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Origins of...

Electron microscopy and viral diagnosis

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The electron microscope (EM) was developed in Germany during the 1930s to visualise objects too small to be resolved clearly by the light microscope[1]; viruses were among the first objects to be seen with it. Stanley and Anderson[2] published pictures of unstained tobacco mosaic virus and other plant viruses in the early 1940s but the contrast was poor and no fine detail was revealed. No further progress in unveiling virus morphology was made until Brenner and Horne[3] showed that salts of some heavy metals would form an electron dense amorphous "glass" around viruses, making their fine structure visible by negative contrast (negative stain). The method of preparation was elaborate at first but has since been refined to mixing a stain with a suitably dilute preparation of virus, placing the mixture on a formvar coated EM grid, drawing off the surplus mixture, and allowing it to dry. This extremely simple method has made it possible to visualise most viruses provided that the concentration was higher than about 10^6 viruses per millilitre.[4]

In the 10 years or so after Brenner and Horne's paper, micrographs of one virus after another were published and these studies confirmed two important facts. First, although preparation might include ultracentrifugation at 100 000 $\times g$, treatment with various stains, drying, and irradiation in the microscope, the morphology revealed by electron microscopy was very consistent and could be used reliably to identify individual structural groups. Second, morphology was closely linked to other physicochemical properties—for example, all viruses with a herpes morphology contain double stranded DNA of a generally similar size and genomic organisation. Although some scientists maintained that all EM images were artefacts, they were consistent enough possibly to be useful in diagnosing viral infections.[5]

The electron microscope as a diagnostic tool

By the mid-1960s smallpox had been eliminated from Europe, North America, and Australasia but was widespread in Africa, Asia, and South America. In the non-endemic areas fewer and fewer people were vaccinated and there was a risk that occasional imported cases could initiate an epidemic. Smallpox had to be distinguished rapidly and reliably from chickenpox to allow genuine cases of smallpox to be isolated and the threat contained. Pox viruses and herpes viruses were totally dissimilar under the EM[6] and it was shown by several groups that more than sufficient virus was present in vesicle fluid from each disease to be seen without difficulty. A considerable number of EMs were then bought for diagnostic laboratories in the United Kingdom and elsewhere. Although imported smallpox was rare, the microscope became an integral part of smallpox diagnosis.[7]

With the elimination of smallpox in 1976 a new role for the diagnostic EM was needed. Other skin lesions were known to be caused by viruses and it was shown that common warts,[8] molluscum contagiosum,[9] and orf lesions[10] all contained enough virus to be visible under the EM in a simple extract. However, none of these had the destructive potential of smallpox and could often be diagnosed clinically. Even as early as 1970 the EM was in danger of reverting to research use only.

By the mid-1970s viruses had been identified as the cause of many diseases, but they were also thought to be involved in the common episodes of diarrhoea and vomiting responsible for substantial morbidity and mortality in children, particularly in the tropics. Nevertheless, although bacterial causes were identified by culture, no pathogenic organisms were recovered from the stools of more than half the patients and "viruses" were blamed. Enteroviruses, adenoviruses, and reoviruses were isolated from stool samples but not frequently enough to determine a causal relation.[11] Kapikian and colleagues in the United States used electron microscopy to investigate an outbreak of gastroenteritis that had occurred in Norwalk, Ohio.[12] They used convalescent sera from infected individuals to aggregate virus particles in stool extracts in the (correct) assumption that such clumps would be easier to see in EM.

This demonstration that virus could be seen in stool extracts attracted little attention, probably because it involved a defined outbreak, required convalescent sera, was labour intensive, and particles seen lacked defining features. It must have seemed a lot of effort for little reward but no one thought it worthwhile to examine stool extracts directly.

The alternative EM preparative technique of fixing, embedding, and thin sectioning tissue was regarded as too elaborate and too slow for routine use in virus diagnosis. However, Bishop and colleagues[13] in Australia examined biopsies by thin section EM taken from children with

