The Virus of Molluscum Contagiosum and its Adsorption to Mouse Embryo Cells in Culture

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SUMMARY

Earlier work suggested that the antiviral activity in extracts from lesions of molluscum contagiosum was due to the molluscum virus present. This has now been supported by showing a direct relationship between inhibitory activity and electron microscopically determined virus particle count in extracts from 14 patients. Using this antiviral activity as a biological marker, an analysis of the adsorption of the molluscum virus to mouse embryo cell monolayers was made. In general, the pattern of adsorption of the molluscum agent, with respect to time, temperature and inoculum volume, was similar to that already recorded in the literature for adsorption to cell cultures of the infectivity of vaccinia virus. There was a short delay between adsorption of molluscum virus and the onset of challenge virus plaque inhibition. Thereafter inhibition increased progressively for several hours.

INTRODUCTION

Extracts from lesions of molluscum contagiosum were recently shown to inhibit plaque production by subsequently added heterologous viruses in cultures of mouse embryo cells (Postlethwaite, 1964). The inhibitory agent was thought to be the molluscum virus itself since activity was sedimented by ultracentrifugation, was weakly neutralized by patients' serum and was destroyed at pH 2. Other properties corresponded to those found by Neva (1962) for an agent, present in similar extracts, which was cytopathogenic for, but not serially transmissible in, human and monkey cell cultures. The present paper describes experiments directly relating the inhibitory activity in lesion extracts to the number of electron microscopically demonstrable molluscum virus particles present; and a detailed study of the adsorption of the molluscum virus to mouse embryo cell monolayers.

METHODS

Molluscum contagiosum extracts. Expressed cores from clinically typical lesions were stored for variable periods at \(-20^\circ\) before extraction into distilled water or McIlvaine's buffer, pH 7.3 (0.004 M), by grinding with sterile sand. The clarified supernatant fluids were again stored at \(-20^\circ\). Each extract was prepared from a group of lesions from one patient. Only one suspension was prepared from each patient. The experiments on adsorption were all made with samples from a single suspension prepared by extracting five lesions from one patient into 10 ml. of
McIlvaine’s buffer.

**Challenge virus.** A stock of vaccinia virus in its eighth mouse embryo passage was prepared by treating infected cultures, after the appearance of a widespread cytopathic effect, with a 50 w ultrasonic apparatus (Measuring & Scientific Equipment Ltd.) for 1 min. After a clarifying centrifugation, samples of the supernatant fluid with a titre of $2\times10^8$ plaque forming units (p.f.u.)/ml. were stored at $-70^\circ$.

**Media, solutions and cell cultures.** Eagle’s basal medium (E), tryptose phosphate broth (T) and inactivated (56°C for 30 min.) calf serum (C) were incorporated into growth (ETC/80:10:10) and maintenance (ETC/95:3:2) media with penicillin, streptomycin and mycostatin at final concentrations of 100 units, 100 μg. and 50 μg. per ml. respectively. Overlay medium for plaque assays consisted of 0.9% (w/v) Noble agar (Difco) in ETC/95:3:2, with incorporation of 1/12,000 neutral red in a second overlay added 2 days later. Secondary mouse embryo cultures were prepared by seeding $1.1\times10^8$ cells in 4 ml. of growth medium into 50 mm. glass or plastic (Sterilin Ltd., Richmond, Surrey, England) Petri dishes. Incubation was at 36.5°C in an atmosphere of 5% (v/v) CO₂ in air. Before use in experiments cultures were washed once with 2 ml. of phosphate buffered saline (Dulbecco & Vogt, 1954). In some experiments sheep serum was substituted for calf serum. Results were not appreciably affected.

