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Enzymes of *Entamoeba histolytica*

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In spite of extensive studies of the pathogenic activities of Entamoeba histolytica *upon animal hosts by many investigators, the factors that govern the pathogenesis of the parasite are still largely unknown. Attempts have been made to determine the factors concerned in the pathogenicity of certain strains of* E. histolytica *from the aspect of parasite itself and from the point of view of its interaction with the host. The enzyme content of the trophozoites of the amoebae examined has been studied in detail, both in the living organism and in its extracts. The total patterns of proteolytic enzymes and of certain other enzymes were determined to establish whether it was possible to define the invasiveness of the various strains of* E. histolytica *already established as pathogenic by other workers or in our laboratories. It was found impossible to distinguish clearly between the so-called " pathogenic " and " non-pathogenic " strains of* E. histolytica *by such means.*

EARLY INTERPRETATIONS OF INVASION

The mechanism by which *Entamoeba histolytica* gains entry into the host tissues is still not fully understood. It is generally believed, however, that the parasite penetrates through the gut epithelium of humans and animals by virtue of histolytic enzymes secreted by the amoebae. This hypothesis was first put forward by Dobell (1919). The evidence in favour of the existence of such enzymes is based on observation of the initial contact with the gut mucosa, and of the histological studies of lytic necrosis in the tissue at later stages, when the amoebae are visible and are often surrounded by a clear area (" lytic area ") separating them from the adjacent host tissue. The earliest superficial mucosal changes, the typical bottle-neck ulcers and the honeycombing destruction of the submucosa seen in amoebic lesions, indicate that lytic action is involved in the development of amoebic ulcers, although it is generally agreed that mechanical action may aid the amoebae in their penetration and migration. The so-called " lytic area " has usually been ascribed to enzymatic cytolysis by the amoebae, but some of these haloes may arise as artefacts during fixation of the tissues (Torpy & Maegraith, 1957).

DEMONSTRATION OF PROTEOLYTIC ENZYMES

Many workers have shown that *E. histolytica* possesses proteolytic activity *in vitro* towards various substrates. In demonstrating these proteolytic activities various methods have been employed. Hallman, Michaelson & De Lamater (1950), Rees et al. (1953), and Kaneko (1956) demonstrated the presence of proteolytic enzymes in the parasite by growing the amoebae in gelatin solutions. Harinasuta & Maegraith (1954, 1958) showed gelatinase activity of *E. histolytica* by placing the amoebae on gelatin film, the activity being indicated by areas of lysis of the gelatin film after intervals of incubation. Nakamura & Edwards (1959) demonstrated the ability of the amoebae to hydrolyse gelatin by employing the micro-test for gelatinase developed by Thirst (1957), which consisted of a thin film of gelatin on a glass slide, and also the agar–gelatin plate test developed by Smith (1957). Hydrolysis of gelatin on the slides or the plates was indicated by the appearance of clear areas. The existence of caseinase activity in *E. histolytica* was first shown by Neal (1956).

The proteolytic activity on gelatin and casein of various strains of *E. histolytica* was demonstrated by Neal (1960) both in living amoebae and extracts. The ability of the amoebae to hydrolyse gelatin was shown by the reduction of viscosity of a gelatin solution, whereas hydrolysis of casein solution was measured by determining the splitting of tyrosine by means of the phenol reagent (Folin & Ciocalteau, 1927). However, the results showed that the invasive character of the amoebae did not seem to be related

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to the proteolytic activity. Of 5 strains freshly isolated from patients with amoebic dysentery, 2 exhibited high proteolytic activity, whereas 3 other pathogenic, freshly isolated strains, as well as 2 non-pathogenic ones, showed low proteolytic activity of the same order. It was concluded that the amoebic proteinase resembles trypsin and that high proteolytic activity may not be required for tissue penetration but merely acccompanies some other factor.

