STUDIES ON THE MITOGENIC PRINCIPLE OF THE LIPOPROTEIN FROM THE OUTER MEMBRANE OF ESCHERICHIA COLI

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Summary - Lipoprotein,from the outer membrane of E. coli,and several derivatives were investigated for lymphocyte stimulating activity in different species. We could show that lipoprotein exhibits activity towards mouse and rat splenocytes and rabbit and bovine lymph node cells; human peripheral blood lymphocytes showed a weak but significant stimulation. Thymocytes of all species were also weakly activated. Altered molecular structures at the C-terminal end of lipoprotein had only small influence on activity, hydrolysis of N-terminal fatty acids abolished mitogenicity.

Lipoprotein from the outer membrane of Escherichia coli has been characterized by V. Braun and coworkers. The polypeptide chain composed of 57 amino acids contains at the N-terminal end 2 ester-linked and 1 amide-linked fatty acids bound to glyceryl-cysteine, and is covalently bound by the C-terminal lysine to the carboxyl group of a diaminopimelate residue of the murein sacculus. In aqueous solution lipoprotein occurs in a highly aggregated form probably due to its α-helical structure with hydrophobic amino acids clustered at one side of the helix, and to its lipid part (1). Melchers et al. found that lipoproteins stimulate murine B-lymphocytes to proliferation and to the development of IgM-secreting plasma cells (2). This study investigates further the stimulating activity of lipoprotein and of derivatives with differing C-terminal residues towards lymphocytes of 5 different species.

EXPERIMENTAL

Lymphocytes. Human lymphocytes were isolated from the blood of adult, normal donors by the Ficoll gradient technique according to Böyum (3). Bovine lymph nodes and thymi were obtained from

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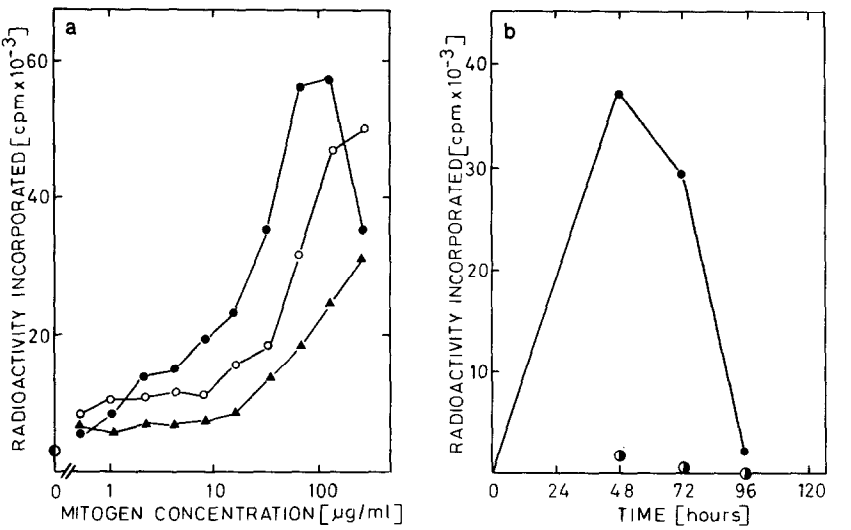
the local slaughter house. Rabbits (Russen, age 12-16 weeks) were purchased from Gassner, Sulzfeld; rats (LEW/Han [Lewis], age 6-10 weeks) from Zentralinstitut für Versuchstierzucht, Hannover, and mice (C57/Bl, age 6-10 weeks) from Gl. Bomholtgard, Denmark. Cell suspensions were prepared as described (4). Mitogens. Murein-lipoprotein was prepared as described by Braun et al. (5,6) from E. coli B/r. Cells were disrupted with glass beads and the cell envelope was solubilized in 4 % boiling sodium dodecyl sulfate. The murein-lipoprotein complex was spun down and washed. - Muropeptide-lipoprotein was prepared from murein lipoprotein by enzymatic digestion with lysozyme and subsequent purification on Sephadex G75 (Pharmacia) (7). Lipoprotein was prepared from murein-lipoprotein by enzymatic digestion with trypsin (20 min, 37°C), precipitated from a solution in 1 % sodium dodecyl sulfate by addition of an equal volume of acetone, and chromatographed on Sephadex G75. - Murein was prepared from murein-lipoprotein by pronase digestion and subsequent mild alkali hydrolysis (0.03 M NaOH, 90 % ethanol, 4 h, 37° C) to destroy residual lipopeptide(8). Alkali treatment of the lipoproteins was performed under the same conditions. The lipopolysaccharide content of the preparations was below 1 % as judged from their content of hydroxymyristic acid, which occurs in lipopolysaccharide but not in lipoprotein (8,9). Before addition to the cultures, the lipoproteins were suspended in RPMI-1640 medium by sonification in a Branson ultrasonic bath (30 min, 37° C). - Concanavalin A (Con A) was prepared from seeds according to Agrawal and Goldstein (10). Stimulation experiments. Cell cultures were performed in Falcon 3040 microtiter plates as described (4). Mitogenicity was measured by the uptake of ^{3}H-thymidine into DNA and by microscopic observation of blast formation (4). All experiments were done at least in duplicates.

