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Proteinases from invasive larvae of the trematode parasite *Schistosoma mansoni* degrade connective-tissue and basement-membrane macromolecules

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Larvae of the human parasite *Schistosoma mansoni*, which invade the vascular system through the skin, secrete proteinases that degrade radioactively labeled extracellular matrices produced by smooth-muscle cells, dermal fibroblasts and endothelial cells. The proteinase purified from one larval form, the cercaria, degrades fibronectin and laminin and is a type-specific collagenase with activity against basement-membrane collagens IV and VIII, but not interstitial collagens I, III and V. The substrate specificity of this enzyme resembles that of the proteolytic enzymes which facilitate tissue invasion by inflammatory cells and tumour cells.

INTRODUCTION

Schistosoma mansoni is one of the trematode parasites that cause schistosomiasis (bilharzia), a disease affecting over 250 million people. The initial step in infection of the human host by *Schistosoma mansoni* is invasion of intact skin by an aquatic larval form, the cercaria. To gain access to the vascular system, cercariae must cross the epidermal basement membrane, the dermal connective-tissue extracellular matrix, and finally an endothelial-cell basement membrane (Lewart & Lee, 1954; Stirewalt, 1974). During this migration, cercariae transform into a second larval form, the schistosomule. Morphological studies demonstrating the dissolution of basement-membrane structure and extracellular matrix at a distance from invading larvae have suggested degradation by secreted larval proteinases (Lewart & Lee, 1954; Bruce *et al.*, 1970). Although proteolytic activity has been known for some time to be a component of secretions from the preacetabular glands of cercariae [reviewed by Stirewalt (1974) and McKerrow *et al.* (1983)], investigation of the role of larval proteinases in tissue invasion has been hampered by a lack of suitable connective-tissue substrates.

We recently adapted to the study of parasite infection an '*in vitro*' model of connective-tissue extracellular matrix previously used to study tumour invasion (McKerrow *et al.*, 1983; Keene *et al.*, 1983). We demonstrated that a serine proteinase secreted by cercariae in response to skin lipid degrades elastin and trypsin-labile glycoproteins in this matrix (McKerrow *et al.*, 1983). The schistosomule initially secretes the same enzyme, but later expresses a surface-associated metalloproteinase that degrades only trypsin-labile glycoproteins in the matrix (Keene *et al.*, 1983). Proteinase inhibitors blocked the matrix degradation, even though they did not affect parasite motility. These findings suggest that proteinases play a critical role in invasion.

We now extend these studies to show that living invasive schistosome larvae degrade the extracellular matrix synthesized by dermal fibroblasts and the basement-membrane-like matrix synthesized by endothelial cells *in vitro*. These '*in vitro*' models allow assay of the proteolytic activity of invasive larvae against a variety of macromolecules in the type of interactive framework in which they are found in the different microenvironments of skin. Building on the results of the extracellular-matrix assays, we undertook more detailed studies of the larval degradation of fibronectin, laminin and specific collagen types, which allowed comparison of larval invasion with proteinase-mediated tissue invasion by tumour cells and inflammatory cells.

EXPERIMENTAL PROCEDURES

Collection of larvae

The procedures we used to maintain the *S. mansoni* life cycle and to collect cercariae were described previously (McKerrow *et al.*, 1983; Keene *et al.*, 1983). Briefly, infected snails (*Biomphalaria glabrata*) were exposed to light to induce shedding of cercariae into sterile distilled water containing gentamicin (100 units/ml) as a bacteriostat. No bacterial contamination was detected in the collection water by aerobic or anaerobic culture, and no contaminating microbial proteinase activity was detected when a number of different assays were used (McKerrow *et al.*, 1983; Campbell *et al.*, 1976).

For the production of schistosomules, the methods of Basch (1981) were used to concentrate and wash the cercariae. Transformation to schistosomules was induced by mechanical shearing as previously described (Keene *et al.*, 1983), and the methods of Basch (1981) were used to establish schistosomule cultures. Cultures were incubated for 24 h to allow full transformation to occur (McKerrow *et al.*, 1983; Keene *et al.*, 1983) before schistosomules were used in assays.

Preparation of extracellular matrices

Extracellular matrices were prepared from rat smooth-muscle cells of the R22 strain, from cloned bovine venous endothelial cells, V_2Cl_2 strain, or from human skin

Abbreviations used: SDS, sodium dodecyl sulphate; DTT, dithiothreitol; MEM, Eagle's minimal essential medium.

fibroblasts, T-1 strain, and were radioactively labelled with *L*-[3,4-^{3}H]proline as previously described (Jones *et al.*, 1979; Jones & Scott-Burden, 1979; DeClerck & Jones, 1980; Bogenmann & Jones, 1983; Laug *et al.*, 1983; Sage, 1984).

