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Antibiotics Enhance Binding by Human Lipid A-Reactive Monoclonal Antibody HA-1A to Smooth Gram-Negative Bacteria

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The effect of antibiotic exposure of phenotypically smooth gram-negative bacteria on binding by the human lipid A-reactive monoclonal antibody HA-1A (trademark of Centocor, Inc.) was examined by liquid-phase immunoassay and by dual-parameter flow cytometry (fluorescence-activated cell sorter [FACS]) analysis. HA-1A exhibited dose-dependent binding to untreated rough gram-negative bacteria such as the *Escherichia coli* D21F2 Re chemotype strain but little binding to untreated smooth strains such as *E. coli* O111:B4, or to gram-positive bacteria. However, overnight incubation of *E. coli* O111:B4 with inhibitory concentrations of ceftazidime produced dose-dependent enhancement of HA-1A binding. Similar augmentation of HA-1A binding was observed when other smooth strains were exposed to cell wall-active agents. Dual-parameter FACS analysis of *E. coli* O111:B4 exposed overnight to two times the MIC of ceftazidime revealed a decrease in forward light scatter, indicating a reduction in average cell size or bacterial fragmentation, accompanied by a striking increase in lipid A-inhibitable HA-1A binding. Moreover, ceftriaxone, but not gentamicin, produced a marked increase in propidium iodide uptake, indicating an increase in bacterial cell permeability, and a corresponding enhancement of HA-1A binding. Antibiotic-induced enhancement of HA-1A binding to smooth strains of gram-negative bacteria thus appears related to specific alterations in bacterial cell morphology resulting in exposure of the epitope recognized by HA-1A.

Recent attention has focused on the specificity and function of antibodies that recognize phylogenetically conserved epitopes in the core and lipid A regions of bacterial endotoxin. Of particular interest is the protective role of such antibodies in naturally acquired immunity to gram-negative bacterial disease and their possible adjunctive role in the treatment of gram-negative sepsis (4, 21, 26). In this context, the human lipid A-reactive monoclonal antibody (MAb) HA-1A (trademark of Centocor, Inc.) is of particular interest because of its current use and ongoing testing in patients with gram-negative bacteremia and septic shock (36).

The ability of at least some anti-core and -lipid A antibodies to recognize corresponding epitopes on wild-type smooth lipopolysaccharide (LPS) or intact bacteria is restricted by the relative inaccessibility of such epitopes to antibody attack due to overlying O polysaccharide and core structures (7, 13, 18, 22, 27, 28). Physicochemical factors other than those inherent in the primary structure of LPS may also critically affect antibody recognition of core- and lipid A-associated epitopes (8). Anti-lipid A antibodies may, for example, react better with LPS associated with the bacterial cell surface than with isolated LPS, and physical or chemical manipulation of the bacteria may further enhance this reactivity (10, 28).

Certain antibiotics, particularly cell wall-active agents, produce profound alterations in the outer membrane structure and cell morphology of exposed bacteria. These effects may expose cryptic epitopes in the core and lipid A regions of cell-associated LPS, as evidenced by the enhanced reactivity of certain core- and lipid A-reactive antibodies with antibiotic-treated bacteria (12, 23, 24). Both cell-associated and cell-free LPS are produced during infection, although the comparative pathogenic functions of these two forms of LPS are unknown. It is clear, however, that at least some and perhaps most antibiotics release LPS from the bacterial surface and possibly alter the antigenic structure of LPS remaining on the cell surface (32, 33).

Because the lipid A-reactive MAb HA-1A is used in septic patients in conjunction with antibiotic therapy, it is critical to understand possible immunochemical interactions between these therapeutic agents. Indeed, potential clinical interactions between antibodies and antibiotic therapies were considered as far back as 50 years ago (2, 35). The purpose of this study was to evaluate binding of HA-1A to gram-negative bacteria exposed in vitro to inhibitory concentrations of antibiotics achieved in vivo in clinical practice and to correlate possible antibiotic-associated differences in HA-1A binding with antibiotic-induced alterations in bacterial cell morphology.

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MATERIALS AND METHODS

Bacterial cultures. The following cultures were obtained from the American Type Culture Collection (ATCC, Rockville, Md.): *Klebsiella pneumoniae* (ATCC 13883), *Serratia marcescens* (ATCC 14757), *Enterobacter aerogenes* (ATCC 13048), *Proteus mirabilis* (ATCC 7002), *Pseudomonas aeruginosa* (ATCC 27853), *Streptococcus pyogenes* (ATCC 19615), *Streptococcus faecalis* (ATCC 19433), and *Staphylococcus epidermidis* (ATCC 14990). *Escherichia coli* O111:B4, *E. coli* J5 (Rc chemotype rough mutant), and *Salmonella minnesota* Rd (rough mutant) were obtained from E. Ziegler (University of California at San Diego, San Diego). *E. coli* D21F2 (Re chemotype rough mutant) was obtained from E. McGroarty (Michigan State University, East Lansing). Cultures of gram-negative isolates from patients enrolled in the HA-1A clinical trial (36) were obtained from Bender Hygienic Laboratory (Albany, N.Y.), which served as a reference laboratory for the HA-1A clinical study. The clinical isolates were sent directly from the microbiology laboratories of the participating medical centers to the reference laboratory, where the culture identification was confirmed and lyophilized stocks were prepared. Clinical isolates were typically subcultured a total of five to seven times prior to analysis of HA-1A binding. For binding studies, cultures were seeded from colonies on agar slants or plates and grown in 15-ml Trypticase soy broth tubes (BBL Microbiology Systems, Cockeysville, Md.) at 37°C with constant shaking. For antibiotic treatment, fresh cultures were grown overnight and then adjusted to an optical density (OD) of 0.600 at 660 nm (ca. 2×10^8 CFU/ml) and incubated for 16 to 18 h in the presence of antibiotic at 37°C with constant shaking. Antibiotics were prepared fresh or stored according to the manufacturer's instructions.

