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Hackett, A.J.

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INHIBITION OF MSV VIRAL FUNCTION BY RIFAMPICIN DERIVATIVES

A. J. Hackett¹, R. B. Owens¹, M. Calvin² and U. Joss²

¹Naval Biomedical Research Laboratory, University of California, Berkeley, and ²Laboratory of Chemical Biodynamics, University of California, Berkeley

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INTRODUCTION

The rifampicins specifically bind with the DNA-dependent RNA polymerase of bacteria and inhibit the RNA chain initiation step (14,20). The enzymes of resistant organisms can be shown to differ in a single amino acid and such a change is sufficient to abolish or limit the binding capacity of the drug (15,18). The mammalian enzymes are not inhibited under conditions which completely inhibit the bacterial enzyme, and for this reason these drugs have been extensively used in clinical medicine (4,17,19,20).

The effects of the rifampicins on viruses are not as well defined. The DNA pox viruses are sensitive to certain derivatives (8,16) at very high concentrations. Resistant strains have been selected, indicating specificity for the inhibition (6). The mechanism of action against vaccinia is not clear but it is definitely not the same as that observed with bacterial systems (10).

Green et al. (7) showed that certain derivatives of rifampicin were active inhibitors of the RNA-dependent DNA polymerase of the RNA tumor viruses. The high concentrations required for inhibition of the enzyme, and the fact that a different but similar enzyme present in normal cells (5,13) was also sensitive to the drugs has made the specificity of the inhibition doubtful (12).

Recently, inhibition by dimethyl-benzyl rifampicin (DMB*) of foci of cells transformed by the murine sarcoma virus [MSV(M)] was demonstrat-

* This was made from a commercial rifampicin and its purity checked by thin layer chromatography. DMB-rifampicin is 2',6'-dimethyl-N(4')-benzyl-N(4')-[desmethyl]rifampicin, previously known as dimethyl-N-benzyl-N-des-methyl rifampicin.

ed (1). We now report further experiments with this compound, at dose levels which do not reduce the replication of the cellular substrate. Significant reduction in the number of focus formations and the yield of infectious virus at such levels of DMB have been observed. However, when all of the cells in a culture are infected with a virus (a multiplicity of 3 to 8 FFU/cell) and exposed to a similar level of DMB, replication proceeds unhampered. The implications of these findings are discussed.

Growth of B/3T3 Cells in the Presence of DMB:

The effect of DMB on the growth of transformed and non-transformed cells over the time period required for the focus inhibition test was determined. The effect of various concentrations of DMB during an 8-day growth period is shown in Figure 1. The MSV-transformed culture, shown with the dotted lines, was less sensitive to DMB toxicity at high doses than were the non-transformed cells. DMB was very toxic at 20 μ g/ml, causing a 100 - 800 fold reduction in cell number by 8 days. At 10 μ g/ml cell growth was reduced by 80-90% and even at 5 μ g/ml the number of attached cells was only 40-60% of the number in control cultures.

A more stringent test, that of efficiency of plating (EOP), measures the percent of developing colonies relative to the number of cells seeded. No effect was found when 100 cells were seeded in the presence of 3 μ g/ml of DMB; the EOP was 9.5% for the drug treated cells and 10% for the controls.

Inhibition of Focus Formation by MSV with DMB:

Table I gives typical results from focus inhibition assays done at intervals during the past six months. Experiments I and II were

done on the B/3T3 cell line obtained from Dr. R. Gilden at passage 60 and passaged 6 times in this laboratory; experiments III and IV utilized a B/3T3 cell line carried by Dr. H. Smith now of this laboratory. The two lines now vary in growth characteristics and morphology, but focus inhibition by DMB is equally effective on both lines.

With DMB present through the 7 days of the experiment, focus formation by MSV is reduced. Total inhibition was observed at 8 - 10 $\mu\text{gm/ml}$ while low levels (2 - 3 $\mu\text{gm/ml}$) reduced focus formation by 50%. Variation at intermediate levels of the drug illustrates differences obtained between experimental trials. The inhibition is consistent and reproducible so that even 50% reduction in foci can be taken as significant, providing enough replicas are included in the experiment.

Effect of Addition of DMB During Different Time Periods on Focus Formation by MSV:

Figure 2 shows the effect of DMB on focus formation during different time periods following initiation of infection. Six $\mu\text{gm/ml}$ was used in the experiment in the lower portion of Fig 2 wherein cells were exposed to the drug for only 24 hr intervals, and 3 $\mu\text{gm/ml}$ was used where the cultures were exposed for longer periods of time. Monolayers (with approximately 1×10^6 cells) were inoculated with an estimated 200 FFU of MSV and the drug was added or removed at the indicated times.

