Research

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Inhibition of LPS-induced systemic and local TNF production by a synthetic anti-endotoxin peptide (SAEP-2)

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Summary Lipopolysaccharide (LPS) exerts its biological activity through the lipid A moiety. We tested the efficiency in inhibiting TNF production in sera and in tissues of mice and in the derma of rabbits challenged with LPS, of a synthetic anti-LPS peptide (SAEP-2) previously shown to specifically detoxify the lipid A region of LPS on the basis of structural similarities with the antibiotic polymyxin B (PMXB). In mice, SAEP-2 (100 μg/mouse, i.v.) injected with various schedules (−30 to +10 min from LPS at 50 ng/mouse, i.v.) significantly inhibited serum TNF as well as liver, spleen and lung-associated TNF. In rabbits, SAEP-2 significantly inhibited TNF produced in dermal tissue and the resulting local hemorrhagic necrosis. The amount of tissue-associated TNF released by LPS challenge in the mouse was up to 6 times that present in the serum and inhibition by SAEP-2 or PMXB accounted for 75% of the total. Direct measurement of the binding kinetics by surface plasmon resonance and molecular filtration at equilibrium revealed that SAEP-2 and PMXB bind to LPS only in the presence of a significant amount of water but that they are unable to bind LPS in undiluted serum. Altogether these findings strongly suggest that inhibition of LPS-induced TNF by SAEP-2 and PMXB may occur in tissues.

INTRODUCTION

Development of novel strategies designed to specifically inhibit the very first events involved in the activation of the complicated pathway responsible for LPS toxic effects, demands an accurate investigation of the molecular mechanisms at the basis of the interaction between the lipid A region of LPS and candidate target receptors.

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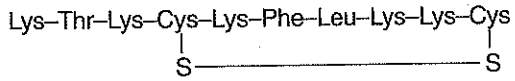
Among the most recent strategies under clinical evaluation, there are natural and recombinant LPS-binding proteins like the bactericidal permeability-increasing protein (BPI) from human neutrophils[1,2] and synthetic anti-endotoxin peptides (SAEP) mimicking the primary and secondary structure of PMXB and predicted as lipid A binding site(s) in the primary sequence of natural LPS-binding proteins.[3–5] Since the toxicity of LPS is known to be essentially mediated by TNF, as demonstrated by the protective activity of anti-TNF antibodies in animal models,[6,7] the aim of the present study was to characterize the inhibitory effect of one of these peptides, SAEP-2 described previously,[3] on LPS-induced TNF production in the sera and tissues of mice. For this purpose, we compared various doses of SAEP-2 (given at a peptide/LPS weight ratio of 20:1–2000:1) and various treatment

schedules (from 120 min before to 60 min after LPS challenge) for their ability to lower TNF levels in the serum. Using the optimal treatment schedule as experimentally determined (LPS at 50 ng/mouse, i.v.; SAEP-2 100 μg/mouse, i.v., at −30 min), we also investigated the ability of the peptide to inhibit TNF production in various organs, including spleen, liver and lungs. In fact, local TNF production in these tissues has been demonstrated[8,9] and multiple organ failure is the final step of an endotoxicosis-related septic process which includes acute hepatic failure as well as respiratory distress syndrome (ARDS).[10,11] Further analysis on the capability of SAEP-2 to inhibit LPS-induced TNF production in tissues has been investigated in rabbits by inhibition of local hemorrhagic dermonecrosis (local Schwartzman reaction). Finally, we have investigated, by two different physicochemical methods, the conditions required for allowing SAEP-2 and PMXB to bind LPS in the presence of serum, in order to explain the most likely mechanism for their prophylactic and therapeutic activities observed in this study.

MATERIALS AND METHODS

Materials

PMXB, Escherichia coli O55:B5 LPS and LPS from Salmonella minnesota Re595 LPS were from Sigma Chemical Co. (St Louis, MO, USA). Neisseria meningitidis A1 LPS was kindly provided by Dr C.M. Tsai, (OB-FDA, Bethesda, MD, USA). SAEP-2 was synthesized and characterized as previously reported.[3] The structure of SAEP-2 is the following:



Animals and treatments

Male CD-1 mice (25–30 g body weight, Charles River, Calco, Como, Italy) were used. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana No. 40, February 18, 1992; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

Mice were treated with LPS and/or peptides (SAEP-2 or PMXB) dissolved in 0.2 ml of sterile, pyrogen-free saline at various doses, as indicated in the text. Control mice received saline alone. 90 min after LPS challenge, animals were bled from the retro-orbital plexus and serum prepared for TNF titration. Then, the animals were sacrificed and spleen, liver and lungs removed and homogenized with an Ultra Turrax rotating blade device in 4 vol, w/v, (liver) or 10 vol (spleen, lungs) of ice-cold saline. The homogenate was then centrifuged for 10 min at 13 000 rpm (spleen, lungs) or 35 min at 15 000 rpm (liver) in a refrigerated centrifuge and the supernatant was used for TNF determination.[12] Preliminary experiments indicated that these were the optimal time points for the determination of this cytokine in LPS-treated mice.[12,13]