**Virus particle counts.** The method of Watson (1962) was modified as follows. The reagents used were 1% (w/v) aqueous phosphotungstic acid adjusted to pH 7.2 with N-KOH, a suspension of polystyrene latex (kindly given by the Dow Chemical Corporation, Midland, Michigan, U.S.A.) calculated to contain $1.25\times10^9$ spheres per ml., of diameter 0.188 μ (Bradley, 1961), and a freshly prepared 1% (w/v) aqueous solution of bovine plasma albumin (Armour Pharmaceutical Company, Eastbourne, England). To 0.1 ml. of the latex spheres were added 0.1 ml. of phosphotungstate, 0.03 ml. of bovine plasma albumin and 0.1 ml. of molluscum extract and the whole was thoroughly mixed. After 8 min. a drop of the suspension was added to each of two carbon-coated grids with a Pasteur pipette. Eight min. later excess fluid was removed with filter paper and the grids dried in air. Molluscum particles and polystyrene spheres were then counted, up to a total of 200 spheres for each grid, by scanning the grids in a Siemens Elmiskop I. Molluscum particles per 2.5 ml. were calculated from the estimated concentration of latex spheres. The counts were made using the single condenser system, a 200 μ condenser aperture, a 50 μ objective aperture and an accelerating voltage of 80 kv. An instrumental magnification of $\times20,000$ was used, together with $\times3$ optical lenses attached to the viewing ports. This facilitated the examination of relatively large fields with sufficient clarity to allow unequivocal identification of virus particles and latex spheres.

**Assay of molluscum inhibitor.** Serial fourfold dilutions of molluscum extracts in 2.5 ml. of ETC/80:10:10 were added to almost confluent, washed 2nd-day monolayer cultures of secondary mouse embryo cells. After incubation at 36.5°C for 24 hr fluids were removed and the cultures were washed twice with 2 ml. of phosphate buffered saline. They were then challenged with 0.1 ml. of vaccinia virus, diluted in ETC/95:3:2 to produce about 100 plaques on control cultures. After adsorption for 2 hr agar overlay (5 ml.) was added and cultures were reincubated. Plaques were counted 2 days later, after the addition of 3.2 ml. of neutral red agar. The inhibitory titre was the reciprocal of that dilution of extract which, containing one unit of activity in 2.5 ml., reduced the challenge virus plaque count by 50%. Seven samples
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of a single extract, stored at $-20^\circ$, were all thawed and titrated on a single occasion: titres were within the range 76,500 to 110,000, giving a mean titre of 93,000 and a coefficient of variation of $13.4\%$. The $95\%$ confidence limits for any single assay were therefore $\pm 27\%$.

RESULTS

Correlation of antiviral activity with molluscum virus particle count in lesion extracts

When examined by negative staining (Pl. 1, fig. 1) molluscum virus appeared in two predominant morphological forms (Williams, Almeida & Howatson, 1962) whose relative proportions varied from preparation to preparation. Since, for vaccinia virus, this morphological difference is artifactual (Harris & Westwood, 1964), counts of total particles were made on two samples from each of 14 separate extracts, each sample being spread on two grids. All 14 extracts were assayed on the same batch of cultures and, after storage at $-20^\circ$, again on another batch of cells. Particle concentration was plotted against inhibitory activity and the straight line indicating a direct linear relationship between the two variables was fitted by inspection (Fig. 1). According to Spearman’s Rank Order, correlation was $0.925 (P < 0.01)$ supporting the view that inhibitory activity in lesion extracts was a property of the molluscum virus they contained. The inhibitory activity will therefore be referred to as molluscum virus.

Fig. 1. Relationship between concentration of virus particles and antiviral activity in molluscum extracts. Each point represents mean data for one extract. Vertical and horizontal lines indicate the variation encountered in the two measurements made for each variable. The straight line drawn through the plotted points depicts the unit slope theoretically expected for a direct linear correlation between the two measured variables.
Adsorption of molluscum virus to mouse embryo cell monolayers

