INFECTION AND IMMUNITY, Dec. 1992, p. 5153-5156
0019-9567/92/125153-04$02.00/0
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Vol. 60, No. 12

Lack of Vessel Wall Elastolysis in Human Invasive Pulmonary Aspergillosis

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Received 21 July 1992/Accepted 21 August 1992

In experimental studies, the apparent ability of *Aspergillus fumigatus* isolates to produce elastase in agar plates correlates with their ability to cause invasive pulmonary aspergillosis in mice pretreated with cortisone. Thus, elastase production may govern the pathogenicity of particular isolates. If this is so, then disruption of the elastic layers within blood vessel walls in invasive aspergillosis would be expected. To test this hypothesis, tissue blocks were prepared from nine patients with invasive pulmonary aspergillosis. Separate but immediately adjacent histological sections were stained by the Grocott and periodic acid-Schiff methods for fungal hyphae and by the elastic van Gieson technique for elastic tissue. Comparison of those segments of vessel walls infiltrated by hyphae with those not infiltrated by hyphae showed no overall loss of elastic tissue. Material from five of the cases was also stained with an unconventional combination of histochemical stains, allowing accurate identification of both fungal hyphae and elastic laminae in the same histological sections. The results showed no more disruption of elastic laminae than would be expected from simple physical displacement of elastic laminae. We conclude that if elastolysis contributes at all to invasion of vessel walls by aspergilli, then it seems to be very localized and/or transient.

Aspergillus fumigatus is the most common cause of invasive aspergillosis in humans, but *A. flavus*, *A. terreus*, and *A. niger* can also cause invasive aspergillosis. Antigenic (2), morphological (11), and DNA (3) differences exist between isolates, and it is likely that there are also differences in pathogenicity between isolates. In one study a correlation was found between elastase production and virulence in a murine model of invasive pulmonary aspergillosis (9). In this study, 75 isolates of *A. fumigatus* collected from the air near a sewage treatment plant in New Jersey were screened for elastase production by two assays. Four isolates were found to not produce elastase in either assay; they were then tested in a murine respiratory model and found to be nonpathogenic, in contrast to other isolates, which led to death. The study concluded that elastase production by *A. fumigatus* was probably a virulence determinant. This conclusion might be generalized to other aspergilli, for some isolates of *A. flavus* and *A. terreus* also produce elastase (16). Indeed, a non-elastase-producing, UV-irradiated *A. flavus* mutant was less virulent than its parent strain but retained its general proteolytic activity (15).

An early study of tissue reactions to invasive aspergillosis suggests "considerable preservation" of the elastic tissue within alveolar walls and indicates that the remains of elastic laminae can be demonstrated within the walls of vessels otherwise rendered histologically structureless by fungal invasion (8). In contrast, a recent review purports to demonstrate extensive elastolysis when *Aspergillus* species hyphae infiltrate across the wall of a pulmonary blood vessel (20). Unfortunately, the former of these studies neither gives the details of the staining method used nor illustrates its claims with appropriate photomicrographs (8), and the latter study uses a stain which may provide misleading results (20). We initially approached the problem by using routine "special" stains of the kind regularly used by diagnostic histopathologists, applying separate stains to parallel sections. Subsequently, we found that an unconventional combination of special stains allowed easier interpretation of sections.

MATERIALS AND METHODS

Tissue sections were obtained from nine patients who, when subjected to autopsy at Manchester Royal Infirmary, had been shown to have invasive pulmonary aspergillosis; details of the underlying diseases are given in Table 1. Six patients had leukemia, and seven were known to have been profoundly neutropenic in the last few days before death; no information on neutropenia was available for the other two. Fungal cultures were not done after autopsy in some cases and were unsuccessful because of bacterial overgrowth in others. Manchester Royal Infirmary is an old building with a long-standing problem of endemic invasive aspergillosis and occasional cases of zygomycosis. Microbiological records were incomplete; *A. fumigatus* was isolated from the sputum of one of the patients described here.

