Metabolic and Functional Stimulation of Lymphocytes and Macrophages by an \textit{Escherichia coli} Extract (OM-89): In Vitro Studies

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\textbf{Summary:} OM-89, a proteinaceous extract from \textit{Escherichia coli} with very low endotoxin content, was tested for its capacity to stimulate in vitro cells involved in the immune response. OM-89 induced a marked proliferation of mouse spleen cells; \textit{E. coli} lipopolysaccharide (LPS) at the same concentration as present in OM-89 was totally ineffective. Passage through nylon wool strongly decreased the OM-89-induced effect, suggesting that the responding lymphocytes were of the B lineage. Exposure of bone marrow-derived macrophages to OM-89 promoted glucose oxidation through the hexose monophosphate shunt pathway and the capacity to generate superoxide upon phorbol myristate acetate (PMA) stimulation. These effects were not blocked by polymyxin B, whereas this compound completely prevented induction of similar metabolic activation by \textit{E. coli} lipopolysaccharide. In addition, OM-89 treatment induced marked PMA-dependent superoxide and hydrogen peroxide release by macrophages from the LPS low responder mouse strain C3H/HeJ. Incubation with recombinant murine interferon-\(\gamma\) and OM-89, but not with either compound alone, led to functional activation, as shown by the killing of tumor target cells, and by the destruction of the intracellular parasite \textit{Leishmania enriettii} by macrophages of both LPS-responsive and unresponsive mouse strains. These experiments indicate that OM-89 can stimulate metabolic and functional activities of lymphocytes and macrophages that are important for host defense. \textbf{Key Words:} Bacterial extract—Macrophage activation—Tumor cell killing—Intracellular killing—Lymphocyte stimulation.
also displayed an increased delayed hypersensitivity response to the skin sensitizer oxazolone (6) and rapid-onset prostaglandin E2 release from Peyer's patches (5). Experiments in vitro indicated that exposure of human peripheral blood lymphocytes to the drug would stimulate their rosetting and natural-killer activity (7) and alleviate the allergic autotoxic destruction observed when white blood cells from patients hypersensitive to wheat are exposed to wheat antigen in the presence of antibody to the latter (8). Finally, exposure of human peripheral blood mononuclear cells to OM-89 led to the synthesis of various cytokines (7).

Among the most interesting findings regarding the effect of OM-89 in humans is the observation that it significantly improves clinical parameters as-sociated with rheumatoid arthritis (e.g., Ritchie index, number of swollen joints, morning stiffness, erythrocyte sedimentation rate, pain) (9-11). In other double-blind clinical trials, the compound was shown at lower dosages to reduce significantly bacteriuria and dysuria in patients suffering from recurrent urinary tract infections (12,13). The experiments presented in this report were designed to investigate further the activity of OM-89 on metabolic and functional parameters of cells involved in the immune response, i.e., lymphocytes and macrophages. Moreover, since OM-89 is obtained from *E. coli* and since some of the earlier described findings are also typical of the effects of endotoxins on immune cells, the possible involvement of bacterial lipopolysaccharide in mediating the observed effects was examined.

**MATERIALS AND METHODS**

**Chemicals, Reagents, Media, and Animals**

Chemicals were purchased as follows: Con A from Pharmacia (Uppsala, Sweden); Dulbecco's medium and fetal bovine serum (FBS) from Seromed (Munich, F.R.G.); lipopolysaccharide (LPS; from *E. coli* 055:B5) from Difco Laboratories (Detroit, MI, U.S.A.); and polymyxin B (PMB) from Sigma (Munich, F.R.G.). The bacterial extract OM-89 was kindly provided by Laboratoires OM (Meyrin/ Geneva, Switzerland). OM-89 is a glycoprotein-rich alkaline extract from *E. coli*. The experiments described hereafter were performed using a single lot of OM-89 (No. 521). All other lots tested, manufactured during the course of several months, were found to display similar activities. The compound was used either as a liquid concentrate containing 20 mg of active substance per ml, or as a lyophilized powder containing 10% of active substance and 90% inert carriers (excipients). Both preparations were tested for endotoxin contamination by the limulus amoebocyte lysate and pyrogenicity assays. They were found to contain less than 60 ppb of endotoxin (60 ng of LPS/ml of liquid concentrate) and less than 1.2 ppm of endotoxin (1.2 µg of LPS/g of lyophilized powder).

Male or female mice of the CBA/T6, C57BL/6, DBA/2, and C3H/OUJ strains indicated were supplied by the animal colony of the Swiss Institute for Experimental Cancer Research (Epalinges, Switzerland), whereas C3H/HeJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, U.S.A.). The animals were used between 8 and 18 weeks of age.

