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UPREGULATION OF ADHESION MOLECULES INDUCED BY BRONCHO-VAXOM ON PHAGOCYTIC CELLS

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Abstract — Whole blood was incubated with the bacterial extract Broncho-Vaxom (OM85) at various concentrations and for different periods of time. Expression of the β_2-integrins (LFA-1, CD11a/CD18; MAC-1, CD11b/CD18; p150,95, CD11c/CD18) and ICAM-1 (CD54) by monocytes and granulocytes was studied using flow cytometry. OM85 enhanced the expression of MAC-1 and ICAM-1 on monocytes and granulocytes in a dose-dependent manner. Maximal expression was achieved with 1 mg/ml bacterial extract. The effect on MAC-1 expression was not due to the low concentration of endotoxin contaminating the preparation (less than 1 ng/mg) since polymyxin-B did not substantially affect the adhesion molecule upregulation induced by OM85. In addition, OM85 enhanced the expression of p150,95 on monocytes and granulocytes, and also increased expression of LFA-1 on monocytes, but not on granulocytes. While MAC-1 and p150,95 expression reached peak values between 1 and 6 h, levels of ICAM-1 rose constantly for 10 h. We suggest that the clinical interest of OM85 in the management of recurrent infections could be related to be upregulation of adhesion molecules induced by this bacterial extract.

The immunomodulator OM85 (Broncho-Vaxom^R) is a lyophilized bacterial extract from common pathogenic bacteria. In uncontrolled clinical trials, it proved to be useful as an adjuvant treatment for infections of the respiratory tract (Debbas & Derenne, 1990; Cvoriscec, Ustar, Pardon, Palecec, Stipic-Markovic & Zimic, 1989; Maestroni & Losa, 1984).

Several mechanisms could be involved in its therapeutic effect. *In vitro* studies have shown that it can enhance T-cell function (Maestroni & Losa, 1984) but its main targets are probably phagocytic cells. Indeed, OM85 stimulates the respiratory oxidative burst of macrophages and the killing of intracellular pathogens (Mauel, Van-Pham, Kreis & Bauer, 1989). Moreover, it is able to induce the release of tumour necrosis factor-alpha (TNF-α), interleukin-1 and interleukin-6 by peripheral blood mononuclear cells (Wybran, Libin & Schandene, 1989).

Adhesion molecules expressed at the surface of phagocytes play a pivotal role in the anti-bacterial defence as demonstrated by the inability of patients with leucocyte adhesion deficiency to clear bacterial infections (reviewed in Anderson & Springer, 1987). These individuals display a selective defect in the expression of a major group of adhesion molecules, the β_2-integrins (LFA-1, CD11a/CD18; MAC-1, CD11b/CD18; p150,95, CD11c/CD18), on leucocytes. The present study was undertaken to analyse *in vitro* the effects of OM85 on the expression of β_2-integrins by granulocytes and monocytes in whole blood. In addition, we looked at the expression of ICAM-1 (CD54), a ligand for LFA-1 and MAC-1 (Wawryk *et al.*, 1989; Diamond *et al.*, 1990).

EXPERIMENTAL PROCEDURES

Bacterial extracts

Broncho-Vaxom (OM85) was supplied by Laboratoires OM (Geneva, Switzerland). It is a bacterial extract obtained by submitting eight microorganisms (*Diplococcus pneumoniae, Haemophilus 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 and finally lyophilized.

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The lyophilizate was dissolved in sterile RPMI-1640 medium (Gibco, Grand Island, U.S.A.) containing 10% LPS-free foetal calf serum (FCS, Myoclone, Gibco). The solutions were then filtered with 0.22 μm Milex-OR filters (Millipore S.A., Molsheim, France) and immediately used for experiments. The LPS content of these preparations, as determined with the *Limulus* assay (LAL-QCL-1000, Whittacker MA. Bioproducts, Walkersville, U.S.A.) was less than 1 ng/ml for solutions containing OM85 at a concentration of 1 mg lyophilizate/ml. LPS of *Escherichia coli* O111: B4 was obtained from Sigma Chemical Co. (St. Louis, U.S.A.).

