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Identification of *Bacillus anthracis* by Using Monoclonal Antibody to Cell Wall Galactose–*N*-Acetylglucosamine Polysaccharide

JOHN W. EZZELL, JR.,[1]* TERESA G. ABSHIRE,[1] STEPHEN F. LITTLE,[1] BURTON C. LIDGERDING,[2] AND CHARLES BROWN[3]

Bacteriology Division,[1] Virology Division,[2] and Pathology Division,[3] U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701-5011

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Guanidine extracts of crude *Bacillus anthracis* cell wall were used to vaccinate BALB/c mice and to develop monoclonal antibody (MAb) to vegetative cell surface antigens. Two hybridomas selected during this study produced immunoglobulin M immunoglobulins, which appear to be directed to an epitope associated with the galactose–*N*-acetyl-D-glucosamine polysaccharide. Both demonstrated specificity in their binding to purified *B. anthracis* cell wall, *o*-stearoyl-polysaccharide conjugates, and intact, nonencapsulated vegetative cells. The interaction of the MAbs with purified polysaccharide was inhibited by 0.5 M galactose and lactose but not by *N*-acetylglucosamine, glutamate, glycine, or glycerol. Inhibition by glucose or sucrose was approximately 75% of that seen with galactose. Electron microscopy showed that both MAbs interacted with the cell wall of vegetative cells as well as with the cortex of spores. Neither MAb reacted with encapsulated vegetative cells, such as those from infected guinea pigs, nor did they react with intact spores. After conjugation to fluorescein isothiocyanate, the MAbs stained intensely all *B. anthracis* strains tested, whereas with two exceptions, none of the strains of 20 other *Bacillus* spp. was stained. The exceptions, strains of *Bacillus cereus*, could be differentiated from *B. anthracis* by being beta-hemolytic on blood agar.

A variety of methods for the identification of *Bacillus anthracis*, the etiologic agent of anthrax, have emerged over the past century. These tests include determinations of colony characteristics on various media and susceptibility to penicillin and gamma-phage (27), interaction with specific lectins (4, 5, 9), demonstration of capsule formation (27, 5), determination of characteristics of spores and vegetative cells (27), carbohydrate fermentation tests (24), fatty acid profiles (18), anthrax toxin production tests, and tests for pathogenicity in laboratory animals (1, 2, 20, 27, 28, 30). Demonstration of the production of the components of the anthrax lethal toxin (i.e., protective antigen [PA] and lethal factor) or edema toxin (i.e., PA and edema factor) (21) by an isolate is perhaps the most reliable means of identifying anthrax strains. However, both toxins are encoded by the 110-megadalton pXO1 plasmid (formerly pPA1 [25]), and strains lacking this plasmid are thereby nontoxigenic. A second, 60-megadalton plasmid, pXO2, encodes the synthesis of the poly-D-glutamyl capsule (10, 32). The products of both plasmids are required for virulence. Although the identification of most *B. anthracis* strains can be made with certainty by using a combination of tests, Brown et al. (1) have proposed that transition strains may bridge the traits used to distinguish *B. anthracis* from the closely related species *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides* (18, 19).

Techniques for the identification of *B. anthracis* on the basis of the interaction of the lectin from *Glycine max* (soybean agglutinin) with a cell wall-associated polysaccharide have been proposed (4, 5, 9). The polysaccharide is composed primarily of D-galactose and *N*-acetyl-D-glucosamine (12, 13) (Gal-NAG polysaccharide) and represents a major portion of the cell wall (13, 26). The early studies describing the identification and purification of the Gal-NAG polysaccharide have been reviewed (3). The polysaccharide has been reported to be essentially unique to *B. anthracis* strains and has been identified only rarely in *B. cereus* (14). For these reasons, Ivanovics and Foldes (14) concluded that the anthrax bacillus is not merely a pathogenic variant of *B. cereus*, as proposed (1), but that the universal presence of the polysaccharide among *B. anthracis* strains indicates its potential for identification. They also proposed that it is the presence of this Gal-NAG polymer in unusual strains of *B. cereus* (possible transition strains; 1) that chemically and serologically distinguishes them from most other *B. cereus* strains.

We describe the development and characterization of monoclonal antibodies (MAbs) to the anthrax polysaccharide. Data which demonstrate their specificity and potential for rapid identification of *B. anthracis* vegetative cells are presented.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains (Table 1) were obtained from the culture collection of the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md.; the American Type Culture Collection, Rockville, Md.; the National Collection of Type Cultures, London, United Kingdom; and the Bacillus Genetics Stock Center, Columbus, Ohio. *B. anthracis* strains (22), cured of their pXO1 toxin plasmid and designated by the prefix Δ, have been described previously (15). The strains, maintained as frozen spore suspensions, were plated on 5% sheep blood agar and incubated at 37°C for 18 to 20 h. Growth from blood agar cultures was used to inoculate liquid R medium (28) buffered with 50 mM Tris hydrochloride, pH 7.5. Cultures (500 ml) in 1-liter sealed screw-top flasks were incubated with shaking at 100 rpm at 37°C for 18 to 20 h.

Cell wall purification. *B. anthracis* Sterne or V770 NP-1R cells ($pXO1^+$ and $pXO2^-$) were grown in R medium, harvested by centrifugation for 15 min at 10,000 × *g*, and suspended at 0.1 g/ml in 0.1 M acetate buffer (1.25 mM

* Corresponding author.

