ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1986, p. 475–480
0066-4804/86/090475-06$02.00/0

Vol. 30, No. 3

Influence of β-Lactam Antibiotics and Ciprofloxacin on Composition and Immunogenicity of *Escherichia coli* Outer Membrane

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Received 21 February 1986/Accepted 12 June 1986

The effects of subinhibitory concentrations of different β-lactam antibiotics and one quinolone on the sedimentation of outer membranes (OMs) of *Escherichia coli* and on the qualitative properties and immunogenicity of OM components were studied. Membranes were prepared by osmotic lysis of plasmolyzed bacteria. OM and cytoplasmic membrane vesicles were separated by sucrose density ultracentrifugation. Two peaks of OM vesicles with different buoyant densities could be isolated; the quantitative contribution of these to the total OM varied, depending upon the growth phase. In early log phase, the OM consisted mainly of lighter material; in late log and stationary phases, the OM consisted mainly of heavier material. Moxalactam, imipenem, and ciprofloxacin inhibited the formation of heavier material in all growth phases. The immunogenicity of OM vesicles was tested in mice by the hemolytic plaque test. The lighter OM material was markedly less immunogenic than the heavier OM material. The vesicles from antibiotic-treated bacteria and those from early-log-phase cells were less immunogenic than vesicles from untreated late-log-phase and stationary-phase bacteria. These changes were found for the immune response against lipopolysaccharides, as well as against OM proteins. Thus, the immunogenicity of OM components seems to be dependent upon the quantitative composition of lighter and heavier compounds, which is strongly influenced by growth phase and treatment with certain antibiotics.

Although the primary targets of most antibiotics are known, there are few reports on the secondary effects of antibiotics on bacteria (6, 28, 34). Such secondary effects are of interest, especially in regard to subinhibitory concentrations of antibiotics which can appear in body fluids and tissues during the course of chemotherapy. Bacteria grown in the presence of subinhibitory concentrations of certain β-lactam antibiotics often show changes in surface-related virulence properties such as serum resistance and adherence (2, 5, 23, 24, 33, 34). It was the first aim of this study to investigate the influence of subinhibitory concentrations of different β-lactam derivatives and a quinolone on the formation of the outer membrane (OM) and on the composition of the main OM components of *Escherichia coli*.

Because we found indications of quantitative changes in the OM, we further analyzed the immunogenicity of the OM. The immunogenic properties of bacterial surface components and the effect of various adjuvants on these properties have been the subject of studies for several years. It was demonstrated that the quantity, as well as the subclass composition, of the immune response against lipopolysaccharide (LPS) from *Proteus mirabilis* can be influenced over a wide range by complexation with proteins, phospholipids, or both (16, 29). In complexes of antigens, especially in liposomes associated with various antigens (1), mutual adjuvant effects, depending upon quality as well as quantitative contribution of the antigens, modulate the immune response against the single constituents of such complexes.

Most studies dealing with the effects of antibiotics on the immune response were concerned with direct influences of the antibiotics on the immune system of the host. To exclude these influences and further effects due to morphological changes and active metabolism of the bacteria from this study, we presented OM vesicles, isolated from bacteria that had been grown in the presence or absence of antibiotics, to mice not influenced by antibiotics and measured the primary immune response.

MATERIALS AND METHODS

Bacteria. *E. coli* WF 96 (serotype O7:K1:H6) and D 509 (serotype O86) were used for the experiments. Bacteria were grown in Mueller-Hinton broth (E. Merck AG, Darmstadt, Federal Republic of Germany) with or without antibiotics and with agitation.

Antibiotics. The following antibiotics were used: ciprofloxacin (Bayer AG, Leverkusen, Federal Republic of Germany), cephaloridine (Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom), imipenem (Merck Sharp & Dohme, Rahway, N.J.), and moxalactam (Eli Lilly & Co., Indianapolis, Ind.).

MIC determination. The MIC determination was performed by microtiter plate assay DIN 58940.