Dermonecrosis in the rabbit

The dorsal region of New Zealand white rabbits (2–3 kg body weight) was shaved about 2 h before treatment, with particular attention to avoid local irritations of the skin. Animals were divided in groups of three rabbits and in each rabbit up to 15 points could be injected in the shaved area, spaced about 2 cm from each other. Injections were performed intradermally by sterile syringe with a 25 gauge needle with a maximum volume injected of 0.2 ml. Complexes were prepared in buffered saline (PBS) by incubation (30 min at 37°C) of LPS (10 μg/dose) with SAEP-2 or PMXB at various stoichiometric ratios (LPS:peptide = 1:10, 1:100, 1:250 w/w) in consideration of previous experiments showing that a significant excess of peptide is necessary for quantitatively inhibiting the local lesion induced by LPS.[4] The dose-volume injected was 0.2 ml. *S. minnesota* Re595 or *N. meningitidis* A1 LPS were injected in the same volume (0.2 ml) at doses of 2.5, 5, 10, 25 μg (respectively 12.5, 25, 50 and 125 μg/ml) as positive controls. Saline was injected as negative control. In each animal, the positive and negative controls were injected in order to avoid inaccurate quantitation of the lesions due to some variability in the entity of the hemorrhagic areas produced in different animals. 72–96 h after injections, each rabbit was inspected for the size of the lesions and sacrificed for collecting the dermal biopsy samples at the injection sites. Each biopsy sample was washed once with sterile saline and twice with ethanol 70% (v/v) and maintained in this alcoholic solution or photographed for later reference.

The area of each lesion due to the complexes injected (when present) was measured and compared to its own reference control and the significance of the inhibition assessed by *t*-test in a minimum of three assays. In selected experiments, rabbits were sacrificed 90 min following the injections and the biopsy samples at the injection sites were removed, homogenized and used for TNF measurement.

TNF determination

TNF was measured by cytotoxicity on L929 cells as previously described,[14] using human recombinant TNF as a standard (Genzyme, Cambridge, MA, USA) and adding the samples to be tested at serial dilutions (1:4, 1:16, 1:64, etc.). The sensitivity of the assay was 0.05 ng/ml. SAEP-2 and PMXB (at concentrations up to 1 mg/ml) did not inhibit the bioactivity of TNF in this assay (data not shown). Specificity of the TNF bioassay was demonstrated in preliminary experiments where a rat-anti-mouse TNF monoclonal antibody[15] inhibited by more than 95% TNF bioactivity in tissues or sera from LPS-treated mice.

Comparison of the binding between PMXB or SAEP-2 and LPS in saline and serum

The capability of PMXB or SAEP-2 to bind LPS in the serum of rabbits and mice and rabbits, as opposed to aqueous solutions, was tested in two different conditions, that is in kinetics and at the equilibrium. In the first approach, surface plasmon resonance analysis (SPR) was performed using the BIAcore™ biosensor system (Pharmacia Biosensor SB, Uppsala, Sweden) which is a proven method to allow real-time interaction between molecules. The use of this system offers, in general, some advantages over conventional methods since monitoring of the interaction is performed as it proceeds. The detection principle of BIAcore™, relies on the optical phenomenon of SPR,[16–18] which detects changes in the refractive index of the solution close to the surface of a sensor chip. Refractive index is directly correlated to the concentration of material dissolved in the medium. By keeping other factors constant, SPR is used in BIAcore™ to measure changes in the concentration of macromolecules in a surface layer of solution in contact with a dextran-coated gold film. One interactant is immobilized on the sensor surface, which forms one wall of a micro flow-cell. Solution containing the other interactant continuously flows over the sensor surface. As molecules from solution bind to the immobilized interactant, a response is followed and expressed as resonance units (RU). Association and dissociation of interacting molecules are followed in a sensorgram where changes in RU are reported by dedicated software as a function of time. PMXB (250 μg/ml in 10 mM sodium acetate pH 4.5) was immobilized on the carboxymethylated sensor chip surface previously activated with a 1:1 mixture of 100 mM N-hydroxy-succinimide (NHS) and 400 mM N-ethyl-N'(dimethyl -aminopropyl) carbodiimide (EDC). Unreacted activated groups were deactivated by injection of 1 M ethanolamine hydrochloride, pH 8.5. All measurements in aqueous system were performed at 25°C in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% BIAcore™ surfactant P20, (HBS P20). Different amounts of freeze-dried LPS, dissolved in HBS P20 or directly in undiluted serum, were passed over the matrix-bound PMXB. Two subsequent injections of 0.5% SDS were used to detach PMXB-bound material after each cycle.

The second approach used to study peptide–LPS binding was based on a modification of equilibrium dialysis. In this case, molecular filtration of mouse serum containing 150 μg/ml of LPS and 75 μg/ml PMXB or SAEP-2 was used and performed on Ultrafree CL filters with a membrane of NMWL ≤ 10 000 cut off (Nikon-Millipore, Kogjo, Japan) after the reaction was allowed to reach equilibrium for 30 min.[3] The serum filtrate, collected by centrifugation at 5000 rpm for 60 min, was processed by HPLC through a C_{18} reverse-phase column and the amount of free PMXB or SAEP-2 detected by comparison with reference solutions, as previously described.[3]

