anesthetized mice via the intranasal route. Two days later, the mice were anesthetized again and challenged with influenza virus via the same route. Morbidity (body weight and disease index) and mortality (percent survival) were documented for 21 days following challenge. All mice surviving at day 21 recovered completely. The influenza virus model highlights the capacity of TLR4 agonists for stimulating early innate immune responses that limit the initial spread of infection, while other TLR-mediated events promote the development of subsequent acquired immune responses. Protective AGPs mediate innate responses that limit the level of virus production during the initial 96 h of infection but do not generally interfere with the host's capacity for mounting antigen-specific adaptive immunity (data not shown).

The level of protection for the AGPs with different-length acyl chains at the R1, R2, and R3 positions is presented for the *Listeria monocytogenes* (Fig. 3A) and influenza virus (Fig. 3B) systems. The striking differences in the protective abilities of AGPs were found to depend heavily on secondary acyl chain length; compounds with 6-carbon (CRX-526) or 7-carbon (CRX-554) acyl chains were essentially inactive, whereas compounds with 10-carbon (CRX-527), 11-carbon (CRX-538), or 12-carbon (CRX-560) acyl chains were highly active. The compound with 14-carbon (CRX-512) acyl chains was found to be active in both systems, though the potency of this compound was diminished relative to the compounds with 10- to 12-carbon acyl chains, since a larger dose (20 μg) was required to obtain maximal protection.

Relative positional importance of secondary acyl chains. To evaluate the relative positional importance of the three secondary acyl-oxy-acyl residues with regard to activation of innate immune effectors, a series of L-seryl compounds in which one of the maximally active 10-carbon acyl chains at R1, R2, or R3 was replaced by an "inactive" 6-carbon acyl chain (CRX-566, CRX-565, or CRX-569, respectively) was evaluated (Table 1). In addition, three AGPs in which two of the three acyl chains at R1, R2, or R3 were replaced by six-carbon acyl chains were synthesized (CRX-568 has six-carbon acyl chains at R1 and R2; CRX-570 has six-carbon acyl chains at R2 and R3; and CRX-567 has six-carbon acyl chains at R1 and R3).

These six hybrid AGPs were evaluated for their capacities for inducing protection against intravenous challenge with *Listeria monocytogenes* or influenza virus. The AGPs with six-carbon acyl chains in two of the three secondary positions (CRX-567, CRX-568, and CRX-570) induced little to no protection in either system (Fig. 4). In the *Listeria* system, the compound with a single six-carbon acyl chain at R1 (CRX-566) was relatively inactive, inducing only slightly better protection than the inactive compounds containing two or three six-carbon secondary acyl chains. CRX-565 and CRX-569, containing a single 6-carbon secondary acyl chain substitution at R2 and R3, respectively, induced significant protection, though the activity of these compounds was reduced compared to the maximally active compound with 10-carbon acyl chains at all three secondary positions (CRX-527) (Fig. 4A). The same general pattern held for the influenza virus system, though only CRX-569, with a single six-carbon acyl chain at R3, induced better protection than the compounds containing two six-carbon acyl chains (Fig. 4B).

