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Inhibition of *Aspergillus fumigatus* Elastase with Monoclonal Antibodies Produced by Using Denatured Elastase as an Immunogen†

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In preparing monoclonal antibodies to the elastase from *Aspergillus fumigatus*, we found that the enzyme was weakly immunogenic in BALB/c mice. Antiserum titers were only 1:1,000 to 1:5,000, and hybridomas secreted nonspecific immunoglobulin M (IgM). Denaturing the elastase in 0.5% sodium dodecyl sulfate at 80°C for 10 min prior to injection increased titers of antiserum against the nondenatured (native) enzyme 10-fold. Of eight hybridomas selected following immunization with the denatured enzyme, seven produced IgG reactive with the native enzyme and one produced nonspecific IgM. The nondenatured immunogen tested again yielded mainly IgM producers. Immunoblots and enzyme-linked immunosorbent assay showed that the IgG monoclonal antibodies were reactive with both the denatured and nondenatured fungal elastases; none cross-reacted with human neutrophil elastase, porcine pancreatic elastase, or *Pseudomonas* elastase. Elastase-specific polyclonal antibody produced in mice inhibited elastase activity beginning at a molar ratio (antibody to elastase) of 4:1, and activity was completely inhibited at 14.5:1. Some individual monoclonal antibodies partially inhibited elastase, but certain pairs, at a molar ratio of each antibody to elastase of 5.4:1, acted synergistically to inhibit the activity completely.

Aspergillus fumigatus, an opportunistic pathogen, is the predominant organism causing invasive pulmonary aspergillosis (36), a serious problem in immunocompromised individuals (5, 36, 37, 53). Previously, we correlated elastase production in environmental strains of *A. fumigatus* with the ability to cause invasive pulmonary aspergillosis in immunocompromised mice (18). Elastases are proteinases with the ability to catalyze the hydrolysis of elastin, the extensively cross-linked structural protein of lung tissue. Indeed, many studies have shown that porcine pancreatic elastase (30, 33, 45, 54), human neutrophil elastase (8, 32, 41, 42), and *Pseudomonas* elastase (2, 10, 14, 28) can cause emphysema in animal models, strongly suggesting that elastases play a vital role in the pathogenesis of various pulmonary diseases.

To define the role of *Aspergillus* elastase in invasive infection, we propose to determine whether murine monoclonal antibodies (MAb) or polyclonal antibodies (PAb) can ameliorate the course of the disease in immunocompromised mice infected with *A. fumigatus* spores. This report deals with production of antibodies for this purpose. Initial attempts to produce specific immunoglobulin G (IgG) antibody by using purified elastase as the immunogen were not successful. Only low antiserum titers were produced in mice, and MAb produced in fusion experiments were all of the IgM type and cross-reacted extensively with other proteins. However, immunization with elastase which had been denatured by being heated in sodium dodecyl sulfate (SDS), a procedure (described by Harlow and Lane [15]) for increasing immunogenicity of proteins, elicited antibody titers 10-fold higher than those elicited by the nondenatured elastase and resulted in production of specific monoclonal IgG reactive with the native (nondenatured) enzyme. Certain pairs of these antibodies were found to act synergistically and to totally inhibit elastase activity in vitro.

MATERIALS AND METHODS

Preparation of antigens. Elastase (32 kDa) from *A. fumigatus* 18, isolated by Kothary et al. (18), was purified 220-fold from culture broth by using ion-exchange and gel filtration chromatographies on a fast-performance liquid chromatography system (11). The purified enzyme produced a single major band on isoelectric focusing (IEF; pI 8.75). Two forms of the elastase, native and denatured, were used in immunizations. The native form was prepared in phosphate-buffered saline (PBS), pH 7.4, at 0.1 to 0.24 mg/ml and was maintained at 4°C before being injected. Denatured elastase (0.1 to 0.24 mg/ml) was prepared in PBS (pH 7.4) containing 0.5% SDS, and the mixture (0.2 to 0.4 ml) was heated at 80°C for 10 min to denature the antigen (15).