TABLE 1. Differentiation of *B. anthracis* from other *Bacillus* spp.[a]

Species and strain	Source	MAb[b] 5G4	MAb[b] 6G4	β-h[c]	PA[d]	phg[e]	cap[f]	mot[g]
Bacillus alvei ATCC 6344	b	–[h]	–	NR[i]	NT[j]	NT	NT	NT
Bacillus amyloliquefaciens	a	–	–	NR	NT	NT	NT	NT
Bacillus anthracis								
(pXO1$^+$, pXO2$^+$) strains[k]								
57 (South Africa, goat); 17T5 (South Africa, kudu); Ames (Iowa, cow); Buffalo (Iowa, buffalo); Colorado, EB1-R3; G-28 (South Africa); LA-2, phage sensitive; Nebraska (Nebraska, cattle); New Hampshire (Manchester); VH (South Africa, human); Vollum; Vollum (British strain); Vollum 1 (South Africa); Vollum 1B	a	4+	4+	–	+	+	+	–
LA-2, phage resistant	a	4+	4+	–	+	–	+	–
M-strain	a	4+	4+	–	+	+	–	–
C-4880C	a	4+	4+	–	+	+	±	–
(pXO1$^+$, pXO2$^-$) strains								
German LVS, Sterne, Texas, V770-NP-1R	a	4+	4+	–	+	+	–	–
STI	a	4+	4+	–	+	–	–	–
V770-2-P	a	4+	4+	–	+	NT	–	NT
(pXO1$^-$, pXO2$^+$) strains								
1928 (cow, Iowa), ΔAmes-1, ΔNH-1, Albia (Iowa)	a	4+	4+	–	–	+	+	–
ATCC 4229 (PVS), 6602 (PVS)	b	4+	4+	–	–	+	+	–
CDC strains								
CDC 607 (Canada, bison), SK-31 (South Africa), SK-61 (California), SK-102 (New Jersey), SK-128 (Massachusetts), SK-162 (Haiti)	c	4+	4+	–	+	+	+	–
CDC 472 (Lebanon), 476 (Pakistan), 569 (Argentina), 572 (Argentina), 763, SK-465 (Iowa, buffalo)	c	4+	4+	–	+	+	–	–
CDC 713, 471 (Pakistan)	c	4+	4+	–	–	+	+	–
CDC 477 (Pakistan), 608 (Canada, bison), 700	c	4+	4+	–	–	+	–	–
CDC 620 (Florida, cow), 621 (Seato Lab)	c	–	–	+	–	–	–	+
CDC 701 (Argentina)	c	–	–	–	–	–	+	–
CDC 474 (Pakistan), 624 (Thailand death), 714 (Thailand soil)	c	–	–	–	–	–	–	–
CDC 503 (Tanganyika)	c	–	–	–	–	–	NT	NT
Bacillus brevis ATCC 8246	b	–	–	NR	NT	NT	NT	NT
Bacillus cereus								
ATCC 246, 9620, 11778, 13472, 19637, 23260	b	–	–	+	–	–	–	+
B10, B33, B74	f	–	–	+	–	–	–	+
BGSC 6A1 (T)[2], 6A2 (T-HT)	e	–	–	+	–	–	–	+
NCTC 926, 2599, 4415, 4415, 7586, 8012, 8035, 8075, 8079, 8096, 9634, 9680, 10024	d	–	–	+	–	–	–	+
NCTC 10320	d	–	–	+	NT	NT	NT	NT
T strain	a	–	–	+	–	–	–	+
USAMRIID	a	–	–	–	–	–	–	+
NCTC 9946	d	–	–	–	–	–	–	+
ATCC 14579	b	–	–	–	–	–	+	+
ATCC 7064	b	4+	4+	+	–	–	–	+
NRS-820	a	4+	4+	+	–	–	–	+
Bacillus circulans ATCC 4513	b	–	–	NR	NT	NT	NT	NT
Bacillus coagulans ATCC 7050	b	–	–	NR	NT	NT	NT	NT
Bacillus eugilitis B-61	f	–	–	NR	NT	NT	NT	NT
Bacillus lentus CDC 683/NRRL B-396	c	–	–	NR	NT	NT	NT	NT
Bacillus licheniformis ATCC 8189, 8480/BGSC 5A1, 9259, 9945a, 12713, 14580	b	–	–	NR	NT	NT	NT	NT
Bacillus macerans ATCC 8244	b	–	–	NR	NT	NT	NT	NT

