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A Defined Fragment of Bacterial Protein I (OmpF) Is a Polyclonal B-Cell Activator

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Protein I from the outer membrane of *Escherichia coli* and other members of the family *Enterobacteriaceae* is a potent mitogen and polyclonal B-lymphocyte activator. To determine the part of the polypeptide responsible for biological activity, we cleaved the molecule into defined polypeptide fragments of approximate molecular weights 24,000, 15,000, 9,000, 7,000, and 3,000 by using the cyanogen bromide method. The fragments were purified by gel permeation chromatography and by preparative polyacrylamide gel electrophoresis. They were investigated for mitogenicity and for the induction of immunoglobulin synthesis in lymphocyte cultures from several inbred mouse strains. The fragment of molecular weight 24,000 turned out to be a potent polyclonal B-lymphocyte activator comparable to native protein I. The low-molecular-weight fragments exhibited only marginal effects. Neither purified T lymphocytes nor thymocytes were activated. Our results show that a defined fragment of protein I is responsible for its lymphocyte-stimulating activity.

A great variety of bacterial surface components, such as protein I (5), protein II* (5, 21), lipopolysaccharide (LPS) (2), and lipoprotein (3, 20) have been shown to cause polyclonal activation of murine B lymphocytes. For LPS and lipoprotein it has been shown that the lipid constituents of the molecules, lipid A or lipopeptides containing two to five amino acids attached to triacylglyceryl cysteine, were responsible for their activity (1, 7). Both lipid compounds have been recently synthesized in a biologically fully active form (6, 18, 29). Protein I, a major protein of the outer membrane of *Escherichia coli* and other members of the family *Enterobacteriaceae*, consists of a polypeptide chain of molecular weight 37,000 devoid of any lipid structures (9). The protein has been previously shown by us to be a potent B-lymphocyte mitogen and a polyclonal activator of immunoglobulin synthesis (5). Lymphocyte stimulation was shown in a variety of LPS responder and nonresponder mouse strains, and the polyclonal activation observed was comparable to the activation induced by lipoprotein or LPS (5). To determine the molecular structure responsible for the lymphocyte-stimulating activity of this mitogen, defined peptide fragments of the molecule were prepared and investigated for biological activity. This publication shows that the lymphocyte-stimulating activity of protein I can be assigned to a major polypeptide fragment of the molecule. The results will be of importance for the further elucidation of the molecular mechanism of the mitogenic B-lymphocyte activation and will provide further insights into the host-parasite relationship during bacterial infections.

MATERIALS AND METHODS

Mice. BALB/c mice were obtained from Ivanovas, Kisslegg, Federal Republic of Germany. C3H/HeJ mice were from Jackson Laboratories, Bar Harbor, Maine. C3H/Tif/Bom *nu/nu* athymic mice were from Bomholtgart, Denmark. The animals (age, 6 to 16 weeks) were sacrificed by cervical dislocation, and the spleen or thymus was excised and macerated gently in a loose-fitting tissue grinder (Braun, Melsungen, Federal Republic of Germany). Cells were filtered through cotton wool and suspended in minimal essential medium.

Incorporation of [^{3}H]thymidine. [^{3}H]thymidine incorporation experiments (4) were carried out in flat-bottomed Falcon 3040 microtiter plates (Becton Dickinson Labware, Oxnard, Calif.) Unless otherwise indicated, lymphocyte cultures were performed for 48 h at a cell density of 3.3×10^6/ml in 0.18-ml portions per well in RPMI 1640 medium (GIBCO Diagnostics, Madison, Wis.) supplemented with 3.3% heat-inactivated fetal calf serum, fresh glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2-mercaptoethanol (5×10^{-5} M). Before harvesting, cultures were pulsed for 24 h by the addition to each well of 0.5 μCi of [^{3}H]thymidine (specific activity, 5 Ci/mmol; Amersham, Braunschweig, Federal Republic of Germany). The cultures were harvested with a Mash-II harvester (Microbiological Associates, Bethesda, Md.), collected on glass fiber filters (Dynatech, Nürtingen, Federal Republic of Germany), and measured by liquid scintillation. Assays were done in triplicate.

Incorporation of [^{3}H]leucine. Cultures (cell density, 3.3×10^6/ml) were done as described for [^{3}H]thymidine incorporation. The cells were pulsed for 24 h by addition of 0.5 μCi of [^{3}H]leucine (specific activity, 50.5 Ci/mmol; Amersham) to each well and then harvested as described above.