Immunization. In preliminary studies, 7-week-old female BALB/c mice (Charles River Breeders, Wilmington, Mass.) were anesthetized by inhalation of methoxyflurane (Pitman-Moore, Inc., Atlanta, Ga.) and injected intraperitoneally (i.p.) with 1.5 μg (70 μl) of native elastase in 135 μl of complete Freund adjuvant (Sigma Chemical Co., St. Louis, Mo.). Booster injections 4 weeks later contained 5.5 μg (100 μl) of elastase in 200 μl of incomplete Freund adjuvant (Sigma). Three and 4 weeks later, mice received 12 μg (75 μl; in PBS) intrasplenically. One week later, splenocytes from one mouse were harvested for fusion with myeloma cells for hybridoma production. After an additional 3 weeks, a second mouse received 20 μg (100 μl) i.p. on each of 2 days prior to harvest of splenocytes for hybridoma production.

An immunization protocol based on that described by Stähli et al. (40) was used in a study that directly compared denatured elastase with native elastase for antibody production. Three mice were immunized with denatured elastase,

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and three were immunized with native enzyme. On day 1, anesthetized mice received a total of 18 μg (83 μl) of the appropriate elastase preparation emulsified in 83 μl of complete Freund adjuvant and injected with 111 μl i.p. and 27.5 μl in each hind foot pad. After 4 weeks, mice were boosted with 20 μg (85 μl) of antigen emulsified in an equal volume of incomplete Freund adjuvant and injected with 81 μl i.p. and 40 μl in each hind foot pad. Three days before fusion, mice received 60 μg (255 μl; in PBS), with 170 μl administered i.p., 43 μl administered intravenously, and 21 μl injected in each hind foot pad. On each of the 2 days prior to fusion, mice were injected with 30 μg (128 μl; in PBS) of antigen, with 85 μl given i.p. and 43 μl given intravenously.

ELISA. Antibodies reactive with native elastase were detected with an enzyme-linked immunosorbent assay (ELISA) based on the general method described by Tijssen (47). Unless otherwise stated, reagent volumes added to wells of 96-well polystyrene enzyme immunoassay plates (GIBCO Laboratories, Grand Island, N.Y.) were 100 μl, and the reaction temperature was maintained at 37°C. Between steps, wells were emptied of reagents and washed three times (NUNC Immuno-Wash; LDP, North Haledon, N.J.) at room temperature with 0.05% Tween 20 in PBS, pH 8.0. Purified elastase (1 μg/ml in PBS, pH 8.0) was added to the wells to coat them with the enzyme. After storage of plates overnight at 4°C, wells were filled with blocking solution (3% bovine serum albumin [BSA; Sigma] or 2% crude ovalbumin [Sigma] in PBS, pH 8.0). After 1 h, wells were washed and test antibody was added. After 2 h, antibody reactive with elastase was detected with horseradish peroxidase-labeled goat anti-mouse IgG. The enzyme substrates were 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) and hydrogen peroxide. All three reagents were from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, Md.), and the procedures used were described by the manufacturer. The A_{405} was measured every 10 min for 30 min in a microplate reader, and the reactivity was compared with those of appropriate controls.

Antibody titer in serum. Blood samples were taken from anesthetized mice immediately prior to immunization, 5 days after the second immunization, and immediately before fusion experiments. These samples were collected from the retroorbital plexus of mice via heparinized capillary tubes (Fisher Scientific, Springfield, N.J.). Dilutions (1:100 to 1:500,000) of the serum, prepared in PBS (pH 8.0), were assayed by ELISA to determine antibody titer to native elastase.

MAb production. Murine hybridomas were produced by the procedure of Galfré and Milstein (12). In brief, 2×10^8 lymphocytes and 2×10^7 myeloma cells (P3-X63-Ag 8.653, repository no. GM 3570; Human Genetic Mutant Cell Repository, Institute of Medical Research, Camden, N.J.) were fused in 50% polyethylene glycol (1,500 molecular weight; M. A. Bioproducts, Walkersville, Md.). Hybridomas were selected under 8% CO_2 as described by Galfré and Milstein (12). Hybridomas testing positive for production of MAb to native elastase were cloned twice by limiting dilution (13). Antibody class and subclass were determined with an isotyping kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by following guidelines of the manufacturer.

Purification of antibodies. Antiserum was dialyzed against 20 mM sodium phosphate buffer, pH 7.7, and applied to a protein G-Sepharose Fast Flow (Sigma) column previously equilibrated in the same buffer. The column was washed (10 bed volumes) with buffer, PAb was eluted with 0.1 M glycine buffer (pH 2.7), and fractions containing PAb were neutralized with 1.0 M Tris HCl (pH 9.0), pooled, and concentrated by using ultrafiltration and then Centriprep 10 and Centricon 10 concentrators (both from Amicon Corp., Danvers, Mass.).

