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TH1-ORIENTATED IMMUNOLOGICAL PROPERTIES OF THE BACTERIAL EXTRACT OM-85-BV

M. Huber[1], H. Mossmann[2], W. G. Bessler[1]

[1]Institute for Molecular Medicine and Cell-Research, AG Tumor Immunology/Vaccine, University Hospital Freiburg, Germany
[2]Max-Planck-Institute for Immunobiology, Freiburg, Germany

Abstract: The bacterial extract OM-85-BV prepared from 21 pathogenic bacterial strains is administered orally to adults and children for the treatment and prevention of recurrent infections of the respiratory tract. We analyzed *in vitro* and *in vivo* the immunomodulatory effects of the extract. The lysate acted as a non specific macrophage activator, inducing NO production as well as the translocation of transcription factor NF-κB into the nucleus in murine bone marrow-derived macrophages. Besides stimulating unspecifically the immune system, a bacteria-specific humoral immune response was revealed. After oral application, a trend to increase bacteria-specific IgG and IgA in serum was observed. Also a marked increase of bacteria specific IgA in saliva as well as in supernatants of Peyer's patches and mesenteric lymph nodes-derived cell cultures was found. The immunomodulatory properties of the extract were also investigated with respect to shifting the Th1/Th2 bias in an *in vivo* allergy model. BALB/c mice were orally immunized with OM-85-BV and subsequently sensitized intraperitoneally with the allergen ovalbumin. The group pretreated with OM-85-BV showed a decrease of both total and ovalbumin specific IgE. Accordingly, in spleen cell supernatants the Th1-associated cytokine IFN-gamma was increased, and the Th2-associated cytokine IL-4 was downregulated. Our findings suggest that the immunoprotective effects of OM-85-BV observed in human beings may be correlated to its Th1 augmenting properties.

Key words: OM-85-BV, Broncho-Vaxom, bacterial extract, NF-κB, nitric oxide

INTRODUCTION

The bacterial extract OM-85-BV, whose active principle contains immunomodulating fractions extracted from 21 bacterial strains occuring in infections of the respiratory tract, has been shown to reduce the incidence of recurrent infections of the upper and lower respiratory tract in both children and adults (Heintz et al., 1989; Paupe 1991; Derenne and Delclaux 1992; Orcel et al., 1994; Jara-Pérez and Berber 2000; Gutiérrez-Tarango and Berber 2001; Schaad et al., 2002). Protection is most likely based on the stimulation of different humoral and cellular immune defense mechanisms. Bacterial extracts have been shown *in vitro* to enhance the production of tumor necrosis factor alpha (TNF-alpha), interleukin-1, interleukin-2, and IFN-gamma. They stimulate the phagocytic and metabolic activity of macrophages and induce B lymphocyte proliferation, activate natural killer cells, and upregulate adhesion molecules (Wybran et al., 1989; Emmerich et al., 1990; Nauck et al., 1991; Duchow et al., 1992; Mauel et al., 1994), and activate dendritic cells (Zelle-Rieser et al., 2001). We also demonstrated in a murine system that OM-85-BV is a polyclonal B cell activator *in vitro*, and that it is also able to act *in vivo* as an immunoadjuvant enhancing the antigen-specific immune response (Bessler et al., 1990; Bessler and Sedelmeier 1993; Bessler et al., 1997). Here we analyze further *in vitro* and *in vivo* the immunostimulatory and immunomodulatory effect of the bacterial extract OM-85-BV.

MATERIAL AND METHODS

Mice and immunostimulants

6- to 10-week old C57Bl/10 ScSn LPS responder, C57Bl/10 ScCr LPS nonresponder, NMRI and BALB/c mice were obtained from the breeding facilities of the Max-Planck-Institut für Immunbiologie, Freiburg, Germany or from the Institut für Ernährungsforschung, Potsdam, Germany. OM-85-BV lyophilizate was obtained from OM PHARMA, Meyrin/Geneva, Switzerland (OM-85-BV is commercially available under the trade name Broncho-Vaxom®). The lyophilized product contains immunostimulating fractions extracted from 21 alkaline-treated bacterial strains frequently found during infections of the respiratory tract.

Determination of spleen cell proliferation

Splenocytes proliferation was determined by measuring ^{3}H-thymidine incorporation into DNA. Cultures were set up in flat bottom microtiter plates in a total volume of 150 µl/well. 3 x 10^5 splenocytes and OM-85-BV at the concentrations indicated were cultured in RPMI 1640 (Gibco, Eggenstein, Germany) containing 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 5% CO_2. Control cultures received medium. After 24 h cells were pulsed for additional 24 h by the addition of ^{3}H-thymidine (23.125 KBq/well). After freezing and thawing the cell culture plates, cell debris were harvested and ^{3}H-thymidine incorporation into DNA was determined by liquid scintillation counting.

Preparation of murine bone marrow-derived macrophages (BMDM)

BMDM were differentiated *in vitro* from bone marrow precursor cells as previously described (Huber et al., 2000). Briefly, murine bone marrow cells were flushed from femur and tibia, washed twice in RPMI 1640 and grown for 11 days in liquid cultures in teflon film bags (SLG, Gauting, Germany) at 37°C and 5% CO_2. 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 10^6 cells/50 µl. After harvesting, the macrophages were washed once, counted and resuspended at 2 x 10^6 cells/ml. For the preparation of L-cell-conditioned medium 1 x 10^5 L929 cells/ml were cultured in 100 ml cell culture flasks (Falcon/Becton Dickinson) in RPMI 1640 with 10% FCS, 4 mM L-glutamine, 1% non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin at 37 °C and 5% CO_2. 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.