Continued on following page

TABLE 1—*Continued*

Species and strain	Source	MAb[b]		β-h[c]	PA[d]	phg[e]	cap[f]	mot[g]
		5G4	6G4					
Bacillus megaterium								
ATCC 14581 (type strain)	b	−	−	+	−	−	−	−
B-64	f	−	−	+	−	−	−	+
BGSC 7A1	e	−	−	−	−	−	−	−
BGSC 7A2 (ATCC 19213)	e	−	−	−	−	−	+	−
CDC 684/NRRL-349S/NRS 234	c	−	−	−	+	+	+	−
CDC 699	c	−	−	−	−	−	−	−
Bacillus mycoides								
CDC 672/NRS 1215, CDC 673/NRS 1316, CDC 694/NRS 911	c	−	−	−	−	−	−	−
CDC 674/NRS 936	c	−	−	−	−	+	−	−
Bacillus pasteur B-84	f	−	−	NR	NT	NT	NT	NT
Bacillus polymyxa								
B-74	f	−	−	NR	NT	NT	NT	NT
ATCC 842	b	−	−	NR	NT	NT	NT	NT
Bacillus pulvifaciens CDC 689/NRS 1283	c	−	−	NR	NT	NT	NT	NT
Bacillus pumilus								
B-83	f	−	−	NR	NT	NT	NT	NT
ATCC 7061	b	−	−	NR	NT	NT	NT	NT
BGSC 8A1 (BP1)	e	−	−	NR	NT	NT	NT	NT
CDC 686/NRS 272	c	−	−	NR	NT	NT	NT	NT
Bacillus sphaericus								
ATCC 14577	b	−	−	NR	NT	NT	NT	NT
BGSC 13A1 (1593), 13A4-(WHO 2297)	e	−	−	NR	NT	NT	NT	NT
Bacillus subtilis								
4051—USAMRIID	a	−	−	NR	NT	NT	NT	NT
ATCC 6051	b	−	−	NR	NT	NT	NT	NT
BGSC 1A2 (SB 491), 2A2-(WB 672), 3A1 (NC1B 3610)	e	−	−	NR	NT	NT	NT	NT
BST1	a	−	−	−	−	−	NT	−
BST-2	a	−	−	−	NT	−	NT	−
CDC 673/NRS 744, CDC 678/ST-72, CDC 709/NRS 624/B-362	c	−	−	NR	NT	NT	NT	NT
globigii	a	−	−	NR	NT	NT	NT	NT
PA1, PA2	a	−	−	−	+	−	NT	−
PY143	a	−	−	NR	NT	NT	NT	NT
Bacillus thuringiensis								
3B11	a	−	−	−	NT	−	NT	+
4042-B	a	−	−	+	NT	−	NT	−
ATCC 10792 (type strain)	b	−	−	+	NT	NT	NT	NT
B-8	f	−	−	+	NT	−	NT	+
BGSC 4A1 (NRRL-B4039)	e	−	−	+	NT	−	NT	+
BGSC 4B1 (CCEB 460)	e	−	−	+	NT	NT	NT	NT
CDC 697/NRS 1440	c	−	−	+	NT	−	NT	+
NCTC 4040	d	−	−	+	−	−	−	+
NCTC 4041	d	−	−	+	−	NT	NT	NT
NCTC 4045	d	−	−	+	−	NT	NT	NT
NCTC 4055	d	−	−	+	−	−	−	+
NCTC 4060	d	−	−	+	NT	−	NT	+

[a] Source code: a, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Md.; b, American Type Culture Collection, Rockville, Md.; c, CDC; d, National Collection of Type Cultures, London, United Kingdom; e, Bacillus Genetics Stock Center, Columbus, Ohio; f, CDE. CDC cultures that were supplied to CDC by other laboratories and had other designations are separated from the CDC numbers by slashes. The state within the United States or the country in which a *B. anthracis* strain was originally isolated (and in some cases, the host) is indicated in parentheses. Strains from the same source and with the same test reactions are grouped.

[b] 5G4, MAb EAI-5G4; 6G6, MAb EAII-6G6.

[c] β-h, Beta hemolysis on blood agar.

[d] PA, PA production in R medium plus heat-inactivated horse serum.

[e] phg, Susceptible to gamma-phage.

[f] cap, Capsule formation on bicarbonate agar in 20% CO_2.

[g] mot, Motility in Craigie tube.

[h] Staining intensity scale ranges from − (no fluorescence) to 4+ (staining equal to that seen with *B. anthracis* Sterne).

[i] NR, Not recorded.

[j] NT, Not tested.

[k] Plasmids possessed by the indicated strains.

$MgSO_4$, pH 5.0) containing 1 μg of DNase and RNase per ml (Sigma Chemical Co., St. Louis, Mo.). Chilled cells were passed through a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) at 32,000 lb/in^2, and the disrupted cell suspension was centrifuged at 10,000 × *g*. The pellet was washed three times in cold 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) plus 2 mM $MgSO_4$ (pH 7.5) at approximately 0.1 g of cells per ml and then washed three times with HEPES-$MgSO_4$ buffer containing 0.1 M NaCl. Microscopy of the material stained with crystal violet revealed an absence of intact cells. Particulate matter was suspended at 0.05 g/ml in phosphate-buffered saline (PBS) (10 mM sodium phosphate in 0.85% NaCl, pH 7.3) and sterilized either by gamma irradiation (3×10^6 rads) while on ice or by autoclaving for 15 min.

Polysaccharide isolation from cell wall. Polysaccharide-peptidoglycan complexes were purified from cell walls by extraction with sodium dodecyl sulfate (SDS) at 90°C. Analysis was as previously described (7). A portion of the material was supplied to the laboratory of R. J. Doyle, University of Louisville, Louisville, Ky., where the GalNAG polysaccharide was isolated and purified.

Immunizations. Female BALB/c mice were immunized with a partially purified 2.5 M guanidine-HCl extract of *B. anthracis* cell walls. Prior to inoculation, the material was dialyzed against distilled water to remove guanidine and maintained lyophilized. The initial vaccination consisted of an intramuscular injection of 10 μg of extract in a 1:1 mixture of Freund complete adjuvant and PBS; a second injection on day 14 contained Freund incomplete adjuvant. On days 21 and 28, the mice were injected intraperitoneally with 10 μg of extract suspension in 0.1 ml of PBS. Intravenous injection was not possible since the extract was insoluble in physiological buffers. On day 42, the antibody titers to the extract were determined by enzyme-linked immunosorbent assay (ELISA) (7).