MAb was purified on protein G-Sepharose Fast Flow essentially as described above except that antibody that did not bind when applied in phosphate buffer was collected, dialyzed against 0.1 M sodium borate buffer (pH 9.0) containing 2.5 M NaCl, and rechromatographed on the protein G column previously equilibrated in the borate-NaCl buffer. MAb was eluted, neutralized, and concentrated as described above.

ELISA and immunoblot analysis of MAb specificity. Reactivity of the MAb was tested against elastases from three sources: human neutrophils, *Pseudomonas aeruginosa* (both gifts from William Hanlon, Merck, Sharp & Dohme Research Laboratories, Rahway, N.J.), and porcine pancreas (Worthington Enzyme, Freehold, N.J.). Some common proteins (BSA, crude and purified ovalbumin, and nonfat dry milk) were also tested for reactivity with MAb. For ELISA, 100 μl of each antigen at concentrations of 1 μg/ml for the elastases and 1 mg/ml for the other proteins was used to coat wells.

All four elastases were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (20) prior to immunoblot analysis. Proteins were denatured and reduced by being boiled in 4% SDS–10% mercaptoethanol and were electrophoresed in a 12.5% polyacrylamide slab gel (Hoefer SE 600 apparatus) with a reservoir buffer consisting of 25 mM Tris (pH 8.3), 192 mM glycine, and 0.1% SDS. The proteins resolved in the gel were fixed in 10% acetic acid–30% methanol and silver stained (31). Proteins in a duplicate gel were transferred electrophoretically (49) onto nitrocellulose paper (BA 83; Schleicher & Schuell, Keene, N.H.) in cooled 25 mM Tris buffer (pH 8.3) containing 192 mM glycine, 0.05% SDS, and 10% methanol. Subsequent steps were performed at 37°C. Between steps, the nitrocellulose sheets were washed (three times for 10 min each time with shaking at room temperature) in Tris-buffered saline (TBS), pH 8.0 (0.05 M Tris, 0.2 M NaCl), containing 0.05% Tween 20. Nitrocellulose sheets were soaked for 1 h, with shaking, in TBS containing 3% BSA to block residual binding sites. The sheets were then immersed in 18 ml of a 1:3 dilution of the test MAb for 3 h at 37°C or overnight at 4°C on a rocking plate. To visualize the MAb reactive with the elastases, sheets were soaked in 60 to 90 ml of alkaline phosphatase-labeled goat anti-mouse IgG for 1 h, washed, and then immersed in 60 to 90 ml of phosphatase substrate (4-bromo-3-chloro indolyl phosphate [16.7 μg/ml] and Nitro Tetrazolium Blue [33.3 μg/ml] in 100 mM Tris [pH 9.0]) until color developed (all three reagents were from Boehringer Mannheim). The reaction was stopped by flooding the nitrocellulose sheets with distilled water.

IEF and zymogram analysis. IEF and staining of proteins in IEF gels were carried out as described elsewhere (11). Zymograms containing 0.5% soluble elastin (Elastin Products Co., Owensville, Mo.) were employed for detecting elastase activity in IEF gels. After a reaction time of 2.5 h at 37°C, residual elastin in the zymogram was fixed, dried, and stained (11).

Proteins in IEF gels were transferred to nitrocellulose for detection of reactivity with MAb. The IEF gel was covered with a sheet of wet (TBS, pH 7.5) nitrocellulose (Schleicher & Schuell). Two sheets of filter paper (GB002; Schleicher & Schuell) were stacked on top of the nitrocellulose sheet and a stack (4 cm) of paper towels was placed on top of the filter

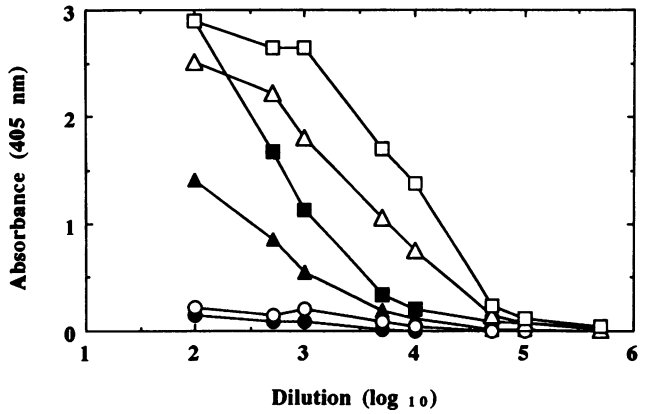

FIG. 1. Antiserum titers for two mice used in fusion experiments to compare denatured elastase (open symbols) and native elastase (closed symbols) as immunogens. Antibody titers before immunizations (circles), 5 days following second immunizations (triangles), and after final immunizations (squares) are compared. A_{405} values were determined by ELISA 30 min after addition of substrate.

