The bacterial extract Broncho-Vaxom protects against respiratory infections - in vivo and in vitro studies

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The bacterial extract Broncho-Vaxom (OM-85 BV) given orally to patients is used for the prevention and treatment of respiratory tract infections. We investigated OM-85 BV efficacy against viral and bacterial infections in murine models. We first evaluated its role of protecting from an A/PR/8/34 (H1N1) influenza virus infection. In a group treated with OM-85 BV all animals survived, compared to 70% in the untreated control. We also investigated on the protection of mice from a *Salmonella typhimurium* infection after the oral administration of OM-85 BV. Here, 100% of the OM-85 BV treated animals survived compared to 58% of the untreated control. We have also shown the capacity of OM-85 BV to behave as a general immunomodulator, e.g. its macrophage stimulatory effect by monitoring the NO release in bone marrow derived macrophages. To further explore the mode of action of OM-85 BV, in vitro analysis of biological material of mouse treated with BV revealed an increase of bacteria specific IgA in supernatants of Peyer's patches and mesenteric lymph nodes derived cell cultures. Thus, this report again demonstrates the antimicrobial activity of Broncho-Vaxom.

Keywords Bacterial extract, Broncho-Vaxom, OM-85 BV, Immunostimulation, Respiratory tract infections

1. Introduction

The bacterial extract Broncho-Vaxom® (OM-85 BV) given orally to patients is indicated in adults and children for immunotherapy, prevention of recurrent infections of the respiratory system and acute and chronic bronchitis, and co-medication in the treatment of acute respiratory infections, and its clinical efficacy has been documented by multicentric, placebo controlled, double blind studies [1-9]. The extract 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. Immunoprotection by the extract is based on the stimulation of different humoral and cellular immune defence mechanisms. Bacterial extracts have been shown *in vitro* to enhance the production of various cytokines including tumor necrosis factor alpha (TNF-alpha), interleukins, and IFN-gamma. The soluble extracts stimulate the phagocytic and metabolic activity of macrophages, activate natural killer cells, and upregulate adhesion molecules also in leukocytes from the lung [10-16 and OM Pharma internal study reports]. Dendritic cells activated with OM-85 BV exhibit an enhanced T cell stimulatory activity [17] and induce a mild and well-shaped hDC activation that may contribute to the generation of a "pre-alert state" in the organism and result protective towards incoming infections [C Parola et al., submitted for publication]. In agreement with past data, we demonstrated in a murine system that OM-85 BV is a polyclonal B cell activator *in vitro*, and that it is able to act as an immunoadjuvant [18]. We also have shown its macrophage stimulatory effect by monitoring the NO release in bone marrow derived macrophages and by determining the translocation of nuclear transcription factor NF-kappa B as an index of cell activation [19]. The aim of this study was to summarize our investigations on the immune stimulatory effect of OM-85 BV and its efficacy against viral and bacterial infections (ref [22] and [27]) in murine models.

2. Material and Methods

6- to 10-week old mice were obtained from the breeding facilities of the Freiburg University Hospital, or the Max-Planck-Institut für Immunbiologie Freiburg, or from the Institut für Ernährungsforschung, Potsdam. OM-85 BV, an soluble alkaline extract from Hemophilus influencae, Diplococcus pneumoniae, Klebsiella pneumoniae, Klebsiella ozaenae, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus viridans, and Neisseria catarrhalis combined with inert admixtures was obtained from OM-Pharma, Meyrin, Switzerland (OM-85 BV is commercially available under the trade name Broncho-Vaxom). If not indicated otherwise, amounts of OM-85 BV confer to the pure dry bacterial extract. Bone marrow derived macrophages (BMDM) were differentiated *in vitro* from bone marrow precursor cells as previously described [20]. The induction of NO in murine macrophages was performed as described in [19]. Influenza A/PR/8/34 (H1N1) was grown as described previously as well as the murine model of aerosol influenza virus infection [21]. A virus concentration of 7.5×10^6 pfu/ml was used for challenge infection. To determine the protective effect of OM-85 BV in a *Salmonella typhimurium* infection model, nineteen C57BL/6 mice were used each for the group treated with OM-85 BV and for the water control group. Mice were treated orally with 15 mg OM-85 BV in 0.5 ml distilled water once a day for 10 consecutive days, and challenged with a suspension of *Salmonella typhimurium* strain 415 by intraperitoneal injection [22]. The ELISA assays for the investigation of potential bacteria specific

