Ferritin in Crohn's disease tissue: detection by electron microscopy

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SUMMARY Using immune electron microscopy, an attempt has been made to visualise viral particles in Crohn's disease tissue and faeces. No particles resembling viruses were observed, but in the absence of a specific antiserum a negative result does not exclude the presence of a virus, particularly if it is sparsely distributed. Compared with controls, an abundance of a 12 nm particle was found in all Crohn's disease tissue and one small intestinal lymphoma. This particle has been identified as ferritin and is unlikely to be of aetiological significance.

The report in 1970 by Mitchell and Rees of a transmissible agent in Crohn's disease renewed interest in a possible viral aetiology for this condition. Since then, this work has been reproduced by some (Cave *et al.*, 1973; Taub *et al.*, 1974a, b; Cave *et al.*, 1975 a, b) but others have consistently failed to repeat it (Bolton *et al.*, 1973; Heatley *et al.*, 1975).

In 1975, Aronson and his colleagues reported the isolation by tissue culture techniques of a viral agent from Crohn's disease tissue which has been tentatively classified as a small RNA virus. It has also been isolated from patients with other intestinal disorders, but in only two of 16 controls (Beeken *et al.*, 1976). Additional evidence for a transmissible agent in inflammatory bowel disease stems from the demonstration of lymphocytotoxic activity in sera from patients with ulcerative colitis and Crohn's disease and their household contacts (Korsmeyer *et al.*, 1974; 1975). Since cold lymphocytotoxins occur in viral disease (Mottironi and Terasaki, 1970), these authors have suggested that this finding may indicate an exposure to a common environmental agent. Despite circumstantial evidence for a viral aetiology, there have been relatively few electron microscopic studies in Crohn's disease (Aluwihare, 1971; O'Connor, 1972; Cook and Turnbull, 1975) and, to our knowledge, until now no attempt has been made to visualise viral particles directly by immune electron microscopy.

Methods

TISSUE SPECIMENS

Twelve resected specimens of histologically confirmed Crohn's disease were examined, 10 from the ileum and two from the colon. One small intestinal lymphoma was also studied. Four control specimens of apparently normal intestine from patients having resections for a strangulated hernia, a small intestinal diverticulum and two for a carcinoma of the colon were examined. All specimens were taken at operation, washed in normal saline to remove luminal debris, snap frozen at -70°C , and subsequently stored at -20°C . All subsequent manipulations, including centrifugation, were conducted at 4°C . Sixty grams of tissue were homogenised for four minutes in an MSE homogeniser in 60 ml glycine/hydrochloric acid buffer at pH 2.9. This pH was selected to prevent complexing of any viral particles with antibody that might be present in the specimen. The homogenate was spun at 2800 g in an MSE 6L centrifuge for 30 minutes to remove particulate matter. The resultant supernatant was spun at 10 000 g for 30 minutes in an MSE superspeed 75, six of the 12 specimens having been previously concentrated 25-fold by the use of a minicon (Amicon Mass. U.S.A.). The second supernatant was spun at 140 000 g for two hours, using a swing-out rotor in an MSE superspeed 75. The pellet so obtained was resuspended in glycine/HCl buffer at pH 2.9 to give a concentrate from which aliquots were taken for (1) direct electron microscopy with negative staining (Almeida and Waterson, 1969); (2) dialysis followed by immune electron microscopy (Almeida and Waterson, 1969); (3) density gradient centrifugation, and (4) storage at 4°C .

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Fig. 1 Particles (12 nm) from a resected specimen of Crohn's disease tissue.
× 250 000

Direct electron microscopy A drop of the concentrate was placed on a formvar carbon-coated grid, partially dried, washed three times in distilled water and finally stained with 3% potassium phosphotungstate at pH 6.5. The grids were then examined, using a Philips 201 electron microscope.

Immune electron microscopy A sample of the concentrate was dialysed against phosphate-buffered saline pH 7.4 for 24 hours to raise the pH and facilitate immune complexing. In the absence of a known antibody to the postulated infectious agent, varying quantities of pooled Crohn's sera were added to equal volumes of the concentrate on the assumption that the serum pool might contain specific antibodies. After standing overnight, a drop of the mixture was placed on a grid and stained and examined as described above.