Induction of NO in murine macrophages

BMDM were harvested, washed once, and resuspended in RPMI 1640 medium supplemented with 10% FCS, 1% non-essential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin. 1 x 10^5 cells were seeded per well into 96-well flat bottom microtiter plates (Falcon, Becton Dickinson) and stimulated with various concentrations of OM-85-BV, in a total volume of 150 µl and incubated for 40 h at 37 °C and 5% CO_2. 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 were 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.

Immunofluorescence analysis of the transcription factor NF-κB

Indirect immunofluorescence analyses were performed as previously described (Ayoub et al., 2000). BMDM of BALB/c mice were seeded at a concentration of 5x10^4 cells/ml (chamber slides 154534, Nunc, Wiesbaden, Germany) and allowed to grow up to 70% confluency. 10 µg OM-85-BV were 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 $MgCl_2$ 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 min with constant gentle agitation and then incubated for 2 h with anti-NF-kB antibody recognizing the NF-κB p65 subunit (Santa Cruz Biotechnology, Heidelberg, Germany), diluted 1:40. Cells were washed five times (5 x 3 min), 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 min), 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 min with constant gentle agitation at room temperature, and finally examined under a fluorescence microscope (Reichert-Jung, Polyvar, Jena, Germany).

Preparation of lymphocytes from spleen, mesenteric lymphnodes, and Peyer`s patches

Mice were sacrificed by cervical dislocation. Spleens were homogenized using a potter. The cell suspension was washed twice by centrifugation (280 x g, 4 °C, 10 min) and resuspended in 20 ml RPMI 1640, containing 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (supplemented RPMI). Preparation of lymphocytes from mesenteric lymphnodes (MLN) or Peyer´s patches: MLN were taken from the peritoneum and dispersed with a scalpel; Peyer´s patches were cut from the small intestine. The organs were incubated, with gentle shaking at 37 °C for 30 min in 20 ml RPMI 1640 containing 0.75 mg/ml $CaCl_2$ x $2H_2O$ and 50 U/ml collagenase type VII, 150 µg DNAseI (Sigma, Deisenhofen, Germany). The partially digested tissues were further homogenized by passing through a syringe. After washing twice, cells were resuspended in 20 ml supplemented RPMI. For cytokine determinations, 3 x 10^6 cells/ml were incubated in the presence of Concanavalin A (ConA, 5 µg/ml) for 50 h. Afterwards, the supernatants were harvested, centrifuged at high speed to remove any remaining cell debris, aliquoted and kept at -20 °C.

Time-resolved fluoroimmunoassay (FIA)

Microtiter plates (Dynex) were coated with 10^9 bacteria/ml. 3 x 10^6 cells/ml, prepared from mesenteric lymphnodes or Peyer's patches of control or immunized BALB/c mice, were applied to the individual wells and incubated for 20 h, 37 °C, 5% CO_2. As detection antibody biotinylated affinity-purified goat-anti-mouse IgA (Biozol) was used.

Cytokine Detection by ELISA

Assays were performed according to the instructions of the manufacturer (BD Bioscience, Franklin Lakes, NJ, USA). In short, anti-cytokine capture antibodies (anti-IFNgamma 5 µg/ml, anti-IL-4 2.5 µg/ml in PBS, 50 µl/well) were added to Immulon4 HBX-96-well microtiter plates (Thermo Life Science), and incubated overnight at 4 °C. The plates were washed five times with PBS containing 0.05% Tween 20 (SLT-Washer, SLT Labinstruments, Germany). Free potential binding sites were blocked by adding 200 µl/well of assay diluent at room temperature in PBS for 1 h, followed by five consecutive washings of the plates with PBS containing 0.05% Tween 20. 50 µl/well of cell super-

natants or standards diluted in medium were added and incubated overnight at 4°C. After washing, the plates were incubated for 1 h at room temperature with the detection dilution (detection antibody and avidin-HRP each 1 : 250 in assay diluent). Again, the plates were washed ten times and 50 µl of TMB substrate (A and B 1 : 1) or 0.4 mg/ml ortho-phenylene-diamine (OPD), 0.02% H_2O_2 (30%) in 0.1 M citrate buffer, pH 4.2, were added and incubated for 20 min at room temperature. The reaction was terminated by 2N H_2SO_4, and photo-metrical determination of the enzymatic reaction was performed in a Dynatech MRX Reader (SLT, Overath, Germany) at 450 nm for TMB or 490 nm for OPD.