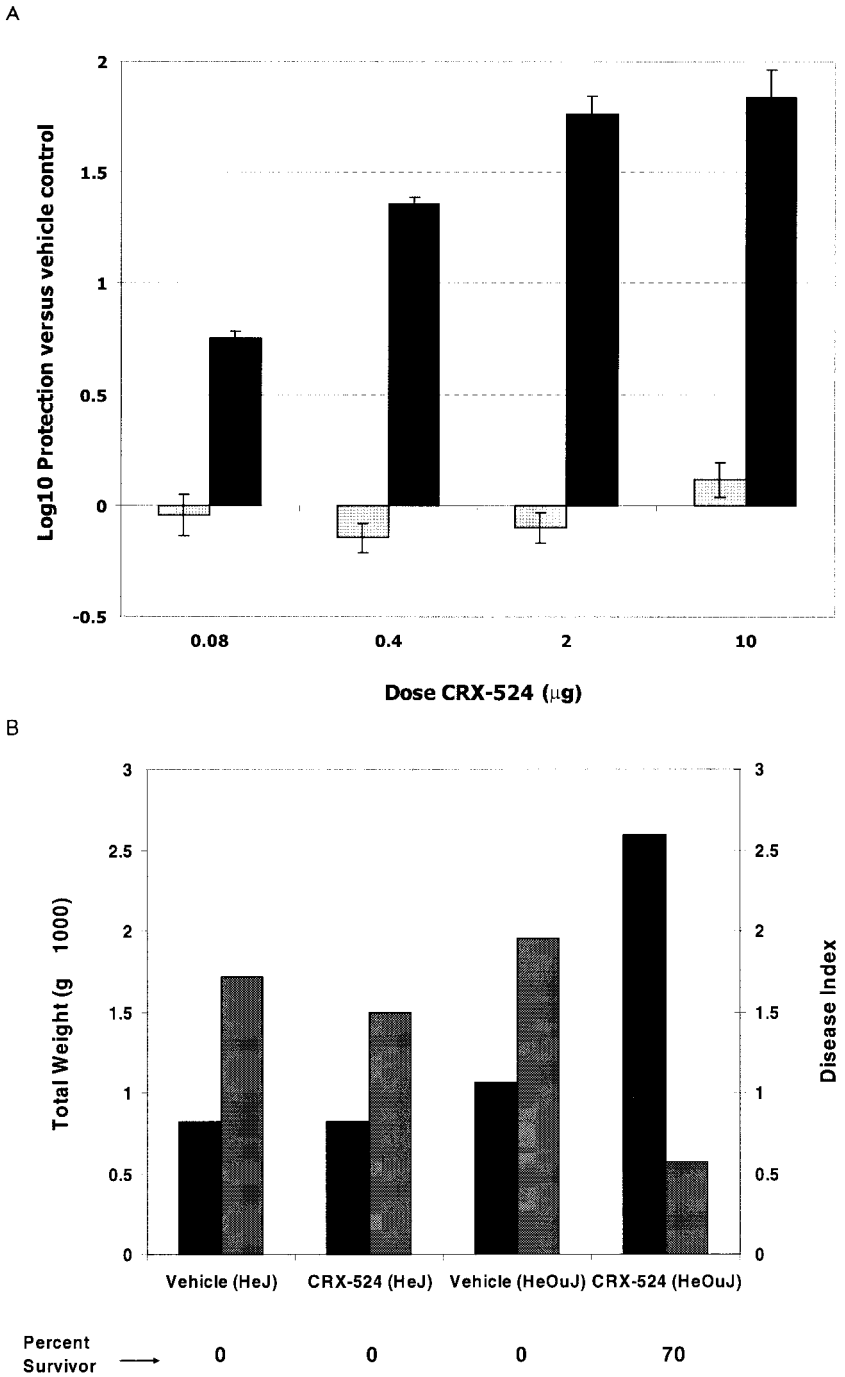

FIG. 2. (A) Induction of nonspecific resistance to challenge with *Listeria monocytogenes* by intravenous pretreatment of TLR4 mutant C3H/HeJ mice (± standard errors of the means [SEM]; hatched bars) or wild-type C3H/HeOUJ mice (plus SEM; filled bars) with CRX-524 (five mice per group). The data are presented as $\log_{10}$ protection versus vehicle control on the *y* axis. The average $\log_{10}$ numbers of splenic CFU in vehicle-treated control mice ± SEM were 7.46 ± 0.08 for C3H/HeJ and 7.61 ± 0.08 for C3H/HeOuJ (no significant difference between C3H/HeJ and C3H/HeOuJ in this regard by Student's *t* test). Compared to vehicle-treated mice, the number of splenic CFU was significantly lower in CRX-524-treated C3H/HeOuJ mice (all doses tested) but not in CRX-524-treated C3H/HeJ mice ($P < 0.05$; Student's *t* test). (B) Induction of nonspecific resistance to intranasal influenza virus challenge by intranasal pretreatment of TLR4 mutant C3H/HeJ mice or wild-type C3H/HeOuJ mice with CRX-524 (five mice per group). Anesthetized mice received 5 μg of CRX-524 in a 20-μl volume via the intranasal/intrapulmonary route. Filled bars, total weights; hatched bars, disease index scores. Percent survival for each group 21 days after infection is presented at the bottom of the graph. Treatment with CRX-524 resulted in a significant increase in survival of C3H/HeOuJ mice but not C3H/HeJ mice, relative to vehicle treatment ($P < 0.05$; Fisher's exact test).