RESULTS

Effect of SAEP-2 and PMXB on LPS-induced serum TNF in vivo

Figure 1, reports the results of experiments where SAEP-2 (Fig. 1A) or PMXB (Fig. 1B) were administered at various doses (1, 10 or 100 μg/mouse, i.v.) 30 min before LPS (50 ng/mouse, i.v.). The results are reported as the percentage of TNF levels detected compared to controls (LPS alone). No significant TNF was detectable in the absence of LPS treatment (data not shown). SAEP-2 significantly inhibited TNF production at the dose of 100 μg/mouse, while with PMXB an inhibition was also observed at the dose of 10 μg/mouse. The possibility that SAEP-2 might inhibit TNF production via an aspecific mechanism was ruled out by experiments where TNF was induced by Gram-positive bacteria. In these experiments, SAEP-2 was given at 100 μg/mouse, i.v., 30 min before heat-killed *Staphylococcus aureus*, 0.2 ml of a 10^{10}/ml suspension, and no inhibition of serum TNF was observed (*Staph. aureus*, 0.63 ± 0.17 ng TNF/ml; SAEP-2 + *Staph. aureus*, 0.57 ± 0.31 ng/ml; data are mean ± SE from 10 mice/group). Figure 2 shows the results of several experiments where SAEP-2 was administered at a dose of 100 μg/mouse, i.v., at different times with respect to LPS (50 ng/mouse, i.v.). In all experiments, serum TNF was measured 90 min after LPS, since previous experiments indicated that this is the peak time for serum TNF induction after injection of LPS.[13,19] The results are expressed as the percentage of TNF levels in respective controls (LPS alone) for each experiment. SAEP-2 effectively inhibited serum TNF levels when administered within −30 and +10 min of LPS administration. Maximal inhibition was

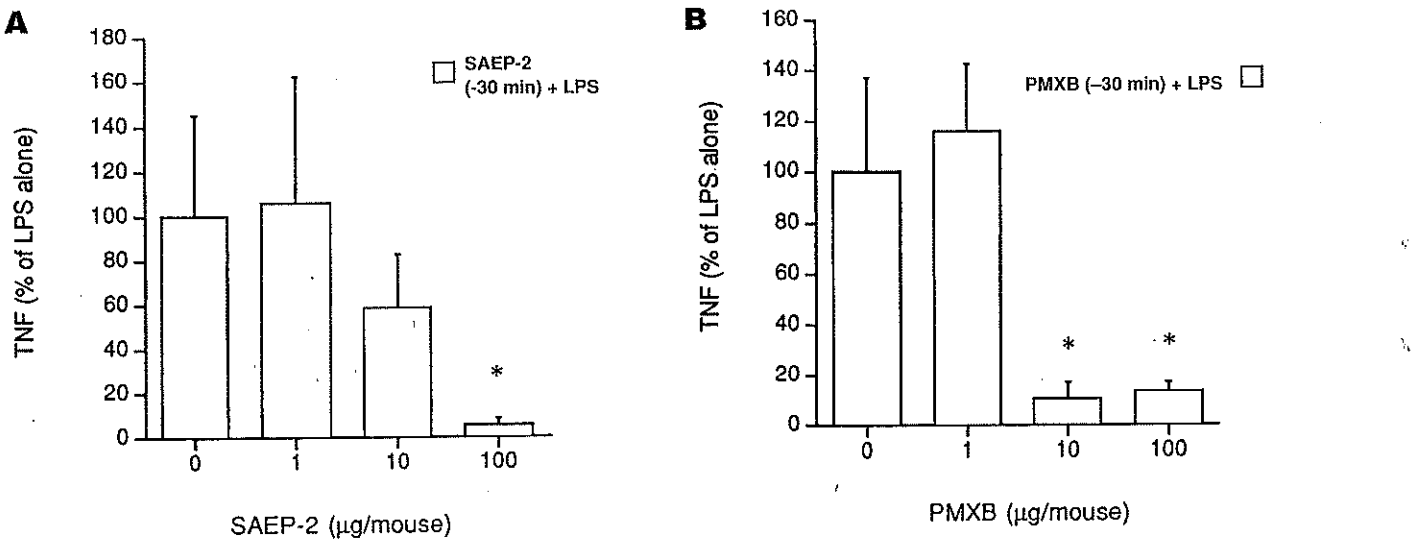


Fig. 1 Dose-response of the effect of SAEP-2 and PMXB on serum TNF levels in LPS-treated mice. SAEP-2 (**A**) or PMXB (**B**) were administered at the indicated doses, 30 min before LPS (50 ng/mouse, i.v.); serum TNF was measured 90 min after LPS challenge. Data are expressed as percent of those observed in mice treated with LPS alone (mean ± SE, $n = 5$). The actual concentration of TNF in mice treated with LPS alone (100% in the figure) was 3.7 ± 1.7 ng/ml. $^*P < 0.05$ versus LPS alone by Student's *t*-test.

observed when SAEP-2 was administered at –30 min (74 ± 7%, mean ± SD, $P < 0.01$, in eight independent experiments using five mice per group; range: 50–95%). Administering SAEP-2 in the range –10/+10 min reduced the TNF levels by 50% and 59%, respectively ($P < 0.05$ in both cases). Administering SAEP-2 earlier (–120 min) or later (from +30 min on) did not reduce TNF levels significantly. An inhibitory effect of SAEP-2 was also observed when it was administered in the same time interval (at –30 or +10 min) by a different route of LPS challenge (SAEP-2, 1000 μg/mouse, i.v.; LPS, 0.5 μg/mouse, i.p.), as shown in Figure 3.

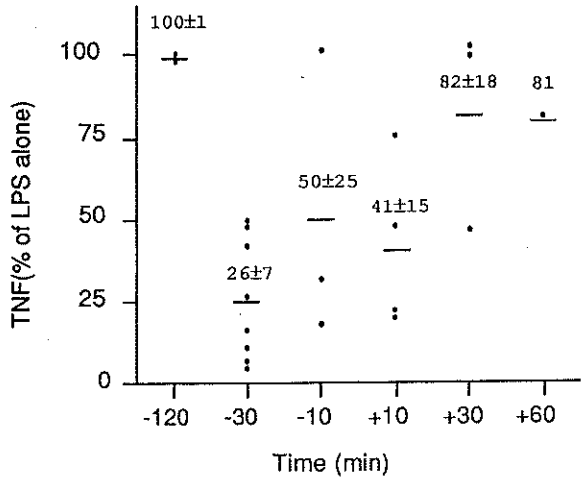


Fig. 2 Inhibition of serum TNF levels by SAEP-2 in LPS-treated mice with different treatment schedules. SAEP-2 was administered at the times indicated respective to LPS (SAEP-2, 100 μg/mouse, i.v.; LPS, 50 ng/mouse, i.v.) and serum TNF was measured 90 min after LPS. Serum TNF levels in mice treated with SAEP-2 and LPS are expressed as percent of those observed in mice treated with LPS alone within the same experiment (mean 4.3 ± 1.4 ng/ml). Each dot represents the results obtained from independent experiments (the number of mice was five per group in each experiment).