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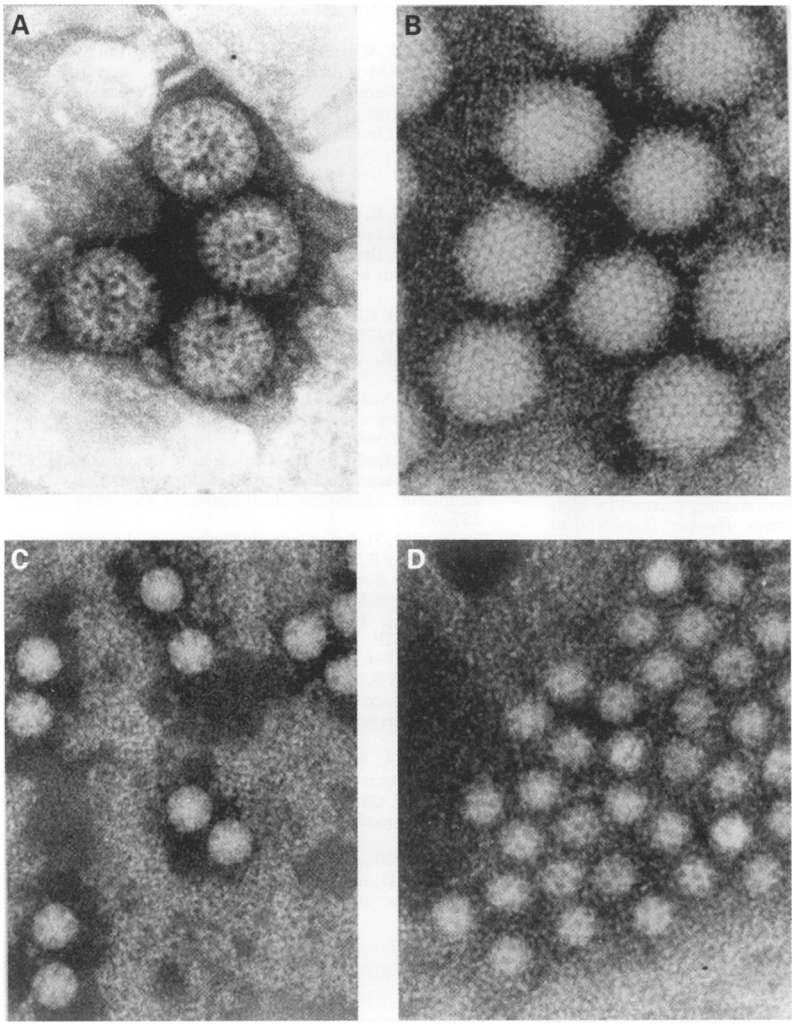

Figure 1 (A) Rotavirus; (B) fastidious adenovirus; (C) astrovirus; (D) calicivirus. All from stool extracts negatively contrasted with 3% potassium phosphotungstate pH 7. Original magnification ×200 000.

possible malabsorption and discovered much larger virus-like particles (approximately 70 nm in diameter compared with 30 nm for the Norwalk virus) present in the cytoplasm of enterocytes. Shortly afterwards, Flewett *et al*[14] showed that these virus particles could be seen directly in stool extracts by negative contrast after elaborate concentration and separation procedures. The virus they found (fig 1A) was later called a rotavirus and is one of the most common of all viruses. It has been found everywhere, with similar viruses infecting the young of a wide variety of animal species.

Initially, rotaviruses were assumed to be the cause of viral diarrhoea but, with simplified preparation methods, other viruses were also found in diarrhoeal stools. Adenoviruses[15] (later found to be types 40 and 41) (fig 1B), astroviruses (fig 1C), caliciviruses (fig 1D),[17] faecal coronaviruses,[18] and a variety of small round viruses[19] (either plain (SRV) or structured (SRSV)) were rapidly identified. Some were associated with outbreaks of vomiting and diarrhoea (often after eating raw or undercooked shellfish) and others with endemic diarrhoea, mostly in children but occasionally in adults and the elderly.

The middle to late 1970s were a heady period as a succession of new viruses were identified, but by the 1980s, new additions had become rarer. Toroviruses[20] and picobirnaviruses[21] were added but their role in causing disease remains uncertain. Even after discovery of all these viruses, there are considerable cases of diarrhoea in which neither a bacterial nor a viral cause is found. The previous observation that these viruses did not grow in cell culture was confirmed even though the numbers present reached astronomical levels (> 10^{12} per gram of faeces). Some were persuaded to grow under research conditions and other techniques were developed to show that all the newcomers had several serotypes. Would further information come from using enhanced methods of detection?

Enhancement

Norwalk virus was found by immune electron microscopy (IEM). The surface fuzziness on the particles was misinterpreted as a coating of antibody but later evidence showed that the virus is naturally fuzzy. IEM was not new in 1970, Anderson and Stanley[22] had used it in their studies of tobacco mosaic virus. Later Almeida and Waterson[23] explored its possibilities but found that it worked best when the amount of virus and the amount of antibody were approximately equivalent. The absence of titrated sera and unknown concentrations of virus has limited its use in routine diagnosis. Moreover, a heavy coating of antibody will obscure surface detail on the virus making identification difficult. Development of the Derrick technique[24] (or solid phase IEM (SPIEM)) showed how antibody might be of practical use by coating the microscope grids with antibody. (This made them sticky and anchored any virus particles, greatly enhancing the number visible.) Suitable antiserum was essential but, because it biased the results towards the virus targeted by the antiserum, SPIEM has not been used routinely but mainly to investigate outbreaks associated with Norwalk-like viruses. Heavy metal labels attached to antibodies (ferritin or colloidal gold[25]) have been tried both in research and diagnostic roles. This should allow the identification of specific antigens on the surface of both virus particles and cellular structures but neither has proved ideal. Ferritin conjugates are notorious for non-specific reactions and colloidal gold labels detach from the antibody in storage giving a very limited shelf life. Nevertheless, antibody plain or labelled can help in identifying "difficult" or low titre virus.