Antibodies. HA-1A was produced at Centocor, Inc., by continuous-perfusion cell culture and was purified from the supernatant fluid by a series of steps involving selective precipitation and a series of chromatographic steps including anion and cation exchange and size exclusion. A control human immunoglobulin M (IgM) myeloma antibody was obtained from Jackson ImmunoResearch (West Grove, Pa.), and control human anti-cytomegalovirus IgM MAb C58 (9) was produced at Centocor, Inc., in a manner similar to production of HA-1A. ^{125}I-labeled sheep F(ab')$_2$ anti-human Ig antibody was obtained from Amersham (Arlington Heights, Ill.), and the specific activity ranged from 5 to 20 μCi/μg. Fluorescein-labeled (fluorescein isothiocyanate [FITC]) goat F(ab')$_2$ anti-human IgM was obtained from Jackson ImmunoResearch or from Organon Teknika (West Chester, Pa.). The murine 9B5.5 anti-HA-1A MAb (14) and control murine MT-412 anti-CD4 MAb (29) were produced at Centocor, Inc., and F(ab')$_2$ fragments were prepared by pepsin digestion and purified by Fast-Flow S Sepharose (Pharmacia, Piscataway, N.J.) ion-exchange chromatography. The murine anti-HA-1A idiotype MAb 9B5.5 has been previously described (14), and recent studies have shown that it binds at or near the antigen-combining site of HA-1A (7a).

Antibiotics. Antibiotics were obtained from the following vendors: ceftazidime, Glaxo (Research Triangle Park, N.C.); imipenem-cilastatin (Imipenem), Merck Sharp & Dohme (West Point, Pa.); ceftriaxone, Hoffmann-La Roche Inc. (Nutley, N.J.); piperacillin, Lederle (Carolina, P.R.); gentamicin, Sigma Chemical Co. (St. Louis, Mo.); ciprofloxacin, Miles, Inc. (West Haven, Conn.); tetracycline, Warner Chilcott Labs (Morris Plains, N.J.); and polymyxin B, Burroughs Wellcome Co. (Research Triangle Park, N.C.).

Radiolabeling of antibodies. HA-1A and control antibody were radiolabeled with Na^{125}I (Amersham or Dupont, NEN Research Products) by using Iodogen beads (Pierce Chemicals, Rockford, Ill.) and passed through a Sephadex G-25 column to remove unreacted ^{125}I iodide. The antibody concentration after iodination was determined by IgM capture enzyme-linked immunosorbent assay (ELISA). The iodination procedure typically yielded ^{125}I-HA-1A or ^{125}I-control antibody with specific activities of ca. 0.1 μCi/μg. The immunoreactive fraction for ^{125}I-HA-1A binding to *E. coli* D21F2 was greater than 90% by the method of Lindmo et al. (16).

MIC determination. MICs for the various antibiotics were determined in a 96-well microtiter format by mixing serial twofold dilutions of antibiotics with 10^8 CFU of the various bacteria per ml and incubating for 16 to 18 h at 37°C in Trypticase soy broth. Growth was monitored by observing the OD at 630 nm in a Dynatech (Chantilly, Va.) MR5000 microtiter plate reader. The bacterial inoculum size was selected to mimic the conditions used in subsequent binding experiments.

Bacterial binding liquid-phase RIA. Bacterial cultures were grown as described above, washed two times in pyrogen-free saline, and adjusted to an OD of 0.600 at 660 nm in a Milton Roy (Rochester, N.Y.) Spectronic 1201 spectrophotometer, using 3% bovine serum albumin (BSA) in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–0.9% NaCl (pH 7.2) (assay buffer) as the diluent. This corresponded to approximately 2×10^8 CFU/ml. Since the antibiotic-treated cultures could not be standardized by bacterial colony counting, these were adjusted to an OD of 0.600 at 660 nm in assay buffer. Each binding experiment was internally controlled by comparison of HA-1A binding to control human IgM. Equal volumes of test bacteria and antibody were incubated together for 1 h at 4°C in a 96-well Removawell reaction plate (Dynatech) that had been blocked for 1 h at 37°C with 10% nonfat dried milk solution. Total volume was 200 μl. All antibody dilutions were made in assay buffer. Bacteria were pelleted by centrifugation at 3,000 rpm for 10 min in a Sorvall (Du Pont Co., Wilmington, Del.) RT 6000 or GLC-2B centrifuge to facilitate washing. After two washes, antibody bound to the bacteria was detected by the addition of approximately 100,000 cpm of the radiolabeled sheep anti-human antibody probe for 1 h at 4°C. After two washes, radioactivity bound to the pelleted bacteria was determined in an LKB Wallac (Turku, Finland) gamma counter by removing the individual reaction wells and counting the radioactivity in each. To facilitate autoclaving of assay plates and disposables used with gram-negative clinical isolates, we adapted the assay to a nonradioactive ELISA format. This was accomplished by substituting an alkaline phosphatase-conjugated goat anti-human IgM antibody (Jackson ImmunoResearch) at 1:1,000 dilution for the radiolabeled probe. After washing and a 20-min paranitrophenylphosphate substrate development, the OD at 410 nm was determined in a Dynatech MR5000 microtiter plate reader. For both radioimmunoassay (RIA) and ELISA formats, assay points were generally run in triplicate.