Hybridomas. Spleen cells from immunized mice were fused with logarithmically growing SP2/0-Ag14 myeloma cells (6). Hybridoma cell cultures were screened by ELISA (7) to determine antibody to the guanidine extract. Fluids that were positive by ELISA were assayed by electrophoretic immunotransblots (Western blots) (7, 31) and indirect fluorescent-antibody (IFA) staining (7). Selected hybridoma cell lines were sent to Salk Institute, Swiftwater, Pa., for ascites fluid production.

Purification of MAb. Ascites fluid (5 ml) was dialyzed against 20 mM Tris hydrochloride (pH 8.0) for 18 to 20 h, clarified by centrifugation (10,000 × *g*, 30 min), and pumped onto a 30-ml column of Affi-Gel Blue (Bio-Rad Laboratories, Rockville Centre, N.Y.). The column was washed with 20 mM Tris buffer, and 2-ml fractions were collected until the A_{280} of the eluant returned to the baseline. The bound antibody was eluted with a 100-ml gradient of 0 to 1.0 M NaCl in 20 mM Tris buffer, pH 8.0. The fractions containing antibody were identified by IFA staining and ELISA. The ELISA was as previously described (7), except that the purified polysaccharide-peptidoglycan complex antigen was bound as follows: 20 μg of cell wall suspended in distilled H_2O was added per well of Linbro polystyrene microdilution plates, dried at 60°C in a convection oven, and fixed with methanol for 5 min. Fractions from the Affi-Gel Blue column that demonstrated the greatest activity were pooled, and the protein concentration of the material was determined by the Pierce BCA protein assay (Pierce Chemical Co., Rockford, Ill.). The assay was performed in microdilution plates (Linbro) with bovine serum albumin as the standard, and the A_{540} was measured with a Bio-Tek EL308 microplate reader (Bio-Tek Instruments, Inc., Burlington, Vt.).

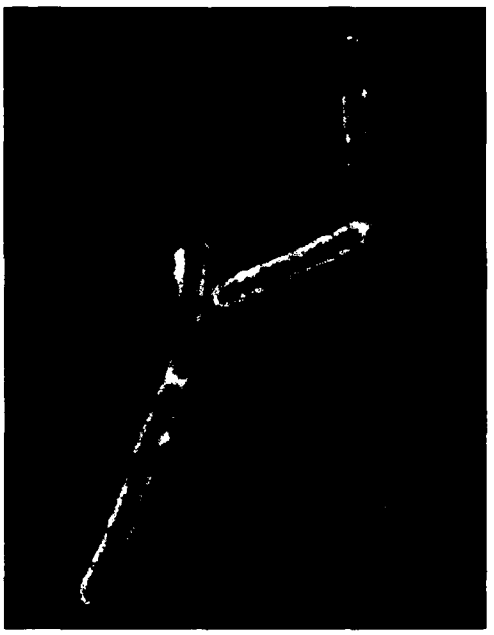

FIG. 1. Sterne cells grown on blood agar and stained with MAb EAII-6G6 conjugated to FITC. Magnification, ×3,600.

MAb conjugation to FITC. Purified MAb was mixed with fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratory, Baltimore, Md.) at a protein-to-FITC ratio of 25:1 (wt/wt) in 0.05 M bicarbonate buffer at pH 9.0. The mixture was stirred slowly for 4 h at ambient temperatures (AT), and unbound FITC was removed by chromatography through a prepacked PD-10 Sephadex G-25M column (Pharmacia/LKB, Piscataway, N.J.) equilibrated with PBS, pH 7.3. FITC incorporation was estimated from the A_{490} after a 1:50 dilution in 0.1 M NaOH. The protein concentration was determined by the BCA protein assay (Pierce). Conjugates contained 3.5 to 5.5 μg of FITC per mg of protein.

Immunofluorescence studies. Growth from *Bacillus* spp. grown for 18 to 20 h at 37°C on blood agar was suspended in PBS, and 5 μl was placed in 4-mm wells of printed slides (Cel-Line Associates, Inc., Newfield, N.J.). Suspensions were diluted to yield 5 to 20 cells per 1,000× oil immersion field. The slides were air dried and fixed in methanol for 5 min. Each spot was washed twice with PBS and blocked with PBS plus 0.5% gelatin (PBSG) for 30 min at 37°C, and then 40 μl of FITC conjugate at 10 μg/ml in PBSG plus 0.3% Tween 20 was added. After incubation at 37°C for 1 h in a humid chamber, the spot wells were washed four times with PBS. For IFA assays, used to screen hybridoma culture fluids, 25 μl of goat anti-mouse immunoglobulin G (IgG), IgA, and IgM-FITC conjugate (Organon Teknika, Malvern, Pa.) was diluted 1:200 in PBSG and added to similar slides. To reduce fading during fluorescence microscopy, cover slips were mounted by using a few drops of 1,4-diazobicyclo-(2,2,2)-octane (Sigma) at 25 mg/ml in 10% PBS plus 90% glycerol, pH 7.4 (17). The intensity of the staining was recorded on a scale ranging from negative for no fluorescence to 4 plus for staining equal to that seen with *B. anthracis* Sterne (Fig. 1) (Table 1).

