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Production and Characterization of Monoclonal Antibodies against the Lethal Factor Component of *Bacillus anthracis* Lethal Toxin

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The lethal toxin of *Bacillus anthracis* consists of two components, protective antigen and lethal factor. Protective antigen is cleaved after binding to cell receptors, yielding a receptor-bound fragment that binds lethal factor. Sixty-one monoclonal antibodies to the lethal factor protein have been characterized for specificity, antibody subtype, and ability to neutralize lethal toxin. Three monoclonal antibodies (10G3, 2E7, and 3F6) neutralized lethal toxin in Fisher 344 rats. However, in a macrophage cytolysis assay, monoclonal antibodies 10G3, 2E7, 10G4, 10D4, 13D10, and 1D8, but not 3F6, were found to neutralize lethal toxin. Binding studies showed that five of the monoclonal antibodies that neutralized lethal toxin in the macrophage assay (10G3, 2E7, 10G4, 10D4, and 13D10) did so by inhibiting the binding of lethal factor to the protective antigen fragment bound to cells. Monoclonal antibody 1D8, which was also able to neutralize lethal toxin activity after lethal factor was prebound to cell-bound protective antigen, only partially inhibited binding of lethal factor to protective antigen. Monoclonal antibody 3F6 did not inhibit the binding of lethal factor to protective antigen. A competitive-binding enzyme-linked immunosorbent assay showed that at least four different antigenic regions on lethal factor were recognized by these seven neutralizing hybridomas. The anomalous behavior of 3F6 suggests that it may induce a conformational change in lethal factor. Differences in neutralizing activity of monoclonal antibodies were related to their relative affinity and epitope specificity and the type of assay.

Collectively, three proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF), compose the two exotoxins of *Bacillus anthracis*, lethal toxin and edema toxin. In several respects, the toxin components interact to fit the pattern of A-B-type protein toxins (12). Individually, these three proteins have no known toxic activity in animals. However, when PA is combined with LF, lethal toxin is formed, and combination of PA with EF forms edema toxin. PA (83 kilodaltons) is cleaved by a cellular protease after binding to a cell receptor (18, 26). The larger, C-terminal, 63.5-kilodalton fragment (PA63) remains bound to the cell receptor. Subsequent binding of either LF or EF leads to expression of either lethal or edema toxin activity, respectively. EF has been shown to be a calcium- and calmodulin-dependent adenylate cyclase (16), and edema toxin produces edema in the skins of animals (2). No enzymatic mechanism of action for LF has been described yet. Lethal toxin causes death in experimental animals (28) and lyses macrophages and macrophagelike cell lines (11, 26).

Monoclonal antibodies (MAbs) have been described for PA (19) and have proven useful for confirmation of the model proposed for interaction of anthrax toxin with cells. This report describes the preparation and characterization of MAbs to LF. In addition to utilizing Fisher 344 rats to test the ability of the LF MAbs to neutralize lethal toxin, we exploited the unique sensitivity of certain macrophage cell lines to lethal toxin (26) and adopted a convenient colorimetric assay for cell viability (13, 21) that can be performed in 96-well microdilution plates to evaluate the ability of LF MAbs to neutralize lethal toxin. This in vitro, cell-based assay allows the testing of individual toxin components in various combinations with MAbs.

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MATERIALS AND METHODS

Abbreviations. DMEM, Dulbecco modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; EMEM, Eagle minimum essential medium with Earle balanced salts solution; HBSS, Hanks balanced salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; IgA, immunoglobulin A; LDH, lactic dehydrogenase; PA63, 63.5-kilodalton, C-terminal fragment purified from trypsin-cleaved PA.

General methods. Several previously reported procedures (19) were used, some with minor changes. An ELISA-based subtype kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was used to determine antibody subtype and subclass specificity. MAb affinity was calculated by using logarithmically transformed data in a modified linear regression analysis computer program (J. R. Lowe, Fed. Proc. 45:1852, 1986). Immunodiffusion plates, used to measure immunoglobulin concentration by radial immunodiffusion, were also prepared with rabbit anti-mouse IgA (Kirkegaard & Perry, Gaithersburg, Md.). Immunoglobulin concentrations were calculated from IgA (Meloy Laboratories, Inc., Springfield, Va.) standard curves after incubation of the plates for 48 h. The monoclonal nature of selected cell lines was identified by isoelectric focusing of purified IgG MAbs. Competition between MAbs for a single antigenic site was measured by a competitive binding ELISA. The percent biotinylated MAb bound in the presence of competing, unlabeled MAb was calculated by the following formula: percent binding = (test well A_{405}/control well A_{405}) × 100.