Hemolytic plaque assays. (i) TNP plaques. The development of immunoglobulin-secreting plasma cells was measured by using a hemolytic plaque assay against densely trinitrophenylated sheep erythrocytes (TNP-SRBC) (24). Lymphocytes were cultured at a cell density of 5×10^6/ml in 1-ml portions in Falcon 2054 culture tubes with various doses of mitogen. After 4 days, 100 μl of the lymphocyte suspension appropriately diluted in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffered Eagle minimal essential medium containing additional glucose (0.45%, wt/vol), 20 μl of guinea pig complement (Serva, Heidelberg, Federal Republic of Germany), and 20 μl of a 30% suspension of TNP-SRBC were quickly added to 300 μl

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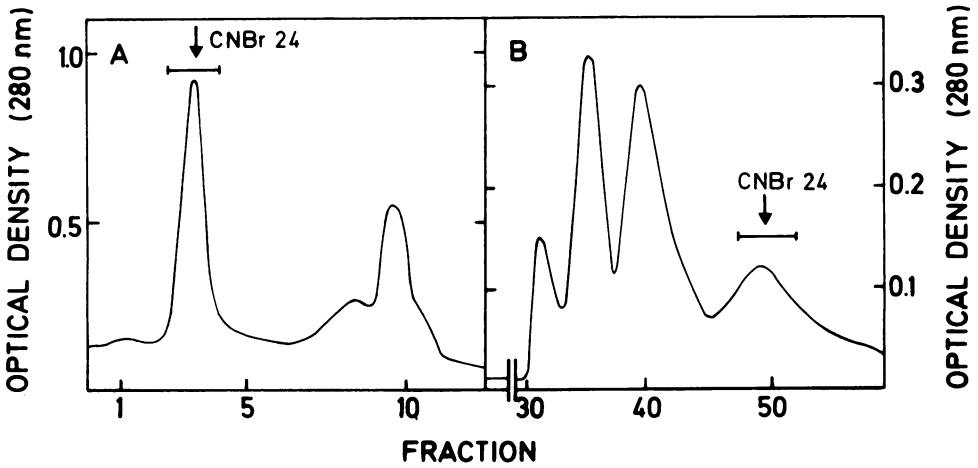

FIG. 1. Purification of CNBr 24 with an Ultrogel ACA 54 gel filtration column (A) and by preparative gel electrophoresis (B) by the Laemmli system (19) with BRL gel apparatus. (A) Protein I (5 mg) was applied to the column after CNBr cleavage and dissolved in 1 ml of elution buffer. (B) Protein I (1.5 mg) was applied to the preparative gel electrophoresis mixture after CNBr fragmentation in 0.3 ml of sample buffer by the procedure of Laemmli (19). Separation was performed at 7 mA. Fractions were collected and lyophilized.

of 0.5% agarose in medium at 45°C. The suspensions were mixed and plated on disposable petri dishes (Greiner, Nürtingen, Federal Republic of Germany). The dishes were kept in a humid atmosphere for 3 h, and plaques were scored with a plaque viewer (Tecnomara, Zürich, Switzerland).

(ii) Protein A plaques. The lymphocytes were tested for the development of immunoglobulin-secreting plasma cells by a hemolytic plaque assay with protein A-coated SRBC (14). The tests were performed as described for the TNP plaque assay, but instead of TNP-SRBC, protein A-coated SRBC were used. Plaques were developed (14) with a rabbit anti-mouse immunoglobulin serum provided by R. B. Johnson. The plated plaques were viewed after 6 h of incubation at 37°C in a humid atmosphere.

Gel electrophoresis. The purity of the protein fractions was determined by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis by the methods of Laemmli (19) and Hashimoto et al. (15). The gels were stained either with Coomassie blue (28) or by a silver-staining method (22). Preparative gel electrophoresis was performed with a gel apparatus (Bethesda Research Laboratories [BRL], Gaithersburg, Md.) by using a 12% polyacrylamide separation gel in the gel system as described by Laemmli (19).

Cyanogen bromide cleavage of protein I. Proteins containing methionine can be cleaved by CNBr. In this process, the methionine residues are transformed into C-terminal homoserine lactone residues (26). For the cleavage, 20 mg of protein I, purified as described previously (16), was dissolved in 2 ml of 70% trifluoroacetic acid (8, 13) or 70% formic acid. A 30- to 100-fold excess of CNBr was added, and the solution was stirred at room temperature for 16 to 20 h. Water (20 ml) was added, and the reaction mixture was lyophilized.