ELISA. Because native polysaccharide binds poorly to polystyrene microdilution plates, the polymer was conju-

gated to stearoyl chloride in the laboratory of R. J. Doyle by the method of Hammerling and Westphal (11). The optimal concentration of the conjugate for coating the wells was determined for each preparation. After addition of the conjugate (100 μl/ml), the plates were incubated at AT for 18 to 20 h. Quench buffer (PBS plus 0.5% gelatin and 5% powdered milk, pH 7.5) was added (300 μl per well), and the plates were incubated at AT for 30 min. The wells were washed four times with PBSG. Tween 20 was omitted because it released the *o*-stearoyl-polysaccharide conjugate from the plates. MAbs were titrated in PBSG and incubated for 1 h at 37°C, and the wells were washed four times with PBSG. Goat anti-mouse IgA, IgG, and IgM-horseradish peroxidase (Cappel Laboratories) and substrate were added, and A_{405} was determined in a Bio-Tek EL308 microplate reader as previously described (7).

Antibody typing. Ascites fluids were diluted 1:100 in PBSG, incubated for 2 h at 37°C in microtiter plates coated with 50 μl of *o*-stearoyl-polysaccharide (100 μg/ml), and blocked with quench buffer. A subtyping kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was used to determine immunoglobulin subtype by ELISA (23).

Immunoelectron microscopy. *Bacillus* spp. were grown 18 to 20 h on blood agar at 37°C, harvested, suspended in PBS, and centrifuged; the pellet was suspended in 2% paraformaldehyde–0.1% glutaraldehyde in 0.01 M phosphate buffer (pH 7.25) for 2 h on ice and then rinsed three times with phosphate buffer. After fixation, the cell pellets were sterilized while on ice by 3×10^6 rads of gamma irradiation. The specimens were embedded in LR White (Polysciences, Inc., Warrington, Pa.), and the resin was cured at 60°C for 18 to 20 h. Thin sections of each specimen were collected on Formvar-coated nickel grids. For immunostaining, the grids were immersed in 4% normal goat serum for 10 min at AT and rinsed gently three times with 0.02 M Tris buffer, pH 7.2. The grids were incubated for 1 h at AT in dilutions of cell culture fluids containing MAb EAI-5G4 or MAb EAII-6G6 in Tris buffer containing 0.1% bovine serum albumin and 0.05% Tween 20, pH 8.1. The grids were rinsed three times with Tris buffer-bovine serum albumin-Tween 20, pH 7.2, for 5 min each and then transferred to 10-nm gold-sphere-labeled goat anti-mouse IgM diluted 1:20 (G-10; Janssen Life Sciences, Piscataway, N.J.) for 1 h at AT. The grids were washed twice with Tris buffer, fixed with 2% glutaraldehyde, rinsed in water, and poststained with 1% uranyl acetate and lead citrate. Specimens were observed with a JEOL (Peabody, Mass.) 100CX electron microscope at 80 kV.

Confirmation of *B. anthracis* strain identity. To confirm the identity of strains for the purposes of comparison with the MAb studies, a number of other tests were performed, including beta hemolysis on 5% sheep blood agar, in vitro PA production, phage sensitivity, motility, and capsule production. Although most *B. anthracis* strains produced weak diffuse hemolysis, especially with extended incubation, colonies were scored positive only if hemolysis was marked and clearly beta hemolytic in cultures incubated less than 18 h. Strains of *Bacillus* species to be tested for PA production were grown on blood agar for 18 to 20 h at 37°C. Growth was suspended in R medium containing 50 mM Tris hydrochloride buffer and diluted to give an optical density of 0.1 at 540 nm. This suspension (50 μl) was inoculated into 0.8 ml of filter-sterilized R medium supplemented with 10% heat-inactivated horse serum. The cultures were added to 1.5-ml screw-top microfuge tubes, sealed, and incubated horizontally at 37°C with shaking. After 20 h, 0.2 ml of 5× extraction buffer was added to each microfuge tube to give final concentrations of 1% SDS, 5 mM 2-mercaptoethanol, and 10 mM EDTA at pH 9.0. The mixtures were placed in a boiling water bath for 5 min and centrifuged in a microcentrifuge (model 12; Beckman Instruments, Inc., Fullerton, Calif.) equipped with a sealed bowl rotor to ensure biocontainment. The supernatant was sterilized by passage through a 0.22-μm Millex GV low-protein-binding filter (Millipore Corp., Bedford, Mass.). The treated samples were subjected to electrophoresis with PhastGel SDS 10/15 gradient gels (Pharmacia/LKB). Proteins were transblotted to nitrocellulose (Bio-Rad Laboratories) for at least 4 h by direct contact, using 20% methanol in 25 mM Tris with 200 mM glycine at pH 8.3 to transfer. The transblotted proteins were analyzed by Western blot with a mixture of ascites fluids containing PA MAbs PA2II-20C5 and PA1-2D3 and diluted 1:1,000 in PBSTG (7). Antibody was detected by goat anti-mouse IgG, IgA, IgM-alkaline phosphatase conjugate (Kirkegaard & Perry, Gaithersburg, Md.), and 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium stain (Bio-Rad Laboratories). Production of PA was scored as (+) or (−) on the basis of the results derived from the method described above or from information obtained from previous reports (7).

