Vol. 121, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1984 Pages 55-61

THE MITOGENIC PRINCIPLE OF ESCHERICHIA COLI LIPOPROTEIN: B-LYMPHOCYTE MITOGENICITY OF THE SYNTHETIC ANALOGUE PALMITOYL-TETRAPEPTIDE (PAM-SER-SER-ASN-ALA)

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Received March 1, 1984

N-Palmitoyl-(S)-seryl-(S)-seryl-(S)-asparaginyl-(S)-alanine (Palmitoyl-tetrapeptide) is an analogue of the N-terminal part of the lipoprotein from the outer membrane of Escherichia coli. It was prepared by chemical synthesis and tested for biological activity in in vitro lymphocyte culture systems. In spleen cells of the inbred mouse strains C3H/HeJ, C3H/He/Bom/nunu, and Balb/c, the compound exhibited stimulatory activity towards B-lymphocytes comparable to the effect of native lipoprotein, as measured by the incorporation of ^{3}H-thymidine and ^{3}H-uridine, and by a hemolytic plaque assay. The B-lymphocyte tumor cell line BCL1 was also activated by the compound. The results demonstrate, that the N-terminal tetrapeptide moiety of lipoprotein, linked to a lipophilic molecule, constitutes by itself a novel B-lymphocyte mitogen.

A variety of surface components from gram negative bacteria such as lipopolysaccharide (LPS) (1), lipoprotein (2,3), protein I and protein II*(4) have been shown to act as lymphocyte mitogens, stimulating murine B cells into both proliferation and immunoglobulin secretion. The part of the lipoprotein molecule responsible for B-cell mitogenicity appears to reside in the three fatty acids containing N-terminal lipopeptide region, which can be prepared by enzymatic cleavage of the molecule (5). We could also demonstrate that this N-terminal lipopeptide constituent of bacterial lipoprotein (Tripalmitoyl-pentapeptide), as prepared by chemical synthesis (6,7), is a mouse B-lymphocyte mitogen (8,9). The N-terminal tetrapeptide moiety Ser-Ser-Asn-Ala is essential for the biological activity of Tripalmitoyl-pentapeptide, since a compound lacking this moiety (Tripalmitoyl-cysteine) is only moderately active (9,10). In this communication we demonstrate that a novel polyclonal B-cell-

Abbreviations: LPS lipopolysaccharide

0006-291X/84 $1.50

activator and mitogen is formed by coupling the peptide Ser-Ser-Asn-Ala to a hydrophobic molecule (palmitic acid). This substance is active towards B-lymphocytes of three inbred mouse strains and towards a lymphoid B cell line.

EXPERIMENTAL

Reagents. Palmitoyl-tetrapeptide was obtained by coupling palmitic acid (Pam-OH) to O-tert-butyl-seryl-O-tert-butyl-seryl-asparaginyl-alanine-tert-butyl ester(H-L-Ser(Bu^t)-Ser(Bu^t)-Asn-Ala-OBu^t) with N,N`-dicyclohexylcarbodiimide in dimethylformamide/dichloromethane (2:1). The resulting Pam-Ser(Bu^t)-Ser(Bu^t)-Asn-Ala-OBu^t was purified on Sephadex LH20 in dichloromethane/methanol (1:1). The tert-butyl groups were removed in trifluoroacetic acid within 30 min. to yield the compound Pam-Ser-Ser-Asn-Ala (Rf = 0.3, silica gel plates, chloroform/methanol/water (65:25:4)). Tripalmitoyl-pentapeptide and the free tetrapeptide Ser-Ser-Asn-Ala were prepared as described (6). Murein-free lipoprotein (FLP) was prepared from E. coli 0111K58 according to Inouye et. al. (11). Before addition to the cultures, the synthetic lipid mitogens as well as lipoprotein were suspended in 20 mM Hepes-buffered Eagles Minimal Essential Medium containing 3.3% heat inactivated fetal calf serum (Seromed, München, Germany) with the aid of sonication (6x10 sec, 100 W, Braun Labsonic 1510 Sonifier, Braun, Melsungen). Concanavalin A was purchased from Pharmacia (Uppsala, Sweden). Lipopolysaccharide (LPS) from E. coli B/r was a gift from W. Scheuer (Tübingen).

