Activity and Specificity of a Mouse Monoclonal Antibody to Ferric Aerobactin

DIDIER LE ROY,1 DOMINIQUE EXPERT,2 ALAIN RAZAFINDRATSITA,3 ALAIN DEROUSSENT,4 JOSE COSME,3 CLAUBE BOHUON,3 AND ANTOINE ANDREMONT1,5

Laboratoire d'Ecologie Microbiene,1* Département de Biologie Clinique,2 and Centre National de la Recherche Scientifique URA 158,3 Institut Gustave-Roussy, 94800 Villejuif, Laboratoire de Pathologie Végétale, Institut National Agronomique, 75005 Paris,2 and Laboratoire de Microbiologie, Faculté de Pharmacie, 92290 Chatenay-Malabry, France

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We isolated a monoclonal antibody directed against the ferric complex of aerobactin purified from Escherichia coli KH576. This antibody, which we designated MAB AERO1, was identified as an immunoglobulin G, subtype 2. A competitive enzyme-linked immunosorbent assay with MAB AERO1 had a limit of 10 nM for the detection of purified ferric aerobactin and allowed detection of the crude aerobactin produced by various members of the family Enterobacteriaceae isolated from cancer patients with bacteremia. The only two other structurally related siderophores recognized by MAB AERO1 were ferric arthrobactin and ferrioxamine B. These results suggest that the epitope recognized by MAB AERO1 was the lysyl moiety of ferric aerobactin. We also showed that MAB AERO1 reduced the growth of an aerobactin-producing strain of E. coli in newborn calf serum, which indicates that it might be effective in reducing the severity of infections caused by bacteria for which the production of aerobactin is an important virulence factor.

Although bacterial virulence is thought to be a multifactorial and polygenic process, the availability of iron is considered crucial for invading bacteria to proliferate in host tissues and body fluids (8, 31). However, in the tissues and fluids of vertebrates, the levels of free iron are much lower than the minimum required for bacterial growth (<10 versus 50 to 5.0 × 10² nM) (12). To circumvent these restrictive conditions, most microorganisms have evolved high-affinity iron transport systems, which consist of producing specific low-molecular-weight ferric iron chelators called siderophores, as well as specific transport systems for siderophore complexes (21). Some of these systems allow bacteria with the ability to remove the vital iron from the host chelating proteins transferrin and lactoferrin during infection and may therefore be of the utmost importance in the pathogenesis of bacterial infections.

For instance, it is known that most members of the family Enterobacteriaceae, which cause episodes of nosocomial bacteremia in France (25) and the United States (5) with a case fatality rate around 25% (5, 31), produce the catechol-type siderophore enterobactin. Several lines of evidence, however, argue against the involvement of enterobactin in either extra- or intracellular microbial proliferation (12). A number of strains of Enterobacteriaceae and some Pseudomonas strains (9) also produce aerobactin, a hydroxamate-type siderophore, which, unlike enterobactin, appears to be an efficient contributor to the extracellular pathogenesis of enterobacteria causing septicaemia (23) and urinary tract infections (23, 32) and to the extracellular-cycle spreading of intracellular pathogens like Shigella spp. (12). The role of aerobactin as a virulence factor has been deduced from the results of experimental infection studies with isogenic aerobactin-producing and nonproducing strains of Escherichia coli (33) or Klebsiella pneumoniae (20). An indication of this role has also been provided by the fact that strains of enterobacteria isolated from the blood or urine of patients with documented infections produce aerobactin more frequently than do strains isolated from the feces of normal subjects (2, 23).

It has been shown that polyclonal antibodies directed against IutA, the outer membrane receptor for ferric aerobactin (FeAero), protected turkeys from experimental E. coli infection (6), but whether the biological activity of FeAero itself would be abolished by a specific antibody has not been determined. We report here the production, activity, and specificity of a mouse monoclonal antibody that reacted in an enzyme-linked immunosorbent assay (ELISA) with the ferric complex of crude aerobactin produced by clinical isolates of the family Enterobacteriaceae and that reduced the growth of an aerobactin-producing strain of E. coli in newborn calf serum.

MATERIALS AND METHODS

Aerobactin was purified as described previously (22) from the E. coli K-12 strain KH576 (nalA rpsL, pColV-K30), from P. H. Williams, cultured by shaking for 24 h at 37°C in Tris medium (29) without FeCl₃ and containing 0.2% glucose and 1 µg of vitamin B₁₂ per ml. FeAero was prepared as described previously (17) and assayed by spectrophotometric reading at 399 nm (15). Purity was determined by thin-layer chromatography on silica gel (7), by fast atom bombardment-mass spectrometry on a double-focusing magnetic instrument (VG 70-250SEQ; VG Instruments, Gentilly, France) equipped with a fast atom bombardment source, and by nuclear magnetic resonance analysis at 250 MHz on a Bruker WM250 analyzer. Each stage of aerobactin purification was checked by a universal chemical assay for siderophores (27) and a chemical assay for hydroxamates (11). Its biological activity was assayed by cross-feeding of the aerobactin-deficient mutant E. coli W0987 (lac araD aroB rpsL thi fepA, pColV-EN41::Tn10) as described previously (2, 13, 26).