***B. anthracis* antigens.** Purified PA, LF, and EF antigens were prepared as previously described (17). PA63 was prepared by cleaving PA with trypsin and separating the fragments by chromatography on Mono Q resin (3). Each protein was at least 95% pure, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Hybridomas. Two different immunization protocols were

used in three cell fusion experiments. For the first two cell fusion experiments, female BALB/c mice were immunized intramuscularly with 12 to 25 μg of LF antigen on days 0, 15, 57, and 82. LF was administered in Freund complete adjuvant (1:1; days 0, 15, 57) or Freund incomplete adjuvant (1:1; day 82). Eighteen weeks later, mice received 25 μg of LF intravenously (i.v.) in 0.01 M sodium phosphate–0.15 M NaCl (pH 7.3). For the third cell fusion experiment, mice were immunized intramuscularly with 1 to 10 μg of LF on days 0, 21, and 49. Freund complete adjuvant was used on day 0 (1:1), and Freund incomplete adjuvant was used on days 21 and 49 (1:1). On day 101, 50 μg of LF was administered i.v. in 0.01 M sodium phosphate–0.15 M NaCl (pH 7.3). Cell fusion experiments were performed 3 days after the i.v. immunization by using spleen cells from a single immunized mouse and SP2/O-Ag14 myeloma cells (8). Hybridoma cultures were screened for reactivity with LF by ELISA (described below), and positive hybridomas were subcloned twice by limiting dilution. Ascites from each hybridoma, produced by injecting approximately 10^6 hybridoma cells intraperitoneally into female BALB/c mice 3 weeks after the intraperitoneal injection of 0.5 ml of pristane (2,6,10,14-tetramethyl pentadecane; Sigma Chemical Co., St. Louis, Mo.), were pooled and clarified by centrifugation, and aliquots were stored at −70°C.

A MAb from one of the hybridoma clones prepared in this report was used for the purification of LF from crude culture supernatant fluids by affinity chromatography (15, 20).

ELISA. The ELISA was as previously described (19), except that bound mouse immunoglobulins were detected with a 1:1,000 dilution of horseradish peroxidase conjugated to goat antibody to mouse IgG, IgM, and IgA (0.5 mg/ml; Kirkegaard and Perry). Absorbance was read on a model EL310 autoreader (Bio-Tek Instruments, Inc., Winooski, Vt.). Wells with an A_{405} of >0.20 were considered positive.

Purification of MAb. Immunoglobulins from selected ascitic fluids were purified by fast protein liquid chromatography by using a 1.0-ml Mono S cation-exchange chromatography column (Pharmacia-LKB, Piscataway, N.J.). Ascitic fluids were diluted 1:2 in buffer A (50 mM acetate buffer [pH 5.0]), centrifuged (10,500 × *g*, 5 min) in a Microfuge 12 (Beckman Instruments, Inc., Fullerton, Calif.), and filtered through a 0.22-μm-pore-size filter before 1-ml volumes were added on the column. Elution was carried out with a 0 to 40% gradient with buffer B (buffer A plus 1 M NaCl [pH 5.0]). Fractions containing specific antibody were identified by ELISA and concentrated either by precipitation with 50% saturated $(NH_4)_2SO_4$ (pH 7.5) or by ultrafiltration (Centricon 10; Amicon, Div. W. R. Grace & Co., Danvers, Mass.). Samples were dialyzed extensively either against 0.1 M $NaHCO_3$–0.15 M NaCl (pH 8.5) buffer if the sample was to be labeled with biotin (see below) or against 10 mM Tris hydrochloride (pH 8.0). One MAb (10D4) formed a precipitate in the 10 mM Tris hydrochloride buffer (pH 8.0) and was dialyzed instead against 0.01 M sodium phosphate–0.15 M NaCl (pH 7.3). Samples were frozen at −70°C.

Protein analysis. Protein concentrations were determined by the method of Redinbaugh and Campbell (24). Mouse IgG was used as the protein standard.

Biotinylation of purified MAb. Purified MAbs were biotinylated by adding 1 mg of biotin (Long Arm) NHS (50 mg/ml in dimethyl sulfoxide; Vector Laboratories, Inc., Burlingame, Calif.) per 10 mg of protein. Biotin (Long Arm) NHS is an *N*-hydroxy-succinimide derivative of biotin with an aminohexanoate spacer arm. After incubation for 2 h at room temperature, the biotinylated MAbs were dialyzed against 10 mM Tris hydrochloride (pH 7.5) and frozen at −70°C. For use, glycerol was added to 50%, and vials were held at −20°C.

In vivo neutralization. Neutralization of lethal toxin was assayed in male Fisher 344 rats (225 to 250 g) by injecting i.v. 1 ml of mixtures of 40 μg of PA, 8 μg of LF, and ascitic fluid containing 1 mg of immunoglobulin of each MAb after preincubation at 37°C for 1 h. Only one rat per MAb was tested initially. Ascites fluids of MAbs that protected the rats were retested at lower concentrations until no protection was observed. Ascites fluids that demonstrated a delayed time to death were retested at a higher concentration (5 mg of immunoglobulin) to determine if protection could be obtained. Rats were observed for 2 days.