HA-1A affinity determination. The affinity of HA-1A binding to gram-negative bacteria was estimated using ^{125}I-radiolabeled HA-1A or control human IgM antibody radiolabeled to a specific activity between 0.1 and 0.4 μCi/μg. Bacteria (10^8 CFU/ml final concentration or equivalent OD at 660 nm) and radiolabeled antibody were incubated at 4°C

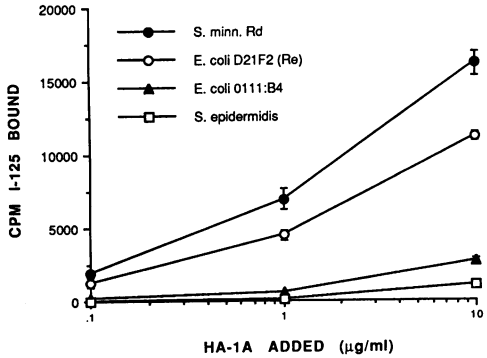

FIG. 1. Binding of HA-1A to strains of live gram-negative and gram-positive organisms by RIA. Binding of a negative-control human IgM (C58, anticytomegalovirus) at 10 μg/ml was as follows: *S. minnesota* Rd = 2,001 cpm; *E. coli* D21F2 = 2,575 cpm; *E. coli* O111:B4 = 2,015 cpm; and *S. epidermidis* = 735 cpm.

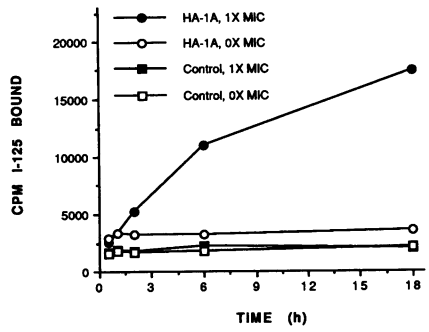

FIG. 3. Binding of HA-1A (10 μg/ml) or control human IgM (10 μg/ml) to *E. coli* O111:B4 that had been treated with ceftazidime (1× MIC) for various periods. MIC of ceftazidime = 8 μg/ml. Binding was also assessed with mock-treated cultures (0× MIC).

in phosphate-buffered saline (PBS)–3% BSA in a total volume of 50 μl in 96-well V-bottom polyvinyl microtiter plates that had been blocked with 10% nonfat dry milk in PBS. After a 24-h incubation, the bacteria were pelleted and a 20-μl aliquot of supernatant was removed and its radioactivity was counted to determine free antibody. Cell pellets were washed three times with 0.05% Tween 80 in PBS, transferred to another 96-well plate, and pelleted. Wells were cut and their radioactivity was counted to determine bound antibody. Nonspecific antibody binding was determined by performing the same experiment with radiolabeled control human IgM antibody, and this varied from 1 to 5% of HA-1A binding at saturation. Molar quantities of bound and free HA-1A were determined from control human IgM-subtracted HA-1A counts per minute values. Average affinity constants were then estimated from a modification of Scatchard analysis (30).

FACS analyses. Fluorescence-activated cell sorter (FACS) analyses of antibody binding to bacteria were performed as previously described (10). Samples were prepared as described above except that FITC-labeled goat anti-human IgM probe (20 μg/ml, final concentration) was substituted for the radiolabeled probe. The final bacterial antigen pellet was fixed in 200 μl of 1% paraformaldehyde, and a 1/100 dilution of the fixed antibody-bacterium complex was analyzed in a Becton Dickinson (Sunnyvale, Calif.) FACscan flow cytometer. For two-color analysis, the DNA-specific fluorochrome propidium iodide (Sigma) was added to the antibody-bacteria complexes at 10 μg/ml for 15 min prior to analysis. HA-1A binding was assessed in comparison to an equal concentration of control human IgM antibody.

Competition studies. The effect of polymyxin B, 9B5.5 anti-idiotype MAb, and purified lipid A on HA-1A binding was assessed by coincubating various concentrations of the inhibitors with a fixed concentration of HA-1A and bacteria. For polymyxin B, bacterial concentration was adjusted to a final level of 10^7 CFU/ml, or an equivalent OD at 660 nm, in order to reduce the total number of lipid A-binding sites in the reaction mixture.

RESULTS

Binding of HA-1A to intact, untreated bacteria. HA-1A exhibited dose-dependent binding to untreated rough gram-negative bacteria by RIA, but little binding to untreated smooth strains such as *E. coli* O111:B4, or to untreated gram-positive bacteria such as *S. epidermidis* (Fig. 1). The average association constant for HA-1A binding to untreated *E. coli* D21F2 Re chemotype bacteria was estimated by Scatchard analysis to be $(3.8 \pm 2.2) \times 10^8\ M^{-1}$, $N = 5$ (data