Tissue blocks taken at autopsy were fixed in 10% formaldehyde and processed to paraffin wax. Routine histological sections stained with hematoxylin and eosin were screened, and an appropriate tissue block was selected from each case. Parallel tissue sections, 4 μm thick, were cut from each block and stained with the periodic acid-Schiff stain, Grocott's modification of Gomori's methenamine-silver stain, and an elastic van Gieson (EVG) stain. The first two stains were performed by published techniques (1), and the EVG stain was performed with commercially available Miller's stain (Raymond A. Lamb, London). Briefly, this involves dehydrating the tissue section by passing it through a series

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TABLE 1. Summary of cases

Case	Age (yr)	Sex	Underlying disease or factor
1	59	M	Acute myeloid leukemia
2	56	F	Non-Hodgkin's lymphoma
3	52	M	Renal transplantation
4	67	M	Acute myeloid leukemia
5	39	F	Acute lymphoblastic leukemia
6	47	M	Idiopathic aplastic anemia
7	70	M	Acute myeloid leukemia
8	24	M	Acute T-lymphoblastic leukemia
9	67	F	Acute myeloid leukemia

of alcohol solutions of increasing concentration, treating it with a 0.5% solution of potassium permanganate for 5 min, and then decolorizing it with 0.5% oxalic acid. Sections are then washed in water, rinsed with methylated spirits, and stained with Miller's stain for 2 h. A conventional van Gieson counterstain was then applied (1).

There was sufficient material from five of these cases for immunohistochemical methods to be used for the identification of *Aspergillus* spp. Paraffin sections (4 μm) of tissues from these five patients were dewaxed in xylene, rehydrated in 100% ethanol, and washed in distilled water. Endogenous peroxidase was blocked with 3% hydrogen peroxide. After further washes with distilled water and phosphate-buffered saline, nonspecific binding sites were blocked with normal horse serum, and the sections were incubated with a monoclonal antibody that recognizes only *Aspergillus* spp. at a concentration of 2 μg/ml (5). The sections were then incubated with biotinylated horse anti-mouse immunoglobulin G and then with peroxidase-linked biotin-avidin (Vectastain ABC; Vector Laboratories, Inc.), and positive-staining hyphae were recognized by incubation with the substrate diaminobenzidine hydrochloride (Sigma, Poole, United Kingdom).

Additional sections of tissues from these five cases were stained by a combination of the Grocott technique and the EVG stain as detailed below.

Preparation of incubating solution. Three solutions are used to prepare incubating solution. Solution A is 5% (wt/vol) sodium tetraborate in distilled water; solution B is 5% (wt/vol) silver nitrate in distilled water; and solution C is 3% (wt/vol) methenamine in distilled water. Add 5 ml of solution B to 100 ml of solution C. A white precipitate will form, which will clear after shaking. Mix 25 ml of this solution with 5 ml of solution A and 25 ml of distilled water and filter it. The filtrate is the incubating solution.

Staining technique. The steps of the staining technique are: (i) dehydrate the tissue section by passing it through a series of alcohol solutions of increasing concentration; (ii) add oxidase in 5% (wt/vol) chromic acid solution for 1 h; (iii) wash the section well in running water; (iv) rinse it in 1% (wt/vol) sodium bisulfite solution (to remove excess chromic acid); (v) wash the section well in running water; (vi) place it in incubating solution at 60°C for 1 h in the dark; (vii) tone it in 0.1% (wt/vol) gold chloride solution for 4 min; (viii) wash it in water; (ix) place the section in 3% (wt/vol) sodium thiosulfate solution for 5 min; (x) rinse it in distilled water; (xi) rinse it in 95% methylated spirits; (xii) place it in Miller's solution for 1 h; (xiii) rinse it in 95% methylated spirits; (xiv) counterstain it in van Geison solution for 2 min; (xv) blot it dry; and (xvi) quickly dehydrate, clear, and mount it.

When the slides are examined, fungal hyphae have a brown-black hue readily distinguished from the blue-black of elastic fibers. Collagen stains red, and myocyte fibers stain yellow.

The length of exposure to the incubating solution (step vi) may have to be modified according to the fixative used and according to the length of time that the tissue was left in the fixative. Overstaining makes the fungi prominent but obscures internal detail. Prolongation of step xii increases the intensity of elastic fiber staining but does not produce staining of other connective-tissue elements.