The future

EMs require major capital investment and they are expensive to maintain. There is an increasing feeling in cost conscious diagnostic laboratories that the days of electron microscopy are numbered. Alternative tests which are simpler (and presumably cheaper) should be replacing these white elephants. Nevertheless the speed of diagnosis (approximately 15 minutes at its best), the versatility of the EM in being able to detect a wide variety of viruses without any prior selection, and that up to nine or more different tests would be needed to replace it, should ensure its continued use. A complete

assessment of its costs will show that it does not have to be prohibitively expensive, particularly as good quality second-hand machines are available. New viruses are still being identified and, as morphology is a good predictor of other properties, EM examination is essential. An EM remains a vital facility in a regional diagnostic laboratory. Without it, investigation of outbreaks are incomplete. Though the heady days of the 1970s may be over when opening a journal seemed always to reveal yet another virus, electron microscopy still provides a diagnosis unmatched for speed and certainty.

1 Ruska E. Über fortschritte im Bau und in der Leistung des magnetischen Elektronemikroscops. *Z Physik* 1934;**87**:580–602.
2 Stanley WM, Anderson TF. A study of purified viruses with the electron microscope. *J Biol Chem* 1941;**139**:325–38.
3 Brenner S, Horne RW. A negative staining method for high resolution microscopy of viruses. *Biochim Biophys Acta* 1959;**34**:103–10.
4 Madeley CR, Field AM. *Virus morphology.* 2nd edn. Edinburgh: Churchill Livingstone, 1988.
5 Nagington J. Electron microscopy in differential diagnosis of poxvirus infections. *BMJ* 1961;**ii**:1499–500.
6 Nagler FPO, Rake G. Use of the electron microscope in diagnosis of variola, vaccinia and varicella. *J Bacteriol* 1948; 55:45–51.
7 Cruickshank JG, Bedson HS, Watson DH. Electron microscopy in the rapid diagnosis of smallpox. *Lancet* 1966;**ii**: 527–30.
8 Almeida JD, Howatson AF, Williams MG. Electron microscope study of human warts, sites of virus production and nature of the inclusion bodies. *J Invest Dermatol* 1962; **38**:337–45.
9 Williams MG, Almeida JD, Howatson AF. Electron microscope studies on viral skin lesions. *Arch Dermatol* 1962;**86**:290–7.
10 Nagington J, Newton AA, Horne RW. The structure of orf virus. *Virology* 1964;**23**:461–72.
11 Madeley CR. Viruses in the stools. *J Clin Pathol* 1979;**32**:1–10.
12 Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM. Visualization by immune electron microscopy of a 27nm particle associated with acute non-bacterial gastroenteritis. *J Virol* 1972;**10**:1075–81.
13 Bishop RF, Davidson GP, Holmes IH, Ruck BJ. Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. *Lancet* 1973;**ii**: 1281–3.
14 Flewett TH, Bryden AS, Davies HA. Virus particles in gastroenteritis. *Lancet* 1973;**ii**:1497.
15 Flewett TH, Bryden AS, Davies HA, Morris CA. Epidemic viral enteritis in a long stay children's ward. *Lancet* 1975;**i**: 4–5.
16 Madeley CR, Cosgrove BP. Viruses in infantile gastroenteritis. *Lancet* 1975;**ii**:124.
17 Madeley CR, Cosgrove BP. Calicivirus in man. *Lancet* 1976;**i**:199–200.
18 Mathan M, Mathan VI, Swaminathan SP, Yesudoss S, Baker SJ. Pleomorphic virus-like particles in human faeces. *Lancet* 1975;**i**:1068–9.
19 Caul EO, Appleton H. The electron microscopical and physical characteristics of small round human fecal viruses: an interim scheme for classification. *J Med Virol* 1982;**9**: 257–65.
20 Beards GM, Hall C, Green J, Flewett TH, Lamouiliatte F, du Pasquier P. An enveloped virus in stools of children and adults with gastroenteritis that resembles the Breda virus of calves. *Lancet* 1984;**i**:1050–2.
21 Pereira HG, Fialho AM, Flewett TH, Teixeira JMS, Andrade ZP. Novel viruses in human faeces. *Lancet* 1988;**ii**:103–4.
22 Anderson TF, Stanley WM. A study by means of the electron microscope of the reaction between tobacco mosaic virus and its antiserum. *J Biol Chem* 1941;**139**:339–44.
23 Almeida JD, Waterson AP. The morphology of virus-antibody interaction. *Adv Virus Res* 1969;**15**:307–38.
24 Derrick KS. Quantitative assay for plant viruses using serologically specific electron microscopy. *Virology* 1973;**56**: 652–3.
25 Pares RD, Whitecross MI. Gold-labelled antibody decoration (GLAD) in the diagnosis of plant viruses by immunoelectron microscopy. *J Immunol Methods* 1982;**51**:23–8.