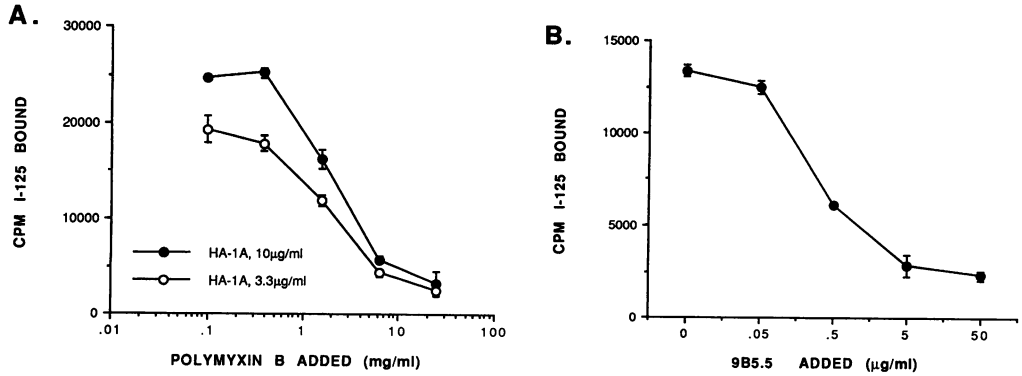

FIG. 2. Competition of HA-1A binding to *E. coli* D21F2 by polymyxin B (A) and an $F(ab')_2$ fragment of murine 9B5.5 anti-HA-1A idiotype MAb (B). (A) Various concentrations of polymyxin B were added to 2×10^7 CFU of bacteria per ml with fixed HA-1A concentrations of 3.3 and 10 μg/ml. (B) Various concentrations of anti-idiotype MAb 9B5.5 were added to bacteria with a fixed concentration of 5 μg of HA-1A per ml. After a 1-h incubation at 4°C, HA-1A binding was detected with a ^{125}I-labeled sheep anti-human antibody probe for both experiments. In panel B, binding of HA-1A with 50 μg of control murine MT-412 $F(ab')_2$ (anti-CD4) per ml added was 11,827 ± 463 cpm.

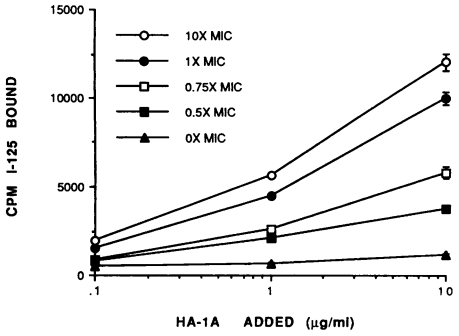

FIG. 4. Binding of HA-1A to *E. coli* O111:B4 that had been treated with various doses of ceftazidime. MIC of ceftazidime = 8 μg/ml. Binding of 10-μg/ml negative control human IgM was 2,882 ± 185 cpm with 10× MIC-treated cultures and 1,263 ± 38 cpm with untreated cultures.

not shown). The reactivity of HA-1A with *E. coli* D21F2 was inhibited in a dose-dependent manner by polymyxin B (Fig. 2A), which binds to the lipid A component of LPS (20). Reactivity was also inhibited by $F(ab')_2$ fragments derived from the HA-1A anti-idiotype MAb 9B5.5 (Fig. 2B), but not by $F(ab')_2$ fragments from a control anti-CD4 MAb of the same murine isotype tested at 50 μg/ml.

Binding of HA-1A to ceftazidime-treated *E. coli* O111:B4. Incubation of the smooth strain, *E. coli* O111:B4, with 1× MIC of ceftazidime at 37°C produced a time-dependent enhancement of HA-1A binding to antibiotic-exposed bacteria (Fig. 3). Enhanced HA-1A binding was evident with cultures exposed to antibiotic for as short as 2 h and appeared to plateau after 18 h of exposure. No enhancement of HA-1A binding was observed with mock-treated cultures, and control IgM showed no binding to either antibiotic- or mock-treated cultures (Fig. 3). Figure 4 shows the effect of antibiotic dose on cultures exposed for 18 h to ceftazidime. Maximal HA-1A binding, observed at approximately 10× MIC of ceftazidime, was similar to that observed for untreated rough strains. HA-1A binding to ceftazidime-treated *E. coli* O111:B4 was inhibited by polymyxin B (Fig. 5A) and by $F(ab')_2$ fragments derived from the HA-1A anti-idiotype MAb 9B5.5 (Fig. 5B), in a manner similar to that observed for rough bacteria. The average association constant estimated for HA-1A binding to the ceftazidime-treated *E. coli* O111:B4 smooth strain was $(6.6 \pm 3.1) \times 10^7\ M^{-1}$, $N = 3$ (data not shown). This was slightly lower than that estimated for HA-1A binding to the *E. coli* D21F2 Re chemotype rough mutant.

Effect of pretreatment of *E. coli* O111:B4 with various antibiotics on HA-1A binding. Overnight incubation of the *E. coli* O111:B4 smooth strain at 37°C in the presence of 10× MIC of various antibiotics resulted in up to a 13-fold increase in HA-1A-binding signal, as measured by RIA (Table 1). The observed increases in HA-1A binding appeared to be greater among bacteria exposed to cell wall-active agents (ceftazidime, ceftriaxone, piperacillin, imipenem) than those treated with antibiotics having intracellular targets (ciprofloxacin, tetracycline). However, some differences in HA-1A binding were also noted among various cell wall-active agents (Table 1).

