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STIMULATION BY A BACTERIAL EXTRACT (BRONCHO-VAXOM) OF THE METABOLIC AND FUNCTIONAL ACTIVITIES OF MURINE MACROPHAGES

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Abstract — Peritoneal and bone marrow-derived macrophages of the C57BL/6 and DBA/2 mouse strains were exposed *in vitro* to increasing concentrations of the bacterial lysate Broncho-Vaxom (BV), in the presence or absence of macrophage-activating factor (MAF)-rich media. Two metabolic pathways and two functional activities of the macrophages were studied. First, oxidative metabolism was found to increase sharply in macrophages incubated with BV, as measured by the catabolism of glucose via the hexose monophosphate shunt pathway, and by the production of the superoxide anion (O_2^-). Both effects were further increased by co-stimulation of macrophages with MAF. Second, exposure to BV together with MAF (or with recombinant murine interferon-γ) led to acquisition by macrophages of the capacity to destroy the intracellular parasite *Leishmania enriettii*; such activated macrophages were also lytic towards P815 mastocytoma indicator target cells. These cytotoxic properties failed to develop in the absence of MAF. The BV-dependent increase in metabolic and functional activities was of the same magnitude as that induced by incubation of macrophages with 10 ng/ml of bacterial lipopolysaccharide (LPS). Residual contamination of BV by endotoxin was however much lower. In addition, polymyxin B, a LPS inhibitor, blocked the effect of LPS without significantly affecting macrophage stimulation by BV. These experiments indicate that BV can markedly stimulate macrophage metabolic and functional parameters that are important for host defense against pathogens and tumors.

The present study was aimed at investigating the possible role played by macrophages in the immunomodulatory activity of the bacterial lysate Broncho-Vaxom (BV; Laboratoires OM, Meyrin/Geneva, Switzerland). BV is used as a polyvalent immunotherapeutical agent active in the treatment of respiratory tract infections, particularly acute and chronic bronchitis (Gsell, 1980; Sequeira, 1980; Messerli, Michetti, Sauser-Hall, Stäubli, Taddei, Weiss, Farine & Fux, 1981; Martin du Pan & Martin du Pan, 1982; Geiser, 1983; Ahrens & Wiedenbach, 1984; Maestroni & Losa, 1984; Palma-Carlos, Palma-Carlos, Inacio & Sousa Uva, 1987). Experimental studies indicate that the compound has an immunopotentiating effect on both cellular and humoral responses. Following oral administration in man, BV has been found to stimulate the mitogenic response of peripheral blood leucocytes to polyclonal activators (Clot & Andary, 1980) and allogeneic lymphocytes (Maestroni & Losa, 1984), and to increase the levels of both salivary IgA and serum IgG and IgM (Puigdollers, Rodés Serna, Hernandez del Rey, Tillo Barruffet & Jofre Torroella, 1980). Treatment of mice *per os* increased the levels of IgA in gut and lung secretions (Bosch, Lucena, Parés & Jofre, 1983), enhanced resistance against experimental infection by streptococci and staphylococci in animals immunosuppressed with cyclophosphamide, and restored serum immunoglobulin levels as well as the number of anti-sheep erythrocyte plaque-forming cells in the spleens of such animals (Bosch, Lucena, Parés & Jofre, 1984). Other effects of BV, including increased numbers of macrophages in the peritoneal cavity of mice following oral administration and inhibition of the allergic autocytotoxicity phenomenon in leucocytes from patients with bronchial asthma (Podleski, 1985), have been reviewed recently (Palma-Carlos *et al.*, 1987).

Resistance of the organism against invading pathogens depends in part on the acquisition by macrophages of enhanced microbicidal properties, a process known as *activation* (North, 1978). Activation may be induced by interaction of

macrophages with soluble lymphokines, such as macrophage-activating factors (MAF), that are secreted by stimulated lymphocytes (Buchmüller & Mauël, 1979), or with various substances of bacterial and non-bacterial origin (Oegmundsdottir & Weir, 1980). Acquisition of enhanced microbicidal and cytolytic properties usually correlates with an increased metabolic activity (Murray, 1984); in particular, activated macrophages display elevated hexose monophosphate shunt levels, and generate toxic oxygen metabolites such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). In view of the clinical and experimental evidence suggesting that BV may increase non-specific resistance against infectious agents, experiments were designed to test whether this compound might induce in macrophages metabolic and/or functional activities known to be associated with the activated state.