STRAIN DIFFERENCES IN ENZYME ACTIVITY

The proteolytic enzyme contents of *E. histolytica* have been further studied in detail both in the living organism and in its extract by Jarumilinta & Maegraith (1961a, 1961b). The first studies were carried out with regard to the proteolytic activity of the trophozoites and freeze–thaw extracts of various strains of *E. histolytica* (5 isolated from patients with amoebic dysentery and proved pathogenic to rats and guinea-pigs, and 4 isolated from human carriers and also proved non-pathogenic to animals) on gelatin, casein, fibrin, haemoglobin and suspensions of epithelial cells from the guinea-pig caecum. A strain of free-living amoeba, *Acanthamoeba* sp., maintained in axenic culture, was also subjected to similar studies. Hydrolysis of gelatin, casein, and haemoglobin was measured by determining the content of *a*-amino nitrogen liberated (by means of ninhydrin reagent, Yemm & Cocking, 1955) after various intervals of incubation (3 h–24 h) with the parasite under optimum conditions of temperature and pH. Control suspensions of associated bacteria and their extracts were also set up in parallel. For the guinea-pig caecal epithelial suspensions, the amino acids liberated after the hydrolysis by the amoebae were determined by 1- and 2-dimensional paper chromatography. Hydrolysis of the epithelial suspensions by trypsin, pepsin and HCl was also studied in parallel. In determining the fibrinolytic activity, a modification of Permin's (1950) fibrin plate method was employed. Trypsin was used as the standard for reference of the fibrinolytic activity of the parasite. The area of dissolved fibrin which appeared after incubation for 22 h was taken as a measure of the activity of the added trophozoites or extracts. It was demonstrated that both living organisms and extracts of all strains of *E. histolytica* examined were capable of hydrolysing all the substrates used, including the guinea-pig gut epithelium, whereas the free-living amoebae lysed only gelatin and casein but not haemoglobin, fibrin or epithelium. The chromatographic patterns of amino acids produced by hydrolysis of the gut epithelium by all the strains of *E. histolytica* and by trypsin were found to be similar. It was thus considered that it might be trypsin (or a trypsin-like enzyme) which provides *E. histolytica* with a potential pathogenic mechanism.

The use of proteins as substrates as described above does not, however, allow differentiation of the different proteinases (endopeptidases) contained in a given mixture. Since Bergmann & Fruton (1941, 1942) had found that it was possible by the use of specific simple peptides to determine and estimate various proteinase (endopeptidase) and exopeptidase activities from an enzyme mixture, Jarumilinta & Maegraith (1961b) studied the action of *E. histolytica* on various synthetic substrates considered specific for proteinases, i.e., trypsin, pepsin and chymotrypsin, and for exopeptidases, i.e., carboxy-, amino-, and dipeptidase. Trophozoites and saline extracts of the same strains of the amoebae as in the studies with the 5 protein substrates described above were used. Control suspensions of associated bacteria of the corresponding strains were included in the experiments. The hydrolysis of these substrates after various intervals of incubation (3 h–24 h) with the parasite under optimum conditions was demonstrated both qualitatively by paper chromatography and quantitatively by estimating the amino group liberated in the reaction. The data obtained showed that *E. histolytica*, whether pathogenic or not, possessed tryptic and peptic but not chymotryptic activity. Non-pathogenic strains of *E. histolytica* possessed carboxy-, amino-, and dipeptidase, whereas pathogenic strains did not contain carboxypeptidase. *Acanthamoeba* sp. possessed only peptic activity and amino- and carboxypeptidase. These results thus confirmed those described above that all strains of *E. histolytica* studied contain trypsin whereas *Acanthamoeba* sp. does not. Both *E. histolytica* and *Acanthamoeba* sp. were shown to possess pepsin. However, digestion of the epithelium by pepsin is regarded as unlikely because the parasites are inactive *in vitro* within the pH range of pepsin activity.

ENZYME ACTIVITY AND STRAIN PATHOGENICITY

It has thus not been possible by the study of these proteolytic enzymes to distinguish between the pathogenic and non-pathogenic strains of *E. histolytica*, since all of them contain trypsin, which is capable

of digesting guinea-pig caecal epithelium within the range of pH in which it is hydrolysed by the parasites. A distinction could, however, be drawn between the potential activity of *E. histolytica* in so far as their tryptic activity is concerned and the absence of this potential activity in the *Acanthamoeba* sp. The potential activity of the enzyme present in the non-pathogenic strains of *E. histolytica* thus appeared to be controlled or limited in some way. This limitation may well depend on the presence of inhibitors in the gut lumen or possibly in the host tissue or even in the parasite itself. It may be that the enzymes of the pathogenic parasites are potentiated by the host environment or that the enzyme inhibitors themselves are inhibited or controlled in these organisms.

