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Neutralizing Antibodies against the Peptide Antibiotic AS-48: Immunocytological Studies

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Antisera against the broad-spectrum peptide antibiotic AS-48 produced by *Enterococcus faecalis* were obtained from immunized rabbits. Appreciable antibody titers were obtained only after repeated immunization, suggesting a feeble antigenicity for AS-48. Upon incubation with AS-48, the antisera neutralized its bacteriolytic action on *E. faecalis* S-47, although the simultaneous addition of AS-48 and serum did not prevent lysis. Crude serum cross-reacted with outer envelope components of enterococci, although specific anti-AS-48 antibodies, purified by affinity chromatography, reacted only with AS-48-treated cells. Labelling with immunofluorescence and colloidal gold particles was carried out on sensitive and resistant bacterial species to determine the interaction of AS-48 with cell structures.

In previous studies (6, 7) we have reported the production of the antibiotic AS-48 by a particular strain of *Enterococcus faecalis*. The purified substance (7.4 kDa) is a basic peptide (pI 10.4) which exhibits a bactericidal effect on gram-positive and gram-negative bacteria (4). This peptide forms pores of 0.7 nm on the cytoplasmic membrane, making it permeable to ions and low-molecular-weight compounds and collapsing the membrane potential (5). Furthermore, AS-48 induces bacteriolysis on several gram-positive bacteria after prolonged incubation, probably as a consequence of primary lesions (4, 7). We have also reported on the genetic location of AS-48 on the conjugative plasmid pMB2 (14), which is being characterized.

Our results indicate that the gastric proteolytic enzymes trypsin and chymotrypsin destroy antibiotic activity. Thus, the role of AS-48 as a chemotherapeutic agent should be restricted to topical use as an antiseptic. Nevertheless, AS-48 could be used as a food preservative, like nisin from *Lactococcus lactis* (10). Nevertheless, the safety of AS-48 for human health needs to be studied before it can be included in a food formulation.

Other basic peptide antibiotics with low molecular weight, such as nisin (10), gallidermin (12), epidermin (1), Pep-5 (16), or subtilin (8), showing structural and functional similarities have been described in gram-positive bacteria, but no studies on the antigenic properties of these peptides, which are important for establishing their safety, have been reported.

The purpose of this work has been to investigate the immunogenicity of AS-48, to study the nature of the antibodies elicited, and to find out about the interaction of AS-48 with the cell structures by means of antibody tracking.

Moreover, anti-AS-48 antibodies are at present being used widely in the cloning and expression of AS-48 genes in *Escherichia coli* to define the fragments of pMB2 that code for this antibiotic.

Preparation of immune sera. The peptide antibiotic AS-48 from crude supernatant of *E. faecalis* S-48 was purified as described elsewhere (6). The AS-48 used as an antigen showed an inhibitory activity against *E. faecalis* S-47 (indicator strain) of 200 arbitrary units/ml (6).

Young adult New Zealand rabbits were injected intramuscularly with 0.5 mg of AS-48 antigen suspended in complete Freund's adjuvant. Booster injections of AS-48 plus incomplete Freund's adjuvant were given 2 weeks later and 5, 7, and 15 weeks after the first shot.

Blood was taken by venipuncture at 0, 3, 8, 10, 16, and 21 weeks. These serum samples were tested for anti-AS-48 antibodies by the enzyme-linked immunosorbent assay (ELISA) indirect method (15). Appreciable titers were obtained after different booster injections, there being neces-

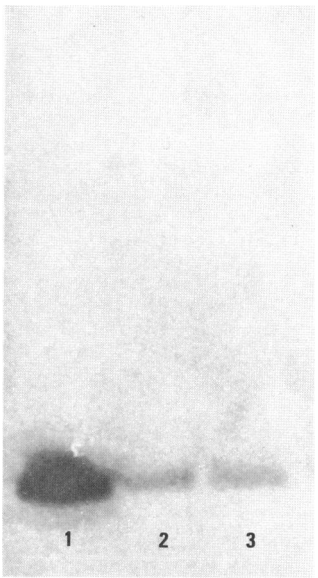

FIG. 1. Recognition of AS-48 by immune serum. Five, 1, and 0.5 μg of AS-48 (lanes 1, 2, and 3, respectively) were electrophoresed and blotted on a nitrocellulose membrane as described in the text. Visualization was carried out with 4-chloro-1-naphthol by using a secondary antibody labelled with peroxidase.