EXPERIMENTAL PROCEDURES

Materials

Broncho-Vaxom (BV) is an extract obtained by submitting eight microorganisms (*Diplococcus pneumoniae, Hemophilus influenzae, Klebsiella pneumoniae, Klebsiella ozaenae, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus viridans* and *Neisseria catarrhalis*) to progressive alkaline lysis. The resulting preparation is purified by means of clarification and filtration procedures. The final dry preparation contains by weight 35% protein, 8% free amino acids, 10% lipids, 8% nucleotides, 2% carbohydrates and approximately 37% salt. Tests for endotoxin contamination, as performed by the *Limulus* amoebocyte lysate assay and pyrogenicity in the rabbit, have consistently yielded less than 1 ng of endotoxin/mg of bacterial extract. The amount of BV quoted in the figures and tables refers to the actual quantity of bacterial extract in the tested product (excluding excipient or other inactive substances).

Mice

Male or female mice of the strains indicated were obtained from the Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland. 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 (Mauël, Buchmüller & Behin, 1978), and suspended in Dulbecco's medium (Seromed, Munich, F.R.G.) supplemented with 10% fetal bovine serum (FBS; Seromed). Alternatively, macrophages used in certain tests were derived by differentiation of bone marrow cells *in vitro* as described by Kelso, Glasebrook, Kanagawa & Brunner (1982). The cells were distributed as follows: for determinations of leishmanicidal activity, extracellular cytolysis of target cells, and superoxide (O_2^-) production, in 96-well microculture plates (Costar 3596, Cambridge, U.S.A.; 10^5 cells in 100 μl/well); for measurements of hexose monophosphate shunt (HMPS), in 24-well tissue culture clusters (Costar 3524; 5×10^5 cells in 0.5 ml/well) or 96-well microplates (Costar 3596; 7×10^4 cells in 100 μl/well.

Infection of macrophage cultures

Amastigotes of *Leishmania enriettii* were prepared from infected guinea pig tissue (Mauël, Behin, Noerjasin & Rowe, 1977); the parasites were then propagated as promastigotes in 5% FBS-supplemented HOSMEM II growth medium (Berens & Marr, 1978), and used within 1 month of the original harvest. Infection of macrophages was achieved by adding 100 μl of promastigote suspension to each microwell containing peritoneal exudate cells in culture at a ratio of 20 parasites per cell, 3 h after plating the cells. The vessels were kept at 37°C for 18 – 20 h to allow for phagocytosis of the parasites. To remove free parasites and non-adherent cells, the infected cultures were then thoroughly washed by flicking off the medium, and filling the wells with warm Hank's 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.

Macrophage activation and measurement of intracellular parasite killing and cytolysis of target cells

Activation of macrophages was obtained by exposing the infected cells to macrophage-activating factor (MAF)-rich supernatants of concanavalin A-stimulated spleen cells from C57BL/6 or CBA/T6 mice (Buchmüller & Mauël, 1979), in the presence or absence of appropriate dilutions of BV. In certain experiments, recombinant murine interferon-γ (Lot No. 2309-24, produced by Genentech Inc. and kindly supplied by Boehringer Ingelheim, Vienna, Austria) was used as an activator instead of MAF. Endotoxin (lipopolysaccharide (LPS) from *E. coli* 055:B5, Difco Laboratories, Detroit, U.S.A.) was added in

certain wells to provide a positive control. Polymyxin B (PMB, Sigma), an inhibitor of endotoxin action, was also added as required by the experimental protocol. After 24 h of incubation at 37°C, the macrophages were lysed with 0.01% sodium dodecyl sulfate (SDS), as previously described (Mauël, 1984), to release the intracellular microorganisms. The wells were then supplemented with 10% FBS-supplemented HOSMEM II medium and parasite growth was recorded by ^{3}H-thymidine deoxy-riboside (TdR) incorporation.

Macrophage activation was also tested by the capacity of the cells to lyse ^{51}Cr-labelled P815 mastocytoma (DBA/2) target cells. To this end, bone marrow-derived macrophages in 96-well microplates were exposed to MAF-rich supernatants, as above, in the presence of dilutions of BV or LPS as a positive control, with or without PMB added. The cultures were then washed and ^{51}Cr-labelled target cells (Brunner, Mauël, Cerottini & Chapuis, 1968) were added. After 20 h, the amount of radioactivity released by target cells incubated with stimulated vs control macrophages was recorded, and the percent specific target cell destruction was calculated (Kelso *et al.*, 1982).