RESULTS

Several vessels invaded by fungus were seen in each case. There was no detectable difference in the amount of elastic tissue revealed by the EVG stain between vessels infiltrated by hyphae and vessels of the same type and size not so infiltrated (Fig. 1). Several vessels were identified in which some radial segments were infiltrated with hyphae whereas immediately adjacent radial segments were not, but even with such stringent internal controls, no evidence of a reduced amount of elastic tissue could be detected in infiltrated areas. The combined EVG-Grocott's stain produced excellent differential staining of fungal hyphae and elastic tissue, and both were readily distinguished from other connective-tissue elements; the relationship of individual hyphae to elastic fibers could thus be seen. Most hyphae produced no visible attenuation or damage to adjacent elastic fibers, and where there was an apparent defect in elastic fibers, careful observation as the plane of focus of the microscope was adjusted indicated that, in the majority of cases, the elastic laminae were simply displaced. Occasionally, hyphae appeared kinked immediately proximal to where they crossed elastic laminae and were very narrowed at the point of crossing itself. This kinking suggests that longitudinal growth of the hyphae continues while the tips of the hyphae are temporarily arrested by elastic fibers.

Of the five sections stained immunohistochemically for *Aspergillus* spp., all five were positive. Thus, six of the nine cases were confirmed as invasive aspergillosis.

DISCUSSION

The architecture of the elastic layers within the pulmonary vasculature has been well characterized, and its demonstration by empirical stains such as the EVG method is standard practice. In our study, we concentrated on arteries of more than 0.5 mm in diameter, which are by definition elastic arteries (10). The elastic laminae of such vessels are entire in infancy but become fragmented into short fibrils by the age of 2 years (2). In our study, we cannot demonstrate any loss of elastic tissue where there is hyphal infiltration of elastic arteries in the lung, and our findings are entirely consistent with the speculation that *Aspergillus* spp. cross vessel walls by simple physical force. Occasionally, hyphae can be seen to have very severe constrictions where they cross elastic laminae, and we believe that if elastolysis is involved in fungal infiltration of blood vessel walls, then the phenomenon is very localized and/or highly transient. This is not in keeping with the demonstration of a powerful diffusable elastase in tests for a putative correlation between elastase production and pathogenicity (16).

Our findings are discrepant with those illustrated by Steele (20), which we ascribe to Steele's reliance on Gomori's methenamine-silver stain. This stain is entirely nonspecific for connective tissues, and longer staining times yield progressively more connective-tissue staining until all are