Monoclonal antibodies

Phycoerythrin (PE)-conjugated monoclonal antibodies (mAb) Leu-M5 directed against CD11b (Mac-1) and Leu-15 against CD11c (p150,95) were purchased from Becton – Dickinson (BD, Mountain View, U.S.A.). Fluorescein-isothiocyanate (FITC)-conjugated antibodies against human CD11a (LFA-1) and human CD54 (ICAM-1) were obtained from Immunotech (Marseille, France). PE- and FITC-conjugated anti-Leu-M3 mAb (Becton – Dickinson) directed against CD14 (Mo2) were used to gate monocytes and granulocytes. All mAbs used were titred using flow cytometry (FACScan, Becton – Dickinson) to determine the concentration that leads to saturation of surface binding sites on granulocytes and monocytes stimulated with LPS (1 μg/ml, 37°C, 4 h). IgG isotype matched control antibodies were obtained from Becton – Dickinson (Mountain View). In all experimental conditions only low and stable background fluorescence was observed after staining with these antibodies.

Incubation of whole blood

Blood obtained from healthy volunteers was collected in syringes containing LPS-free heparin (Heparin Novo, Novo Industri A/S, Bagsvaerd, Denmark). Samples of 1.35 ml whole blood were then incubated with 150 μl of solubilized bacterial extract at 37°C in polystyrene tubes (Falcon^{R} 2051, BD). As controls, samples of whole blood were incubated either alone, with RPMI-medium or with LPS. In some experiments polymyxin-B (Sigma Chemical Co.) was added at a final concentration of 100 μg/ml.

Flow cytometry analysis

After incubation, aliquots of 200 μl whole blood were washed in phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin (Behring Werke AG, Marburg, F.R.G.). Pellets were then incubated simultaneously with 10 μl of anti-CD14 mAb and 10 μl of mAb directed against the antigen to be studied (30 min at 4°C in the dark). After lysis of red blood cells (FACS Lysing Solution, BD), white blood cells were washed and resuspended in 300 μl paraformaldehyde solution (1%).

Analysis with the FACScan flow cytometer was performed using forward light scatter and side scatter to acquire data of monocytes and granulocytes only (10^4 events). Fluorescence of populations was then studied using CD14 and side scatter properties to gate monocytes and granulocytes. Changes in the expression of CD14 during our experiments did not interfere with the gating of cells. Standardization of fluorescence signals with fluorescein-isothiocyanate- and phycoerythrin-coated microbeads (Alignment Microbeads Standards™, Flow Cytometry Standards Corporation, FCSC, U.S.A.) (Vogt, Cross, Henderson & Phillips, 1989) indicated that the mean fluorescence channels measured related linearly to the number of fluorescein and phycoerythrin molecules per cell. Simply cellular^{R} microbeads (FCSC) coated with a defined quantity of polyclonal goat-anti-mouse antibodies were incubated with each of the mAbs used to transform mean fluorescence channels into the number of monoclonal mouse immunoglobulins bound on the cell surface (IgG/cell).

Data analysis

Statistical comparisons were made using Wilcoxon's rank sum test.

RESULTS

Increased expression of β_2-integrins and ICAM-1 on monocytes and granulocytes stimulated in whole blood with various doses of OM85

Preliminary *in vitro* experiments indicated that OM85 enhanced expression of MAC-1 and ICAM-1 on monocytes and granulocytes in a dose-dependent manner (Fig. 1). Maximal effects were achieved when the bacterial extract was used at a concentration of 1 mg lyophilizate/ml. LPS at a concentration of 1 μg/ml was used as the positive control. This concentration was, therefore, chosen for further experiments. Since LPS is known to increase β_2-integrin expression on leucocytes, it was important to show that the effect of OM85 was not due to small amounts of endotoxin in the preparations. As shown in Table 1, polymyxin-B