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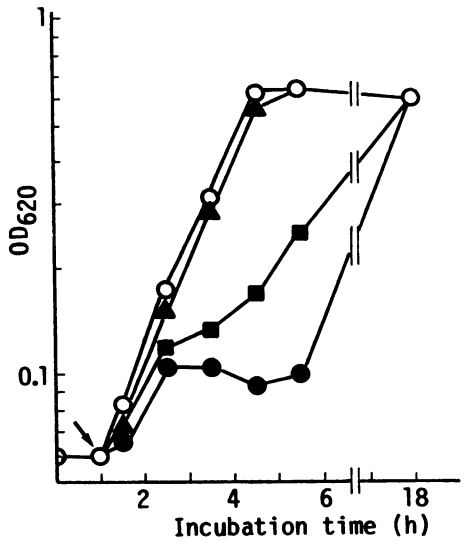

FIG. 2. Neutralization of the bacteriolytic effect of AS-48 on *E. faecalis* S-47 by anti-AS-48 immune serum. Sixty microliters of AS-48 (200 arbitrary units/ml) (●) or 60 μl of AS-48 previously incubated for 1 h with 60 (▲) or 6 (■) μl of serum was added to 6-ml aliquots of S-47 cultures. ○, control culture with serum alone added. Simultaneous addition of AS-48 and immune serum gave results coincident with those with AS-48 alone. The arrow indicates the addition of AS-48 or serum. OD_{620}, optical density at 620 nm.

sary intervals of inactivity between them, because of the low molecular mass of AS-48.

The saturating amount of antigen was determined to be 2 μg in ELISA experiments using different amounts of purified AS-48 (0.1, 0.25, 0.5, 1, 2, 3, and 4 μg in 200 μl of phosphate-buffered saline [PBS] per well against a 1/500 dilution of immune serum).

The rabbits were bled 4 or 5 weeks after the booster dose, and the separated serum was fractionated by sodium sulfate precipitation and then chromatographed on a Whatman DE52 column according to the method of Johnstone and Thorpe (11). Anti-AS-48 antibodies were further purified by affinity chromatography on CNBr-Sepharose 4B (Pharmacia, Uppsala, Sweden) coupled to AS-48 according to the manufacturer's procedure. The purity of AS-48 used for coupling to CNBr-Sepharose 4B was greater than 99% (according to reverse-phase high-performance liquid chromatography and electrophoresis data [3]), and its biological activity was estimated as 1,000 arbitrary units/ml.

Immunological detection of AS-48 on nitrocellulose membranes. Samples (5, 1, and 0.5 μg) of AS-48 were electrophoresed in a sodium dodecyl sulfate (SDS)-polyacrylamide gel according to the method of Laemmli (13). Slabs of 14% (30:0.15, acrylamide–*N,N*-methylenebisacrylamide) with a 4.5% stacking gel were used.

After electrophoresis, the proteins were transferred to nitrocellulose sheets (Schleicher and Schuell; 0.45-μm pore size, code BA85) as described by Towbin et al. (17) by using a Novablot kit (LKB Products, Bromma, Sweden). The transfer buffer (continuous system) consisted of 25 mM Tris, 192 mM glycine, 0.06% SDS, and 12% methanol, pH 8.3. The transfer was carried out at a constant current of 0.8 mA/cm^2 of the transfer unit for 1 h.

Proteins bound to nitrocellulose were visualized according to the method of Towbin et al. (17) after having first been reacted with the primary antibody (1/100 dilution) and then with a horseradish peroxidase-labelled secondary antibody (1/1,000 dilution; Institut Pasteur, Paris, France).