Preliminary experiments

To study the development of inhibitory activity under conditions of the standard assay procedure 2.5 ml. volumes of a dilution of molluscum extract or of diluent only (ETC/80:10:10) were added to pairs of cultures at intervals from 24 to 2 hr before challenge. Graphs a and b refer to cultures exposed to 2.5 ml. of M16/8000 for varying periods before challenge. Graphs c and d refer to an experiment in which, after adsorption for 2 hr of 0.2 ml. of M16/1000, cultures were washed and reincubated with 2.5 ml. of medium to a total of the number of hours indicated before challenge (the 2 hr point was challenged immediately after adsorption, the 0 hr point had no contact with molluscum virus). Control cultures for each point received medium only. Graphs a and c show challenge (vaccinia) virus plaque counts on control (O - - O) and treated (● - - ●) cultures. Graphs b and d represent plaque counts on treated cultures as percentages of control counts.

Table 1. The influence of inoculum volume on adsorption of molluscum virus during 2 hr at 36.5°

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Inoculum volume (ml.)</th>
<th>Effective adsorption (reciprocal of 50% inhibitory titre with s.e.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5</td>
<td>7,790 (± 1630)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>4,530 (± 1177)</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>24,500 (± 1490)</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>10,760 (± 788)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>7,970 (± 991)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>8,440 (± 649)</td>
</tr>
</tbody>
</table>

After adsorption cultures were washed and incubated with fresh medium for 22 hr before challenge.
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washing and challenge with vaccinia virus. The dilution of extract chosen was calculated to reduce challenge virus plaque count by 90 to 95%. Whilst cultures treated for 12 hr showed little inhibition of plaque count, resistance to challenge virus increased markedly thereafter, reaching its maximum in 18 hr (Fig. 2a, b). To see whether this acquired resistance was accompanied by a loss of molluscum virus from that initially added, the sequentially removed inocula were added for 24 hr to fresh cultures, which were then challenged with vaccinia virus. In all, plaque numbers were reduced by 95 to 99% relative to controls, as they were following exposure of monolayers for 24 hr to the initial inoculum. This apparent failure to detect loss of molluscum virus from the inoculum, despite manifest gain of resistance by cells, may result from an excess of virus in the inoculum, inefficient adsorption of virus from a large volume, elution of adsorbed virus, or the cellular production of a second inhibitor which balanced the loss of virus from the initial inoculum. Experiments concerned with adsorption will be described in the sections which follow. Other aspects will be treated separately.

The effect on induced cellular resistance of varying the volume of molluscum inoculum

In two experiments triplicate cultures were exposed for 2 hr at 36.5° to different volumes of serial fourfold dilutions of molluscum virus. After adsorption the inocula were removed and the cultures were washed twice and incubated for 22 hr at 36.5° with 2·5 ml. of fresh medium before challenge with vaccinia virus. Detailed analysis was not attempted but the proportionate activity taken up during 2 hr was clearly greater from the smaller volumes (Table I).

Delay in the manifestation of induced resistance after adsorption

The time course shown in Fig. 2a, b for the development of resistance represents the kinetics of adsorption, under these conditions, as modified by any subsequent period required for the resistance to become manifest. To delineate the latter more clearly, a constant short period of adsorption of a uniform dose of molluscum extract was followed by challenge after different periods of subsequent incubation. Monolayers were exposed, at intervals from 24 to 2 hr before challenge, to 0·2 ml. of a suitable dilution of extract or of diluent. After adsorption for 2 hr the residual inoculum was removed and the cultures were washed twice and reincubated with 2·5 ml. of ETC/80:10:10 before simultaneous challenge of all cultures with vaccinia virus. Inhibition of challenge virus was not detected until about 4 hr after exposure to molluscum virus (Fig. 2c, d). Thereafter inhibition increased, reaching a maximum by 24 hr. It should therefore be possible to determine the adsorption kinetics of this agent by challenging 24 hr after different adsorption periods as defined above.

