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Carumonam Enhances Reactivity of *Escherichia coli* with Mono- and Polyclonal Antisera to Rough Mutant *Escherichia coli* J5

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Escherichia coli O111 reacts only slightly with antiserum to its rough mutant E. coli J5 in an enzyme-linked immunosorbent assay. When E. coli O111 was grown in the presence of sub-MICs of the monocyclic β-lactam antibiotic carumonam, however, the enzyme-linked immunosorbent assay titer increased from 1,280 to 81,920. When the bacteria were grown in the presence of carumonam, the titer that was obtained with antiserum against E. coli O111 was not affected. This reaction was abolished after this antiserum was absorbed with E. coli J5 in the case of the carumonam-treated strain, whereas this absorption did not affect the reaction with E. coli O111. Thus, the O-antigenic side chain of E. coli O111 seems to be affected if this strain is cultured in the presence of carumonam. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a relative loss of the O polysaccharide in E. coli O111 when this strain was grown in the presence of carumonam. Also, a much stronger reaction of the antibiotic-affected lipopolysaccharide with a monoclonal antibody against E. coli J5 lipopolysaccharide was shown in immunoblots. The results of this study indicate that there is a synergism between certain antibiotics and monoclonal antibodies, something that could have clinical implications.

Antibodies against core glycolipids of *Escherichia coli* J5, a Rc mutant of *E. coli* O111:B4, or to the Re mutant of *Salmonella minnesota* cross-react with the core glycolipid of various otherwise unrelated gram-negative bacteria (13, 25). Antisera to these mutant strains have therefore been used in protection studies with heterologous bacteria in animals (3, 27), and the prevention and the improved outcome of gram-negative septic shock in human subjects have been shown (1, 28). Also, some monoclonal antibodies (MAbs) against *E. coli* J5 that show cross-reactivity have been described (6, 7, 16, 20). However, Gigliotti and Shenep (10) showed that antibodies that are reactive with the J5 core glycolipid are unable to bind to the parent strain *E. coli* O111 unless the O-antigenic polysaccharides are first removed.

It has been suggested that antibiotics that are present in subinhibitory concentrations interfere with the reactivity of bacteria with host defenses (24, 26). During studies on the effect of subinhibitory concentrations of several β-lactam antibiotics on bacterial growth, we observed that certain bacteria only reacted with anti-core antibodies after growth in the presence of low concentrations of carumonam. Carumonam {Ro 17-2301; AMA-1080; (3*S*,4*S*)-3-[2-(2-aminothiazol-4-yl)-(*Z*)-2-carboxy-methoxyiminoacetamido]-4-carbamoyloxymethyl-2-azetidinone-1-sulfonic acid [12]} is a monocyclic β-lactam antibiotic with a bactericidal activity against gram-negative bacteria; this activity is carried out by binding to penicillin-binding protein 3, which is comparable to that which occurs with aztreonam (2, 4, 8, 18, 22). Here we describe results of experiments that show that subinhibitory concentrations of carumonam influence the reactivity of *E. coli* O111 with poly- and monoclonal antibodies against the rough (R) mutant *E. coli* J5.

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MATERIALS AND METHODS

Bacteria. *E. coli* O111 and its Rc mutant *E. coli* J5 were used in this study. *E. coli* O111 was a gift from P. A. M. Guinée (National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands) and was originally selected in the Max Planck Institut für Immunobiologie, Freiburg im Breisgau, Federal Republic of Germany, and provided by the Institut Pasteur, Paris, France. *E. coli* J5 was a gift from M. P. Glauser (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). This strain lacks both the enzyme UDP-galactose-epimerase and the ability to incorporate exogenous galactose into its lipopolysaccharide (LPS).

Growth media. The MIC of carumonam for *E. coli* O111 was determined with twofold dilutions in Giston broth (Difco Laboratories, Detroit, Mich.). Thereafter, bacteria were cultured in the presence of half the MIC of carumonam in Giston broth for 18 h at 37°C (CAR-O111). After the bacteria were washed, they always showed large, filamentous forms.