paper. A 3-kg weight was placed on top of the stack to facilitate transfer of the proteins onto the nitrocellulose by capillary diffusion. After 3 h at room temperature, the sheet was cut into strips, and proteins binding with MAb were detected as described above for immunoblot analysis.

RESULTS

Preliminary experiments to produce MAb specifically reactive with *A. fumigatus* elastase were not successful. After immunization, the antiserum titer in one mouse reached only 1:1,000, and although a second mouse had received additional i.p. injections, the titer was still only 1:5,000. In separate experiments, the spleen lymphocytes from these two mice were fused with myeloma cells. Of 2,400 wells that showed hybridoma growth, only 12 tested positively for antibody reactive with elastase. The nine hybridoma lines that were cloned and stabilized all produced IgM that reacted strongly with the blocking agents (BSA, nonfat dry milk) used in ELISA and immunoblot.

Evidently, the fungal elastase is weakly antigenic in mice. We decided to test the possibility that denaturing the elastase by heating it in SDS, a procedure for improving immunogenicity, would facilitate production of highly specific MAb to the elastase. Three mice were immunized with the denatured elastase, and as a comparison, three mice were immunized with the native (nondenatured) elastase. Care was taken to ensure that amounts, routes, and timing of injections were the same in both sets of mice and that the antigen was thoroughly emulsified in adjuvant. Five days following boost injections, the antibody titers in mice immunized with denatured elastase were at least 10-fold higher than titers in mice injected with the native enzyme.

After the second injections, the mouse with the highest antibody titer for elastase in each group was selected for final immunizations. Figure 1 shows the antibody titers in serum taken from the two selected mice before immunization and after each injection. The titer against the native elastase after the final injection was 10-fold higher in the mouse immunized with the denatured elastase than in the mouse immunized with native elastase.

Splenocytes were harvested from the two mice, and separate fusion experiments were performed, with care taken to duplicate experimental conditions. Splenocytes from the mouse immunized with the denatured immunogen yielded eight hybridomas that produced MAb reactive with native elastase: seven produced IgG, and one produced IgM. On cloning, only six lines (five producing IgG and one producing IgM) continued to grow and produce antibody. The fusion using splenocytes from the mouse immunized with native enzyme yielded 24 hybridomas that produced MAb reactive with the native elastase, but only 12 retained activity on transfer to fresh medium; 2 of these produced IgG, and 10 produced IgM. Only 3 of the 12 could be stabilized and cloned; 2 of these 3 produced IgG.

The MAb from stabilized cell lines were tested by ELISA for reactivity against different elastases and common protein blocking agents (Table 1). Cell lines BB11, KD5, GD11, and MB8, which had been produced with denatured elastase as the immunogen, produced MAb reactive specifically with *A. fumigatus* elastase. These four MAb did not cross-react with other elastases and proteins tested, and this specificity was confirmed by immunoblot analysis. Figure 2 shows immunoblot analysis for two of these MAb (BB11 and KD5); the other two MAb showed similar results. All four cell lines were IgG producers. A fifth cell line (A) produced IgG reactive with fungal elastase but also somewhat cross-reactive with other proteins. Cell line HB8 produced IgM, which reacted strongly with all of the proteins tested. In contrast (Table 2), nine cell lines resulting from immunization with native elastase produced antibody reacting with *A.*

TABLE 1. Specificity of MAb prepared by using denatured elastase as immunogen

Proteins tested	Relative reactivity of MAb measured by ELISA[a]					
	A (IgG)	KD5 (IgG1)	GD11 (IgG1)	HB8 (IgM)	BB11 (IgG2A)	MB8 (IgG2A)
Elastases						
A. fumigatus	+++	+++++	+++++	+++++	+++++	+++++
Porcine	++	−	−	+++++	−	+
Human	+	−	−	+++++	−	+
P. aeruginosa	+	−	−	+++++	−	−
Other proteins						
Egg white	++	−	−	++++	−	−
Ovalbumin	++	−	+	++++	−	−
BSA	+	−	−	+	−	−
Dry milk	+	−	−	+	−	−

[a] Hybridoma strains and immunoglobulin classes are given. ELISA values (A_{405}) were measured 30 min after substrate addition. Symbols indicate A_{405} values: −, <0.2; +, 0.2 to 0.4; ++, 0.4 to 0.7; +++, 0.7 to 1.0; ++++, 1.0 to 1.5; +++++, >1.5.