IgG/IgA-ELISA

Assays were performed as previously described (Sedelmeier and Bessler, 1995). Sera were tested by ELISA for bacteria-specific antibodies. Bacterial antigens (lysates from a mixture of all bacterial strains used for the preparation of OM-85-BV) suspended in 50 µl aliquots of 0.1 M carbonate buffer pH 9.6, were coupled to the surface of 96-well standard ELISA plates (M129A,F; Dynatech, Denkendorf, Germany) by incubating for 2 h at 37 °C and overnight at 4 °C in a H_2O saturated atmosphere. The plates were washed three times with PBS containing 0.2% Tween 20 (SLT-Washer, SLT Labinstuments, Germany). Free potential binding sites were blocked by adding 200 µl/well of 1 % bovine serum albumin (BSA in PBS) at 37°C for 2 h, followed by threefold washing of the plates with PBS containing 0.2 % Tween 20. 50 µl/well of the appropriately diluted pooled antisera or saliva in PBS/1 % BSA containing 0.1 % Tween 20 were then transfered to the coated microtiter plates, which were incubated for 3 h at 37 °C. Saliva was collected 3-5 min after the i.p. injection of 0.5% Pilocarpin (Sigma, Deisenhofen, Germany). After repeated washing 50 µl RAMIgG-POD or GAMIgA-POD (peroxidase conjugated rabbit-anti mouse immunoglobulin G or peroxidase conjugated goat-anti mouse immunoglobulin A), dissolved in PBS/1% BSA, were added into the wells at a dilution of 1 : 1000. The plates were incubated for 2 h at 37 °C and washed three times with PBS containing 0.2 % Tween 20. After the addition of 100 µl/well of substrate solution (0.4 mg/ml ortho-phenylene-diamine (OPD), 0.02% H_2O_2 (30%) in 0.1 M citrate buffer, pH 4.2), the plates were incubated for 20 min at room temperature and the reactions were stopped by adding 50 µl of 2N H_2SO_4. Photometrical determination of the enzymatic reaction was performed in a Dynatech MRX Reader at 490 nm.

IgE-ELISA

Sera were assayed for ovalbumin-specific IgE and total IgE. ELISA plates (Immulon4 HBX, Dynex, Denkendorf, Germany) were coated with ovalbumin (50 µg/ml) in 0.1 M carbonate buffer pH 9.6, or for the determination of total IgE with anti-IgE (1 : 250) in PBS by incubating for 2 h at 37 °C and overnight at 4 °C in a H_2O saturated atmosphere. The plates were washed five times with PBS containing 0.05% Tween 20 (SLT-Washer). Free potential binding sites were blocked by adding 200 µl/well of 1% bovine serum albumin (BSA in PBS) at room temperature for 2 h, and the plates were washed five times with PBS containing 0.05% Tween 20. 100 µl/well of the appropriately diluted pooled antisera in PBS/1% BSA were then transfered to the coated microtiter plates, which were incubated for 2 h at room temperature and overnight at 4 °C. After repeated washings, 100 µl biotinylated anti-mouse IgE (biotin conjugated anti-mouse-immunoglobulin E) dissolved in PBS/1% BSA were added into the wells at a dilution of 1 : 500. After incubation for 1 h at room temperature, plates were washed again five times followed by incubation with 100 µl/well streptavidin-HRP 1:1000 in PBS/1% BSA for 1 h at room temperature and washed again. After the addition of 100 µl/well of substrate solution (TMB substance A and B, 1 : 1), the plates were incubated for 20 min at room temperature, and the reaction was stopped by 2N H_2SO_4. Photometrical determination of the enzymatic reaction was performed in a Dynatech MRX Reader at 450 nm.

RESULTS

We first investigated the unspecific activation/stimulation of the defense system by OM-85-BV *in vitro* in a murine model. The results of spleen cell proliferation experiments are demonstrated in Figure 1. Cells from three inbred mouse strains, BALB/c, C57Bl/10 ScSn LPS responder, and C57Bl/10 ScCr LPS nonresponder, showed clearly increased ^{3}H-thymidine incorporation after stimulation with 16 - 2000 µg/ml OM-85-BV. The response induced by OM-85-BV in the LPS nonresponder splenocytes was diminished, suggesting that OM-85-BV does not share the same mechanism of action than LPS. Note that for all strains, maximum activity was found around 400 µg/ml.

We also investigated the macrophage stimulatory effect of OM-85-BV by monitoring the NO release in bone marrow-derived macrophages (BMDM). Cells were prepared from the bone marrow of BALB/c mice, and the NO release was measured after a 40 h incubation period. OM-85-BV induced a pronounced NO release from BMDM within a broad concentration ranging from 25 µg/ml to 2.5 mg/ml (Fig. 2). Maximal activity was reached at 250 µg/ml. The macrophage stimulatory effect of OM-85-BV was also examined by determining the translocation of nuclear transcription factor NF-κB as an index of cell activation. As can be seen in Fig. 3B, BMDM from BALB/c mice incubated for 2 h with only 10 µg/ml OM-85-BV showed a strong nuclear fluorescence, due to an OM-85-BV-dependent translocation of NF-κB into the cell nucleus. In contrast, Fig. 3A 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). The results presented so far show clearly that OM-85-BV activates macrophages *in vitro* from concentrations as low as 10 µg/ml in the NF-κB translocation assay, whereas the maximal activity in the proliferation assay (see above) is reached at 400 µg/ml.