Maximum inhibition of focus formation was 50% when the drug was present during the 24 to 74 hr period of the experiment. Removal of

DMB before 48 hours or addition after 48 hours resulted in much less inhibition. At 6 $\mu\text{gm/ml}$ maximum focus inhibition (50%) occurred when the drug was present during the 24 to 48 hr period also. Again, the presence of the drug during the first 24 hours, or after 72 hours of virus growth, produced less inhibition. At both dose levels the critical time period for reduction in focus formation for MSV is the 24 to 48 hour period following initiation of infection. Total cell growth was reduced only 1 to 12 percent under these conditions.

Effect of DMB (3 $\mu\text{gm/ml}$) on Yield of Infectious Virus:

The yield of virus differs, depending on the multiplicity of infection (MOI). With low MOI the yield is reduced in the presence of DMB while at high MOI the yield does not differ from the control cultures. The yield of infectious virus produced under conditions used to demonstrate focus inhibition (low MOI) was measured at each 24 hour interval, following infection. No virus was detectable at 24 and 48 hours in either the control or drug treated cultures. At 72 hours the DMB treated cultures yielded only 15% as much virus as the controls. At the end of the experiments, the yield was 18% of the controls. Thus, a significant reduction of infectious virus production results from the presence of the drug during the first 72 hours after infection with a low multiplicity of MSV.

The amount of MSV produced in B/3T3 cells was not reduced significantly in the presence of DMB under conditions of high multiplicity of infection. In this experiment subconfluent monolayers were infected with an estimated 3 to 8 FFU per cell, and after 1 hour adsorption at

37C, the inoculum was removed and growth medium containing 10 $\mu\text{g}/\text{ml}$ DMB was added. Virus progeny were labelled with ^3H -uridine 4 hours prior to harvesting. The radioactivity recovered in peak fractions, which banded in the 1.18 - 1.16 g/ml region, of sucrose gradients was used to measure the amount of virus produced at four hour intervals during the first 24 hrs post-infection. Progeny virus produced during the 44 - 48 and 68 - 72 hr periods were also measured. The shape of the growth curves with or without the drug was the same both during the first cycle of virus growth (approx 12 hours), and throughout the remainder of the 72 hr experiment. Neither the time course of the infection, nor the quantity of labelled virus particles released, is influenced by the presence of the drug under conditions of high multiplicity.

Mechanism of Action of DMB:

In order to test whether the antibiotic is incorporated into the virion when it is present during virus replication, virus was grown in the presence of 3 $\mu\text{g}/\text{ml}$ of tritium-labelled DMB (^3H -DMB). A companion culture was grown with an equivalent level of unlabelled DMB and with ^{32}P (as inorganic phosphate). A portion of the yield from the ^{32}P -labelled culture was purified by centrifugation through sucrose gradients, the band harvested and the radioactivity in the virus-containing fraction determined. A sample of the remaining crude ^{32}P harvest, sufficient to give about 200 counts per minute ^{32}P in the peak fraction, was added to 4.5 ml of the crude harvest from the ^3H -DMB-containing yield. The pool was purified, banded and acid precipitable counts obtained from the gradient fractions. Figure 3 shows the counts obtained for both the ^{32}P -labelled marker virus, and for

the virus grown in the presence of ^3H -DMB. Because of the very low level, and apparent heterogeneity of the counts throughout the whole gradient, we conclude that there was no appreciable irreversible binding of the drug by the virus. Based on the specific activity of ^3H in the drug, and the estimated number of physical particles in the sample (10^{11}), one would expect a distinct peak to be observed if as few as 2 - 10 DMB-molecules were bound per virion.

The kinetics of heat inactivation of the virus under conditions which prevailed during the focus inhibition test was studied. In the presence of either 6 or 10 $\mu\text{g}/\text{ml}$ of DMB, the surviving fraction of MSV did not significantly differ from the control preparation after 24 hrs at 37C.

DISCUSSION

Robinson and Robinson (11) have pointed out that the toxic effects of a drug on cultured cells may increase with time of exposure, and that even slight toxic effects may have a direct effect on the ability of an RNA tumor virus to transform cells. Experiments which require exposure to the drug over a prolonged period of time should be interpreted with caution. Our experiments to determine the effects of various dose levels of DMB on cellular growth rate and efficiency of plating have shown that, over an 8 to 10 day period, 3 $\mu\text{g}/\text{ml}$ has no detectable effect on cellular replication. Furthermore, the inhibition of focus formation can be effected by exposure to DMB for only 24 hrs providing it is present during the sensitive time period, 36 to 72 hrs post infection.