ELISAs. Enzyme-linked immunosorbent assays (ELISAs) were performed as described elsewhere (6). Viable *E. coli* O111 or CAR-O111 was used to coat the microtiter plates; and the reactivity with normal rabbit serum, rabbit anti-J5 serum, and rabbit anti-O111 serum was measured spectrophotometrically at 450 nm in a spectrophotometer (Titertek Multiscan; Flow Laboratories, Inc., McLean, Va.). An optical density below 0.200 was considered to be negative.

Antisera. Antisera against *E. coli* O111 or *E. coli* J5 were prepared by immunizing rabbits with heat-killed organisms (1 h, 100°C) by the method described by Kaufmann (14).

Absorption of antisera. Bacteria were grown on Giston agar (prepared from Bacto-Peptone; Difco) plates (diameter, 15 cm) for 18 h at 37°C, harvested with physiological salt

solution, and centrifuged. Bacteria were washed once, transferred to polypropylene vials (Biovials; Beckman Instruments, Inc., Chicago, Ill.), centrifuged once more, and incubated with 1.2 ml of antiserum for 60 min at 37°C on a rotator (25 rpm). The suspension was transferred to Eppendorf vials (Sarstedt BV, Eindhoven, The Netherlands) and centrifuged for 10 min at 10,000 rpm. The absorption procedure was repeated twice.

MAbs. BALB/c mice were immunized with heat-killed *E. coli* J5. Spleen cells were fused with mouse myeloma cell line Sp 2/0-Ag 14 (19) as described by Köhler and Milstein (15) with the modification described by Fazekas de St. Groth and Scheidegger (9). Hybridomas were subcloned twice by the limiting dilution method and screened for antibody activity of supernatants in an ELISA by using heat-killed bacteria as the antigen. Ascites fluid containing anti-J5 MAbs were obtained from BALB/c mice that were treated with pristane (Janssen Chimica, Beerse, Belgium) and subsequently injected with 5×10^5 cells of the hybridomas that were selected.

Electrophoretic transfer and immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed with bacterial cell walls that were sonicated for 1 min at 4°C, as described previously (7), and treated with proteinase K (1 h, 60°C). The Western blot technique was applied by the method described by Burnette (5). After electrophoresis in the Trans-Blot system (Bio-Rad Laboratories, Richmond, Calif.), gels were immediately transferred to nitrocellulose paper (Schleicher & Schüll, Dassel, Federal Republic of Germany) for 3 h at constant voltage (35 V). Afterward the remaining gels were stained to check the effectiveness of LPS transfer to nitrocellulose. To block unbound sites, the nitrocellulose paper was soaked for 1 h at 37°C in Tris-buffered saline (10 mM) containing 0.05% Tween 20 and 3% gelatin. The nitrocellulose sheets were incubated with a mouse immunoglobulin M (IgM) MAb against *E. coli* J5 that has been shown to be reactive with the core glycolipid of *E. coli* J5 and not with the O polysaccharide of *E. coli* O111 (6). The MAb was diluted 1:1,000 in Tris-buffered saline (10 mM) containing 0.05% Tween 20 for 1 h at 37°C under gentle shaking. After three washing procedures and incubation for 15 min in Tris-buffered saline (10 mM) containing 0.05% Tween 20, the paper sheets were incubated for 1 h at 37°C in horseradish peroxidase-conjugated goat anti-rabbit IgM (Nordic Immunology, Tilburg, The Netherlands) that was diluted 1:7,000 in Tris-buffered saline (10 mM) containing 0.05% Tween 20. After another three washing procedures, the sheets were placed into 30 ml of buffer (100 mM citric acid, 200 mM Na_2HPO_4 [pH 5]), 10 ml of ethanol containing 20 mg of 3,3',5,5'-tetramethylbenzidine (Sigma Chemical Co., St. Louis, Mo.), and 80 mg of dioctylsulfosuccinate sodium salt (Serva, Heidelberg, Federal Republic of Germany) to color the bands. The reaction was started with 0.02 ml of H_2O_2 and stopped with water after 1 to 5 min.