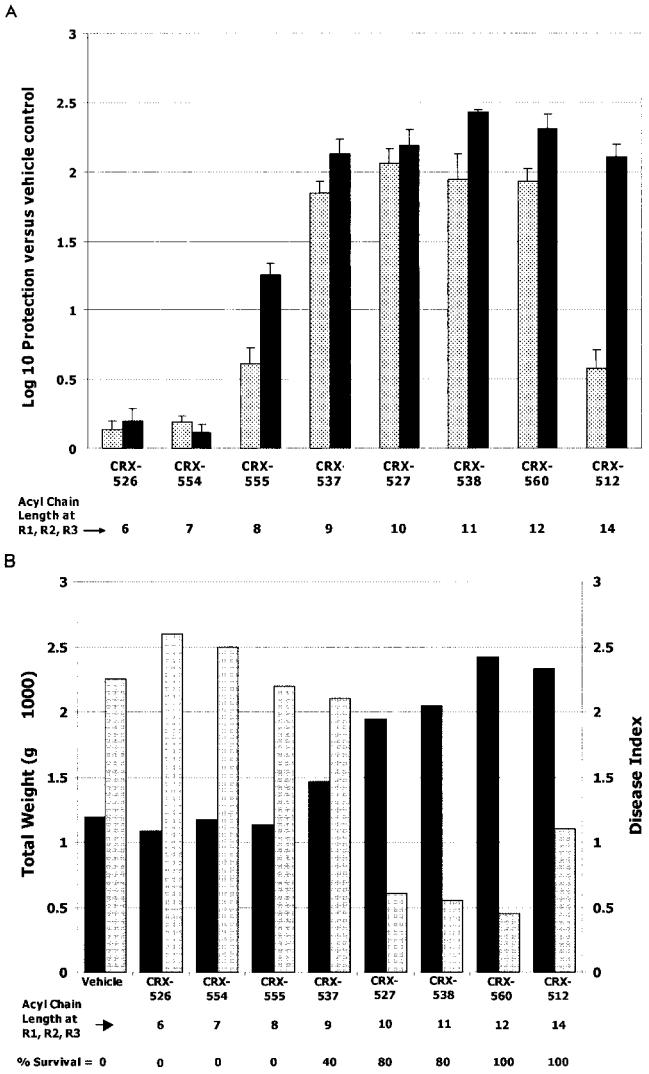

FIG. 3. (A) Induction of nonspecific resistance to challenge with *Listeria monocytogenes* (plus SEM [error bars]) by intravenous pretreatment with AGP compounds (hatched bars, 1-μg dose; filled bars, 20-μg dose) that differ in acyl chain length at positions R1, R2, and R3 (five BALB/c mice per group). The average $\log_{10}$ number of splenic CFU in vehicle-treated control mice ± SEM was 7.51 ± 0.13. At either dose tested, only CRX-526 and CRX-554 failed to result in significantly lower splenic CFU than the vehicle control ($P < 0.05$; Student's *t* test). (B) Induction of nonspecific resistance to intranasal influenza virus challenge by intranasal pretreatment with AGP compounds that differ in acyl chain lengths at positions R1, R2, and R3 (five BALB/c mice per group). Filled bars, total weights; hatched bars, disease index scores. Percent survival for each group 21 days after infection is presented at the bottom of the graph. Treatment with AGPs having secondary fatty acids containing nine or more carbon atoms resulted in a significant increase in survival relative to vehicle treatment ($P < 0.05$; Fisher's exact test).