The antigen-antibody complexes on the blotted membranes were visualized as a single band (Fig. 1) with 4-chloro-1-naphthol (3 mg of 4-chloro-1-naphthol per ml of methanol–0.05 M Tris-HCl [10/50], pH 7.7, containing 0.01% [vol/vol] hydrogen peroxide). This band, in the original gel, can be clearly distinguished by the tracking dye.

Neutralization experiments. To test whether the fractionated immune serum neutralized the bacteriolytic activity of the antibiotic AS-48, different amounts (6 and 60 μl) of the immune serum were incubated at 37°C for 60 min with 60 μl of the AS-48 preparation (200 arbitrary units/ml) prior to its addition to early-log-phase cultures (optical density at 620 nm, 0.075) of *E. faecalis* S-47 (6 ml of BHI-T). Controls were carried out without antibiotic. In another S-47 culture, AS-48 (60 μl) and immune serum (60 μl) were added simultaneously. The optical density at 620 nm of the cultures was monitored at regular intervals with a Spectronic 20 spectrophotometer.

The protective capacity of the serum is shown in Fig. 2. Preincubation of AS-48 with 60 μl of antibodies prevented its bacteriolytic action on *E. faecalis* S-47. The simultaneous addition of immunoglobulins and AS-48, however, did not show any protection. This might be put down to the rapid absorption of the antibiotic on the cell surface, as suggested by the fact that its lethal action on cells can be detected after 1 min of contact (4).

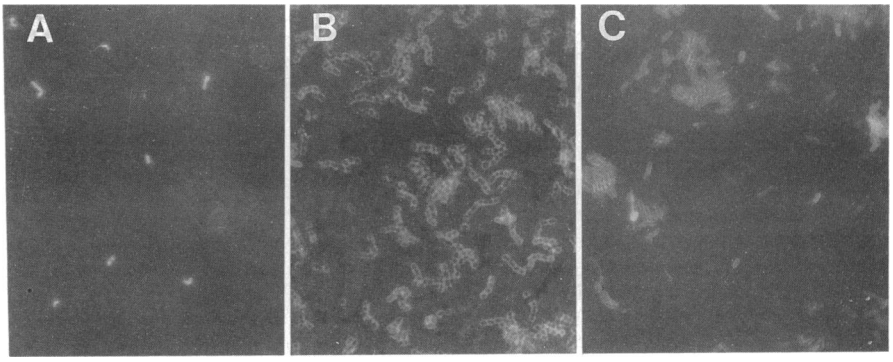

FIG. 3. Photomicrographs of indirect fluorescent antibody preparations specific for several sensitive bacterial species treated with high-purity AS-48 after fixation with 2% glutaraldehyde. (A) *E. faecalis* S-47. (B) *S. aureus* ATCC 8. (C) *B. subtilis* CECT 397.