Colorimetric assay for cell viability. An in vitro colorimetric assay, developed to assess cell growth, survival, and viability (13, 21), was modified for use as a neutralization assay. J774A.1 murine macrophage cells, obtained from the American Type Culture Collection, Rockville, Md., were cultured in medium consisting of EMEM, 0.45% glucose, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 5% heat-inactivated fetal bovine serum. Cells were scraped from confluent cultures, suspended to 4×10^5 to 5×10^5 cells per ml in the above medium supplemented with 0.025 M HEPES, plated in 96-well plates (100 μl per well), and incubated overnight at 37°C. Three different neutralization protocols were developed to study interactions among J774A.1 cells, PA, LF, and MAb. First, ascitic fluid containing MAb was diluted in lethal toxin (400 ng of PA per ml plus 40 ng of LF per ml), incubated for 1 h (37°C), and subsequently added to the J774A.1 cells. All MAbs were tested in this assay. Second, J774A.1 cells were preincubated with either PA or PA63 (400 ng/ml) at 4°C for 2 h. In separate 96-well plates, ascitic fluid containing MAb was diluted in LF (40 ng/ml) and incubated for 1 h at 37°C. After the PA or PA63 was aspirated from the cells and the wells were washed with 200 μl of incubation medium, MAb dilutions in LF were added. Third, J774A.1 cells were sequentially incubated with PA63 (800 ng/ml) and LF (80 ng/ml) at 4°C for 2 h each. Cells were washed after each incubation step with 200 μl of incubation medium. Dilutions of ascitic fluids containing MAb in incubation medium were then added. Only selected MAbs were tested by the last two protocols.

After addition of the MAb dilutions to the cells, the plates were incubated for 3.5 to 4 h at 37°C. Twenty microliters of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue, Sigma) at 5 mg/ml in HBSS with 10 mM HEPES was then added to each well, and the plates were reincubated. After an overnight incubation, the plates were centrifuged (900 × *g*, 3 min), contents of each well were aspirated, and 100 μl of acidified isopropanol (9.6 ml of isopropanol plus 0.4 ml of 1 M HCl) was added to each well. After 1 h, the contents of each well were mixed thoroughly, and the A_{540} was measured on a Bio-Tek model EL 310 autoreader. Assays were repeated to determine the endpoint titer of those MAbs that neutralized the lethal toxin. The percent neutralization was calculated from the following formula: percent neutralization = $100 \times [(A_{540}$ toxin plus MAb)/(A_{540} MAb)].

Each calculation was performed by using the average A_{540} of pairs of wells receiving either toxin plus MAb or MAb alone. The A_{450} of wells containing only incubation medium averaged about 0.800. For comparative purposes, the neutralizing ability of MAbs was arbitrarily chosen as that concentration of ascites immunoglobulin (micrograms per

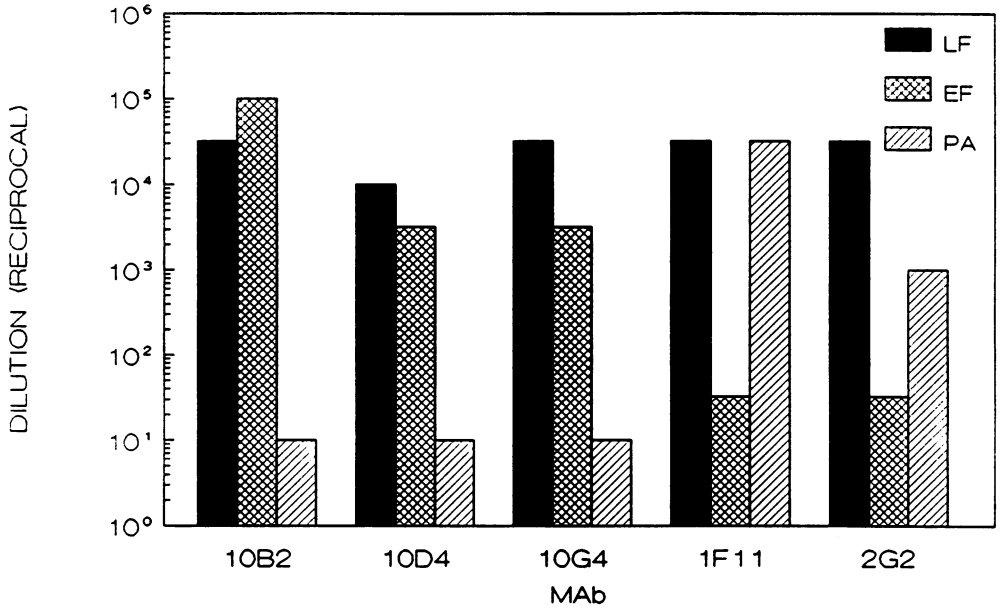

FIG. 1. ELISA titers of LF MAbs that reacted with EF and PA antigens.

milliliter) in the test wells that gave a value of ca. 80% in the above formula.

Stimulation of cell growth, even in the presence of toxin, was occasionally observed with MAbs that contained low concentrations of immunoglobulin. This activity was rapidly lost upon further dilution and was therefore attributed to the high concentration of serum proteins in the MAb sample and not considered an ability to neutralize lethal toxin. These results are not shown.

