DISTINCTIVE EFFECTS OF INHIBITORS OF MITOCHONDRIAL FUNCTION ON ROUS SARCOMA VIRUS REPLICATION AND MALIGNANT TRANSFORMATION

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SUMMARY: The effects of chloramphenicol and ethidium bromide on Rous sarcoma virus replication and transformation were studied. Chloramphenicol suppressed virus production with little effect on focus formation. Ethidium bromide inhibited focus formation up to 95% when cells were treated 24 hours prior to, or within the first 48 hours after infection, but failed to suppress virus replication significantly. These results suggest that virus replication and cell conversion require a function(s) having the antibiotic sensitivity resembling that of mitochondria.

Previous studies in this laboratory(1) demonstrated that Rifampicin (Rif) inhibits both malignant transformation and virus production in chick embryo fibroblast (CEF) cultures infected with the Bryan strain of Rous sarcoma virus (RSV). The susceptibility of infected cells to inhibition of focus formation by Rif was limited to the 24 hour period from day 1 to day 2 after infection. Although an inhibition of 50-75% was consistently observed, oncogenic transformation could not be completely inhibited. Because Rif has no effect on the RNA tumor virus polymerase *in vitro*(2) and does not inhibit nuclear RNA polymerases(3), it was postulated that the inhibition observed in these experiments might be the result of an effect on mitochondrial RNA polymerase (4,5). Therefore we investigated the effect of two other inhibitors of mitochondrial function, chloramphenicol (CAP) and ethidium bromide (EB) on oncogenic transformation. The data demonstrate that a short exposure to EB inhibits focus formation without significantly decreasing virus production or cell growth. Chloramphenicol, on the other hand, inhibits virus replication with only a minimal suppressive effect on focus formation. These data support

the suggestion that the mitochondrion plays a significant role in virus production and malignant transformation in this model system.

METHODS: The Bryan high-titer strain of RSV was used in all experiments. Chick embryo fibroblast cultures were maintained on F12 medium(6). Stock solutions of CAP and EB were prepared in F12 medium and stored in the dark at -20° C.

Assay of the effects of inhibitors on malignant transformation was carried out using focus formation under agar(7). Second passage CEF, infected at day 0 with 100 focus forming units (FFU) of virus per plate, were refed after adsorption with F12 medium containing Bryan antiserum. At various times thereafter, inhibitors were added for 24 hour intervals as described in Fig.2. Following treatment, control and treated plates were overlaid with agar and observed for focus formation at 10 and in some cases at 16 days after infection. Virus production studies were carried out by infecting cultures at a multiplicity of 0.1-0.2 FFU per cell, refeeding after adsorption with F12 medium containing no antiserum and exposing to the inhibitor for 24 hour periods. Following treatment, fluids were harvested and titrated for infectivity(8).

Cell growth was measured by pooling cells in the culture fluid with cells from the trypsinized monolayer and counting in a Coulter Counter. Viability was measured by dye exclusion with erythrosin B(9).

RESULTS AND DISCUSSION:

Effect of chloramphenicol on focus formation and virus production - If Rif suppressed focus formation and virus production by inhibiting mitochondrial RNA polymerase, it was postulated that CAP, a specific inhibitor of 70s ribosomal protein synthesis(10), might also affect these functions during the Rif-sensitive period between day 1 and day 2 after infection. As shown in Fig. 1, treatment with concentrations of CAP ranging from 5-75 µg/ml for 24 hours between day 1 and 2 causes only a small decrease in focus formation. Despite minimal suppression of focus formation by CAP, virus production is significantly inhibited, an effect directly proportional to antibiotic concentration (Fig. 1).

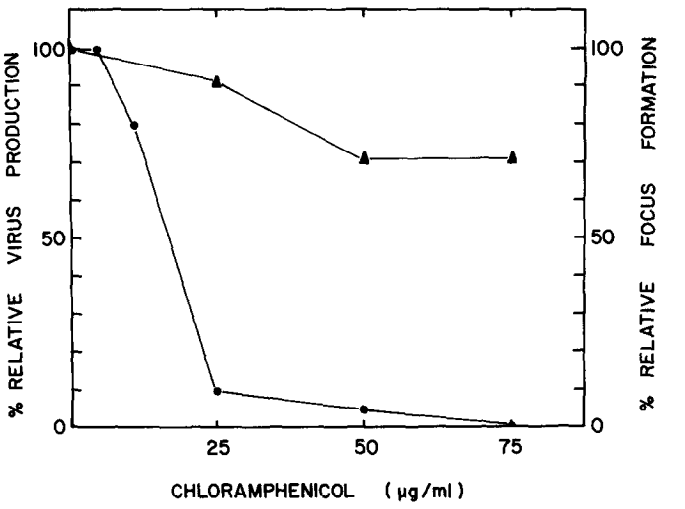

Figure 1. Effect of chloramphenicol(CAP) on focus formation (▲——▲) and virus production (●——●) in RSV infected cells. Second passage CEF were infected at day 0 for assay of focus formation(100 ffu/plate) or virus production(0.1 - 0.2 ffu/cell) as described in Methods. Cultures were treated with CAP for a 24 hour period between day 1-2 after infection. Following treatment, control and treated assay plates were overlaid with F12 medium and agar for focus assay. Also on day 2, fluids were harvested from plates infected at high multiplicity and titrated for infectivity.

A similar inhibition of virus production is obverved following CAP treatment between day 2-3 and day 3-4 after infection in the absence of an effect on focus formation. Thus, CAP is a more effective inhibitor of virus production than Rif. This finding suggests that the synthesis of some protein on mitochondrial ribosomes is required for the production of infectious RSV.

