Effect of Rifampicin and Two of Its Derivatives on Cells Infected With Moloney Sarcoma Virus

(rifazine/2',6'-dimethyl-N(4')-benzyl-N(4')-[desmethyl]rifampicin)

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**ABSTRACT** It is shown that rifampicin, and especially the related antibiotic 2',6'-dimethyl-N(4')-benzyl-N(4')-[desmethyl]rifampicin (DMB-rifampicin) can inhibit focus formation by Moloney sarcoma virus on BALB/3T3 tissue cultures. At 10 μg/ml DMB-rifampicin totally inhibits focus formation while reducing virus replication by at least a factor of fifty and cell proliferation by only a factor of three. These observations, taken together with those of others, suggest a role for an RNA-dependent DNA polymerase and the gene for its synthesis both in normal cell processes and in the transformation process.

Rifampicin and its derivatives constitute a group of antibiotics that have been developed particularly for use against mycobacteria. Their mode of action involves the bacterial RNA polymerase (1). After the discovery that these drugs could also inhibit the replication of certain viruses, particularly adenovirus and vaccinia (2-4), it became of interest to explore the extent and possibly determine the nature of this antiviral activity. Toward this end, we obtained some samples of these materials in April of 1970* whose structures are shown in Fig. 1.

There followed the discovery of the RNA-dependent DNA polymerase in oncogenic RNA virus by Temin and Mizutani (5) and by Baltimore (6). An extensive discussion ensued about the distribution of this enzyme (particularly in virions and in cells from a variety of tumors) and the inhibition of the enzyme by some of the rifampicin derivatives (7-10).

It thus became clear that the possibility was real that one or more of these rifampicin derivatives could inhibit the transformation of cells from the normal into the neoplastic state. We therefore undertook to determine whether or not such a transformation could be affected by some of the derivatives which we had available.

Such a possibility had already been suggested by the experiments of Diggelmann and Weissmann (11), in which Rous sarcoma virus transformation of chick fibroblast monolayers had been inhibited by rifampicin at a concentration of 60 μg/ml. Such concentrations of rifampicin were completely toxic to our cell lines. The evidence of Green, presented at the Paris meeting on oncogenic viruses in November 1970, suggested that some of the derivatives of rifampicyn might be more effective (9). We were interested not only in the possibility of preventing the transformation, but, ultimately, of affecting the transformed cells as well.

**MATERIALS AND METHODS**

Cell cultures

BALB/3T3 cells were kindly sent to us by R. Gilden, Flow Laboratories, Inc., Rockville, Md. Cultures were grown in 250-ml plastic flasks in growth medium consisting of Eagle's minimal essential medium (Grand Island Biological Co., Richmond, Calif.) with 10% fetal bovine serum. Cell counts were performed in a Coulter counter. The medium was replaced weekly with fresh medium. Cell cultures were passaged every 4 days to maintain them in logarithmic growth.

**Abbreviations:**
- DMB-rifampicin, 2',6'-dimethyl-N(4')-benzyl-N(4')-[desmethyl]rifampicin, previously known as dimethyl-N-benzyl-N-des-methyl rifampicin; FFU, focus-forming units.
- Rifazine, 2',6'-dimethyl-N(4')-benzyl-N(4')-N-des-methyl rifampicin.

*The samples of rifampicin and its derivatives were kindly supplied by Drs. P. Sensi and G. Lancini of Gruppo Lepetit, Milan, Italy. It must be noted that the new drugs used by Subak-Sharpe (3) contained at least 1% of impurities. It is unknown whether such impurities constitute an active component of the drugs we utilized.