It is apparent (Fig 2) that DMB is not effective during the first 24 hrs after infection. In contrast to the findings of Carter et al. (2) who found that the streptovaricins (compounds structurally similar to the rifampicins) are most effective during the first 24 hr period following MSV infection, DMB is most effective when present during the 36 to 72 hr period post infection. This is similar to results reported for rifampicin inhibition (at much larger dosage) of focus formation by Rous sarcoma virus (RSV) in chick cells (3,11).

Transformation probably takes place during the first 72 hr period after infection. If the drug is added after the 72 hr period, the number of foci observed at 7 days is not appreciably affected. There was no effect on cellular replication under any of the conditions described in Figure 2. However, virus replication was about 85% inhibited by the drug at the 3 μ gm/ml level under conditions of low multiplicity of infection.

This is in contrast to the apparent failure of the drug to inhibit virus replication at high multiplicity of infection. Although transformation takes place under these conditions, as observed in the whole culture, the efficiency of transformation is unknown. Experiments not yet performed, utilizing infectious center assay techniques, may yield information on the transformation efficiency when cells are infected with a high multiplicity of virus.

The mechanism of action of DMB is still not clear. We have shown that contact between the drug and the virion, under conditions which prevail during focus formation, does not inactivate the infectivity of the virus. DMB is not incorporated into the virion when it is present during viral replication; and, the physical characteristics of the virion, grown in the presence of DMB, remain unaltered.

It would appear that the explanation of direct action of the drug on the RNA-dependent DNA polymerase (RDP) is too simple; under conditions of low multiplicity infection, direct action may be involved. At high multiplicity the interactions among several infectious units of MSV and unknown, but larger, numbers of MLV in each cell may overcome the concentration-dependent inhibition of RDP.

It may be that transformation is not an obligatory concomitant of the viral replication cycle, but rather a probabilistic event branching from it. If such is the case, it may be possible to interfere with transformation without interfering with virus replication. This would imply one or more enzymes involved in the transformation process and sensitive to the drug. Experiments to determine this are in progress.

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TABLE I
FOCUS INHIBITION OF MSV IN B/3T3 CELLS BY DMB

<u>Experiment Number</u>	<u>DMB* (μgm/ml)</u>	<u>Average # Foci</u>	<u>% of Control Foci</u>
I	0	43	100
	5	6	14
	10	0	0
II	0	597	100
	2	301	50
	5	16	2
III	0	150	100
	3	70	47
IV	0	159	100
	6	13	8
	8	0	0

* Drug was present in the medium throughout experiment, extending over 7 days. Details of experimental method published previously (1).

Figure 1

Effect of dimethyl-benzyl rifampicin (DMB) on Growth of B/3T3 Cells.

Cells were seeded at 5×10^5 /flask in medium containing the appropriate amounts of DMB. Control cultures received equivalent amounts of DMSO. All cultures were fluid changed on day 4. Duplicate cultures from each test group were trypsin-dispersed and counted in a Coulter counter on the 4th and 8th days after seeding.

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FIGURE 1

Figure 2

Inhibition of MSV Focus Formation by DMB During Different Time Periods.

Cultures with $1-2 \times 10^6$ cells were infected with an estimated 200 FFU of MSV. DMB at 3-6 $\mu\text{g}/\text{ml}$ was added to or removed from duplicate cultures at various intervals during the 7-day growth period. Paired control cultures received equivalent amounts of DMSO at the same time intervals. Growth medium was renewed on all cultures at 72 hours with appropriate drug dosages included. Cultures treated with 6 $\mu\text{g}/\text{ml}$ of DMB were exposed to the drug for periods no longer than 24 hrs during the first 3 days of growth. The average number of foci and cells in the paired cultures of each group were determined at 7 days and expressed as a percentage of the number found in the respective control pair.

The shaded area indicates the time interval when inhibition was most effective.

INHIBITION OF MSV-FOCUS FORMATION BY DMB DURING DIFFERENT TIME PERIODS

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FIGURE 2

Figure 3

Isopycnic Banding of MSV Grown in the Presence of Tritium-labelled dimethyl-benzyl rifampicin (^3H -DMB).

^3H -DMB prepared by U.J. Methods for synthesis will be published elsewhere. Specific activity of the compound was 60 mCi/mM in the benzene ring. Cultures of B/3T3 were infected with MSV at a multiplicity of 3-8 FFU/cell. ^3H -DMB (3 $\mu\text{gm/ml}$) was added to the growth medium in one culture, and the same level of unlabelled DMB, with ^{32}P as inorganic phosphate (20 $\mu\text{Ci/ml}$) was added to the second culture. Fluids were harvested at 72 hrs.

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FIGURE 3

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