**Macrophage Cultures**

Exudate cells were harvested from the peritoneal cavity of mice after elicitation by starch (14); alternatively, macrophages were obtained by differentiation in vitro of bone marrow cells (15) as indicated in the text. For determinations of leishmanicidal activity, extracellular cytolysis of target cells, and O2− or H2O2 production, macrophages were suspended in N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid-buffered Dulbecco's medium (DH) supplemented with 5% FBS, and then distributed in 96-well microculture plates (Costar No. 3596. Cambridge, MA, U.S.A.; 105 cells in 100 µl/well except for the H2O2 assay: 4 × 104 cells in 100 µl/well). For measurements of hexose monophosphate shunt (HMPS) levels, the cells were seeded in 24-well tissue clusters (Costar No. 3524; 5 × 105 cells in 0.5 ml/well).

**Spleen Cell Cultures**

Spleen cells were obtained from mice as described elsewhere (16) and suspended in DH medium enriched by addition of l-arginine (200 mg/L), l-asparagine (36 mg/L), l-glutamine (800 mg/L), folic acid (10 mg/L), 5 × 10−5 M 2-mercaptoethanol, and supplemented with 0.5% normal mouse serum (17). Proliferation assays were adapted from Louis et al. (18). Briefly, the cells were distributed in 96-well microtiter plates (Costar No. 3596; 5 × 105 cells/well in 100 µl of medium). Serial dilutions of
OM-89 or other stimulants (in the same medium) were then added, to reach a total volume of 200 µl/well. After 3 days at 37°C, [3H]methylthymidine ([3H]TdR; 2 Ci/mmol; The Radiochemical Centre, [Amersham, England]; 0.5 µCi in 20 µl/well) was added for 16-20 h. The cells were then harvested by means of an automated harvester (Automash, Dynatech, Zürich, Switzerland) and the amount of [3H]TdR incorporated was determined by liquid scintillation counting. In some experiments, T cells were removed from the spleen cell suspension by treatment with a cytotoxic anti-Thy-1 monoclonal antibody (kindly provided by Dr. H. Robson MacDonald) and rabbit complement (Low-Tox-M, CL 3051, Cedarlane Laboratories, Ontario, Canada) as described elsewhere (19). Conversely, the lymphocyte suspension was enriched in T cells by passage through nylon wool (20). The resulting population was analyzed using an anti-Thy-1 monoclonal antibody (kindly supplied by Dr. H. R. MacDonald) and a fluoresceinated goat anti-rat Ig (Tago Inc., Burlingame, CA, U.S.A.) as previously described (17), followed by passage through a fluorescence-activated cell sorter (Becton Dickinson, Sunnyvale, CA, U.S.A.) as previously described (21). The percentage of living T cells after nylon wool treatment of spleen cells was always greater than 85%. Proliferation assays using nylon wool-purified or anti-Thy-1 antibody-treated cell populations were performed in the presence of syngeneic irradiated (2,000 R) filler spleen cells (5 × 10⁵ cells/well) as a source of accessory cells. Control experiments indicated that irradiated filler cells failed to incorporate [3H]TdR when exposed to either Con A or OM-89.

Assessment of Macrophage Activation by Intracellular Killing of Leishmania Parasites

Promastigotes of Leishmania enrietti were prepared from infected guinea pig tissue as previously described (22), and used within 1 month of the original harvest. Infection of macrophages was achieved by adding 100 µl of promastigote suspension to each macrophage-containing microwell at a ratio of 20 parasites per cell, 3 h after plating the latter. The vessels were kept at 37°C for 18-20 h to allow for phagocytosis of the parasites. To remove free parasites and nonadherent cells, the infected cultures were then thoroughly washed by flicking off the medium, and filling the wells with warm Hanks' balanced salt solution (HBSS) using a multichannel pipette. The procedure was repeated twice. Microscopic examination of stained preparations indicated a rate of infection of over 85% of the macrophages. Activation of the macrophages was obtained by exposing the infected cells to recombinant murine interferon-γ (r-Mu-IFN-γ; lot no. 2309-24, produced by Genentech Inc. and kindly supplied by Boehringer Ingelheim, Vienna, Austria), in the presence or absence of appropriate dilutions of OM-89 or LPS. After 24 h of incubation at 37°C, the macrophages were lysed with 0.01% sodium dodecyl sulfate to release the intracellular microorganisms (23). The wells were then supplemented with 5% FBS-enriched HOSMEM II medium (24) and [3H]methylthymidine ([3H]TdR; 2 Ci/mmol; The Radiochemical Centre, Amersham, England); 0.5 µCi in 20 µl/well) was added for 24 h. Counts were expressed as percent intracellular parasite destruction in activated macrophage cultures as determined by comparison with [3H]TdR incorporation in parasites released from control, nonactivated cultures.