Preparation of OM vesicles. Membranes were prepared by the method of Kroll et al. (18). To obtain membranes from late-logarithmic-phase cells, bacteria were grown in 1.2 liters of Mueller-Hinton broth on an orbital incubator in the presence of different subinhibitory concentrations of ciprofloxacin, cephaloridine, moxalactam, and imipenem. To obtain maximum comparability, all samples were grown in parallel and inoculated (1:200) from the same overnight culture. Optical density (578 nm) of the samples was checked

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every 30 min. The administered subinhibitory concentrations caused no changes in the growth curves. At an optical density of 0.5, the samples were rapidly cooled to about 4°C in a carbon dioxide-methanol bath, and bacteria were harvested by centrifugation. Subsequently, bacteria were suspended in 5 ml of 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.2]) containing 0.75 M sucrose and 50 μg of egg white lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml. After 10 min of incubation, the plasmolyzed bacteria were lysed by transfer into 120 ml of distilled water containing 1 mg of DNase I (Sigma). Membranes were separated from soluble material by ultracentrifugation (1 h at 130,000 × *g* and 4°C). Subsequently, OM and cytoplasmic membrane (CM) vesicles were isolated by sucrose density ultracentrifugation (16 h at 120,000 × *g* and 4°C). During fractionation of the gradients, the A_{278} was continuously monitored with a Uvicord (LKB Instruments, Inc., Rockville, Md.). The density of the fractions was determined by refractometry. To obtain membranes from early-logarithmic-phase cells, bacteria were grown to an optical density of 0.1 and treated as described above. To obtain membranes from stationary-phase cells, an overnight culture (optical density = 1.5) was used.

OM-containing fractions were combined and diluted with at least 5 volumes of water. OMs were recollected by ultracentrifugation (1 h at 130,000 × *g* and 4°C) and washed once in water. Afterwards, membranes were suspended in distilled water and dried under reduced pressure. The isolated membranes (approximately 30 mg per sample) were weighed, distributed in aliquots, and dried again. Drying was chosen to avoid membrane surface alterations due to freezing. Electron microscopy performed after resuspension showed a morphologically homogeneous population of vesicles.

Extraction of LPS, OM proteins, and phospholipids. LPS was extracted by the phenol water method and further purified by fractional Cetavlon precipitation by procedure III of Westphal and Jann (36). To obtain an OM protein extract for coating of sheep erythrocytes, OMs were treated with 2% deoxycholate, LPS was removed by ultracentrifugation, and the supernatant was dialyzed against water. A 2-keto-3-deoxyoctulosonic acid (KDO) determination of the extract showed the preparations to be contaminated with less than 1% LPS. Phospholipids were extracted by the method of Folch et al. (4).

Analytical methods. Protein was determined by the method of Markwell et al. (20), and KDO was measured by the method of Karkhanis et al. (17). Discontinuous sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed as described by Lugtenberg et al. (19) with 11% acrylamide in the separating gel. Protein (60 μg) and LPS corresponding to 0.5 μg of KDO were applied to each slot of the gel. The LPS gels were stained by the method of Karch et al. (15), and protein gels were stained with Coomassie brilliant blue R-250 (LKB).

Enzyme assay. NADH oxidase activity was determined by use of the method of Osborn et al. (25).

Immunization. A 75-μg (dry weight) amount of OM vesicles was suspended in 0.5 ml of sterile 0.9% NaCl solution. Directly after vigorous agitation, suspensions were injected intraperitoneally into 8- to 10-week-old male specific-pathogen-free B6D2 mice (Zentralinstitut für Versuchstierzucht, Hanover, Federal Republic of Germany). The 75-μg dose was chosen because for vesicles from untreated late-logarithmic-phase control (Co-LL) bacteria it gave optimal responses against LPS and OM proteins. All animals were injected and killed between 8 and 10 a.m. to reduce the effects of circadian rhythmicity.

