A Reappraisal of the Effect of Actinomycin D and Cordycepin on the Multiplication of Cowpea Mosaic Virus in Cowpea Protoplasts

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SUMMARY

Actinomycin D (AD) administered to cowpea mosaic virus-infected cowpea protoplasts immediately after inoculation inhibited virus multiplication, whereas late in the incubation period neither virus multiplication nor host DNA transcription were affected. The data obtained suggested that neither virus uncoating nor encapsidation were inhibited by AD. The inhibition of virus multiplication was manifested as a decrease in the level of progeny viral nucleoproteins and (+) and (−) viral RNAs. Cordycepin strongly inhibited coat protein production and (+) and (−) viral RNA synthesis throughout the incubation period.

INTRODUCTION

Actinomycin D (AD) inhibits DNA-directed RNA synthesis and it has been used to show that the replication of several RNA viruses depends upon continuing host gene expression (Alblas & Bol, 1977; Morris-Krsinich et al., 1979; Motoyoshi & Hull, 1974; Otsuki et al., 1974). Previously, it was reported (Rottier et al., 1979) that during the early stages of infection of cowpea protoplasts, the multiplication of cowpea mosaic virus (CPMV) was sensitive to AD. It was noted, however, that inhibition had little effect upon the accumulation of virus-specific proteins despite there being an absence of progeny nucleoprotein particles. Apparently, all the virus coat protein was in the form of empty capsids and none of the translated RNA was encapsidated.

Recently, it was suggested that the uncoating of the virus particles and/or an event occurring within a short time after uncoating, could be the AD-sensitive step of virus multiplication (Mayo & Barker, 1983). AD did not decrease the proportion of tobacco protoplasts which became infected upon inoculation with tobacco mosaic virus (TMV) or tobacco rattle virus (TRV) RNAs, whereas it was inhibitory when virions were used in the inoculation. Studies carried out with alfalfa mosaic virus-infected cowpea protoplasts (Nassuth et al., 1983) and animal cells infected with Sindbis virus (Baric et al., 1983) indicate that the synthesis of viral (+) and (−) RNAs can be affected by AD. In both cases the sensitive step appeared to be during or before (−) RNA synthesis.

Cordycepin (3′-deoxyadenosine) is another inhibitor of RNA synthesis (Beach & Ross, 1978). A similarity has been noted (Mayo & Barker, 1983) between cordycepin and AD in that the inhibition of virus multiplication was less, in both cases, if there was a delay between inoculation and the addition of the inhibitors.

For the work presented here it was proposed to investigate further and to compare the effects of AD and cordycepin on virus multiplication, paying particular attention to the inhibition of host metabolism over the course of the infection.

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METHODS

Virus and protoplasts. CPMV, Nigerian isolate, was purified from infected cowpea plants [Vigna unguiculata (L.) Walp. cv. Blackeye Early Ramshorn] as described by van Kammen & de Jager (1978). The CPMV components were isolated by three successive equilibrium gradient centrifugations in 40% (w/w) CsCl in 0.02 M-potassium phosphate buffer pH 8.5, in a Beckman Ti50 rotor for 18 h at 140000 g. Fractions were collected through the side of the tubes with a syringe needle.

Cowpea mesophyll protoplasts were isolated as described by Hibi et al. (1975), except that 10mM-CaCl₂ was included in the enzyme digestion mixture. After three washes in 0-6 M-mannitol, isolated protoplasts were inoculated with CPMV, or isolated CPMV B-component or isolated viral RNA, using 30% polyethylene glycol (PEG) as uptake inducer (Maule, 1983) with 20 μg of virus or 100 μg of viral RNA per 10⁶ protoplasts. After the addition of PEG, inoculated protoplasts were incubated for 5 min at room temperature when using virus or 10 min on ice when using viral RNA. Protoplasts were washed three times with 0.6 M-mannitol, 10 mM-CaCl₂, and incubated at a concentration of 5 × 10⁵ protoplasts/ml in a simple salts medium (Rottier et al., 1979) at 25 °C with continuous illumination. AD (Sigma) at 5 to 25 μg/ml or cordycepin (Sigma) at 0.5 to 100 μg/ml was added at the beginning of the incubation period unless stated otherwise. Controls without inhibitors were always included. The percentage of infected protoplasts was estimated by indirect fluorescent antibody staining (Maule et al., 1980). Viability was assessed using phenosafranine (Widholm, 1972).

