Vaccines94

MODERN APPROACHES TO NEW VACCINES INCLUDING PREVENTION OF AIDS

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Molecular Requirements of Peptide Structures Binding to the Lipid-A Region of Bacterial Endotoxins

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The lipid-A moiety of endotoxin (lipopolysaccharide [LPS]), the major antigen of the outer membrane of gram-negative bacteria, is responsible for the toxic effects of LPS in mammalians. Lipid A is a structurally well-defined glycophospholipid, quite homogeneous among heterologous LPSs, that optimally binds to synthetic peptides mimicking the primary and secondary structures of the peptide antibiotic Polymyxin B (PmB). The binding thermodynamic is comparable to that of an antigen-antibody reaction and occurs with LPS in micellar form. Binding results in the detoxification of lipid A "in vitro" and "in vivo" as estimated by different biochemical and biological criteria. The features of these synthetic peptides have therefore been used for selecting candidate lipid-A-binding sequences in the primary structure of LPS-binding proteins originating from species phylogenetically different and carrying out different biological functions. The selected sequence-related synthetic peptides bound to lipid A of heterologous LPSs, inhibiting its toxic effects by a mechanism suggesting competition with receptor proteins. These results open new strategies for treatment of LPS-mediated diseases by novel peptide-based drugs and also introduce a rational approach to the design of nontoxic anti-LPS vaccines delivered to the host's immunosystem as LPS-peptide micelles, either native or covalently conjugated to peptides or to T-cell-dependent carrier proteins. The main objectives of such LPS-based vaccines, for induction of a specific immune response against the complete and native structure of LPS, are focused on prevention of gram-negative bacteremia and endotoxemia.

DISCUSSION

Peptide Selection from the Sequences of Proteins Binding to Heterologous LPSs

The molecular features required by synthetic peptides for optimal binding and detoxification of lipid A (Rustici et al. 1993) have provided unique opportunities for searching lipid-A-specific homologous amino acid sequences in the primary structure of natural polypeptides reported in the literature to bind heterologous LPSs specifically. Among these are CD14, the 55-kD LPS receptor-protein existing in soluble form in the serum as well as anchored on the cell membrane of monocytes and macrophages; LBP, *LPS-b*inding *p*rotein, a 60-kD plasma glycoprotein; BPI, *b*actericidal *p*ermeability-*i*ncreasing protein, a 55-kD glycoprotein isolated from polymorphonuclear leukocytes; LALF,

Table 1
Characteristics of Synthetic Peptides Selected from the Primary Amino Acid Sequences of Natural Polypeptides

Peptides	*Rc/h*	*Selectivity*	*LAL inhibition (w/w)*
$CD14_{67-75}$ Human V-K-A-L-R-V-R-R-L	1.00	0.02	100
$CD14_{68-78}$ Mouse K-S-L-S-L-K-R-L-T-V-R	1.00	0.03	200
LBP_{92-100} Human (Rabbit) K-V-R-K-S(A)-F-F(L)-K(R)-L	1.00	0.07	100
$LBP_{376-384}$ Human (Rabbit) F-L-K-P-G-K(R)-V(L)-K(Q)-V	0.75	0.04	100
BPI_{27-34} Human K-E-L-K-R-I-K-I	1.33	0.04	100
BPI_{90-98} Human K-W-K-A-Q-K-R-F-L	1.33	0.03	100
BPI_{90-99} Human K-W-K-A-Q-K-R-F-L-K	1.67	0.20	10
$LALF_{41-51}$ Crab K-R-L-K-W-K-Y-K-G-K-F	1.50	1.83	1
LEBP-PI_{5-17} Crab C-Q-S-W-K-S-S-E-I-R-C-G-K S----------S	1.50	0.05	100
LEBP-PI_{86-98} Crab C-R-Q-H-G-T-Y-I-N-C-L-H-V S----------S	0.25	0.00	>1000
$BPI_{153-160}$ Human I-Q-L-P-H-K-K-I	0.67	0.00	>1000
$LALF_{33-41}$ Crab H-Y-R-I-N-P-T-V-K	0.67	0.00	>1000

