

SELECTIVE INHIBITION OF ROUS SARCOMA VIRUS PRODUCTION IN TRANSFORMED CHICK FIBROBLASTS BY TWO RIFAMYCIN DERIVATIVES

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1. Introduction

Rifampicin prevents the formation of foci of Rous cells (phenotypically transformed chick embryo fibroblasts) at concentrations which do not affect virus replication [1]. However, focus inhibition may not be due to a specific action of the drug on cell transformation but to the toxicity of the doses used for tissue culture cells [2], and notably for transformed cells [3].

On the other hand, rifampicin is not an inhibitor *in vitro* of mammalian DNA-dependent RNA polymerase [4], nor of RNA-dependent DNA polymerase of RNA tumor viruses [5] and, therefore, it does not appear to be a good candidate for interfering specifically with the replication of RNA tumor viruses if this replication involves a DNA intermediate, as suggested by *in vitro* studies [6–10] and by the recent finding of infectious DNA in RSV-transformed cells [11, 12].

New rifamycin derivatives have now been synthesized which are powerful inhibitors of the RNA-dependent DNA polymerase of MSV [13]. Especially active are the 3-oxime derivatives such as AF/05 and AF/013 which have also been shown to inhibit DNA-dependent RNA polymerase by preventing initiation, but not elongation of the initiated RNA

chains [14, 15]. We have examined the effect of the rifamycins AF/05 and AF/013 on the replication of a clonal isolate of Schmidt-Ruppin strain Rous sarcoma virus (SR4) in chick embryo fibroblasts and have found that they do not inhibit transformation of infected cells, as measured by focus formation, at nontoxic doses, whereas virus production is reduced markedly in transformed, but not in newly infected untransformed cells.

2. Experimental

Medium: Standard growth medium was double strength Eagle's MEM supplemented with nonessential amino acids (also double strength), 5% calf serum, 10% tryptose phosphate broth (Difco), and the usual antibiotics (penicillin and streptomycin).

3. Results and discussion

As seen in fig. 1, the growth of subcultured fibroblasts obtained from whole chick embryos [16] is not affected by AF/05 and AF/013 concentrations up to 20 µg/ml and their cloning efficiency is equally resistant to AF/013. Focus formation following in-

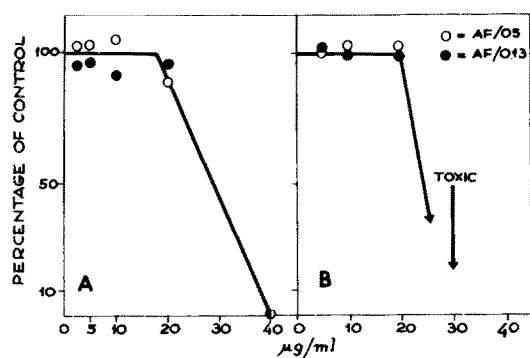


Fig. 1. A) Two days growth of subcultured chick embryo fibroblasts (seeded at 2×10^6 cells/plate (Falcon, 6 cm)) in medium containing rifamycin AF/05 (\circ) or AF/013 (\bullet) at various concentrations. Cells were counted after trypsinization. Untreated control cells grew up to 7.5×10^6 /plate (mean of two plates). B) Formation of foci of Rous cells in monolayers of subcultured fibroblasts (2×10^6 /plate) infected 1 day after seeding with about 100 FFU of SR4 and incubated in the presence of various concentrations of AF/05 and AF/013, added 2 hr before infection. The infected cultures were incubated 5 days at 37° under medium gelled with agar (Difco, 0.7%) and containing the antibiotics, then 3 more days under normal gelled medium.

fection with SR4 is not affected either by antibiotic concentrations which do not affect cell growth when the monolayer cultures are exposed to the drugs 2 hr before infection and grown subsequently for 5 days in their presence and 3 more days in their absence; however, the mean diameter of foci is reduced 2-fold compared to controls, and up to 6-fold when rifamycin AF/013 is present in the medium for 8 days from the time of infection.

On the other hand, the production of free virus 2 days after infection of the cells is not inhibited by the two rifamycin derivatives added at the time of infection, but the 2 days virus production of established transformed Rous cells is strongly reduced by the two compounds at concentrations which do not significantly affect multiplication of the transformed cells (fig. 2). The production of virus becomes sensitive to AF/013 between the second and third day after infection which corresponds to the appearance of Rous cells (fig. 3).

Inhibition of virus production also depends on the proportion of Rous cells: when this proportion is over 90%, virus production is reduced about 10-fold,