Importance of a negatively charged functional group at position R4 on the aglycon component of AGPs. AGPs differing only in the chemical composition of the functional group R4 (Fig. 1 and Table 1) within the aglycon portion of the molecule were used to further evaluate the structure-activity relationships of these synthetic TLR4 agonists. All AGPs for this experiment contained 14-carbon primary acyl chains and 10-carbon secondary acyl chains at R1, R2, and R3. The aminoalkyl AGP (CRX-524) carries a hydrogen atom at R4, while the L-seryl AGP (CRX-527) contains a carboxyl residue at this position. The other AGPs tested in this experiment contain a carboxamide residue (CRX-522), a hydroxymethyl residue (CRX-545), or a carboxyethyl residue (CRX-573) at R4. At a dose of 1 μg, CRX-527 was found to induce significantly better protection than the other four compounds (Fig. 5). Consistent with this result was the finding that the dose of CRX-527 required to prevent death in 50% of mice challenged with a lethal dose of *Listeria* (the 50% effective dose) was 10 to 20 times lower than the 50% effective dose for any of the four other compounds (data not shown).

Importance of acyl-oxy-acyl juxtaposition. A family of AGPs with an ω-aminoalkanol-based aglycon unit (termed the aminoalkyl family) whose members differ in the length of the carbon-based linker arm between the sugar moiety and aglycon amino group (Table 1) was used to evaluate the importance of the relative proximity of the three acyl-oxy-acyl residues and/or the conformation of the AGP backbone on the ability of these compounds to induce innate immune responses. Members of the aminoalkyl AGP family have two-carbon atom (CRX-529), three-carbon atom (CRX-525), four-carbon atom (CRX-557), or six-carbon atom (CRX-571) linkers between the sugar moiety (containing two acyl-oxy-acyl residues) and the aglycon unit (containing the third acyl-oxy-acyl residue). These compounds were compared with regard to their capacities for inducing innate resistance to challenge with *Listeria*.

The compounds used in this experiment all contain 14-carbon secondary acyl chains at R1, R2, and R3. Because a 1-μg dose of AGPs with 14-carbon secondary acyl chains has consistently been found to elicit significantly lower levels of protection than 1 μg of otherwise identical compounds containing 10-carbon secondary acyl chains (Fig. 3A), lower levels of protection (ca. 0.5 $\log_{10}$ units) induced by even the most potent of these AGPs was not unexpected (Fig. 6). Despite this limitation, the experiment demonstrated that the level of protection diminishes as the linker length between the sugar moiety and the aglycon unit is increased. Whereas the AGP with a two-carbon linker afforded the greatest level of protection, compounds with linkers of three to six carbons provided successively lower levels of protection. The lower activity of the compounds with longer linkers could be due to the structural alterations in the AGP backbone, changes in the spatial orientation of the acyl-oxy-acyl residues, or both.

DISCUSSION

LPS and monophosphoryl lipid A were both previously shown to enhance nonspecific resistance to challenges with viral and bacterial pathogens, albeit by an unknown mechanism (19, 39). We now know that both molecules work by activation of TLR4, which presumably evolved to recognize LPS as a danger signal and to activate appropriate innate and adaptive immune responses (1). TLR4 activation leads to the initiation of the MyD88-dependent intracellular signaling pathway, which results in the activation of NF-κB, a transcription factor responsible for promoting, among other things, the production of multiple inflammatory cytokines (e.g., interleukin 6 [IL-6], IL-8, and IL-12). LPS exposure also results in the

