INFECTION AND IMMUNITY, June 1980, p. 818-823
0019-9567/80/06-0818/06$02.00/0

Vol. 28, No. 3

Mitogenicity of a Lipid-Deficient Lipoprotein from a Mutant *Escherichia coli* Strain

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From the mutant bacterial strain *Escherichia coli* JE5511 *lpp lpm*, muropeptide-containing and muropeptide-free lipoproteins were prepared. By gas chromatography and by infrared spectroscopy we showed that the products were deficient in the two ester-bound N-terminal fatty acids, but still carried the amide-linked fatty acid. Mutant lipoproteins were tested for mitogenicity in lipopolysaccharide nonresponder C3H/HeJ mice by incorporation of [^{3}H]thymidine and [^{3}H]uridine and by hemolytic plaque assays for immunoglobulin-secreting plasma cells. Our results showed that the mutant lipoproteins still exhibited marked mitogenicity toward mouse B-lymphocytes, although the activity of the products was reduced in comparison to the wild-type lipoprotein. Thus, the presence of one fatty acid in the N-terminal part of lipoprotein is sufficient to bring about mitogenicity.

The lipoprotein from the outer membrane of *Escherichia coli* is a mitogen toward mouse, rat, rabbit, and calf lymphocytes (2, 4, 12). It was characterized and sequenced by V. Braun and co-workers. It is a major protein of the bacterial cell wall; its polypeptide chain is composed of 58 amino acids and contains, at the N-terminal end, two ester-linked and one amide-linked fatty acids bound to glycerylcysteine. The protein is convalently 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 both to its α-helical structure with hydrophobic amino acids clustered at one side of the helix, and to its lipid part (6). The conformation of lipoprotein is very stable. Denaturation of the lipoprotein by heat, 8 M urea, or sodium dodecyl sulfate is fully reversible in the pH range from 5 to 12 (7).

By pronase treatment of lipoprotein, it was possible to prepare lipopeptide fragments containing two to five amino acids bound to diacylglyceryl-*N*-acyl-cysteinthio-ether (10). These products still showed polyclonal B-lymphocyte activation comparable to the native molecule. Thus, the N-terminal lipopeptide of lipoprotein containing three fatty acids is responsible for its mitogenicity (5). Recently, an *E. coli* mutant strain was described, possessing a mutant lipoprotein lacking the two ester-bound fatty acids (14, 16). It was then possible to test if the presence of the residual amide-linked fatty acid, bound to the lipoprotein polypeptide carrier, would be sufficient to induce mitogenesis. This paper describes the lymphocyte-activating properties of *E. coli* JE5511 *lpp lpm* mutant lipoprotein in several inbred mouse strains.

MATERIALS AND METHODS

Lymphocytes. Mice (C3H/HeJ) were purchased from Jackson Laboratories, Bar Harbor, Maine, and C57BL/6J/Bom nu/nu mice were purchased from Bomhaltgard, Ry, Denmark. The animals were between 8 and 12 weeks of age. They were sacrified by cervical dislocation, and thymus or spleen were removed immediately after killing. The organs were placed in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered Eagle minimal essential medium (Flow Laboratories, Rockville, Md.), dissected free of adherent tissue, cut into small pieces, and macerated gently in a loose-fitting glass tissue grinder. Tissue remnants were removed by filtering cells through a small column of nylon fibers (Leukopak; Fenwal Laboratories, Morton Grove, Ill.). After the cells were washed twice, they were suspended in 25 mM HEPES-buffered RPMI 1640 medium (GIBCO Diagnostics, Madison, Wis.).

Reagents. Lipoprotein was prepared from *E. coli* wild-type strains B/r 0111K58 and JE5549 (*lpp lpm*$^+$, F'*lpp*$^+$) both carrying the complete, three fatty acids-containing lipoprotein, and from the *lpm* mutant *E. coli* JE5511 (HfrC *man lpp lpm pps*) provided to us by Y. Hirota. In short, cells were disrupted with glass beads and the cell envelope was separated by differential centrifugation and solubilized in 4% boiling sodium dodecyl sulfate. The murein-lipoprotein complex was spun down and muropeptide-containing lipoprotein or muropeptide-free lipoprotein were isolated from murein-lipoprotein by enzymatic digestion (7). Additionally, the free form of lipoprotein (free lipoprotein) was obtained from the supernatant by the method of Inouye et al. (11). Purified lipoprotein preparations were suspended by sonication in distilled water and lyophilized. Before addition to cell cultures,

lipoprotein was suspended in RPMI 1640 medium by short sonication (1 min, 100 W, BRAUN Labsonic 1510 Sonifier, BRAUN, Melsungen, Germany). Lipopolysaccharide (LPS) was a gift of C. Galanos (Freiburg). Concanavalin A was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