Lymphocyte cultivation. Spleen cells from inbred mice (female, 8-12 weeks of age) were used. C3H/HeJ mice, which are LPS non-responders (12), were from the Jackson Laboratories, Bar Harbor, Maine, USA, and athymic C3H/Tif/Bom/nunu mice were from Bomholtgard, Ry, Denmark. Balb/c mice were from Ivanovas, Kissleg, Germany. Mice were sacrificed by cervical dislocation. The spleens were excised, dissected free of adherent tissue and macerated gently in a loose-fitting glass tissue grinder with 20 mM Hepes-buffered Eagles Minimal Essential Medium (Flow Laboratories, Meckenheim/Bonn, Germany). Tissue remnants were removed by filtration through cotton wool. After washing, the cells were suspended in either 25 mM Hepes-buffered RPMI 1640 medium (Gibco, Glasgow, Scotland) for subsequent use in radiolabel incorporation studies, or in 20 mM Hepes-buffered Eagles Minimal Essential Medium supplemented with 0.45% glucose for the hemolytic plaque assays. The BCL1 lymphoid B-cell line (obtained from Dr. W. O. Weigle, Scripps Clinic and Research Foundation, La Jolla, Ca., USA) was maintained in RPMI 1640 medium containing fresh glutamine (2mM), penicillin (100 U/ml), streptomycin (100 µg/m), and 2-mercaptoethanol (5×10^{-5} M) (culture medium), further supplemented with 1% non essential amino acids (Gibco) and 15% heat inactivated fetal calf serum.

Lymphocyte stimulation. Radiolabel incorporation experiments were performed in flat bottom Falcon 3040 microtiter plates (Falcon Plastics, Oxnard , Ca, USA) in culture medium supplemented with 3.3% heat inactivated fetal calf serum. Thymidine incorporation into DNA was determined by labelling splenocyte cultures (3.3×10^6 cells/ml) 24 h before harvesting with 0.5 µCi of ^{3}H-thymidine (Amersham) of specific activity 5 Ci/mMol (185 GBq/mMol). In the ^{3}H-uridine incorporation experiments, cells were cultured at a density of 10^7/ml, and pulsed for 24 h before harvesting by the addition to each well of 0.5 µCi ^{3}H-uridine (Amersham) of the specific activity 5 Ci/mMol (185 GBq/mMol). Hemolytic plaque assays were performed in Falcon 2045 culture tubes and, in alteration of the published procedure (6), at a cell density of 5×10^6/ ml in culture medium supplemented with 10 % heat inactivated fetal calf serum.

RESULTS AND DISCUSSION

The novel synthetic lipopeptide segment N-palmitoyl-(L)-seryl-seryl-asparaginyl-alanine (Palmitoyl-tetrapeptide) has the same amino acid sequence as the

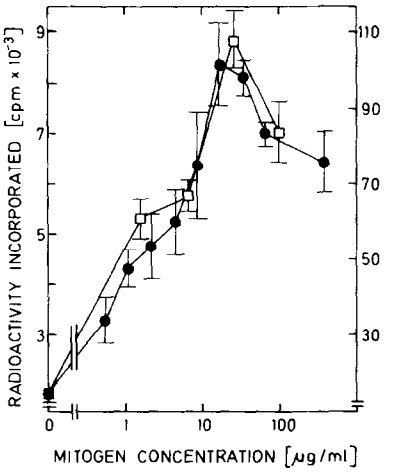

Fig.1. Dose response curves in splenocyte cultures of C3H/HeJ mice for ^{3}H-thymidine incorporation (circles) and for the induction of plaque forming cells (squares). Duration of cultivation 72 h. Shaded square: control cultures without the addition of mitogen. Means of triplicate experiments +/- standard deviations.