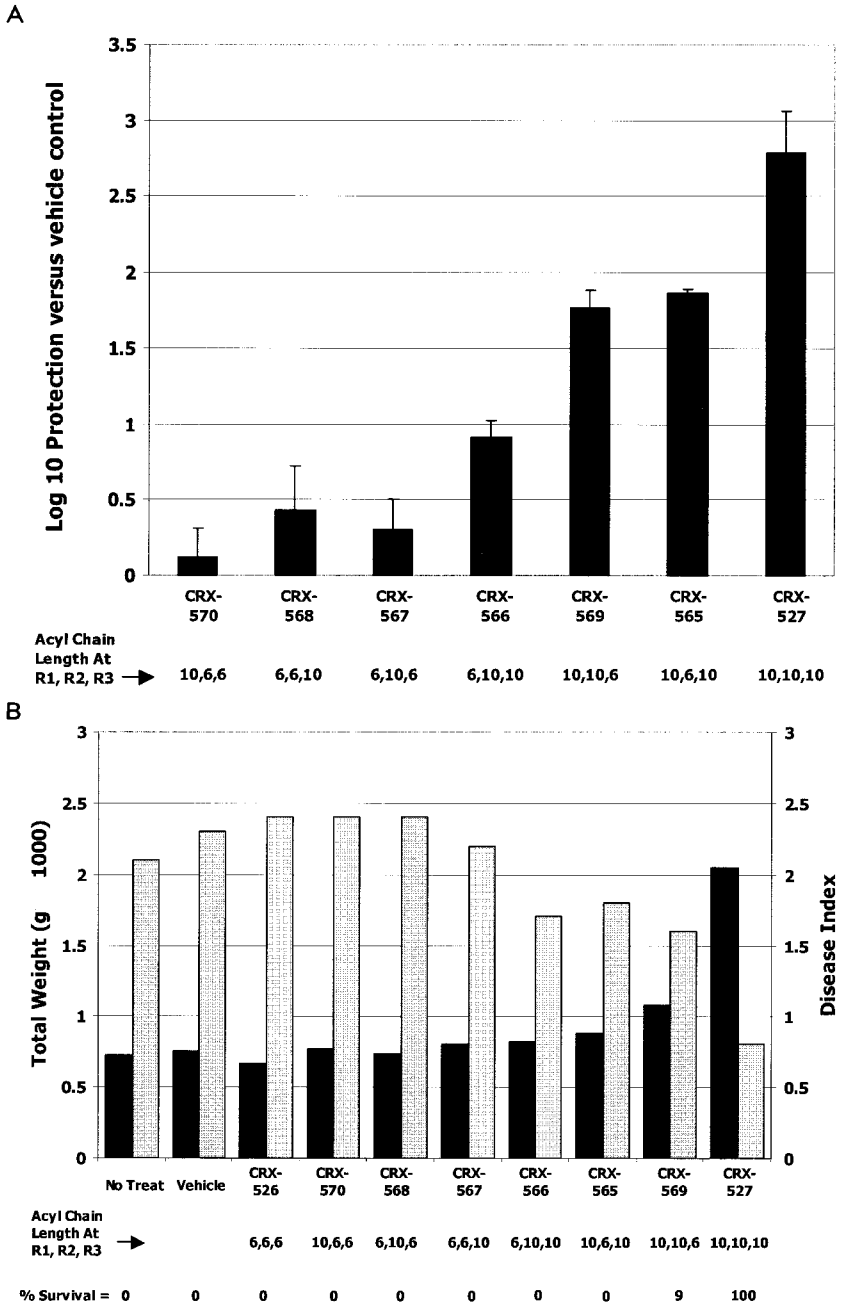

FIG. 4. (A) Induction of nonspecific resistance to challenge with *Listeria monocytogenes* (plus SEM [error bars]) by intravenous pretreatment with AGP compounds (1-μg dose) that have one or two inactive six-carbon acyl chains at positions R1, R2, and/or R3 (five BALB/c mice per group). The average $\log_{10}$ number of splenic CFU in vehicle-treated control mice ± SEM was 7.38 ± 0.17. Relative to vehicle-treated mice, splenic CFU were significantly lower in mice treated with AGPs containing zero or one six-carbon secondary fatty acid but not in mice treated with compounds containing two six-carbon secondary fatty acids ($P < 0.05$; Student's t test). (B) Induction of nonspecific resistance to intranasal influenza virus challenge by intranasal pretreatment with AGP compounds that differ in acyl chain lengths at positions R1, R2, and R3 (five BALB/c mice per group). Filled bars, total weights; hatched bars, disease index scores. Percent survival for each group 21 days after infection is presented at the bottom of the graph. Only CRX-527 treatment resulted in a significant increase in survival relative to vehicle treatment ($P < 0.05$; Fisher's exact test). No treat, no treatment.