Adsorption kinetics by direct challenge of monolayers following exposure to small volumes of inhibitor

Mouse embryo monolayers were washed and exposed to 0·2 ml. of ETC/80:10:10 or of graded doses of a molluscum extract treated by ultrasonic vibration. Adsorption was continued at 36·5° for 4 hr, cultures being rocked ½-hourly to ensure even distribution of the inoculum. At intervals the residual inoculum was removed from pairs of cultures in each dose series and pooled, and the cultures were washed twice and reincubated at 36·5° with 2·5 ml. of ETC/80:10:10. Cultures were challenged with
vaccinia virus after 24 hr. The removed molluscum inocula were stored at -20° and subsequently added to fresh assay plates to determine the residual inhibitory activity. In a similar experiment adsorption was at 4° to 6° for 6 hr. In this experiment, after sequential removal of the inocula the cultures were washed, received 0.2 ml. of ETC/80:10:10 and were then returned to 4° until adsorption was completed in the 6 hr group. They were then all made up to 2.5 ml. with ETC/80:10:10 and incubated for 24 hr at 36.5° before challenge.

The degree and rate of plaque reduction in treated cultures were related to the concentration of molluscum virus applied (Fig. 3). Adsorption was more rapid at the higher temperature, having virtually ceased after 1 or 2 hr at 36.5° but only after 4 to 6 hr at 4°. The increase in resistance to challenge virus with increasing concentrations of molluscum virus showed that, within this dosage range, saturation of cells with the latter could not explain the ceiling of inhibitory activity ultimately reached. Nor was the effect due to uptake of all available molluscum virus. The unadsorbed inocula, removed at each interval during the adsorption sequence, were added to further monolayers for 24 hr before challenge. In each instance the degree of plaque reduction was similar to that caused by the original inoculum. Since similar results

Fig. 3. Adsorption kinetics by direct challenge of molluscum-treated cultures. Cultures were exposed, at either 36.5° or 4 to 6°, to 0.2 ml. of dilutions of M16 extract (reciprocals of dilutions indicated in graphs). At the intervals noted, cultures were washed and incubated at 36.5° for 24 hr before challenge with vaccinia virus. In the 4° experiment, the residual unadsorbed inocula, pooled from four cultures for each point, were added to fresh cultures in 0.2 ml. amounts. After 2 hr these cultures were made up to 2.5 ml. with medium and challenged after 24 hr at 36.5°. The upper and lower interrupted lines on the graph for the 4° experiment relate to the depressed plaque counts obtained by assaying the unadsorbed inocula in this way.

were obtained at 4° (Fig. 3) it is unlikely that, during adsorption, loss of molluscum virus from the inoculum was masked by the release of some cellular product with antiviral activity. A stage of reversible adsorption was also unlikely since no significant elution of cell-bound virus was shown during incubation at 36.5°.

It seemed more likely that even when adsorption had effectively ceased (Fig. 3) the techniques used could not discriminate quantitatively between the residual unadsorbed virus and that in the original inoculum. A molluscum dose-response curve
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plotted from the data of Fig. 3, showed that a twofold difference in molluscum virus concentration never affected challenge virus plaque count by more than 16%, and by progressively less than this at the extremes of the sigmoid curve (Fig. 4). Clearly, much molluscum virus may be taken up during adsorption without the unadsorbed residue differing detectably in amount from that in the inoculum. Further adsorption experiments were therefore performed in which the samples for each interval studied were assayed by the standard 50% inhibitory end-point technique. By thus measuring virus concentration it was possible to relate quantitatively the virus in the inoculum with the residual unadsorbed virus and with that bound to cells. The latter was released for titration by treating monolayers with ultrasonic vibrations.

![Graph of Dose-response curve of M16 extract](image)

**Fig. 4.** Dose–response curve of M16 extract obtained from the experiment recorded in Fig. 3. The points plotted represent the data relating to the 2 hour adsorption period at 36.5°C for the several dilutions of extract used.