TABLE 1. Antibacterial activities of the antibiotics

Antibiotic	MIC (μg/ml) for strain:	
	WF 96	D 509
Ciprofloxacin	0.063	0.0075
Cephaloridine	8	16
Imipenem	0.25	0.125
Moxalactam	0.25	0.125

Assay of antibody-producing cells. Antibody-producing cells secreting immunoglobulin M (IgM) antibodies specific for LPS from serotype O7:K1:H6 and OM proteins were detected by a modification of the thin-layer version of the localized hemolysis in gel technique (21) by using sheep erythrocytes (BAG, Lich, Federal Republic of Germany) sensitized with either alkali-treated LPS from serotype O7:K1:H6 (16) or OM proteins from *E. coli* WF 96 (chromium chloride coupling [7]) as indicator cells. Briefly, 50 μl of a 15% (vol/vol) suspension of sensitized sheep erythrocytes and 50 μl of a 1:3 dilution of guinea pig serum in minimal essential medium-Hanks balanced salt solution without carbonate (Biochrom, Berlin, Federal Republic of Germany) supplemented with 1.16 mM Na_2HPO_4 and 1.19 mM $MgCl_2$ (MHP buffer) were added to 600 μl of 0.6% (wt/vol) LGT agarose (Miles Laboratories, Inc., Elkhart, Ind.) in MHP which had been kept at 37°C. Immediately after this, 0.1 ml of a dilution of spleen cells in MHP was added. The mixture was poured directly into a petri dish. After gelling of the layer (approximately 15 min at ambient temperature), plates were incubated at 37°C for 3 h. Afterwards, plaques were counted under standardized light conditions. Representative plaques were checked for a central lymphocyte. Values obtained for individual mice (plaque-forming cells [PFC] per spleen) were corrected by subtraction of a small number of sheep erythrocyte-specific PFC, so that only LPS- and OM protein-specific PFC values are given in the report. PFC responses were determined on day 5 after immunization. Since values for PFC per spleen are log normally distributed (9), data are given as mean $\log_{10}$ (PFC/spleen) ± standard deviation for groups containing 8 to 10 identically treated mice.

Statistics. Student's *t* test was applied to the normally distributed logarithmic values.

RESULTS

The MICs of the β-lactam derivatives tested and of ciprofloxacin are shown in Table 1.

Distribution of membrane vesicles in sucrose density gradients. (i) Distribution in various growth phases. The whole-membrane mixtures (prepared by osmotic lysis of plasmolyzed bacteria and subsequent ultracentrifugation) after sucrose density ultracentrifugation generated four bands (Fig. 1). The two bands with buoyant densities of 1.28 and 1.25 g/cm^3 were identified as OM material because they contained the bulk of LPS and major OM proteins and only low NADH oxidase activity. The highest NADH oxidase activity was found in a band with a buoyant density of 1.17 g/cm^3. The fourth peak, with a buoyant density of 1.14 g/cm^3, contained little NADH oxidase activity but most likely represents further CM material because of its low content of LPS and major OM proteins and its SDS-PAGE pattern, which is very similar to that of the main CM peak

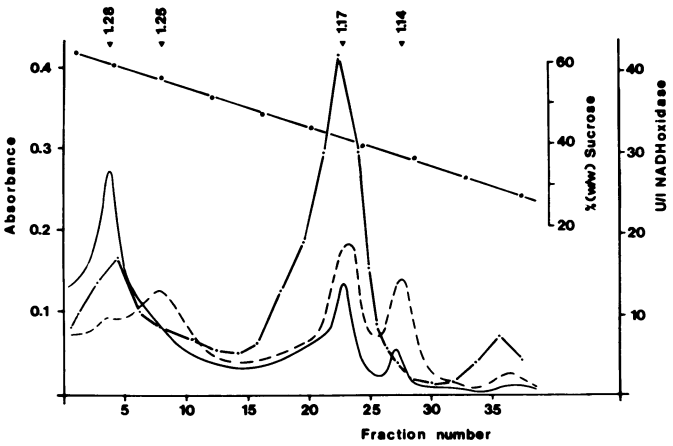

FIG. 1. Distribution of membrane vesicles at A_{280} of *E. coli* D 509 grown for 150 min (——) and for 6 h (– – –) after sucrose density ultracentrifugation. –·–·–, NADH oxidase activity.

(Fig. 2). Depending on the growth phase, different amounts of the two OM fractions could be isolated. Early-log-phase cells produced more OM material with a buoyant density of 1.25 g/cm^3 than of 1.28 g/cm^3. In contrast, from late-log-phase and stationary-phase cells, the denser OM material could almost exclusively be isolated (Fig. 1). These effects are not due to a contamination of the OM material with CM material or vice versa, since OM fractions showed a low activity of NADH oxidase as compared with the CM fraction (Fig. 1) and since the CM fraction contained only relatively small amounts of major OM proteins (Fig. 2).