Citraconylation. The solubility of hydrophobic proteins is increased by the citraconylation procedure (12). Protein (10 to 100 mg) was suspended in phosphate-buffered saline (pH 8 to 8.5). While the mixture was stirred, 160 μl of citraconic anhydride (E. Merck AG., Darmstadt, Federal Republic of Germany) was added in 20-μl portions at time intervals of 5 min. The pH was held constant at approximately 8 to 9 by titration with 1 N NaOH. The reaction mixture was stirred for 1 h at room temperature. Afterwards, an additional 140 μl of citraconic anhydride was added in 20-μl portions as described above. At the end of the reaction, the solution was dialyzed against 20 mM NH_4HCO_3 to remove excess citraconic anhydride. The citraconylated proteins were lyophilized.

Gel filtration of CNBr fragments of protein I. A column (1 by 50 cm) packed with Ultrogel AcA 54 (LKB, Gräfeling, Federal Republic of Germany) was used for the gel filtration of CNBr fragments of protein I. Protein samples (5 mg/ml) were applied to the column equilibrated with 50 mM Tris hydrochloride buffer (pH 8.1) containing 8 M urea (8).

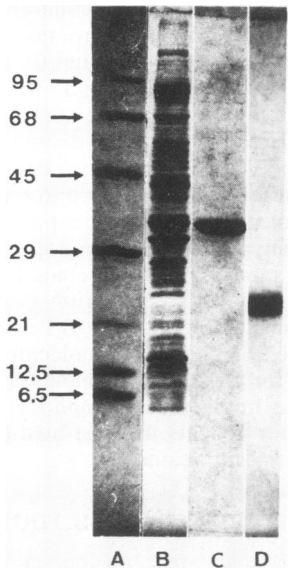

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gradient (10 to 20%) gel electrophoresis, by the procedure of Laemmli (19), of protein I and CNBr 24. Lane A, Molecular mass standards: α-gelatine (95 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbanhydrase (29 kDa), trypsin inhibitor, soja (21 kDa), cytochrome *c* (12.5 kDa), trypsin inhibitor, lung (6.5 kDa). Lane B, *E. coli* lysate. Lane C, Purified protein I. Lane D, Purified CNBr 24. Staining was performed with Coomassie blue.

RESULTS

The bacterial mitogen protein I represents a polypeptide composed of 349 amino acids (10). The molecule, containing four methionine residues, was fragmented by CNBr cleavage into five oligopeptides, of approximate molecular weights 24,000, 15,000, 9,000, 7,000, and 3,000, which were investigated for their lymphocyte-stimulating activity. The fragments obtained were citraconylated and subjected to gel filtration on an ACA 54 column. Separation by gel filtration could be achieved only after citraconylation to avoid aggregation of the material (8). A fragment of 24,000 daltons (Da) (CNBr 24) was eluted from the column as a defined peak (fractions 3 and 4), whereas the lower-molecular-weight components could not be separated by the column and were eluted in fractions 8 through 12 (Fig. 1A). The mixture of the citraconylated cyanogen bromide fragments of protein I was also applied to preparative gel electrophoresis (Fig. 1B). CNBr 24 was eluted as a homogenous band in fractions 48 through 52. The peaks in fractions 30 through 42 represent the low-molecular-weight fragments. Figure 2 shows electropherograms of protein I and CNBr 24, obtained by preparative gel electrophoresis (Fig. 1B). Staining with Coomassie blue resulted in a single polypeptide band, indicating the purity of CNBr 24 (Fig. 2). Figure 3 shows electropherograms of CNBr 24, made with the electrophoretic system of Hashimoto et al. (15), which exhibits high resulting power in the 1,500- to 25,000-Da range. Using this system to exclude possible contaminations with low-molecular-weight proteins, we were able to show that the CNBr 24 fragment was homogenous by sensitive silver staining (22).

Native protein I and the CNBr fragments were investigated for their ability to activate murine B lymphocytes. The mitogenic effects of the compounds can be seen from the dose-response plots in Fig. 4. Protein I induced proliferation, evident from the increased uptake of [^{3}H]thymidine into DNA, starting at concentrations around 10 μg/ml. The optimal dose for stimulation was around 100 μg/ml. CNBr 24 exhibited a reduced mitogenic activity, although optimal stimulatory activity was also found at concentrations around 100 μg/ml. The mixture of the low-molecular-weight frag-

