Skin Penetration by *Necator americanus* Larvae

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Abstract. Skin penetration by *Necator americanus* larvae has been investigated in vitro. Larvae were able to penetrate completely human skin from both the epidermal and dermal directions; their passage through the epidermis was marked by cellular destruction. Removal of chloroform soluble skin lipids affected both the percentage of larvae invading and the percentage exsheathing. The larvae released an enzyme at about 37°C, which showed peak activity against azocoll at 37°C and pH 8. It is suggested that initial invasion is a mechanical process and that the enzyme is functional in passage through the germinal layers of the epidermis.

Introduction

Although thin, the outer layers of mammalian skin present a highly efficient barrier to the great majority of infective micro-organisms; specialised mechanisms have been evolved to traverse them. Penetration by schistosome cercariae has been shown to be an enzymic process (Stirewalt 1963, 1966, 1971), but rather less work has been devoted to skin penetration by nematode larvae. Matthews (1972, 1975) found no evidence for enzyme activity and proposed a mechanical penetration mechanism for *Ancylostoma tubaeforme*, and Lee (1972) has suggested that the damage found at the electron microscope level when *Nippostrongylus brasiliensis* penetrated rat skin need not have been the result of enzymic degradation. *Necator americanus* larvae release an azocoll-positive secretion and show other differences from *A. tubaeforme* during invasion (Matthews 1975, 1977). This paper examines the pattern of invasion by *N. americanus* and the in vitro release and azocoll activity of the enzyme.

Materials and Methods

Human and rabbit skin, excised at autopsy, was immediately frozen and stored at −20°C until required. Chloroform soluble skin surface lipids were removed as described by Clegg (1969) and replaced by applying half the extract to half the area of skin from which it had been removed.
and allowing the chloroform to evaporate completely. In vitro penetration tests were conducted using the method described by Matthews (1972). Infective third stage *N. americanus* larvae isolated from charcoal cultures of human faeces were washed and stored in water until required. Fifty or 100 infective larvae in 0.15 ml water were transferred to either the epidermal or dermal surface of the skin or the surface of No. 4 Whatman filter paper membranes. Tissues were either digested in acid pepsin and the released larvae counted, or fixed in 70% alcohol and processed for histological examination. Serial 10 μm sections were cut and the location of the larvae within the skin recorded (Matthews 1975).

Enzyme activity was assessed using azocoll. Samples of $2 \times 10^3$ ensheathed third stage larvae were washed thoroughly by centrifugation, twice in water and twice in 1.5% saline in 0.05 M phosphate buffer pH 6.8 (PBS) and suspended in 5 ml PBS in 10 ml centrifuge tubes; 25 mg azocoll (Calbiochem) was added to each tube and incubated at a range of temperatures. At periodic intervals the tubes were centrifuged, the supernatant carefully removed and its absorbance measured spectrophotometrically at 520 nm. At the end of the experiment the viability of the larvae was checked by assessing activity.

To separate enzyme release and activity $15 \times 10^3$ washed larvae were incubated at 37°C in 15 ml PBS for 4 h without substrate. Aliquots of 2 ml of the centrifuged supernatant were diluted to 5 ml with PBS, 25 mg azocoll was added to each tube and reincubated at various temperatures for a further 4 h, the absorbance of the supernatant was measured at 520 nm.

The optimum pH for enzyme activity was determined on the centrifuged supernatant from $12 \times 10^3$ washed larvae incubated for 4 h in 12 ml PBS. The solution was divided into 1.3 ml samples, diluted to 5 ml with distilled water and the pH adjusted with either HCl or KOH. Azocoll (25 mg) was added to each tube and incubated at 37°C for a further 4 h; after centrifugation the absorbance of the supernatant was measured at 520 nm.

Control tubes containing substrate and PBS from the final larval wash were included with all tests and their values subtracted from the test results.

### Results

The percentage of larvae entering skin and filter paper membranes in vitro in 4 h is illustrated in Fig. 1. There were some differences in detail between the different membranes but the pattern of invasion was similar for both natural and artificial membranes. Initial invasion occurred within 5 min of exposure and there was a rapid increase in the number entering the membranes over the next 1–2 h. The rate of invasion decreased at longer times leading to a flattening of the curves. Larvae that had not penetrated were recovered from the membrane surface at the end of the experiment. These larvae remained active and were not obviously different from control larvae maintained at room temperature except that many recovered from the skin surface had exsheathed.

Histological examination of skin sections showed disruption of the germinal layers of the epidermis around the anterior of invading larvae (Fig. 2). The damage extended to a region about 100 μm in diameter. All larvae that penetrated were exsheathed; discarded sheaths were recovered from the skin surface but none were seen within the skin.

The influence of human skin lipids on penetration and exsheathment is illustrated in Fig. 3. Removal of the chloroform soluble lipid fraction resulted in a marked decrease in the percentage of larvae entering. Replacement of the lipid resulted in an increase in the number penetrating but did not restore it to the original level. The effect of lipids on exsheathment was most marked after 30 min but there was a significant ($P=0.05$) decrease in the number of sheaths recovered from skin surfaces lacking lipids at each time. Spontaneous