Assessment of Macrophage Activation by Extracellular Cytolysis of Target Cells

Macrophage activation was also tested by the capacity of the cells to lyse 51Cr-labeled P815 mastocytoma (DBA/2) target cells (15,25). To this end, bone marrow-derived macrophages from C57BL/6 mice in 96-well microplates were exposed for 24 h at 37°C to macrophage activating factor (MAF)-rich supernatants from Con A-stimulated spleen cell cultures (26), in the presence of dilutions of OM-89 or of LPS (10 ng/ml) as a positive control. The cultures were then washed, and 51Cr-labeled target cells (10⁴ cells/well) were added. After 20 h, the amount of radioactivity released by the target cells undergoing lysis by the activated macrophages was determined as described (15).

Measurement of Hexose Monophosphate Shunt Activity

HMPS activity was determined by measuring the amount of radioactive carbon dioxide (14CO₂) evolved by macrophages from (1-14C)-D-glucose (3.94 mCi/mmol; The Radiochemical Centre, Amersham, England) using a modification of a technique previously described (27). Briefly, C57BL/6 bone marrow-derived macrophages in 24-well Costar clusters were stimulated by incubation with serial dilutions of OM-89 for 24 h at 37°C. The cultures were then thoroughly washed with glucose-free Eagle's medium, and supplemented with 300 µl/well of glucose-free medium.
RESULTS

Stimulation of Lymphocyte Proliferation by OM-89

Mouse spleen cells were incubated with increasing concentrations of OM-89 or of LPS. Stimulation of cell proliferation was induced by both agents, whereas the carrier was completely inactive (Fig. 1). Of major interest, the effect of OM-89 was clearly unrelated to endotoxin contamination. Indeed, spleen cell proliferation induced by OM-89 at a concentration of 300 µg/ml was identical to that achieved by exposure to 50 µg/ml of LPS, although endotoxin contamination in OM-89 did not exceed 0.4 ng/ml, a concentration at which LPS was totally ineffective (not shown). Moreover, when the respective proliferative effects of OM-89 and LPS were tested on cells of the LPS low responder C3H/HeJ mouse strain, a clear difference was observed between both substances, i.e., a high level of proliferation was obtained upon incubation with OM-89, whereas LPS elicited little response (Fig. 2).

Passage of the responding cell population through nylon wool strongly inhibited OM-89-induced proliferation without affecting reactivity to the T-cell mitogen concanavalin A (Con A); conversely, removal of Thy-1+ lymphocytes by treatment with a specific antibody and complement substantially decreased stimulation by Con A while the response to OM-89 was not impaired (data not shown). These results suggest that the cells proliferating upon treatment with OM-89 were of the B lineage.

Stimulation by OM-89 of Metabolic Parameters of Macrophage Activation: HMPS Levels and Production of Oxygen Metabolites

Macrophages in culture were exposed for 24 h to increasing concentrations of OM-89, and then tested for HMPS activity by measuring their capacity to oxidize (1-[14C])-d-glucose to 14CO2 (cf. the Materials and Methods section). Treatment with OM-89 stimulated markedly this metabolic pathway (Fig. 3). A significant enhancement of HMPS over control values was detectable using 10 µg/ml of the substance; stimulation further increased over a 1,000-fold concentration range, to reach plateau values at around 10 mg/ml. Simultaneous incubation with PMB (5 µg/ml) failed to affect significantly this dose–response curve. Under the same conditions, however, PMB was able to block completely
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FIG. 1. Stimulation of spleen cell proliferation by OM-89. Spleen cells from C57BL/6 mice were incubated with serial concentrations of OM-89 (prepared from the lyophilized powder) or the carrier (excipients) alone (left panel). The proliferative response was determined by $[^{3}H]$Tdr uptake after 72 h and compared to that achieved using *E. coli* lipopolysaccharide (LPS; right panel). Means of triplicate determinations ± SD.

the HMPS stimulation induced by incubation of macrophages with LPS from *E. coli* (Fig. 4). To assess the possible effect of OM-89 on the production of oxygen metabolites by macrophages, the cells were treated with appropriate concentrations of OM-89 during 24 h, and then tested for their capacity to release superoxide ($O_{2}^{-}$) upon triggering with PMA. As shown in Fig. 5, exposure to OM-89 markedly enhanced the production of this metabolite. Such effect was not inhibited by PMB, which,

FIG. 2. Stimulation by OM-89 of the proliferation of spleen cells from LPS low responder C3H/HeJ mice. Spleen cells were incubated with serial concentrations of OM-89 (left; prepared from the liquid concentrate) or of LPS (right). The proliferative response was determined by $[^{3}H]$Tdr uptake after 72 h. Means of triplicate determinations ± SD.