* Corresponding author.
Purified FeAero was coupled to bovine serum albumin (BSA; Sigma) and to thyroglobulin (Sigma) in molar ratios of 50:1 and 300:1, respectively, as described previously (18, 30). Coupling was confirmed by thin-layer chromatography as indicated above and by absorption spectrum analysis with an UVikon 860 spectrophotometer (Kontron AG, Zurich, Switzerland).

The ELISA for detection of anti-FeAERO antibodies was performed as described previously (4) in polystyrene 96-well microtiter plates (Nunc, Roskilde, Denmark) coated with 100 μl of the BSA conjugate of FeAero (1 μg/ml in phosphate-buffered saline [PBS], pH 7.4) per well.

BALB/c mice (Centre d’Elevage René Janvier, Le Genest Saint-Isle, France) were primed with 100-μl subcutaneous injections of a 0.5-mg/ml solution of the thyroglobulin conjugate in PBS as described previously (14). Three days after the last injection, the spleen cells of the two mice with the highest anti-FeAero antibody serum titers were fused with the mouse myeloma cell line P3-NSI-Ag4-1 as described previously (3). Three hybridomas that secreted antibodies to FeAero were identified and cloned by limiting dilutions. The isotypes of the three resulting antibodies were identified as immunoglobulin G2 (IgG2) by using rabbit anti-mouse isotype immunoglobulin-peroxidase conjugates (Nordic Immunological Laboratories, Tilburg, The Netherlands).

The hybridoma producing the monoclonal antibody with the highest affinity for FeAero according to the ELISA was amplified by production of ascitic fluid in locally bred nude mice; the antibody was purified as described previously (4) and named MAb AERO1.

To define the specificity of MAb AERO1, its competitive affinity for three different groups of preparations containing siderophores was studied with the ELISA. The first group of preparations comprised nine purified chelating agents: nonferric aerobactin, FeAero, ferrioxamine B (the desferral ferric complex; CIBA-GEIGY Corp., Summit, N.J.), ferric α,α′-dipyridyl (Sigma), ferric ethylenediamine di(o-hydroxyphenylacetic acid) (EDDHA; Sigma), ferric pseudobactin (obtained from J. S. Buyer [10]), ferric coprogen (obtained from J. B. Neilands [21]), ferric citrate (Sigma), and ovotransferrin (Sigma). The second group was made up of three crude preparations of siderophores: ferric shizokinen from a ferrated culture supernatant of Bacillus megaterium (obtained from J. B. Neilands [21]), grown overnight at 37°C in Tris medium and assayed by a specific hydroxamate test (11); ferric arthrobactin from ferrated culture supernatant of Arthrobacter pasceens ATCC 13346, grown overnight at 20°C in Tris medium and assayed by the same test; and ferric enterobactin from a ferrated culture supernatant of E. coli RW193(pITSS5) (24), grown overnight at 37°C in Tris medium and assayed as described by Arnow (1). The third group of preparations containing siderophores comprised crude preparations of FeAero from ferrated culture supernatants of the following members of the family Enterobacteriaceae, isolated in our laboratory from cancer patients with bacteremia: E. coli, four strains; Klebsiella spp., four strains; Salmonella sp., one strain; Serratia sp., two strains; Citrobacter sp., one strain; and Enterobacter sp., two strains. Strains were grown overnight in Tris medium at 37°C and were positive for hydroxamate in the Csaky chemical assay (11) and in a cross-feeding assay with E. coli WO897.

In practice, 100-μl samples of serial dilutions of each preparation were incubated for 1 h at 37°C with 100 μl of MAB AERO1 (10⁻⁵ mg/ml) diluted in PBS–Tween–1% BSA–200 μM EDDHA, and the residual activity of MAB AERO1 was assayed by the ELISA as described above.

Inhibition by MAB AERO1 of the growth of an aerobactin-producing strain of E. coli was assayed by measuring the growth rate of E. coli BN3040 (F⁺, proC leuB trpE thi entA cin), from J. B. Neilands, in a medium containing either 10% ascitic fluid of MAB AERO1 or an anti-tetanus toxoid-unrelated monoclonal IgG (obtained from D. Bellet), or 10% physiological saline in heat-inactivated newborn calf serum (GIBCO Laboratories, Grand Island, N.Y.). The cultures were incubated at 37°C with shaking, and viable bacteria were counted at various times by plating appropriate dilutions on nutrient agar.

RESULTS

Thin-layer chromatography analysis and nuclear magnetic resonance spectroscopy showed that aerobactin was purified to homogeneity (data not shown). Mass spectral analysis showed peaks at m/z values of 565 (M⁺H⁺), 587 (M⁺Na⁺), and 603 (M⁺K⁺), in agreement with a molecular mass of 565 daltons for aerobactin. Visible light and UV spectroscopy and thin-layer chromatography showed that FeAero had been efficiently coupled to thyroglobulin and to BSA.