RESULTS

As shown by Melchers et al. in a serum-free tube culture system (2), both lipoprotein and muropeptide-lipoprotein are mitogenic towards mouse spleen cells. In our serum-containing culture system, a similar stimulation was obtained. Additionally we could show that murein-lipoprotein is a potent mitogen. We found stimulating activity of all compounds starting at concentrations of 1-10 µg/ml and increasing with higher mitogen concentrations. While for lipoprotein the dose response optimum is located around 100 µg/ml, both muropeptide-lipoprotein and murein-lipoprotein exhibited maximal stimulating activity above 100 µg/ml (Fig. 1a). The kinetics of stimulation of DNA synthesis in mouse splenocytes are depicted in fig. 1b. Thymi-



Figs. 1-4. Thymidine uptake (cmp/7.5 x 10^5 cultured cells) in lymphocytes after stimulation with lipoprotein [●], muropeptide-lipoprotein [▲], and murein-lipoprotein [○]. [◐] = control. Fig. 1. Mouse spleen cells, (a) dose response plot, cells incubated for 60 h, and (b) kinetics of stimulation, mitogen concentration 65 ug/ml. Cultured performed in the presence of 6.6 % human pool serum.

Tab. 1. Effect of alkali treatment on the stimulation of thymidine uptake (cpm/7.5 x 10^5 cultured cells) by lipoproteins in C 57/Bl mouse spleen cells. Means of 2 determinations.

TREATMENT OF LYMPHOCYTES	NOT ALKALI-TREATED	ALKALI-TREATED	CONTROL WITHOUT MITOGEN
LIPOPROTEIN (65 µg/ml)	43 500	1 170	1 260
MUROPEPTIDE-LIPOPROTEIN (200 µg/ml	9 200	950	260
MUREIN-LIPOPROTEIN (200 µg/ml)	25 500	280	260

dine incorporation peaks between 48-72 hours after the addition of trypsin-lipoprotein. Free murein has no significant stimulating effect. After alkali treatment, the stimulating activity

Tab. 2. Stimulation of thymidine incorporation into DNA in splenocytes of Balb/c nu/nu mice by lipoproteins. Cpm per 6 x 10^5 cultured cells incubated in the presence of 6.6 % human pool serum for 61 hours. Means of 6 determinations.

MITOGEN CONC. [µg/ml]	LIPOPROTEIN	MUROPEPTIDE-LIPOPROTEIN	MUREIN-LIPOPROTEIN	CON A
0	1 300	1 300	1 300	1 300
1	3 700	1 800	2 050	1 350
10	6 450	3 850	3 400	1 550
100	15 400	6 150	8 950	900

Tab. 3. Thymidine uptake (cpm/7.5 x 10^5 cultured cells) in several stimulation experiments performed with trypsin-lipoprotein, muropeptide-lipoprotein, murein-lipoprotein (each 50 µg/ml), and Con A (4 µg/ml). - = not done. * = mitogen concentration 10 µg/ml. For specific culture conditions compare legend to fig. 1-4. Human lymphocytes were cultured in the presence of 15 % human pool serum for 72 hours. Means of 4 determinations.

