Studies on the Poxvirus Cotia

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SUMMARY

The poxvirus Cotia was studied by electron microscopy and by serological and biochemical analyses. Thin-sectioned preparations of infected Vero cells indicated that Cotia virus morphogenesis was similar to other mammalian poxviruses; unique filamentous structures and inclusion matrices were apparent in the cytoplasm. Complement fixation tests that included purified Cotia virions showed a reciprocal cross-reaction with rabbit myxoma virus and no cross-reaction with vaccinia virus. Serological results coupled with gradient polyacrylamide gel electropherograms of the structural proteins of purified Cotia, vaccinia, myxoma and fibroma viruses suggested that Cotia virus was similar to the latter two viruses. Agarose gel electropherograms of cleavage fragments of each of these virus DNAs digested with three separate restriction endonucleases showed that each of these viruses had a unique DNA gel profile.

INTRODUCTION

Cotia virus was isolated in 1961 at the Instituto Adolfo Lutz, Sao Paulo, Brazil, from brain specimens from 3-day-old laboratory mice that had been placed as sentinels for arbovirus activity in a local forest area (Cotia County, Sao Paulo) during the hot, rainy season. The virus was isolated from 40 separate mouse brain specimens collected from February to April in 1961 and was suspected of having been mechanically transmitted by mosquitoes to the sentinel mice (de Souza Lopes et al. 1965). Because of the circumstances surrounding its original isolation, the virus was included in the International Catalogue of Arboviruses (Berge, 1975).

Cotia virions were first visualized as poxviruses in preliminary electron microscopy studies in 1965. About the same time, initial serological studies on this virus by immunodiffusion (ID) and complement fixation (CF) tests, which included batteries of virus diagnostic reagents, failed to show any significant relationship between Cotia virus and the poxviruses ectromelia, rabbitpox, cowpox, monkeypox, fowlpox and yabapox, or certain viruses representative of other virus families. In these studies a faint cross-reaction by ID was observed with vaccinia virus (F. A. Murphy & E. C. Borden, unpublished data; R. E. Shope, personal communication). The virus was separately shown to have a significant serological relationship to viruses isolated from several genera of mosquitoes in French Guiana (Serie, 1970,

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1971a), to a virus (No. BeAn 58058) isolated from an *Oryzomys* rat in Para, Brazil (Woodall, 1967) and to a virus isolated from a blood specimen taken from a human in French Guiana (Serie, 1971b; Berge, 1975). Recently Ueda et al. (1978) reported that Cotia virus reacted only with homologous antisera in reciprocal neutralization tests with vaccinia virus and that Cotia virus antisera did not neutralize the infectivity of goatpox, fowlpox, myxoma or tanapox viruses. Further, they observed an antigenic relationship between Cotia and vaccinia viruses by ID and by immunofluorescence (FA) tests.

In this report we present the results of our electron microscopic observations of thin-sectioned Cotia virus infected Vero cells and of our serological and biochemical studies in which we compared Cotia virus with selected members of the genus *Orthopoxvirus* and the genus *Leporipoxvirus*.

**METHODS**

**Viruses.** Cotia virus (ninth passage in suckling mice) was obtained from the collections of the Center for Disease Control (CDC), Atlanta, Georgia, and the WHO International Reference Center for Arboviruses, Yale University, New Haven, Connecticut. The virus stock was further passaged five times in the brains of suckling mice and adapted to grow in cell cultures before our tests were done. The identity of our stock virus preparation was confirmed by complement fixation tests with Cotia virus antisera prepared against a reference virus seed stock.

The Lausanne strain of myxoma virus and the Kasza strain of fibroma virus were obtained from the American Type Culture Collection. Other poxviruses, including the Lister strain of vaccinia virus, were from our own virus repository.

**Cell cultures.** Continuous lines of RK-13 and Vero cells were grown in a reinforced (Bablanian *et al.* 1965) Eagle’s minimal essential medium that contained 10% foetal bovine serum. Cells were infected with virus at an input multiplicity of 0.01 to 1 and the cultures were incubated at 35.5 °C in standard MEM supplemented with non-essential amino acids and 2% serum.