production of defensins, which comprise several distinct families of antibacterial, antifungal, and antiviral peptides (2). In addition to activating the MyD88-dependent signaling, TLR3 and TLR4 agonists activate the TRIF-dependent pathway (also known as the MyD88-independent pathway), which results in the expression of interferon regulatory factor 3 and production of alpha/beta interferons (particularly beta interferon [37]), as well as the activation of mitogen-activated protein kinases and NF-κB (16), production of inducible nitric oxide synthase, and functional maturation of dendritic cells (1).

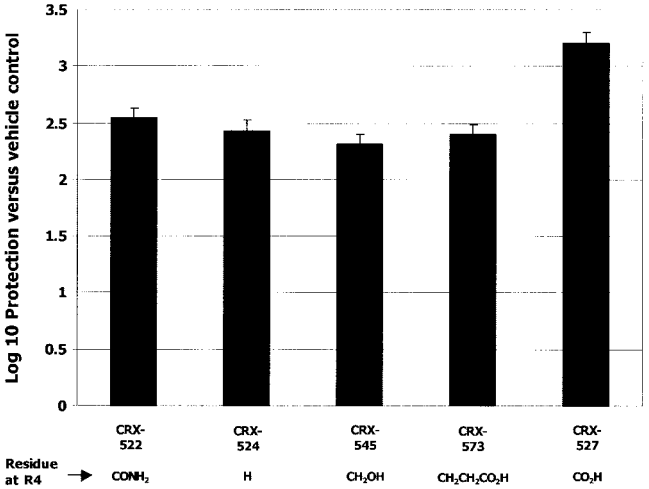

FIG. 5. Induction of nonspecific resistance to challenge with *Listeria monocytogenes* (plus SEM [error bars]) by intravenous pretreatment with AGP compounds (1-μg dose) that have various functional groups at position R4 on the aglycon unit (five BALB/c mice per group). The average $\log_{10}$ number of splenic CFU in vehicle-treated control mice ± SEM was 7.98 ± 0.13. All compounds tested resulted in significantly lower numbers of splenic CFU than the vehicle control ($P < 0.05$ using Student's *t* test).

Induction of beta interferon appears to be crucial for the antiviral effects associated with activation of TLR3 and TLR4, since mice deficient in TRIF are highly susceptible to viral infection (11). Mice deficient in either MyD88 or TRIF produce lower levels of immune effectors, and mice deficient in both are completely nonresponsive to LPS (45). Thus, TLR4 activation results in short-term antimicrobial effects, which can be observed within minutes to hours of exposure to microbial