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Stimulation of hexose monophosphate shunt activity of macrophages by OM-89. Bone marrow-derived macrophages from C57BL/6 mice were incubated with serial concentrations of OM-89 (prepared from the liquid concentrate) in the presence or absence of polymyxin B (5 pg/ml). After 24 h, HMPS activity was determined by counting the radioactivity in $^{14}$CO$_2$ evolved by the cells from (1-[$^{14}$C])-D-glucose. Means of three determinations $\pm$ SD.

however, completely prevented LPS-induced superoxide production (data not shown). The capacity of OM-89 to stimulate the production of oxygen metabolites was further tested on macrophages of the LPS low responder mouse strain C3H/HeJ. Such cells reacted to OM-89 treatment by a vigorous burst of $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ release, under conditions where response to LPS was minimal (Fig. 6).

Stimulation by OM-89 of Macrophage Functional Activities: Extracellular Cytolysis of Tumor Cells and Intracellular Killing of Microorganisms

The capacity of OM-89 to stimulate functional activities in macrophages was first tested by measuring extracellular cytolysis of tumor target cells. Exposure of bone marrow-derived macrophages for 24 h to OM-89 up to 3.0 mg/ml failed to stimulate macrophage cytotoxicity (data not shown). In a second series of experiments, macrophages were incubated with increasing concentrations of OM-89 in the presence of supernatant fluids from Con A-stimulated lymphocytes (a source of macrophage activating factor), prior to being challenged with $^{51}$Cr-labeled target cells. As shown in Fig. 7, a dose-dependent lysis of the tumor cells could be observed. A highly significant effect was detectable using as little as 10 $\mu$g/ml of OM-89; no cytotoxic activity was observed in macrophages exposed to MAE in the absence of OM-89. A possible effect of OM-89 on intracellular killing of microorganisms by macrophages was then examined. To this end, macrophages from the LPS low responder mouse strain C3H/HeJ as well as from the LPS high responder strain C57BL/6 were allowed to phagocytize Leishmania enriettii, a protozoan parasite that specifically infects mononuclear phagocytes (cf. the Discussion section), before being exposed to r-Mu-IFN-$\gamma$ and to dilutions of OM-89 known to stimulate metabolic parameters of activation such as the HMPS pathway and $\text{O}_2^-$ release. Results of such an experiment are presented in Fig. 8. Whereas treatment with OM-89 alone failed to stimulate macrophage microbicidal activity, simultaneous incubation with OM-89 and r-Mu-IFN-$\gamma$ led to intracellular destruction of the microorganisms within 24 h in macrophages from both mouse strains. It should be noted that LPS up to a concentration of 100 ng/ml was totally inefficient as a coactivator of C3H/HeJ macrophages; in the contrary, exposure of these cells to r-Mu-IFN-$\gamma$ (100 U/ml) in the presence of 1 mg/ml of OM-89 led to destruction of 75% of the microorganisms. Since the endotoxin content of added OM-89 was below 0.3 ng/ml, the effect of the bacterial

FIG. 3. Stimulation of hexose monophosphate shunt activity of macrophages by OM-89; comparison with LPS. Bone marrow-derived macrophages from C3H/HeJ mice were exposed to either OM-89 (500 pg/ml, prepared from the liquid concentrate), LPS (10 ng/ml), or control DH medium, in the presence or absence of polymyxin B (4 pg/ml). After 24 h, HMPS activity was determined by counting radioactivity in $^{14}$CO$_2$ evolved by the macrophages from (1-[$^{14}$C])-D-glucose. Means of three determinations $\pm$ SD.
extract cannot be ascribed to LPS contamination of the preparation.