RESULTS

Influence of carumonam on the reaction between antiserum and bacteria. The reaction of *E. coli* O111 and CAR-O111 with normal rabbit serum and antiserum raised against *E. coli* J5 and *E. coli* O111 is shown in Fig. 1. *E. coli* O111 reacted only slightly better with the J5 antiserum, as compared with normal rabbit serum. However, when *E. coli* was grown in the presence of subinhibitory concentrations of carumonam, the antiserum sample was able to react with the bacteria even at low concentrations. Antiserum against *E. coli* O111 reacted with both *E. coli* O111 and CAR-O111. These findings may indicate that low concentrations of carumonam interfere with the synthesis of the LPS and that this leads to the more pronounced exposure of the core glycolipid, so that antibodies against the core fraction (as in antiserum against *E. coli* J5) are able to react with the carumonam-treated bacteria.

Reactivity of strains with absorbed antisera. The ELISA titers of *E. coli* O111 with the various antisera were diminished when these serum samples were absorbed with *E. coli* O111 (Fig. 2). When the same experiment was performed with CAR-O111, the pattern of the reactivity with the absorbed O111 antiserum sample was different. Whereas absorption of this serum sample with *E. coli* O111 decreased the titer against the normal *E. coli* O111 strain from 20,480 to 160, the reactivity against CAR-O111 was only reduced from 10,240 to 2,560. Absorption of the O111 antiserum sample with *E. coli* J5 did not give a decrease in its reactivity with *E. coli* O111. In contrast, the titer of the O111 antiserum sample that was absorbed with *E. coli* J5 against CAR-O111 decreased from 10,240 to 640. Thus, it appears that the decrease in titer is more pronounced when the antibodies that are directed against the core glycolipid are absorbed out of the serum than when the antibodies against the O polysaccharide are absorbed. It could be that the accessible antigenic sites of a carumonam-treated *E. coli* O111 are located primarily in the core glycolipid region and not in the O-polysaccharide fraction, suggesting a relative loss of O polysaccharide in the CAR-O111.

SDS-gel electrophoresis of LPS. To determine whether carumonam interfered with the O-antigen formation, cell walls were isolated, treated with proteinase K, and subjected to SDS-gel electrophoresis. On a dry weight basis, all lanes were inoculated with the same amount of cell walls of *E. coli* O111 or CAR-O111. Cell walls of *E. coli* O111 produced many O-antigenic bands but only a small band in the core region. In contrast, the CAR-O111 cell walls produced only some bands in the O-antigenic region but a large zone of bands in the core glycolipid area, which was comparable to that of cell walls of *E. coli* J5 (Fig. 3).

Reactivity of MAbs with LPSs after LPS immunoblotting.

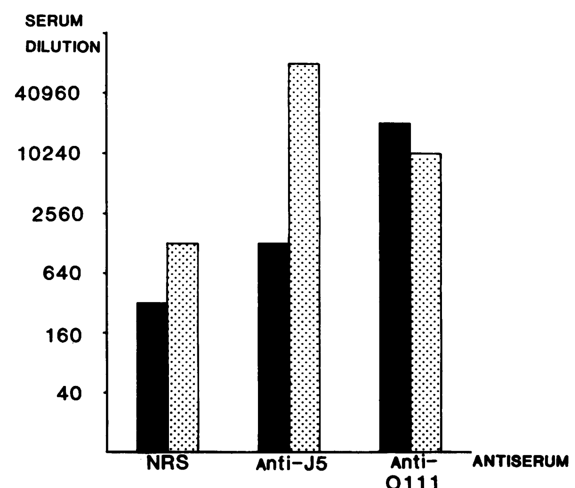

FIG. 1. ELISA titers of viable *E. coli* O111 and CAR-O111 isolates with normal rabbit serum (NRS), rabbit anti-J5 serum, and rabbit anti-O111 serum. Symbols: ■, *E. coli* O111; ▨, CAR-O111.

