**IMMUNOSTIMULATORY ACTIVITY OF THE BACTERIAL EXTRACT OM-89**


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**Abstract:** The bacterial extract OM-89 used for the prevention and treatment of recurrent urinary tract infections constitutes an effective immunostimulant in vitro and in vivo. Here we demonstrate that OM-89 shows mitogenic properties towards murine spleen cell cultures from LPS responder and non-responder mice. In macrophages the extract induces the translocation of NF-κB into the cell nucleus and RNI (radical nitrogen intermediates) release, which could be attributed to single fractions of the extract. Our findings on the in vitro immunostimulatory effect of OM-89, as well as its immunogenic and adjuvant properties, are of importance for understanding its therapeutic efficacy as demonstrated in clinical studies.

**Key words:** bacterial extract; proliferation; NF-κB; nitric oxide; LPS responder; LPS non-responder; gel elution

**INTRODUCTION**

The bacterial extract OM-89, which contains immunostimulatory components extracted from 18 alkaline treated, uropathogenic *Escherichia coli* strains has been shown to reduce the incidence of recurrent infections of the lower urinary tract in both children and adults (Frey et al. 1986; Hachen et al. 1990; Tammen et al. 1990). This protection is most likely based on the stimulation of different immune defense mechanisms: bacterial extracts have been shown in vitro to enhance the production of tumor necrosis factor α (TNF-α), interleukin-1, interleukin-2, and γ-interferon by human peripheral blood mononuclear cells (Wybran et al. 1989). The extract also stimulates the phagocytic activity of macrophages and activates natural killer (NK) cells (Wybran et al. 1989; Van Pham et al. 1990). We have shown in a mouse model, using an in vitro cell culture system, that OM-89 acts as a polyclonal B-lymphocyte activator in mice (Bessler et al. 1991). OM-89 administered orally to mice enhances the IgA levels in intestinal secretions (Bessler et al. 1999). We have also demonstrated the in vivo immunogenicity of the extract in mice after parenteral and oral administration (Bessler et al. 1991; Sedelmeier et al. 1995). We could show the reactivity of immune sera against the 18 *E. coli* strains used for the preparation of the extract, and against defined bacterial cell wall components (Baier et al. 1997a). In addition the sera recognized a variety of other pathogenic bacterial strains (Huber et al. accepted). Here we demonstrate data on the in vitro immunostimulatory effect of OM-89. The extract is shown to exhibit a mitogenic effect towards murine spleen cells of different LPS responder and non-responder mouse strains. In macrophage cell cultures we show that OM-89 induces NF-κB translocation and RNI-release, this activity can be attributed to defined fractions of the extract.

**MATERIAL AND METHODS**

**MICE AND IMMUNOSTIMULANTS**

Mice (female, 6-8 weeks of age) were obtained from the Max-Planck-Institut für Immunobiologie, Freiburg, Germany; OM-89 (batch no. 10118/C) was obtained from OM-PHARMA, Geneva, Switzerland (OM-89 is commercially available under the trade name Uro-Vaxom®). The lyophilized product contains immunostimulating fractions extracted from 18 uropathogenic *Escherichia coli* strains. LPS from *Salmonella abortus equi* was a gift from C. Galanos, Freiburg, Germany. Recombinant mouse IFN-γ was purchased from Pharmingen/Becton Dickinson, Hamburg, Germany.

**POLCYCLONAL LYMPHOCYTE ACTIVATION IN VITRO**

Stimulation experiments using murine splenocytes were performed in flat bottom microtiter plates (Falcon/Becton Dickinson, Heidelberg, Germany) in 100 μl aliquots of culture medium at a cell density of 3 x 10⁶/ml, as previously described (Bessler et al. 1985). After 24 h of incubation with different concentrations of OM-89 (50 μl/well), the cells were pulsed for another 24 h with [³H]thymidine (23.1 kBq/well, specific activity 1.85 x 10¹⁴ Bq/mol, Amersham, Braunschweig, Germany). The cell membranes were broken by freezing and thawing, and the cells were harvested with an automatic cell harvester (Pharmacia LKB, Freiburg, Germany), collected on glass fiber filters.
(Pharmacia LKB), and dried overnight. Radioactivity was measured by liquid scintillation (Betaplate, Pharmacia LKB).