The significance of the presence of carboxypeptidase in non-pathogenic strains of *E. histolytica* and *Acanthamoeba* sp. and its absence from the pathogenic strains is not immediately obvious. It is possible, however, that in the final digestion of protein by the pathogenic strains of *E. histolytica* some residual peptide moiety may remain, which plays a part in controlling the over-all proteolytic activity of the parasite. Jarumilinta & Maegraith (1964, unpublished) carried out some investigations to clarify this point, but were not able to arrive at any conclusion.

In Liverpool, later, Nonglak, in an unpublished work, confirmed that 2 other pathogenic strains of *E. histolytica* possessed no carboxypeptidase activity whereas 2 non-pathogenic strains of *E. histolytica* and 1 strain of *Entamoeba coli* did.

" SPREADING FACTOR " ENZYME (HYALURONIDASE)

After *E. histolytica* has penetrated the gut epithelium into the submucous tissue (either by proteolytic enzyme, mechanical penetration or through an area of epithelial damage) the subsequent spreading of the successful organism into the deeper tissue may well depend on the activity of the enzymes of the " spreading factor " type including hyaluronidase, an enzyme capable of disaggregating and depolymerizing hyaluronic acid which Meyer et al. (1941) demonstrated to be a common component of the so-called ground substance of the connective tissue. Attempts by many workers to demonstrate hyaluronidase in *E. histolytica* have met with variable success. By employing the viscosimetric method of Swyer & Emmens (1947), Townshend (1948) failed to detect hyaluronidase activity of *E. histolytica*. Using a turbidimetric method, Bradin (1953) failed to demonstrate activity of this enzyme in 5 strains of *E. histolytica* which had been maintained *in vitro* over a long period, but did demonstrate activity in the same strains recovered from an experimentally induced hamster-liver abscess. De Lamater et al. (1954) failed to detect any hyaluronidase activity in *E. histolytica* grown anaerobically and aerobically and in strains of amoebae passaged through hamster liver. Neal (1960) also failed to detect the enzyme in 2 strains of *E. histolytica* isolated from human cases of amoebic dysentery. Determination of this enzyme in cultures of *E. histolytica* was also carried out by Jarumilinta & Maegraith (1960) and by Jarumilinta (1962). They first demonstrated hyaluronidase activity in the living form and extracts of 3 strains of *E. histolytica* isolated from patients with amoebic dysentery by the viscosity-reduction method, turbidity-reduction method, and a chemical method (estimation of liberated *N*-acetylglucosamine) employing sodium hyaluronate as substrate. There was a possibility that the hyaluronidase demonstrated in these experiments was derived in some way from the joint action of the parasite and the associated bacteria, but none of the bacteria associated with the amoebic strains in themselves showed any hyaluronidase activity. The differences between these findings and those of other workers are probably due to differences in strains and techniques employed.

HYALURONIDASE IN RELATION TO PATHOGENICITY

In order to find whether there was any correlation between the existence of hyaluronidase activity and the pathogenicity of the parasite, Jarumilinta (1962) further examined the enzyme activity of 14 strains of *E. histolytica*; 10 of these were isolated from human cases of amoebic dysentery and were pathogenic to rats and guinea-pigs, and the 4 others were from human carriers and were non-pathogenic to the animals; 1 strain of *Acanthamoeba* sp. was also studied. The results showed that of the pathogenic strains, 8 contained hyaluronidase, while 2 did not. All the non-pathogenic strains and the *Acanthamoeba* exhibited no hyaluronidase activity. Among the pathogenic strains containing hyaluronidase it was also found that there was no correlation between the degree of activity and the average grade of lesion produced in guinea-pigs by a given strain.

Since hyaluronidase was found only in strains of *E. histolytica* which were known to be pathogenic, it was at first thought that the enzyme might be an integral part of the pathogenic process of the

organism. Unfortunately, however, 2 pathogenic strains were found not to contain the enzyme. It was therefore concluded that hyaluronidase as such was not essential to pathogenicity, and that the parasite was capable of spreading in the tissue whether hyaluronidase was present or not. This was emphasized by the results of other experiments in which it was shown that the introduction of trophozoites of a non-pathogenic hyaluronidase-negative strain of *E. histolytica* into the submucous tissue of guinea-pig caecum, did not result in lesions even when purified hyaluronidase was added to the inoculum. Moreover, approximately the same grades of lesions were produced by the introduction of trophozoites of a pathogenic hyaluronidase-negative strain into the submucous tissues with and without purified hyaluronidase. Organisms recovered from the lesions produced by them did not show any hyaluronidase activity, indicating that the hyaluronidase-negative pathogenic strain of *E. histolytica* was unable to develop the enzyme when introduced into the caecal submucous host tissue.

