Synthetic Peptide Segments from the *Escherichia coli* Porin OmpF Constitute Leukocyte Activators

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Various surface compounds of gram-negative bacteria constitute mitogens and polyclonal activators for murine B lymphocytes. This was shown previously for lipopolysaccharide (1), bacterial lipoprotein (2, 18), and porins extracted from *Escherichia coli* (4) and *Salmonella typhimurium* (25–27). For several leukocyte-activating bacterial cell wall components, the molecular structures responsible for biological activity have been determined and prepared by chemical synthesis. For bacterial lipoprotein, we have synthesized the N-terminal lipopeptide PamCys-Ser-Ser-AsnAla (28) and shown it to be responsible for the B lymphocyte-activating properties (3), as well as for the adjuvanticity (5, 15, 17) and macrophage-stimulating effects (11, 12) of the native molecule. Also, the part of lipopolysaccharide responsible for most of its biological activity, lipid A, has been synthesized in a biologically active form (8), and synthetic muramyl dipeptides derived from bacterial murein constitute potent macrophage activators (24).

Porins play an important role in the molecular sieving system of the outer membrane of *Enterobacteriaceae* (20). They act as receptors for bacteriophages, and as trimers, they form diffusion channels for hydrophilic substrates (21, 23). In an attempt to elucidate the structures responsible for the lymphocyte-activating effects of the porin OmpF from *E. coli*, we prepared a cyanogen bromide fragment of 24 kilodaltons (kDa) (26, 27), and a protease digest of the porin containing peptides of 8 kdaltons or less (26). These compounds were able to activate B lymphocyte proliferation and immunoglobulin secretion. On the basis of these findings and with the amino acid sequence of OmpF (20), we used computer-assisted epitope analysis to determine the parts of the membrane protein exposed on the surface and thus accessible for lymphocytes. Three of the predicted sequences were chosen for peptide synthesis, and the synthetic oligopeptide derivatives prepared were tested for biological activity. The peptides were able to stimulate the proliferation of murine B lymphocytes and their differentiation into immunoglobulin-secreting cells and, on a smaller scale, to induce tumor cytotoxicity of murine bone marrow-derived macrophages.

**Protein purification, epitope analysis, and peptide synthesis.**

The porin OmpF from *E. coli* was isolated by the method described by Hindenach and Henning (10). Surface-exposed areas of the molecule were predicted by using computer-assisted epitope analysis (EPICON 89 program), which calculates a weighted sum of different parameters called Fazit (Fig. 1). Three peptides were selected: OmpF (153–174) (containing amino acids 153 to 174), OmpF (157–174), and OmpF (275–285) were synthesized and tested. Like the native protein, the segments were mitogenic for BALB/c splenocytes and induced B lymphocyte differentiation into antibody-producing plasma cells and tumor cytotoxicity of macrophages against the fibroblast cell line L929. We thus demonstrated that defined peptide segments are responsible for the leukocyte-activating properties of a major bacterial surface protein.

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Harbor, Maine) mice were sacrificed by cervical dislocation, and spleen cells were prepared as described previously (25). Erythrocytes were lysed by treatment with Gey's solution. Polyclonal lymphocyte activation was measured as described previously (3). Briefly, lymphocytes were cultured in flat-bottom Falcon 3040 microtiter plates (Becton Dickinson, Oxnard, Calif.) at a density of 3.3 x 10⁶ cells per ml for 48 h in RPMI 1640 (GIBCO Diagnostics, Karlsruhe, Federal Republic of Germany) supplemented with 3.3% fetal calf serum, antibiotics, and 2-mercaptoethanol (5 x 10⁻⁵ M) in the presence of varying doses of stimulant. During the last 24 h, the cultures were pulsed with 0.625 μCi of [³H]thymidine per well (185 GBq/mmol; Amersham, Braunschweig, Federal Republic of Germany) and harvested onto glass fiber filters. Incorporated radioactivity was measured by liquid scintillation counting on a β-plate counter (Pharmacia/LKB, Freiburg, Federal Republic of Germany). The development of lymphocytes into immunoglobulin-secreting cells was measured by using a direct hemolytic plaque assay against highly trinitrophenylated sheep erythrocytes (TNP-SRBC). Lymphocytes were cultured at 5 x 10⁶ cells per culture in flat-bottom microtiter plates in the presence of stimulant and assayed on day 5 for the development of immunoglobulin-secreting cells. The supernatants of these cultures were assayed by enzyme-linked immunosorbent assay with TNP-SRBC coupled to enzyme-linked immunosorbent assay microtiter plates (MR 93; Dynatech, Denkendorf, Federal Republic of Germany) with poly-L-lysine (Serva, Heidelberg, Federal Republic of Germany), as previously described (17). Bone marrow-derived macrophages were prepared as described previously (12). Tumor cytotoxicity of the macrophages against cell lines L929 and P815 was determined by a [³H]thymidine release assay, as described by Meltzer (19). Results are expressed as the per-
The spontaneous lysis percentage of exposed areas of the labeled target cells was calculated as follows: 
\[ \text{specific lysis} = \frac{(\text{cpm}_{\text{exp}} - \text{cpm}_{\text{spont}})}{(\text{cpm}_{\text{total}} - \text{cpm}_{\text{spont}})} \times 100 \]