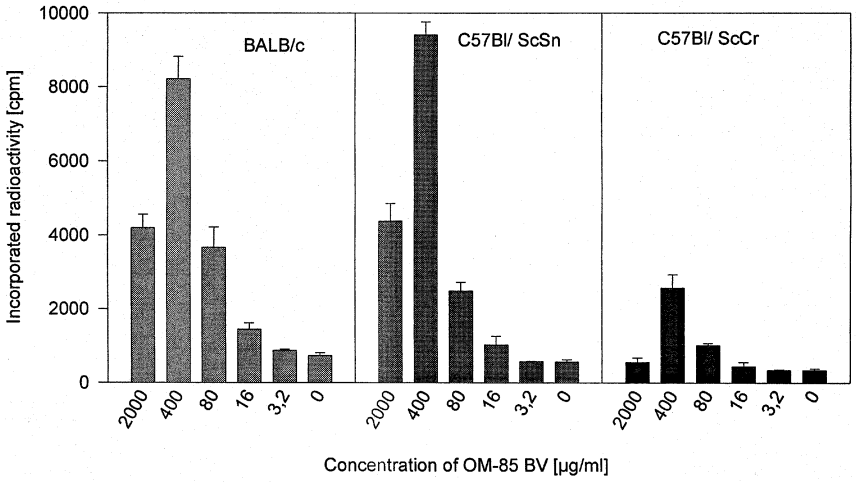

Fig. 1. Splenocyte activation induced by OM-85-BV. Dose-response dependent ^{3}[H]-thymidine incorporation in 3×10^5 splenocytes from BALB/c, C57Bl/10 ScSn LPS responder, and C57Bl/10 ScCr LPS nonresponder mice measured after incubating cultures for 48 h in the presence of OM-85-BV. Values represent means ± SD of triplicate cultures. Assays were done at least two times.

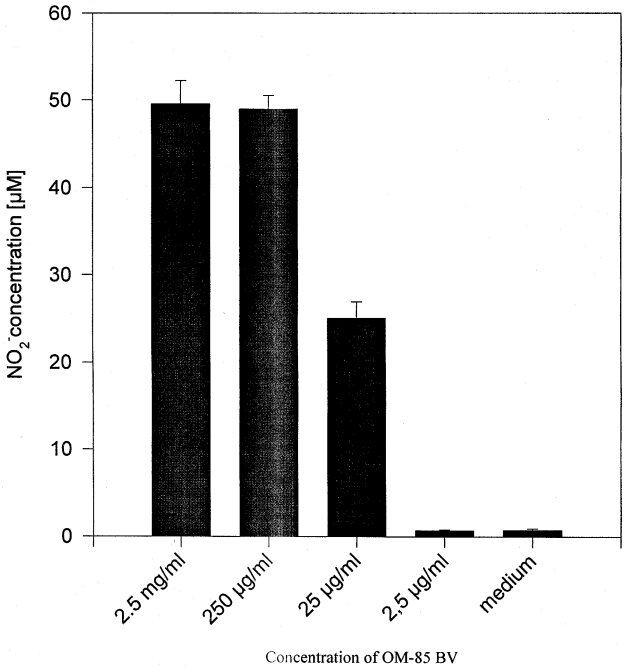

Fig. 2. Induction of nitric oxide release in bone marrow-derived murine macrophages by OM-85-BV. Dose-response for nitric oxide release of 10^5 BMDM / well for 40 h in the presence of OM-85-BV. Values represent means ± SD of triplicate cultures. Assays were done at least two times.

We next investigated *in vivo* if the oral administration of OM-85-BV would induce an enhancement of bacteria-specific antibodies in serum. In Figure 4, the serum IgG response after the administration of the extract is shown in NMRI mice kept in SPF conditions. Control animals exhibited serum IgG levels, which were only slightly increased after the immunization with the bacterial extract (Fig. 4 A), and we found an increase of bacteria specific serum IgA in the group immunized with OM-85-BV (Fig. 4 B). In contrast to this slight increase in serum immunoglobulins, a marked increase of bacteria specific IgA was found in supernatants of Peyers patches and mesenteric lymph nodes-derived cell cultures (Fig. 5).

Next we measured IgA levels in saliva. BALB/c mice were immunized with the bacterial extract, and bacteria-specific IgA concentrations in saliva were determined three days after the last immunization. Mice immunized with the unrelated control antigen tetanus toxoid showed no bacteria-specific IgA, whereas animals orally immunized with OM-85-BV (2 x 10 daily immunizations) exhibited increased bacteria specific saliva IgA levels (Fig. 6). This increase was found both in mice having received only bacterial extract and in mice having received bacterial extract in combination with unrelated antigen.

Finally, the immunomodulatory properties of the extract were investigated with respect to shifting the Th1/Th2 bias in an *in vivo* allergy model. BALB/c

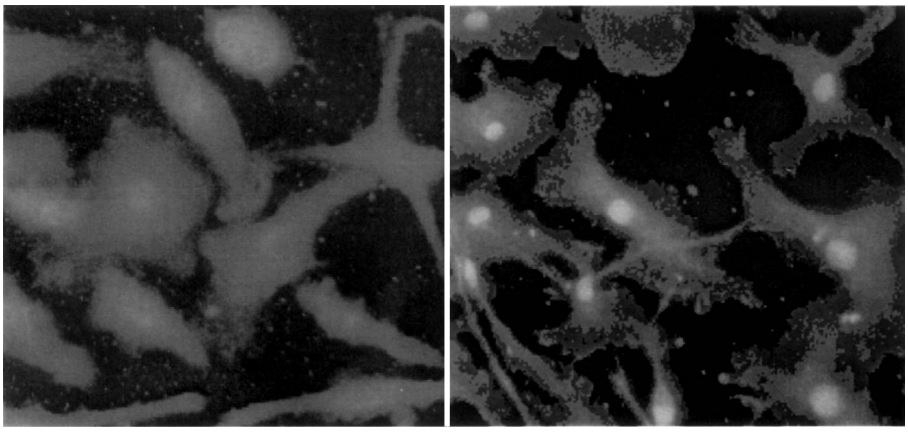

Fig. 3. OM-85-BV induces translocation of NF-kB. Bone marrow-derived macrophages from BALB/c mice (5×10^4 cells/ml) were stimulated for 2 h with medium (A) or 10 µg/ml OM-85-BV (B). After fixation and permeabilization, cells were immunostained with anti-NF-κB-p65-antibodies and visualized with biotinylated goat-anti-rabbit IgG and fluorescein isothiocyanate-conjugated avidin. Assays were done at least two times.