Measurement of HMPS activity

HMPS activity was determined as described previously (Mauël, Schnyder & Baggiolini, 1984) from the amount of radioactive carbon dioxide ($^{14}CO_2$) evolved by macrophages from (1-^{14}C)-D-glucose (3.94 Ci/mmol; The Radiochemical Centre, Amersham, U.K.). In brief, macrophage cultures were washed and exposed to 300 μl of serum-free medium (glucose- and bicarbonate-free Eagles' medium) containing 0.1 μCi of (1-^{14}C)-D-glucose. The wells were immediately sealed with a silicone rubber disk fitted with a piece of filter paper moistened with 2.0 M NaOH. After 90 min at 37°C, 50 μl of 1.0 M H_2SO_4 were injected into each well through the disks to release the radioactive carbon dioxide evolved in the reaction. After 15 min at room temperature, the filters were removed, dried and counted. In certain experiments, this technique was adapted for use with 96-well microplates. Each well was supplemented with 40 nCi of (1-^{14}C)-D-glucose in 70 μl medium, and incubation time extended to 120 min.

Measurement of O_2^- production

Superoxide production was determined in microwell cultures by the cytochrome *c* reduction assay as described by Pick & Mizel (1981). Briefly, washed cells were incubated with 200 μg of ferricytochrome *c* (from horse heart, type VI; Sigma) and 50 ng of phorbol myristate acetate (PMA; Sigma) in 100 μl of HBSS per well. Wells used as blanks were supplemented with 30 μg/ml of superoxide dismutase (SOD; Sigma) in the reaction medium. Cytochrome *c* reduction was measured at 550 nm in a micro ELISA reader (Easy Reader EAR 340, Kontron Analytik, Switzerland), using a 492 nm reference filter. Results were expressed as the difference in absorbance (ΔE) between cultures incubated in the absence or presence of SOD (−SOD or +SOD, respectively) at each time point (t_i) relative to the beginning of the measurements (t_0), according to the formula:

$$\Delta E = \Delta E_{t_i - t_0}(-\mathrm{SOD}) - \Delta E_{t_i - t_0}(+\mathrm{SOD}).$$

Statistical analysis

Where required, the significance of differences in activity between preparations was determined by the *t*-test for independent variables (Student's *t*-test).

RESULTS

1. *Stimulation of macrophage hexose monophosphate shunt activity by BV*

Treatment with the bacterial extract stimulated markedly HMPS activity in bone marrow-derived macrophages (Fig. 1). In several experiments, a significant effect was observed at concentrations of bacterial extract as low as 10 ng/ml, corresponding to an endotoxin content of less than 10 pg/ml. A similar stimulation was observed when the cells were exposed to *E. coli* lipopolysaccharide; in this case, the minimum concentration required to achieve HMPS stimulation was, however, 300 pg/ml [in the experiment shown, radioactivity values for $^{14}CO_2$ evolved by macrophages exposed to this concentration of LPS were 1455 ± 113 counts/min as against 392 ± 55 counts/min for macrophages incubated with control medium only, a significant (P = 0.0005) difference]. Moreover, addition of 10 μg/ml of polymyxin B (PMB) to the incubation fluids was sufficient to reduce to background levels the HMPS activity obtained with 3 ng/ml of LPS. PMB failed however to decrease HMPS levels in macrophages exposed to BV at any concentration of this compound (Fig. 1).

To further ascertain that the stimulatory effect of BV was not due to endotoxin contamination, LPS in a bovine serum albumin solution was submitted to