**Fig. 1.** Structures of rifampicin, DMB-rifampicin, and rifazine.
**Table 1. Inhibition of focus formation and Moloney sarcoma virus replication by rifampicin and DMB-rifampicin**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Conc (µg/ml)</th>
<th>Virus yield per flask (×10^4 FFU)</th>
<th>Foci in cultures (FFU/flask)</th>
<th>% FFU of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>308</td>
<td>1210</td>
<td>100</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>5</td>
<td>401</td>
<td>1490</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>141</td>
<td>683</td>
<td>56</td>
</tr>
<tr>
<td>DMB-rifampicin</td>
<td>5</td>
<td>11</td>
<td>240</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.09</td>
<td>0*</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

BALB/3T3 cultures, seeded with 1 × 10^6 cells 24 hr previously, were infected with approximately 1200 FFU/flask. After a 90-min adsorption period, freshly prepared rifampicin or DMB-rifampicin was added at 5 and 10 µg/ml in growth medium. Me2SO was added to a concentration of 1% w/v to the controls. 3 days after inoculation, the cultures were fluid-changed with the same medium. Foci appearing in the cultures at day 6 were counted and expressed as FFU/flask. In addition, the supernatant fluid was assayed for the yield of infectious virus. The figures are an average of two flasks per group.

*While we saw no identifiable foci, there may have been some too small to recognize.*

were made with a Coulter counter after suspending the cells with trypsin–EDTA and diluting in growth medium.

**Virus stock**

Moloney murine sarcoma virus was obtained from J. Moloney, National Institutes of Health, as a tumor homogenate. It has been passaged four times in a Swiss-derived high-passage mouse embryo cell line and assayed for focus-forming units (FFU) in BALB/3T3 cells. The virus pool used in these experiments titered 8.5 × 10^4 FFU/ml.

Vesicular stomatitis virus, New Jersey serotype, and methods used for its growth and assay, have been described (12).

**Assay of Moloney virus**

A modification of the method described by Hartley and Rowe (13) was used for the focus assay. Flasks were seeded with 1–2 × 10^6 cells in 25 ml of growth medium and incubated at 37°C for 24 hr. After the removal of fluids, virus was introduced in 0.5 ml of growth medium and allowed to adsorb on the monolayer for 90 min at 37°C. 25 ml of growth medium was then added and the cultures were returned to the incubator. After 3 days the cultures were fluid-changed, and foci of transformed cells were counted at day 7.

The antibiotics were dissolved in dimethylsulfoxide at 1 mg/ml. In the control experiments (absence of drug) the equivalent amount of MeSO was added without drug.

**RESULTS AND DISCUSSION**

Our first exploratory experiments defined the concentration region of useful activity of the drugs in the tissue cultures. Concentrations ≥ 20 µg/ml of DMB-rifampicin produced grossly visible toxic effects. Even 10 µg/ml of DMB-rifampicin seemed to slow the growth of both transformed and non-transformed cells during the early period, but both cell lines showed a net gain in number on the seventh day after inoculation. Rifampicin itself had little effect at 10 µg/ml (see Table 2).

The results of one experiment are shown in Table 1. It is quite clear that DMB-rifampicin is a potent inhibitor of the transformation process at 10 µg/ml. No foci were visible even though the control shows over 1000 foci/flask. It is also clear that the drug inhibits virus replication as well. Rifampicin at 10 µg/ml also shows a slight inhibition of focus formation as well as some inhibition of virus replication. A more explicit and broader experiment was then performed, using a single dose of drug at a concentration of 10 µg/ml, the result of which are shown in Table 2. Here it is again quite clear that the most potent drug we have so far studied is the DMB-rifampicin, which totally inhibits focus formation at 10 µg/ml. That not all RNA viruses are subject to this inhibition is demonstrated by the fact that vesicular stomatitis virus, which is not oncogenic but cytopathic and does not carry the RNA-

**Table 2. Effect of rifampicin, rifazine, and DMB-rifampicin on cellular and viral replication in BALB/3T3 cells infected with Moloney sarcoma virus (MSV)**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cells</th>
<th>Yield of MSV (FFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per flask (×10^-4)</td>
<td>% of control</td>
</tr>
<tr>
<td>Uninfected control</td>
<td>9.9</td>
<td>100</td>
</tr>
<tr>
<td>Infected control</td>
<td>8.4</td>
<td>100</td>
</tr>
<tr>
<td>Rifampicin + MSV</td>
<td>5.9</td>
<td>60</td>
</tr>
<tr>
<td>Rifazine + MSV</td>
<td>6.3</td>
<td>63</td>
</tr>
<tr>
<td>DMB-rifampicin + MSV</td>
<td>3.1</td>
<td>32</td>
</tr>
</tbody>
</table>

The same procedure as described in Table 1 was utilized to infect BALB/3T3 cultures with an estimated dose of 500 FFU of MSV. The antibiotics were added to the growth media (including fluid change) at a final concentration of 10 µg/ml. FFU were counted, the supernatant fluid was assayed for yield of infectious virus, and the number of cells per flask was counted at day 7. These data are from the last of eight separate experiments conducted. Although the figures varied between experiments, the data have followed a consistent pattern.