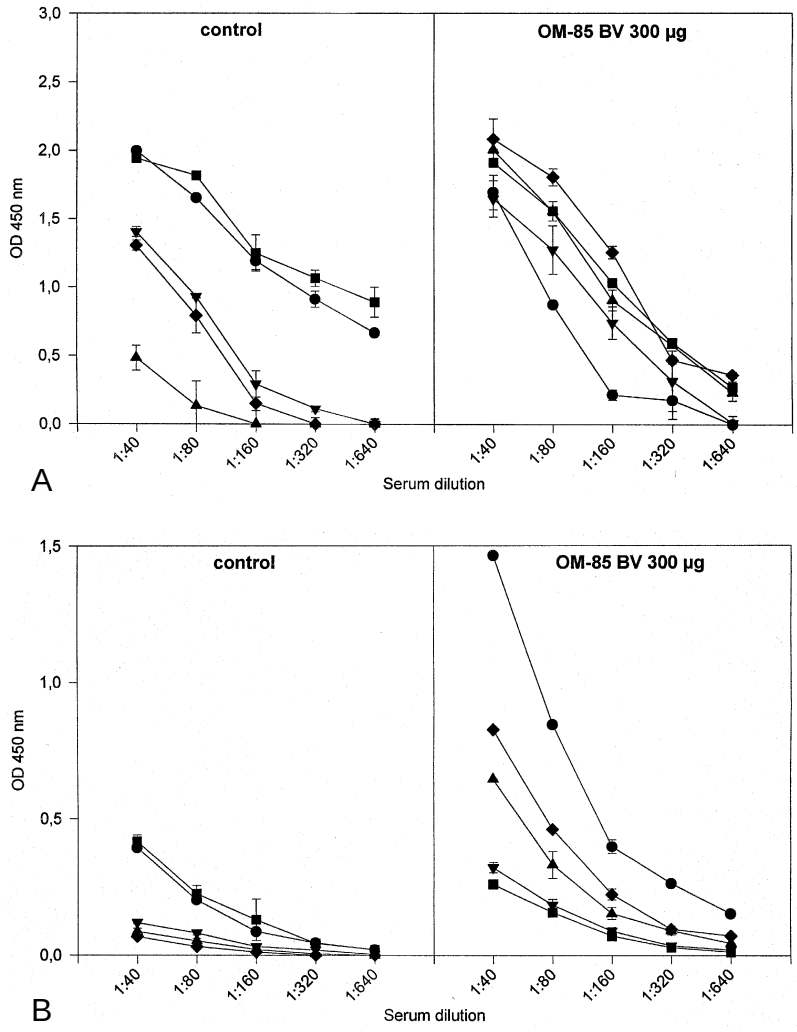

Fig. 4. Oral immunization with OM-85-BV. Determination of bacteria-specific serum IgG and IgA. Groups of 5 mice were immunized orally with 0 or 300 µg of OM-85-BV on days 1, 2, 3, 4, 5, 8, 9, 10, 11, 12 and bled on day 20. Bacteria-specific serum IgG (A) or IgA (B) was measured in NMRI mice kept in SPF conditions. One experiment, means of duplicate determinations ± SD.

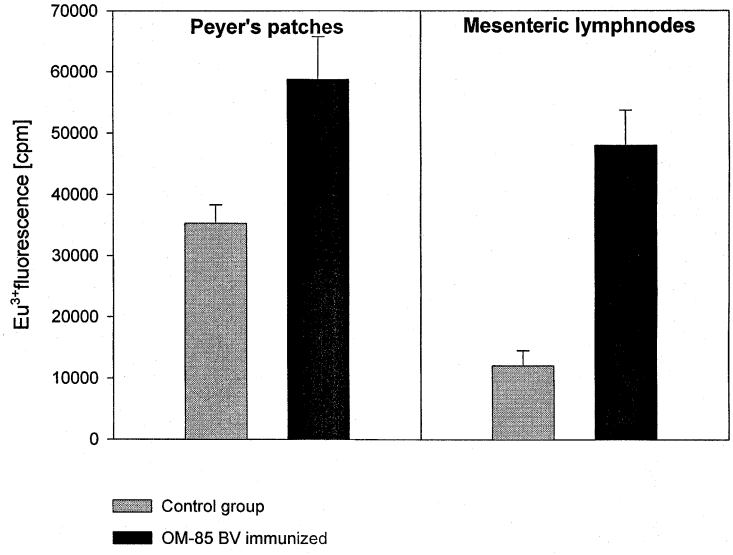

Fig. 5. Oral immunization with OM-85-BV. Determination of bacteria-specific IgA in supernatants of Peyers patches and mesenteric lymph nodes of BALB/c mice. Groups of 5 BALB/c mice were immunized on days 1-10, and bacteria-specific IgA concentrations in supernatants of Peyers patches and mesenteric lymph nodes were determined on day 11. Grey columns: control immunizations. Black columns: oral immunizations with 300 µg OM-85-BV. One experiment, means of duplicate determinations.