**Electron microscopy.** Thin sections of Vero cells infected with Cotia virus were prepared and examined by methods previously described (Murphy *et al.* 1971).

**Serology.** Complement fixation tests were performed according to a standardized microtitre system (Casey *et al.* 1965). Anti-myxoma virus sera and a crude CF antigen preparation were donated by Dr I. Marshall and Dr F. Fenner, The Australian National University, Canberra. Anti-Cotia virus mouse ascitic fluid was prepared by hyperimmunizing animals with an infectious suckling mouse brain preparation of Cotia virus.

**Virus purification.** Virions were purified from the cytoplasm of infected cells by velocity sedimentation in 20 to 40% (w/w) sucrose density gradients (Joklik, 1962a, b; Holowczak & Joklik, 1967; Esposito *et al.* 1977, 1978).

**Virion proteins.** The structural proteins in preparations of purified virus particles were resolved with an SDS-discontinuous polyacrylamide slab gel electrophoresis system. Seven to 20% linear gradient resolving gel slabs (140 × 280 × 1.5 mm) were cast with the Laemmli (1970) tris-buffered system, essentially as described by Chin & Maizel (1976). The separated proteins in the gels were stained with Coomassie blue as previously described (Obijeski *et al.* 1973; Esposito *et al.* 1978).

**Virion DNA.** Virion DNA in purified virus preparations was released by digesting the particles in a solution that contained Proteinase-K, N-lauroyl sarcosinate and 2-mercaptoethanol and then were adjusted to 1 M-NaCl as previously described (Esposito *et al.* 1978). DNA in the digests was extracted twice with a phenol-chloroform-isoamyl alcohol (50:48:2) mixture that was saturated with 0.1 M-tris buffer, pH 8.9, and contained 0.1% 8-hydroxy-
quinoline. The aqueous phase was dialysed against 25 mM-tris buffer, pH 7.6, concentrated when necessary against polyethylene glycol-20000, dialysed again, then extracted twice with chloroform. The isolated virus DNAs were subsequently cleaved with restriction enzymes and the fragments separated in a Studier submerged gel-slab apparatus (Aquebogue Machine and Repair Shop, Aquebogue, New York) by agarose-ethidium bromide gel electrophoresis essentially as described previously (Esposito et al. 1978) but with a tris-phosphate-EDTA buffer (Loening, 1969).

RESULTS

Electron microscope study

In a late harvest series (6 and 9 days after inoculation) of Vero cells infected with Cotia virus at low input multiplicity, morphological features characteristic of mammalian poxvirus development (Bergoin & Dales, 1971; Dales, 1973) were observed. Two types of cell inclusion were seen; one was a granular viroplasmic matrix or type B inclusion commonly seen in the mammalian poxvirus infections and the second was a unique, less compact inclusion composed of bead-like subunits arranged in clusters of filamentous strands (Fig. 1a). Another prominent feature of Cotia virus-infected Vero cells was long, linear arrays of filaments, cross-striated in appearance, with a spacing of approx. 9.5 nm (Fig. 1b). They appeared to be excess or aberrant virus membrane material reminiscent of structures reported in rabbit fibroma virus (Prose et al. 1971). No type A inclusions (Kato et al. 1963; Ichihashi et al. 1971) were found in our cell preparations.

Reciprocal complement fixation tests

Reciprocal CF tests that included a purified preparation of Cotia virions as antigen revealed a serological relationship between Cotia virus and the leporipoxviruses Australian and Venezuelan myxoma viruses (Table 1). Cotia virus mouse ascitic fluid showed homologous reactivity and, to a lesser degree, reacted with a crude antigen preparation of myxoma virus. No cross-reactivity was observed when purified vaccinia virions were used. Two myxoma antibody preparations showed homologous reactivity and reacted with Cotia virions. [The presence of the common poxvirus 'NP' antigen (Woodroofe & Fenner, 1962) or its antibody were not specifically identified with the reagent preparations used here.]

