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RAPID IDENTIFICATION OF VIRUSES BY A SIMPLE INDIRECT IMMUNE ELECTRON MICROSCOPY TECHNIQUE USING FERRITIN-LABELLED ANTIBODIES

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A simple indirect immune electron microscopy technique using ferritin-labelled antibodies and negative staining to rapidly visualize and identify human viruses is described. The increased electron density given by the ferritin molecules, which also served as a reliable marker, has greatly facilitated virus detection.

INTRODUCTION

In 1941, Stanley and Anderson and Von Ardenne et al. were the first to independently use immuno-electron microscopy (IEM) in order to gain an insight into the nature of antigen–antibody reactions. With the development of electron microscope technology, similar studies, later performed by Lafferty and Oertelis (1963) and Almeida et al. (1963), significantly contributed to the knowledge of lattice formation and antibody molecules. IEM has also been used to study the interaction between IgM molecules and bacterial or viral antigens (Feinstein and Munn, 1966; Svehag and Bloth, 1967), the effect of complement on viral particles (Berry and Almeida, 1968; Almeida and Laurence, 1969; Jensen et al., 1980), as well as the localization and the number of different antigenic sites on viruses (Hummeler et al., 1962; Höglund, 1967; Brown and Smale, 1970; Yanagida and Ahmad-Zadeh, 1970).

Furthermore, in addition to the serotyping of various viruses (Penney et al., 1972; Anderson and Doane, 1973; Lutton, 1973; Kelen and McLeod, 1974; Vasal and Ray, 1974; Kelen et al., 1976; Doane and Anderson, 1977; Fauvel et al., 1977), IEM has facilitated the detection and identification of elusive or fastidious agents such as rubella virus (Best et al., 1967), hog cholera virus (Ritchie and Fernelius, 1967), rhinoviruses (Kapikian et al., 1972a), coronaviruses (Kapikian et al., 1973), wart viruses (Almeida and Goffe, 1965), hepatitis A and B viruses (Bayer et al., 1968; Feinestone et al., 1973), the Norwalk agent (Kapikian et al., 1972b), a parvovirus-like agent (Paver et al., 1973), and rotaviruses (Flewett et al., 1974). Direct IEM, where specific antisera were substituted by pools of human γ-globulins modifying the Anderson and Doane (1973) method of serum-in-agar
diffusion, was used successfully in our laboratory as a first step approach in viral diagnosis (Berthiaume et al., 1981). We now report the development of an indirect IEM technique, using ferritin-labelled antibodies and negative staining, to visualize rapidly and identify viruses. This technique is more rapid and simpler than pre-existing indirect IEM methods and has been successfully used with three groups of viruses of different size and morphology taken from clinical or cell cultured samples. The applications and advantages of this technique are also discussed.

MATERIAL AND METHODS

Cell cultured adenovirus type 1 and coxsackievirus B-5, and rotaviruses from infantile gastroenteritis faeces were used throughout this study. In the latter case, faeces were prepared for virus identification as follows: faeces were mixed with phosphate buffer saline (PBS) to give a final 20% (w/v) suspension and were then centrifuged at 1000 g for 5 min to sediment larger debris, and further clarified by a second centrifugation at 10,000 g for 20 min. Then, 30 µl of the supernatant were mixed for 15 min at room temperature in a humid chamber with 10 µl of rabbit anti-bovine rotavirus serum diluted 1/40 in PBS, known to cross-react with human rotavirus (Woode et al., 1976) (kindly provided by R. Assaf, Institut Armand-Frappier). To this suspension, 10 µl of ferritin-labelled goat anti-rabbit IgG diluted 1/50 in PBS were subsequently added and incubated for another 15 min. The whole mixture was then deposited in a microtiter well half-filled with 1% agar in distilled water. A carbon formvar-coated grid was overlaid, face downward, on the mixture which was allowed to almost completely diffuse in the agar. The preparations were then picked up, stained with 3% phosphotungstic acid (PTA), pH 6.8, and examined with Philips EM 300 or 200 electron microscopes. Supernatants of adenovirus type I or coxsackievirus B-5 infected cell cultures were used without any purification and were treated as described above, but using commercial rabbit anti-adenovirus type I and anti-coxsackievirus B-5 sera (Microbiological Associates, Bethesda, MD).

The conjugation procedure of ferritin to goat anti-rabbit IgG was essentially that of De Petris and Raff (1972), except that unlabelled antibodies were removed by chromatography on a 2.6 X 90 cm Bio-Gel A 1.5 m (Bio-Rad, Richmond, CA) column, equilibrated and eluted with 0.1 M Tris(hydroxymethyl)aminomethane—0.05 M HCl buffer, pH 7.6, containing 0.02% sodium azide. The first eluted peak contained the goat-IgG-ferritin conjugate, a very small amount of free ferritin, and small aggregates. These aggregates are formed when using the one-stage glutaraldehyde conjugation technique (Otto et al., 1973), and are known to bind antigen (Batsford et al., 1979).