Many MAbs were raised against the core glycolipid of *E. coli* J5. Most of these MAbs did not react with the LPS of *E. coli* O111. We then tested whether these antibodies reacted with CAR-O111 LPS. Indeed, these MAbs did not react with the LPS of *E. coli* O111 (Fig. 4). A strong reactivity between these antibodies and CAR-O111 LPS was observed, however, after the LPS was transferred to nitrocellulose, again indicating that treatment of the bacteria with subinhibitory concentrations of carumonam gives rise to relatively more core glycolipid antigens.

DISCUSSION

Gram-negative bacteria are responsible for many serious nosocomial infections. The high mortality rate from gram-negative bacteremia can be explained partly by the lowered resistance of patients whose immunity is impaired as a result of disease or immunosuppressive drugs and partly by the failure of antibiotics against these bacteria. An important factor in the failure of antibiotics is the endotoxin, which is not affected by antibiotics. It has been shown that the toxic part of the LPS is the lipid A fraction, which is common to all LPS molecules. A protective effect against the toxic effects of the endotoxin has been shown for polyclonal (1, 28) as well as for monoclonal (21) antibodies. Although MAbs that can bind to glycolipids of many different bacteria have been described, some investigators could not show cross-reactivity (10, 20). Also, antibodies against core glycolipid have not appeared to be effective in every study (11, 17, 23). It is possible that the O-polysaccharide side chain of gram-negative bacteria prevents binding of such antibodies against core glycolipid and that these antibodies are not optimally effective against strains with complete O antigens.

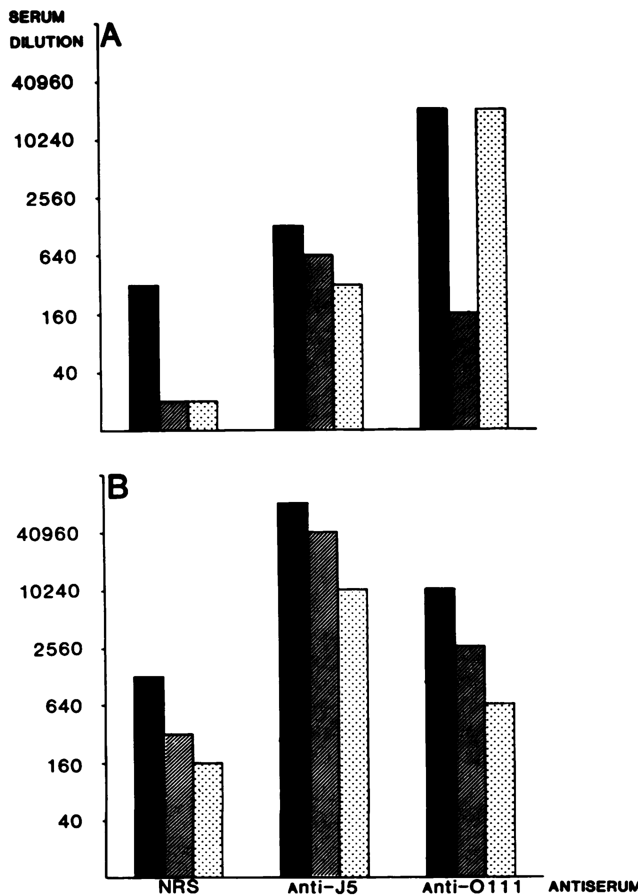

FIG. 2. ELISA titers of viable *E. coli* O111 grown without carumonam or in the presence of one-half the MIC of carumonam, with various antisera (NRS, normal rabbit serum), with or without absorption. (A) *E. coli* O111; (B) CAR-O111. Symbols: ■, nonabsorbed serum; ▨, serum absorbed with *E. coli* O111; ⊞, serum absorbed with *E. coli* J5.