**CULTURE CONDITIONS FOR THE MACROPHAGE CELL LINE RAW 264.7**

The mouse macrophage cell line RAW 264.7 was cultured in tissue culture dishes (Falcon/Becton Dickinson) using RPMI 1640 (Gibco, Paisley, UK) supplemented with 2.5 g/l glucose, 10% heat-inactivated FCS, 1% non-essential amino acids, 100 U/ml penicillin/100 µg/ml streptomycin (all from Seromed Biochrom KG, Berlin, Germany) and allowed to grow up to 70% confluency. 10 pg OM-89 was added for 2 h

**PREPARATION OF MURINE BONE MARROW-DERIVED MACROPHAGES (BMDM)**

BMDM were differentiated in vitro from bone marrow precursor cells as previously described (Hoffmann et al. 1996). Briefly, bone marrow cells were flushed from femur and tibia of 6- to 8-week-old mice, washed twice in RPMI 1640 (Gibco) and grown for 11 days in liquid cultures in teflon film bags (SLG, Gating, Germany) at 37 °C and 5% CO₂. The culture medium consisted of RPMI 1640 supplemented with 15% L-cell-conditioned medium as a source of M-CSF, 10% heat inactivated FCS (Gibco), 5% heat inactivated horse serum, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin and 5 x 10⁻⁵ M 2-mercaptoethanol (all from Seromed Biochrom KG). Cultures were set up with 6 x 10⁶ cells/50 ml. After harvesting, the macrophages were washed once, counted and resuspended at 2 x 10⁶ cells/ml. For the preparation of L-cell-conditioned medium 1 x 10³ 1929 cells/ml were cultured in 100 ml cell culture flasks (Falcon/Becton Dickinson) in RPMI 1640 with 10% FCS, 4mM L-glutamine, 1% non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin at 37 °C and 5% CO₂. After 7 days, the culture supernatants were harvested, cleared from cell debris by centrifugation (1500 x g, 4 °C, 15 min) and stored at -20 °C.

**IMMUNOFLUORESCENCE ANALYSIS OF THE TRANSCRIPTION FACTOR NF-κB**

Indirect immunofluorescence analyses were performed as previously described (Beranger et al. 1994). Bone marrow-derived macrophages of Balb/c mice were seeded at a concentration of 5 x 10⁴ cells/ml (chamber slides, 154534, Nunc, Wiesbaden, Germany) and allowed to grow up to 70% confluence. 10 µg OM-89 was added for 2 h at room temperature. Cells were washed with PBS and fixed in 3.5% paraformaldehyde in PBS for 15 min at room temperature. Permeabilization of the cells was performed by incubation with 0.5% saponin, 0.2% BSA, 2% FCS and 5 mM MgCl₂ in PBS for 10 min at room temperature. All further washings and incubations were carried out at room temperature in the same buffer containing 0.05% saponin. Cells were washed 3 times for 3 minutes with constant gentle agitation and then incubated for 2 h with anti-NF-κB antibody recognizing the NF-κB p65 subunit (Santa Cruz Biotechnology, Heidelberg, Germany), diluted 1:40. Cells were washed five times (5 x 3 minutes), and incubated for 2 h with an anti-mouse IgG biotin-conjugated secondary antibody (Sigma, Deisenhofen, Germany), which was diluted 1:50. After washing (5 x 3 minutes), FITC-labeled avidin (Sigma), diluted 1:50, was added, and cells were further incubated for 2 h in the dark. Subsequently, cells were washed six times for 3 minutes with constant gentle agitation at room temperature, and finally examined under a fluorescence microscope (Reichert-Jung, Polyvar, Jena, Germany).

**INDUCTION OF NO IN MURINE MACROPHAGES**

Mature BMDM were harvested, washed once, and resuspended in RPMI 1640 medium (Gibco) supplemented with 10% FCS, 1% non-essential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin. 1 x 10⁵ cells were seeded per well into 96-well flat bottom microtiter plates (Falcon, Becton Dickinson) and stimulated with various concentrations of the OM-89 (25 pg/ml - 2.5 mg/ml), as indicated in the results section, in a total volume of 150 µl. RAW 264.7 cells were harvested, washed once, and seeded at a density of 5 x 10⁴ cells/well (100 µl) into the wells of 96-well microtiter plates. After 24 h of incubation at 37 °C and 5% CO₂, 50 µl OM-89 (dissolved in supplemented medium) was added per well and the plates were incubated for further 42 h. Culture supernatants were harvested after 40 or 42 h and checked for nitrite concentration. Assays were performed in triplicate.