Gas chromatography. The determination of the fatty acid composition was done by gas chromatography as described (14). The fatty acid composition of the murein-lipoprotein complex and the free lipoprotein from wild-type and mutant strains was determined on 1- to 3-mg lipoprotein samples. Pentadecanoic acid methyl ester was added as internal standard. Samples were hydrolyzed under nitrogen for 18 h at 105°C in double-distilled 5.7 N HCl. The hydrolysates were extracted with *n*-hexan, the *n*-hexan phase was evaporated, and the remainder was converted to the fatty acid methyl esters by treatment with diazomethane in ether. The concentrations of the fatty acid methyl esters were determined by gas chromatography (Varian Aerograph 1400 with a 180-cm-long, 2-mm-diameter column filled with OV 1 on Gas-Chrom Q, 100 to 120 mesh, Serva Heidelberg, 170°C). The exact lipoprotein concentration was determined by amino acid analysis of the extracted hydrolysate.

[^{3}H]thymidine incorporation. Experiments were carried out in Falcon 3040 microtiter plates (Falcon Plastics, Oxnard, Calif.). Unless otherwise indicated, lymphocyte cultures (cell density, 3.3×10^6/ml in 180-μl portions per well) were performed in RPMI 1640 medium supplemented with 3.3% inactivated human AB serum (GIBCO), 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 (Amersham-Buchler, Braunschweig, Germany) of specific activity 40 mCi/mmol. The cultures were harvested with a Mash II harvester (Microbiological Associates, Bethesda, Md.), collected on glass-fiber filters (Dynatech, Nürtingen, Germany), and measured by liquid scintillation. Assays were done at least in duplicate. During the experiments, cell viability was determined by dye exclusion with trypan blue (0.2%).

[^{3}H]uridine incorporation. Cultures were performed in microtiter plates as described above. An amount of 10^7 cells per ml was cultured in 0.18 ml of HEPES-buffered Dulbecco minimal essential medium (GIBCO) supplemented with 2.5 mg of defatted albumin per ml as described (5). The cells were pulsed for 4 h before harvesting by the addition to each well of 0.2 μCi of [^{3}H]uridine (Amersham-Buchler) of the specific activity 40 mCi/mmol and harvested as described for [^{3}H]thymidine incorporation.

Hemolytic plaque assay. Mouse spleen cells were tested for the development of immunoglobulin-secreting plasma cells by a modifed hemolytic plaque assay, using protein A-coated erythrocytes as described by Gronowicz et al. (9). Lymphocytes were cultured at a cell density of 3×10^6 cells per ml in 0.55-ml amounts in Falcon 2054 culture tubes with different doses of mitogens. After 4 days, 100 μl of the lymphocyte suspensions were appropriately diluted in phosphate-buffered saline, and 20 μl of guniea pig complement (Serva) and 20 μl of a 30% suspension of staphylococcal protein A-coupled sheep erythrocytes (9) were added quickly to 300 μl of 0.5% agarose in phosphate-buffered saline at 45°C. The suspensions were mixed and plated on microscopic slides. The slides were kept in a humid atmosphere for 5 h at 37°C; plaques were observed by microscopy.

RESULTS

In Table 1, the fatty acid compositions of the free and of the murein-bound forms of both wild-type and mutant lipoproteins used by us are listed, as determined by gas chromatography. The absolute amount of total fatty acids per lipoprotein molecule was 0.84 for the JE5511 murein-lipoprotein complex and 0.76 mol fatty acids per mol of JE5511 free lipoprotein in the mutant, compared with a value of 2.84 in the wild type. Whereas wild-type lipoprotein contains three fatty acid residues, the mutant lipoprotein apparently carries only one.