The sensitivity of bacterial strains to gamma-phage (27) was determined by spreading an inoculum (0.5 McFarland standard) in nutrient broth on blood agar. After the surface had dried and 5 μl of gamma-phage was added to the center of the inoculated blood agar, the plate was incubated for 18 to 20 h at 37°C and then inspected for plaque formation.

RESULTS AND DISCUSSION

MAb to vegetative cell-extracted antigens. The majority of the MAbs developed against the guanidine-extracted antigens stained vegetative cells by IFA assay in an irregular, patchy manner (data not presented) and were not specific for *B. anthracis*, in that a few *B. thuringiensis* strains also stained. These MAbs generally reacted on Western blots with a 93-kilodalton surface protein termed extractable antigen 1 (EA1) (7). Of special interest was a small percentage of MAbs which specifically stained *B. anthracis* vegetative cells uniformly and intensely by IFA assay (Fig. 1). In addition, none of the specific MAbs interacted on Western blots with any of the proteins extracted with SDS from whole *B. anthracis* cells (7).

Two of the specific MAbs, designated 5G4 and 6G6, were selected for further study. Unlike MAbs to the EA1 protein, which were typed as IgG, both of the specific MAbs were IgM with kappa light chains and, as described below, appear to be directed to the galactose-*N*-acetylglucosamine polysaccharide associated with *B. anthracis* cell walls.

Interaction of polysaccharide MAbs with spores and encapsulated vegetative cells. Refractile and presumably dormant spores, as seen under phase microscopy (Fig. 2A), were not stained by the MAbs when viewed under fluorescence (Fig. 2B). Interestingly, the central portion of what appeared to be germinating spores (Fig. 2A) was intensely stained with polysaccharide MAbs (Fig. 2B). A comparison of Fig. 2A and B revealed that the ends of the germinating spores did not stain, suggesting that the ends are protected by the residual spore coat. Our data support the premise that *B. anthracis* spores germinate in a manner similar to that of other *Bacillus* spp. That is, after elongation of the core of the emerging vegetative cell within the germinating spore, the spore coat splits equatorially along the long axis and then separates away to both terminals of the elongated core, where it remains attached (33). This is consistent with the