Incorporation of radioactive precursors. To assess the effect of AD and cordycepin on host RNA synthesis, healthy protoplasts were labelled with 1 μCi/ml of [5,6-³H]uridine (Amersham) and its incorporation into RNA was measured after precipitation from 10% trichloroacetic acid (TCA).

Measurement of virus nucleoproteins. Healthy and infected protoplasts, labelled with 10 μCi/ml [³H]uridine, were collected by centrifugation, homogenized with 400 μl/10⁵ protoplasts of 10 mM-potassium phosphate pH 7.0 and aliquots corresponding to 2.5 × 10⁵ protoplasts were layered on 15 to 40% (w/v) sucrose gradients. Gradients were centrifuged for 3 h at 160000 g in a SW40 rotor. Fractions were collected and their radioactivity was measured by scintillation counting. Purified virus was centrifuged in the same conditions and the positions of M- and B-components were estimated from the A₂₆₀ of the fractions. The sum of the radioactivities of peak fractions was calculated and AD inhibition was expressed as a percentage of the untreated controls.

Measurement of viral RNA. RNA was extracted from pelleted protoplasts (5 × 10⁵ to 7.5 × 10⁵) with 400 μl 5% Sarkosyl (Sigma) in 40 mM-Tris-Cl, 80 mM-NaCl and 4 mM-EDTA, pH 8.5, extracted with 450 μl phenol-chloroform (8:1) and precipitated from 70% ethanol. In the spot hybridization assay, RNA extracted from unlabelled protoplasts was spotted onto nitrocellulose filters (Thomas, 1980) and probed with ³²P-cDNA. Alternatively, total RNA was denatured with formamide in the presence of glyoxal (Covey et al., 1983) and electrophoresed on 1% agarose gels at 5 V/cm for 5 h in 10 mM-sodium phosphate, 1 mM-EDTA, pH 7.0. After electrophoresis, the gels were blotted onto nitrocellulose filters (Thomas, 1980) and probed with ³²P-cDNA or ³²P-CPMV RNA. Hybridizations were carried out at 65 °C in 3 × SSC (SSC is 0.15 M-NaCl, 15 mM-trisodium citrate, pH 7.2). After washing with 2 × SSC, 0.1% SDS and 0.1 × SSC, 0.1% SDS at 65 °C, filters were autoradiographed at -70 °C with intensifying screens. For quantification, the autoradiographs were scanned with a Beckman DU-8B spectrophotometer and the relative peak areas were estimated. Alternatively, if the spot hybridization assay was used, radioactive areas were cut from the nitrocellulose filters and assayed by scintillation counting or the autoradiographs were scanned as described.

Preparation of radioactive probes. Complementary DNA was prepared to CPMV RNA by random priming with calf thymus DNA (Taylor et al., 1976). ³²P-CPMV RNA was prepared by polynucleotide kinase labelling of fragmented CPMV RNA (Maxam & Gilbert, 1980).

RESULTS

Effect of AD on viral RNA synthesis

Viral RNA synthesis decreased when AD (5 to 25 μg/ml) was present within 1 h after inoculation. The synthesis of both species of genomic (+) RNA was inhibited (Fig. 1): B-RNA synthesis was slightly, but consistently, more inhibited than M-RNA. This difference in inhibition varied from experiment to experiment, but was in the range of 10 to 30% more inhibition for B-RNA compared to M-RNA. When protoplasts were inoculated with isolated B-component, the replication of B-RNA was also inhibited. Similar observations were made for virus (−) RNA synthesis. Synthesis of both species of (−) RNA was inhibited by AD after inoculation with virus, as was that of (−) B-RNA after inoculation with isolated B-component (Fig. 1).
Inhibition of CPMV in cowpea protoplasts

Fig. 1. Effect of AD on the production of CPMV RNAs in cowpea protoplasts. Northern blots of CPMV RNAs from mock-inoculated (H) or infected (I) protoplasts 48 h after inoculation with complete virus (a and b), or B-component alone (c and d). Gel tracks contained RNA from 2.5 × 10^6 (a and c) or 5.0 × 10^6 (b and d) protoplasts. Blots (a) and (c) were hybridized with 32P-cDNA, to probe for (+) sense viral RNA. Blots (b) and (d) were hybridized with 5' end-labelled 32P-viral RNA to probe for (−) sense viral RNA. Protoplasts were incubated in the presence of the indicated concentrations (μg/ml) of AD.