Effect of ceftazidime pretreatment of various bacteria on HA-1A binding. Overnight incubation of various species of phenotypically smooth gram-negative bacteria in the presence of 10× MIC of ceftazidime produced up to 12-fold increases in HA-1A-binding signal, as measured by RIA (Table 2). In contrast, no increased binding by HA-1A was observed for gram-positive organisms exposed to similar, inhibitory concentrations of ceftazidime (Table 2). Data obtained by ELISA of HA-1A binding to imipenem-treated compared with untreated bacterial isolates from patients with gram-negative sepsis documented the consistency of antibiotic-induced enhancement of HA-1A binding to phylogenetically diverse, phenotypically smooth gram-negative bacteria. In this assay format, up to a 100-fold increase in HA-1A-binding signal was measured in antibiotic-exposed compared with untreated isolates (Table 3). Similar results were obtained in the ELISA format with acid polysaccharide-encapsulated *E. coli* O111a, O111b, and O86:K1 (data not shown), indicating that these strains are also susceptible to antibiotic-enhanced HA-1A binding.

FACS analysis of HA-1A binding to antibiotic-treated bacteria. Dual-parameter FACS analyses were used to further describe the antibiotic-induced enhancement of HA-1A binding to exposed bacteria and to relate this effect to changes in bacterial cell or particle size (forward light scatter) and permeability (propidium iodide staining). *E. coli*

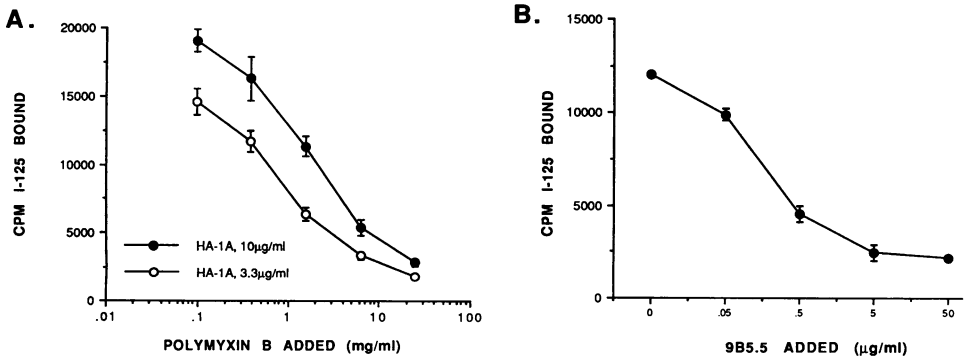

FIG. 5. Competition of HA-1A binding 80-μg/ml ceftazidime-treated (10× MIC) *E. coli* O111:B4 by polymyxin B (A) and an $F(ab')_2$ fragment of murine 9B5.5 anti-HA-1A idiotype MAb (B). (A) Various concentrations of polymyxin B were added to the equivalent of 2×10^7 CFU of bacteria per ml with fixed HA-1A concentrations of 3.3 and 10 μg/ml. (B) Various concentrations of anti-idiotype MAb 9B5.5 were added to a fixed concentration of 5 μg of HA-1A per ml. After a 1-h incubation at 4°C, HA-1A binding was detected with ^{125}I-labeled sheep anti-human antibody probe for both experiments. In panel B, binding of HA-1A with 50 μg of control murine $F(ab')_2$ (anti-CD4) per ml added was 10,157 ± 1,311 cpm.

TABLE 1. Binding of HA-1A to *E. coli* O111:B4 treated with various antibiotics, measured by RIA

Antibiotic (mg/ml)[a]	HA-1A bound (of ^{125}I)[b]			Control IgM bound (of ^{125}I)[b]		
	Untreated	+ Antibiotic	Signal ratio[c]	Untreated	+ Antibiotic	Signal ratio[c]
Ceftazidime (0.08)	6,137 ± 164	26,234 ± 653	4.3	2,725 ± 125	3,714 ± 276	1.4
Ceftriaxone (0.08)	7,579 ± 695	19,771 ± 2,894	2.6	3,745 ± 402	3,719 ± 145	1.0
Piperacillin (0.01)	3,345 ± 502	45,454 ± 2,455	13.6	4,820 ± 743	7,337 ± 640	1.5
Imipenem (0.10)	5,312 ± 201	25,946 ± 874	4.9	3,584 ± 101	5,205 ± 131	1.5
Ciprofloxacin (0.10)	2,524 ± 480	6,373 ± 649	2.5	4,089 ± 133	4,406 ± 196	1.1
Tetracycline (0.01)	3,310 ± 278	4,826 ± 62	1.5	4,020 ± 251	5,305 ± 60	1.3

[a] Concentrations of antibiotic are equal to 10 times the MIC.
[b] Binding of 10 μg of HA-1A per ml or control human IgM. Data are averages of triplicate determinations ± standard deviations.
[c] Signal ratios are of ^{125}I bound with antibiotic-treated cultures/of ^{125}I bound with control untreated cultures.

O111:B4 incubated overnight in the presence of a subinhibitory concentration (0.5× MIC) of ceftazidime showed an increase in forward light scatter consistent with the formation of globular forms (17), while no net increase in HA-1A binding was observed (Fig. 6). In contrast, when *E. coli* O111:B4 was exposed overnight to an inhibitory concentration (2× MIC) of ceftazidime, a decrease in forward light scatter, indicating a reduction in average cell size or bacterial fragmentation, was accompanied by a striking increase in HA-1A binding (Fig. 6). The enhanced reactivity of HA-1A with ceftazidime-treated *E. coli* O111:B4 was inhibited in a dose-dependent manner by purified *E. coli* J5 lipid A (Fig. 7).