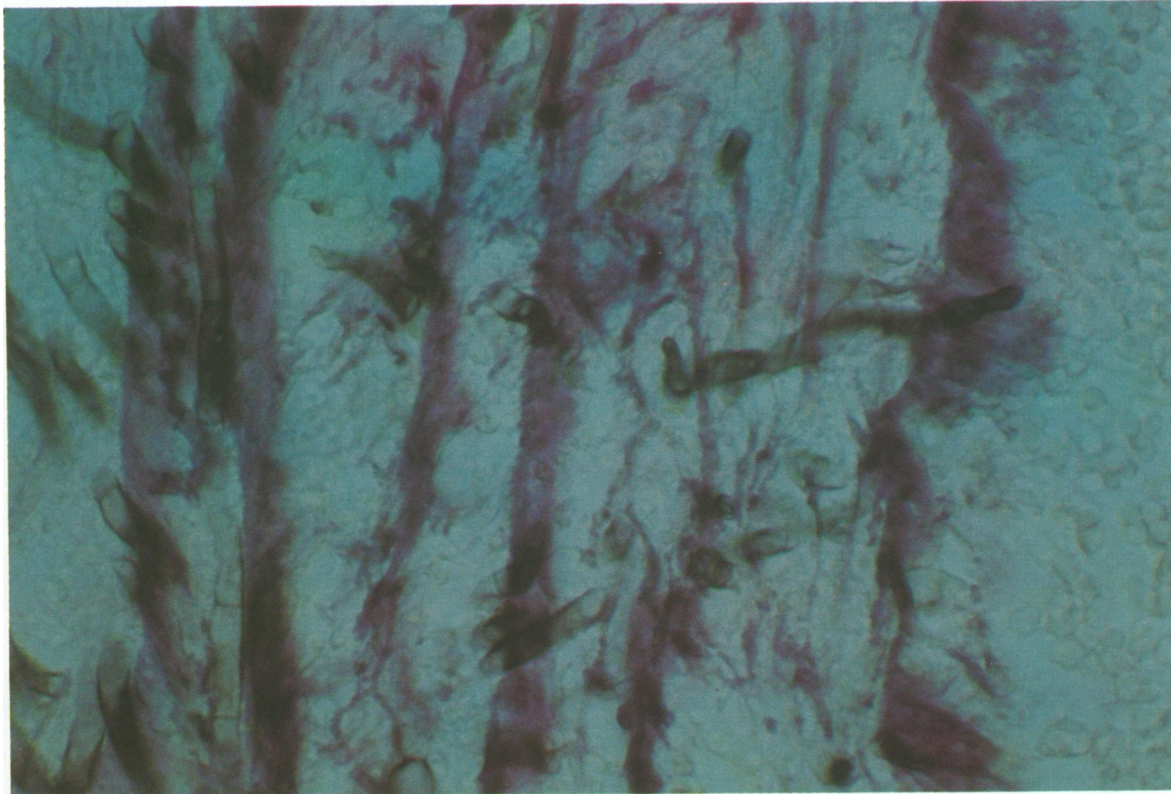

FIG. 1. Section of a small pulmonary artery. The lumen is to the left, and elastic fibers run vertically within this field; the photograph is overilluminated to provide clarity, and the elastic fibers appear blue-grey. The fungal hyphae are brown-black and cross the elastic fibers without any evidence of damage to the latter. Combined Grocott's-EVG stain. Magnification, ×730.

stained. Another possibility is that Steele's results are a misinterpretation of hyphal infiltration of a previously necrotic segment of vessel wall. We have observed loss of the elastic tissue in alveolar walls because of ischemic necrosis in the absence of fungi. It cannot be assumed that all loss of elastic tissue implies elastolysis by some extrinsic agent.

Although only one of our cases was confirmed by culture, tissues from a further five cases stained positively when examined with a non-cross-reactive monoclonal antibody. This antibody recognizes only three species of the genus *Aspergillus*, *A. fumigatus*, *A. flavus*, and *A. niger*, and no other fungi, confirming that five of these infections were caused by *Aspergillus* spp. (5). We have little doubt that the remaining unconfirmed cases were invasive aspergillosis. Culture confirmation at autopsy is not always possible, and invasive aspergillosis is by far the most common septate hyphal fungal infection in the United Kingdom, such organisms as *Fusarium* spp., *Pseudallescheria boydii*, and *Alternaria* spp. being exceedingly rare. We have sufficient experience of zygomycosis to be sure that this condition was not present in any of the cases described in this report.

It may be that the elastase activity of *A. fumigatus* is merely one enzymatic activity among several. For example, neutrophil and *Pseudomonas aeruginosa* elastases have been shown to degrade a variety of proteins, including complement (19), immunoglobulin G (4), collagen (12), and α_1 proteinase inhibitor (13). Neutrophil elastase has also been shown to degrade many connective-tissue proteins, including fibrinogen (21), collagen (7), and proteoglycan (12). Recently, the elastase of *A. fumigatus* has been purified by two groups and shown to be a serine proteinase with a requirement for divalent cations (6, 14) and able to degrade casein, hemoglobin, and albumin (14). Thus, it is possible that the presence or absence of elastase does correlate with virulence but that it is not this particular enzymatic activity that is important.

Another possibility is that the production of elastase is merely a nonspecific marker for another virulence factor. One possibility along these lines is a linkage of two genes on a particular chromosome that results in frequent coexistence of elastase with another virulence factor.

The third possible explanation is that the demonstration of elastase activity in vitro is merely an in vitro phenomenon and has no relevance in vivo. The authors of the original work (9) did two different assays for elastase. In both situations, the assays were somewhat crude, because elastic tissue is essentially insoluble. Growth on the elastin-containing medium is possible only if elastase is produced because the medium has a very low nitrogen content. This may stimulate the production of elastase in vitro, which is not what happens in vivo.

Thus, the role of an elastin-degrading enzyme in the pathogenicity of *Aspergillus* spp. in the context of human invasive aspergillosis is at best unclear. Further work needs to address this apparent dichotomy between our findings and those published previously, with respect to the pathogenicity of elastase-producing isolates of *Aspergillus* spp.

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