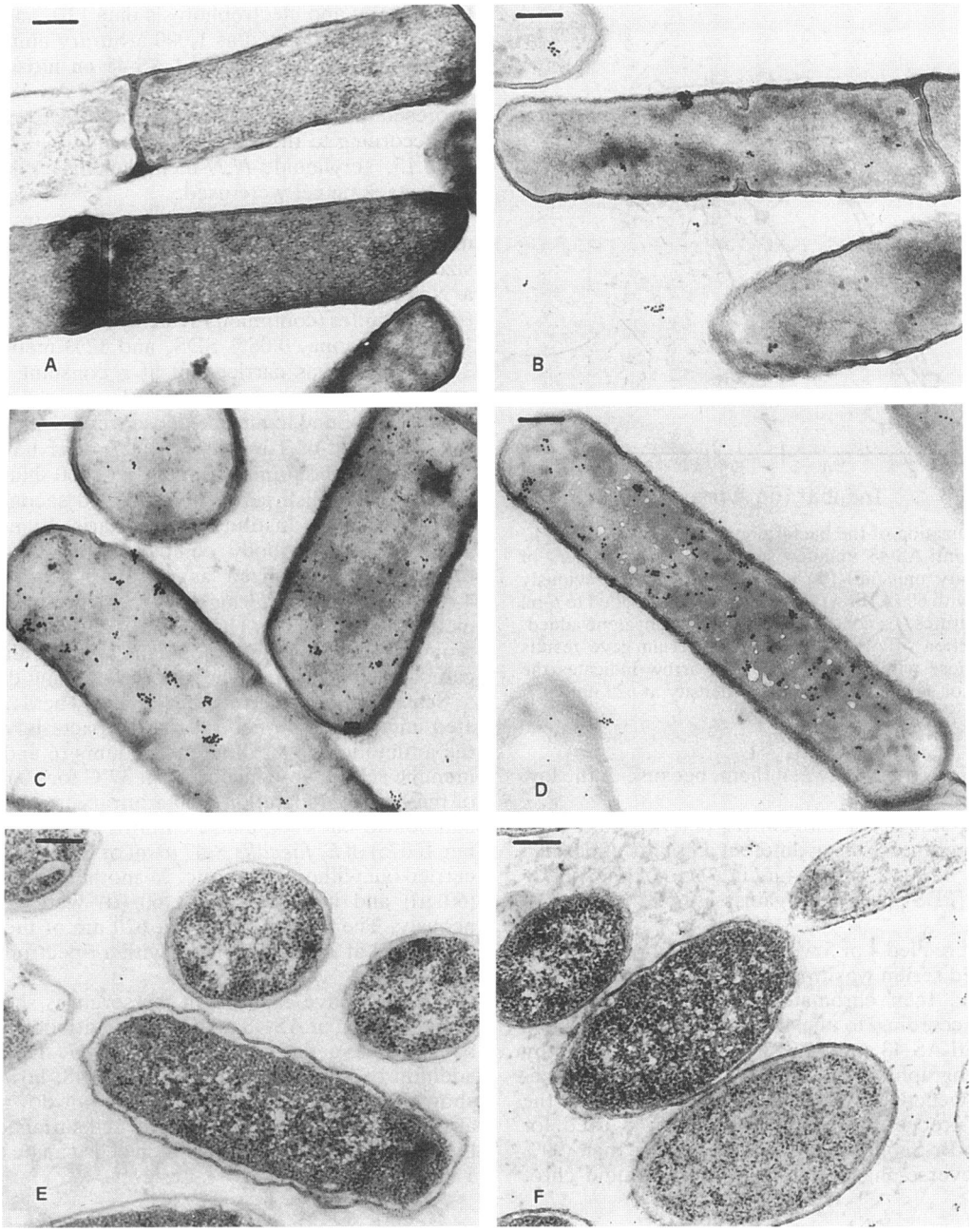

FIG. 4. Electron micrographs of ultrathin sections of *B. subtilis* CECT 397 (A, B, C, and D) and *E. coli* Delbruck (E and F) treated with high-purity AS-48, reacted with immune serum, and conjugated with anti-rabbit immunoglobulin G-gold (10-nm-diameter gold particles). (A) Untreated cells. (B and C) Cells treated with AS-48 for 15 and 90 min, respectively, before being embedded. (D) Cells treated with AS-48 for 15 min after being embedded. (E) Cells treated with AS-48 for 90 min before being embedded. (F) Cells treated with AS-48 for 15 min after being embedded. Bars: A, B, D, E, and F, 40 nm; C, 32 nm.

Neutralization of antibiotic activity may be attributed to steric hindrance of the antibody-antigen complexes or to direct binding of the antibodies to the active site of the peptide AS-48.