**Adsorption kinetics by titration of residual unadsorbed virus and of cell-bound virus released by ultrasonic treatment**

The recovery of inhibitory activity, by ultrasonic vibration, from cultures to which molluscum virus had been adsorbed was studied as follows. Chilled washed monolayers were exposed to 0.15 ml. of a suitable molluscum dilution at 4 to 6°C with rocking of the cultures ½-hourly. It was assumed that no loss or gain of total activity would occur at this temperature. Two hours later the unadsorbed inoculum was removed and stored at −20°C, after pooling with 3 washing fluids of ET/80:10 in a total volume of 3.6 ml. The washed monolayers received 2.5 ml. of ET/80:10 and were also stored at −20°C. The frozen cultures were thawed later and the cell debris, dislodged by pipetting, was pooled. From this pool samples were distributed to glass containers and each was treated by ultrasonic vibration for a different period. A portion was then assayed directly. Other samples were centrifuged at 1200 g for 7 min. The supernatant
fluids and the deposits, resuspended in medium by ultrasonic treatment for 60 sec., were also assayed. In calculating absolute amounts of virus the inhibitory titres of the inocula, washings and disrupted cellular materials were converted to units, a unit being defined as the amount of activity in 2.5 ml. at the 50% inhibitory end-point dilution. In three experiments (Table 2) the inhibitory activity recovered after a single cycle of freezing and thawing was not enhanced by ultrasonic treatment. However the proportion of activity which sedimented during centrifugation appeared to diminish and a grossly particulate suspension became visibly homogeneous during ultrasonic treatment for up to 60 sec. Even after treatment for 120 to 180 sec. 15 to 25% of the previously cell-bound inhibitory activity sedimented under these conditions.

Table 2. Recovery of adsorbed molluscum virus by ultrasonic treatment

<table>
<thead>
<tr>
<th>Duration of ultrasonic treatment (sec.)</th>
<th>No. of experiments contributing to mean values indicated</th>
<th>%* adsorbed activity recovered in:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Supernatant fluid after slow centrifugation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total volumes treated (a)</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>132*†</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>91</td>
</tr>
<tr>
<td>40</td>
<td>1</td>
<td>58</td>
</tr>
<tr>
<td>60</td>
<td>3</td>
<td>65</td>
</tr>
<tr>
<td>90</td>
<td>1</td>
<td>74</td>
</tr>
<tr>
<td>120</td>
<td>2</td>
<td>76</td>
</tr>
<tr>
<td>180</td>
<td>1</td>
<td>53</td>
</tr>
<tr>
<td>Controls‡</td>
<td>2</td>
<td>&lt; 30†</td>
</tr>
</tbody>
</table>

Mouse embryo cell monolayers with adsorbed molluscum virus and 2.4 ml. of ET/80:10 were frozen at -20°. After thawing, samples of the pooled culture materials were subjected to ultrasonic vibration for different periods. Samples of the total volumes treated and of the centrifuged fractions indicated were assayed for inhibitory activity. The results of three experiments were combined, mean values being calculated when identical periods of ultrasonic treatment were used in different experiments.

* 100% is the adsorbed activity as calculated by the difference between that in the inoculum and that in the unadsorbed residue and washing fluids after adsorption.
† One experiment only.
‡ Cultures inoculated with diluent only.