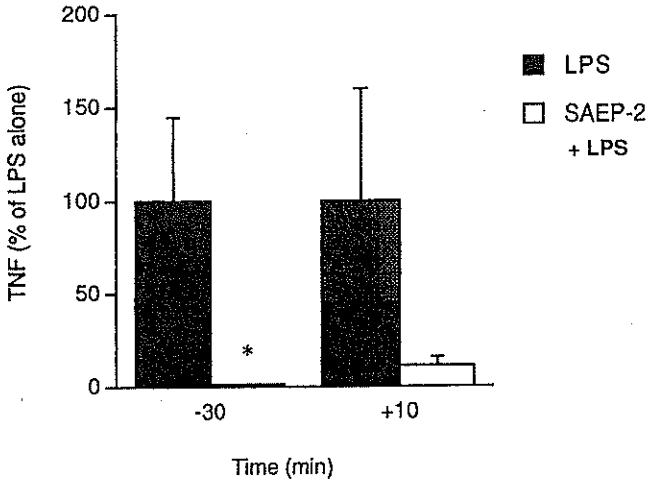


Fig. 3 Inhibition of serum TNF levels by SAEP-2 in intraperitoneally LPS-treated mice. SAEP-2 was administered at –30 min or +10 min, respectively, to LPS (SAEP-2, 1000 μg/mouse, i.v.; LPS, 0.5 μg/mouse, i.p.). Serum TNF was measured 90 min after LPS. Data are expressed as percent of the mean ± SE ($n = 5$) of those observed in mice treated with LPS alone (100% TNF release with LPS alone was 2.3 ± 1.1 ng/ml). $^*P < 0.05$ versus LPS alone by Student's *t*-test.

SAEP-2 and PMXB inhibit LPS-induced TNF production in mouse tissues in vivo

We studied the effect of SAEP-2 or PMXB (100 μg/mouse, i.v., 30 min before LPS challenge) on the induction of tissue-associated TNF 90 min after LPS (50 ng/mouse, i.v.) in liver, spleen and lungs. Figure 4 reports the results of experiments showing that SAEP-2 (Fig. 4A) and PMXB (Fig. 4B) significantly inhibited TNF levels in the tissues. Mean TNF concentrations in the organs of each of 5 animals, following LPS alone (100% of TNF release) were: 7.0 ± 0.7 ng/g in the lung; 10.1 ± 1.7 ng/g in the liver; 63.3 ± 10.7 ng/g in the spleen. Inhibition by SAEP-2 was 50%, 65% and 80%, respectively, and comparable to that obtained with PMXB. It should be noted that a quantitative balance comparing the TNF found in the tissue homogenates of the organs considered and that measured in the serum of each animal (6.6 ± 2.1 ng/ml) was in favour of the former. In fact, within the uncertainty due to the variability of the assays, the total amount of tissue-associated TNF released by LPS challenge in the organs considered of each animal was 6.3 times higher than that present in the serum (1.4 ng in the lung, 20 ng in the liver, 9.5 ng in the spleen, total of 30.9 ng versus 4.9 ng in the serum). Inhibition of TNF by SAEP-2 and PMXB, prophylactically administered, accounted for about 75% of the total (tissues + serum) released in each animal (mean of 26.9 ng inhibited versus 35.8 ng released). Contaminant serum TNF present in the residual amount of blood in the organs after exsanguination, known to be very little and ranging from 47 μl/g in the liver to 166 μl/g in the spleen,[20] could only account for less than 5% of the total amount found in the tissues of each animal.

SAEP-2 and PMXB inhibit LPS-induced dermonecrosis and TNF production in rabbits in vivo

In these experiments, SAEP-2 was tested for its ability to inhibit LPS-induced dermonecrosis. For this purpose, two R-chemotypes of LPS were injected intradermally in a total volume of 0.2 ml alone or complexed with varying amounts of PMXB or SAEP-2. The results of all samples assayed can be summarized as follows: the size of the hemorrhagic necrotic areas produced by the two R-chemotype LPS of the study (*N. meningitidis* A1 and *S. minnesota* Re595), were related to the dose injected and ranged from an average diameter of 1–2 mm for the low dose (12.5 μg/ml) to an average diameter of 6–8 mm for the high dose (125 μg/ml). Complete inhibition of hemorrhagic necrosis at the selected doses of the study was related to the amount of SAEP-2 or PMXB in excess to that stoichiometrically necessary for inhibition of LAL clotting.[3] Examples of these results are shown in Figure 5. Analysis of the reproducibility of the experiments has shown that complete inhibition of the hemorrhagic necrotic reaction occurred with a ratio SAEP-2:LPS = 250 (w/w) in 12 out of the 16 assays performed (75%) while in the remaining assays (25%) the inhibition was greater than 80% ($P < 0.01$ against its positive control). PMXB gave complete inhibition in 11 out of the 14 experiments performed (79%) with a ratio PMXB:LPS = 100 (w/w) while in the remaining assays (21%) the inhibition was greater than 80% ($P < 0.01$ against its positive control).