antibodies and cytokines were performed as previously described [18, 19]. In short: mice were sacrificed by cervical dislocation. Spleens were homogenized using a potter. Mesenteric lymph nodes (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. Süpernatants were then harvested, centrifuged at high speed to remove any remaining cell debris, aliquoted and kept at -20°C until cytokine determination. For IgA detection by a time-resolved fluoroimmuno assay (FIA*),* microtiter plates (Dynex) were coated with 10^9 bacteria/ml. 3 x 10^6 cells/ml, prepared from mesenteric lymph nodes 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. For cytokine detection by ELISA, anti-cytokine capture antibodies (anti-IFN gamma 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 supernatants 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 asssay 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, was added and incubated for 20 min at room temperature. The reaction was terminated by 2N H_2SO_4, and photometrical 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.

3. Results

As an indication of cell activation, we 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 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. 1). Maximal activity was reached at an extract concentration of 250 µg/ml.

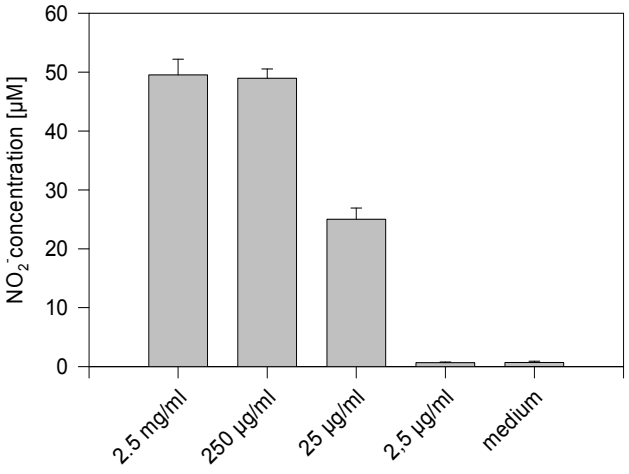

Fig. 1 Induction of NO release in bone marrow derived murine macrophages by OM-85 BV. Dose response plot for 10^5 BMDM / well for 40 h in the presence of OM-85 BV. Values represent means ± SD of triplicate cultures. Adapted from [19].

We next investigated on the immunogenicity of the soluble bacterial extract. While per os administration of OM-85 BV is the usual route in patients and does not generate a significantly increase in bacteria specific antibody response in serum (data not shown), we investigated if repeated intraperitoneal (ip) injections of OM-85 BV would yield specific serum Ig antibodies in our murine model. BALB/c mice were immunized in 2 week intervals 12 times with 8.75 µg OM-85 BV and bled on day 166. Compared to the sera of untreated control mice, the antiserum of OM-85 BV immunized animals showed an elevated response to all bacterial strains used for the preparation of the extract (Fig. 2, one experiment, groups of 5 mice. Adapted from [22]).