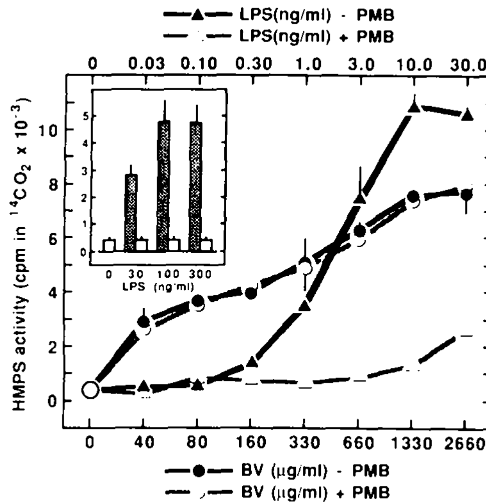

Fig. 1. Hexose monophosphate shunt stimulation in macrophages by BV. Bone marrow-derived macrophages from C57BL/6 mice were plated in 96-well microplates, then exposed for 24 h to increasing concentrations of BV or lipopolysaccharide in the presence or absence of polymyxin B (10 µg/ml), prior to being tested for HMPS activity by measuring the amount of $^{14}CO_2$ evolved from (1 – ^{14}C)-D-glucose. Inset: in a separate experiment, macrophages were exposed to increasing concentrations of alkali-treated (empty bars) or control LPS (filled bars) for 24 h, then tested for HMPS activity. Standard deviations (S.D.) are indicated by vertical bars above or below symbols; when not shown, S.D. fall within symbols. Using LPS, at concentrations of 0.3 ng/ml or above, the differences between cultures incubated in the presence and absence of PMB were significant (P = 0.0005 at 0.3 ng/ml of LPS, all other conditions: P<0.0001). On the contrary, there were no significant differences between cultures treated with BV in the presence or absence of PMB. BV, Broncho-Vaxom; LPS, lipopolysaccharide; PMB, polymyxin B.

Table 1. Effect of MAF on BV-induced HMPS stimulation in mouse macrophages

Concentration of BV (µg/ml)	HMPS activity * in macrophages exposed to BV	
	In control medium	In MAF-rich medium[†]
0	0.46 ± 0.12	1.23 ± 0.17
25	0.52 ± 0.50	4.62 ± 0.68
50	0.95 ± 0.15	6.48 ± 0.50
100	1.37 ± 0.10	6.12 ± 0.33

Peritoneal exudate macrophages from DBA/2 mice were plated in 24-well Costar clusters and exposed to various incubation media. After 24 h, they were tested for HMPS activity by measuring the amount of $^{14}CO_2$ evolved from (1 – ^{14}C)-D-glucose.
*Activities are expressed in counts/min × 10^{-3}.
[†]MAF, macrophage-activating factor-rich conditioned medium from Con A-stimulated spleen cells, used at a 1:16 dilution.

the same alkaline incubation as used for producing BV (cf. Experimental Procedures). The resulting preparation failed to elicit any stimulation of HMPS activity in macrophages up to a concentration of 300 ng/ml (Fig. 1, inset).

Incubation of macrophages with BV for more than 24 h did not increase further HMPS activity (data not shown). Although exposure to BV alone was sufficient to raise significantly HMPS levels in macrophages, the effect of the compound was markedly increased when the cells were simultaneously incubated with MAF-rich medium (Table 1). Under these conditions, HMPS stimulation by BV was detectable in macrophages at concentrations which were no longer active when tested in absence of the MAF-rich fluids.

2. *Stimulation of superoxide production by macrophages exposed to BV*

Incubation of macrophages in medium containing BV stimulated the capacity of the cells to generate superoxide upon challenge with phorbol myristate acetate (Fig. 2). Although macrophage stimulation by BV (at the dilution tested) was lower than achieved by addition of 10 ng/ml of LPS to the incubation media, PMB failed to decrease significantly this effect (Fig. 2A). On the contrary, the antibiotic markedly depressed the LPS-induced response.