Fig. 2. Effect of AD on uncoating of CPMV in vivo. Spot hybridization for CPMV (+) RNA in protoplasts inoculated with CPMV virus particles (a), CPMV RNA (b) or mock-inoculated (c). For (a) each spot is equivalent to 1-25 × 10^5 protoplasts; for (b) and (c) each spot is equivalent to 2-5 × 10^5 protoplasts. Protoplasts were incubated for 60 h in the presence of the indicated concentrations (μg/ml) of AD.

Effect of AD on virus uncoating

Total (+) RNA synthesis was inhibited to similar extents by AD when the same batch of protoplasts was inoculated with either virus or viral RNA (Fig. 2). It seems that avoiding virus uncoating by inoculation with viral RNA does not prevent RNA synthesis being sensitive to AD.

Effect of AD on viral coat protein synthesis and encapsidation of viral RNA

Fluorescent antibody staining supported the observation made previously (Rottier et al., 1979) that viral coat protein synthesis is less sensitive than virus replication to AD. In fact, in no experiment was there more than a 30% reduction in the number of protoplasts showing fluorescence as compared with untreated controls, for AD concentrations as high as 25 μg/ml. In higher AD concentrations the protoplasts showed reduced viability.
As with previous work (Rottier et al., 1979), AD administered soon after inoculation caused a drastic reduction in the amount of progeny nucleoprotein particles synthesized. However, it was clear that a complete inhibition of virus synthesis did not occur. Labelled material that sedimented in sucrose gradients to the same positions as M- and B-components, was always present in the homogenates of CPMV-inoculated protoplasts, 48 or 55 h after inoculation. No such material was found in healthy protoplasts (Fig. 3). There was a larger decrease in the amount of B-component compared to M-component. If it is assumed that the pool size of intracellular precursors is not changed by AD treatment, then the values corresponding to the experiment in Fig. 3 show that B-component was reduced to 15 and 10% of untreated control, with 10 and 25 μg/ml AD, respectively. M-component was reduced to 22 and 18% of the untreated control, in the same experiment. Overall reduction of progeny CPMV particles was to 19 and 14% of the untreated control. Spot hybridization showed the inhibition of total viral RNA to be similar. Total incorporation of [3H]uridine into TCA-insoluble material was reduced to 15 and 5% of the untreated control with 10 and 25 μg/ml AD, respectively. The greater overall level of inhibition could be attributed to a greater specific inhibition of ribosomal RNA over viral components, although in these experiments virus infection itself stimulated the synthesis of ribosomes (Fig. 3).

**Effect of AD added late in the incubation period**

As has been previously reported (Rottier et al., 1979), addition of AD to infected protoplasts 12 to 16 h after inoculation failed to inhibit CPMV multiplication. However, a series of control experiments where we examined the inhibition of incorporation of [3H]uridine into TCA-insoluble material showed that host RNA synthesis was not affected by AD when this inhibitor was added 16 h after protoplast isolation (Fig. 4). The same result was obtained with infected protoplasts (not shown). Incubation of protoplasts in a medium containing 1% cellulase did not alter this result. In other experiments, a decrease of host sensitivity to AD was observed as early as 6 h after protoplast isolation.
Inhibition of CPMV in cowpea protoplasts

Fig. 4. Effect of AD on host transcription. Incorporation of [3H]uridine into healthy protoplasts incubated without AD (●), or with 5 μg/ml AD added immediately (□), 5 h (△) or 16 h (▲) after isolation. Protoplasts were labelled with 1 μCi/ml of [3H]uridine 16 h after isolation, and incorporation into TCA-insoluble material by 5 × 10⁴ protoplasts was measured at subsequent times.