The spontaneous release (cpm\text{spont}) was determined by lysis of target cells in wells containing only labeled target cells. The total release (cpm\text{total}) was determined by lytic agents with 0.5% sodium dodecyl sulfate. The \(^{3}H\)thymidine release induced by each agent was determined as cpm\text{exp}. Tumor necrosis factor secretion was determined by a cytolysis assay of the fibroblast cell line L929, as previously described (12).

In order to define more closely the regions responsible for the mitogenic properties of OmpF, we predicted surface-exposed areas of the molecule by using computer-assisted epitope analysis (Fig. 1) on the basis of the amino acid sequence of OmpF determined by Mizuno et al. (20). We used the program EPICON 89, combining the predictions obtained from the analysis of the parameters of hydrophilicity (14), hydropathy (6), acrophilicity (13), and flexibility (16) together with conformational predictions (10). Antigenicity was calculated by using parameters expressing a comparison of the amino acid composition of known sequential epitopes and that of the whole sequence of various membrane-associated proteins (F. Gombert et al., unpublished data). The possibility of finding suitable epitopes was further enhanced by using different parameters for globular and membrane proteins (10; Gombert et al., unpublished data). A particular weighting of the single predictions was introduced, which resulted in the Fazit plot (Fig. 1) demonstrating the most probable antigenic sites. We selected and synthesized three peptide segments corresponding to two regions of the molecule. The peptides were OmpF (153–174) and OmpF (157–174), the latter being a peptide lacking the four hydrophobic amino acids on the amino end of the peptide OmpF (153–174), and OmpF (275–285). Figure 2 shows the polyclonal lymphocyte activation induced by OmpF by using BALB/c splenocytes. The lymphocytes reacted in a dose-dependent manner in respect to both proliferation, as measured by \(^{3}H\)thymidine incorporation, and differentiation into immunoglobulin-secreting cells, as

![FIG. 3. Incorporation of \(^{3}H\)thymidine in BALB/c splenocytes after 48 h of culture. Incorporation was induced by synthetic oligopeptides OmpF (153–174) (■), OmpF (157–174) (□), and OmpF (275–285) (○). Values are the means of triplicate determinations.](image)

![FIG. 4. Generation of hemolytic plaques against TNP-SRBC on day 5 after stimulation of BALB/c splenocytes with the synthetic oligopeptides OmpF (153–174) (■), OmpF (157–174) (□), and OmpF (275–285) (○). Values are the means of triplicate determinations ± standard deviations.](image)

![FIG. 5. Generation of immunoglobulin M antibodies specific for TNP-SRBC detected by enzyme-linked immunosorbent assay in supernatants of BALB/c splenocyte cultures after 5 days of stimulation with the synthetic oligopeptides OmpF (153–174) (■), OmpF (157–174) (□), and OmpF (275–285) (○). Values are the means of triplicate determinations ± standard deviations.](image)
Specific lysis of radiolabeled L929 target cells by BALB/c (top) and C3H/HeJ (bottom) bone marrow-derived macrophages (day 9) stimulated by the native OmpF (Protein I) or the oligopeptides OmpF (153–174) and (157–174). Bone marrow-derived macrophages (4 × 10⁵ per well) were incubated for 2 h with various concentrations of the stimulants and washed twice afterwards. Lipopolysaccharide at a concentration of 50 μg/ml was put in as a control. Labeled L929 cells (4 × 10⁴) were then added to each well, resulting in an E:T (effector to target) ratio of 10:1. The percentage of specific lysis was determined after 48 h. Values are the means of triplicate determinations ± standard deviations.
measured by a hemolytic plaque assay using TNP-SRBC, thus confirming our previous findings (4, 27).