After cloning, amplification, and purification, the limit of FeAero detection by MAB AERO1 was 10 nM (10⁻¹⁰ mol in a 100-μl sample) in the competitive ELISA (Fig. 1A). In this assay, in which the affinity of MAB AERO1 for various siderophores was studied, this antibody also reacted with ferric arthrobactin and ferrioxamine B, although to a lesser extent than with FeAero. It did not react at all with nonferric aerobactin or with ferric shizokinen, ferric pseudobactin, ferric coprogen, ferric citrate (Fig. 1), ovotransferrin, ferric α,α′-dipyridyl, or ferric EDDHA (data not shown).

The ELISA with MAB AERO1 permitted the detection of FeAero in the ferrated supernatants from low-iron cultures of all of the aerobactin-producing members of the family Enterobacteriaceae tested. FeAero titers in the supernatants ranged from 10 to 100 μM, depending on the strain, and no species-related pattern of aerobactin production was observed (Fig. 2).

Last, we found that MAB AERO1 limited the growth of E. coli BN3040 in newborn calf serum, whereas the growth curve obtained with the anti-tetanus toxoid monoclonal IgG was similar to that obtained in the absence of any antibody (Fig. 3).

DISCUSSION

This is the first report on the production of a monoclonal antibody to FeAero; as far as we know, only monoclonal antibodies directed against the siderophore pseudobactin from Pseudomonas putida B10 have been described (10).

Here, MAB AERO1 provided a sensitive assay (10 nM) for the detection of the ferric complex of aerobactin. We believe that this is also the first report of the inhibition of the growth of an E. coli strain by a monoclonal antibody directed toward a bacterial metabolite. Previous investigations only showed that stimulation by the siderophore enterochelin of the growth of an Ent⁺ strain of E. coli could be blocked by an enterochelin-specific immunoglobulin contained in normal human serum (19). We chose to assay the biological activity of MAB AERO1 under iron-limiting conditions, using newborn calf serum, and not in synthetic media, so as to reproduce as closely as possible the in vivo conditions that prevail during extracellular infections. The E. coli BN3040 strain used in
this experiment was a mutant in enterobactin synthesis and was fully dependent on aerobactin to chelate iron.

The fact that MAb AERO1 displayed no significant affinity for nonferric aerobactin highlights the importance of the ferric ion in the recognized epitope. Furthermore, the fact that MAb AERO1 recognized ferrioxamine B and ferric citrate suggests that a common epitope is present on FeAero, in addition to these two siderophores. Shizokinen, which differs from aerobactin and arthrobactin by its carbon chain, in which \((\text{CH}_2)_4\) is replaced by \((\text{CH}_2)_2\), was not recognized by MAb AERO1. Therefore, the length of the carbon chain appeared to be an important feature of the conformational structure of the epitope on FeAero recognized by MAb AERO1. Taken together, these data suggest that this epitope is the iron-bound moiety of the lysyl-

FIG. 1. Competitive ELISA with MAb AERO1 with various purified siderophores (aerobactin [□], FeAero [△], ferrioxamine B [■], ferric pseudobactin [●], ferric coprogen [×], ferric citrate [+] (A) and dilutions of ferrated culture supernatants of various siderophore-producing strains [aerobactin from E. coli KH576(pColV-K30) (●), arthrobactin from A. pascens (+), shizokinen from B. megaterium (■), and enterobactin from E. coli RW193(pITS55) (○)].

FIG. 2. Concentrations of FeAero in low-iron culture supernatants of various aerobactin-producing members of the family Enterobacteriaceae. Concentrations were calculated from the readings obtained with known concentrations of purified FeAero included in each ELISA. The thick bars are means of three different assays, and the thin bars indicate the standard errors of the means.

FIG. 3. Effect of 10% ascitic fluid containing MAb AERO1 (M) on the growth rate of E. coli BN3040 in heat-inactivated newborn calf serum. Controls were physiological saline (▲) and an antitetanus toxoid monoclonal IgG (■). Data are means of three assays ± the standard errors of the means.
modified residue shared by the three siderophores that reacted with MAb AERO1.

Since neither arthrobactin nor ferrooxamine B, the two siderophores cross-reacting with MAb AERO1, is produced by members of the family Enterobacteriaceae, the competitive ELISA described in this paper provides a simple means of detecting aerobactin production by members of this bacterial family. Its specificity and sensitivity compare favorably with those of the bioassay and chemical assay (unpublished results). Thus, our results show that MAb AERO1 recognized the aerobactin produced by at least 14 separate strains from six different genera of the family Enterobacteriaceae. All of these strains were isolated from blood infections contracted by cancer patients.

Several reports have indicated that the use of passive immunization with monoclonal or polyclonal antibodies was promising for the treatment of severe gram-negative (34) and gram-positive infections (16, 28). Given that MAb AERO1 reduced the growth rate of an aerobactin-dependent mutant strain of E. coli in vitro, it now seems logical to investigate its possible efficacy in reducing the severity of infections caused by bacteria for which the production of aerobactin is an important virulence factor.

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