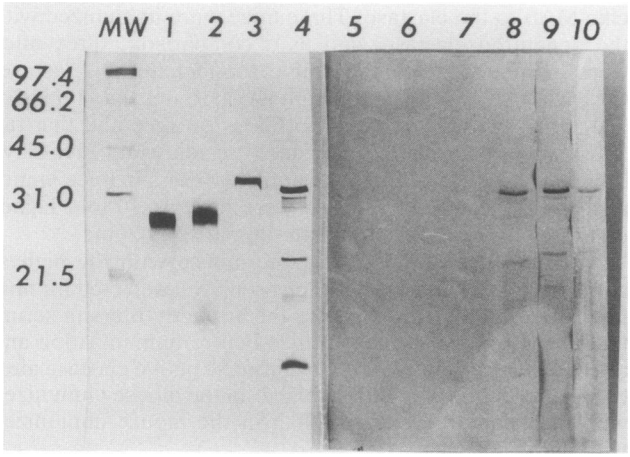

FIG. 2. Silver-stained SDS-PAGE and immunoblots of elastases from various sources. One-microgram amounts were applied to an SDS-PAGE gel (lanes 1 to 4), and proteins were transferred electrophoretically to nitrocellulose sheets for immunoblots (lanes 5 to 10). Porcine pancreatic elastase (lanes 1 and 5), human neutrophil elastase (lanes 2 and 6), *Pseudomonas* elastase (lanes 3 and 7), and *A. fumigatus* elastase (lane 4, 8, 9, and 10) were assayed. Bands in immunoblots were visualized by reaction with MAb BB11 (lanes 5 to 8), CCIII 19 (lane 9), or KD5 (lane 10). Negative reactions of MAb CCIII 19 and KD5 are not shown. Lane MW, molecular mass standards (in kilodaltons).

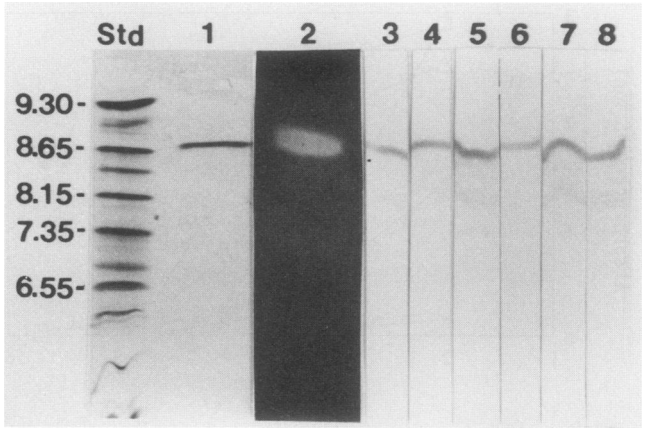

FIG. 3. Analysis of purified fungal elastase by IEF. Lane 1, IEF gel stained with Coomassie brilliant blue R250; lane 2, zymogram with soluble elastin as substrate; lanes 3, 4, 5, 6, 7, and 8, immunoblots of the IEF gel visualized by using MAb KD5, MB8, BB11, A, GD11, and CCIII 19, respectively. Lane std, molecular mass standards (in kilodaltons).

fumigatus elastase but also cross-reacting with all proteins tested. All but two of these cell lines (CVI B3 and CCIII 19) produced IgM. Again, most of these IgM-producing cell lines were unstable and could not be cloned. Only one cell line (CCIII 19) produced IgG that reacted specifically with *A. fumigatus* elastase, and this was confirmed by immunoblot analysis (Fig. 2).

The specificity of the MAb was confirmed by immunoblot analysis of purified elastase subjected to IEF (Fig. 3). Zymograms, made with soluble elastin as substrate, showed that elastase activity corresponded to a stained (Coomassie brilliant blue R250) protein with a pI of 8.75. All of the MAb that were reactive with elastase in ELISA also reacted with this protein in immunoblot.