LDH assay. The ability of ascitic fluid MAbs to neutralize lethal toxin was also determined by measuring the amounts of LDH present in control or toxin-treated cell monolayers. J774A.1 cells (2×10^5 to 4×10^5/ml) were grown in 24-well tissue culture plates in medium containing EMEM, 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml to 80 to 90% confluency. A 200-μl sample of each monoclonal antibody (500 μg of immunoglobulin per ml) was incubated with 100 μl of LF (250 ng/ml) for 1 h at 37°C with shaking. A 350-μl sample of PA (250 ng/ml) and 150 μl of the MAb-plus-LF mixture were added to the cells in duplicate wells and incubated for 18 h at 37°C. LDH retained in the cells was assayed as described previously (11). Results were expressed as percent neutralization, determined as follows: 100 × [(LDH with toxin plus MAb − LDH with toxin)/(LDH without toxin or MAb − LDH with toxin)]. The LDH in cells incubated with toxin alone was ≤10% of that of control cells not exposed to toxin.

Binding studies with ^{125}I-LF. Binding studies with radioiodinated LF were based upon previously reported studies (3). L6 cells (2×10^5 to 4×10^5/ml; obtained from the American Type Culture Collection) cultured in triplicate 24-well plates were washed with cold HBSS for 5 min before 500 μl of PA (500 ng/ml) diluted in EMEM medium (EMEM [without sodium bicarbonate], 1% bovine serum albumin, 20 mM HEPES, 100 U of penicillin per ml, 100 μg of streptomycin per ml) was added to each well. The plates were incubated for 18 h at 4°C on ice. In a separate 24-well plate, 200 μl of each ascitic fluid containing MAb (500 μg of immunoglobulin per ml), 40 ng of ^{125}I-LF (specific activity, 1×10^7 to 2×10^7 cpm/μg), and EMEM medium to bring the volume of each well to 500 μl was incubated with shaking for 2 h at 4°C. After this incubation, each well received 500 μl of EMEM medium. The L6 cells were washed twice with cold HBSS, and 400 μl of the MAb-plus-^{125}I-LF mixture was added per well. After incubation for 6 h at 4°C on ice, the wells were then washed three times with cold HBSS, the cells were solubilized with 1 ml of 0.1 N NaOH, and radioactivity was counted. The data were reported as percent inhibition of the binding occurring in control wells incubated in the absence of MAb, which was approximately 10,000 cpm per well.

RESULTS

MAb characterization. Sixty-three MAbs to the LF component of the lethal toxin of *B. anthracis* were obtained from three separate fusions by using the SP2/O myeloma cell line and spleen cells from mice immunized with LF antigen. Of the 63 MAbs, 58 were specific for LF and did not bind to PA or EF, as determined by ELISA. Three MAbs (10B2, 10D4, and 10G4) cross-reacted with EF, and two (1F11 and 2G2) cross-reacted with PA (Fig. 1). A detailed analysis of the cross-reactions of these five MAbs will be presented elsewhere. Isotyping of the MAbs demonstrated that 43 were IgG clones (28 IgG1, 13 IgG2a, and 2 IgG3), 18 were IgM clones, and 2 were IgA clones. Isoelectric focusing analysis, performed on IgG clones of similar isotypes located in closely adjacent wells on the initial fusion plate, demonstrated unique banding patterns and pI differences among all MAbs tested except for two pairs of clones (3E6-3F6 and 10E4-10F4) (data not shown). Isoelectric focusing analysis of mixtures of these two clone pairs demonstrated their apparent identity (data not shown). Although 3E6 and 3F6 probably are identical, they were both carried through subsequent analysis. The results indicate that approximately 61 distinct MAb clones were obtained against LF. Isoelectric focusing was not performed on the IgM clones.

Protection assays. Neutralization assays demonstrated that four MAbs (3F6, 3E6, 2E7, and 10G3) protected Fisher 344 male rats after injection i.v. with mixtures of these MAb

TABLE 1. Percent survival of Fisher 344 rats injected with lethal toxin and MAb immunoglobulin

MAb	Survival after injection with immunoglobulin			
	1 mg		5 mg	
	No. of survivors/ total (%)	MTTD[a] (h)	No. of survivors/ total (%)	MTTD (h)
3F6	5/5 (100)		2/2 (100)	
3E6	0/2 (0)	2.1 ± 1.1	3/3 (100)	
2E7	3/7 (43)	10.5 ± 8.5	8/8 (100)	
10G3	2/4 (50)	5.8 ± 0.9	4/4 (100)	
1D8	0/2 (0)	2.0 ± 0.1	0/4 (0)	2.8 ± 0.1

[a] MTTD, Mean time to death. Mean time to death for the other 59 MAbs ranged from 0.9 to 1.75 h. Control rats averaged 1.1 ± 0.1 h mean time to death.