Production of nitric oxide was determined by measuring nitrite, a stable metabolite of nitric oxide, in culture supernatants using the Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine in 2.5% phosphoric acid): 100 µl culture supernatant was mixed with 100 µl Griess reagent, and the absorbance at 550 nm was measured using a Dynatec MRX ELISA plate reader (Denkendorf, Germany). Nitrite concentrations were calculated using sodium nitrite as a standard.

**FRACTIONATION OF OM-89 AND GEL-ELUTION**

OM-89 (30 mg/ml) was separated in a 12% non-reducing SDS-PAGE (non-reducing sample buffer: 125 mM Tris/HCl, 10% glycerin, 3.3% SDS, 0.05% bromphenol blue). For the estimation of molecular weights a prestained molecular weight marker was used (Bio-Rad, Munich, Germany). Polyacrylamid gel electrophoresis was performed at 200 V for 40 min at room temperature using a Ready Gel Cell (Bio-Rad). After electrophoresis one gel was stained for protein. The second gel was transferred into the the Mini Whole Gel Eluter
(Bio-Rad). Proteins were eluted according to Andersen et al. (1992). Briefly, the gel was removed from the electrophoresis chamber, placed on a glass plate, and fitted by the use of a template provided by the manufacturer. The Mini Whole Gel Eluter chamber was filled with buffer, the gel transferred and overlaid with soaked filter paper. Electroelution was performed at 100 mA for 30 min at room temperature. The resulting fractions (14 in total) were diluted 2:3 in sample buffer (125 mM Tris/HCl, 10% glycerol, 3.3% SDS, 2 mM DTT, 0.05% bromophenol blue) and submitted to a further SDS-PAGE (12%). Subsequently, the proteins were visualized by silver staining (Blum et al. 1987).

**RESULTS**

Fig. 1 demonstrates the mitogenic lymphocyte activation of spleen cells of different mouse strains by the bacterial extract OM-89. At concentrations above 3 μg/ml OM-89 induced a marked increase of [3H]-thymidine incorporation in BALB/c splenocytes (Fig. 1a). Similar results were obtained with splenocytes from C57Bl/10ScSn-mice (LPS responder). When OM-89 was tested on splenocytes from C57Bl/10ScCr-mice (LPS non-responder), the effect was considerably reduced; however, above a concentration of 80 μg/ml an enhanced incorporation of radioactivity was detected. As a control we tested the bacterial mitogen LPS (Fig. 1b):

![Graph showing mitogenic splenocyte activation induced by OM-89 and LPS](image-url)

*Fig. 1. Mitogenic splenocyte activation induced by OM-89 and LPS.* Dose-response dependent [3H]-thymidine incorporation in $3 \times 10^5$ splenocytes from BALB/c mice, C57Bl/10ScSn mice and C57Bl/10ScCr mice was measured after incubating cultures for 48 h in the presence of OM-89 (a) or LPS (b). Values represent means ± SD of triplicate cultures.
splenocytes from BALB/c and C57Bl/10ScSn exhibited a pronounced reaction to LPS, as detected by the enhanced incorporation of [3H]-thymidine using 0.01-10 μg/ml of LPS for 48 h. In contrast, the LPS non-responder C57Bl/10ScCr mice were not reactive.

We further investigated the in vitro stimulatory effect of OM-89 on macrophages: we examined the translocation of the nuclear transcription factor NF-κB as an index of cell activation. As can be seen in Fig. 2a, bone marrow derived macrophages (BMDM) from BALB/c mice incubated for 2 hours with 10 μg/ml OM-89 showed a strong nuclear fluorescence, due to an OM-89 dependent translocation of NF-κB into the cell nucleus. Fig. 2b shows control macrophages incubated with medium alone, resulting in an uniform distribution of the NF-κB complex (IκB/p50-p65) throughout the cytoplasm, indicating no translocation of NF-κB (p50-p65) into the nucleus.