Elastase-specific PAb was purified on protein G. Various amounts were incubated with elastase for 30 min and then assayed for inhibition of purified elastase (Fig. 4). The amount of enzyme assayed was always 1 μg in 750 μl of reaction mixture. Elastase activity decreased compared with that of controls containing nonspecific PAb only when the molar ratio of PAb to enzyme exceeded 4:1 (20:1 on a weight basis). Complete elastase inhibition was observed when the PAb was increased to a molar ratio of 14.5:1. Nonspecific PAb did not inhibit elastase at any molar ratio tested; instead, it appeared to increase activity, as did specific IgG at molar ratios below about 5:1. BSA (50 μg), substituted for IgG in a similarly conducted experiment, enhanced elastase activity 25% over the level of the control with buffer alone, indicating that the apparent increase in activity was not unique to IgG.

Various concentrations of each of the five MAb, each purified on protein G, were tested individually for their effects on elastase activity. As with PAb, lower concentrations of these antibodies generally enhanced elastase activity; four MAb increased elastase activity by 40 to 78% when tested against an equal molar amount of enzyme, and only one MAb (KD5) showed slightly decreased activity (91% of control). Two MAb (GD11 and CCIII 19) and perhaps a third (KD5) decreased elastase activity when tested individually at higher concentrations. None of these MAb completely

TABLE 2. Specificity of MAb prepared by using native elastase as immunogen

Proteins tested	Relative reactivity of MAb measured by ELISA[a]									
	CVI B3 (IgG)	CI 2F5 (IgM)	CCIII 19 (IgG1)	4G9 (IgG-IgM)	6C4 (IgM)	8C6 (IgM)	9G5 (IgM)	10B10 (IgG-IgM)	2F4 (IgM)	1B10 (IgM)
Elastases										
A. fumigatus	+++++	++	+++++	++	++++	++	+	+	++	+
Porcine	+++++	+	−	+	+++	+++	+++	+	+	+
Human	+++++	+	−	++	+++	+++	++	+	+	+
P. aeruginosa	+++++	+	−	+	++++	++	+++	+	+	+
Other proteins										
Egg white	++++	++	+	++	+++	++	+++	+	+	+
Ovalbumin	++++	+	−	+++	++	+	+	+	+	+
BSA	−	−	−	++	+	+	+	+	+	+
Dry milk	+	−	−	++	+	+	+	+	+	+

[a] Hybridoma strains and immunoglobulin types are given. C in a MAb designation indicates that the cell line was cloned. ELISA values (A_{405}) were determined 30 min after substrate addition. Symbols indicate A_{405} values: −, <0.2; +, 0.2 to 0.4; ++, 0.4 to 0.7; +++, 0.7 to 1.0; ++++, 1.0 to 1.5; +++++, >1.5.

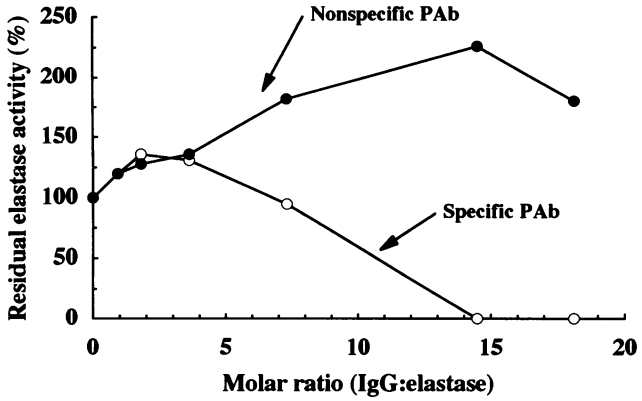

FIG. 4. Comparison of the effects of elastase-specific and nonspecific murine PAb on elastase activity. Mixtures containing PAb (0 to 85 μg) and 1 μg of elastase were prepared in 50 mM sodium borate buffer (pH 7.5) to a final volume of 500 μl. The PAb was tested at molar ratios of IgG to elastase ranging from 0 to 18. After 30 min at 37°C, 250 μl (5 mg in buffer) of elastin-Congo red was added, and after 30 min, the amount of dye released was measured spectrophotometrically (11). Residual activity is reported as percentage of a control which contained elastase but no PAb.

inhibited elastase at the highest molar ratio of MAb to elastase tested, 10.7:1, the lowest activity observed being 58% that of the control (Table 3). However, pairs of MAb, each with a MAb to elastase molar ratio of 5.4:1, acted synergistically to inhibit activity. Most pairs significantly decreased activity, and two pairs inhibited it totally.