ascites and lethal toxin (Table 1). Lethal toxin (40 μg of PA and 8 μg of LF, approximately 13 50% lethal doses [10]) was neutralized by 1 mg of 3F6 or 5 mg of 3E6, 2E7, and 10G3 ascites immunoglobulin. A few Fisher 344 rats, however, did survive at 1 mg of 2E7 (three out of seven) and 10G3 (two out of four) ascites immunoglobulin. These rats appeared ill (lethargic, rapid respiration rate, and raised fur) for 4 to 5 h after toxin challenge before eventually recovering. MAb 3E6 did not appear to be able to neutralize lethal toxin as effectively as 3F6 did at similar immunoglobulin concentrations. The difference may be attributed to immunoglobulin concentrations which were measured as total ascites immunoglobulin and not specific MAb immunoglobulin (5). Although 1D8 did not protect rats, there was a significant delay of time to death (mean time to death, 2.8 h at 5 mg of immunoglobulin).

Incubation of the 63 MAbs at immunoglobulin concentrations up to 1 mg/ml with lethal toxin (400 ng of PA plus 40 ng of LF; approximately 27 times the concentration of LF that inhibited 50% of cell growth and viability [personal observations]) prior to exposure to cells demonstrated that neutralization was achieved with six different MAbs: 10G3 (3 μg of ascites immunoglobulin), 2E7 (30 μg of ascites immunoglobulin), 10D4, 10G4, and 13D10 (250 μg of ascites immunoglobulin), and 1D8 (500 μg of ascites immunoglobulin) (Fig. 2). We did not observe neutralization of lethal toxin in cell culture with either 3F6 or 3E6 at 1 mg of ascites immunoglobulin. However, three clones (10D4, 10G4, and 13D10) that did not protect rats against death with lethal toxin at the highest dose tested (1 mg of immunoglobulin) or show an appreciable delay of time to death neutralized lethal toxin in the cell culture assay. In addition, the clone that delayed the time to death of rats (1D8) also neutralized lethal toxin in the cell culture assay. Identical results were obtained when MAbs were preincubated with LF (40 ng/ml) and then added to cells with bound PA or PA63 (400 ng/ml). Again, 3F6 and 3E6 did not neutralize lethal toxin. When MAbs were added to cells to which LF was allowed to bind to PA by sequentially incubating J774A.1 cells with PA63 (800 ng/ml) and then LF (80 ng/ml) at 4°C for 2 h each, neutralization of lethal toxin activity was observed only with 1D8 (600 μg of ascites immunoglobulin at 80% MAb control).

Neutralization of lethal toxin was also demonstrated by the LDH assay in which J774A.1 cells cultured in 24-well plates were exposed to lethal toxin pretreated with MAbs. The percent neutralization at 100 μg of MAb immunoglobulin, measured as a percentage of control wells, indicated that 2E7 (102%), 10G3 (87%), 10G4 (51%), 10D4 (52%), 13D10 (51%), and 1D8 (89%) neutralized lethal toxin. MAbs 3F6 (5%) and 3E6 (6%) did not appear to neutralize lethal toxin in this assay. The percent neutralizing activity for the remaining MAbs ranged from 3.2 to 23%.

Competitive binding assay. The competitive ELISA was performed to determine whether the MAbs that neutralized lethal toxin in either the in vivo or in vitro assays were directed against overlapping or nonoverlapping antigenic determinants. Four different competition groups could be

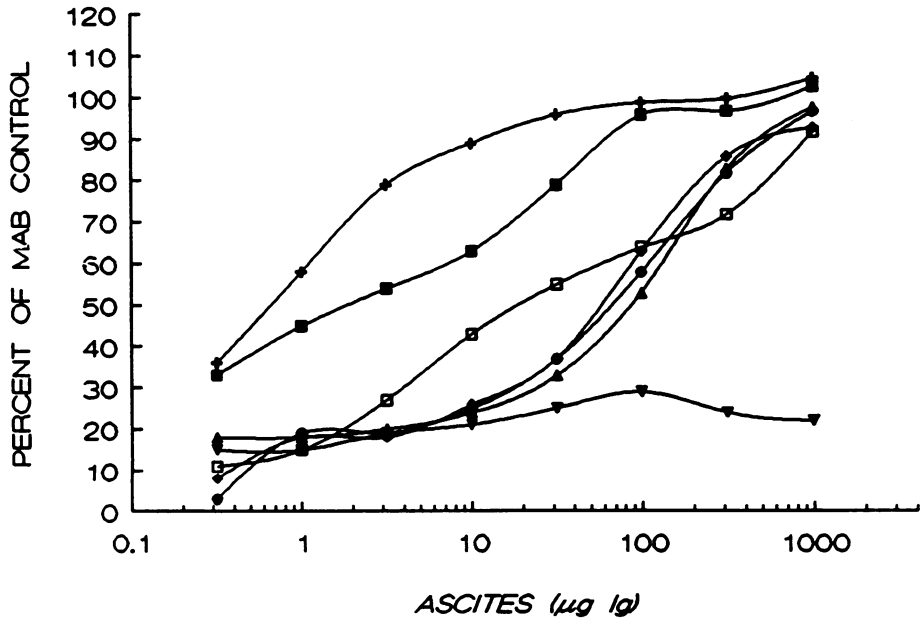

FIG. 2. Ability of LF MAbs to neutralize lethal toxin activity. A colorimetric assay was used to calculate the percentage of J774A.1 cells surviving in the presence of lethal toxin (400 ng of PA per ml and 40 ng of LF per ml) preincubated with MAbs before addition to cells. Symbols: +, 10G3; ■, 2E7; ◆, 10D4; ▲, 10G4; ●, 13D10; ▼, 3F6; □, 1D8. Ig, Immunoglobulin.