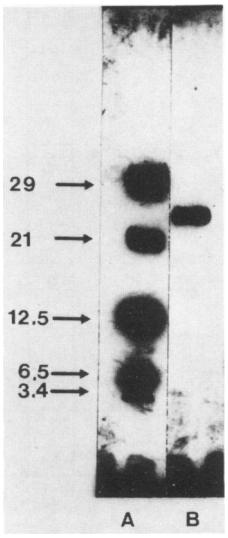

FIG. 3. SDS-polyacrylamide gradient (10 to 18%) electrophoresis confirming the purity of CNBr 24 in the SDS-polyacrylamide gel electrophoresis system by the procedure of Hashimoto et al. (15). Lane A, Molecular mass standards: carbanhydrase (29 kDa); trypsin inhibitor, soja (21 kDa), cytochrome *c* (12.5 kDa), trypsin inhibitor, lung (6.5 kDa), insulin, β-chain (3.4 kDa). Lane B, Purified CNBr 24. Silver staining was performed by the method of Mornay (22).

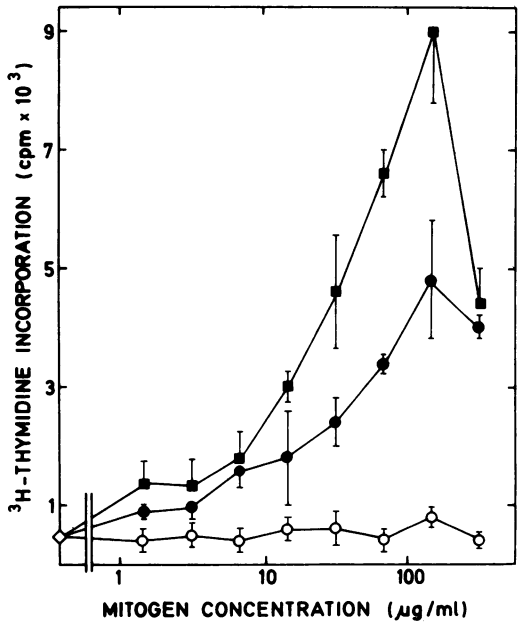

FIG. 4. Dose-response curves for [^{3}H]thymidine incorporation in splenocytes of BALB/c mice cultured for 48 h with protein I (■), CNBr 24 (●), and a mixture of the low-molecular-weight CNBr fragments (○). Values are means of triplicate determinations ± standard deviations.

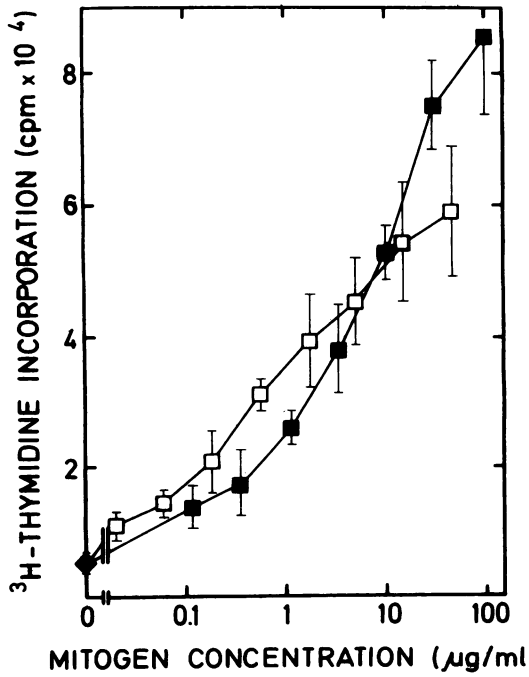

FIG. 5. Dose-response curves for [^{3}H]thymidine incorporation in splenocytes of BALB/c mice cultured for 48 h with protein I (■) and a mixture of all CNBr fragments (molecular weight, 24,000, 15,000, 9,000, 7,000, and 3,000) (□). Values are means of triplicate determinations ± standard deviations.

TABLE 1. Stimulation of [^{3}H]thymidine incorporation in splenocytes of C3H/HeJ mice by protein I, CNBr 24, and Con A[a]

Mitogen concn (μg/mg)	Incorporation of [^{3}H]thymidine (cpm)		
	Protein I	CNBr 24	Con A
137	7,584 ± 1,209	4,980 ± 752	64 ± 24
34	7,492 ± 1,867	4,490 ± 437	454 ± 242
8.5	3,748 ± 1,110	2,150 ± 292	5,813 ± 2,412
2.1	2,201 ± 365	1,455 ± 217	31,916 ± 1,327
0	963 ± 116	963 ± 116	963 ± 116

[a] Duration of activation, 48 h. Values are means of triplicate determinations ± standard deviations.