The collagen types present in the endothelial cell matrices were described previously (Laug *et al.*, 1983). Analyses kindly done by Dr. Yves A. DeClerck indicated that collagen types I and III were present in the smooth-muscle and fibroblast matrices.

Extracellular matrix from the R22 smooth-muscle strain was used because it contains elastin as well as trypsin-labile glycoprotein and types I and III collagen. Elastin fibres in the dermis form an extensive network that represents a potential barrier to tissue invasion by multicellular organisms (Smith *et al.*, 1982). The human fibroblast and bovine endothelial cells used in the present study do not deposit insoluble matrix elastin in culture (DeClerk & Jones, 1980; Laug *et al.*, 1983), but they produce fibronectin, laminin and several of the collagens found in the skin (Sage & Bornstein, 1982; Sage, 1984).

For studies of matrix degradation, cercariae or schistosomules were concentrated to 1000–2000/ml in Eagle's minimal essential medium (MEM) containing 100 μg of gentamicin/ml. They were then introduced into 16 mm- or 32 mm-diameter tissue-culture wells containing the radioactively labelled matrix and incubated at 37 °C in 5% CO_2. The progress of degradation was monitored by assaying 50 μl samples for the release of radioactivity. At the end of the experiment, the remaining reaction mixture was removed and the specific degradation of non-collagenous glycoprotein, elastin and collagen was estimated as the percentage of the trypsin-, pancreatic-elastase-, or clostridial-collagenase-solubilized material in controls (McKerrow *et al.*, 1983). Both ultrastructural and biochemical analyses of matrices at each step of this sequential enzyme digestion (Werb *et al.*, 1980; Laug *et al.*, 1983) provide evidence that trypsin liberates primarily non-collagenous glycoprotein, elastase almost exclusively elastin, and collagenase the remaining collagens.

Purification of cercarial proteinase

The procedures used to purify and characterize the proteinase secreted by cercariae (McKerrow *et al.*, 1985) or present in cercarial extracts (Landsperger *et al.*, 1982) were described previously. For these studies, pooled cercarial secretions were freeze-dried and then chromatographed on Ultragel AcA54 (LKB), followed by an isoelectric-focusing column (Mono P; Pharmacia). Proteolytic activity was assayed by the degradation of Azocoll and [^{3}H]elastin (McKerrow *et al.*, 1985). The purified enzyme migrated as a silver-staining single band at M_r 30000 on SDS/polyacrylamide-gel electrophoresis (McKerrow *et al.*, 1985).

Purification of collagens

Type I procollagen (bovine fibroblast), Type III procollagen (bovine fibroblast), Type IV procollagen (murine teratocarcinoma), and Type VIII procollagen (EC1) (bovine aortic endothelial cell) were purified from [^{3}H]proline-labelled culture-medium proteins by ion-exchange chromatography, under conditions in which the triple-helical conformation of the native molecules was retained (Sage *et al.*, 1980; Sage & Bornstein, 1982). Types IV and V collagen were purified from tissue by pepsin extraction as previously described (Sage & Bornstein, 1979). Type I collagen was solubilized, without proteolysis, from tail tendon of lathyritic rats.

Collagen-degradation assays

Purified enzyme was concentrated (Prodicon; Biomolecular Dynamics, Beaverton, OR, U.S.A.) to approx. 0.7 mg/ml in 50–100 mM-Tris/HCl, pH 7.5, containing 100 mM-NaCl. An equal volume of Laemmli (1970) buffer containing 50 mM-DTT was added to each digest and its incubated control; the reaction products were resolved by SDS/polyacrylamide-gel electrophoresis on a 5%-stacking/10%-separating gel, and proteins were stained with Coomassie Blue. The enzyme/substrate ratio (mol/mol) was approx. 1:100.

Assay of degradation of laminin and fibronectin

A 40 μg portion of mouse laminin (BRL) and 10 μg (100000 c.p.m.) of native [^{3}H]pro-fibronectin (human glomerular endothelial cells) were incubated with 7 μg and 1 μg of cercarial enzyme respectively for 3 h at 37 °C in 20 mM-Tris/HCl buffer (pH 9)/100 mM-NaCl. An equal volume of Laemmli buffer containing 100 mM-DTT was added to the digest and its incubated control. Products were resolved on a 5%-stacking/10%-separating gel for fibronectin and both a 4%/5% gel as well as a 4%/10% gel for laminin in order to detect both undegraded laminin and any degradation product from M_r 10000 to M_r 400000. Protein was detected by silver stain (laminin) or fluorescent autoradiography (fibronectin).