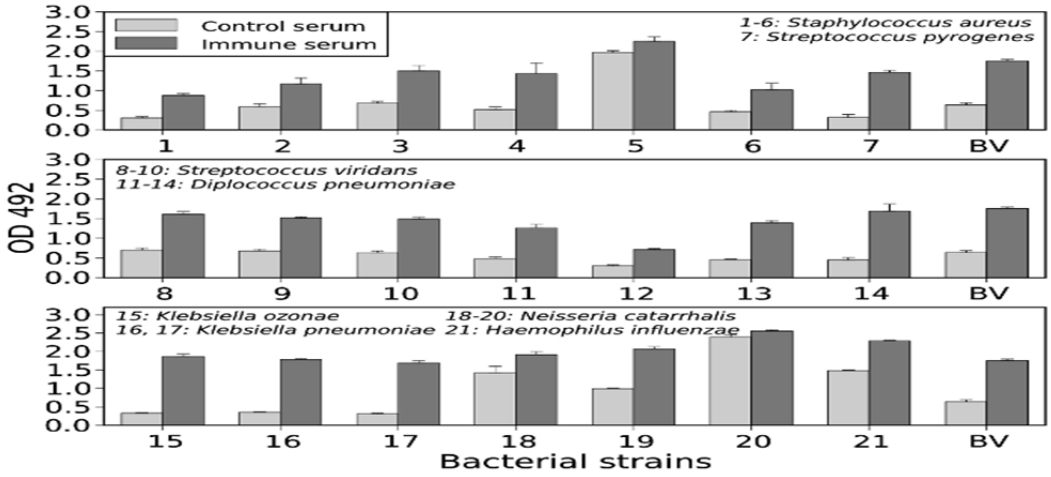

Fig. 2 Intraperitoneal immunization of BALB/c mice with OM-85 BV. Determination of bacteria specific serum Ig. Mice were immunized ip in 2 week intervals 12 times with 8.75 µg OM-85 BV and bled on day 166. Bacteria specific serum Ig was measured by ELISA. Means of duplicate determinations ± SD. BV: Positive control wells coated with OM-85 BV. One experiment, groups of 5 mice. Adapted from [22].

We then investigated if the multiple dosing of oral administration of OM-85 BV would induce an enhancement of bacteria specific antibodies in serum in our mouse model. In Fig. 3, the serum IgA response after the administration of the extract is shown in NMRI mice kept in SPF like conditions. Control animals exhibited only slightly increased serum IgA levels, however, we found an increase of bacteria specific serum IgA in 3 of 5 mice in the immunized group. In contrast to this slight increase, a more pronounced increase of bacteria specific IgA was found in supernatants of Peyer`s patches and mesenteric lymph nodes derived cell cultures (Fig. 4).

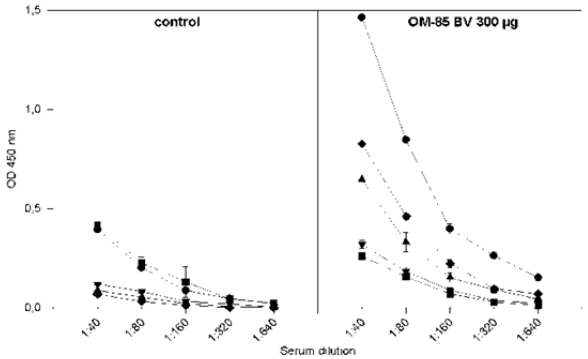

Fig. 3 Determination of bacteria specific IgA in serum after multiple oral immunizations with OM-85 BV. Groups of 5 mice were immunized orally with 0 or 52.5 µg of OM-85 BV (corresponding to 300 µg of OM-85 BV lyophilizate, which contains as active principle 17.5% dry bacterial extract) on days 1,2,3,4,5,8,9,10,11,12 and bled on day 20. Serum IgA was measured by ELISA. One experiment, means of duplicate determinations ± SD. Adapted from [19].

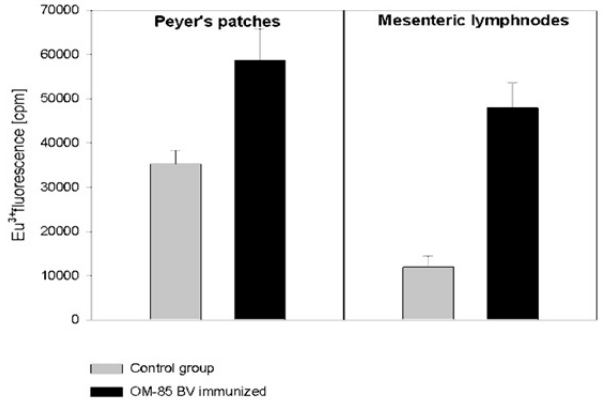

Fig. 4 Determination of bacteria specific IgA in supernatants of Peyer's patches and mesenteric lymph nodes derived cell cultures of BALB/c mice after multiple oral immunizations with 0 or 52.5 µg OM-85 BV. Groups of 5 BALB/c mice were immunized on days 1-10, and bacteria specific IgA concentrations in the culture supernatants were determined on day 11 by a time-resolved fluoroimmuno assay on bacteria coated microtiter plates. Means of duplicate determinations Adapted from [19].