N-terminal lipopeptide fragment of the bacterial mitogen lipoprotein (13). The product is, however, lacking the three fatty acids containing S-glyceryl-L-cysteine region of the native lipopeptide, which was replaced by palmitic acid as a lipophilic constituent. The induction of DNA synthesis by this amphiphilic Palmitoyl-tetrapeptide was followed in C3H/HeJ mouse splenocytes (Fig. 1, closed circles); after 72 h of cultivation the compound increased ^{3}H-thymidine incorporation starting at concentrations below 1 µg/ml, and the dose optimal for stimulation amounted to 20-30 µg/ml. The ability of the Palmitoyl-tetrapeptide to polyclonally stimulate B-lymphocytes into immunoglobulin secretion was assessed using a hemolytic plaque assay. Our results (Fig. 1, squares) demonstrate that the number of plaque forming cells against densely trinitrophenylated sheep red blood cells increased markedly after stimulation of mouse spleen cells with the synthetic mitogen. The dose response plot exhibited a similar pattern as shown above for the thymidine incorporation experiment. A comparable stimulation of DNA synthesis and immunoglobulin secretion has previously been shown for lipoprotein- and LPS-stimulated mouse splenocyte cultures and for cultures activated with synthetic Tripalmitoyl-pentapeptide (8-10); palmitic acid had no stimulatory effect (9).

From the experiments presented in Fig. 1 it is obvious that Palmitoyl-tetra-

peptide (closed circles) constitutes a novel lymphocyte mitogen. Since the C3H/HeJ mouse strain used in the experiments is genetically non-responsive to endotoxin (12), any contaminating LPS is not responsible for the effects. The stimulating action of Palmitoyl-tetrapeptide is, however, less pronounced than the effect of native lipoprotein (2,3) or of the synthetic Tripalmitoyl-pentapeptide (8-10), which both carry membrane anchoring structures containing three fatty acids. Thus, while the amphiphilic properties due to the presence of one fatty acid seem to be sufficient for lymphocyte activation, the increased hydrophobicity due to three fatty acid residues is likely to favour mitogen - cell interactions and to further increase the stimulatory effect. We obtained similar results with lipoproteins carrying 1 as well as 3 fatty acids, which were isolated from bacterial mutants (14).

The results of the hemolytic plaque assay (Fig. 1, squares) further demonstrate that Palmitoyl-tetrapeptide acts as a polyclonal activator for lymphocytes of the B-cell lineage. These data are confirmed by stimulation experiments using athymic C3H/Tif/ Bom/nunu mouse spleen cells, which are lacking mature T-lymphocytes (Fig.2). In this mouse strain Palmitoyl-tetrapeptide (closed circles) activated the incorporation of thymidine into DNA comparable to C3H/HeJ mice. As expected, the T-cell mitogen Concanavalin A

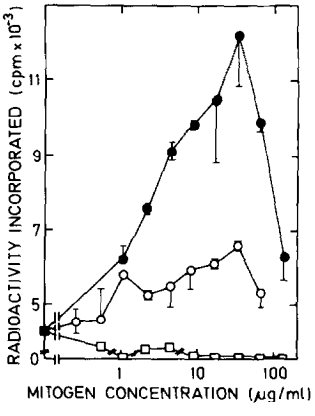

Fig. 2. Dose response curves for the incorporation of ^{3}H-thymidine into DNA in athymic C3H/Tif/Bom/nunu mouse splenocytes cultivated for 48 h in the presence of Pam-Ser-Ser-Asn-Ala (closed circles), of the tetrapeptide Ser-Ser-Asn-Ala (open circles), and of Concanavalin A (open squares). Shaded square: control cultures without the addition of mitogen. Means of triplicate experiments +/- standard deviations.

Table 1. Stimulation of ^{3}H-uridine incorporation into RNA in splenocytes of Balb/c mice by Palmitoyl-tetrapeptide, Tripalmitoyl-pentapeptide and Concanavalin A. Cpm per 1.3 X 10^{6} cultured cells incubated for 48 hours in the presence of 3.3 % fetal calf serum.

Mitogen concentration (µg/ml)	Radioactivity incorporated (cpm)		
	Palmitoyl-tetrapeptide	Tripalmitoyl-pentapeptide	Concanavalin A
137	3542 +/- 193	6718 +/- 341	478 +/- 68
34	5395 +/- 200	5790 +/- 297	2276 +/- 176
8.5	4403 +/- 703	6045 +/- 1326	12725 +/- 1478
2.1	3709 +/- 267	4583 +/- 757	16553 +/- 1643
0.55	n. d.	3421 +/- 633	11766 +/- 592
0	2419 +/- 716	2419 +/- 716	2419 +/- 716

n. d. = not determined.