DISCUSSION

In early experiments with the native elastase as immunogen, antiserum titers remained relatively low, even after several injections. The hybridomas prepared following these immunizations all produced nonspecific IgM MAb, indicating that a secondary immune response (35) was not elicited. A number of possibilities might explain these earlier results. Elastases, which are found in many organisms, including mammals (4, 16, 22, 38, 46), bacteria (19, 25, 50, 51), schistosomes (23, 24), and fungi (18, 27, 34), may be conserved structurally and thus be essentially weak immunogens (52). If the native fungal elastase is structurally similar to murine elastase, then it may not be sufficiently foreign to induce a vigorous immune response. A second possibility is that the fungal elastase does not trigger a strong immunological response because it is bound structurally by natural elastase inhibitors present in the mouse (23, 42). Another possibility is that the fungal elastase may simply be poorly immunogenic in its native form, as are other monomeric, water-soluble proteins (13).

We sought to improve immunogenicity by heating the elastase in SDS, as several investigators (3, 9, 43) had previously reported success with this procedure. There are reports that immunization with antigens heated in SDS (9, 43) or even with antigen separated on but not removed from SDS gels (1, 6, 7, 17, 21, 48) results in highly specific antisera that react equally well with both the denatured and the nondenatured proteins. For example, Dahl and Bignami (9) studied the glial fibrillary acidic protein, which was only weakly immunogenic in its native form. The investigators found that denaturation of the antigen in SDS prior to injection resulted in specific antiserum that reacted with complete identity with the native protein on assay in double-diffusion gels. Boulard and Lecroisey (6) used SDS-PAGE slices containing protein as immunogen, and in immunoelectrophoresis, the resulting antisera produced identical precipitin bands against the native and denatured proteins. Although heating in SDS frequently results in production of desired antibodies, this is not always true, as other investigators (3, 26, 39) have found that some proteins lose their antigenicity after this treatment.

We found that immunogenicity can be greatly enhanced by this treatment, as the antiserum titers in mice immunized with the denatured elastase had titers 10-fold higher than those in mice immunized with the native elastase. Also, seven of eight hybridomas from the fusion experiment with the mouse immunized with denatured elastase produced IgG MAb, demonstrating that a successful secondary response had been induced with this immunogen. Perhaps because of the very weak immunogenicity of the native form of *A. fumigatus* elastase, denaturation in SDS may have actually exposed previously hidden antigenic determinants, eliciting an overall stronger immune response. Four of the five cell lines that were eventually stabilized produced specific antibodies that did not cross-react with other antigens tested. The majority of tested cell lines, which were generated following immunization with native elastase as the immunogen, produced nonspecific IgM MAb.

Because the specific MAb from both fusions reacted with the native as well as the denatured elastases, they must recognize epitopes that are not destroyed by treatment with heat or detergents. These results suggest that the epitopes are linear rather than conformational, as heating in the presence of detergents would alter the conformation of the elastase (21). The five specific MAb from stabilized cell lines (BB11, MB8, KD5, GD11, and CCIII 19) from the two fusion experiments recognize only the fungal elastase and do not cross-react with other elastases in either ELISA (Table 1 and 2) or immunoblot (Fig. 2). These data suggest that there must be significant differences between the elastases, at least in their amino acid sequences. This is likely, as bacterial

TABLE 3. Effect of MAb on elastase activity[a]

MAb	Residual elastase activity (%)
MB8	114
BB11	110
KD5	87
CCIII 19	77
GD11	58
MB8 + CCIII 19	81
MB8 + BB11	47
BB11 + CCIII 19	37
KD5 + CCIII 19	37
KD5 + BB11	32
KD5 + MB8	27
GD11 + BB11	27
GD11 + KD5	18
GD11 + MB8	0
GD11 + CCIII 19	0

[a] A mixture containing a total of 100 μg of MAb (50 μg for each MAb in pairs) and 2 μg of elastase was prepared in 50 mM sodium borate buffer (pH 7.5) to give a final volume of 500 μl. The molar ratio of MAb to elastase was 10.7:1 in each mixture. After 20 min at 37°C, 250 μl (5 mg in buffer) of elastin-Congo red was added, and after an additional 30 min, the amount of dye released was measured spectrophotometrically (11). Residual elastase activity is reported as percentage of a control which contained elastase but no MAb.

elastase is a metalloenzyme, while *A. fumigatus* elastase is a serine protease (11). Human neutrophil elastase and porcine pancreatic elastase are both serine proteases, so some homology might be expected, although no cross-reactivity was seen.