RESULTS

Degradation of extracellular matrices by live larvae

As has been observed by others (Stirewalt *et al.*, 1983), transfer from water to Eagle's minimal essential medium (MEM) at 37 °C induced cercariae to transform to

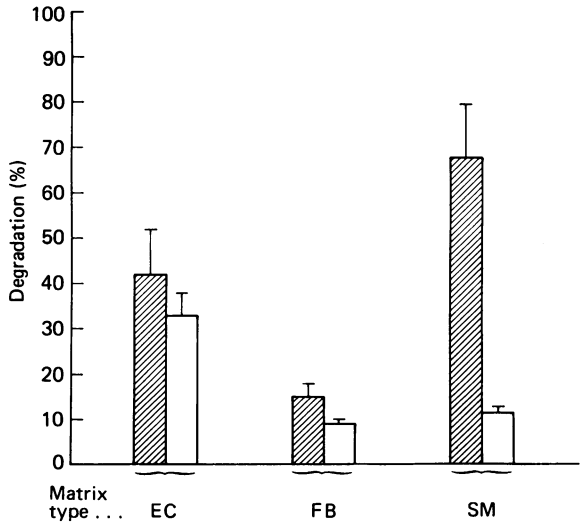

Fig. 1. Total matrix degradation by 1000 cercariae (hatched bars) or schistosomules (open bars) incubated with radioactively labelled matrices for 48 h at 37 °C

Abbreviations used: EC, endothelial-cell matrix; FB, fibroblast matrix; SM, smooth-muscle-cell matrix. Error bars represent S.D. of three to nine data points.

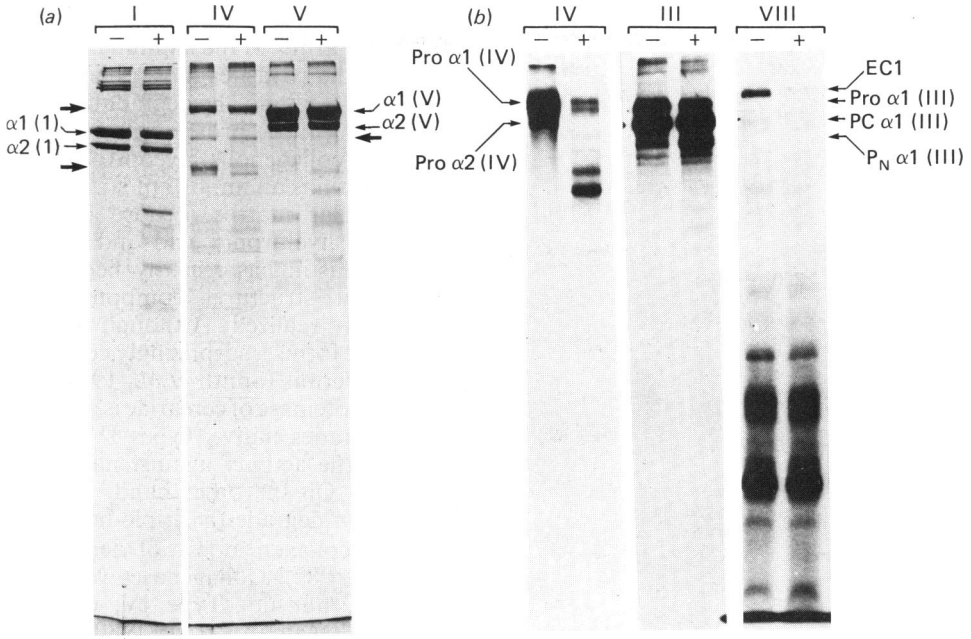

Fig. 2. Activity of purified cercarial enzyme toward (*a*) native collagen and (*b*) procollagen types