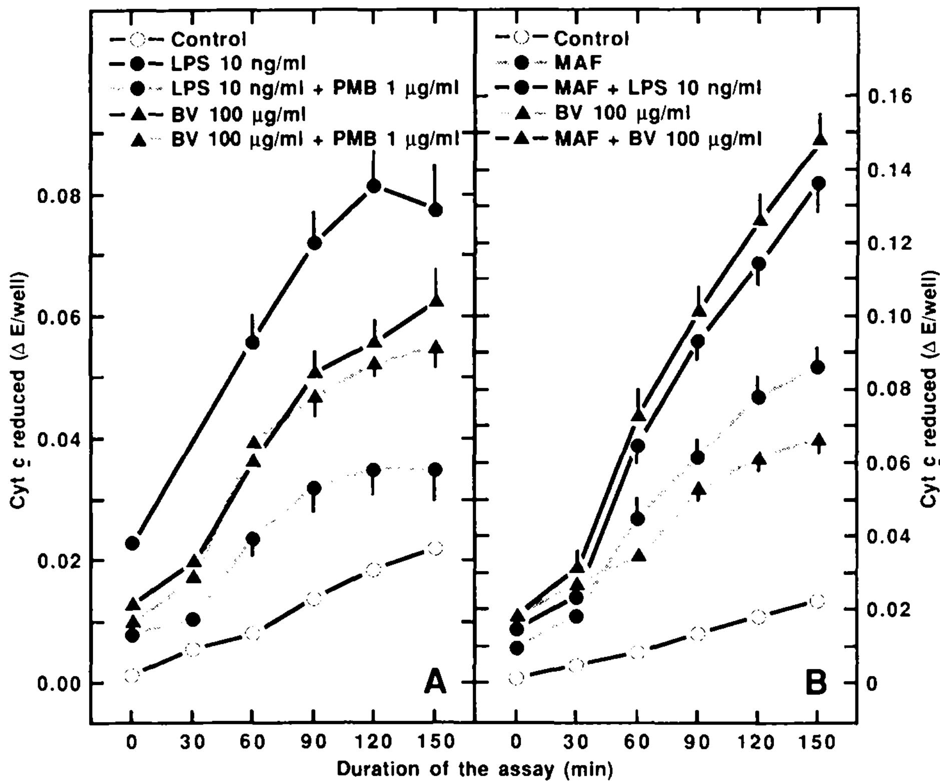

Fig. 2. Priming for superoxide anion ($O_2^{\cdot -}$) production in macrophages exposed to BV. Peritoneal exudate cells from DBA/2 mice were incubated with BV (100 µg/ml) or LPS, or to a 1:16 dilution of MAF-rich medium in the presence or absence of the above two compounds. After 24 h, the cells were exposed to phorbol myristate acetate in the presence of cytochrome c, and reduction of the latter compound followed spectrophotometrically (cf. Experimental Procedures). S.D., cf. legend to Fig. 1A. Differences between measurements of O_2^- production by macrophages exposed to LPS in the absence vs presence of PMB were significant, whereas differences between cultures treated with BV in the absence vs presence of PMB were not. The differences between measurements of O_2^- production by macrophages exposed to MAF or to BV alone vs MAF + BV were all significant.

Exposure of macrophages to MAF primed the cells for increased secretion of superoxide upon PMA stimulation (Fig. 2B). This effect was further enhanced by simultaneous addition of BV, confirming that the compound behaves as a potentiator of macrophage activation induced by MAF.

3. *Stimulation of macrophage microbicidal and cytolytic activities by BV*

Peritoneal macrophages were infected with *L. enriettii* parasites, then activated by exposure to MAF-rich media in the presence or absence of BV or LPS. BV failed to activate macrophages by itself for intracellular killing of the microorganisms (Fig. 3, control). Full intracellular killing was achieved however by simultaneous treatment of the macrophages with both MAF and BV, or with MAF and LPS. BV was active at complementing MAF for parasite killing in the same concentration range that induced HMPS stimulation and O_2^- production (Fig. 4), and was as efficient as LPS at 10 ng/ml. While the effect of LPS was fully reversed by PMB, this drug failed to interfere with the activity of BV at any concentration tested (Figs 3 and 4). A similar coactivating effect of BV was observed when macrophages were treated with recombinant interferon-γ instead of crude MAF (Fig. 4A, inset).

To determine whether BV might similarly complement MAF for induction in macrophages of extracellular cytolytic activity, peritoneal cells were exposed to the reaction mixture (MAF + BV) for 24 h, then incubated together with ^{51}Cr-labelled

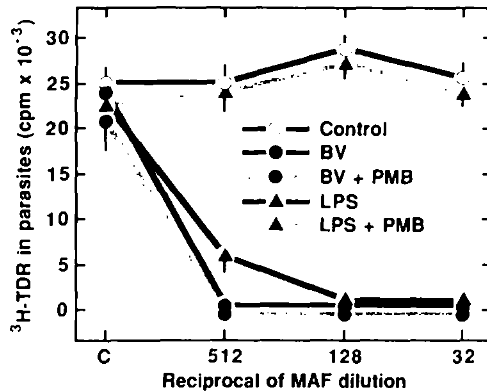

Fig. 3. Stimulation of intracellular killing of *Leishmania enriettii* in macrophages exposed to MAF and BV. Peritoneal exudate cells from C57BL/6 mice were infected with *L. enriettii*, then exposed to MAF in the presence or absence of BV (100 μg/ml) or LPS (10 ng/ml) for 24 h. Intracellular parasite survival was then determined by sodium dodecyl sulphate lysis of the infected cells, and ^{3}H-TdR incorporation following multiplication of the released parasites. S.D., cf. legend to Fig. 1. The differences between cultures exposed to MAF and LPS *in the presence of PMB*, and control cultures incubated with MAF alone (no parasite killing) are not significant. Differences between cultures incubated with BV in the absence vs presence of PMB are not significant. "Control", cultures exposed to MAF in the absence of either BV or LPS. C, BV or LPS diluted in plain medium instead of MAF.