Finally, we 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 BV. Cytokine secretions of 3 x 10^6 cells/ml were determined *ex vivo* (Fig. 5). We found the Th1-specific cytokine IFN-γ upregulated and the Th2-specific cytokine IL-4 downregulated.

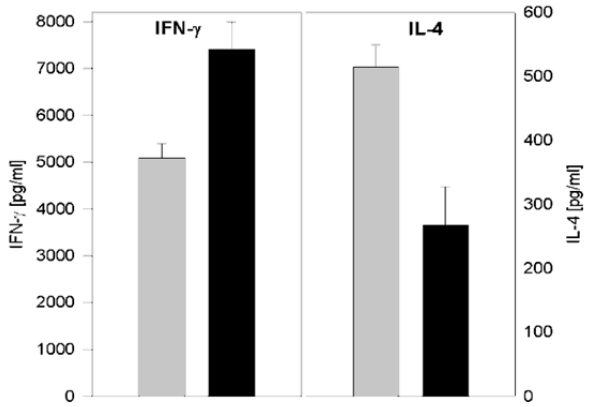

Fig. 5 Oral immunization with 52.5 µg OM-85 BV induces upregulation of IFN-gamma and downregulation of IL-4. Cytokines were determined in spleen cell supernatants of BALB/c mice. Groups of 3 BALB/c mice were orally immunized on day 1/3/5, 17/19, 29/31/33, 43/45/47, 65/66/68, 78/80/82, 92/94/96, 113/115/120 and sacrificed on day 121. Spleen cells were prepared and cytokine secretion of 3 x 10^6 cells/ml after *ex vivo* stimulation with 5 µg/ml ConA was determined. Control group: grey columns; OM-85 BV immunized group: black columns. One experiment, averages of fourfold determinations ± SD. One experiment, groups of 5 mice. Adapted from [19].

We then investigated the role of OM-85 BV administered to mice by the oral route to induce protection against an aerosol A/PR/8/34 (H1N1) influenza virus infection. In the group treated with 1.75 mg/mouse OM-85 BV, all animals survived, compared to 70% in the untreated control group and the group treated with 175 µg/mouse (Fig. 6). Due to the small number of animals, these findings just indicate a trend towards protection (Cochran-Armitage Trend Test, p=0.084). However, the clinical symptoms of surviving animals support the conclusion that the dose of 1.75 mg was able to confer some protection: In animals treated with 1.75 mg extract/mouse, clinical signs occurred at least two days later than in controls and in a less severe form. Moreover, upon a second virus infection three weeks after the first one, a markedly increased antibody induction was observed in the extract treated group [22].

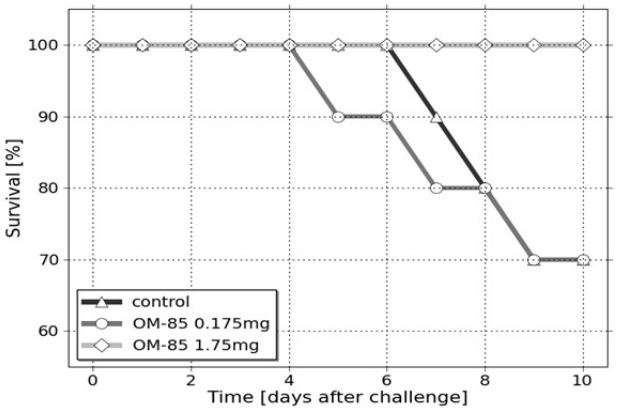

Fig. 6 Survival of BALB/c mice from A/PR/8/34 (H1N1) influenza virus infection. Mice were orally treated with different doses of OM-85 BV for 10 days and then challenged with influenza virus. All animals of the group treated with 1.75 mg/mouse survived. The group receiving 175 µg of the extract and the control group showed partial survival. Adapted from [22].