Figure 1 shows the dose-response plots of stimulation experiments performed with wild-type lipoproteins and mutant lipoproteins on C3H/HeJ LPS nonresponder (15) mouse spleen cells; we tested free lipoproteins (a) as well as muropeptides-containing lipoproteins (b). LPS from *Salmonella abortus equi* was tested as a control. For all lipoprotein compounds, we found thymidine incorporation into deoxyribonucleic acid starting at mitogen concentrations of about 1 μg/ml; the optimal dose for stimulation amounted to around 100 μg/ml for both wild-type and mutant lipoproteins. Wild-type lipoproteins, however, exhibited a more pronounced mitogenic activity than mutant lipoproteins. For both wild-type and mutant lipoprotein, incorporation appeared to peak 48 to 72 h after the addition of the mitogens; similar results were obtained in spleen cells of C57BL/10 and DBA mice, which are LPS responders (data not shown).

To determine if the mutant lipoproteins are also B-lymphocyte mitogens, spleen cells of congenitally athymic mice were treated with the compounds. As seen from Table 2, mutant lipoproteins were potent mitogens for these cells. Mutant lipoproteins were also able to stimulate B-lymphocytes to the development of immunoglobulin-secreting plasma cells. Table 3 shows the results of a single-cell plaque assay performed with the compounds. Either free mutant lipoprotein or muropeptide-containing mutant lipoprotein activated C3H/HeJ B-lymphocytes in a less pronounced manner than the wild-type lipoproteins (Table 3). In contrast, in thymocytes of C3H/HeJ mice, only a marginal increase in thymidine incorporation could be found for the mutant lipoproteins (Table 4), whereas concanavalin A, which was used as a control, ex-

TABLE 1. *Moles of fatty acids per mole of lipoprotein*[a]

Lipoprotein	Mol of fatty acid/mol of lipoprotein					
	$C_{14:0}$	$C_{16:0}$	C_{16} unsaturated	C_{17} cyclo	$C_{18:0}$	C_{18} unsaturated
Free lipoprotein						
JE5511	0.07	0.58	0.05	<0.01	0.02	0.04
JE5549	0.27	1.61	0.28	0.11	0.08	0.49
Murein-lipoprotein complex						
JE5511	0.12	0.53	0.06	< 0.01	0.03	0.10
JE5549	0.27	1.72	0.18	0.08	0.03	0.56

[a] Fatty acids were determined from hydrolysates of free lipoprotein and murein-lipoprotein complexes isolated from merodiploid wild-type stem JE5549 (*lpp lpm*$^+$, F'*lpp*$^+$) and mutant JE5511 (*lpp lpm*). The figures are the average of three determinations made with two independently isolated samples.

hibited marked stimulatory activity toward the cells at a concentration of 4.3 μg/ml.

Mutant lipoprotein was also able to induce the incorporation of [^{3}H]uridine into ribonucleic acid. Figure 2 shows the dose-response curves for mutant and for wild-type free lipoproteins. Comparable to the stimulation of deoxyribonucleic acid synthesis, optimal incorporation of uridine was observed at mitogen concentrations around 100 μg/ml in C3H/HeJ mouse splenocytes. Concanavalin A was found to activate the cells to a similar degree.

In a last set of experiments, we tested if the addition of serum was required for the mutant lipoproteins to act as mitogens. Table 5 presents the results of experiments performed with C3H/HeJ mouse splenocytes under serum-free conditions. As shown previously for wild-type lipoprotein (5), the presence of serum was not necessary for the activity of the mutant mitogens at cell densities ranging from 3.3×10^6 to 6.6×10^6 cells per ml. A concanavalin A control experiment was also performed; the mitogen exhibited a marked activity at a concentration of 2.1 μg/ml.

DISCUSSION

It is possible to remove two of the three fatty acids from lipoprotein and from lipopeptide fragments by mild alkali hydrolysis (5, 10). When we treated lipoprotein with this method, a water-soluble molecule was obtained, which had almost lost its mitogenic activity as measured by thymidine incorporation experiments (4). These results are in agreement with the findings of Melchers et al. (12), who showed a reduction of plaque-forming cell activity of lipoprotein after alkali treatment. It is, however, possible that alkali treatment, besides cleaving off the ester-bound fatty acid, causes additional changes of the lipoprotein molecule. A further degradation of the molecule might be possible. Thus, to elucidate the role of the lipoprotein fatty acids for mitogenic stimulation, we isolated a mutant lipoprotein from *E. coli* JE5511, which is lacking the two ester-bound fatty acids at the N-terminal part of the molecule (Table 1).