Density gradient centrifugation One millilitre of the concentrate was placed on a solution of 4 ml caesium chloride (0.625 g/ml) made up in glycine/HCl buffer pH 2.9, which gave a density gradient between 1.28 and 1.60 after 24 hours centrifugation at 140 000 *g* using a swing-out rotor in an MSE superspeed 75. The gradient was collected in 0.25 ml fractions, the density of each fraction was estimated by measuring the refractive index and then reading the density from a calibration curve. A drop from each

fraction was taken for direct electron microscopy and the rest dialysed and treated for immune electron microscopy as described above.

STOOL SPECIMENS

Stool specimens were obtained from nine patients with Crohn's disease and two normal controls and stored at -20°C . Using a method similar to that described by Flewett *et al.* (1974), faeces were suspended 10% weight by volume in phosphate-buffered saline pH 7.4. The suspension was homogenised for three minutes in an MSE homogeniser and then centrifuged at 2800 *g* in an MSE 6L centrifuge. The supernatant was then spun at 10 000 *g* in an MSE superspeed 75 and the resultant supernatant concentrated 25-fold in a minicon. Faecal concentrate (0.5 ml) was mixed with 0.2 ml of Crohn's or normal serum, allowed to stand overnight, and then centrifuged at 140 000 *g* for two hours. The pellet was resuspended in phosphate-buffered saline and placed on a grid as before. The technique was verified using a known positive stool containing rotavirus (Dr T. H. Flewett, Birmingham).

Results

TISSUE SPECIMENS

No consistent viral particles common to the Crohn's disease tissue were observed by either direct or

immune electron microscopy of any of the fractions or concentrates. A small 12 nm particle of density 1.45 was found in abundance in all the Crohn's specimens examined (Fig. 1) and in the small intestinal lymphoma. By contrast, after lengthy scanning only very occasional particles which appeared to be morphologically similar to those illustrated were seen in two of the controls but not in the remainder. The particles were not clumped by Crohn's or normal sera.

STOOL SPECIMENS

Phages and bacterial flagellae were a common finding and in one case probable enteroviruses were seen. But again no consistent viral or phage particle common to Crohn's disease was visualised, nor did there appear to be any specific clumping by sera.

Discussion

In the present study, we have been unable to demonstrate viral particles peculiar to Crohn's disease in intestinal tissue or stools, although the methods used have been successful in identifying core particles of hepatitis B from liver homogenates (Almeida *et al.*, 1970) and viruses from stool specimens (Flewett *et al.*, 1974). Despite our inability to isolate a virus in Crohn's disease, it should be emphasised that this does not exclude the

possibility of a virus being present, particularly if it is small and in low concentration. It would seem from the data at present available that, if a virus were of importance in the pathogenesis of Crohn's disease, then it could be a common agent to which some individuals have an abnormal response. By analogy with HBsAg positive liver disease, tissue damage in Crohn's disease may be variable, only certain cells being affected, and homogenisation of whole tissue would much dilute the virus, making recognition difficult even if followed by a concentration process. To visualise viral particles from a suspension, they need to be present at a concentration of $10^6/\text{ml}$ before one obtains a distribution of one virus per grid square, assuming a 200-mesh grid (Flewett and Boxall, 1976). Consequently, small viruses in low concentration can easily escape detection. By immune electron microscopy, viral particles are agglutinated by a process of complexing, thus making recognition easier and enhancing detection. The problem when applied to Crohn's disease is that it is not known whether specific antibody is present in the serum and at what concentration. To try to overcome this problem, we used various combinations of sera from acute untreated cases, chronic cases, and those who were in apparent remission, but with negative results.

The 12 nm particle isolated is too small for any known virus. Almeida (personal communication)

Fig. 2 Particles (12 nm) of horse spleen ferritin. $\times 250\,000$.

suggested that these particles resemble ferritin, and we have confirmed this in two ways. Firstly, negative staining and electron microscopy of purified horse spleen ferritin revealed particles of identical morphology (Fig. 2). Secondly, radioimmunoassay demonstrated ferritin in those fractions of the gradient containing the particles, the levels showing a close correlation with the particle count. It is of interest that ferritin has been demonstrated in Burkitt's lymphoma and Hodgkin's disease tissue (Eshhar *et al.*, 1974; Order *et al.*, 1974), where it was originally thought to be a tumour-associated antigen (Order *et al.*, 1971; 1972.) Certain diseased cells, such as leukaemic cells (White *et al.*, 1974), have an abnormally high rate of ferritin synthesis, and we think it possible that increased synthesis may also explain our findings of a high ferritin content in Crohn's tissue.

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