(ii) Distribution after growth in various concentrations of antibiotics. The gradient profiles of membranes isolated from early-stationary-phase bacteria (*E. coli* D 509) grown in one-fourth and one-sixth the MIC of moxalactam and from control bacteria are shown in Fig. 3. The antibiotic induced a dose-dependent quantitative shift from the heavier to the lighter OM peak; although the OMs of control bacteria consisted predominantly of heavy OM material, incubation in one-sixth the MIC of moxalactam induced a strong reduction of the heavier material and an increase of the lighter one, and after incubation in one-fourth the MIC only the lighter material could be isolated. Similar results were found for the other antibiotics tested, with the exception of cephaloridine. Exactly the same results were found for *E. coli* WF 96.

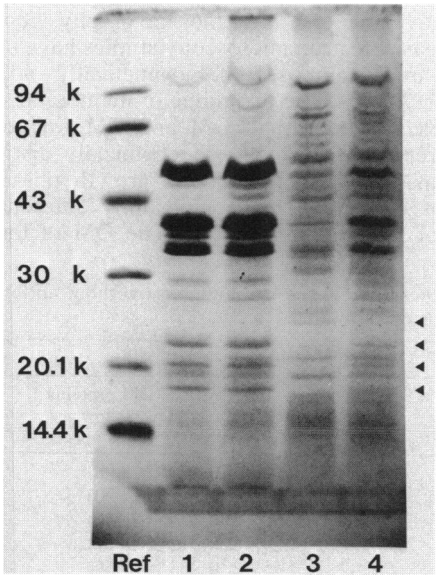

FIG. 2. SDS-PAGE of the OM proteins of *E. coli* D 509 corresponding to the peak with a buoyant density of 1.28 (lane 1) and 1.25 (lane 2) g/cm^3 and of the CM proteins corresponding to the peak with a buoyant density of 1.17 (lane 3) and 1.14 (lane 4) g/cm^3 after sucrose density centrifugation of whole membranes. Arrowheads indicate differences in the protein composition of the first and second CM peaks. Ref, Reference proteins; molecular weights (in thousands) are indicated.

OM proteins, LPS, and phospholipids. OM proteins and LPS were analyzed by SDS-PAGE. The OM proteins (major and minor) of cells grown in subinhibitory concentrations of the antibiotics used did not exhibit any differences from those of control cells (Fig. 4a and b). This was shown for both strains tested. In addition to this, none of these strains showed significant alterations of the LPS pattern after antibiotic treatment (Fig. 4a and b). According to Palva and Mäkelä (26) and Goldman and Leive (8), the multitude of bands in the SDS gel represents LPS molecules containing different numbers of the repeating units of their O side chains (i.e., the fastest-migrating band has only a complete core oligosaccharide, the next band has a core oligosaccharide plus one repeating unit, and so on). This means that none of the antibiotics tested had any influence on the degree of polymerization of the O side chain. This is valid for very low concentrations up to concentrations near the MIC. The phospholipids of antibiotic-treated and untreated bacteria showed identical migration properties in thin-layer chromatography.

Effects of antibiotics on immunogenicity. For the immunizations, 75 μg (dry weight) of OM vesicles isolated from late-logarithmic-phase antibiotic-treated and untreated bacteria was used. The IgM-producing cell response against LPS and OM proteins was measured by a plaque assay on day 5. The immune response against LPS and OM proteins of vesicles prepared from late-logarithmic-phase bacteria grown in the presence of one-fourth the MIC of each of moxalactam, imipenem, and ciprofloxacin was significantly lower than the response against vesicles from late-logarithmic-phase control cells (Co-LL; Table 2). The amount of the reduction of the PFC response was dependent on the