The mutant lipoprotein, which carried only one amide-bound fatty acid, was still mitogenically active toward mouse lymphocytes. The stimulatory effect of mutant lipoprotein on B-lymphocytes was assured by testing the compounds on congenitally athymice mice (Table 2) and could furthermore be confirmed by a hemolytic plaque assay (Table 3). The effects of the products towards thymocytes are listed in Table 4. Even at very high protein concentrations, no effect could be found. Since thymocytes contain only minor amounts of mature T-lymphocytes, further experiments will be performed with enriched T-cell preparations. Mutant lipoprotein acts as a B-lymphocyte mitogen equally well toward LPS-responsive mouse strains (C57BL/10 stimulation index, 4.7 and DBA stimulation index, 14.8 [determined at a mitogen concentration of 68 μg/ml]) as it acts toward the LPS nonresponder strain C3H/HeJ, thus showing a similar mode of action as wild-type lipoprotein. To activate lymphocytes, the presence of serum is not required (Table 5). Similar results were shown for wild-type lipoprotein (5) and for the B-lymphocyte mitogen protein I from *E. coli* (3). Our data indicate that the presence of one fatty acid on the lipoprotein molecule is sufficient to induce mitogenicity. Mutant lipoprotein constitutes a mitogen with almost the same properties as wild-type lipoprotein.

The exact mechanism of lymphocyte activation by B-lymphocyte mitogens is still unknown. The first step involves the binding to the plasma membrane of their target cells. For LPS and lipoprotein, binding is probably mediated by the lipid parts of the molecules, which are essential for activation (1, 5). Evidence has been presented that LPS interacts with a specific receptor structure on the B-lymphocyte membrane (8). For lipoprotein and lipopeptides, lympho-

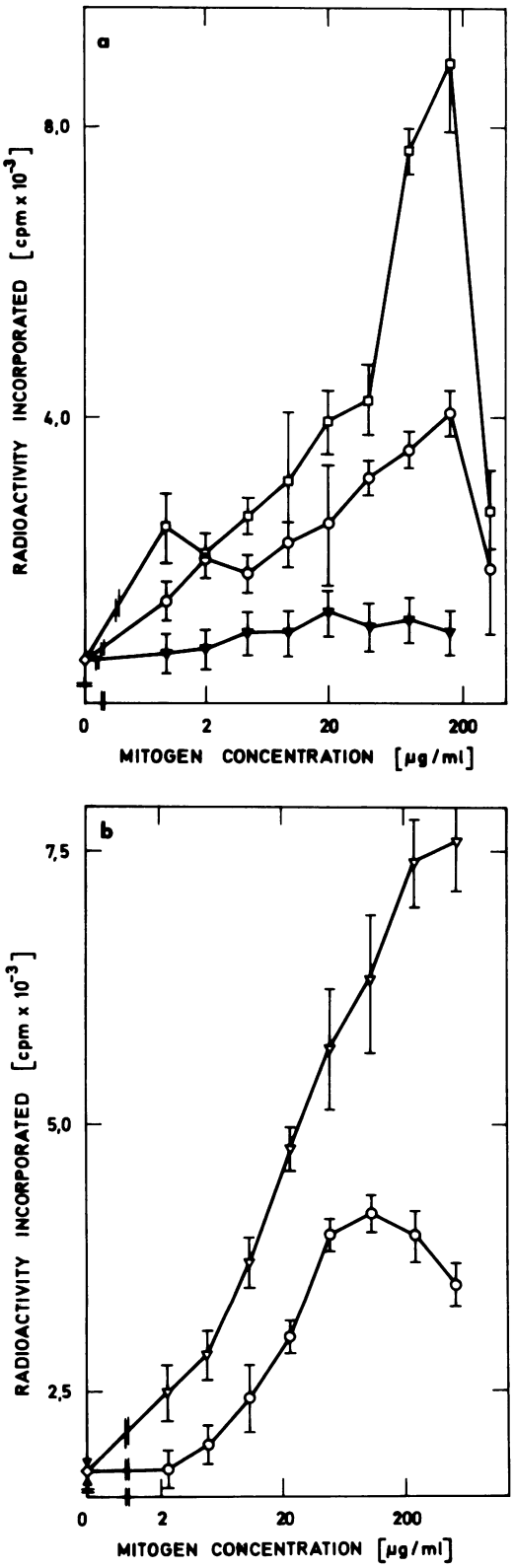

TABLE 2. *Stimulation of [³H]thymidine incorporation in splenocytes of C57BL/6 J/Bom nu/nu mice by mutant muropeptide lipoprotein and mutant free lipoprotein isolated from E. coli JE5511*[a]