Adsorption kinetics were studied in four experiments, with addition of 0.1 or 0.2 ml. of a molluscum extract, or of medium, to chilled monolayers at 4 to 6°. At intervals medium was added to pairs of cultures and the diluted unadsorbed material was removed and pooled. After one or two further rinses, the re-fed cultures and all washings were stored at -20°. Later they were assayed for inhibitory activity, the thawed cultures being first treated by ultrasonic vibration, usually for 1 min. In all experiments inhibitory activity was lost from the added inoculum as adsorption proceeded (Fig. 5). Concurrently, increasing amounts of activity were recovered from the disrupted monolayers. After 2 hr, when adsorption had slowed considerably, nearly 40% of the input activity was still recovered free from cells. Because of the quantitative variations between experiments the influence of cultural conditions was studied. Results were not systematically affected by the use of glass or plastic Petri dishes, 2- or 3-day-old cultures, calf or sheep serum in the diluents, molluscum inocula
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Differing in activity from 11.4 to 660 units per culture, or by ultrasonic treatment of the inocula. However, three features which may all relate to the nature and firmness of the union of virus and cell were noted. In Expts 1 and 2 mean values for activity recovered in a second wash after adsorption were 10.8 and 7.3\% respectively of that first rinsed from the monolayers. Whilst this may have been free virus inadequately removed during the first rinse, the possibility of an unstable bond between virus and cells at 4° could not be excluded. However, when materials from these experiments were assayed again, after further storage at -20°, titres of the initially cell-bound activity were, on average, 2.2 and 1.8 times greater for experiments 1 and 2 respectively, whilst values for the unadsorbed materials were only 1.2 and 0.9 times the initially determined titres. Although these materials were treated by ultrasonic vibration before assay, it seems likely that firm binding of virus to cell fragments may account for some of the inconsistencies. The sedimentation of much activity during slow centrifugation of disrupted cell materials (Table 2 and Fig. 5, Expt 4) supports this view. No inhibitory activity was ever detected in the medium of control cultures but up to about 2 units were regularly found associated with the cells.

**DISCUSSION**

These experiments support the earlier conclusions (Postlethwaite, 1964) that the agent in molluscum extracts responsible for initiating antiviral activity in mouse embryo cultures is the molluscum virus itself. Preliminary studies have not shown for molluscum virus the variable effects on particle morphology noted for vaccinia virus by Harris & Westwood (1964). It is still possible therefore that different morphological types may...
contribute unequally to the overall activity. But if all are equivalent in interfering capacity, then $1.77 \times 10^6$ particles in 2.5 ml. constitute one unit of 50% inhibitory activity in a culture containing, at the time of challenge, approximately 1.5 to $2.0 \times 10^6$ cells. Particle counting was more reproducible (Fig. 1) and quicker than the biological assay though the latter was the more sensitive technique. Whilst many grid fields had to be scanned to detect a few virus particles at a concentration of $10^8$ per 2.5 ml., such a preparation had a 50% inhibitory titre of 1/57.

The rate of attachment of molluscum virus to cells and the influence of inoculum volume and temperature were generally similar to findings with vaccinia virus (Youngner, 1956; Allison & Valentine, 1960; Postlethwaite, 1960). The quantitative discrepancies noted (Fig. 5) could not be fully explained; but varying sensitivity of the biological assay and the presence of weak inhibitory activity in extracts of control cultures may have been significant. If so, then repetition of the experiments by the more reproducible though less sensitive particle counting technique may eliminate the inconsistencies.

The effect of temperature in the experiments recorded in Fig. 3 may have been complex. After adsorption the cultures were incubated at 36.5°C before challenge. The initial period at 4°C or 36.5°C may therefore have differentially affected not only adsorption of molluscum virus but also any subsequent stages which it may have undergone. Though no direct evidence was obtained for any stage beyond that of adsorption, the marked rate differences noted support this view since adsorption itself should depend only on the absolute temperature. By limiting adsorption to a period of 2 hr at 36.5°C, a "post-adsorption delay" of approximately 4 to 6 hr occurred before resistance to challenge virus became manifest (Fig. 2). If, at 36.5°C, penetration occurred rapidly as with vaccinia virus (Postlethwaite, 1960), then this delay would mainly have represented a period between penetration and the development of effective interference. Afterwards resistance increased progressively for several hr. Growth studies of challenge virus in molluscum-inhibited cultures (to be reported) revealed a similar post-adsorption delay, with subsequent progressive effect, in the appearance of viral inhibition. These findings suggest a degree of asynchrony in the development of resistance which warrants further investigation.

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REFERENCES

Adsorption of molluscum contagiosum virus


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EXPLANATION OF PLATE

Electron micrograph of virus particles from a molluscum extract. The single particles in the upper half show details of the two major morphological types commonly present. In the lower half, particles are seen together with polystyrene latex spheres of diameter 1260 Å.