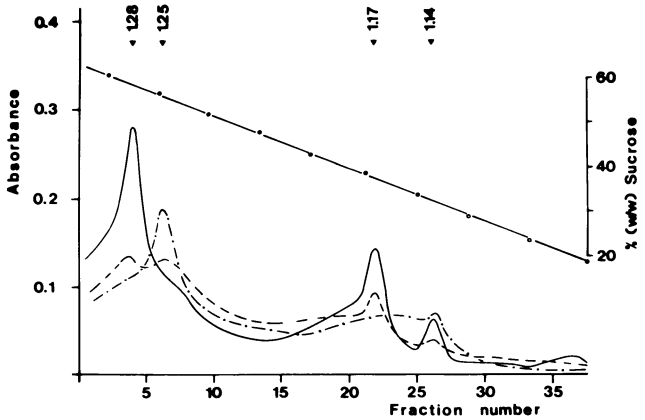

FIG. 3. Distribution of membrane vesicles at A_{280} of *E. coli* D 509 grown in the absence of moxalactam (——) or in the presence of one-sixth (– – –) or one-fourth (–·–·–) the MIC of moxalactam after sucrose density ultracentrifugation.

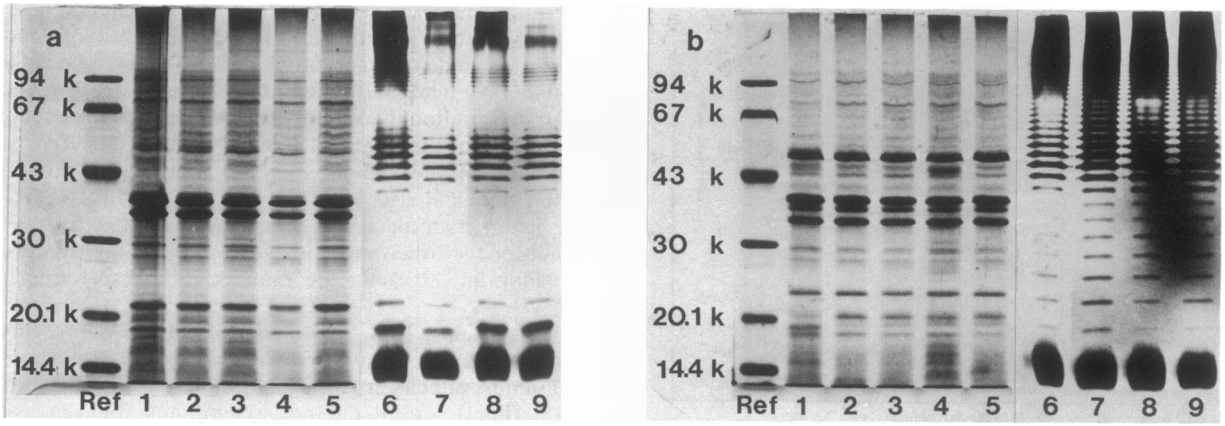

FIG. 4. SDS-PAGE of OM proteins (lanes 1 to 5) and LPSs (lanes 6 to 9) of *E. coli* WF 96 (a) and D 509 (b) grown in the absence of drug (lanes 1 and 6) and in the presence of one-fourth the MIC of cephaloridine (lane 2), ciprofloxacin (lanes 3 and 7), imipenem (lanes 4 and 8), and moxalactam (lanes 5 and 9). Ref, Reference proteins; molecular weights (in thousands) are indicated.

concentration of the antibiotic. When 1/16 the MIC of moxalactam was applied, the response against LPS equalled the value for Co-LL vesicles; the response against OM proteins was also higher than that for 1/4 the MIC with vesicles from bacteria grown in the presence of moxalactam but still differed significantly from the value for Co-LL vesicles. Similar dose dependency was found for imipenem and ciprofloxacin (Table 2). Growth of bacteria in cephaloridine-containing medium had no significant influence on the immunogenicity of the OM.

Effects of growth phase on immunogenicity. The immune response against Co-LL vesicles was compared with that against vesicles from untreated (control) early-logarithmic-phase bacteria and from untreated bacteria from a stationary-phase culture (Table 3). Vesicles from untreated early-logarithmic-phase bacteria, like vesicles from antibiotic-treated bacteria, induced significantly lower PFC responses against OM proteins and LPS than did Co-LL vesicles. The immunogenicity of Co-LL vesicles and of vesicles from bacteria from a stationary-phase culture did not differ significantly.