TABLE 2. Competitive antibody-binding ELISA between selected biotinylated and unlabeled neutralizing MAbs

Unlabeled MAb	% Biotinylated MAb bound[a]							
	3E6	3F6	2E7	10G3	10G4	10D4	13D10	1D8
3E6	**14**	7	294	211	90	97	84	218
3F6	4	**4**	408	208	81	100	62	209
2E7	71	63	**7**	0	60	54	42	96
10G3	43	40	4	**0**	29	42	32	67
10G4	82	95	8	18	**58**	46	27	71
10D4	62	76	1	9	37	**21**	31	58
13D10	73	88	11	21	34	33	**32**	41
1D8	38	40	145	143	33	23	18	**1**

[a] In the presence of a saturating concentration of unlabeled MAb. Determinant groups are identified by boxes.

identified on the basis of competitive binding experiments among the eight neutralizing MAbs (Table 2). Similar antigenic regions were recognized by 3E6 and 3F6; 2E7 and 10G3; and 10G4, 10D4, and 13D10. MAb 1D8 reciprocally blocked only itself. We observed nonreciprocal or one-way inhibition by 10G4, 10D4, and 13D10 by blocking the binding of 2E7 and 10G3, both from the same determinant group. MAbs 3E6 and 3F6 enhanced binding of 2E7, 10G3, and 1D8, which suggested a conformational change in LF (27). MAb 1D8 also slightly enhanced the binding of 2E7 and 10G3. The low reciprocal percent values for 10G4 and 13D10 are reflected by their low affinity (see Table 3). The antigenic determinant groups were further studied in a competitive ELISA in which biotinylated 10G3 or 3F6 was tested against various concentrations of unlabeled, competing MAb (Fig. 3). MAbs 10D4, 10G4, and 13D10 showed partial recognition with 10G3, especially at higher immunoglobulin concentrations (Fig. 3a). However, 3F6 (as well as 3E6) and 1D8 not only bound to a different antigenic site but again enhanced the A_{405} value of biotinylated 10G3. The initial binding of 3F6 (or 3E6) or 1D8 to LF increased the apparent affinity of biotinylated 10G3 for LF. Similar results were observed with biotinylated 2E7 instead of 10G3. Data from the competitive ELISA with biotinylated 3F6 (Fig. 3b; similar results were obtained with 3E6) demonstrated recognition of the same antigenic region by only 3F6.

Binding studies. Binding studies showed that the five MAbs that neutralized lethal toxin activity by interacting with LF in solution in the cellular assays (10G3, 2E7, 10D4, 10G4, and 13D10) inhibited binding of ^{125}I-labeled LF to PA bound to cell receptors from 70 to 97% (Table 3). MAb 1D8, which was able to neutralize lethal toxin activity in the cellular assays even after LF was prebound to cell-bound PA, inhibited the binding of ^{125}I-labeled LF to PA bound to cell receptors by 41%. The binding inhibition for both 3F6 and 3E6, which did not neutralize in the cellular assays, was 7%. Several other MAbs (3E3, 5E5, 5C8, 8F2, 9E5, and 9F10) blocked the binding of ^{125}I-LF to PA from 21 to 32% (data not shown). However, these MAbs did not appreciably delay the time to death of rats injected with lethal toxin and 1 mg of ascites immunoglobulin (range, 1 to 1.75 h) and did not demonstrate neutralization in the macrophage cytolytic assays.

Table 3 summarizes the properties of these MAbs. The eight neutralizing MAbs also can be placed into one of three groups based upon the relative affinity measurements (32): high (≤0.2 μg), moderate (≥0.2 to ≤1 μg), and low (>1 μg) affinity. Affinity measurements give a quantitative estimate of the antibody concentration required to achieve 50% plateau binding (30, 31). We observed neutralization of lethal toxin by MAbs of moderate and low affinity.