We also investigated the macrophage stimulatory effect of OM-89 by monitoring the NO release in primary murine macrophage cell cultures derived from LPS responder and non-responder strains. Macrophages were prepared from the bone marrow of BALB/c, C57Bl/10ScSn and C57Bl/10ScCr mice, and the NO release was measured after a 40 h incubation period. OM-89 induced a pronounced NO release from BMDM of the LPS responder mice BALB/c and C57Bl/10ScSn within a concentration ranging from 2.5 pg/ml to 2.5 mg/ml (Fig. 3a). The effect was less pronounced in the LPS non-responder strain C57Bl/10ScCr. As a positive control we measured the induction of the NO release by LPS. As seen in Fig. 3b, a marked enhancement of the NO-release after incubation with LPS was observed in BMDM from LPS responder mice. As expected, in the LPS non-responder mice no NO release could be observed.

We also determined the NO release from the murine macrophage cell line RAW 264.7. As seen in Fig. 4, OM-89 at concentrations between 2.5 ng/ml and 250 ng/ml enhanced the NO-release from the cell line RAW 264.7 after a stimulation
Fig. 3. Induction of nitric oxide release in bone marrow derived murine macrophages by OM-89 and LPS. Dose-response dependent nitric oxide release of $1 \times 10^5$ BMDM / well was measured after incubation for 40 h in the presence of OM-89 (a) and LPS (b). Values represent means ± SD of triplicate cultures.

period of 42 h in a dose dependent manner. At concentrations between 0.25 μg/ml and 250 μg/ml, OM-89 exhibited a pronounced stimulatory effect; the NO amounts obtained were similar to that induced by interferon-γ (2.5 U/ml) which was used as control.

In order to identify the bacterial component(s) responsible for its biological activity, the bacterial extract OM-89 was submitted to SDS-PAGE and subsequent gel elution, resulting in different fractions containing bacterial components of a defined molecular weight range, as shown in Fig. 5a. Each fraction was assayed for its ability to induce NO-release in BMDM. Fig. 5b shows the results obtained: only fraction 4 and 5, containing compounds of molecular masses between 40 and 80 kDa, showed a strong stimulatory effect. The NO-release induced by the other fractions was marginal.

DISCUSSION

The bacterial extract OM-89 was investigated with respect to its immunostimulatory properties in vitro. We could show that OM-89 is able to act as a polyclonal lymphocyte activator, since the ex-
Fig. 4. OM-89-induced release of nitric oxide from the murine macrophage cell line RAW 264.7. Dose-response dependent nitric oxide release of 5 x 10^4 RAW 264.7 cells/well was measured after 42 h in the presence of OM-89. Values represent means ± SD of triplicate cultures.

Fig. 5. Induction of nitric oxide release in murine BMDM by fractions of OM-89 obtained after separation (SDS-PAGE) and gel elution. (a) OM-89 (30 mg/ml) was separated in 12% SDS-PAGE and the gel was electro-eluted on a Mini Whole Gel Eluter. Aliquots (10 µl) of fractions 1-14, were diluted 2:3 with sample buffer, and analyzed by SDS-PAGE (12%); proteins were visualized by silver staining. (b) Nitric oxide release of 1 x 10^5 BMDM/well was measured after a 40 h incubation period in the presence of either 50 µl aliquots from each OM-89 fraction or 25 µg/ml OM-89. Values represent means ± SD of triplicate cultures. (n.d. = not detectable)
tract exhibited a stimulatory effect on spleen cell cultures of LPS responder BALB/c, C57Bl/10ScSn mice, and also of LPS non-responder C57Bl/10ScCr mice. These results indicate that the stimulatory effect of OM-89 is due to an active principle different from LPS. This was confirmed by further investigations, performed by us and other groups, using additional LPS non-responder mouse strains (Bessler et al. 1991; van Pham et al. 1990).

We also determined the immunostimulatory properties of the extract by measuring the NF-κB translocation into the nucleus. NF-κB, a key transcription factor of lymphocytes and macrophages, belongs to the Rel family with important regulatory functions in the immune system (Neurath and Pettersson et al. 1997) including cell division and differentiation (Gerondakis et al. 1998). NF-κB controls genes encoding cytokines, chemokines, interferons, MHC proteins, growth factors and cell adhesion molecules (Baueuerle and Henkel 1995). NF-κB is a heterodimer, composed of p50 and p65 subunits. In unstimulated cells, NF-κB is located in its inactive form (bound to a member of the IκB family of inhibitory proteins) in the cytoplasm. Upon stimulation, IκB is phosphorylated by cellular kinase complexes known as IKK. This leads to its degradation and translocation of NF-κB (p50-p65) to the nucleus. We here demonstrate by immunofluorescence using an antibody against the subunit p65 of NF-κB that the staining pattern in control macrophages was predominantly distributed in the cytoplasm, whereas in cells stimulated with OM-89 (10 μg/ml) a distinct nuclear fluorescence was observed. This indicates an OM-89 induced nuclear translocation of NF-κB (p50-p65) and thus macrophage activation.