Electrophoresis of structural proteins

The observation that Cotia virus and myxoma virus gave an apparent cross-reaction in CF tests led us to compare the virion structural proteins by SDS-polyacrylamide gel electrophoresis (PAGE).

Approximately 40 proteins could be resolved for each virus with the gradient gel system used here (Fig. 2). The virion proteins were not in equimolar distribution and ranged in mol. wt. from 8000 to 200000. Cotia virus and the leporipoxviruses each have a characteristic, large 200000 mol. wt. structural protein not observed with purified preparations of vaccinia virus.

Inspection of Fig. 2 shows that the protein PAGE profile for Cotia virus bears more likeness to the patterns of the purified leporipoxviruses myxoma and fibroma viruses than to vaccinia virus. The protein gel patterns of the leporipoxviruses greatly resemble each other. In one experiment (results not shown) we have compared the protein profile of Cotia virus with those of the orthopoxviruses variola, cowpox and monkeypox. These orthopoxvirus electropherograms were quite similar to that of vaccinia virus but not of Cotia virus (for further poxvirus gel profiles for comparison, see Obijeski et al. 1973; Arita & Tagaya, 1977; Esposito et al. 1977; Ikuta et al. 1978a, b; Harper et al. 1979). A resemblance
Fig. 1. Thin sections of Vero cells infected with Cotia virus. (a) Mature virus particles in the cytoplasm and within vacuoles of the Golgi and endoplasmic reticulum; arrow, Cotia inclusion body. (b) Linear array of filaments, cross-striated in appearance, with a spacing of approx. 9.5 nm.
Table 1. *Relationship of Cotia and myxoma viruses by reciprocal complement fixation testing*

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Cotia*</th>
<th>Myxoma† (Australia)</th>
<th>Myxoma‡ (Venezuela)</th>
<th>Vaccinia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotia§</td>
<td>256</td>
<td>32</td>
<td>&gt;64</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Myxoma∥</td>
<td>64</td>
<td>256</td>
<td>512</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Vaccinia§</td>
<td>&lt;8</td>
<td>&lt;8</td>
<td>&lt;8</td>
<td>512</td>
</tr>
<tr>
<td>RK-13 cells</td>
<td>&lt;8</td>
<td>&lt;8</td>
<td>&lt;8</td>
<td>&lt;8</td>
</tr>
</tbody>
</table>

* Hyperimmune mouse ascitic fluid.
† Gamma globulin fraction of rabbit serum collected from animals convalescing from myxomatosis and given subsequent hyperimmunizations.
‡ Serum from rabbits hyperimmunized with virus grown on chicken embryo chorioallantoic membranes.
§ Purified preparation of virus particles from infected RK-13 cells.
∥ Homogenate of a local lesion from a rabbit infected with myxoma virus (Brazil/Campinas/1949/I, Lausanne strain).

in patterns certainly does not prove the identity of this virus; however, it seems likely that these similarities in profile reflect qualitatively the extent of viral relatedness.

**DNA fragment electrophoresis**

In seeking further taxonomic data, we used electrophoretic analysis of cleaved virus DNA in order to examine the genomes of Cotia virus, the leporipoxviruses and the orthopoxviruses. Preparations of DNA from Cotia, myxoma, fibroma and vaccinia viruses were digested separately with the restriction endonucleases BamHI, SalI and HindIII and the cleaved DNA fragments in the digests were separated by agarose gel electrophoresis. Fig. 3 shows the electrophoresis patterns obtained by this analysis. Fragments which range in mol. wt. from 30 to 0.5 million were resolved. The DNA fragment migration patterns did not indicate that there was any relationship among the four viruses examined here. Comparison of the present work with that of others indicated that Cotia virus and the leporipoxviruses were each unique and that vaccinia virus resembled other orthopoxviruses (for cowpox, variola, whitepox and monkeypox virus DNA patterns see Esposito et al. 1978; for rabbitpox, ectromelia and fowlpox viruses see Muller et al. 1978; for *Molluscum contagiosum* virus see Parr et al. 1977; for stomatitis papulosa virus see Menna et al. 1979).