* See Table 1.
DNA-dependent DNA polymerase (14), is in no way affected by this drug in its ability to replicate on BALB/3T3 cell tissue culture. A 24-hr control showed 1.9 × 10^8 plaque-forming units/ml, while the system containing 10 μg/ml of DMB showed 2.2 × 10^8 plaque-forming units/ml. The interaction of the rifampicins with the RNA-dependent DNA polymerase in vitro (9) displays the same pattern of sensitivity as is shown in these experiments which test the intracellular effects of the drugs, namely, that rifampicin is a less active inhibitor than DMB-rifampicin. The specificity of the effect is further indicated by the failure (data not shown) to inhibit vesicular stomatitis virus.

It is also important to note that cell proliferation itself is somewhat inhibited at 10 μg/ml of DMB-rifampicin, although only by about 60%. It is unlikely that a net increase in cell number occurred during the 24-hr period between seeding and introduction of the drug (15). The 3-fold increase in cell number (from 1 × 10^6 to 3.1–3.5 × 10^6 on day 7) in the cultures containing DMB-rifampicin probably occurred after introduction of the drug; this is supported by the fact that 10^4 FFU were produced in these cultures. That the inhibitory action of DMB on focus formation and on the production of infectious virus is solely a function of the reduced number of cellular divisions seems unlikely since cell number increased 3-fold, while focus formation was apparently totally inhibited. A more detailed exploration of this effect, both in time and in quantity, must be made and eventually related to the molecular effects of the drug on the enzyme involved.

It is interesting to note in this connection that Scolnick et al. (16) have recently reported the presence in nontransformed BALB/3T3 cells of a small amount of RNA-dependent DNA polymerase which responded to the poly(A)·poly(dT) template (16). This in itself might be enough to account for our observation of reduced cell multiplication in the presence of the drug. However, it is altogether likely that other enzymes crucial to cell multiplication are also inhibited.

Another interesting observation, reported by Spiegelman (17), is that a monocytic leukemia virus carried in an ascitic form in a rat and induced by treatment with dimethylbenzanthracene has an enzyme very similar to the one found in human leukemia cells. Spiegelman has reported the presence of a similar activity in various embryonic tissues (18, 19). It seems that this enzyme, a DNA polymerase that is dependent on hybrid RNA·DNA, may be common to cells that are growing and dividing rapidly. In fact, it may be an especially facile supplementary route for replacing DNA via the DNA to RNA to DNA route, particularly in view of the questions that are being raised regarding the function of the Kornberg enzyme in DNA replication (20).

All of this tends to support the notion of a gene for this enzyme, and for other aspects of transformed cells, which may very well be present in an unexpressed form in what we believe to be normal cells (21). Expression of such genes, then, may be triggered either by chemicals, perhaps even by radiation, and by viruses, with the last one possibly introducing new information into the cell as well. It remains to be seen how far such hypotheses can be developed in molecular terms.

NOTE ADDED IN PROOF
Recent data show focus inhibition without reduction in cell replication by exposure to a more highly purified sample of DMB-rifampicin (3 μg/ml) for 72 hr after infection with MSV, in an experiment analogous to that described in Table 2.

The work described in this paper was sponsored, in part, by the U.S. Atomic Energy Commission and, in part, by Contract no. PH 43-65-13 between the Regents of the University of California and the National Cancer Institute (Special Virus Cancer Program). One of us, URJ, is a fellow of the Stiftung für Stipendien auf dem Gebiete der Chemie, Basle, Switzerland.

17. Spiegelman, S., by special permission, private communication.