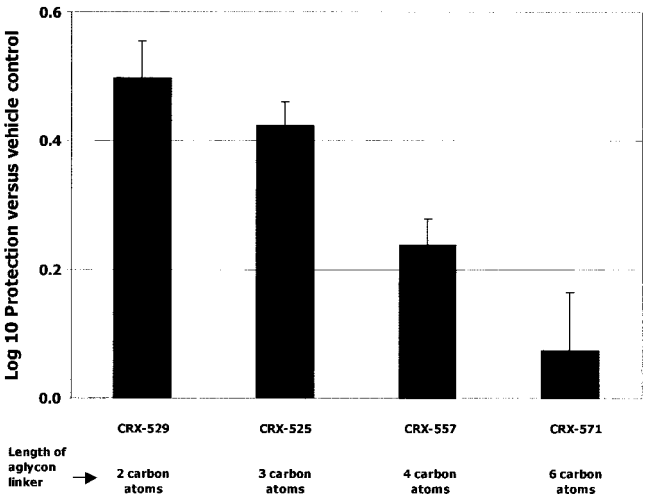

FIG. 6. Induction of nonspecific resistance to challenge with *Listeria monocytogenes* (plus SEM [error bars]) by intravenous pretreatment with AGP compounds (1-μg dose) that have different spacer lengths between the sugar moiety and aglycon moieties (five BALB/c mice per group). The average $\log_{10}$ number of splenic CFU in vehicle-treated control mice ± SEM was 7.03 ± 0.07. Only CRX-571 treatment did not result in significantly lower numbers of splenic CFU relative to vehicle treatment ($P < 0.05$; Student's *t* test).

components, as well as long-term effects on the adaptive response as dictated by the tenor of the cytokine environment in which antigens are presented. The latter effect is thought to play the greatest role in determining the adjuvant effects of TLR agonists (15). It is likely that the AGP-induced protective effects observed in this study are mediated by at least a subset of the effector molecules elicited by LPS.

Although the precise mechanism of action for the protective effect of AGPs in these murine infectious challenge models has not been elucidated, a pleiotropic effect that involves multiple immune mechanisms is suggested by the findings that serum concentrations of numerous inflammatory cytokines and chemokines increase (e.g., tumor necrosis factor alpha, IL-6, KC, RANTES, macrophage inflammatory protein 1α, and IP-10) and various cell lineages become activated (shown by increased CD69 expression on NK cells, macrophage, dendritic cells, and B cells) within hours of AGP administration (data not shown). While the production of gamma interferon during the immune response to *Listeria monocytogenes* is ultimately required for bacterial clearance, significant levels of this cytokine are not detectable in the serum of mice up to 24 h post-intravenous or post-intranasal AGP administration. This finding suggests that gamma interferon production is associated with the more slowly developing adaptive immune response and that the protection observed within hours of AGP administration results from a distinct mechanism. How the various cytokines and activated cell subsets mediate the protection induced by AGPs is currently being investigated.

Two AGPs were previously reported to induce protective innate immune responses when administered prior to bacterial or viral challenge in mice, though neither the structural features responsible for this activity nor its dependence on TLR4 were described (3). In this study, structural variants of these compounds were used to investigate the TLR requirement and the structure-activity relationship of lipid A-mediated induction of enhanced innate resistance to infectious challenge. The protective effect is clearly mediated by TLR4, since LPS-hyporesponsive C3H/HeJ mice with a mutation in TLR4 did not develop resistance to infection with *Listeria* or influenza virus after treatment with doses of AGP that were effective at producing strong protection in wild-type C3H/HeOuJ mice (Fig. 2). With regard to TLR specificity, AGPs do not appear to activate cells via interaction with TLR2, since treatment of TLR2-transfected 293 cells with several AGPs induced no NF-κB activity (data not shown). AGPs are currently being evaluated for potential interaction with other TLRs.