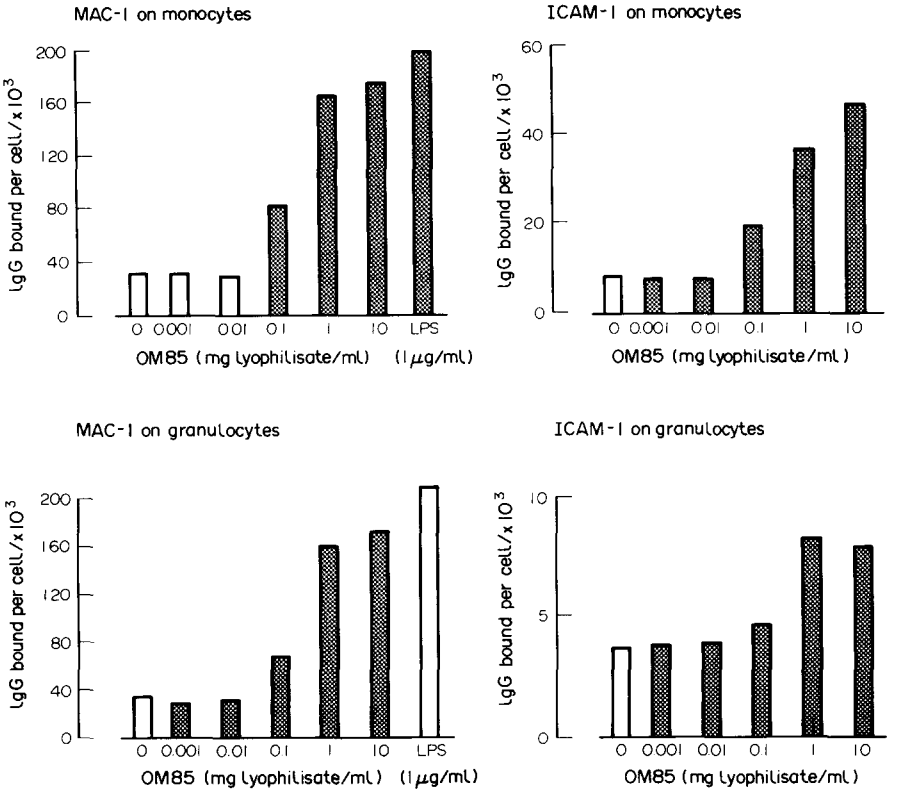

Fig. 1. Dose-dependent increase of MAC-1 and ICAM-1 expression on monocytes and granulocytes stimulated with various doses of OM85. Whole blood from a single individual was incubated for 4 h at 37°C with medium alone, various concentrations of bacterial extract or LPS. Expression of adhesion molecules is expressed as the number of IgG-molecules bound per cell (see Experimental Procedures).

Table 1. Effects of polymyxin-B on the hyperexpression of MAC-1 induced by OM85 or LPS

Stimulus	Polymyxin-B	MAC-1* Monocytes	MAC-1* Granulocytes
RPMI-medium	–	72	72
	+	76	70
LPS (1 μg/ml)	–	318	378
	+	77	50
OM85 (1 mg/ml)	–	294	282
	+	283	207

Whole blood was incubated for 4 h at 37°C with the bacterial extract, LPS or medium alone. When indicated polymyxin-B was added at a final concentration of 100 μg/ml.
*Expressed as IgG bound per cell × 10^3.

completely abrogated the LPS-induced MAC-1 hyperexpression, while the effect of OM85 was not substantially affected.

Data presented in Table 2 indicate that OM85 also induces a 50 – 70% increase in LFA-1 expression on monocytes, while no significant effect was observed on granulocytes. The expression of the third β_2-integrin, p150,95, was also enhanced by OM85, both on monocytes and granulocytes.