In these experiments, the synthetic oligopeptides were investigated for their biological effects on murine spleenocytes. Figure 3 shows the results of a [3H]thymidine incorporation assay using BALB/c spleenocytes cultured for 48 h in the presence of various doses of the peptide segments. OmpF (153–174), OmpF (157–174), and OmpF (275–285) all induced lymphocyte proliferation in a dose-dependent manner, though at different molar efficiencies. On a molar basis, the peptide OmpF (153–174) was three to four times more efficient than the more hydrophilic peptide OmpF (157–174). The segment OmpF (275–285) was less active than OmpF (157–174). As a negative control, we used a randomly chosen peptide of comparable length consisting of 28 amino acids (secretin). This segment exhibited only marginal stimulatory effects towards lymphocytes (control, 6,009 ± 364 cpm; secretin, 120, 30, and 7 μmol/ml resulted in 6,382 ± 807, 7,466 ± 1,173, and 8,373 ± 859 cpm, respectively).

We also tested the ability of the peptides to induce lymphocyte differentiation into immunoglobulin-secreting cells. Cultures were performed with BALB/c spleenocytes (5 × 10⁶ per culture) and different concentrations of the peptides. Figure 4 indicates the number of anti-TNP plaques induced by the peptides after 5 days of culture. The peptides OmpF (153–174) and OmpF (157–174) both induced differentiation of resting B lymphocytes into plaque-forming cells with optimal doses of around 200 μmol/ml. The peptide OmpF (275–285) induced immunoglobulin-secreting cells in an amount comparable to that induced by OmpF (153–174), with an optimum dose of around 500 μmol/ml. These data were confirmed by testing the SRBC-specific immunoglobulin content in the culture supernatants. By using TNP-coated SRBC in an enzyme-linked immunosorbent assay both OmpF (153–174) and OmpF (157–174) were able to induce the production of anti-TNP-SRBC immunoglobulin M (Fig. 5).

Also, OmpF (275–285) was able to induce immunoglobulin M secretion comparable to the plaque assay data with the same optimum concentration. In all assays performed, the peptides differed in their efficiencies of stimulation. The most efficient substance proved to be peptide OmpF (153–174), which was superior to peptide OmpF (157–174), which lacked four N-terminal hydrophobic amino acids. This could be due to the fact that the more hydrophobic segment OmpF (153–174) was better able to interact with the B-cell membrane. The segment OmpF (275–285), being more hydrophilic than the peptides described above, exhibited reduced activity. Comparing these data with those described in Fig. 2, OmpF protein turned out to be considerably more active than the synthetic peptide segments. These findings may be explained by the contribution to mitogenicity by secondary folding structures of the native protein (23), by the presence of multiple biologically active segments on the native molecule, or by inaccurate selection of the peptide segments to be synthesized. The requirement of higher molar amounts of a synthetic partial sequence, compared with its native protein, to obtain comparable biological effects, is also reported for other peptides (e.g., for an interleukin-1β peptide with adjuvant activity [22]).

In addition to B-cell activation, preliminary experiments indicate that protein I as well as the selected segments (153–174) and (157–174) are also macrophage activators. We measured the protein- and peptide-induced tumor cytotoxicity of bone marrow-derived macrophages against the L929 fibroblast and the P815 mastocytoma cell line in a [3H]thymidine release assay. With BALB/c macrophages as effector cells against L929 (Fig. 6), the porin OmpF as well as the segments 153–174 and 157–174 enhanced cytolytic activity of macrophages in a dose-dependent manner. At a concentration of 10.8 nmol/ml, almost 70% tumor cell lysis was found for the porin OmpF, whereas its segments led to a threefold increase in cytotoxicity compared with control macrophages. In order to exclude effects of lipopolysaccharide in the system, C3H/HeJ macrophages were used as effector cells. Comparable results were obtained with OmpF, leading to approximately 65% lysis of L929 cells at a stimulator concentration of 10.8 nmol/ml (Fig. 7). The segment 153–174 showed a twofold increase in cytotoxicity at the highest doses tested. In both assays, the macrophages stimulated with OmpF or the segments showed only marginal capability to lyse the tumor necrosis factor-insensitive cell line P815 (data not shown). To further elucidate the role of soluble factors in this system, we also looked for cytotoxic activity in the supernatants of macrophages activated with OmpF (10.8 nmol/ml) for 2 h. Preliminary results of cytolyis assays on L929 indicate that the macrophages released a cytotoxic factor (64 U/ml) into the supernatant which is likely to be tumor necrosis factor, since the activity could be totally abolished by a 1:40 dilution of a specific anti-murine tumor necrosis factor α antiserum (generous gift of M.-L. Lohmann-Matthes, Hannover).

Additional studies on the macrophage-activating properties of OmpF and its segments, as well as studies on the immunoadjuvant properties of the compounds, are in progress in our laboratory.

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