The precise nature of the interactions of lipid A with TLR4 and with other molecules, such as LBP, CD14, and MD-2, in the recognition pathway is currently an area of intense investigation. Several studies have highlighted the important roles of accessory molecules, especially MD-2, in LPS recognition (7, 40, 41). The emerging consensus is that LBP interacts with aggregates of LPS and promotes the formation of CD14/LPS monomeric complexes, which then facilitate the docking of LPS acyl chains into a hydrophobic pocket within MD-2; the negatively charged residues of the carbohydrate backbone of lipid A interact with the positively charged residues along the edge of the pocket. This interaction presumably leads to a conformational change in MD-2 that locks the two entities together as a stable, soluble complex (8). The MD-2/LPS com-

plex is then able to bind TLR4 on the cell surface and promote aggregation of the receptors into lipid rafts. Once a critical mass of aggregated TLR4 molecules is achieved, intracellular adaptor molecules are activated, and the subsequent induction of signaling pathways results in the expression of a range of cellular activities associated with the innate immune response. We showed previously that recognition of AGPs requires both TLR4 and MD-2 (35). In this study, we found that CRX-527, an AGP with two negatively charged residues on its backbone (i.e., the phosphate residue on the sugar and the carboxyl group at R4), induces stronger protection than other AGPs (CRX-522, CRX-524, and CRX-545) harboring a single negatively charged residue (Fig. 5). CRX-573, which contains a carboxyethyl group at R4, was also found to be less protective than CRX-527. Because the carboxyl group in CRX-573 is more distant from the phosphate group on the sugar moiety due to the intervening ethyl group, the weaker activity induced by this AGP suggests that the distance between the ionizable group at R4 and the negatively charged phosphate residue on the sugar moiety is a critical determinant of binding to MD-2. This result is not unexpected, considering the importance of charged amino acid orientation within binding pockets for receptor/ligand interactions (26). We also found that increasing the length of the AGP backbone with carbon-based linkers led to diminished protective activity (Fig. 6). Such changes might affect the strength of the interaction between the acyl chains of the AGP and the hydrophobic pocket of MD-2. Our findings from an in vitro study (35) and the in vivo studies described herein are thus consistent with each other and are supportive of the hypothesis that positively charged residues on the edge of the hydrophobic pocket of MD-2 (40) are important for binding to lipid A or its mimetics.

Importantly, we found that secondary acyl chain length is the most critical determinant of protective activity of AGPs when combined with a constant primary acyl chain length of 14 carbons. While AGPs with 8- to 14-carbon acyl chains at positions R1, R2, and R3 induced strong protection against *Listeria* or influenza virus challenge, compounds with 6- or 7-carbon acyl chains at these positions were inactive (Fig. 3). The positional importance of the three acyl-oxy-acyl residues attached to the AGP backbone was demonstrated in the *Listeria* system. In this regard, an AGP with a six-carbon acyl chain at R1 (CRX-566) was relatively inactive compared to compounds containing six-carbon acyl chains at R2 or R3 (CRX-565 and CRX-569), both of which induced moderate levels of protection (Fig. 4A). We have observed similar differences in activity between these compounds in cytokine induction assays, both in vivo in mice (data not shown) and in vitro with human or murine cell lines and/or peripheral blood leukocytes (13). In the influenza virus system, however, differences in activity between AGPs with single six-carbon secondary acyl chains at R1, R2, or R3 were not as apparent (Fig. 4B), which may mean that the influenza virus system is not as sensitive for quantifying differences in activity between compounds. Taken together, our results indicate that the lengths of the secondary acyl chains, particularly at R1, are critical determinants for productive interaction with MD-2. Although there is still much to learn about the binding of lipid A molecules to the MD-2/TLR4 complex, our findings suggest that this library of synthetic lipid A mimetics will ultimately be useful for dissecting the essential features of this interaction.

The finding that the AGPs are active in the airways further suggests that MD-2 and TLR4 are accessible in this compartment and that TLR4 agonists may have a unique therapeutic role in the modulation of innate immune responses when administered directly to the respiratory mucosa. TLR4 agonist activity can be potentially exploited to provide short-term resistance to infectious challenge such as might occur in the setting of exposure to biothreat agents or epidemic infections. Indeed, the accessibility of TLR4 in the airways may allow relatively small doses of drug to be used compared to what would have to be administered systemically to obtain the same effect. Future studies of this unique family of TLR4 agonists will likely yield important insights into their potential clinical applications.

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