Amino acids are represented in one-letter code. Peptides were synthesized and analyzed for selectivity according to the method of Rustici et al. (1993). Rc/h value defines the ratio between aliphatic cationic (at physiologic pH) and hydrophobic amino acids present in a given sequence. The amino acid solvent parameter values, assigned by Levitt (1976), equal to or greater than +1.5 kcal/mole (lysine and arginine) and −1.5 kcal/mole (valine, isoleucine, leucine, tyrosine, phenylalanine, and tryptophan) were considered for calculating the ratio. LAL inhibition indicates the minimal ratio peptide:LPS (w/w) inhibiting clotting with 0.125 EU/ml (0.04 ng/ml) of *E. coli* 055:B5 LPS. All peptides have been randomly tested, with comparable results, using heterologous LPSs isolated from the following bacterial strains: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Neisseria meningitidis* group B, *Escherichia coli* 055:B5, *Salmonella typhi*, *Vibrio cholerae* Inaba 569B, and *Bordetella pertussis*.

Limulus amebocytes lysate factor, the 15-kD anticoagulant anti-LPS factor of the *Limulus polyphemus* hemolymph; and LEBP-PI, *Limulus* endotoxin-binding protein–protease inhibitor, a 12-kD anti-LPS protein of *Limulus* hemocytes with anti-trypsin activity. The synthetic peptides selected from the sequences of the five phylogenetically different proteins are shown in Table 1.

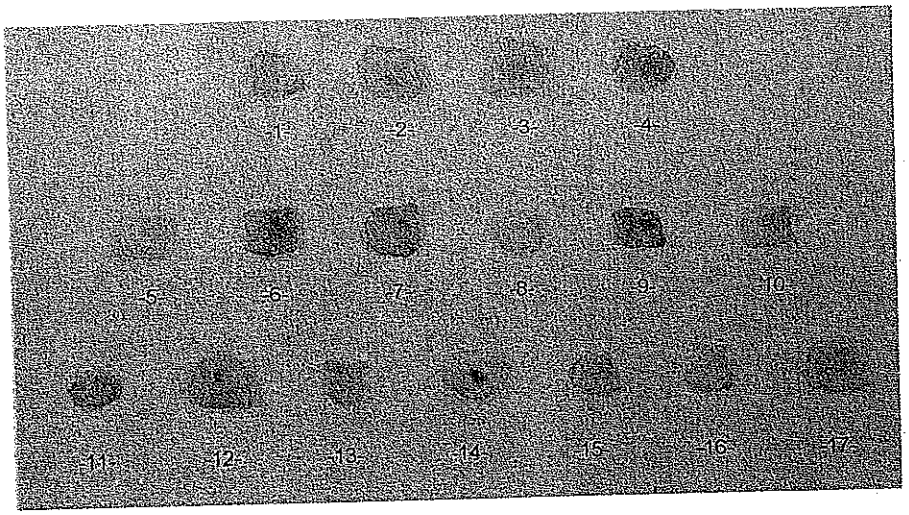

Figure 1
Local hemorrhagic necrosis in the skin of rabbits; 0.2 ml of saline containing LPS from *Salmonella minnesota*, Re595 mutant, was injected intradermally in the dorsal region of New Zealand white rabbits (shaved 4–6 hr before injection) at concentrations ranging from 25 to 125 μg/ml. Preformed complexes of comparable amounts of LPSs with synthetic peptides at different LPS:peptide ratios (w/w) were injected in the same volume. Hemorrhagic necrosis for complexes and controls were evaluated 96 hr after injection. (*1*) Saline; (*2–4*) Re-LPS at 25, 75, and 125 μg/ml, respectively. Re-LPS at 125 μg/ml in complex with: (*5*) PmB (1:100); (*6*) the lipid-A nonbinding/nonneutralizing peptide 4 (1:100), as positive control; (*7*) reference peptide 1 (1:10); (*8*) peptide 1 (1:100); (*9*) reference peptide 3 (1:10); (*10*) peptide 3 (1:100). The structures of the reference peptides 1, 3, and 4 have been described previously (Rustici et al. 1993). Re-LPS at 75 μg/ml in complex with the peptides: (*11*) human $CD14_{67-75}$ (1:100); (*12*) LBP_{92-100} (1:10); (*13*) LBP_{92-100} (1:100); (*14*) BPI_{27-34} (1:10); (*15*) BPI_{27-34} (1:100); (*16*) $LALF_{41-51}$ (1:100); (*17*) LEBP-PI_{5-17} (1:100).