(squares) was not active in this experiment. The tetrapeptide moiety Ser-Ser-Asn-Ala alone (open circles) was only marginally active emphasizing the importance of the anchoring hydrophobic constituent.

As a further parameter of cell activation, the incorporation of ^{3}H-uridine into RNA induced by Palmitoyl-tetrapeptide, was determined. The compound was able to markedly increase uridine incorporation at mitogen concentrations ranging from 2.1 to 137 µg/ml and thus exhibited an activity comparable to synthetic Tripalmitoyl-pentapeptide (Table 1). In addition, Palmitoyl-tetrapeptide was also able to stimulate the protein synthesis of the cells, as determined by ^{3}H-leucine incorporation studies (not shown). These data are also in agreement with the results published for native lipoprotein (3).

Summarizing the first part of our results, Palmitoyl-tetrapeptide with the molecular mass of 616 was able to polyclonally activate murine B-lymphocytes from both LPS-responder and nonresponder strains and had, in all aspects tested, a lymphocyte stimulatory activity comparable, but slightly inferior, to native lipoprotein (molecular mass 7200) (2,3) or LPS. There are several reports that lymphoid B-cell lines can also be activated by B-cell mitogens like LPS to increase DNA synthesis and IgM-secretion (comp. 15,16). Bacterial lipoprotein has been shown to activate the B cell tumor line BCL1 (17), and the synthetic lipoprotein analogue Tripalmitoyl-pentapeptide was active towards the *in vitro* and *in vivo* BCL1 cell lines (16). Here, we examined the

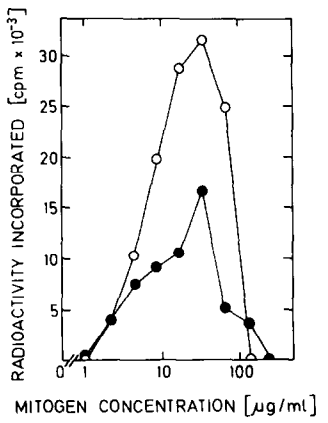

Fig. 3. Dose response plots for the incorporation of ^{3}H-thymidine into DNA in BCL1 cell cultures. Cells were cultured for 48 h at a density of 1.25 x 10^{5}/ml in the presence of Palmitoyl-tetrapeptide (closed circles) and Tripalmitoyl-pentapeptide (open circles). The control value of the cultures without mitogen (25,465 cpm) was subtracted from each value. Means of duplicate experiments +/- ranges.

ability of Pam-Ser-Ser-Asn-Ala to activate the *in vitro* BCL1 cell line, which exhibits the phenotype of immature B-lymphocytes (19,20). As shown in Fig. 3, Palmitoyl-tetrapeptide was able to markedly enhance the incorporation of ^{3}H-thymidine at mitogen concentrations above 2 µg/ml. Optimal stimulation was obtained around 30 µg/ml; mitogen doses above 100 µg/ml had only a marginal effect. This dose response pattern is very similar to that obtained for Tripalmitoyl-pentapeptide (open circles, comp. 18). The results indicate that the BCL1 line might serve as a model for the further investigation of lymphocyte activation by bacterial B-cell mitogens.

In this communication we could demonstrate that the highly pure, synthetically prepared amphiphilic compound Pam-Ser-Ser-Asn-Ala is a stimulatory agent towards primary and transformed mouse B-lymphocytes. The exact mechanism of the lymphocyte activation process is still unknown, but the first step seems to involve the interaction of the mitogens with the plasma membrane by insertion of their lipophilic constituents into lipid bilayer regions of the membrane and, possibly, by additional binding to surface receptors. For lipoprotein and its synthetic analogues we presented evidence that they are binding to histocompatibility-2 complex proteins of mouse leukocytes (21,22). We assume that the mitogenic Palmitoyl-tetrapeptide will be a valuable tool

for further investigations into the molecular mechanisms of lymphocyte activation.

ACKNOWLEDGMENT

We thank Prof. V. Braun for generous support. This investigation was in part funded by the Deutsche Forschungsgemeinschaft (SFB 76).

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