Effect of cordycepin on viral coat protein synthesis

The proportion of protoplasts that showed fluorescence after 48 h of incubation decreased with increasing levels of cordycepin. Protoplasts infected with CPMV and incubated with 10 μg/ml of cordycepin still showed about 3% infection compared to around 90% for the untreated control. No protoplasts showed fluorescence after incubation with 50 μg/ml of cordycepin.

No reduction in protoplast viability was observed after incubation in these concentrations of cordycepin, although with 50 μg/ml the incorporation of [3H]uridine into TCA-insoluble material was completely blocked during the first 18 h of incubation.

Effect of cordycepin on viral RNA synthesis

Cordycepin concentrations greater than 1 μg/ml were highly inhibitory for viral RNA synthesis though at lower concentrations viral RNA synthesis sometimes seemed to be stimulated. Complete inhibition of RNA synthesis was achieved with 50 μg/ml of cordycepin. Both M- and B-RNA were equally inhibited by cordycepin. Synthesis of (−) RNA appeared to be less sensitive than (+) RNA to inhibition by low concentrations of cordycepin. The RNA synthesized in the presence of cordycepin appeared to be of genomic size (Fig. 5).

Effect of cordycepin added late in the incubation period

The addition of 50 μg/ml of cordycepin 18 h after inoculation, when the synthesizing apparatus is established and progeny viral RNA is already present, immediately stopped viral RNA replication (Fig. 6).

DISCUSSION

AD administered to cowpea mosaic virus-infected protoplasts immediately after inoculation inhibits virus multiplication. There is indirect evidence that uncoating of the CPMV virions is not specially sensitive to AD, since inoculation with isolated viral RNA did not protect the virus multiplication against AD. This result is different from the results reported for TMV and TRV (Mayo & Barker, 1983) but similar to the situation reported for Sindbis virus (Baric et al., 1983).

The results presented suggest that encapsidation of viral RNA is also not inhibited by AD. The proportion of total viral RNA (estimated by spot hybridization) to encapsidated viral RNA (components separated on sucrose gradients) is similar whether or not AD is present. The fact that we were always able to detect viral nucleoproteins in CPMV-infected protoplasts in the
presence of AD, is not in agreement with the results reported by Rottier et al. (1979). This difference may be explained by the higher level of label incorporation in our experiments leading to a higher sensitivity of the assay.

The synthesis of (+) and (−) RNAs was affected by AD, although coat protein synthesis was less inhibited. Low levels of inhibition of coat protein synthesis and of other virus-specific proteins in the presence of AD (Rottier et al., 1979), may be explained by the fact that AD seems to stabilize mRNAs (Cereghini et al., 1979; Kessler-Icekson et al., 1978), possibly through an association of mRNAs with a host protein (Dreyfuss et al., 1984). A lower level of viral RNA

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Fig. 5. Effect of cordycepin on the production of CPMV RNAs in cowpea protoplasts. Northern blots of CPMV RNAs from mock-inoculated (H) or infected (I) protoplasts 60 h after inoculation with CPMV. Protoplasts were incubated in the presence of indicated concentrations (µg/ml) of cordycepin. (a) RNA from 2.5 x 10⁵ protoplasts hybridized with ³²P-cDNA to viral RNA to detect (+) sense RNA. (b) RNA from 5 x 10⁵ protoplasts hybridized with viral 5' end-labelled ³²P-RNA to detect (−) sense RNA.

Fig. 6. Effect of cordycepin, added late in infection, on the production of CPMV RNAs. Protoplasts inoculated with CPMV were incubated without cordycepin (●) or with 50 µg/ml cordycepin (○). The RNA extracted from protoplasts was used in a spot hybridization assay with RNA from 2.5 x 10⁵ protoplasts/spot and the blot was probed with ³²P-cDNA. The radioactive squares were cut and scintillation counted.
would in this case be balanced by a higher stability, leading to a higher ratio of protein to mRNA. Alternatively, a separate pool of translated viral RNA may be maintained despite considerably reduced RNA synthesis.