In a second study [22] on the anti-infective efficacy of OM-85 BV, an experimental model of *S. typhimurium* bacterial infection in mice was used. Mice were pretreated orally for 10 days with 15 mg/mouse OM-85 BV. Animals were challenged intraperitoneally with *S. typhimurium* the day after the end of pretreatment. The follow-up observation was performed for 21 days after infection. 100% of the OM-85 BV treated mice survived, compared to 58% of the untreated mice (Fig. 6).

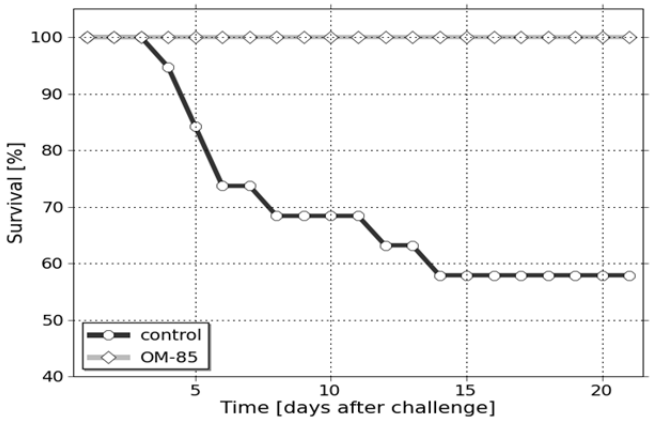

Fig. 7 Death records and defence efficacy of OM-85 BV in *S. typhimurium* infection. Mice pretreated orally for 10 days with 15 mg/mouse OM-85 BV or water were challenged intraperitoneally with *S. typhimurium* (10^4 CFU per mouse) the day after the end of pretreatment. The follow-up observation was performed during 21 days post-infection. The dynamics of death in the groups is shown. 100% of the OM-85 BV treated mice survived, compared to 58% of the untreated mice. Adapted from [22].

4. Discussion

The use of the bacterial extract OM-85 BV for the prevention and therapy of respiratory tract infections and its safety in human patients is founded on a large body of clinical experience [1-9]. While experiments addressing the immunogenicity of OM-85 BV requires further investigation (some performed only once or using different routes of administration), they clearly confirm the capacity of this soluble bacterial extract to elicit a dual immune response. On the one hand the sensing and activation of the innate cell-mediated response together with a general increase of the antibody response from various lymphoid cells and organs, and on the other hand an impact on the adaptive immune cell response as demonstrated by of a decrease of a prototypic Th2 cytokine.

Since OM-85 BV is used orally we expect activation of the mucosal immune system and, in particular, the Gut Associated lymphoid Tissue (GALT). Accordingly, we could demonstrate in mice an increase of IgA in supernatants of cell cultures derived from Peyer's patches and mesenteric lymph nodes. The data correspond to the findings of Puigdollers *et al*. [24] and Emmerich *et al.*[11] describing increased salivary IgA production in man. Interestingly, the bacteria specific IgE titer does not seem to increase after the administration of OM-85 BV [25], and also in human patients reduced IgE titers were found after the administration of OM-85 BV [11]. Also, 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 [15, 16]. Accordingly, we also determined the level of IFN-γ and IL-4 in spleen cell supernatants of mice orally immunized with OM-85 BV using a time and dose regiment similar as used in human patients. After ConA stimulation of the cell cultures which is currently use to activate T cells, 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. While these data have been only performed once and as such have to be interpreted with care, they clearly demonstrate a trend and correspond to findings showing the enhancement of systemic Th1 immunity in newborn rats receiving orally the bacterial extract [26]. The findings suggest that the protective effects of OM-85 BV may also be correlated to its Th1 balancing properties.

The soluble bacterial extract was also able *in vitro* to act as a mitogen for splenocytes in LPS responder and nonresponder mice [18] as well as being a strong activator of macrophages as e.g. shown by the release of NO. Further to this, we also demonstrated in a murine model that OM-85 BV was able to elicit immunogenic properties when changing the route of normal administration. Repeated ip applications to mice induced OM-85 BV specific antibodies which, in this particular case and to a variable degree, recognized all 21 bacterial strains used for the preparation of the soluble extract, thus yielding evidences that alkaline treatment of the bacteria during the preparation of the soluble extract may positively impact on the immunogenicity. The antibodies also recognized the 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 [23]. Alltogether, these results confirm the general model recently described by Rozy and colleagues [28], which is the consecutive mounting of non-specific macrophage stimulation with the intensification of phagocytosis and increased production of pro-inflammatory cytokines resulting in intensive, more effective combined specific and non-specific resistance to infection.