TABLE 2. Stimulation of [^{3}H]leucine incorporation in splenocytes of BALB/c mice by protein I and CNBR 24[a]

Mitogen concn (μg/ml)	Incorporation of [^{3}H]leucine (cpm)	
	Protein I	CNBr 24
273	1,009 ± 29	708 ± 163
137	746 ± 234	693 ± 156
68	948 ± 59	608 ± 83
34	861 ± 142	703 ± 113
8.5	592 ± 141	634 ± 117
1.1	452 ± 68	554 ± 55
0	353 ± 68	353 ± 68

[a] Duration of activation, 34 h. Values are means of triplicate determinations ± standard deviations.

ments as obtained by gel filtration (Fig. 1A, fractions 8 through 10) was only marginally active. To test whether the low-molecular-weight pool of peptides was able to suppress the proliferative response induced by CNBr 24 or protein I, a mixture of all cyanogen bromide fragments (molecular weights, 24,000, 15,000, 9,000, 7,000, and 3,000) was investigated on BALB/c splenocyte cultures (Fig. 5). This mixture induced a proliferative response comparable to that of native protein I at the lower concentrations and was only slightly less active at the higher concentrations. To ensure that the stimulatory effect was not due to minor contamination with endotoxin, protein I and CNBr 24 were also tested for mitogenicity in the C3H/HeJ LPS-nonresponder mouse strain (27). An activation comparable to the results obtained with BALB/c splenocytes was achieved (Table 1). The mixture of the low-molecular-weight fragments was not active in this system (data not shown).

To determine the kinetics of stimulation for protein I and CNBr 24, lymphocyte activation was examined 24, 48, 72, and 96 h after the addition of the mitogens (Fig. 6). Optimal incorporation of thymidine into the DNA of spleen cells was achieved after an incubation period of 48 h. Protein I and CNBr 24 brought about a similar degree of stimulation in BALB/c splenocytes, although the stimulating effect of protein I was more pronounced than that of CNBr 24 (Fig. 6).

To test the influence of protein I and CNBr 24 on protein biosynthesis, the incorporation of [^{3}H]leucine in BALB/c

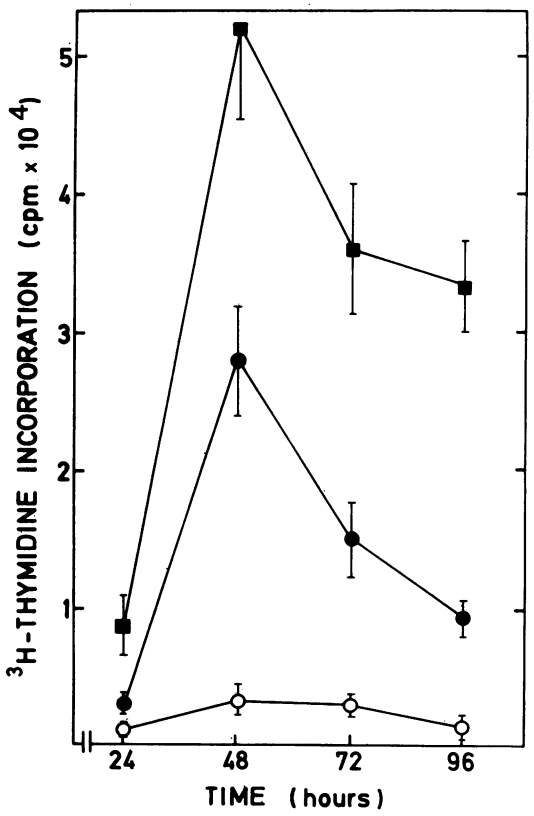

FIG. 6. Kinetics of stimulation of [^{3}H]thymidine incorporation in BALB/c splenocytes. Spleen cells were cultured with protein I (■) and CNBr 24 (●) at a concentration of 273 μg/ml for 24, 48, 72, and 96 h. At 24 h before harvesting, 0.5 μCi of [^{3}H]thymidine was added to the cultures. Control cultures without mitogens (○). Values are means of triplicate determinations ± standard deviations.