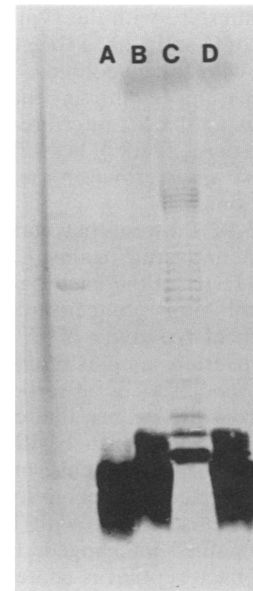

FIG. 3. SDS-polyacrylamide gel electrophoresis of proteinase K-treated cell walls of *E. coli* O111 and J5. Lane A, *E. coli* J5; lane B, CAR-J5; lane C, *E. coli* O111; lane D, CAR-O111.

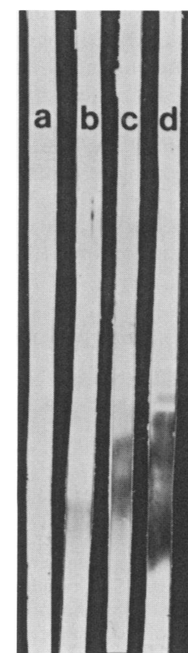

FIG. 4. LPS immunoblotting with mouse MAbs against *E. coli* J5 core glycolipid. Lanes a and b, *E. coli* O111; lanes c and d, CAR-O111.

Antibiotics that interfere with the synthesis of O antigens or with the binding of O antigens to the glycolipid could help antibodies to react with the glycolipids.

In this study we have shown that carumonam, a monocyclic β-lactam antibiotic, interferes with the assembly of LPS and that the core glycolipids of *E. coli* O111 become exposed when the strain is grown in the presence of subinhibitory concentrations.

Results of ELISAs showed that, first, compared with normal rabbit serum, antiserum against *E. coli* J5 binds much better to *E. coli* O111 when the bacteria are cultured in the presence of a subinhibitory concentration of carumonam. Second, the pattern of reactivity of *E. coli* O111 bacteria with three O111 antiserum samples (nonabsorbed, absorbed with *E. coli* O111 bacteria, or absorbed with *E. coli* J5 bacteria) differed from the pattern that evolved when these serum samples were tested in an ELISA with CAR-O111 bacteria. Absorption of O111 antiserum with *E. coli* J5 bacteria strongly affected its reactivity with CAR-O111 bacteria, whereas the reactivity with bacteria that were grown normally remained unchanged. It must be realized that the amount of bacteria that is coated onto the wells of the ELISA plate is difficult to standardize. Thus, one cannot compare absolute titers of the same serum sample with different strains, e.g., *E. coli* O111 versus CAR-O111. Nevertheless, the reactivity of individual strains with different serum samples can be compared. Also, the pattern of reactivity of various antisera with different strains can be studied.

From the results of the ELISAs it can be concluded that CAR-O111 behaves serologically like the Rc mutant *E. coli* J5 more than like *E. coli* O111, thereby suggesting that growth of *E. coli* O111 in the presence of sub-MICs of carumonam leads to a relative loss of O-antigenic side chains.

It was shown by SDS-polyacrylamide gel electrophoresis that this is indeed the case. In addition, the results of immunoblotting experiments with a MAb directed against the core glycolipid of *E. coli* J5 offered strong confirmation that the LPS pattern of CAR-O111 compared with that of *E. coli* O111 represents an enhanced ratio of core glycolipid molecules (incomplete LPS), as opposed to complete O-antigen-containing LPS molecules. The clinical impact of the results of this study may be that there is a synergism between certain antimicrobial agents and antiserum against rough mutants.

Thus, to test the efficacy of antisera against R strains in studies in humans, antimicrobial treatment must be standardized in patients.

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