DISCUSSION

The studies described in this report were aimed at analyzing the effect of the *E. coli* extract OM-89 on the function of cells of the immune system. In a first set of experiments, the compound was found to stimulate markedly the proliferation of spleen cells in culture. Following filtration of the splenocytes through nylon wool, a procedure known to remove immunoglobulin-bearing lymphocytes (20), the response to OM-89 of cells in the effluent was strongly impaired (data not shown). Since nylon wool also traps cells of the monocyte–macrophage...
series, which might respond to OM-89 by producing factors required for lymphocyte growth, proliferation assays were performed in the presence of irradiated filler splenocytes as described (31). Failure of the effluent cells (mostly T-lymphocytes) to respond to OM-89 under these conditions suggests that OM-89 acts on cells of the B lineage, although a cooperative effect of monocytes/macrophages on the OM-89-dependent B-cell proliferation cannot be excluded. That T cells are not a main target of OM-89 was indicated by the observation that treatment of splenocytes by an anti-Thy-1 antiserum and complement, which markedly depressed the reactivity of the resulting population to the T-cell mitogen Con A, failed to interfere with the OM-89-induced proliferative response (data not shown).

Since B cells are known to proliferate in vitro after stimulation by endotoxin (32,33), it was important to evaluate the possible role of endotoxin in the OM-89-mediated effects. Attempts at blocking a putative endotoxin contamination of the bacterial extract using PMB, an agent known to bind to and neutralize endotoxin by interacting with its lipid A moiety, were inconclusive, due to the toxicity of PMB for lymphocytes under the present experimental conditions (data not shown). As indicated in Fig. 1, incubation of lymphoid cells with 3.0 mg/ml of OM-89 led to a stimulation comparable to that achieved by treatment with 50 μg/ml of purified LPS. Since the endotoxin content of lyophilized OM-89 used in this assay was less than 1.2 ppm, the actual endotoxin contamination was more than four orders of magnitude lower than the concentration required to obtain a stimulation level similar to OM-89. Moreover, spleen cells from C3H/HeJ mice (low responder to LPS) were adequately stimulated by OM-89 while reacting poorly to endotoxin, further suggesting that thymidine uptake by splenocytes exposed to OM-89 in culture was independent of possible endotoxin contamination of the extract.

The effect of OM-89 on cells of the monocyte-macrophage series was then examined. A strong stimulation of hexose monophosphate shunt activity by treatment with the extract was observed. This remained essentially unaffected by PMB, whereas similar HMPS stimulation induced by exposure to LPS was completely blocked by the inhibitor. In addition, incubation of OM-89 with macrophages from LPS responder (e.g., C3H/OUJ) as well as from LPS low responder (e.g., C3H/HeJ) mice led to priming of the cells for enhanced production of superoxide (O$_2^-$) and hydrogen peroxide upon triggering with PMA. HMPS stimulation and production of oxygen metabolites by macrophages are indicative of respiratory burst activity and are known to correlate with an increased capacity to destroy intracellular microorganisms (for review,

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see ref. 34). It was thus of interest to determine whether incubation with OM-89 might similarly stimulate functional activities associated with increased oxidative metabolism in macrophages.

Killing of the protozoan parasite Leishmania enriettii was chosen as an index of macrophage activation. L. enriettii and other members of the same genus are obligate intracellular pathogens that, in their mammalian hosts, reside within cells of the mononuclear phagocyte system. Host recovery is thought to depend on macrophage activation resulting from an immune response of the cell-mediated type (35). Intracellular parasite destruction in cultured macrophages can be achieved through activation in vitro following exposure of macrophages to macrophage-activating factor/interferon-γ (MAF/IFN-γ) in the presence of LPS (22) to electron carriers such as methylene blue (23,27) or to immune lymphocytes (17,19). Parasite killing in this system is correlated with respiratory burst activity and can be inhibited by scavengers of oxygen metabolites such as aminotriazole and cytochrome c, suggesting a role for oxygen derivatives in the activation of macrophages (36). When L. enriettii-infected macrophages were treated with OM-89 alone, no intracellular killing could be observed. Incubation with both r-Mu-IFN-γ and OM-89 in macrophages from both LPS-responsive and unresponsive mouse strains led, however, to rapid parasite destruction, indicating that the bacterial extract behaved as a strong coactivator capable of complementing efficiently the lymphokine, an effect unrelated to the minimal endotoxin content of the preparation. OM-89 also acted synergistically with MAF to activate macrophages for the extracellular cytolysis of tumor target cells, thus confirming the efficacy of the product as a coactivator of macrophages. The mechanisms of target destruction in such a system, however, are not fully understood and the nature of the molecule(s) and/or metabolism(s) that are triggered by OM-89 in this case therefore remains unknown.

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REFERENCES

17. Pham TV, MacDonald HR, Mauel J. Macrophage activation in vitro by lymphocytes from Leishmania major infected healer and non-healer mice. Parasite Immunol 1988;10:353-68.
20. Julius MH, Simpson E, Herzenberg LA. A rapid method for

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