In a second type of dual-parameter FACS analysis, binding of fluorescein-labeled HA-1A to *E. coli* O111:B4 was correlated with propidium iodide uptake after overnight exposure of the bacteria to 10× MIC of the cell wall-active antibiotic ceftriaxone, 10× MIC of gentamicin, or no antibiotic (Fig. 8). The DNA-specific fluorochrome propidium iodide is taken up by permeabilized cells (10, 31). As indicated in Fig. 8, ceftriaxone produced a striking increase in bacterial cell permeability (propidium iodide uptake), while gentamicin did not. This increase in the permeability of ceftriaxone-treated bacteria was accompanied by a corresponding enhancement of HA-1A binding. Gentamicin pretreatment, in contrast, produced no increase in antibody binding (Fig. 8). There was thus a strong correlation between antibiotic-induced bacterial cell permeabilization and enhanced HA-1A binding. The majority (82%) of bacteria exposed overnight to 10× MIC of ceftriaxone, under the conditions of this experiment, exhibited both increased cell permeability and increased HA-1A binding.

DISCUSSION

HA-1A is a human IgM lipid A-reactive MAb intended for clinical use in patients with gram-negative bacteremia and septic shock (5, 6, 7a, 34, 36). As documented in this study, HA-1A binds to rough bacteria, while exhibiting limited reactivity with phenotypically smooth organisms grown under conventional in vitro conditions. The antibody demonstrates enhanced binding, however, to phenotypically smooth bacteria that have been exposed to inhibitory concentrations of certain antibiotics, particularly cell wall-active agents. For example, binding by HA-1A to *E. coli* O111:B4 after overnight exposure of the organism to inhibitory concentrations of ceftazidime was comparable to HA-1A binding to the untreated Re chemotype rough mutant *E. coli* D21F2. In both cases, HA-1A binding was characterized by similar functional affinity and was inhibitable by purified lipid A (data shown for *E. coli* O111:B4 but not D21F2), polymyxin B, and $F(ab')_2$ fragments of an HA-1A anti-idiotype MAb. These data suggest that HA-1A reactivity with antibiotic-treated smooth bacteria was based on antibody recognition of a lipid A-associated epitope indistinguishable from that recognized by HA-1A on untreated rough organisms.

Enhanced HA-1A binding to antibiotic-modified smooth bacteria was associated with alterations in bacterial cell morphology, as documented by dual-parameter FACS analyses. There was a strong correlation, for example, between increased fluorescence associated with ceftazidime-induced HA-1A binding to *E. coli* O111:B4 and a decrease in forward light scatter, indicating preferential antibody binding to small or fragmented bacteria. Similarly, ceftriaxone and other cell

TABLE 2. Binding of HA-1A to various strains of bacteria treated with ceftazidime, measured by RIA

Organism	Strain	Antibiotic concn[a] (mg/ml)	HA-1A bound (cpm of ^{125}I)[b]			Control IgM bound (cpm of ^{125}I)[b]		
			Untreated	+ Antibiotic	Signal ratio[c]	Untreated	+ Antibiotic	Signal ratio[c]
E. coli	O111:B4	0.08	3,071 ± 125	10,568 ± 876	3.4	1,304 ± 40	1,247 ± 69	1.0
K. pneumoniae	ATCC 13883	0.04	1,056 ± 77	13,642 ± 1,406	12.9	1,676 ± 69	2,250 ± 246	1.3
S. marcescens	ATCC 14757	12.5	1,378 ± 54	10,282 ± 1,809	7.4	2,043 ± 141	1,564 ± 27	0.8
E. aerogenes	ATCC 13048	1.25	961 ± 100	11,527 ± 511	12.0	1,276 ± 190	1,227 ± 106	1.0
P. mirabilis	ATCC 7002	0.30	1,953 ± 310	6,103 ± 981	3.1	1,380 ± 30	1,951 ± 119	1.4
P. aeruginosa	ATCC 27853	15	2,418 ± 168	9,846 ± 1,184	4.1	2,546 ± 232	3,087 ± 168	1.2
S. pyogenes	ATCC 19615	50	1,530 ± 106	1,356 ± 85	0.9	1,062 ± 70	1,080 ± 68	1.0
S. faecalis	ATCC 19433	50	1,301 ± 91	719 ± 103	0.6	750 ± 73	855 ± 76	1.1

[a] Concentrations of ceftazidime are equal to 10 times the MIC.
[b] Binding of 10 μg of HA-1A per ml or control human IgM. Data are averages of triplicate determinations ± standard deviations.
[c] Signal ratios are of ^{125}I bound with antibiotic-treated cultures/of ^{125}I bound with control untreated cultures.

TABLE 3. Binding of HA-1A to gram-negative clinical isolates treated with imipenem, measured by ELISA