DISCUSSION

Six MAbs (10G3, 2E7, 10G4, 10D4, 13D10, and 1D8) neutralized lethal toxin in both macrophage assays. Five of these MAbs (10G3, 2E7, 10G4, 10D4, and 13D10) appeared to do so by inhibiting the binding of LF to cell-bound PA. This was demonstrated directly by the ability of these MAbs to inhibit the binding of ^{125}I-LF to cell-bound PA and is supported by the results of the colorimetric assay, in which these MAbs were unable to protect cells to which PA and LF had been prebound. Among these MAbs, there appeared to be a correlation between their ability to protect macrophages in vitro and rats in vivo. The MAbs that were most active in neutralizing toxin in vitro (10G3 and 2E7) had higher relative binding affinities and protected rats. Those MAbs which were less active in both in vitro neutralization assays (10G4, 10D4, and 13D10) had lower binding affinities and did not protect rats. It appears as if the in vitro assays were more sensitive for these MAbs. This may be explained by the different ratios of MAb immunoglobulin:LF used in each assay. The ratio was 1 mg:8 μg in the rat assay, 1 mg:0.04 μg in the colorimetric assay, and 100 μg:0.025 μg in the LDH assay. The 32- and 200-fold-higher ratios of MAb immunoglobulin:LF in the LDH and colorimetric assays, respectively, may have enabled detection of neutralizing activities in those MAbs with lower binding affinities.

MAb 1D8 was unique in being the only MAb able to neutralize lethal toxin after PA and LF were sequentially prebound to cells. We can only speculate that the binding site for 1D8 is different from the PA-binding site on LF, as 1D8 did not reciprocally compete in the competitive ELISA with any of those MAbs that inhibited binding of ^{125}I-LF to PA and was unable to inhibit completely the binding of ^{125}I-LF to cell-bound PA.

We did not understand why MAb 3F6 was able to protect rats in vivo but was unable to neutralize lethal toxin activity in the in vitro macrophage assays. The inability of 3F6 to block LF binding to cell-bound PA suggests that it may be binding to the presumed enzymatically active region of LF. We were unable to evaluate the ability of 3F6 to neutralize enzymatic activity of LF since the nature of this activity is not yet known. However, it would be expected that such binding would be detected in the in vitro neutralization assays if cytolysis is due to the postulated enzymatic activity of LF. It may be relevant that MAb and polyclonal antibodies to other toxins that inhibit enzymatic activity but do not neutralize toxicity have been described (7, 22). This may be explained by the removal of antibodies after endocytosis with restoration of toxic activity (7), which could occur in macrophages in vitro but not in other target cells in rats. This inability of 3F6 to neutralize toxin in vitro also suggests that macrophage cytotoxicity may not be a direct correlate of lethality in the rat model. There may be other cell targets and mechanisms responsible for death in the rats. It is also possible that 3F6, when given to rats, directs the toxin to other cells which are resistant to lethal toxin, or it may affect the clearance of the lethal toxin in other unknown ways.

The apparent inability of the remaining MAbs to inhibit binding of ^{125}I-LF to PA or neutralize lethal toxin activity may suggest that they do not bind to regions of LF involved in binding to PA or the postulated enzymatic site. However, it is also possible that some of these MAbs may be unable to bind LF in solution since these MAbs were selected by an