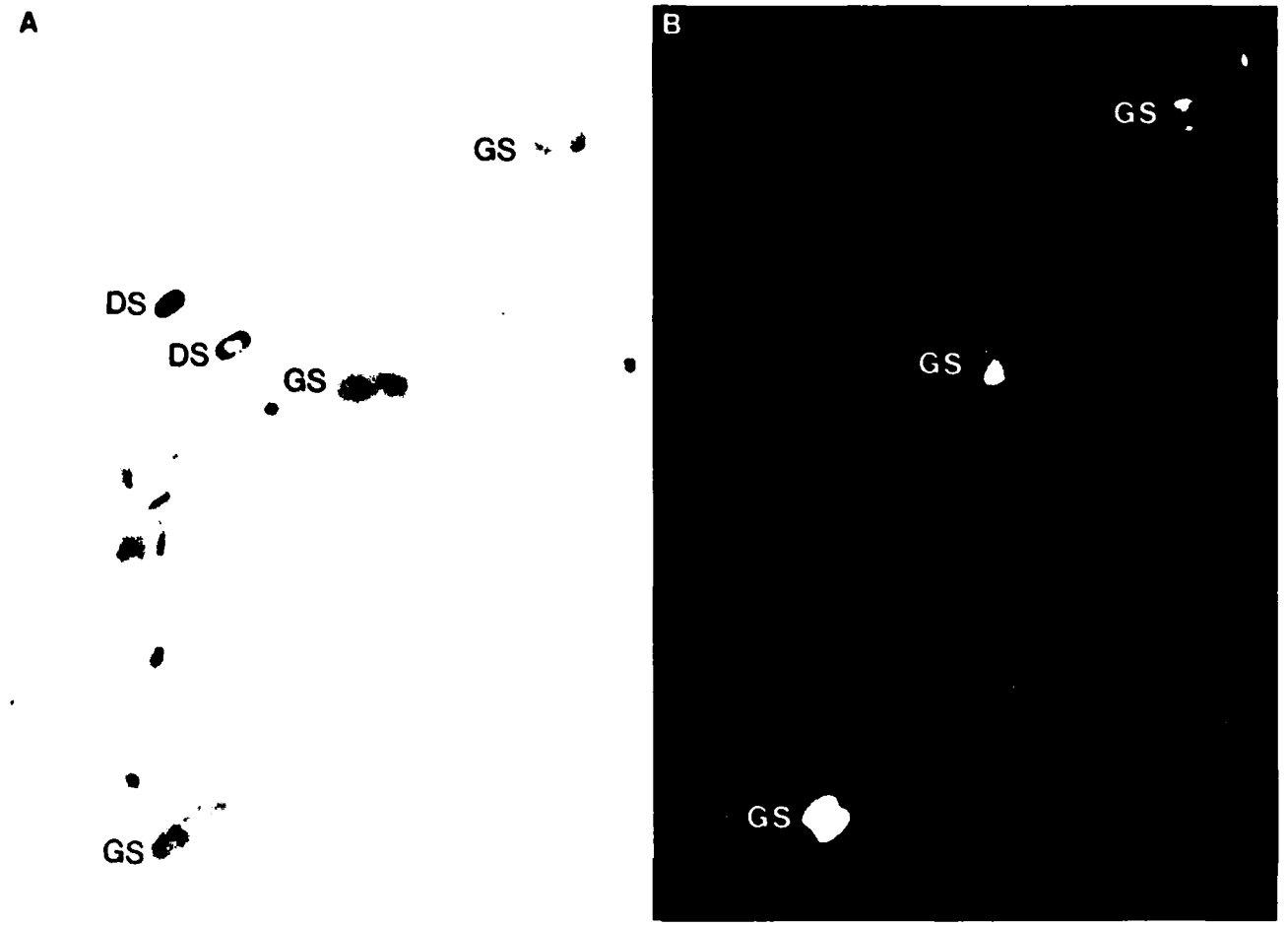

FIG. 2. Spores from *B. anthracis* Ames stained by IFA assay with MAb EAII-6G6. Both germinating (GS) and dormant (DS) spores are shown as viewed by phase (A) and fluorescence (B) microscopy. Magnification, ×3,600.

reported retention of the spore coat as polar caps by *B. anthracis*, *B. cereus*, and *B. mycoides* vegetative cells after spore germination (2). These data suggest that the polysaccharide MAbs can be used for indirect identification of *B. anthracis* spores by first incubating the spores under appropriate culture conditions to induce germination and then staining the emerging vegetative cells.

To determine the usefulness of the two MAbs for identification of *B. anthracis* cells in clinical samples, blood was obtained from female Hartley guinea pigs that were terminally ill with anthrax or had just succumbed to lethal intramuscular challenge with 4,000 *B. anthracis* Ames spores. Attempts to stain the heavily encapsulated Ames cells failed, thereby supporting earlier observations (7) that both the EA1 protein and the Gal-NAG polysaccharide antigens were not expressed on the surface of capsulated vegetative cells.

Immunogold electron microscopy. Thin sections of *B. anthracis* Sterne and *B. subtilis* PA1 (strain 1S53 into which the PA gene was cloned, using cloning vector pUB110) (16) were treated with the hybridoma cell culture fluids containing polysaccharide MAbs 5G4 and 6G6. Although the fixative used did not provide optimal fixation, it did protect the antigenicity of the preparations and thereby permitted immunostaining. Both MAbs clearly stained the cell walls and septae of the Sterne strain vegetative cells (Fig. 3). Neither MAb stained the cell walls of the *B. subtilis* strain which lacked the Gal-NAG polysaccharide, and there was no staining of the *B. anthracis* cell wall by the gold conjugate when the polysaccharide MAb was omitted (data not shown). The ΔSterne strain, which lacked both pXO1 and pXO2 plasmids, stained identically to Sterne, thereby supporting the premise that the polysaccharide was encoded chromosomally. The observation that intact spores were not stained by IFA was consistent with immunogold electron microscopy studies in that the MAbs reacted only with the spore cortex but did not react with the external spore coat or exosporium. The staining of the cortex by the polysaccharide MAbs was not surprising, since the cortex is derived from the cell wall of the sporangium. The internal layers of the formaldehyde-fixed spores were generally more discernable in those lacking an exosporium (Fig. 4A), perhaps due to the protection of the underlying structures from the fixative by this outer structure (Fig. 4B).

Determination of MAb interaction with Gal-NAG polysaccharide. Cell wall peptidoglycan fragments were prepared by disruption and SDS extraction of cell walls (7) from *B. anthracis* Sterne, Ames, Vollum-1B, and V770-NP-1R; from *B. cereus* ATCC 4415 and ATCC 23260; and from *B. thuringiensis* NCTC 4041 and NCTC 4045. Suspensions of the peptidoglycan fragments were analyzed by IFA staining with the polysaccharide MAbs. Both MAbs stained pepti-

FIG. 3. Electron micrographs of *B. anthracis* Sterne vegetative cells treated with polysaccharide MAb EAI-5G4 followed by goat anti-mouse IgM labeled with 10-nm gold spheres (Au). CW, Cell wall; S, septum. Magnification, ×27,000.

FIG. 4. Electron micrographs of *B. anthracis* Sterne spores without (A) and with (B) exosporium, treated with polysaccharide MAb EAI-5G4 followed with goat anti-mouse IgM labeled with 10-nm gold spheres (Au). CX, Cortex; SC, spore coat; EX, exosporium; C, core. Magnifications: A, ×42,000; B, ×37,000.

doglycan fragments from the *B. anthracis* strains but did not stain those from the other two species. The complexes were devoid of protein, lipid, and nucleic acid (7). Since the three species possessed the same type of peptidoglycan, characterized as having directly cross-linked *m*-diaminopimelic acid (29), it is possible that the MAbs were directed to some unique nonprotein, nonlipid, and nonnucleic acid component associated with the *B. anthracis* peptidoglycan complex (possibly the Gal-NAG polysaccharide).

Both MAbs selected for study were tested by ELISA for their interaction with *o*-stearoyl-polysaccharide conjugate. In the presence of PBSG, MAb 5G4 interacted well with the conjugate (Fig. 5). Both 0.5 M galactose and lactose strongly inhibited the reactivity of the MAb with the polysaccharide conjugate, whereas 0.5 M glutamate and NAG weakly inhibited the reaction compared with the PBSG control. In other experiments, glucose and sucrose inhibited the reaction approximately 75% of that seen with galactose and lactose, whereas glycine and glycerol were not inhibitory. The data indicated that the MAbs were directed, at least in part, to pyranose ring moieties. The inhibition by glucose but not by NAG also suggested that pyranose carbon in position 2 cannot be substituted for binding to occur. When the experiments were repeated with MAbs (PA2II-20C5 and PA1-2D3) specific for the PA anthrax toxin component, there was no inhibition by the sugars of antibody binding to microtiter plate wells coated with PA (data not shown). This indicated that the effect of the carbohydrates was not a nonspecific effect on antibody-antigen interactions.