Mitogen concn (µg/ml)	cpm per 6 × 10⁵ cultured cells of		
	Muropeptide lipoprotein JE5511	Free lipoprotein JE5511	Free lipoprotein wild-type (B/r)[a]
273	3,260 ± 50	1,835 ± 80	3,770 ± 100
137	2,095 ± 90	2,460 ± 100	3,705 ± 65
68	1,290 ± 125	2,310 ± 100	3,000 ± 80
34	780 ± 35	1,840 ± 70	2,290 ± 15
17	—	—	1,830 ± 200
0	360 ± 60	360 ± 60	360 ± 60

[a] Control, stimulation by free lipoprotein isolated from the wild-type strain *E. coli* B/r. Cells were incubated for 48 h in the presence of 3.3% human AB serum. —, Not done.

TABLE 3. *Stimulation of B-cells of C3H/HeJ mice to the development of plaque-forming cells by muropeptide lipoprotein (isolated from E. coli wild-type strain B/r), free lipoprotein (isolated from E. coli wild-type strain 0111K58), and by muropeptide lipoprotein and free lipoprotein isolated from the lpm mutant E. coli JE5511*

Concn (µg/ml) of mitogen[a]	No. of plaque-forming cells[b]			
	Muropeptide lipoprotein (B/r)	Free lipoprotein (011K58)	Muropeptide lipoprotein (JE5511)	Free lipoprotein (JE5511)
180	89 ± 4	63 ± 6	30 ± 2	40 ± 2
90	48 ± 3	42 ± 2	35 ± 1	62 ± 3
45	20 ± 3	19 ± 4	16 ± 2	43 ± 2
0	10 ± 3	10 ± 3	10 ± 3	10 ± 3

[a] An amount of 4 × 10⁶ cells per ml were cultured for 96 h in the presence of the mitogens and then tested in a single-cell plaque assay.

[b] Plaque-forming cells per 3 × 10⁴ recovered spleen cells tested for viability by the trypan blue dye exclusion test.

cyte receptor structures, which probably interact with the lipid part of lipoproteins, are currently characterized in our laboratory (A. Frey and W. G. Bessler, manuscript in preparation). The mitogenicity of the mutant lipoproteins carrying one fatty acid can probably be explained by their tendency to form aggregates in aqueous solutions, due mainly to their α-helical

FIG. 1. *Dose-response curves for [³H]thymidine incorporation. (a) Wild-type free lipoprotein from E. coli 0111K58 (□), mutant-free lipoprotein from E. coli JE5511 (○). Control, LPS from S. abortus equi (▼). (b) Muropeptide lipoprotein isolated from E. coli wild-type strain B/r (▽), mutant muropeptide lipoprotein isolated from E. coli JE5511 (○). Cell cultures were performed for 72 h with 3.3% inactivated human AB serum.*

structure with hydrophobic amino acids clustered at one side of the helix, and to the residual one fatty acid. The residual amide-linked fatty acids of the aggregates seems to be sufficient to bring about cell binding and activation.

TABLE 4. *Stimulation of [^{3}H]thymidine in corporation into deoxyribonucleic acid in thymocytes of C3H/HeJ mice by mutant muropeptide lipoprotein and mutant free lipoprotein isolated from E. coli JE5511*[a]

Mitogen concn (μg/ml)	cpm per 1.33 × 10^6 cultured cells for mitogen:		
	Muropeptide lipoprotein JE5511	Free lipoprotein JE5511	Concanavalin A
273	267 ± 19	268 ± 25	—
68	243 ± 19	269 ± 23	—
17	264 ± 20	266 ± 31	—
4.3	241 ± 22	264 ± 41	12,300 ± 860
0	260 ± 30	260 ± 30	260 ± 60

[a] Control, stimulation by concanavalin A. Cultured cells were incubated for 48 h in the presence of 3.3% human AB serum. —, Not done.