mice were orally immunized with the bacterial extract, subsequently they were sensitized intraperitoneally with the allergen ovalbumin in combination with the lipopeptide adjuvant P_3CSK_4. In response to allergen administration, the control group showed an increase in both ovalbumin specific serum IgE (Fig. 7) and total serum IgE (Fig. 8). In contrast, the OM-85-BV-treated group showed downregulation of both ovalbumin-specific and total IgE. We further investigated the possible Th1/Th2 regulatory effect of OM-85-BV by the determination of IFN-γ and IL-4 in spleen cell supernatants after the oral immunization with OM-85-

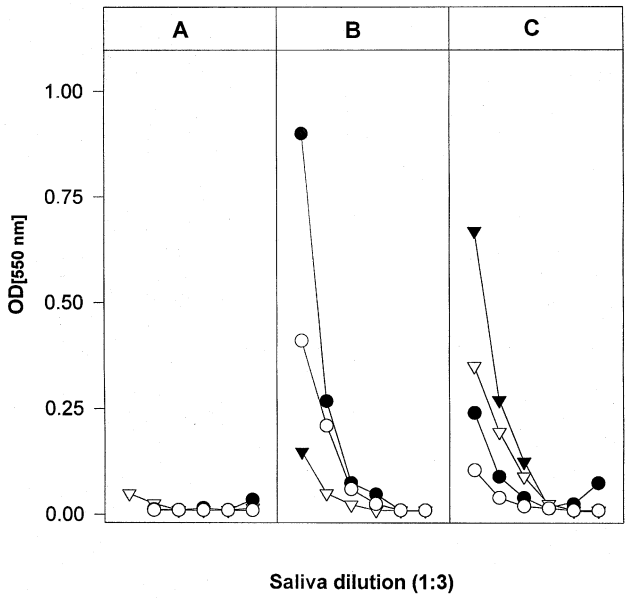

Fig. 6. Oral immunization with OM-85-BV. Determination of bacteria-specific IgA in saliva. Groups of 3-4 BALB/c mice were immunized, and bacteria specific IgA concentrations in saliva were determined. A. Control immunization with an unrelated antigen (Tetanus toxoid, 0.5 μg i.p. on days 1 and 43). B. Oral immunization with 8 mg/immunization OM-85-BV (2x10 daily immunizations starting on days 1 and 43) in combination with the injection of unrelated antigen (Tetanus toxoid, 0.5 μg i.p. on days 1 and 43). C. Oral Immunization with 8 mg/immunization OM-85-BV (2x10 daily immunizations starting on days 1 and 43). Saliva was taken on day 54. One experiment, means of duplicate determinations.

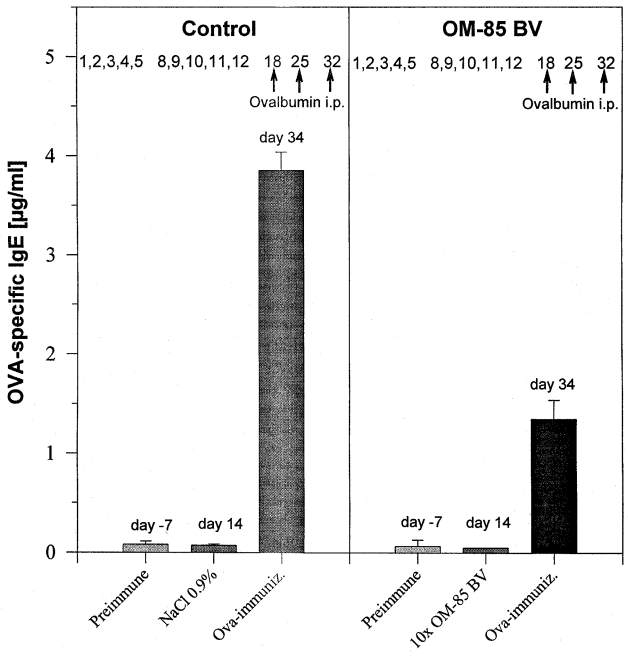

Fig. 7. Decrease of OVA-specific serum IgE after oral treatment with OM-85-BV and OVA immunization. Groups of 3 BALB/c mice were first immunized orally with OM-85-BV on day 1, 2, 3, 4, 5 / 8, 9, 10, 11, 12 and then intraperitoneally with ovalbumin on day 18, 25 and 32. Bleedings were done for preimmune serum, on day 14 and 34. Pool sera were tested for OVA-specific IgE by ELISA. One experiment, means of duplicate determinations ± SD.

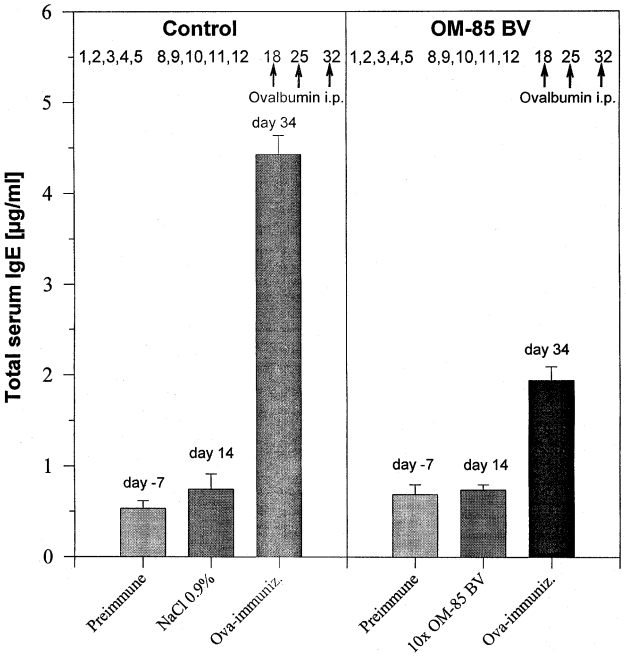

Fig. 8. Decrease of total serum IgE after oral treatment with OM-85-BV and OVA immunization. Groups of 3 BALB/c mice were first mmunized orally with OM-85-BV on day 1, 2, 3, 4, 5 / 8, 9, 10, 11, 12 and then intraperitoneally with ovalbumin on day 18, 25 and 32. Bleedings were done for preimmune serum, on day 14 and 34. Pool sera were tested for total IgE by ELISA. One experiment, means of duplicate determinations.