Gram-negative isolate (isolate no.)	Antibiotic concn[a] (mg/ml)	HA-1A bound (OD at 410 nm)[b]			Control IgM bound (OD at 410 nm)[b]		
		Untreated	+ Antibiotic	Signal ratio[c]	Untreated	+ Antibiotic	Signal ratio[c]
E. coli							
0720	0.02	0.000	1.236	NC	0.000	0.000	NC
1050	0.02	0.000	0.964	NC	0.000	0.000	1
2839	0.10	0.037	0.565	15.3	0.017	0.047	2.8
8927	0.06	0.000	1.379	NC	0.000	0.038	NC
P. aeruginosa							
0506	0.01	0.107	0.521	4.9	0.032	0.021	0.7
3314	0.10	0.261	0.423	1.6	0.191	0.056	0.3
3503	0.10	0.060	1.248	20.8	0.036	0.034	0.9
S. marcescens							
0617	0.25	0.202	0.810	4.0	0.183	0.000	NC
2837	0.06	0.020	0.237	11.9	0.017	0.010	0.6
0715	0.02	0.013	0.607	46.7	0.015	0.032	2.1
E. aerogenes							
0714	0.08	0.016	1.587	99.2	0.027	0.018	0.7
3311	0.30	0.003	0.708	236	0.021	0.021	1
E. cloacae 0717	0.20	0.164	1.040	6.3	0.041	0.105	2.6
K. pneumoniae 3509	0.03	0.000	0.815	NC	0.000	0.000	NC
K. oxytoca 8911	0.02	0.043	0.587	13.7	0.016	0.010	0.6

[a] Cultures were treated with a dose of imipenem equal to 10 times the MIC.
[b] Binding of 10 μg of HA-1A per ml or control human IgM as detected with alkaline phosphatase-labeled goat anti-human IgM. Data are the averages of triplicate determinations with background (binding of anti-human IgM antibody probe alone) subtracted.
[c] Signal ratios are OD at 410 nm of antibiotic-treated cultures/OD at 410 nm of control untreated cultures. NC, not calculated.

wall-active antibiotics, but not gentamicin, which targets bacterial protein synthesis, produced a marked increment in propidium iodide uptake, indicating an increase in bacterial cell permeability, in association with enhanced HA-1A binding. Together, these data suggest that antibiotic-induced enhancement of HA-1A binding to smooth strains of gram-negative bacteria was related to specific alterations in bacterial cell morphology resulting in exposure of the lipid A epitope recognized by HA-1A.

The analyses done in this study involved a number of different antibiotics and bacterial isolates. The former represented a sampling of cell wall-active drugs as well as agents with intracellular sites of action, and the latter included gram-negative organisms from diverse clinical sources and phylogenetic groups. While cell wall-active agents appeared, in general, to enhance HA-1A binding more than non-cell wall-active antibiotics, measurable differences in the relative magnitude of this effect were noted among different cell wall-active agents as well. More comprehensive comparative evaluations of antibiotics representing different antibiotic classes are required to document differential effects on HA-1A binding. The finding of low levels of HA-1A binding to untreated smooth phenotype gram-negative bacteria in the present study differed from some previously reported findings with this antibody (34). While the reasons for this discrepancy are unclear, it may in part be due to differences in assay format and antigen form, since these factors have been shown to profoundly influence in vitro binding interactions of anti-LPS antibodies (3, 8).

The observed enhancement by antibiotics of HA-1A binding to phenotypically smooth gram-negative bacteria may be a paradigm for other factors or processes encountered by bacteria in the infected host that are capable of modifying the antigenicity of LPS or other bacterial cell-associated structures. Alterations in LPS antigenicity may accompany different phases of normal bacterial growth (11, 19, 28) or result from the complex in vivo actions or interactions of host factors such as antibody, complement, phagocytic cells, and administered antibiotics (1, 25). For example, exposure of bacteria to non-cell wall-active antibiotics for periods longer than in the present study, as might occur in vivo, could lead to the eventual disruption of bacterial membrane integrity and exposure of reactive lipid A epitopes. Any of these factors, acting singly or in combination, may influence the presentation of deep LPS structures to the immune system as well as the accessibility of these structures to therapeutically administered antibodies such as HA-1A.

It is clear from the antibiotic-induced enhancement of HA-1A binding to smooth bacteria (and/or bacterial fragments) that certain antibiotics are capable of modifying the antigenic structure or presentation of LPS on the bacterial cell surface. It is as yet unclear whether similar modifications affect LPS released from the bacterial surface, i.e., free LPS. The functional consequences of enhanced HA-1A binding to antibiotic-modified intact or fragmented bacteria require further elucidation. Moreover, the potential augmentation of HA-1A binding to antibiotic-released, free LPS requires documentation.

It is possible that HA-1A bound to the surfaces of antibiotic-exposed bacteria could participate in the complement-mediated bacteriolysis or opsonophagocytic killing of those bacteria. However, the results of dual-parameter FACS analyses presented here suggest that the antibiotic-induced