DISCUSSION

Until now, little was known about the influence of subinhibitory concentrations of β-lactam antibiotics on the cell envelope of gram-negative bacteria. James (11) described an influence of mecillinam on the formation of certain OM proteins. Taylor et al. (34) described changes in the expression of O side chains and the formation of an acidic capsule polysaccharide in *E. coli*. Kadurugamuwa et al. (12, 13) reported that cephalosporins, at a concentration below that influencing growth rate, reduced the production of enterochelin and capsule formation of iron-depleted *Klebsiella pneumoniae* but had no effect on the OM protein profiles of the bacteria. Stephens et al. (32) noticed a marked reduction of the expression of fimbriae after growth of *Neisseria gonorrhoeae* and *Neisseria meningitidis* in subinhibitory concentrations of penicillin and tetracycline.

With the antibiotics tested, we could not detect qualitative changes in the main OM components LPS, proteins, and phospholipids. Moxalactam, imipenem, and ciprofloxacin, however, influenced the formation of the OM. The OM of gram-negative bacteria is a mosaic of physicochemically distinct areas. Electron microscopy studies have shown that there are areas composed of biogenetically old and new material (3, 22, 30). The technique of membrane separation used enabled us to isolate OM and CM fractions which probably represent such physicochemically distinct membrane areas. Depending upon the growth phase, *E. coli* synthesizes OM areas with significantly different buoyant densities of 1.28 and 1.25 g/cm^3. The OM of logarithmic-

TABLE 2. IgM PFC responses in mice to OM vesicles isolated from *E. coli* WF 96 grown to the late log phase without antibiotics or in the presence of subinhibitory concentrations of antibiotics

Vesicle prepn	IgM PFC response against:			
	LPS		OM proteins	
	PFC/spleen[a]	$P <$[b]:	PFC/spleen[a]	$P <$[b]:
Co-LL	3.974 ± 0.239 (10,700)		4.520 ± 0.175 (35,608)	
Moxalactam (1/4 the MIC)	3.507 ± 0.164 (3,414)	0.0005	3.978 ± 0.447 (13,000)	0.005
Moxalactam (1/12 the MIC)	3.702 ± 0.272 (5,880)	0.05	4.084 ± 0.450 (17,420)	0.005
Moxalactam (1/16 the MIC)	3.812 ± 0.373 (8,700)	NS[c]	4.291 ± 0.112 (2,000)	0.01
Cephaloridine (1/4 the MIC)	4.003 ± 0.389 (13,729)	NS	4.553 ± 0.263 (41,666)	NS
Ciprofloxacin (1/4 the MIC)	3.648 ± 0.313 (5,343)	0.025	4.046 ± 0.310 (13,017)	0.0005
Ciprofloxacin (1/8 the MIC)	ND[d]		4.373 ± 0.191 (25,660)	NS
Imipenem (1/4 the MIC)	3.540 ± 0.507 (5,488)	0.025	4.138 ± 0.224 (15,267)	0.001
Imipinem (1/10 the MIC)	ND		4.232 ± 0.234 (19,720)	NS

[a] Mean $\log_{10}$ (PFC/spleen) ± standard deviation. The arithmetic means of PFC per spleen are in parentheses.
[b] Significance of difference from value for Co-LL (Student's *t* test).
[c] NS, Not significant ($P > 0.05$).
[d] ND, Not determined.

TABLE 3. IgM PFC responses to OM vesicles isolated from *E. coli* WF 96 grown to different growth phases

Vesicle prepn	IgM PFC response against:			
	LPS		OM proteins	
	PFC/spleen[a]	$P <$[b]:	PFC/spleen[a]	$P <$[b]:
Co-EL[c]	3.600 ± 0.166 (4,475)	0.001	3.606 ± 0.355 (5,167)	0.0005
Co-LL	3.974 ± 0.239 (10,700)		4.520 ± 0.175 (35,608)	
Co-ST[d]	4.024 ± 0.101 (10,775)	NS[e]	ND[f]	

[a] Mean $\log_{10}$ (PFC/spleen) ± standard deviation. The arithmetic means of PFC per spleen are in parentheses.
[b] Significance of difference from value for Co-LL (Student's *t* test).
[c] Co-EL, Early-logarithmic-phase control bacteria.
[d] Co-ST, Stationary-phase control bacteria.
[e] NS, Not significant ($P > 0.05$).
[f] ND, Not determined.

phase bacteria mainly consisted of the light material; the OM of stationary-phase cells consisted almost exclusively of the heavy material. From late-logarithmic-phase bacteria, both fractions were isolated. Because β-lactam antibiotics (with the exception of cephaloridine) and the quinolone ciprofloxacin inhibited the formation of the heavy OM material and because this heavy material was predominantly formed in the stationary phase, it seems likely that these antibiotics can inhibit maturation processes in the OM.