Experiments to demonstrate inhibition of elastase by elastase-specific PAb or MAb showed that enzyme activity was stimulated rather than inhibited at low concentrations of antibody. However, nonspecific PAb and even BSA also stimulated enzyme activity. The apparent increase in elastase activity is probably an experimental artifact caused by stabilization of the enzyme by extraneous protein in the reaction mixture. The controls used for calculation of 100% activity consisted of an equivalent amount of enzyme incubated in buffer alone for 20 min prior to addition of elastin as substrate. We have found that elastase can lose 10% or more of its activity when incubated in buffer for this length of time. Incubation of elastase with 50 μg of BSA for 20 min before the elastase was added to the reaction mixture increased elastase activity 25% over that of the control level. Elastase is autoproteolytic (11), and the presence of other proteins may allow the enzyme to bind these proteins rather than degrade itself.

Only MAb GD11 and CCIII 19 significantly reduced elastase activity when tested individually. At a MAb to elastase molar ratio of 10.7:1, the enzyme activities were 58 and 77% that of the control without antibody. Pairs of antibodies tested at molar ratios of 5.4:1 for each MAb to elastase acted synergistically and inhibited elastase activity 19 to 100%, depending on the pair, which suggests that the antibodies bind to different epitopes. Pairs including GD11 demonstrated the highest inhibitory effect (73 to 100%). The much greater inhibition by pairs than by individual MAb suggests that inhibition is caused by steric interference or alteration in enzyme conformation rather than by binding at and blocking of the active site.

The increased effectiveness of MAb pairs for inhibition of elastase activity is similar to the observations of Suzuki et al. (44), who found that four MAb which partially inhibited (73 to 87% inhibition) porcine creatine kinase MM isoenzyme at a molar ratio of 1,000:1 acted in concert in certain combinations (1,000:1 for each MAb to enzyme) to inhibit the enzyme 95 to 100%. A fifth MAb could by itself totally inhibit the kinase activity, but again only at a molar ratio of 1,000:1. Individual elastase-specific MAb that only partially inhibited the enzyme at a molar ratio of 10.7:1, the highest ratio tested, might inhibit 100% of the activity at some higher concentration.

We have observed that the fungal elastase can degrade nonspecific IgG under conditions used to test for inhibition of the enzyme (data not shown), and degradation of IgG could have affected the ability of the MAb to inhibit elastase activity. However, as nonspecific IgG did not inhibit activity (Fig. 4), it seems reasonable to conclude that the inhibitory PAb and MAb were blocking elastase activity by specific interaction with the elastase rather than by competing with elastin as the substrate.

Other investigators have reported inhibition of enzyme activity at lower ratios of MAb to enzyme than we describe here. For example, Pfeiffer et al. (29) isolated a battery of MAb reactive with asparagine synthetase. Although some MAb were not inhibitory, others inhibited 85 to 100% of the asparagine synthetase activity at a 6.6:1 molar ratio of MAb to enzyme. The inhibitory MAb did not necessarily affect the glutaminase activity exhibited by the same enzyme.

The MAb pair combinations GD11-MB8 and GD11-CCIII 19 were more effective in completely inhibiting elastase activity than was the elastase-specific PAb. This is probably because the serum contained a high proportion of irrelevant PAb. The elastase-specific PAb had been purified on protein G but was not affinity purified on elastase. Harlow and Lane (15) estimate that even in hyperimmune animals, only 1/10 of circulating antibodies are specific for the particular immunogen.

In conclusion, the results presented here suggest strongly that denaturation made the elastase more recognizable by the mouse immune system, resulting in a more vigorous immune response and the production of specific IgG antibody. We acknowledge that to prove this point conclusively would require immunization and hybridoma production from a much larger group of animals. We have shown that PAb and certain pairs of MAb act in vitro to inhibit elastase activity. Experiments are in progress to determine if elastase-specific antibodies are able to ameliorate *A. fumigatus* infections in immunocompromised mice.

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