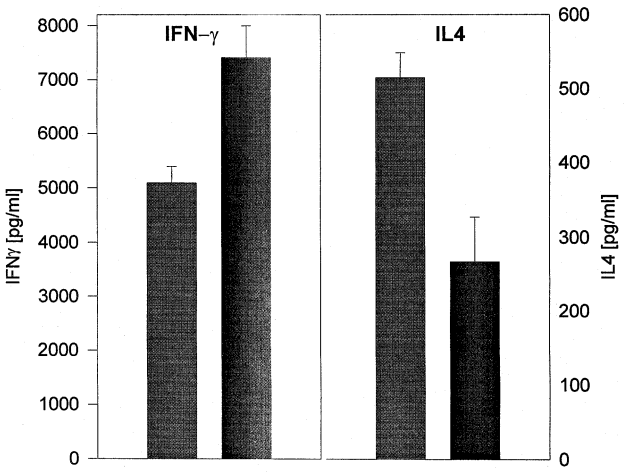

Fig. 9. Oral immunization with OM-85-BV induces upregulation of IFN-gamma and downregulation of IL-4. Cytokines were determined in spleen cell supernatants of BALB/c mice. Groups of 3 BALB/c mice were orally immunized on day 1/3/5, 17/19, 29/31/33, 43/45/47, 65/66/68, 78/80/82, 92/94/96, 113/115/120 and sacrificed on day 121. Spleen cells were prepared (control group - grey columns, OM-85-BV immunized - black columns) and cytokine secretions of $3x10^6$ cells/ml after *in vitro* stimulation with ConA (5 μg/ml) were determined. One experiment, averages of four fold determinations ± SD.

BV. Cytokine secretions of 3 x 10^6 cells/ml were determined *ex vivo* (Fig. 9). We found the Th1-specific cytokine IFN-γ upregulated and the Th2-specific cytokine IL-4 downregulated.

Discussion

The bacterial extract OM-85-BV (Broncho-Vaxom®) is used for the prevention and treatment of recurrent respiratory tract infections and consists of immunostimulating components derived from 8 bacterial species or 21 bacterial strains frequently responsible for these infections. The product is obtained by alkaline extraction of heat-killed bacteria and contains mostly protein and peptide constituents. In previous experiments, OM-85-BV was tested in BALB/c mice after intraperitoneal administration (Bessler and Sedelmeier, 1993; Bessler et al., 1997). We found that OM-85-BV is an immunogen, as repeated injections yielded OM-85-BV-specific antibodies. Thus, alkaline treatment of the bacteria during the preparation of the extract did not abolish the immunogenicity of the bacterial components. The antiserum recognized, to a variable degree, all 21 bacterial strains used for the preparation of the extract (Bessler and Sedelmeier, 1993; Sedelmeier and Bessler, 1995). Major reactivity was found against *Klebsiella*, *Streptococcus*, *Diplococcus* and *Neisseria*. We also determined the serum Ig isotypes induced after repeated immunizations with OM-85-BV. A marked IgG response and a less pronounced IgM response were seen. The antisera recognized the typical bacterial cell wall components porin and the N-terminus of lipoprotein, which are common constituents of Gram negative bacteria, and murein, which is present both in Gram negative and Gram positive bacteria (Sedelmeier and Bessler, 1995). We also have previously demonstrated the adjuvanticity (Bessler et al., 1990 and 1997) of the extract.

Here, the bacterial extract was further investigated with respect to its immunostimulatory properties *in vitro* and *in vivo*. In a first set of experiments we could demonstrate that OM-85-BV is able to act as an *in vitro* activator for splenocytes in LPS responder and LPS nonresponder mice being active over a wide concentration range. Stimulatory activity for other strains has been described by us (Bessler et al., 1990). Cells from BALB/c and C57Bl/10ScSn mice were activated to a similar extent; cells from C57Bl/10ScCr mice were stimulated to a minor degree. These mice exhibit a mutation in the LPS gene locus on chromosome 4, and 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 signaling. C57Bl/10ScCr strains do not express TLR-4 at all, due to a genomic deletion (Poltorak et al. 1998). Thus the results of our investigations suggest that OM-85-BV also contains immunostimulatory compounds different from LPS; the bacterial extract probably acts mainly, but not exclusively via the TLR-4 cascade (data not shown). From previous results some of these compounds have been characterized serologically as bacterial lipopeptides, porins and muropeptide fragments (Sedelmeier et al. 1995; Bessler et al., 1997). Also heat shock proteins, which are constituents of the extract (Jacquier-Sarlin et al., 1996), have been described as adjuvants (Suzue et al. 1996; Schirmbeck et al. 1999).