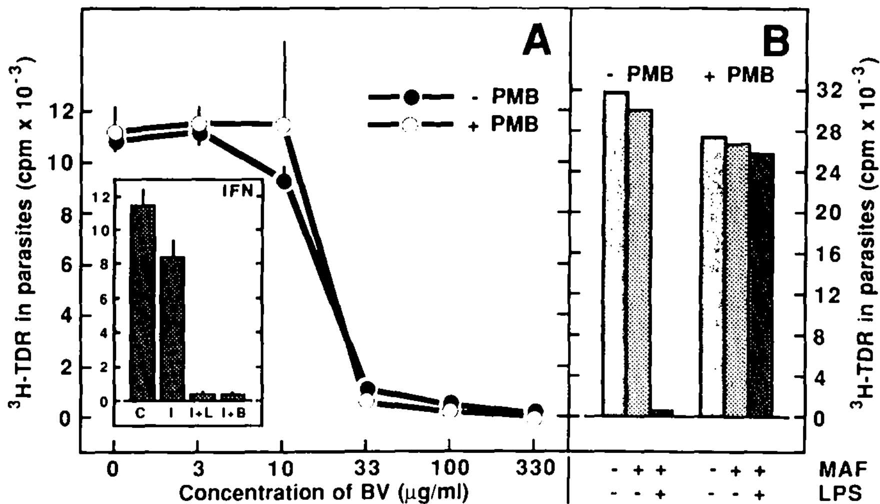

Fig. 4. Stimulation of intracellular killing of *L. enriettii* in mouse peritoneal macrophages by BV: dose–response. A. Peritoneal exudate cells from C57BL/6 mice were infected with *L. enriettii*, then exposed to a 1:16 dilution of MAF-rich medium in the presence of increasing concentrations of BV, with or without PMB (10 μg/ml) added. N.B., in the absence of BV, parasite survival in macrophages exposed to MAF alone was identical to that in macrophages incubated with control non-activating medium. Inset: macrophages were treated with recombinant murine interferon-γ (IFN; 3 U/ml) without (I) or with added LPS (10 ng/ml; I + L) or BV (100 μg/ml; I + B). C, control cells incubated with non-activating medium. Intracellular survival was determined after 24 h, by measuring ^{3}H-TdR incorporation in the parasites following sodium dodecyl sulphate lysis of the infected cells. S.D., cf. legend to Fig. 1. The differences between cultures treated without and with PMB are not significant. B. Macrophages were treated in parallel with MAF and LPS (10 ng/ml) to ascertain the activity of PMB on the LPS-induced microbicidal activity.

target cells. As shown in Fig. 5A, this resulted in a dose-dependent lysis of the target cells; in the absence of MAF, BV failed to activate macrophages (data not shown). While PMB blocked the induction of cytolytic activity in macrophages incubated with MAF + LPS (Fig. 5B), it did not interfere with the