(*a*) A 10 μg sample of each native collagen preparation in Tris/saline buffer was incubated with enzyme (approx. 1:100 enzyme/substrate ratio) for 2 h at 37 °C. Reduced SDS/polyacrylamide gels are shown prepared as described in the Experimental procedures section. Collagen types are indicated, as well as control (−) and enzyme-incubated samples (+). The α1 and α2 chains of Types I and V collagen have been identified; unlabelled arrows refer to the three major pepsin-resistant components of Type IV, of M_r 140000, 100000 and 70000. (*b*) Approx. 2–4 μg (50000 c.p.m.) of each native [^{3}H]proline-labelled procollagen preparation in Tris/saline buffer was incubated with enzyme (1:100 enzyme/substrate ratio) for 3 h at 37 °C. Samples were further processed as in (*a*), and cleavage products were detected by fluorescent autoradiography. Type VIII procollagen is indicated (EC1, M_r 177000); procollagen chains (Pro) and processed intermediate forms (P_C, P_N) of types III and IV procollagen are identified.

schistosomules and release preacetabular-gland contents. Fig. 1 compares the total degradation of each type of matrix by cercariae and schistosomules. Both larval forms degraded a significant portion of the endothelial-cell matrix (cercariae, 40±10%; schistosomules, 32±5%) and the smooth-muscle-cell matrix (cercariae, 68±12%; schistosomules, 12±1%). There was less degradation of the dermal fibroblast matrix (cercariae, 14±4%; schistosomules, 8±1%).

The reasons for the differences in total matrix degradation were apparent when degradation of the specific connective-tissue macromolecules constituting the matrices was analysed. Cercariae degraded more of the smooth-muscle-cell matrix because its principle protein component is elastase-sensitive and the serine proteinase secreted by cercariae has significant elastinolytic activity (McKerrow *et al.*, 1985). Neither larval form degraded very much of the clostridial collagenase-sensitive material in the fibroblast matrix (cercariae, 5±6%; schistosomules, 0±1%) or smooth-muscle matrix (cercariae, 5±5%; schistosomules, 0±2%). On the other hand, both larval forms degraded 18±5% of the collagenase-sensitive protein in the endothelial-cell matrix. Cercariae degraded more than 50%, and schistosomules more than 30%, of the trypsin-labile glycoproteins in all three matrices.

Degradation of collagen, fibronectin and laminin by the cercarial proteinase

More specific assessment of the substrate specificity of cercariae was next undertaken with a purified sample of the serine proteinase secreted by cercariae. The cercarial proteinase was found to be a type-specific collagenase (Fig. 2). There was minimal degradation of native Type I collagen, pepsin-extracted Type IV collagen, Type III procollagen, or Type V collagen. However, there was significant degradation of Types IV and VIII procollagen. Specific fragments of M_r 60000–70000 were produced from Type IV collagen, but discrete proteolysis fragments from Type VIII collagen were not observed. Although the cleavage patterns shown in Fig. 2 resulted from different reaction conditions, a series of incubations for different durations, at different enzyme-to-substrate weight ratios, and at 22 and 30 °C produced equivalent results (results not shown). The minimal cleavage (< 5%) observed for Types I and V collagens was most likely due to a limited degree of unfolding of the collagen triple helix at 37 °C.

The cercarial proteinase also degraded both fibronectin and laminin (Fig. 3). Laminin was degraded to fragments of M_r approx. 40000–50000.

DISCUSSION

We can now present a more detailed picture of how proteinases facilitate skin and vascular invasion by a multicellular parasite. Previous work showed that *S. mansoni* cercariae are stimulated to penetrate skin by interaction with medium-chain-length fatty acids in skin surface lipid (Stirewalt, 1974; McKerrow *et al.*, 1983). A proteolytic enzyme released from preacetabular glands first degrades keratin at the skin surface (Tzeng *et al.*, 1983). Cercariae (now transforming into schistosomules)

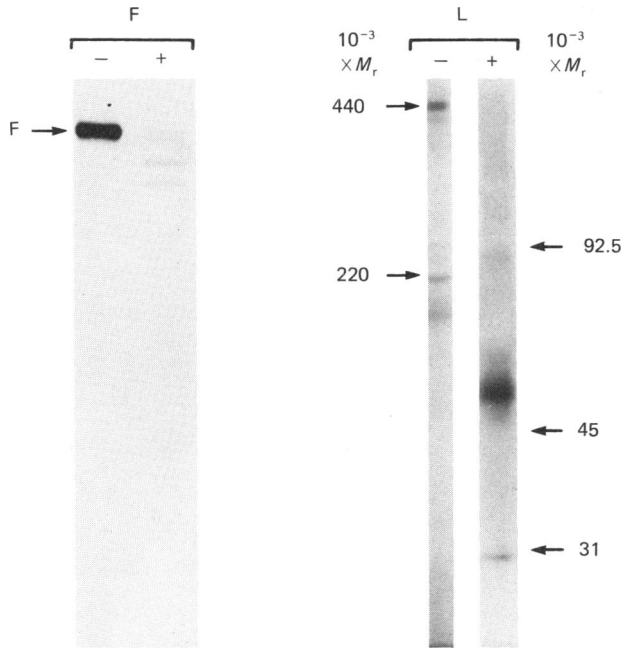

Fig. 3. Activity of purified cercarial enzymes toward fibronectin (F) and laminin (L)