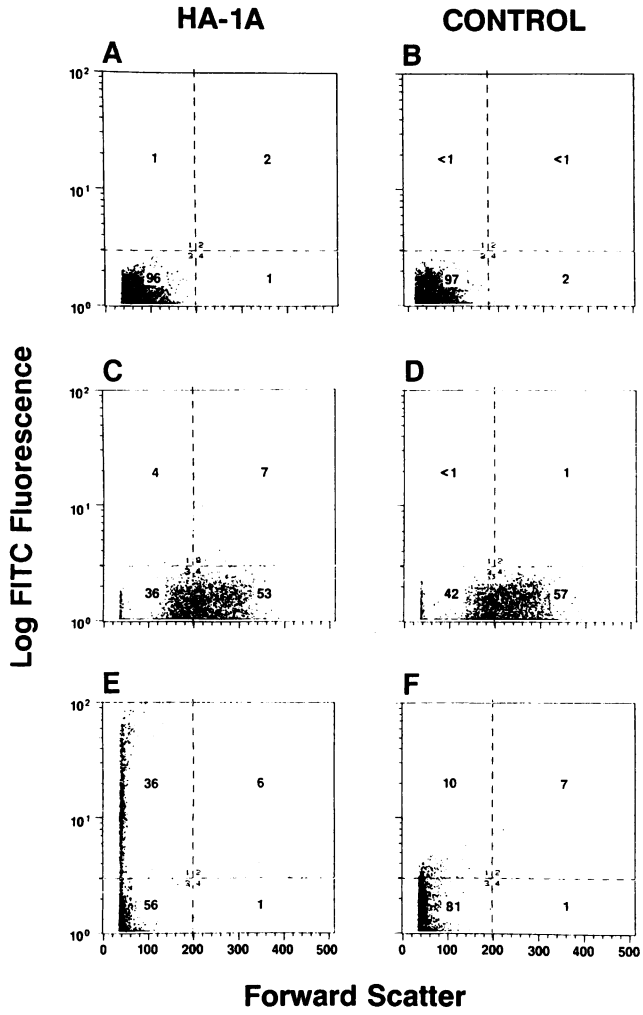

FIG. 6. FACS analysis of 10 μg of HA-1A per ml (A, C, and E) and negative control human IgM (B, D, and F) binding to untreated (A and B), 4 μg/ml (0.5× MIC) ceftazidime-treated (C and D), and 16-μg/ml (2× MIC) ceftazidime-treated (E and F) *E. coli* O111:B4. After a 1-h incubation at 4°C, HA-1A binding was detected with an FITC-labeled goat anti-human antibody. Numbers are percentage of cells in each quadrant.

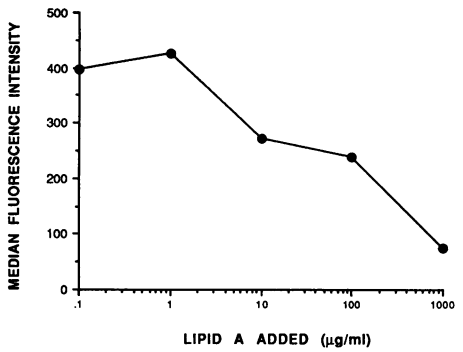

FIG. 7. Competition of HA-1A binding to 80-μg/ml (10× MIC) ceftazidime-treated *E. coli* O111:B4 by lipid A in the FACS format. Various concentrations of *E. coli* J5 lipid A were added to ca. 10^8 CFU of bacteria per ml with a fixed HA-1A concentration of 10 μg/ml. After a 1-h incubation at 4°C, HA-1A binding was detected with an FITC-labeled goat anti-human antibody.

enhancement of HA-1A binding is closely associated with changes in bacterial cell morphology, i.e., permeabilization and possible cell fragmentation, which indicate loss of cell viability. If this apparent loss of cell viability preceded HA-1A binding, the latter would not contribute significantly to bacterial killing. However, the antibiotic-enhanced recognition by HA-1A of corresponding epitopes on nonviable bacteria, bacterial fragments, or free LPS released from bacteria might result in their effective opsonization and eventual detoxification and/or disposal. In support of this hypothesis is the recent observation that HA-1A forms immune complexes with free LPS, deposits C3b on these complexes, and mediates complement-dependent attachment (i.e., immune adherence) of the opsonized complexes to CR1 receptors on human erythrocytes and polymorphonuclear leukocytes (15). These preliminary in vitro findings suggest that HA-1A and possibly other LPS antibodies may play an important humoral role in the transport and disposal of free or membrane-bound LPS through fixed tissue macrophages and polymorphonuclear leukocytes. Antibiotics could participate in this or other antibody-mediated processes by facilitating the recognition by HA-1A or other antibodies of previously unexposed epitopes on lipid A or other structural elements of the LPS core region, respectively.

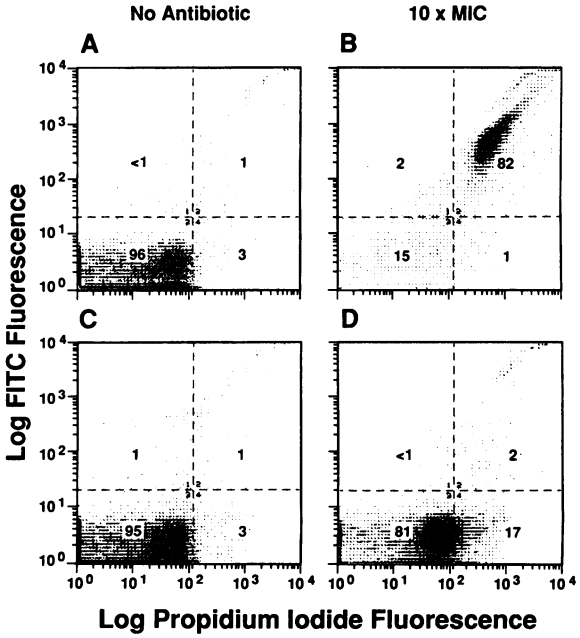

FIG. 8. Dual-fluorescence FACS histograms showing relation between HA-1A (FITC) binding and loss of cell wall integrity (propidium iodide uptake) in 80-μg/ml (10× MIC) ceftriaxone (B)- and 150-μg/ml (10× MIC) gentamicin (D)-treated versus untreated (A and C) *E. coli* O111:B4 cultures. HA-1A (10 μg/ml) was added to the bacterial cultures for 1 h at 4°C, after which binding was determined with an FITC-labeled goat anti-human antibody. Propidium iodide was added during the last 15 min of the 1-h incubation with the FITC-labeled antibody. Numbers are percentage of cells in each quadrant.

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