Macrophage activity is mediated e.g. by cytokines or reactive oxygen and nitrogen intermediates (ROI and RNI) (Ding et al. 1988). RNI include nitrite (NO_2-), nitric oxide (NO) and nitrogen dioxide (NO_2). 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; Hauschildt et al. 1990a; Lu et al. 1996). Here, we investigated the release of nitric oxide from BMDM after stimulation with OM-85-BV. Our data show that OM-85-BV strongly induces, in a dose-dependent manner, the release of NO from macrophages (comp. Bessler et al., 1997). These results are comparable to the NO release detected after stimulation of macrophages with IFN-γ or other bacterial derivates, such as LPS, MDP and lipopeptides (Hauschildt et al. 1990 b; Chen et al. 1996, Terenzi et al. 1995).

We also determined the macrophage 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 (Baeuerle 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 I-kB family of inhibitory proteins) in the cytoplasm. Upon stimulation, I-κB is phosphorylated by cellular kinase complexes known as IKK. This leads to its degradation and translocation of NF-kB (p50-p65) to the nucleus. We here demonstrate by immunofluorescence using an antibody against the subunit p65 of NF-kB that the staining pattern in control macrophages was predominantly distributed in the cytoplasm, whereas in cells stimulated with OM-85-BV a distinct nuclear fluorescence was observed. This indicates an OM-85-BV-induced nuclear translocation of NF-kB (p50-p65) and thus macrophage activation.

Since OM-85-BV is used orally in patients to treat or to prevent recurrent bacterial infections of the respiratory tract, we investigated, in our mouse model, if the oral administration of the extract would induce an enhancement of bacteria-specific antibodies in serum or saliva. In Figure 4, the serum IgG response after the administration of the extract is shown in NMRI mice kept in SPF conditions. As seen from the figure, control animals exhibited low serum IgG levels. After the immunization with the bacterial extract IgG levels were slightly increased. We also determined IgG1 and IgG2a in sera of OM-85-BV-immunized mice and found a slight increase in bacteria-specific IgG2a, and no significant change in bacteria-specific IgG1 (data not shown).

OM-85-BV is given by the oral route. Thus, we expect activation of the mucosal immune system, espe-

cially an increase of IgA. We could demonstrate, in SPF mice, an increase in bacteria-specific serum IgA in 4 out of 5 mice. An increase of bacteria-specific IgA was also found in supernatants of cell cultures derived from Peyer's patches and mesenteric lymph nodes of orally immunized mice. The determination of bacteria-specific IgA in saliva after the oral immunization with OM-85-BV alone, or in combination with an unrelated antigen, also showed a rise in IgA in 2 out of 3 mice. Thus, an increase in bacteria-specific IgA could be demonstrated for several mouse strains and in several organs. The data correspond to the findings of Puigdollers et al. (1980) describing increased salivary IgA production in man stimulated by OM-85-BV.

Finally, in a last set of experiments, in an *in vivo* mouse allergy model, we orally immunized BALB/c mice with the bacterial extract, and subsequently sensitized them intraperitoneally with the allergen ovalbumin in combination with the lipopeptide adjuvant P_3CSK_4. Remarkably, we could show a downregulation of both total and ovalbumin specific IgE in the OM-85-BV pretreated animals. Accordingly, in previous experiments it was shown that the bacteria specific IgE titer does not increase after immunization with OM-85-BV (Bessler und Sedelmeier, 1993). In humans reduced IgE titers were found after immunization with OM-85-BV (Emmerich et al., 1990). The IgE synthesis is inhibited by proinflammatory cytokines, e.g. IFN-γ, TNF-α, IL-1β, IL-6 und IL-8, which were shown to be induced after stimulation with OM-85-BV (Wybran et al., 1990; Keul et al., 1996; Byl et al., 1998). Accordingly, we also dermined IFN-γ and IL-4 in spleen cell supernatants of mice orally immunized with OM-85-BV. After ConA stimulation of the cell cultures we could demonstrate an upregulation of the Th1-specific cytokine IFN-γ and the downregulation of the Th2-specific cytokine IL-4 indicating a slight bias towards a Th1 immune response. This corresponds to findings of another group who found selective enhancement of systemic Th1 immunity in newborn rats receiving orally the bacterial extract (Bowman and Holt, 2001).

In summary, we were able to show *in vitro* immunostimulatory effects of OM-85-BV, such as activation of murine splenocytes, translocation of NF-κB and induction of RNI-release in murine macrophages. Here, OM-85-BV acts mainly, but not exclusively via TLR-4. In addition, we found bacteria specific IgG and IgA immunostimulatory properties of the bacterial extract with a slight bias towards a Th1 immune response. Our data are reflecting the stimulating properties of OM-85-BV with respect to two stages of the host defense against microbial infections. After the nonspecific immune response has been activated, an immunological memory takes place, as shown by increased production of antibacterial IgG and IgA antibodies in sera, saliva, Peyer´s patches, and mesenteric lymph nodes. The effects obtained in the ovalbumin model of sensibilization, together with the increased production of IFN-γ and decreased IL-4 levels in spleen supernatants, suggest that the immunoprotective effects of OM-85-BV observed in human beings may be correlated to its Th1 augmenting properties.

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Address for correspondence:
Prof. Dr. Wolfgang G. Bessler
Institut für Molekulare Medizin und Zellforschung
AK Tumorimmunologie/Vakzine
Universitätsklinikum Freiburg
D-79104 Freiburg, Germany
Tel. ++49-761-203-5500
Fax ++49-761-203-5492
e-mail: wolfgang.bessler@mol-med.uni-freiburg.de