Further evidence for this assumption is provided by the fact that the distinct OM fractions in pulse-chase experiments with [2-^{3}H]glycerol as precursor of phospholipids show different labeling kinetics (data not shown). Horne et al. (10) have described a secretion of lipids from several species of bacteria by inhibition of peptidoglycan synthesis. To test whether our results were due to a selective loss of OM material with a higher buoyant density, we tested the culture supernatant for the OM material and did not find any differences between controls and antibiotic-treated bacteria.

Because of the indications of quantitative changes in the OM of antibiotic-treated bacteria, we further analyzed the immunogenicity of the OM. We used OM vesicles for the immunization to avoid superposition of the effects of other antibiotic interactions on the immune system, such as a direct influence on the host immune system and the morphological changes of the bacteria.

OM components of vesicles from bacteria grown in imipenem-, moxalactam-, or ciprofloxacin-containing medium were markedly less immunogenic than components of Co-LL vesicles. This effect was dependent on the concentrations of the antibiotic used. Responses against OM proteins and LPS behaved principally in the same way, with a relatively constant ratio between PFC value for anti-protein and anti-LPS response. A lower immunogenicity of OM components was also found for OM vesicles from early-logarithmic-phase control cells, whereas immunogenicity of vesicles from stationary-phase control cells did not differ significantly from that of Co-LL cells.

The data on immune response can be related to the findings on membrane profiles in sucrose density gradients. The two OM areas isolated on the basis of their different buoyant densities are differently immunogenic ($P < 0.0005$). The predominance of the light OM material in vesicles from antibiotic-treated cells and early-logarithmic-phase control cells could explain why these vesicles are less immunogenic than Co-LL vesicles.

Our explanation for the different immunogenicities of the two OM areas at present is a hypothesis. Because no qualitative differences in proteins, LPS, and phospholipids are induced by antibiotic treatment, the differences in the buoyant densities seem to be due to a different quantitative composition. The quantitative composition of liposomes associated with various antigens has a strong influence on their immunogenicity. Karch and Nixdorff (16) showed that complexation of LPS from *P. mirabilis* with the 39,000-molecular-weight OM protein leads to an increased immunogenicity of both antigens. Other proteins, too, such as bovine serum albumin, methylated bovine serum albumin, and chymotrypsinogen, as well as phospholipids, were shown to exhibit strong adjuvant effects on the immune response against LPS (14). In addition to these adjuvant effects, the immune responses against antigens which are incorporated into liposomes are influenced by the antigen/phospholipid ratios and also by the physicochemical properties of the lipid components which determine membrane fluidity (35).

Adjuvant effects like these are probably responsible for the differences in immunogenicity between the two OM areas. The described effects could not be shown for cephaloridine. Obviously, they are not induced by all β-lactam antibiotics. The selective inhibition of penicillin-binding protein 1b by cephaloridine does not induce changes of immunogenicity. Because the described effects can also be induced by ciprofloxacin, a gyrase inhibitor, it seems likely that the change in the bacterial surface, probably leading to the reduction of immunogenicity, is a process which can be started by several mechanisms. That there are mutual interactions between peptidoglycan synthesis (as the primary target of β-lactam antibiotics) and membrane biosynthesis has been described by Rogers and Thurman (28). Growth in subinhibitory concentrations of certain antibiotics can apparently change the immunogenic properties of bacteria, probably by inhibition of maturation processes in the OM. The question of whether these effects play any role in therapeutic situations cannot yet be decided. The reported data, however, give further evidence that, in addition to their primary effects on target enzymes, antibiotics interfere with other parts of bacterial metabolism and can induce marked changes in immunogenicity.

ACKNOWLEDGMENTS

We are very grateful to Susanne Wendt and Brigitte Andres for excellent technical assistance and to Ursula Heuzeroth for typing the manuscript.

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