LPS injected at a dose of 50 μg/ml induced local release of TNF at levels of 21.6 ± 2.9 ng/g of tissue. Injection of LPS in complex with SAEP-2 or PMXB resulted in a significantly reduced release of TNF in the derma (Table 1).

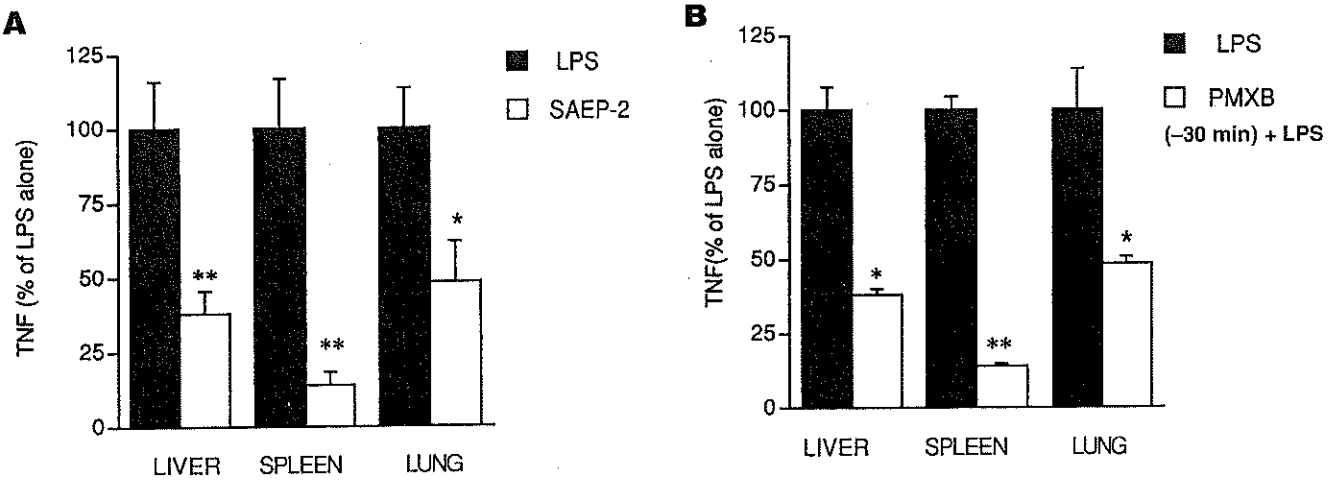


Fig. 4 Inhibition of spleen, liver and lung TNF levels by SAEP-2 and PMXB in LPS-treated mice. Groups of 5 mice each were treated with LPS (50 ng/mouse, i.v.) with and without SAEP-2 (**A**) or PMXB (**B**) at the dose of 100 μg/mouse, i.v., 30 min before LPS challenge. Tissue-associated TNF was measured 90 min after LPS challenge. Data are expressed as percent of those observed in mice treated with LPS alone (mean ± SE, $n = 5$). $^*P < 0.05$; $^{**}P < 0.01$ versus LPS alone by Student's *t*-test.

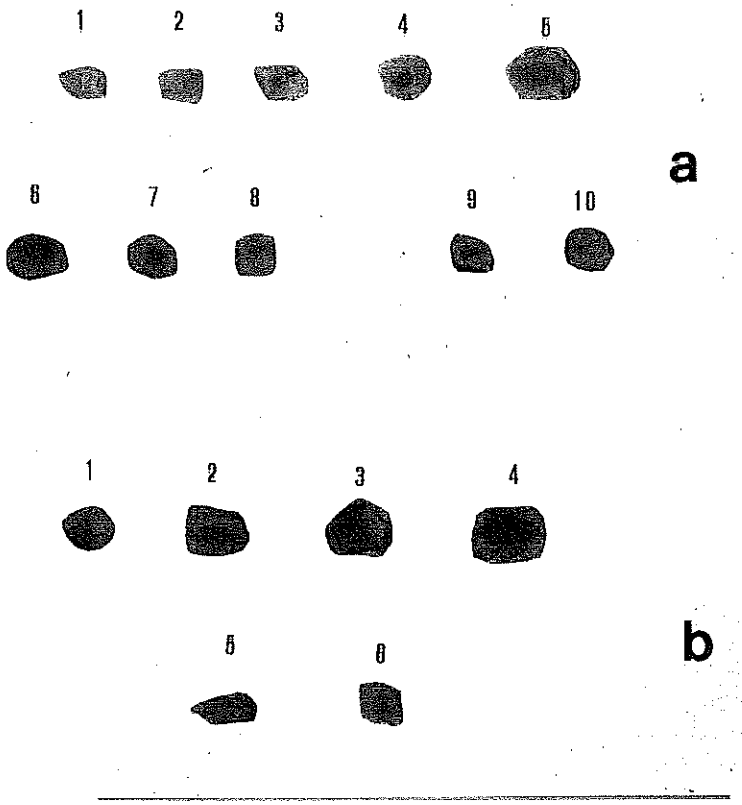


Fig. 5 Inhibition of hemorrhagic necrosis induced by different doses of two bacterial LPS and by complexes of LPS with SAEP-2 or PMXB in the skin of rabbits. **Group a:** (1) saline; (2–5) *S. minnesota* Re595 LPS injected respectively at a concentration of 12.5, 25, 50, 125 µg/ml, volume injected was 0.2 ml; (6) Re595 LPS (10 µg) + SAEP-2 (1:10 w/w); (7) Re595 LPS (10 µg) + SAEP-2 (1:100 w/w); (8) Re595 LPS (10 µg) + SAEP-2 (1:250 w/w); (9) Re595 LPS (10 µg) + PMXB (1:10 w/w); (10) Re595 LPS (10 µg) + PMXB (1:100 w/w). **Group b:** (1–4) *N. meningitidis* A1 LPS at the respective doses of 12.5, 25, 50, 125 µg/ml, volume injected was 0.2 ml; (5) A1 LPS (10 µg) + SAEP-2 (1:250 w/w); (6) A1 LPS (10 µg) + PMXB (1:100 w/w).