Table 2 is a list of the approximate values for the mol. wt. of the four virus DNA genomes calculated by summation of the apparent mol. wt. of the DNA fragments shown in Fig. 3. Visual approximations of fragment fluorescence intensity were made to quantify the relative molarities of the fragments in order to obtain the mol. wt. estimates. The values obtained for Cotia virus indicated that this virus has a genome size similar to that of vaccinia virus. A significant variance in genome DNA mol. wt. was found when the leporipoxviruses were fragmented with each enzyme. This variance suggested to us that many small, unresolved digest fragments were present which made precise measurement of mol. wt. difficult.

**DISCUSSION**

Taken together, preliminary virological studies in this and other laboratories, the recent report of Ueda et al. (1978) and the results of this report indicate that Cotia virus does not fit snugly into established poxvirus taxons. The data suggest that Cotia virus has characteristics of both the orthopoxviruses and the leporipoxviruses as well as distinct ones of its own. The virus appears to be more closely related to the leporipoxviruses as suggested by
Fig. 2. Polyacrylamide linear gradient (7 to 20 %) gel electrophoresis profiles of proteins in purified preparations of vaccinia virus, Lister (V); rabbit fibroma virus, Kasza (F); Cotia virus (C); and rabbit myxoma virus, Lausanne (M). Intracytoplasmic virus particles from infected RK-13 cells were purified by velocity sedimentation in sucrose density gradients and heat-dissociated in a tris-SDS buffer that contained 2-mercaptoethanol as described in the text. Proteins in these preparations were separated by electrophoresis in a discontinuous slab gel system run at 12 mA for 16 h. Purified vesicular stomatitis virus, Indiana (Vs) was included as a protein mol. wt. marker. Stained by Coomassie blue.
Fig. 3. Agarose (0.8 %) gel electrophoresis of cleavage fragments in restriction endonuclease digests of isolated virion DNA. Abbreviations: V, vaccinia virus, Lister; C, Cotia virus; F, rabbit fibroma virus, Kasza; M, rabbit myxoma virus, Lausanne. The DNA from virions purified from infected RK-13 cells was extracted as described in the text and subsequently cleaved into fragments with either the restriction endonucleases SalI, HindIII or BamHI. DNA fragments were separated by electrophoresis at 0.7 V/cm for 16 h in a submerged-slab gel (5 × 230 × 220 mm) system that contained 0.7 μg/ml of ethidium bromide in a neutral tris-phosphate-EDTA buffer. Vaccinia virus, Lister HindIII DNA fragments and lambda phage BamHI and HindIII DNA fragments served as size markers for the mol. wt. scale. Ultraviolet light illuminated gels were photographed through a Wratten 23A filter (the photographic tone difference is due to exposure-time variation for better illustration of faint bands).
Table 2. Molecular weight estimates of virion DNA

<table>
<thead>
<tr>
<th>Virus</th>
<th>Restriction endonuclease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HindIII</td>
</tr>
<tr>
<td>Cotia</td>
<td>120 (13)*</td>
</tr>
<tr>
<td>Vaccinia (Lister)</td>
<td>125 (17)</td>
</tr>
<tr>
<td>Fibroma (Kasza)</td>
<td>161 (9)</td>
</tr>
<tr>
<td>Myxoma (Lausanne)</td>
<td>138 (15)</td>
</tr>
</tbody>
</table>

* Mol. wt. x 10^6 calculated by summation of the mol. wt. of the individual restriction endonuclease fragments of each virus DNA resolved by agarose-ethidium bromide gel electrophoresis (Fig. 3). The total number of fragments resolved that includes fragments of apparent molarity > 1 and ≤ 1 are indicated within parentheses.

Our serological data and protein gel results; however, more comparative studies such as a DNA cross-hybridization analysis would be required before a final placement can be assigned.