Binding of Peptides to Lipid A

Binding of the selected peptides to lipid A of heterologous LPSs was assessed and measured by the value of selectivity, which expresses the ratio between the affinity constant value of PmB and that of each synthetic peptide, in competition analysis (Rustici et al. 1993). Binding-related detoxification efficiency was measured "in vitro" by inhibition of the lipid-A-activated enzymatic cascade, leading to a clot in the *Limulus* amebocyte lysate (LAL) assay, and "in vivo" by inhibition of lipid-A-induced local hemorrhagic necrosis in the skin of rabbits. Inhibition of the LAL assay is highly sensitive in estimating differences of LPS activity at femtomolar levels and correlates with the inhibition of cytokine-mediated pyrogenicity in rabbits. Hemorrhagic necrosis is a highly specific lipid-A-mediated local and general reaction, historically described for LPS (Shwartzman 1928), whose severity is dependent on the dose of lipid A injected (Fig. 1). This pathological process, due to the lipid-A-induced release of tumor necrosis factor (TNF) and interferon-γ (IFN-γ), the two mediators of LPS-induced shock and lethality, may therefore serve as a quantitative measure "in vivo" of the competitive inhibitory activity of the peptides with cell-bearing receptor proteins of derma, an area rich in leukocytes and macrophages expressing high concentrations of CD14 receptor protein. As shown in Figure 1, both the reference and the selected synthetic peptides with a value of Rc/h equal to or greater than 0.75 were able to inhibit competitively the lipid-A-induced hemorrhagic necrosis in rabbits at doses as high as 75–125 μg/ml of LPS.

Synthesis of Nontoxic LPS–Protein Conjugates in Micellar Form

LPS released from bacterial cells into aqueous media is recovered in the form of high-molecular-weight (>10^6) micelles stabilized by divalent cations of calcium/magnesium. In this form, LPS expresses the highest activity in the LAL assay and activates the enzymatic reaction (clotting) at concentrations as low as 40 pg/ml. PmB inhibits the LPS-induced enzymatic reaction. LPS treated with a calcium/magnesium chelating reagent, such as 0.1 M sodium citrate buffer (pH 6.90), lacks the micellar configuration and shows a molecular weight of <10^4 in gel chromatography. In this form, LPS activates the enzymatic cascade of the LAL assay only at a concentration of 4 ng/ml, with a reduction of activity equal to or greater than 100 times. PmB does not bind LPS in this nonmicellar form and does not inhibit the LAL reaction. Since it is well known that PmB inhibits LPS toxicity "in vivo," it is reasonable to speculate that the immunosystem recognizes LPS in the micellar form. For this purpose, it may be worth recalling that calcium/magnesium ions are present in the plasma and in the lymph at concentrations ranging between 25 and 95 mg/liter. On the basis of these considerations, we have covalently linked a T-cell-dependent antigen such as the mutant protein CRM197 (Porro et al. 1985) to native LPSs and have detoxified the resulting LPS-protein conjugates by synthetic peptide binding to the lipid-A moiety of the conjugated LPSs in micellar form. According to this protocol, four conjugates were synthesized, with LPSs from the strains of *Escherichia coli* 055:B5, *Salmonella typhi*, *Shigella flexneri* 1A, and *Neisseria meningitidis* group B. Detoxification of the conjugates by synthetic peptides occurred in all cases with an efficiency equal to 99.8%, as estimated by inhibition of clotting in the LAL assay, in direct competition. Lipid A–peptide and LPS–protein peptide complexes (peptide:LPS = 50 w/w) injected intradermally in rabbits, at doses up to 125 μg LPS/ml, did not show any sign of local hemorrhagic necrosis when compared to reference preparations of lipid A or LPS. After three subcutaneous injections of 5 μg LPS/dose of each of the four LPS conjugates, given 2 weeks apart, mice were bled, and the IgG and IgM immune responses to different structural regions of LPSs were determined by inhibition ELISA. IgG and IgM antibodies were induced against the different structural regions of the heterologous LPSs. In particular, we have focused our attention on the specificity of the immune response for the lipid-A region, in order to evaluate the cross-reactivity of the antibodies generated. Figure 2, as an example for *E. coli* 055:B5 and *N. meningitidis* group-B LPS-protein conjugate antigens, shows the specificity of the induced IgG and IgM isotype antibodies for lipid A. Both IgG and IgM isotypes, purified by affinity chromatography, were able to inhibit competitively the binding of the most affine synthetic peptide binding to the binding site of lipid A, as quantitatively estimated by high-performance liquid chromatography (HPLC) analysis (Rustici et al. 1993). IgG and IgM antibodies induced against either LPS of *E. coli* 055:B5 (S-LPS) or *N. meningitidis* group B (R-LPS) specifically displaced the synthetic peptide 2 from the lipid-A-binding site of purified lipid A from *B. pertussis* and *S. minnesota* Re 595 (Fig. 2A,B). A similar specificity of binding was also determined for lipid A of R-LPS of *N. meningitidis* group B (Fig. 2C,D) and S-LPSs of *S. typhi*, *P. aeruginosa*, *K. pneumoniae*, *E. coli* 055:B5, and *V. cholerae* Inaba 569B (Fig. 2E,F). The molecular mechanism by which the induced antibodies bind to lipid A and competitively inhibit binding of synthetic peptides to the lipid-A-binding site has not been investigated. Inhibition by specific filling of the binding site or by sterical hindrance will be the two main directions followed in our future studies to elucidate this mechanism.