Physicochemical requirements for the binding of PMXB and SAEP-2 to LPS

In aqueous solution, PMXB and SAEP-2 bind to lipid A of LPS with high affinity.[3,21] SPR studies have shown the kinetics of the interaction between PMXB and LPS in aqueous solution (Fig. 6). However no binding of PMXB to LPS was detected in the serum (Fig. 7). Even allowing the reaction between PMXB or SAEP-2 and LPS to reach equilibrium in the conditions reported[3] did not result in any detectable binding either by SPR (data not shown) or by molecular filtration, where the total amount of the peptides added to serum was recovered in the filtrate (Table 2). Murine serum was used in the experiments reported in Table 2. Experiments with human or rabbit serum gave comparable results (data not shown). In order to restore a significant binding activity between PMXB and LPS, serum had to be diluted significantly with saline (90–95% v/v), depending on the concentration of LPS (Fig. 8).

Table 1 Inhibition of LPS-induced dermal TNF production in rabbits by SAEP-2 or PMXB

Sample	TNF (ng/g skin)	% inhibition
Saline	0.9 ± 0.2	—
Re LPS 595	21.6 ± 2.9	0
LPS + SAEP-2 (1:250, w/w)	1.9 ± 0.2	92*
LPS + PMXB (1:100, w/w)	3.7 ± 0.4	83*

TNF was measured in the skin biopsy samples 90 min after the local injection of ReLPS 595 (10 µg/0.2 ml) alone or complexed with peptides. Data are mean ± SD ($n = 3$). * $P < 0.01$ vs ReLPS

Table 2 Influence of mouse serum on the stability of the complex PMXB/LPS or SAEP-2/LPS

Concentration of Re595 LPS(mg/ml)	% serum	% free PMXB or SAEP-2
0.15	0	0
0.15	10	0
0.15	15	12 ± 1.5
0.15	20	95 ± 5

Data are mean ± SE from three independent experiments.

DISCUSSION

The data reported here confirm and extend those previously described with LPS-binding peptides related to the structure of PMXB.[3] In particular, SAEP-2 and PMXB were able to significantly inhibit LPS-induced TNF production in several tissues when given prophylactically and therapeutically. This is particularly important in the light of recent clinical studies showing that tissue-associated TNF plays the most significant contribution to the appearance of circulating TNF levels in patients with ARDS.[22]

The ineffectiveness of SAEP-2 and PMXB when given in single injection as early as 2 h before LPS challenge can be explained by the physiological clearance of the peptides that would need multiple administrations or continuous infusion for sustaining an efficacious concentration, similarly to the common antibiotic treatment. Also, the lack of effect observed when the peptides are given 30 min after LPS challenge is in agreement with earlier reports indicating that just a short time of exposure to LPS is sufficient to trigger TNF gene expression in vivo and in vitro.[8,23]

In support of these observations, data obtained in our laboratory from the pharmacokinetics of peptides in the mouse and rabbit, have shown that, although SAEP-2 shows a half-life time ($T_{1/2}$) in whole blood in vitro of 50