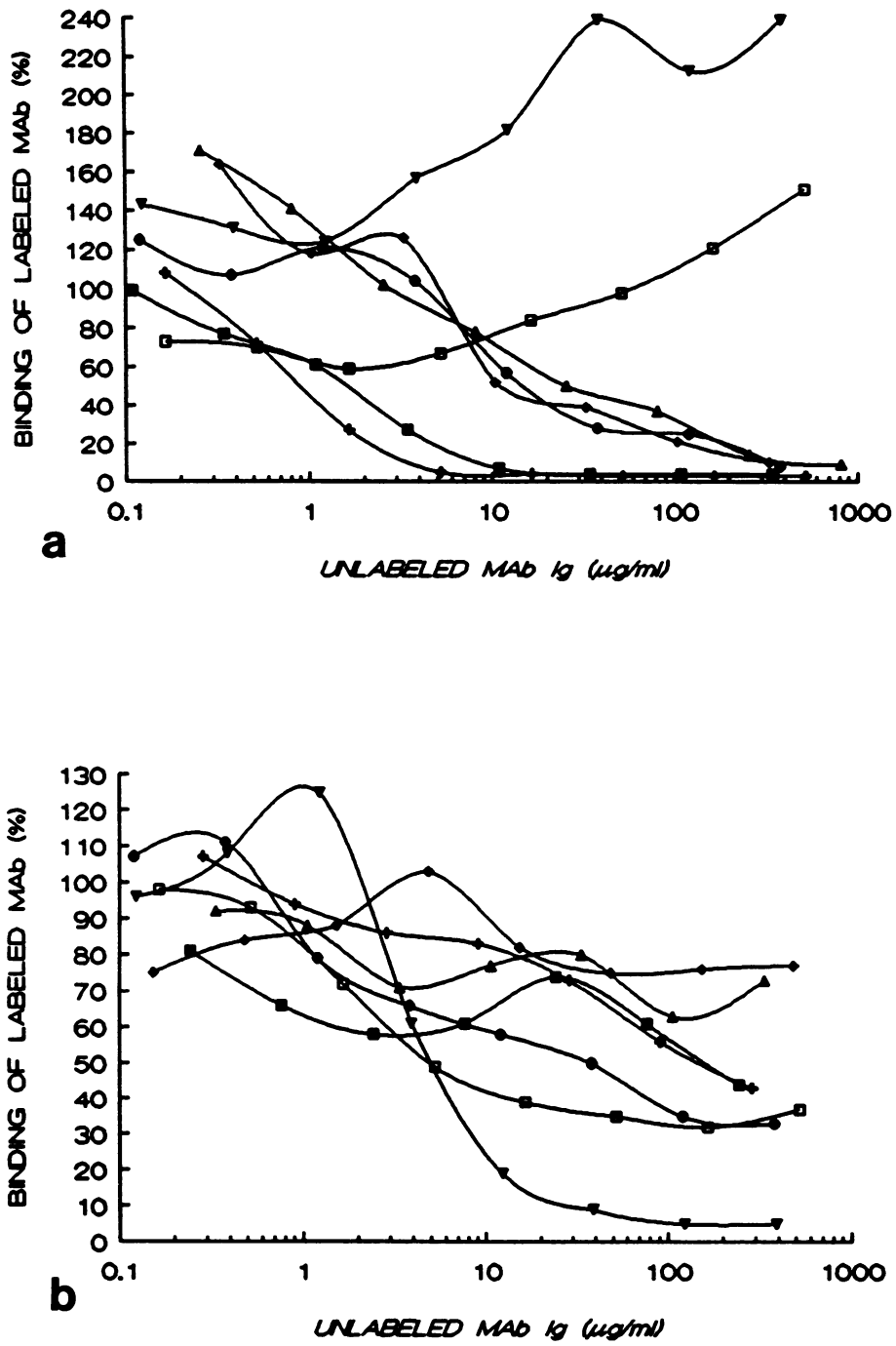

FIG. 3. Competitive antibody-binding assay with biotinylated and unlabeled MAbs. The binding of biotinylated 10G3 (a) or 3F6 (b) was measured in the presence of different concentrations of competing, unlabeled MAbs. Symbols: +, 10G3; ■, 2E7; ◆, 10D4; ▲, 10G4; ●, 13D10; ▼, 3F6; □, 1D8. Ig, Immunoglobulin.

ELISA system in which the antigen, bound to plastic, may be altered in some way (29).

Competitive binding assays using MAbs have been useful in defining antigenic regions on proteins. This study identified four antigenic regions of the LF protein capable of inducing seven neutralizing MAbs. We observed reciprocal and nonreciprocal competition and synergistic, or enhanced binding, interactions. Nonreciprocal binding has been attributed to several factors, including conformational changes, steric hindrance, and avidity differences (1, 6, 14, 23). Synergistic binding, as with 10G3, 2E7, and 1D8 in the presence of 3F6, suggests either an interaction among the Fc regions of the antibody molecules (9) or conformational changes of the antigen (27). Since the synergistic binding was nonreciprocal, the observed effect was probably due to a conformational change (27) in the LF protein.

Until recently, very little homology was thought to exist between LF and EF proteins. However, a comparison of the

TABLE 3. Summary of properties of neutralizing LF MAbs

Hybridoma clone	Antibody subtype	Neutralization			% Binding inhibition[d]	Affinity[e]
		Rats[a]	MTT[b]	LDH[c]		
2E7	IgG1	5	30	102	97	1.3
3E6	IgG1	5	>1,000	7	7	0.2
3F6	IgG1	1	>1,000	3	7	0.6
10G3	IgG1	5	3	87	95	0.3
10D4	IgG3	Neg	250	52	88	7.9
10G4	IgG3	Neg	250	51	77	4.6
13D10	IgG1	Neg	250	51	70	7.8
1D8	IgG1	>5	500	89	41	0.2

[a] Amount (milligrams) of immunoglobulin required to neutralize lethal toxin in rats (Table 1). Neg, Negative.
[b] Amount (micrograms) of immunoglobulin required to neutralize lethal toxin as measured by the reduction of MTT (colorimetric macrophage assay) (Fig. 3).
[c] Percent neutralization of lethal toxin in the LDH assay (see text).
[d] Inhibition of binding of ^{125}I-LF to PA bound on L6 cells.
[e] Amount (micrograms) of immunoglobulin required for 50% plateau binding by ELISA.

deduced amino acid sequences of EF and LF suggests that there are several regions of homology in the N termini of these proteins (4, 25; J. R. Lowe, Ph.D. thesis, University of Kansas, Kansas City, 1989). We identified three LF MAbs that cross-reacted with EF (10D4, 10G4, and 10B2) by ELISA. Two of these MAbs (10D4 and 10G4), which blocked the binding of LF to PA, may recognize antigenic determinants within these regions of homology. There was no inhibition of LF binding with MAb 10B2, suggesting that LF and EF may also share a determinant that does not involve an LF binding site to cell-bound PA. The two MAbs that best blocked binding of LF to PA (2E7 and 10G3) and were assumed to be reacting at a binding site did not cross-react with EF.

It is anticipated that our MAbs should prove useful for studies of antigenic structure and function of LF, interactions of LF with PA, and possible relationships between LF and EF.

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