RESULTS

Rotaviruses from infantile gastroenteritis faeces were easily detected by the indirect IEM technique. Rotaviruses rarely appeared as single particles but in aggregates. A relative-
ly low magnification of a large typical immune complex of up to 100 particles is shown in Fig. 1b. Aggregates like the one presented were detected at a much lower magnification, due to the heavy tagging of ferritin molecules surrounding the viruses, and which greatly increased the electron density of the immune complexes. A square grid containing numerous dark spots was generally first located, and then examined at higher magnification for the identification of rotaviruses on the basis of their morphology. Since almost every rotavirus was found within large electron dense complexes in a positive sample, the absence of rotaviruses in the aggregated conjugate was considered a negative sample. Viral immune complexes containing few rotaviruses were also easily detected as shown in Fig. 1c. A heavy tagging of ferritin was frequently seen among the various sizes of immune complexes of rotaviruses. This is to be expected, since the aggregates of ferritin-marked antibodies generated by the one-step conjugation technique have the capacity to interact with their corresponding antigen, being the rabbit antibodies fixed on rotaviruses. This interaction was very profitable, since it enabled an easy detection of rotaviruses as illustrated in Fig. 1d. In all instances, rotavirus morphology was faintly masked, but distinctive capsomeres were easily observed on the particles. Moreover, the slightly altered thin double membrane or part of it, indicating the typical rotavirus morphology, was frequently visualized on many particles within the complexes. Also, there was no unspecific attachment of the ferritin-labelled goat anti-rabbit IgG to rotaviruses when the use of homologous antiserum was omitted in the first step, indicating the specificity of the ferritin conjugate. Such an example is presented in Fig. 1a which shows an isolated rotavirus free of ferritin.

Ferritin-tagged immune complexes of cell cultured adenovirus type 1 or coxsackievirus B-5 were also observed using the same approach. Here again, the greater inherent density given by the ferritin molecules to the immune complexes has greatly facilitated viral detection and identification. Adenovirus morphology with its characteristic dot-like capsomeres was still clearly visible as illustrated in Fig. 2a. The latter micrograph also clearly shows how aggregates of ferritin-tagged antibodies can improve the detection of viruses, especially in low concentrations when the formation of aggregated viruses by homologous antiserum is improbable. Indirect IEM using ferritin-labelled antibodies appeared also useful in the case of small viruses without clear surface morphological characteristics. Coxsackieviruses B-5, which fall in this category, were easily identified as shown in Fig. 2b. Ferritin molecules served as a reliable marker for the differentiation of viruses from non-viral material. As before, the ferritin conjugate did not interact with adenoviruses type 1 or coxsackieviruses B-5 when these viruses were used without their homologous rabbit antiserum.

DISCUSSION

Electron microscopy and IEM are nowadays well established procedures for the detection and identification of viruses from various clinical specimens or infected cell cultures (Kjedsberg, 1980; Doane, 1980). The method is fast and represents a truly catch-
Fig. 2. Useful applications of the indirect IEM technique using ferritin-labelled antibodies. a) Viruses in low concentration sharply stand out from the background as seen here with an isolated adenovirus type 1 heavily surrounded by aggregates of ferritin-tagged antibodies. b) Small viruses without distinctive morphology such as the coxsackieviruses B-5 are easily detected by this approach. PTA staining. Bars, 100 nm.

Fig. 1. Rotaviruses detected by indirect IEM with ferritin-labelled antibodies. a) Control reaction showing that without using rabbit homologous serum in the first step, the ferritin-labelled goat anti-rabbit IgG aggregate is seen unattached to the isolated rotavirus. b) Large immune complex of rotaviruses seen at low magnification. c, d) The heavy tagging of ferritin on small aggregates of rotaviruses is seen here at high magnification. PTA staining. Bars, 100 nm.
all system for viruses. Virus detection by indirect IEM with unlabelled antibodies has previously been described. Two of these procedures (Edwards et al., 1975; Walters et al., 1975) involved two 1-h centrifugations with an overnight incubation, while another (Fournier et al., 1978), similar to ours, did not concentrate the viruses. Indirect IEM with ferritin-labelled antibodies and negative staining has also been reported, but to verify the precise attachment of IgG on chromatin (Bustin et al., 1976), influenza virus (Patterson et al., 1975), or hepatitis B core antigen (Huang and Neurath, 1979), and most importantly, in all instances, very few molecules of ferritin could be seen around these structures. However, the technique we have described for virus detection and identification bypasses centrifugation steps, concentrates viruses on agar, enables the detection of highly labelled viral aggregates and give results within an hour. Rotaviruses from faeces and cell cultured adenoviruses type 1 or coxsackieviruses B-5 were all easily detected using this technique. Due to the difficulties of in vitro propagation, direct electron microscopy has become a widely used technique for the detection of human rotaviruses in faeces. However, a 100-fold increase in sensitivity was associated with this indirect IEM technique when compared to direct electron microscopy (Trépanier, 1979), but presumably this could be extended to the cell cultured viruses used in this study. The use of this technique would also appear very useful in the later cases, since these viruses can often be identified by electron microscopy 24 h before any cytopathic effect is seen by light microscopy (Doane, 1980). Furthermore, there is no reason to believe that this technique could not be extended to other viruses as well. Interesting applications of this indirect IEM technique would be the detection of viruses from samples containing a very low concentration of viruses. The heavy tagging of ferritin molecules around individual viruses would permit their rapid detection, as shown with adenovirus type 1. Also, since ferritin molecules represent a reliable marker, viruses with uncharacterized morphology or small viruses, already known as well as new ones, that can be easily mistaken as cellular debris, would be identified with more certainty using this technique, thus eliminating ambiguous observations.

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