It is generally accepted that sensitivity to AD is indicative of the involvement of host DNA-dependent RNA synthesis in virus multiplication (Alblas & Bol, 1977; Nassuth et al., 1983; Rottier et al., 1979). The RNA-dependent RNA polymerase involved in CPMV replication seems to be virus-coded (van der Meer et al., 1984), and should not be affected by AD. Since CPMV has been shown to have many features in common with picornaviruses (Franssen et al., 1984), it may be that multiplication of CPMV depends on the presence of a host factor, as was shown for poliovirus (Dasgupta et al., 1980) and encephalomyocarditis virus (Dimitrieva et al., 1979). If so, then AD would block the transcription of the host factor mRNA. The host factor in poliovirus multiplication seems to be involved in priming (−) RNA synthesis from (+) RNA templates (Dasgupta, 1983). If a similar situation exists with CPMV multiplication, AD would be expected to inhibit virus multiplication for as long as (−) RNA synthesis is going on (unless the host factor was very stable). We have observed that in our protoplast system (−) B-RNA is still increasing as late as 96 h post-inoculation (A. de Varennes & A. J. Maule, unpublished results).

AD does not inhibit CPMV multiplication if added late in infection, and the same situation has been reported to occur with other viruses (Nassuth et al., 1983; Otsuki et al., 1974). We have shown, however, that at least in the case of CPMV multiplication in cowpea protoplasts, no conclusions can be drawn from experiments where AD is added late in infection. In fact, host transcription ceases to be sensitive to the presence of AD in the incubation medium, sometimes as early as 6 h after isolation of protoplasts. The possibility that the cell wall presents a barrier to the uptake of AD can be discounted, as AD remains ineffective when protoplasts are incubated with 1% cellulase, even though the protoplasts are still susceptible to virus inoculation (A. de Varennes & A. J. Maule, unpublished results). Further experiments, designed to measure the uptake of AD into protoplasts after different periods in culture, would indicate whether protoplasts develop a specific permeability barrier to AD.

The reason why B-RNA replication is more inhibited than that of M-RNA is at present unknown. Differential inhibition of viral RNA species has been reported for alfalfa mosaic virus as well (Nassuth et al., 1983). This may reflect a competition for the low amount of host factor available in the presence of AD.

The results presented in this paper showed that cordycepin inhibits CPMV multiplication and can shut off completely the expression of the virus. Triphosphorylated cordycepin (3′dATP) has been reported to inhibit RNA synthesis in vitro by plant virus replicases (White & Dawson, 1979) and picornavirus polymerase complexes (Panicali & Nair, 1978). It was reported previously that cordycepin is quickly phosphorylated to 3′dATP in animal cells (Maale et al., 1975), and it is possible that in plant cells a similar reaction may occur. Cordycepin or cordycepin triphosphate probably affects CPMV multiplication directly by inhibition of the virus-coded RNA-dependent RNA polymerase. If the inhibition is caused by premature termination of RNA synthesis, it is interesting to note that full-size RNA, rather than heterogeneous lengths, are found in infected protoplasts incubated in the presence of low concentrations of cordycepin. The same situation was reported for poliovirus (Nair & Panicali, 1976). It is possible that less than full-length single-stranded RNA will not be encapsidated and will degrade in vivo. Unencapsidated CPMV RNAs have been shown to be unstable in vivo (A. de Varennes & A. J. Maule, unpublished data). (−) RNAs formed in the presence of cordycepin are full-length as well. This is probably due to the fact that double-stranded replicative forms and replicative intermediates will be protected from degradation in vivo, but incomplete (−) RNA will not be functionally active. The reason why (−) RNA seems to be more resistant to cordycepin is not known.

Cordycepin will at the same time inhibit host transcription or, at lower concentrations, possibly only polyadenylation (Kuznetov et al., 1983). It is probably impossible to distinguish between a direct effect on virus multiplication and indirect effects due to an inhibition of mRNA synthesis for a hypothetical host factor, and consequently cordycepin may not be useful
for the investigation of the role of host gene expression in CPMV multiplication. Cowpea protoplasts are permeable to cordycepin until at least 18 h after inoculation, whereas they seem not to be to AD. Whether this is related to a selective barrier to AD at the cell surface is at present not known.

A. de Varennes holds a studentship from the National Institute of Scientific Research, Portugal. J. Shaw was on sabbatical leave from the University of Kentucky.

REFERENCES


Inhibition of CPMV in cowpea protoplasts


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