SPECIES	MOUSE	RAT	RABBIT	CALF	MAN	MAN
SOURCE OF LYMPHOCYTES	THYMUS	THYMUS	THYMUS	THYMUS	THYMUS	PERIPHERAL BLOOD
LIPOPROTEIN	890	1 120	4 840*	4 950	645	510*
MUROPEPTIDE-LIPOPROTEIN	270	-	5 180*	-	690	-
MUREIN-LIPOPROTEIN	380	865	-	-	510	625*
CON A	13 600	-	52 500	21 200	3 200	20 200
CONTROL	130	390	1 800	2 600	435	275

of the compounds is abolished (Tab. 1). In order to determine if all our preparations were mitogenic towards B-lymphocytes, spleen cells of Balb/c nu/nu mice were treated with the compounds. As seen from table 2 a significant stimulation was achieved. Mouse thymus cells showed a weak stimulation by the different lipoproteins, compared to the strong stimulatory effect

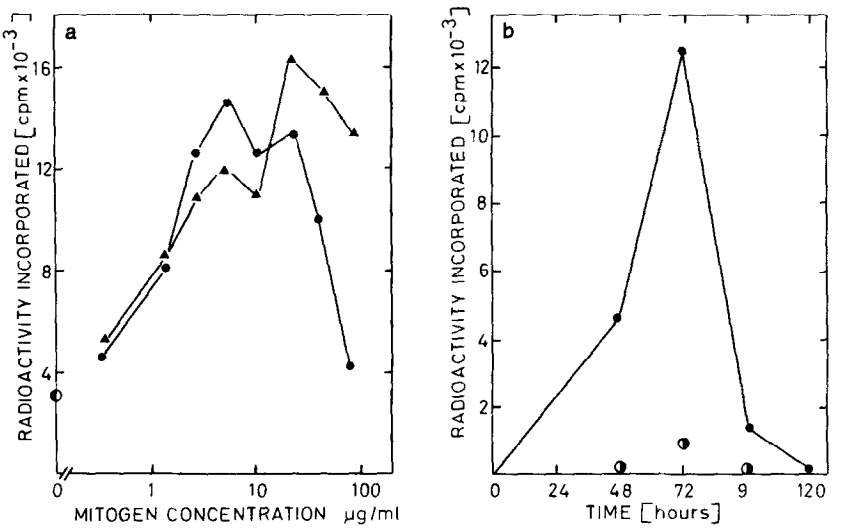


Fig. 2. Rabbit mesenterial lymph node cells, (a) dose response plot, cells incubated for 60 h, and (b) kinetics of stimulation, mitogen concentration 5 ug/ml; 10 % fetal calf serum.

of Con A (Tab. 3). In these and the following experiments, thymidine incorporation was accompanied by blast formation. In some cases, thymidine incorporation into the nucleus of stimulated blast cells was made visible by radioautography (data not shown).

The mitogenicity of both lipoprotein and muropeptide-lipoprotein towards rabbit mesenterial lymph node lymphocytes is shown in fig. 2. The mitogens exhibited dose optima around 5 µg/ml and between 20 and 50 µg/ml. Murein-lipoprotein exhibited a comparable stimulating activity in doses around 100 µg/ml (data not shown). The kinetics show maximal thymidine incorporation 72 hours after the addition of lipoprotein (Figure 2b). Rabbit thymocytes were also activated by lipoproteins, although a far lower degree of stimulation than by Con A was obtained (Tab. 3). - The dose response curves for both lipoprotein and murein-lipoprotein in rat and bovine lymphocytes