Time-dependent expression of MAC-1 on monocytes and granulocytes

Prior to incubation, the average number of anti-MAC-1 mAb bound per monocyte was 22,000 ± 9700 IgG/cell (mean ± 1 S.D. of five different donors). Incubation of whole blood alone or with RPMI-medium did not induce any significant increase in the expression of MAC-1. OM85 at concentrations of 1 mg lyophilizate/ml led to a sustained increase of MAC-1 expression peaking between 1 and 6 h (1 h: 96,000 ± 37,000; 3 h: 129,500 ± 76,000; 6 h: 133,000 ± 58,000; $P<0.01$ as

Table 2. Effects of OM85 on the expression of LFA-1 and p150,95

	LFA-1*		p150,95*	
Stimulus	Monocytes	Granulocytes	Monocytes	Granulocytes
RPMI-medium	92 ± 43	44 ± 12	12 ± 3	6 ± 1
OM85 (1 mg/ml)	158 ± 51†	46 ± 15‡	44 ± 20†	18 ± 7†

Whole blood was incubated for 1 h at 37°C with the bacterial extract or medium alone.
*Expressed as the mean number of IgG bound per cell × 10^3 in two separate experiments on five different donors († $P<0.05$; ‡ not significant).

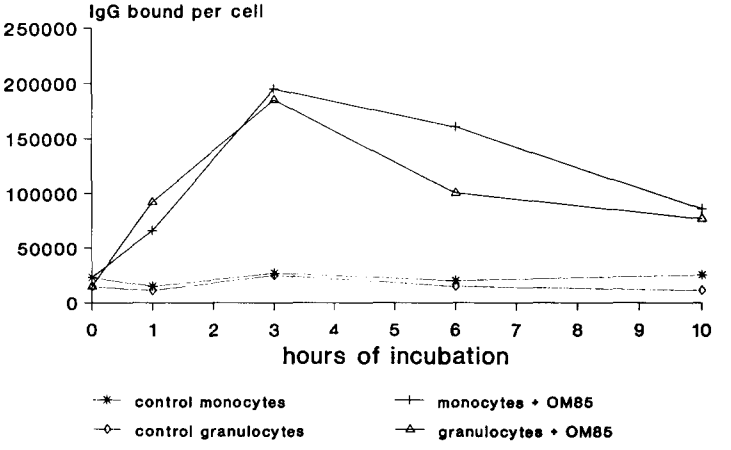

Fig. 2. Time-dependent expression of MAC-1 on granulocytes and monocytes treated with OM85. Whole blood was incubated at 37°C with the bacterial extract at a concentration of 1 mg/ml. Expression of adhesion molecules is expressed as the number of IgG-molecules bound per cell (see Experimental Procedures) in a single representative experiment.

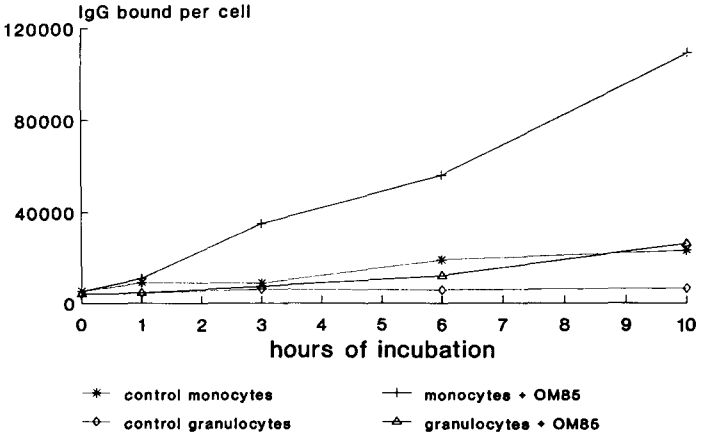

Fig. 3. Time-dependent expression of ICAM-1 on monocytes and granulocytes treated with OM85. Whole blood was incubated at 37°C with the bacterial extract at a concentration of 1 mg/ml. Expression of adhesion molecules is expressed as the number of IgG-molecules bound per cell (see Experimental Procedures) in a single representative experiment.

compared with prestimulation values). A representative experiment on a single donor is depicted in Fig. 2.

The effect of OM85 on MAC-1 expression by granulocytes was similar to that observed with monocytes (Fig. 2). Baseline values were 20,000 ± 10,600 IgG/cell and, again, a maximal increase was obtained after 1–6 h (1 h: 104,500 ± 36,000; 3 h: 100,000 ± 62,000; 6 h: 113,600 ± 50,000; $P<0.01$ as compared with prestimulation values).