As OM-85 BV is sold since many years to prevent recurrent respiratory tract infections, many reports confirm the stimulation of host defence mechanisms in the lung by OM-85 BV [1-9]. At the preclinical level, the immune enhancing effect of the extract in a viral system has been shown in the past including work from our team. In one of these studies, we orally administered 350 µg/mouse OM-85 BV for 5 consecutive days in combination with an intraperitoneal vaccination with the commercial influenza virus vaccine Mutagrip on days 1 and 8. The results showed that OM-85 BV acted as an immune enhancer for the influenza vaccine [18]. Accordingly, using comparable doses of 175 and 1750 µg/mouse, we investigated the effect of the extract in an established murine model of aerosol influenza virus infection.

We administered OM-85 BV to mice by the oral route to stimulate protective mucosal immunity. In our study, all animals treated with 1750 µg/mouse OM-85 BV survived, compared to 70% in the untreated control group. Moreover, OM-85 BV reduced clinical symptom scores, and enhanced the hemagglutination inhibition antibody titer [22]. Since bacterial infections often superimpose viral lung infections, and since OM-85 BV is also used to prevent bacterial infections of the respiratory tract, an in vivo study was performed to investigate the anti-infective efficacy of OM-85 BV using an experimental model of S. typhimurium infection [22]. Since preliminary studies (not shown) indicated that only high doses of the soluble extract would show a pronounced effect, mice were pretreated orally for 10 days with the high dose of 15 mg/mouse OM-85 BV and were challenged a day after the end of pretreatment. A follow up observation was performed for 21 days after infection. The group treated with the extract showed a survival rate of 100%, compared to 58% in the control group. While the effects in the case of influenza have to be due to an unspecific immune stimulation which confirm previous data showing a general increase of the immune response, Salmonella belongs to the same family (Enterobacteriaceae) as the Klebsiella strain fragments present in the soluble extract.

Seven steps to defeat respiratory tract infections by OM-85 BV

1. Oral intake of the soluble bacterial extract
2. Contents of the OM-85 BV capsule are released within the small intestine
3. The bacterial fragments are taken up by M cells of Peyer`s patches from the GALT
4. Bacterial fragments are presented to cells of the immune system by antigen presenting cells (APCs)
5. Sequential activation of B cells, T cells, monocytes/macrophages
6. From GALT to MALT: GALT sensing towards MALT (distal) immune response
7. Elimination of pathogenic microorganisms from the respiratory system by immune cells and antibody- mediated response

Tab. 1 Suggested mechanism of the antimicrobial action of Broncho-Vaxom

Our findings and the reports of other groups are demonstrating the mode of action of the bacterial extract with respect to two stages of its efficacy against microbial infections (comp. [27]). After oral administration the contents of the OM-85 BV containing capsule are released within the small intestine. Based on the preliminary data obtained with Peyer`s patches, OM-85 is presumably taken up by the surrounding M-cells which are part of the Peyer`s patches from the GALT and known to present pathogen to the immune system. Here, in a first immediate step, a nonspecific macrophage, monocyte, dendritic cell, granulocyte and polyclonal B cell activation is induced fighting the beginning microbial infection. In a second step and with the help of multiple administrations of OM-85, an antigen specific immune response is taking place as shown by the increased production of antibacterial antibodies in serum, saliva, PP and mesenteric lymph nodes (comp. [28]). While this last statement has to be confirmed as it originates from data collected following a different route of administration, it brings evidence of the role of the antibody response in the most probably numerous mechanisms of actions of OM-85. Also, the observed increased production of IFN-γ and decreased IL-4 levels in murine spleen supernatants suggest that the immunoprotective effects of OM-85-BV observed in human beings may also be correlated to its Th1 balancing properties. Thus, this report again demonstrates the antimicrobial activity of Broncho-Vaxom as documented by the clinical studies.

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