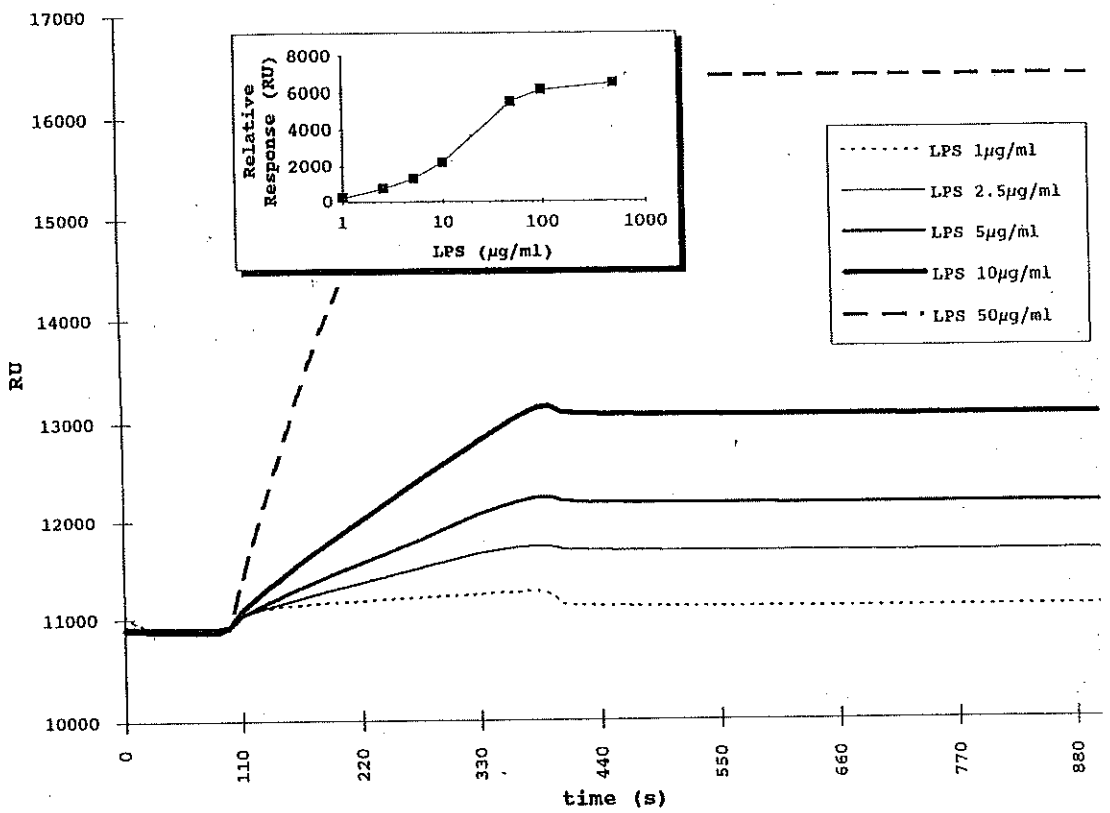


Fig. 6 SPR analysis of Re595LPS-PMXB binding kinetic in aqueous solution. Different concentrations of LPS were dissolved in HBS P20, 35 µl of each sample were injected over the matrix-bound PMXB at a flow rate of 7 µl/min. The dissociation phase was followed for 400 s in each cycle. The RU recorded at the end of the dissociation phase in each cycle after deducting the baseline RU value were reported as relative response in the function of LPS concentration (square).

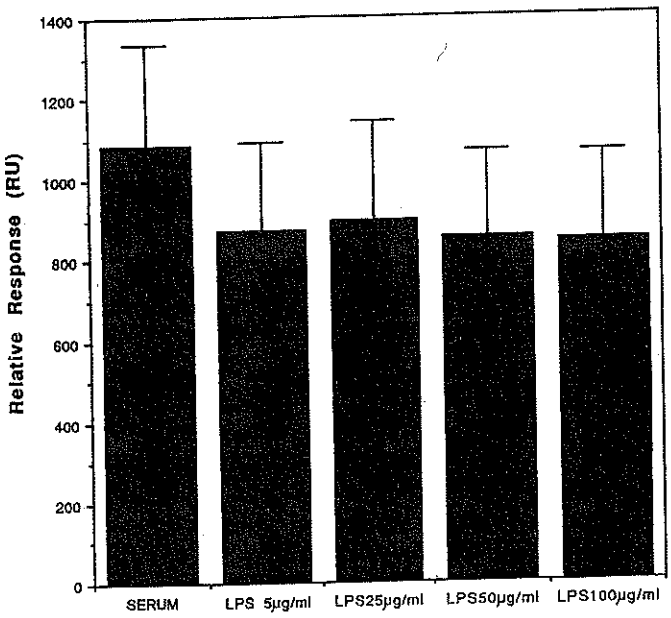


Fig. 7 Binding of serum components to PMXB in Re595-containing undiluted mouse or rabbit serum as detected by SPR. Different amounts of LPS were directly dissolved in undiluted serum, 35 µl of each sample were injected over the matrix-bound PMXB at a flow rate of 7 µl/min. The reported results are the average of the relative response recorded at the end of a 400 s dissociation phase in seven independent experiments.

min,[3] the $T_{1/2}$ value in vivo (i.v. injection) is about 5 min. 30 min after i.v. injection of the peptides, that is when LPS challenge is given, no traces of any of the two peptides were present in the plasma of both species (data not shown), at least at a detection level of 2.5 µg/ml, the sensitivity of our reverse-phase HPLC system.[3] The calculated apparent volume of distribution in the mouse for SAEP-2 was 209 ml/kg, an observation that parallels the well known diffusion of PMXB into tissues.[24]

For this purpose we have also shown that the localized dermal lesions in the rabbit, produced in a dose-dependent manner by R-chemotype LPS,[25] such as *S. minnesota* Re595 and *N. meningitidis* A1, can be inhibited by SAEP-2