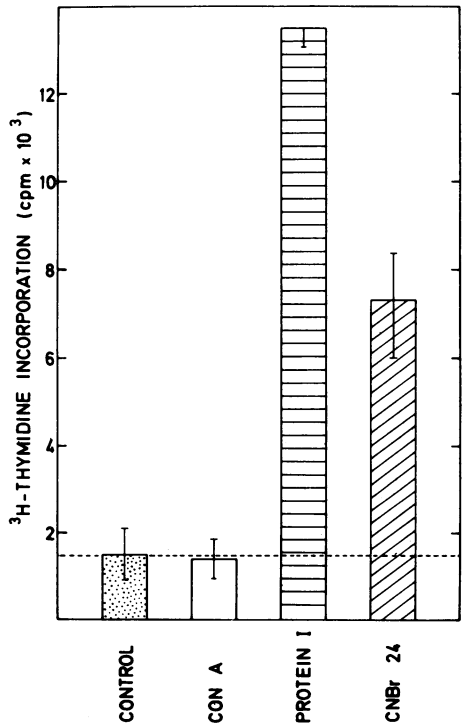

FIG. 7. Incorporation of [^{3}H]thymidine in C3H/Tif/Bom *nu/nu* splenocytes. Stimulation with Con A (2 μg/ml), with protein I (137 μg/ml), and with CNBr 24 (137 μg/ml). Cells were cultured for 48 h. Values are means of triplicate determinations ± standard deviations.

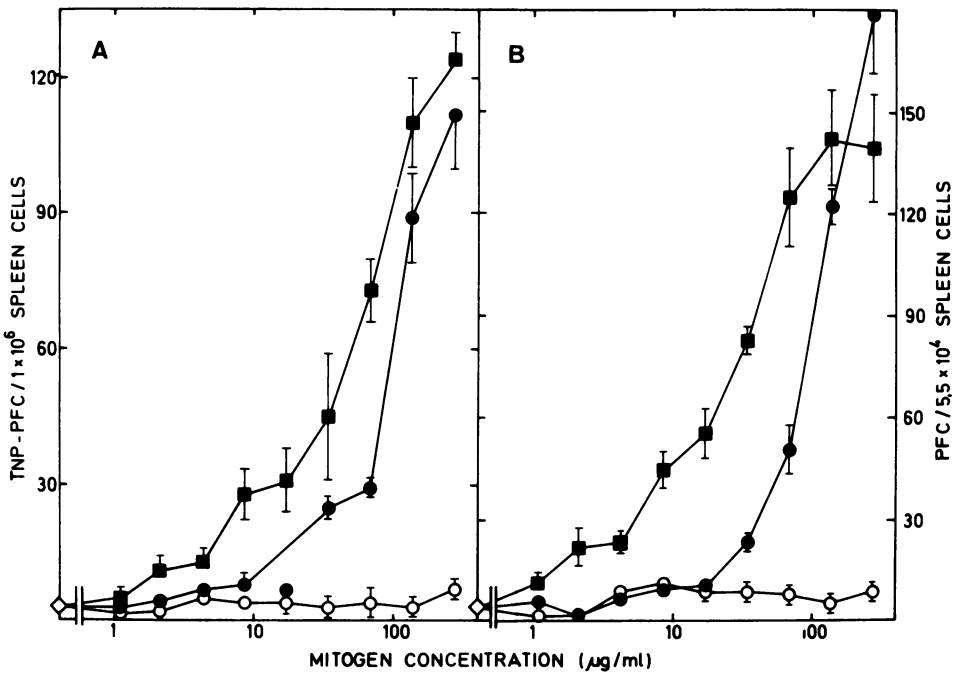

FIG. 8. Stimulation of B cells of BALB/c mice to the development of plaque-forming cells by protein I (■), CNBr 24 (●), and a mixture of the low-molecular-weight CNBr fragments (○) of protein I. (A) TNP plaque assay (23). (B) Protein A plaque assay (14). Values are means of triplicate determinations ± standard deviations.

splenocytes was measured. Both substances stimulated the biosynthesis of proteins in a comparable manner (Table 2), starting at a mitogen concentration around 10 µg/ml and reaching an optimum around 150 to 250 µg/ml.

To establish whether the compounds were mitogenic for B lymphocytes, the effect of protein I and CNBr 24 on spleen cells of athymic nude mice (C3H/Tif/Bom *nu/nu*) was investigated. Testing the incorporation of [^{3}H]thymidine in response to the mitogens, a similar pattern as seen above occurred. Figure 7 shows the stimulation results for protein I, CNBr 24, and concanavalin A (Con A) at optimal mitogen concentrations (Con A, 2 µg/ml; protein I, 137 µg/ml; CNBr 24, 137 µg/ml). Protein I and CNBr 24 were active mitogens, whereas the T-cell mitogen Con A, as expected, was inactive. The data indicate that protein I and CNBr 24 are B-cell mitogens. To determine whether the compounds were also able to stimulate B lymphocytes into immunoglobulin secretion, hemolytic plaque assays were performed with BALB/c mouse spleen cell cultures. CNBr 24 and protein I, but not the low-molecular-weight CNBr fragments, induced a marked increase in the number of plaque-forming cells against TNP-SRBC over a broad concentration range (20 to 200 µg/ml) (Fig. 8A) The protein A plaque assay (Fig. 8B) detecting the immunoglobulin-producing plasma cells in the culture showed similar data.