Immunocytochemical assays. (i) Study on sensitive cells by means of indirect immunofluorescence. A first assay was carried out with the sensitive strain *E. faecalis* S-47. Prior to treatment with the antiserum, the cells were permeabilized according to the method of Hasek et al. (9), with the following modifications: cells were fixed with glutaraldehyde (2%) for 30 min, treated with an excess of AS-48 for 15 min, and extensively washed with distilled water before being placed onto slides and allowed to air dry. The slides were rehydrated in an alcohol series (99, 96, and 90%) and then were twice rinsed with 0.1 M PBS, pH 7.0 and preincubated in 1% bovine serum albumin (BSA) in PBS for 20 min at room temperature. Afterward, the cells were incubated with the fractionated serum (diluted 1:100 in PBS plus 1% BSA)

for 48 h at 4°C and then were washed three times in PBS. Washed cells were incubated for 30 min at 37°C with goat anti-rabbit gamma globulin conjugated with fluorescein isothiocyanate (Institut Pasteur) diluted 1:50 in PBS plus Evan's blue (1:10,000), extensively washed in PBS and filtered distilled water, and finally covered with a drop of 50% glycerol in McIlvaine buffer, pH 7.2, plus *n*-propyl gallate (to reduce the photobleaching). Preparations were observed under a Leitz Dialux 22/22EB microscope equipped for epifluorescence microscopy and were photographed with a Wild MPS 51S camera using Kodak film (400 ASA).

Control samples of preimmune serum and untreated cells were also tested. Nevertheless, positive immunofluorescence obtained in all cases clearly demonstrated cross-reaction of the immune serum with components of cellular envelopes of the enterococci.

Thus, the experiment was repeated with specific antibodies obtained from affinity chromatography of crude serum and also by using sensitive *Bacillus subtilis* CECT 397 and *Staphylococcus aureus* ATCC 8 cells. Resistant bacteria (*E. coli* Delbruck and *Salmonella typhimurium* LT2) were also tested.

Sensitive cells treated with AS-48 had a bright uninterrupted outline around the surface (Fig. 3), while both resistant and untreated cells showed no fluorescence (result not shown). These results suggest the existence of binding sites for AS-48 in the bacterial wall which may be important in the first steps of the action of this antibiotic (penetration of bacterial envelopes) as well as in the induction of autolysis.

(ii) Electron microscopy. Examinations by transmission electron microscopy were made with the sensitive strain *B. subtilis* CECT 397 and also with the resistant strain *E. coli* Delbruck according to the method developed by Galli et al. (2).

Cells were treated with purified AS-48 in an excess of the amount required to induce lysis (data not shown) 5, 15, 30, or 90 min before or 15 min after being embedding into Spurr's resin. Embedded cells were sectioned with a Ultracut E Richter Ultramicrotome, mounted on uncoated nickel grids, treated immunocytochemically (dilutions of 1/200 of specific anti-AS-48 serum and 1/25 of goat anti-rabbit immunoglobulin G conjugated with colloidal gold particles with 10-nm diameters [Sigma]) and viewed under a Zeiss 902 electron microscope.

Transmission electron microscopy examination of samples of *B. subtilis* CECT 397 treated with AS-48 before and after embedding revealed few gold particles on the cell surface, most of the label being associated with cytoplasmic material (Fig. 4). Several dense accumulations were also located at the cell membrane (Fig. 4B and C). However, when a resistant strain was used for this experiment, preembedded cells showed no labelling on either the inner or outer envelopes (Fig. 4E), and only a few gold particles could be seen with nonspecific distribution in preparations of resistant cells subjected to a postembedding treatment (Fig. 4F).

These results indicate that AS-48 enters the cytoplasm of intact sensitive cells and, at the same time, has a large capacity for binding to cytoplasmic components, as shown by the results obtained after treating sectioned cells of *B. subtilis* CECT 397 (Fig. 4D).

The depolarizing effect exerted by AS-48 on cell membranes (5) may probably be interpreted as being a consequence of nonspecific membrane disorganization as AS-48 enters the cytoplasm. Although this effect alone is sufficient to induce death, secondary effects exerted at the cytoplasmic level should not be ruled out.

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