In a comparison of our electron micrographs on the intracellular morphogenesis of Cotia virus with those reported for the genus Leporipoxvirus, rabbit fibroma virus (Prose et al. 1971) and for other mammalian poxviruses (Bergoin & Dales, 1971; Dales, 1973), we found the striated filaments shown in Fig. 1(b) are quite similar to the ‘long, twisting intracytoplasmic lamellated inclusions’ described for fibroma virus. Cotia viroplasmic matrices resemble type B inclusion bodies common in mammalian poxvirus infected cells. The Cotia–Vero cell inclusion shown in Fig. 1(a) illustrates a unique feature of Cotia virus infected cells; Ueda et al. (1978) have termed this structure a ‘Cotia body’. The beadlike structures in these inclusions appeared much like the ‘zipper-like assemblies’ of polyribosomes described at the periphery of type-A inclusions in cowpox virus-infected HeLa cells (Ichihashi et al. 1971).

We compared the structural proteins of virions representative of two poxvirus genera with Cotia virus by PAGE and found Cotia virus was similar in pattern to the leporipoxviruses. The significance of similar gel protein profiles in taxonomy of the complex poxviruses has not yet been fully established. PAGE has been most useful recently for differentiating orthopoxviruses at the genus-species levels (Arita & Tagaya, 1977; Esposito et al. 1977; Ikuta et al. 1978a, b; Harper et al. 1979). Certainly the Cotia virus PAGE protein profile suggests that this virus more closely resembles the myxoma and fibroma viruses than vaccinia virus; only a few co-migrating protein bands are found when vaccinia and Cotia virus are compared. The significance of defining the extent of virus relatedness by comparing protein electropherograms certainly remains unclear until the procedure is standardized and a more complete spectrum of poxvirus profiles is developed. Comparisons of more leporipoxviruses from different geographic areas also deserve further attention as more strains become available.

An interesting speculation which may be of epidemiological importance may be made from the results of serological and protein studies on Cotia virus. On the basis of our CF data, Cotia virions cross-reacted with myxoma virus. Our earlier ID results and the ID and FA results reported by Ueda et al. (1978) showed that Cotia virus cross-reacts with vaccinia virus. Obviously the different tests have detected different antigens expressed by Cotia virus. The speculation is that Cotia virus (presumed to be vector-borne) might represent a natural orthopoxvirus-leporipoxvirus recombinant; further analyses, of course, would test the credibility of this conjecture.

Virion DNA restriction fragment electropherograms have recently proved useful for identifying orthopoxvirus species and strains (Esposito et al. 1978; Muller et al. 1978). Within the orthopoxvirus group the method seems efficacious for taxonomy to a point where DNA base sequence homologies reach a low degree. Recent restriction endonuclease
Cotia virus

maps for various orthopoxviruses (Wittek et al. 1977; L. Archard, personal communication) have indicated that a central region of the genome is conserved intragenically and that the terminal regions reflect interspecific differences. In contrast, among Molluscum contagiosum isolates (Parr et al. 1977) and the parapoxvirus group (D. Schumperli, personal communication) largely dissimilar DNA patterns were found among members related in other characteristics. When DNA fragment profiles for vaccinia, Cotia, myxoma and fibroma viruses were compared (Fig. 3), no similarities were observed. Myxoma and fibroma viruses are reported to be immunologically cross-protective (Fenner & Ratcliffe, 1965) and in this study we found they have a similar PAGE profile. The DNA of the leporipoxviruses and Cotia virus might have analogous restriction characteristics to the Molluscum isolates and the parapoxviruses. If so, the use of restriction patterns in poxvirus taxonomy may be limited.

We are grateful to Dr Robert E. Shope, Yale University, for his participation in the preliminary investigations of Cotia virus and to Dr Frank Fenner, Dr Ian Marshall, Dr David Regnery and Dr Herbert Wenner for their contribution to this work. We also thank Mrs M. Stapp for her excellent technical help.

REFERENCES


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