It has thus been possible to demonstrate hyaluronidase in a number of pathogenic strains of *E. histolytica*, but the above experiments have not established any correlation between the presence of this enzyme in the parasite and its pathogenicity. However, it is noteworthy that all strains examined which contained hyaluronidase were in fact pathogenic to human and animal hosts. On the other hand although all the non-pathogenic amoebae examined had no hyaluronidase, the absence of this enzyme did not necessarily indicate non-pathogenicity, since 2 pathogenic strains also did not contain it.

SIGNIFICANCE OF THE OBSERVATIONS

It has been concluded therefore that the study of the pattern of enzymes in the amoebae is not in itself sufficient to determine whether or not the parasite concerned would be pathogenic when brought into relation with the host tissue. It was possible, however, to differentiate between *E. histolytica* as a group and *Acanthamoeba*, indicating that the genetic enzyme patterns of both pathogenic and non-pathogenic strains of *E. histolytica* as a group differed from those of known non-pathogenic free-living organism.

Other enzymes reported to be present in cultures of *E. histolytica* are amylase (Hallman & De Lamater, 1953; Hilker, Sherman & White, 1957; Baernstein, Rees & Reardon, 1954) phosphomonoesterase (Blumenthal, Michaelson & De Lamater, 1955), glutaminase (Nakamura & Goldstein, 1957), maltase (Hilker, Sherman & White, 1957), esterase (Hallman, De Lamater & Michaelson, 1955), succinic dehydrogenase (Seaman, 1953). The significance of these enzymes with regard to the pathogenicity of *E. histolytica* has not yet been studied.

A cytotoxic effect of trophozoites of various strains of *E. histolytica*, pathogenic and non-pathogenic, on leucocytes of human, sheep, rabbit, chicken, guinea-pig, hamster, rat and mouse origin, was detected by Jarumilinta & Kradolfer (1964). *Entamoeba coli*, *Acanthamoeba*, and an unidentified (non-pathogenic) amoeba do not exhibit the activity. Here again, the results indicated that although the leucocytotoxic action of *E. histolytica* makes it possible to distinguish *E. histolytica* from *E. coli*, *Acanthamoeba*, and the unidentified amoeba, they do not provide an answer to the question of pathogenicity, since in this respect there is no apparent distinction between the strains known to be pathogenic and those non-pathogenic to man and animals. Since *E. histolytica* has been shown to display a variety of enzymatic activities, the parasite might be assumed to act enzymatically upon the leucocyte. However, as trypsin at a high concentration did not show leucocytotoxic activity under the same experimental conditions, it was concluded that the causative agent responsible for the leucocytotoxic reaction was not trypsin which is known to be produced by *E. histolytica*.

As the process by which leucocytes are damaged in response to contact with *E. histolytica* is marked by rapid and extensive lysis of cytoplasmic granules, it seems reasonable to assume that the noxa of *E. histolytica* may disrupt the lysosomes in the leucocytes, thereby releasing hydrolytic enzymes which cause digestion of other structural elements in the leucocytes, and finally result in generalized cell damage and death. Further studies are needed to clarify the nature and significance of amoebic leucocytotoxic factors.

It was clear that a study of the parasite alone would not, at this stage, be adequate to distinguish pathogenicity, and for the present the pathogenicity must continue to be established only in terms of the host–parasite relationship.

RÉSUMÉ

LES ENZYMES D'*ENTAMOEBA HISTOLYTICA*

Bien qu'on ait démontré qu'*Entamoeba histolytica* possède des enzymes protéolytiques et peut faire preuve d'une activité hyaluronidasique et de propriétés leucocytotoxiques, on ne peut actuellement se fonder sur l'étude de ces caractères pour établir une distinction entre souches pathogènes et non pathogènes. Les deux variétés contiennent en effet de la trypsine et de la pepsine et toutes deux ont une activité leucocytotoxique. Quant à la présence de carboxypeptidase dans les souches non pathogènes d'*E. histolytica* et à son absence dans les souches pathogènes, elles n'ont encore reçu aucune explication. En outre, si toutes les souches non pathogènes étudiées ne contiennent pas d'hyaluronidase, certaines souches pathogènes sont également dépourvues de cette enzyme.

Dans l'état actuel des connaissances, la pathogénicité d'*E. histolytica* ne peut être déterminée que par l'étude de la relation hôte parasite.

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