M_r values for laminin chains and standard M_r markers are indicated. These are reduced SDS/polyacrylamide gels prepared as described in the Experimental procedures section. Laminin incubated without enzyme is shown on a 4%/5% reduced gel, whereas cleavage fragments are shown on a 4%/10% reduced gel because no larger fragments were present.

may directly cross the epidermis or may first migrate down a hair shaft. Most of the subsequent proteolytic activity of invading larvae is also due to the preacetabular-gland proteinase of the cercariae, which diffuses widely ahead of the invading larvae (Stirewalt, 1982) and may be secreted for at least 24 h after skin penetration (Keene *et al.*, 1983). Nevertheless, other surface-associated proteinases of the schistosomule in concert with the cercarial enzyme may facilitate subsequent vascular invasion (Auriault *et al.*, 1982; Keene *et al.*, 1983). This concept is supported by our current finding that 3-day-old schistosomules, which are essentially devoid of preacetabular-gland contents (Keene *et al.*, 1983), were as effective as cercariae at degrading the basement-membrane-like extracellular matrix of endothelial cells.

At the epidermal/dermal interface, basement-membrane degradation, seen in electron-microscopic studies, can now be correlated biochemically with degradation of native Types IV and VIII collagen and laminin. Basement-membrane degradation would facilitate parasite invasion not only of the epidermal basement membrane, but also of small dermal vessels. Degradation of Type IV collagen may itself be a mechanism for increasing capillary permeability (Timpl & Martin, 1982). Furthermore, laminin is an important constituent of basement membrane because of its interactions with Type IV collagen, heparan sulphate and adjacent cells (Timpl & Martin, 1982). The 50000-M_r fragments seen in laminin degradation (Fig. 3) are similar in size to those produced by other serine proteinases and could correspond to cleavage of a globular heparin-binding region from the 'arms' of the laminin molecule (Ott *et al.*, 1982).

After the parasites have entered the dermis, invasion would be facilitated by degradation of elastin (McKerrow *et al.*, 1983, 1985), proteoglycan (Landsperger *et al.*, 1982) and non-collagenous glycoproteins such as fibronectin. Fibronectin is an important structural component of the extracellular matrix because of its interactions with collagen, hyaluronic acid and cells (Timpl & Martin, 1982). Elastin has recently been shown to be a more important structural component of skin than was previously realized. Although a minor component by weight, it forms a surprisingly complex network throughout the dermis (Smith *et al.*, 1982).

The proteinase of cercariae is a type-specific collagenase that degrades native Types IV and VIII collagen, but shows little activity against native Types I, III, and V collagen. On the other hand, human skin collagenase, which can degrade the triple-helical portions of Types I and III collagen, does not degrade Type IV (Timpl & Martin, 1982). Collagenases which specifically degrade Type V, but not Type IV, collagen have also been reported (Mainardi *et al.*, 1980; Liotta *et al.*, 1981). The ability of the cercarial proteinase to degrade native Type IV procollagen, but not pepsin-extracted Type IV collagen, suggests that it degrades the molecule in globular non-triple-helical regions. Type VIII collagen also appears to contain interruptions within the triple helix and is degraded by a number of enzymes (Sage *et al.*, 1983). A collagenase isolated from malignant-tumour cells also degrades Type IV collagen and has no activity against Types I and III (Liotta *et al.*, 1980). However, this enzyme cleaves Type IV collagen in a triple-helical rather than a globular region (Fessler *et al.*, 1984). The pattern of degradation of Type IV collagen by the cercarial proteinase is more like the degradation of Type IV collagen by mast-cell proteinase and leucocyte elastase (reviewed by Timpl & Martin, 1982).

Thus the mechanisms underlying parasite invasion, cellular infiltration during inflammation and tumour invasion may have considerable overlap in terms of proteolytic-enzyme substrate specificity, although specific sites of macromolecular cleavage may be unique to each agent. Identifying which macromolecules are degraded in all three phenomena may give clues as to which are key components in maintaining tissue integrity.

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