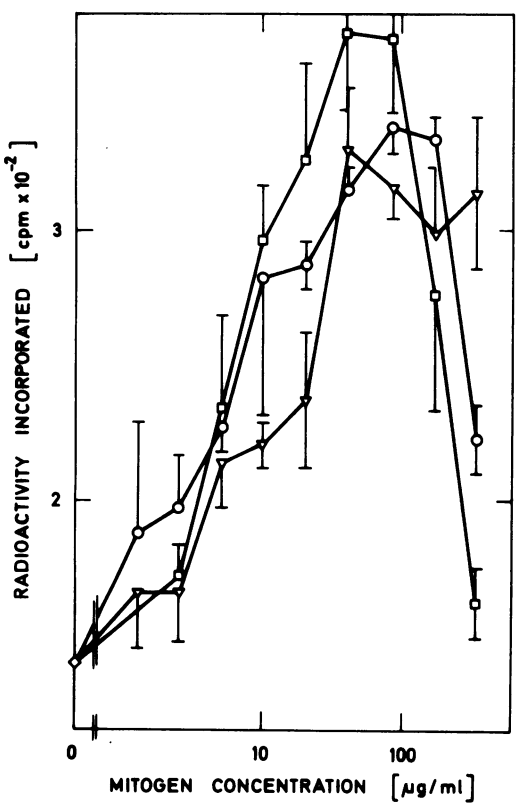

FIG. 2. *Dose-response curves for [^{3}H]uridine (cpm/1.8 × 10^6 cultured cells) uptake in C3H/HeJ spleen cells. Cultures were performed for 24 h in medium supplemented with 2.5 mg of defatted albumin per ml with mutant free lipoprotein from E. coli JE5511* (▽), *wild-type free lipoprotein isolated from E. coli 0111K58* (○), *and concanavalin A* (□).

TABLE 5. *Stimulation of [^{3}H]thymidine incorporation in C3H/HeJ mouse splenocytes*[a]

Mitogen concn (μg/ml)	cpm per indicated no. of cells (density in ml^{-1}) of mitogen:		
	Muropeptide lipoprotein JE5511 (6.6 × 10^6)	Muropeptide lipoprotein JE5511 (3.3 × 10^6)	Concanavalin A (3.3 × 10^6)
273	1,729 ± 301	1,490 ± 97	—
34	1,510 ± 155	1,093 ± 101	—
2.1	853 ± 67	676 ± 56	3,889 ± 547
0	415 ± 60	217 ± 79	217 ± 79

[a] Cultures were performed without addition of serum for 72 h. For serum-containing cultures, compare Fig. 1. —, Not done.

A similar B-lymphocyte mitogenicity is known for other compounds carrying only one fatty acid. When we treated lipopeptide with alkali, a significant mitogenic activity remained in the lipopeptides carrying one fatty acid (5). Mitogenic activity could also be shown for several lipids synthesized by us consisting of one amino acid and one fatty acid (J. Cybulla et al., Biochem. Biophys. Res. Commun., in press). This is in agreement with the findings of Rosenstreich et al., who attributed B-lymphocyte activity to a small glycolipid, *N*-acetyl-galactosamine, substituted with palmitic acid (13). Thus, several systems are known where highly aggregated structures carrying one fatty acid per monomer are sufficient to induce polyclonal activation of lymphocytes.

The events after the binding of lipoprotein to the target cells are still unclear. One mode of action for B-cell mitogens might be to bind to and to intercalate within the lipid region of the target cell membrane, thus initiating membrane perturbations and a repositioning of membrane components. It is noteworthy that wild-type and mutant lipoproteins seem to exhibit a similar physicochemical property in the membrane. Determination of molecular conformation by circular dichroism and infrared spectroscopy in sodium dodecyl sulfate solutions showed the same results for both proteins. The α-helical content below the critical micellar concentration was 75%; content above this concentration was 54% (14). As lipoprotein is a membrane protein and detergents in micellar solutions reflect the surrounding membrane more truly than any aqueous buffer system (17), the same behavior of mutant and wild-type lipoproteins in sodium dodecyl sulfate solution should most probably reflect their identical conformation in the membrane. After the binding to and intercalation within the membrane of the B-lymphocyte, li-

poprotein-induced membrane changes could lead to the generation of cell activating signals (5).

ACKNOWLEDGMENTS

We thank V. Braun for helpful discussions and support. The excellent technical assistance of Brunhilde Ottenbreit is gratefully acknowledged.

This investigation was supported by the Deutsche Forschungsgemeinschaft (SFB 76).

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