Specificity of the polysaccharide MAbs. We evaluated the specificity of polysaccharide MAbs for *B. anthracis* cells by IFA staining. All the well-characterized *B. anthracis* strains used at our institute stained intensely with both MAbs. Strains lacking both pXO1 and pXO2 plasmids also stained strongly, indicating that the synthesis of the polysaccharide was encoded chromosomally.

Some of the strains obtained from the *Bacillus* culture collection of the Centers for Disease Control (CDC), Atlanta, Ga., did not possess traits consistent with *B. anthracis*. Strains CDC 620 and CDC 621 were determined previously by CDC not to be *B. anthracis* (criteria for determinations were not available). In addition to strains CDC 620 and CDC 621, strains CDC 474, CDC 503, CDC 624, CDC 701, and CDC 714 did not react with either of the MAbs, produce PA, or exhibit phage susceptibility. Except for being nonhemolytic, these strains did not resemble *B. anthracis* and were concluded to be identified incorrectly. Two *B. cereus* strains of special interest were NRS-820 and ATCC 7064. Both strains reacted intensely with the Gal-NAG polysaccharide MAbs yet resembled other *B. cereus* strains in that they were strongly beta-hemolytic, failed to produce PA and capsule, and were resistant to gamma-phage. These strains may represent transition forms between *B. anthracis* and *B. cereus* in that they clearly possess traits of both species. Although *Bacillus megaterium* CDC 684 NRRL 3495 NRS 234 did not react with either polysaccharide MAb, we nevertheless recommend its reclassification to *B. anthracis* because it was nonhemolytic, produced PA and poly-D-glutamyl capsule, and was susceptible to phage.

Our data support the observations of Ivanovics and Foldes

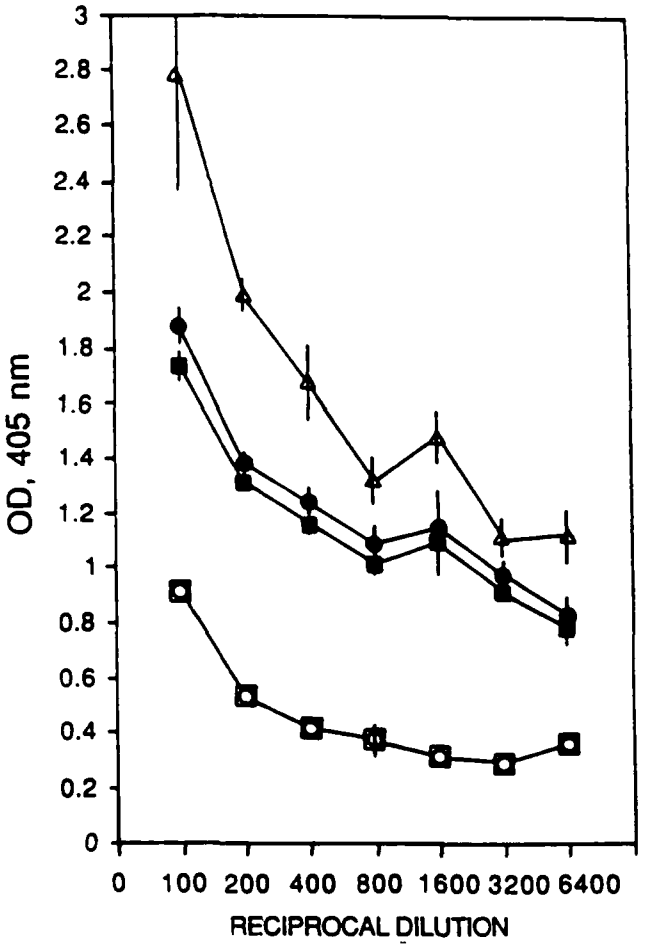

FIG. 5. MAb EAI-5G4 titrated against *o*-stearoyl-polysaccharide conjugate in microtiter plates in the presence of PBSG (△) and 0.5 M galactose (○), lactose (□), *N*-acetyl-D-glucosamine (●), or sodium glutamate (■). Vertical bars indicate the standard deviation (n = 4).

(14) that the Gal-NAG polysaccharide distinguished *B. anthracis* from *B. cereus*, and we concur that it should be used taxonomically (14). Except possibly for the misidentified *B. megaterium* strain discussed, all anthrax strains tested to date possess the Gal-NAG polysaccharide. We therefore propose that the species *B. anthracis* be defined as including those strains that possess the Gal-NAG polysaccharide, are predominantly nonhemolytic, are gamma-phage susceptible, and are nonmotile. Isolates should also be tested for poly-D-glutamyl capsule and PA production, with the understanding that certain strains may lack either or both of these traits. We disagree with past attempts to designate *B. anthracis* as a variant of *B. cereus* (1, 8, 29), since most *B. cereus* strains are motile and hemolytic, lack the Gal-NAG polysaccharide, and are not susceptible to gamma-phage.

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