Effect of ethidium bromide on focus formation and virus production - Ethidium bromide, a phenanthridine dye, is a specific inhibitor of mitochondrial DNA synthesis(11), and transcription (12,13). Nass(11) has demonstrated that EB at a concentration of 1 µg/ml causes degradation of closed circular DNA and loss of cristae in L-cell mitochondria after 24-48 hours of treatment. The effect of EB on focus formation is shown in Fig. 2. Maximum suppression occurs when EB is present for 24 hours prior to infection (day -1 to day 0) or the two 24 hour periods after infection (day 0-1 or day 1-2). The diminishing effect observed at later times in infection is similar to the Rif pattern of inhibition

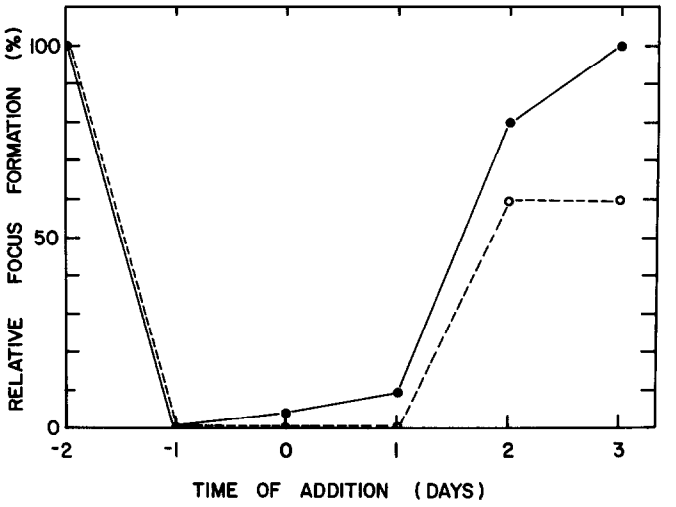

Figure 2. Effect of ethidium bromide (EB) on focus formation in RSV infected cells. CEF cultures were treated with EB(1 μg/ml) for 24 hour periods from day -2 prior to infection until day 4 after infection. Multiple plates were used so that each assay plate received only a single 24 hour exposure to the inhibitor. In these experiments, the day of infection is designated as day 0. The results of two different experiments using cells from two separate embryos are plotted as a function of the time of EB addition. In the case of cells treated before infection, the day -2 pretreated cultures were first passage cells exposed during the 24 hour period prior to plating cells for assay. The day -1 cultures were second passage cells treated during the 24 hour period immediately before infection, in the assay dishes themselves. Following infection of control and pretreated plates, cultures were overlaid with F12-agar medium for the focus assay. Cultures to be treated at later times after infection, together with their respective control cultures, were refed after infection with F12 medium containing Bryan antiserum. Following each 24 hour EB treatment, control and treated plates were overlaid with agar for focus assay and observed at 10 and in some cases at 16 days after infection.

(1). The suppression of focus formation by EB is permanent in that extended incubation of the assay plates (until day 16 after infection) did not alter the inhibition observed at day 10. In contrast to this dramatic inhibition of focus formation, there is no significant inhibition of virus production by EB (Table 1).

Because the inhibition of focus formation by EB could be due to an effect on cell viability or growth rate (14,15), experiments were carried out to study these parameters of cell function. Doses of 1 μg/ml EB for 24 hours did not reduce cell viability. Cell growth in liquid medium and under agar was followed over a 10 day period after EB treatment of normal and infected cells (0.1-0.2 FFU/cell). Although it was not possible to quantitate the growth rate of

TABLE 1

Time of EB Treatment	Virus Titer (FFU/ml) Day 1	Day 2	Day 3	Day 4
Control	3.5×10^4	1.6×10^6	1.4×10^6	1.1×10^6
EB (Day 0-1)	4.0×10^4	N.D.	7.0×10^5	N.D.
EB (Day 1-2)		9.1×10^5	9.5×10^5	5.9×10^5
EB (Day 2-3)			1.3×10^6	N.D.
EB (Day 3-4)				7.5×10^5

Table 1. Titer of Bryan RSV in focus forming units (ffu/ml) as determined by infectivity assay of fluids harvested immediately following EB treatment and for several days thereafter.

infected cells under agar because of the adherence of the transformed monolayer to the agar overlay, the growth rate of infected cells in liquid medium was not significantly altered by EB treatment. Furthermore, the growth of uninfected cells treated with EB in liquid medium or under agar was comparable to that observed in control cultures. In view of these data, and the observation that EB is only effective at early times after infection, it is unlikely that the inhibition is due to a non-specific effect on cell division.

These data demonstrate the distinctive effects of mitochondrial inhibitors on RSV focus formation and virus production. Rifampicin partially inhibits both parameters of viral infection during a critical time period between day 1 and day 2 after infection. Chloramphenicol has a minimal effect on focus formation but inhibits the production of infectious virus. This effect of CAP is similar to that of 5-fluorouracil on murine sarcoma virus(16). Finally, EB suppresses focus formation when used either 24 hours prior to infection or within 2 days thereafter but does not alter virus production. The results indicate that virus replication and oncogenic transformation in RSV-infected cells is contingent upon some function having an antibiotic sensitivity resembling that of mitochondria. Mitochondrial involvement in the replication

of the Schmidt-Ruppin strain of RSV has been suggested recently(17,18), however specific association of the virus with mitochondria has not been shown. The recent demonstration that EB (20 μg/ml) inhibits reverse transcription of murine leukemia virus in vitro(19) could account for the suppression of focus formation observed here. However, normal levels of infectious virus observed in these experiments would not be expected if provirus formation were blocked. Work is now in progress to study more directly the role of mitochondria in neoplastic transformation by RSV.

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