SUMMARY AND CONCLUSIONS

The molecular mechanisms involved in the recognition of bacterial LPS by receptor proteins of specialized cells and antibodies of the immunosystem of mammalians have

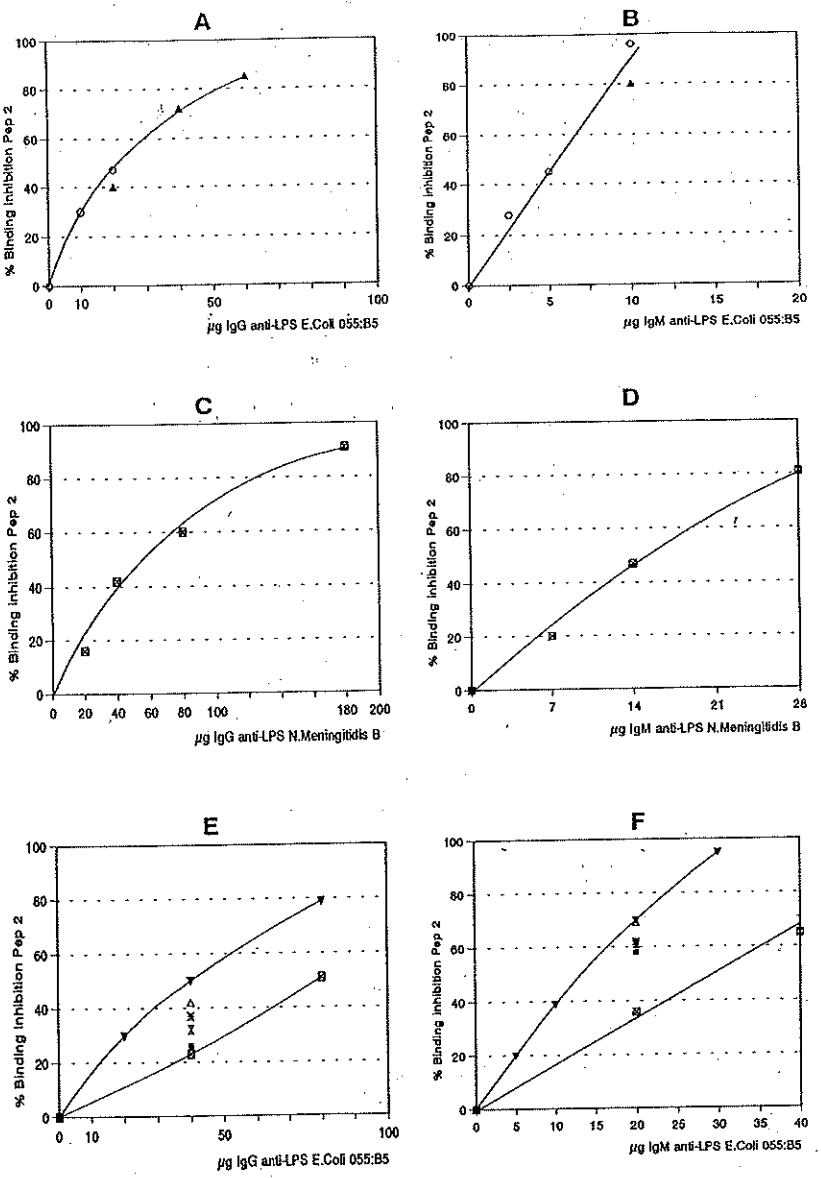

Figure 2
Immune response of mice vaccinated with LPS-conjugate vaccines: specificity of IgG and IgM isotype antibodies for the lipid A moiety of heterologous LPSs. Peptide 2 (16 µg) (Rustici et al. 1993) was added to a solution containing purified lipid A from *B. pertussis* (*closed triangles*) (25 µg) or Re-LPS of *S. minnesota* mutant 595 (*open circles*) (20 µg) pre-incubated with purified IgG (*A*) or IgM (*B*) antibodies induced against LPS conjugates of *E. coli* 055:B5. After separation of the complex by molecular filtration at a molecular weight of $<10^4$, free peptide 2 was quantitatively detected by HPLC analysis. Similar assays were performed with IgG and IgM antibodies (*C,D*) induced by R-LPS conjugates of *N. meningitidis* group B, competing for the homologous R-LPS (*open boxes with x*) (60 µg), as well as with IgG and IgM antibodies (*E,F*) induced by S-LPS conjugates of *E. coli* 055:B5, competing for the following R- and S-LPSs: *N. meningitidis* group B (*open boxes with x*) (60 µg), *S. typhi* (*inverted closed triangles*) (110 µg); *K. pneumoniae* (*closed boxes*) (110 µg); *P. aeruginosa* (*asterisk*) (110 µg); *V. cholerae* Inaba 569B (*open triangles*) (110 µg); *E. coli* 055:B5 (*hourglasses*) (110 µg).

not yet been elucidated. The results of our study are suggesting that natural polypeptides use a lipid-A-specific homogeneous and thermodynamically efficient recognition system based on cationic and amphipathic amino acid sequences of defined size and composition. As shown in our previous work (Rustici et al. 1993) and by synthesis of various peptides in our laboratory, a minimal sequence of six to seven amino acid residues containing a minimum of three aliphatic cationic amino acids, showing a ratio Rc/h equal to or greater