Time-dependent expression of ICAM-1 on monocytes and granulocytes

Incubation of whole blood with OM85 led to a progressive rise of ICAM-1 expression on monocytes which was maximal at the end of the observation period (Fig. 3). Indeed the values measured after 10 h of incubation in five different donors (119,100 ± 20,400 IgG/cell) were 15- to 16-fold higher than prestimulation values (7900 ± 4100 IgG/cell; $P<0.01$ as compared with preincubation values). The effect of the bacterial extract on the expression of ICAM-1 on granulocytes was less marked but still significant (Fig. 3). A 4- to 5-fold rise was observed after 10 h of incubation as compared with preincubation values (preincubation: 4100 ± 400 IgG/cell; OM85: 17,000 ± 4800 IgG/cell; $P<0.01$).

DISCUSSION

The experiments presented in this paper first indicate that the bacterial extract Broncho-Vaxom (OM85) induces a dose-dependent hyperexpression of MAC-1 (CD11b/CD18) on phagocytic cells. This adhesion molecule, known as the complement receptor CR3, is important for the transendothelial migration of both neutrophils and monocytes (Doerschuk, Winn, Coxson & Harlan, 1990; Anderson, Rothlein, Marlin, Krater & Smith, 1990; Prieto, Beatty, Clark & Patarroyo, 1988) and for the phagocytic properties of these cells (reviewed in Brown, 1991). Upregulation of MAC-1 has been described previously in response to LPS, f-mlp, TNF-α or C5a (Yancey *et al.*, 1985; Werfel, Koch & Götze, 1989; Miller, Bainton, Borregaard & Springer, 1987; McLeish, Wellhausen & Dean, 1987). These stimuli appear to mobilize an intracellular pool of complement receptors (Miller *et al.*, 1987). Such a mechanism could also account for the rapid increase of MAC-1 induced by OM85. Although we confirmed previous reports indicating that OM85 contains small amounts of LPS (less than 1 ng/mg of lyophilizate), experiments using polymyxin-B established that the effect of the bacterial extract is essentially maintained in the presence of this LPS inhibitor. OM85 also induced an upregulation of p150,95, known as the complement receptor CR4, on monocytes and granulocytes. Although the role of this molecule has been less studied than that of MAC-1, p150,95 is also involved in phagocytosis (reviewed in Brown, 1991) and adhesion to endothelial cells (Keizer, Te Velde, Schwarting, Figdor & De Vries, 1987). The expression of LFA-1 (CD11a/CD18) on monocytes was also moderately increased under the influence of the bacterial extract so that all three β_2-integrins appear to be upregulated on phagocytic cells stimulated with OM85.

OM85 bacterial extract also enhanced the expression of ICAM-1, particularly on monocytes. The kinetics of changes in ICAM-1 expression suggest a *de novo* synthesis of the protein. Since ICAM-1 has been shown to be involved together with class II MHC molecules in the presentation of antigens (Altmann, Hogg, Trowsdale & Wilkinson, 1989), one can think that the bacterial extracts could also favour the induction of specific immune responses.

OM85 may, thus, enhance anti-bacterial defences in several ways since they were found previously to activate the oxidative respiratory burst of phagocytes (Mauel *et al.*, 1989) and to stimulate the production of monokines including TNF-α (Wybran *et al.*, 1989). Using LPS as a standard, we found that the same doses of OM85 were much more effective in upregulating adhesion molecules than in inducing TNF-α secretion (data not shown).

It is, thus, possible that the apparent beneficial effect of the OM85 bacterial extract may at least in part be due to its action on the adhesion molecules of phagocytes, allowing these cells to reach more efficiently the sites of infection and amplifying their microbicidal activity. However, the doses used in our *in vitro* experiments were relatively high and the concentrations obtained *in vivo* after oral administration are unknown. Further *in vivo* studies are therefore required to evaluate the clinical relevance of our findings.

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