Macrophage activity is mediated e.g. by cytokines, reactive oxygen and nitrogen intermediates (ROI and RNI) (Ding et al. 1988). RNI include nitrite (NO2−), nitric oxide (NO) and nitrogen dioxide (NO2). BMDM have been shown to respond to IFN-γ and bacterial components such as lipopeptides, muramyl-dipeptide (MDP) and LPS by releasing cytokines and RNI (Hoffmann et al. 1988; Haushild et al. 1990a; Lu et al. 1996). Here, we investigated the production and release of nitric oxide from BMDM and the murine macrophage cell line RAW 267.4 after stimulation with OM-89 or with different fractions of this extract. Our and other data (Mauel and Riblet 1991; Huber et al. 1995) show that OM-89 strongly induces, in a dose dependent manner, the release of NO from macrophages. These results are comparable to the NO release detected after stimulation of macrophages with optimal concentrations of IFN-γ or other bacterial derivatives, such as LPS, MDP and lipopeptides (Haushild et al. 1990 b; Chen et al. 1996, Terenzi et al. 1995). BMDM from BALB/c and C57Bl/10ScSn (LPS responder) were activated to a similar extent; BMDM from C57Bl/10ScCr (LPS non-responder) were activated to a minor degree. These mice, which exhibit a mutation in the LPS gene locus on chromosome 4, are highly resistant to all LPS effects (Coutinho and Meo 1978; Watson and Riblet 1974). A null mutation in the LPS gene, which is homologous to human Toll-like-receptor 4 (TLR 4), in C3H/Hej and C57Bl/10ScCr mice, is responsible for the defective LPS signalling. The LPS hyporesponsive mice strain C3H/Hej has a point mutation in the TLR4 gene leading to an amino acid exchange at position 712 of the TIR domaine. In contrast C57Bl/10ScCr strains do not express TLR4 at all due to a genomic deletion (Poltorak et al. 1998). Thus the results of our investigations suggest that OM-89 contains immunostimulatory compounds different from LPS. From previous results some of these compounds have been characterized serologically as bacterial lipopeptides, porins and muropeptide fragments (Sedelmeyer et al. 1995). Also heat shock proteins, which are constituents of the extract, have been described as adjuvants (Suzue et al. 1996; Schirmeck et al. 1999). In order to further characterize the active principles of OM-89, we tested different fractions for their ability to induce NO-release in BMDM. OM-89 was submitted to SDS-PAGE in order to separate its compounds according to their molecular weight. 14 different fractions were obtained after subsequent gel elution, each covering a distinct molecular weight range. In addition, the gel eluter acts as an electrodialyzer removing SDS from protein, leaving them in a non-toxic physiological buffer, allowing the resulting samples to be used directly in cellular assays (Andersen et al. 1993). Activity monitored by NO-release in BMDM was induced only by fractions 4 and 5, containing proteins corresponding to a molecular mass of 40-80 kDa. Presently, we are working on the purification, isolation and characterization of the active component(s) present in fraction 4 and 5.

In summary, we were able to show in vitro immunostimulatory effects of OM-89, such as activation of murine splenocytes, translocation of NF-κB and induction of RNI-release in murine macrophages. Taken together with the immunostimulatory properties of the bacterial extract shown earlier (Baier et al. 1997 a and b, Huber ef al. accepted) our data are reflecting the stimulating properties of OM-89 with respect to both stages of the host defense against microbial infections. First, the innate immune response is enhanced leading to a stimulation of the unspecific defense system. Second, the antigen specific adaptive immune response is activated resulting in specific antibodies. Our findings are of importance for understanding the therapeutic effect of OM-89 as shown in clinical studies performed with patients with chronic urinary tract infections (Tammen et al. 1990 and 1988, Hachen 1990, Rugendorff 1992).

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