To test whether T lymphocytes were also activated by protein I and CNBr 24, the effect of the compounds on the incorporation of [^{3}H]thymidine into the DNA of thymocytes was investigated. Figure 9 shows the results of a stimulation experiment testing BALB/c thymocytes. CNBr 24 and protein I showed only a marginal stimulatory effect toward the cells, whereas Con A, used as a positive control, was a potent mitogen.

In a last series of experiments, we examined the in vivo effects of protein I and CNBr 24 on murine spleen cells. A marked enlargement of the spleen was apparent 3 days after the intravenous administration of 35 µg of protein I or CNBr 24 per mouse (data not shown). Protein I and CNBr 24 were able to cause polyclonal stimulation of splenic B lymphocytes into immunoglobulin secretion, as the number of plaque-forming cells against TNP-SRBC increased markedly (Fig. 10A). CNBr 24 induced about a twofold increase in plaque numbers compared with those of control mice and was therefore less active than native protein I, which induced a three- to fourfold stimulation in plaque numbers. In

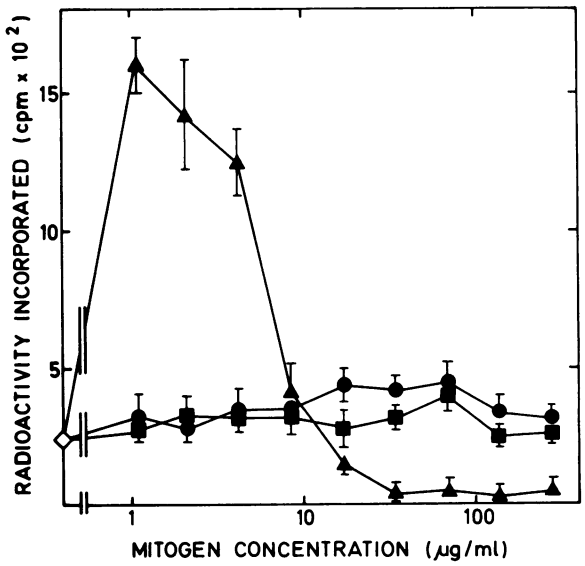

FIG. 9. Dose-response curves for [^{3}H]thymidine incorporation in BALB/c mouse thymocytes cultured for 48 h at a cell density of 10^7/ml in the presence of Con A (▲), protein I (■), and CNBr 24 (●).

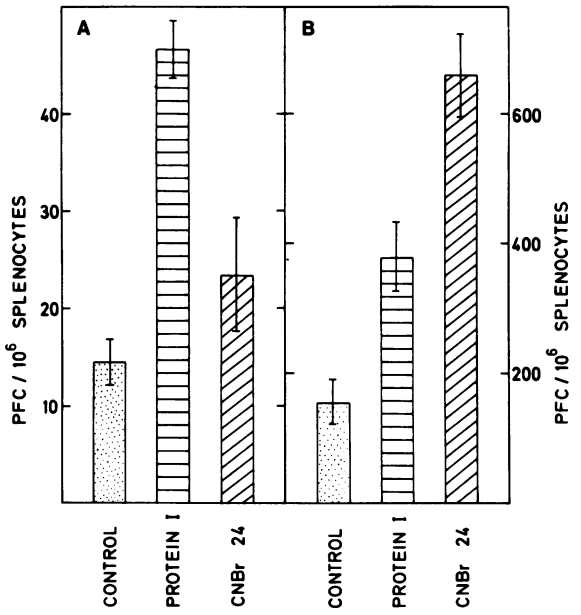

FIG. 10. Stimulation of B cells of BALB/c mice to the development of plaque-forming cells in vivo. Two mice in each group were injected intraperitoneally with protein I at 35 μg per mouse and CNBr 24 at 35 μg per mouse and sacrified after 3 days. The splenocytes of each group were pooled and used in a TNP plaque assay (A) and in a protein A plaque assay (B) as shown in Fig. 7. Values are means of triplicate determinations ± standard deviations.

this assay *E. coli* LPS was included as a positive control and exhibited an effect comparable to that of protein I (data not shown). In the plaque assay against protein A-coated SRBC (Fig. 10B), CNBr 24 was more active (fourfold) than protein I (twofold).