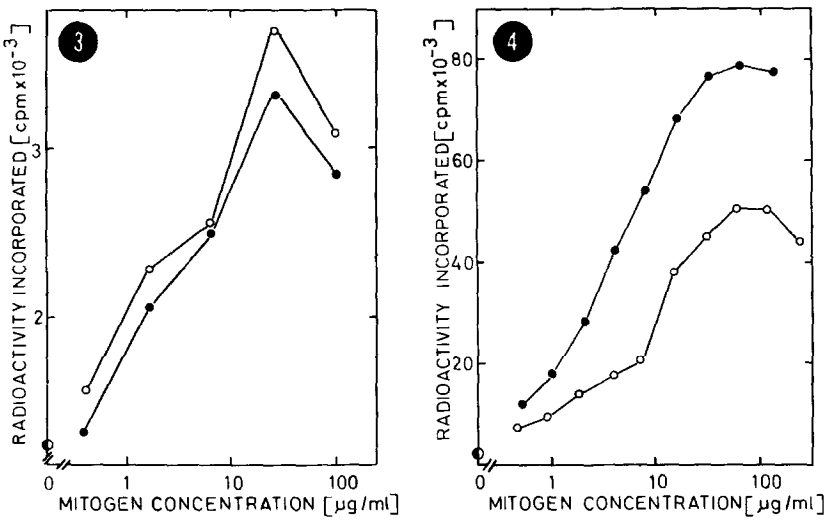


Fig. 3. Rat splenocytes, dose response plot, cells incubated for 54 h; 10 % isologous serum.

Fig. 4. Bovine lymph node lymphocytes, incubation time 72 h; 15 % fetal calf serum.

are depicted in figs. 3 and 4; muropeptide-lipoprotein exhibited a comparable activity (data not shown). Compared to the effect of Con A, lipoproteins stimulated rat thymocytes only weakly (Tab. 3). - Human thymocytes and human peripheral blood lymphocytes showed a weak but significant stimulation by the mitogens (Tab. 3).

DISCUSSION

This study shows that 3 lipoprotein preparations differing only in the content of C-terminal muropeptide and murein components are mitogenic towards mouse, rat, rabbit, and bovine lymphocytes. Additionally, a weak but significant stimulation of human peripheral blood lymphocytes could be obtained; human lymphocytes of other origin still have to be tested. A similar species specific stimulation is also found for lipopolysaccharide, which is a potent mitogen in rodents (11) but is almost

totally inactive towards human peripheral blood lymphocytes (12).

The mitogenicity of both lipoprotein and muropeptide-lipoprotein towards mouse B-lymphocytes has been reported (2). Additionally, we could show that murein-lipoprotein is a potent mitogen towards C57/Bl mouse splenocytes; the mitogenicity towards B-lymphocytes was shown in Balb/c nu/nu mice. Towards C3H/HeJ mice, a non-responder strain towards lipopolysaccharide (13), all 3 preparations were also active (W. Bessler, unpublished).

Lipoproteins exhibited weak stimulatory activity towards the thymocytes of all species tested. Thus, they seem to be acting as both B and T-lymphocyte mitogens; the effect on T-cells, however, is weak compared to the strong thymocyte stimulating activity of Con A. Because thymocytes contain only minor amounts of mature T-lymphocytes further experiments will be performed on enriched T-cell preparations.

Our investigations could help to elucidate the mitogenic principle of the lipoprotein molecule: as seen from the figures and from tables 2 and 3, alterations at the C-terminal region of lipoprotein alter only slightly its mitogenicity. Thus, the presence of muropeptide components, or of the whole murein sacculus, does not adversely affect the stimulating activity of the active lipoprotein. However, alkali-treatment of lipoproteins, which brings about the almost quantitative removal of 2 of the 3 fatty acids at the N-terminal lipopeptide end (8), abolishes the stimulatory activity, as tested in the mouse system (Tab. 1). Thus, the mitogenic principle of lipoprotein seems to reside in the N-terminal region of the molecule. This view is supported by our recent findings that N-terminal lipopeptide fragments

obtained by pronase digestion still exhibit stimulating activity (14). It is interesting to compare our results to those of Andersson et al. (10) obtained with lipopolysaccharide: they found a decrease in B-lymphocyte mitogenic activity when they removed the ester-linked fatty acids from the lipid A-component of lipopolysaccharide.

When carrying out alkali-treatment we additionally obtain a disaggregation of the mitogens that originally formed large aggregates in the aqueous solution (14). The role of this aggregate formation for mitogenicity is being investigated in our laboratory at the moment.

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