than 0.5, is required for binding, whereas high-affinity binding is reached with sequences of ten or more amino acids with Rc/h values equal to or greater than 1.0. Sequence-related synthetic peptides, selected on the basis of these characteristics from the primary sequences of five proteins originating from species phylogenetically different and carrying out different biological functions, have in fact experimentally shown the capability of inhibiting LPS-related toxicity "in vivo," by a quantitative assay, with a mechanism that is strongly suggestive of competition with receptor proteins. The dimensions estimated for the lipid-A-binding site by a highly affine synthetic peptide (Rustici et al. 1993), in agreement with those reported for the structure of lipid A by X-ray diffraction (Kastowsky et al. 1992), are comparable to the epitopes recognized by monospecific antibodies (Cygler et al. 1991; Nnalue et al. 1992) and by major histocompatibility complex (MHC) class I molecules (Parker et al. 1992), suggesting some parallelism between the recognition mechanism proposed for LPS-binding proteins and that used by the immune recognition. These results have offered a rationale for the design of peptide-based drugs and lipid A–peptide complex-based vaccines for the prevention and treatment of LPS-mediated diseases. For instance, treatment of animals with peptides before or shortly after LPS injection intravenously does result in the dose-response inhibition of cytokine release (TNF and IL-6) and in a significant increase of the survival rate. In parallel, immunization of animals by peptide-detoxified LPS-protein conjugates in micellar form does result in the induction of lipid-A-specific IgG and IgM antibodies specifically binding to the lipid-A region of heterologous R- and S-LPSs. Future studies will be directed to clarify the biological functionality of these antibodies for prevention of gram-negative bacteremia and endotoxemia.

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