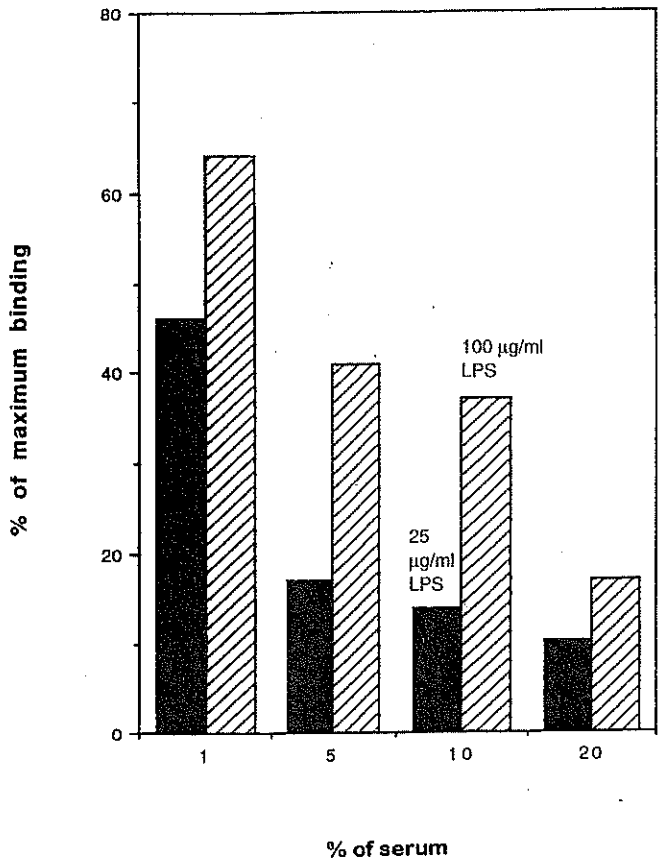


Fig. 8 Influence of mouse or rabbit serum on the SPR-detected Re595 LPS binding to PMXB. 25 µg/ml (solid black bars) and 100 µg/ml (hatched bars) of LPS dissolved in HBS P20 were injected over the matrix-bound PMXB, as described in the caption to Figure 5, in the presence of increasing amount of serum. The RU recorded in each cycle at the end of the dissociation phase were compared with the RU obtained in identical conditions in the absence of serum (considered as 100% binding). The RU obtained, after injection in identical conditions of the corresponding amount of serum in the absence of LPS were subtracted to each point.

at appropriate stoichiometry. It should be noted that the high dose of LPS administered locally in the rabbit experiments, although unlikely present in septic conditions, is consistent with the use of LPS in humans as antigenic component in vaccines for prevention of bacterial meningitis.[26] At least in vitro, SAEPs have shown various degrees of proteolytic resistance depending on their structures[3] and this could explain the need for a consistent excess of peptide in an area rich of serine proteases for complete inhibition of LPS-induced dermonecrosis. However, the fact that a comparable excess was also needed for PMXB, which is proteolytically resistant, would suggest that the dose of peptide required may be determined by binding competition with LPS receptor proteins on target cells. In this respect, experiments with synthetic peptides predicted as LPS-binding site(s) in the primary structure of various LPS-binding proteins, including the receptor protein CD14, have given comparable results.[4,27] Inhibition of the severe local endotoxic effects was paralleled, as expected, by inhibition of local TNF levels. The high levels of local TNF induced by LPS in the rabbit, likely due to the involvement of resident macrophages, are explicable with the high dose of LPS locally injected as well as to the sensitivity of this animal specie to LPS, quite comparable to humans in this respect.

Altogether, these findings strongly suggest that the detoxification activity of SAEP-2 and PMXB in vivo may occur at the tissue level. In further support of the extravascular activity of SAEP-2 and PMXB, it is important to note that the binding activity of these peptides for the lipid A region of LPS in aqueous solutions, detected by accurate physicochemical methods, such as competition at equilibrium and SPR, could not be observed in undiluted serum. To significantly restore the binding activity of PMXB and SAEP-2 for LPS, serum had to be diluted at least 1:6 (v/v). One likely effect of dilution is to decrease the concentration of the plasma components (likely proteins and lipoproteins) known to bind PMXB. Thus, these findings also question the popular concept that PMXB abrogates the toxic effects of LPS in whole blood by forming a stable complex, and suggest caution on the implications of in vitro studies performed in whole blood or undiluted serum, where PMXB may show efficacy likely acting through other less specific mechanisms, e.g. inhibition of protein kinase C.[28,29] As we have shown, stability of the complex between PMXB or SAEP-2 and LPS requires the presence of a significant amount of water, a condition which in vivo could be more properly found in the cellular and interstitial liquids. Also, the lack of complex formation between SAEP-2 or PMXB and LPS in undiluted serum well supports the reported failure of a synthetic peptide with comparable physicochemical features, to inhibit LPS-induced cytokine release in whole blood in vitro, in contrast to its activity in vivo.[5]

Therefore, inhibition of tissue TNF production seems very important, not only in view of the pathogenic relevance of tissue TNF in LPS-induced multiple organ failure, but also when one considers the possibility that the most significant amount of circulating TNF due to endotoxicosis might be produced by tissue macrophages rather than by circulating leukocytes. Indeed, this possibility has been recently shown by clinical studies conducted in patients with ARDS[22] and is supported by experimental data on the distribution of [^{125}I]-labeled LPS incubated in whole blood or injected in mice. When [^{125}I]-labeled LPS is incubated with whole blood, a very small amount of it binds to cell components (mainly monocytes) while the major part of LPS is found in the serum fraction bound to lipoproteins.[30] When injected in mice,

LPS quickly leaves the bloodstream and mainly localizes in the liver, lungs and the spleen within 60 min from its administration and accumulates in these organs for several days,[31] where it can be intracellularly detoxified in tissues by lipid A-specific acyloxyacyl hydrolases.[32].

In conclusion, our data provide evidence that, following an LPS challenge, the most significant amount of TNF is present in organ tissues and either SAEP-2 or PMXB inhibit significantly its local and systemic levels. Although one cannot completely exclude the possibility that a greater accumulation of TNF in tissues than plasma could reflect differences in clearance of TNF from these compartments, the anti-LPS activity of SAEP in tissues seems supported by the following experimental observations: (i) interference of serum in the formation of a stable complex between LPS and the peptides; (ii) the biological activity of the peptides, related to the balance of TNF inhibited in serum and organ tissues, which occurs in a time-frame of treatment (–30 min from LPS challenge) much broader than their $T_{1/2}$ values estimated in the bloodstream (5 min); (iii) the calculated distribution volume of SAEP-2 in vivo, indicating the extravasal diffusion of the peptide as well known for PMXB.[24]

Finally, although serum TNF levels do not correlate with lethality in dose-response experiments, since maximal circulating levels of TNF are already observed at sublethal LPS doses with no further increase when LPS is given at a lethal dose,[9] the results of this study suggest that tissue-associated TNF is often overlooked in studies designed to elucidate the biological mechanisms triggered by endotoxicosis.

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