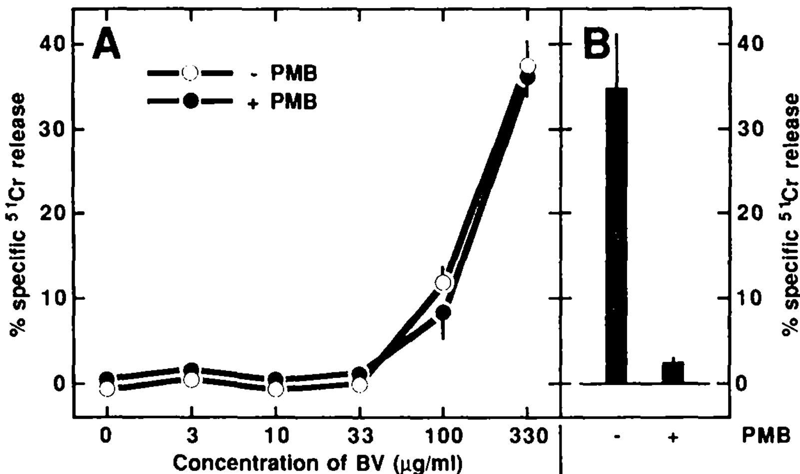

Fig. 5. Extracellular cytolysis of tumour target cells by macrophages exposed to BV. Bone marrow-derived macrophages from C57BL/6 mice were exposed to a 1:16 dilution of MAF-rich medium in the presence or absence of increasing concentrations of BV, with or without added PMB (10 μg/ml). A. After 24 h, the macrophages were challenged with ^{51}Cr-labelled P 815 mastocytoma cells; specific ^{51}Cr release was determined after 18 h. S.D., cf. legend to Fig. 1. None of the differences between cultures incubated without and with PMB are significant. B. Macrophages were activated in parallel by MAF + LPS (10 ng/ml) in the presence or absence of PMB to ascertain the activity of PMB on the LPS-induced cytotoxic activity.

effect of BV (Fig. 5A). When both LPS and BV were used to supplement MAF, a slight increase in target cell killing was observed over that attained with MAF in the presence of BV alone; addition of PMB brought macrophage cytotoxicity back to the level achieved when using MAF + BV.

DISCUSSION

Activation of macrophages is an important physiological mechanism whereby phagocytes acquire enhanced microbicidal and cytolytic properties, that correlate with stimulation of oxidative metabolic pathways. Activation can be induced by a variety of stimuli, including products of microbial origin (Oegmundsdottir & Weir, 1980). Since the bacterial extract BV has been shown to display immunostimulatory properties *in vivo* (see above), it was of interest to determine whether the macrophage might be a target cell for the active principle(s) in this product. Indeed, BV stimulated macrophage oxidative metabolism, as measured by increased HMPS activity and production of the superoxide anion. Intracellular killing by activated macrophages is thought to depend, at least in part, upon the generation of O_2 metabolites, which are highly toxic for microorganisms (Murray, 1984). Interestingly, although BV did stimulate by itself macrophage respiratory burst, it failed to induce any microbicidal or cytolytic activities when used alone. When macrophages were exposed to both MAF and BV however, oxidative metabolism was further enhanced and both intracellular killing and extracellular cytolysis of tumor target cells could be demonstrated.

BV thus appears to act as a "trigger" (Nacy, Oster, James & Meltzer, 1984) required for expression of functional activities in macrophages primed with MAF or interferon-γ. In this sense, its activity is similar to that of endotoxin, which has been shown to be synergistic with MAF for acquisition of cytotoxic activities by macrophages (Pace & Russell, 1981; Nacy *et al.* 1984; Mauël & Buchmüller-Rouiller, 1987). Several lines of evidence strongly suggest, however, that the effect of BV is not due to LPS. First, quality controls during manufacture include routine testing for endotoxin, which has been found to be present in BV at concentrations below 1 ng/mg of product (1 ppm). As indicated under Materials, production of BV involves alkaline lysis of the bacteria. Alkaline treatment is known to hydrolyse the ester bonds between fatty acids and sugars in the lipid A molecule, resulting in loss of toxicity. As shown in this report, incubation of LPS under the same conditions as used for the preparation of BV led to complete inactivation of the molecule, as determined by the HMPS stimulation assay.

Second, the antibiotic polymyxin B, which blocks LPS by forming a complex with the lipid A portion

of the molecule (Morrison & Jacobs, 1976), was unable to inhibit either the metabolic or the functional activities elicited by BV, under conditions where a similar degree of activity induced by LPS was fully inhibited. BV itself did not block the effect of PMB, since this antibiotic was able to counteract the effect of added LPS even in the presence of the immunostimulant.

Third, macrophage stimulation could be achieved by extracts prepared from only the Gram-positive bacteria used for the preparation of BV, at the exclusion of Gram-negative microorganisms (data not shown). This renders very unlikely the participation of endotoxin in the observed effects of BV on macrophages *in vitro*.

The results presented in this paper indicate that the bacterial extract BV can strongly potentiate metabolic and functional activities of macrophages in synergism with MAF, leading to the acquisition of cytotoxic properties *in vitro* against tumor cells and intracellular microbes. This effect appears to be independent of residual contamination by endotoxin. The mechanisms of this activity are currently under investigation.

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