DISCUSSION

To determine the molecular structure of protein I responsible for mitogenicity, we prepared several defined fragments by cyanogen bromide cleavage of the molecule by the method published by Chen et al. (8). The high-molecular-weight fragment CNBr 24 was able to induce proliferation in LPS-responder as well as in LPS-nonresponder mouse splenocytes (Fig. 4; Table 1). In contrast, a mixture of the low-molecular-weight fragments showed no stimulatory effect. The ability of CNBr 24 to stimulate B lymphocytes is evident from Fig. 7. Spleen cells from athymic nude mice, which are devoid of maturated T lymphocytes, could be activated to a similar degree as shown by spleen cells from conventional mice. These data show that CNBr 24 is a T-cell-independent B-lymphocyte mitogen. In addition, the ability of the fragment to nonspecifically stimulate B lymphocytes to immunoglobulin secretion is evident from Fig. 8. The marginal stimulatory effect of the compound toward thymocytes is shown in Fig. 9; experiments with enriched T-lymphocyte preparations from spleens showed similar results (data not shown).

Our results therefore show that the 24,000-molecular-weight CNBr fragment of bacterial protein I was able to activate murine B lymphocytes from both LPS-responder and LPS-nonresponder mouse strains and had, in all aspects tested, a mitogenic and polyclonal activating effect comparable to that of the B-lymphocyte mitogen protein I, although protein I was in some assays more active than CNBr 24. In all experiments, a mixture of the low-molecular-weight (15,000, 9,000, 7,000, and 3,000) compounds showed no stimulatory or supressive activity (Fig. 4 and 5). We therefore assume that the structure responsible for the polyclonal stimulatory activity of protein I is located on the 24,000-molecular-weight fragment of the protein. It is, however, possible that an oligopeptide fragment enhancing the mitogenic activity of CNBr 24 is lost during the fragmentation, which would explain the reduced activity of the CNBr 24 fragment compared with that of protein I in some experiments.

Interestingly, stimulatory activity was achieved without the involvement of a lipid part, which is required for activity in lipoprotein or LPS (1, 7). We assume that the hydrophobic amino acid sequences found in protein I (9) serve to anchor the compound in the lymphocyte plasma membrane, presenting an active moiety of the molecule to putative binding proteins in the lymphocyte plasma membrane. The existence of binding proteins for lipoprotein involving major histocompatibility class 2 products has been shown by our group (25). Yokoyama et al. (30) have also presented evidence for the binding of LPS to major histocompatibility class 2 complex proteins, and Coutinho et al. (11) have published serological evidence for LPS receptors on leukocytes.

Earlier studies performed by Peavy et al. have shown that LPS, when administered to mice, induces splenomegaly and cellular proliferation (23). Johnson et al. (17) showed that lipoprotein and synthetic derivatives produced similar effects. When CNBr 24 or protein I was injected into BALB/c mice, the spleens of these immunized mice had, by day 3, almost doubled in weight. The fragment also induced polyclonal stimulation of B lymphocytes into immunoglobulin secretion in vivo (Fig. 10A and B), as the number of plaque-forming cells against TNP-SRBC as well as against protein A-coated SRBC increased markedly. The fact that CNBr 24 was a more powerful mitogen in the induction of protein A plaques, whereas in the stimulation of TNP plaques protein I was more active, leaves open the possibility that the activating properties in vivo of protein I and CNBr 24 are not identical. This could be due to a potential loss of an enhancing or directing part during the fragmentation of protein I. When the in vivo response to these mitogens was measured by using untreated erythrocytes, a similar stimulation pattern was observed, although the actual number of plaque-forming cells was about 10% of those against TNP-SRBC (unpublished observations). These findings provide evidence that these bacterial surface components may have a significant influence on the host-parasite relationship, particularly prior to the establishment of specific humoral and cellular responses. They also show that the stimulatory activity of the compounds in vivo complements the effects observed in vitro.

In summary, a defined fragment of protein I, the peptide fragment CNBr 24, has been shown to be a potent B-lymphocyte activator in vitro as well as in vivo. The product could be a valuable tool for continuing the investigation of the molecular mechanism of B-lymphocyte activation. To determine more precisely the molecular region of CNBr 24 responsible for B-cell activation, the product has recently been further fragmented by enzymatic digestion. The mixture of fragments obtained after digestion with either trypsin or *Staphylococcus aureus* V8 protease still exhibited a marked stimulatory effect (data not shown). We are presently engaged in verifying and characterizing the active subfragments of CNBr 24.

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