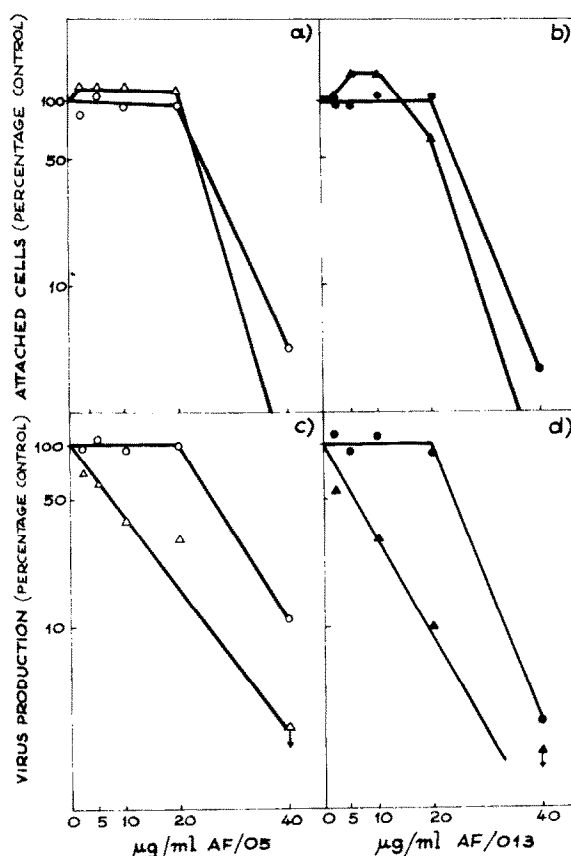


Fig. 2. Growth and two days virus production of fibroblasts infected de novo with SR4 (0.1 FFU/cell) and of Rous cells in the presence of AF/05 (open symbols) and AF/013 (filled symbols) \circ , \bullet : normal cells; Δ , \blacktriangle : Rous cells ($> 90\%$ of the total culture). Cell growth was measured as in fig. 1 and virus production by the focus assay of free virus.

whereas when it is only about 50% inhibition is only 2-fold. On the other hand, Rous cells do not detach preferentially after treatment with the antibiotic.

The inhibition of virus production is not due either to a decreased infectivity of the virus produced in the presence of the drugs since, as shown in fig. 4, the decrease of FFUs of virus is paralleled by a decrease of the number of virus particles released in the medium, as detected by labelling with $[^3\text{H}]$ uridine and centrifuging in a sucrose gradient [17]. It can also be seen that overall RNA synthesis in Rous cells is much less affected by AF/013 than virus production and that DNA synthesis is not significantly affected.

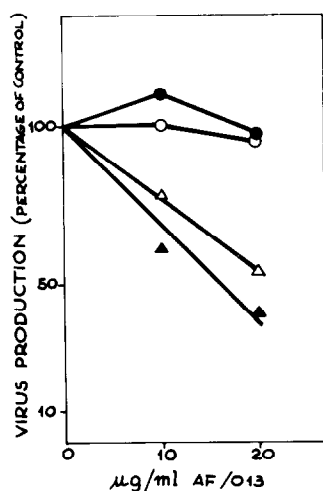


Fig. 3. Inhibition of virus production by different concentrations of AF/013 as a function of time after infection. Replicate fibroblast monolayers were infected with SR4 (0.1 FFU/cell) and treated with AF/013 for the following times: 0 to 1 day: (●); 1 to 2 days: (○); 2 to 3 days: (▲); 6 to 7 days: (△). Results are expressed as percent of the production of the respective untreated controls after the period of contact.

In order to demonstrate that the sensitivity of virus production only depends on the transformed phenotype of infected cells, advantage was taken of the characteristics of a temperature-sensitive mutant of SR4, FU-19, which fails to convert infected cells to Rous cells at 41° although it retains its capacity to replicate at this temperature. Furthermore, Rous cells appearing at 37° in infected cultures recover a phenotype within a few hours when cultures are shifted to 41° , and no macromolecular synthesis is required for this "detransformation" [18]. After infection of most cells with FU-19 or SR4 (controls), cultures were incubated at 37° which led to transformation of about half the cells within 4–5 days. The plates were then divided and incubated either at 37° or at 41° for one more day; Rous cells thus almost completely disappeared by "detransformation" in the FU-19 infected plates shifted at 41° . The cultures were then incubated another day in the presence of different concentrations of AF/013, and free virus titrated. As seen in fig. 5, no inhibition of virus production by the rifamycin occurred in detransformed FU-19-infected cells, whereas virus produc-

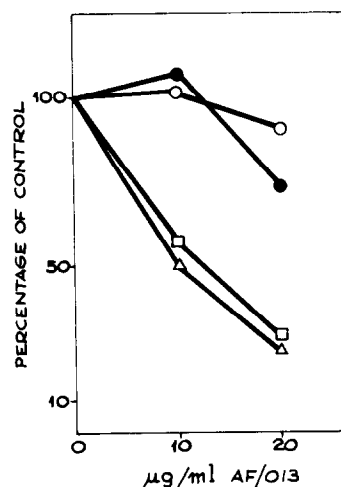


Fig. 4. Incorporation of [^3H] uridine (25 Ci/mmole) and [^3H]thymidine (25 Ci/mmole) by Rous cells (about 70% of the total culture) and free virus released in the medium, in the presence of various concentrations of AF/013. Medium containing 30 $\mu\text{Ci/ml}$ of [^3H]uridine or 12 $\mu\text{Ci/ml}$ of [^3H]thymidine was added to transformed cultures in the presence of the antibiotic; after 24 hr incubation at 37° , the medium was harvested and centrifuged 15 min at 8000 rpm; 1.5 ml of the clarified supernatant was layered on top of a sucrose gradient and centrifuged for determination of the [^3H]uridine incorporated into virus particles [17] (△). In parallel, an aliquot was titrated for FFUs (○). The radioactivity incorporated in the cell RNA and DNA (●: [^3H]uridine; ○: [^3H]thymidine) was determined by a new procedure (De Carli et al., in preparation).

tion was inhibited in transformed cultures maintained at 37° .

These findings demonstrate that the two Rifamycins inhibit the replication of RSV at doses which do not affect cell growth, but only in transformed cells. Therefore, the transformed phenotype either favours penetration of the antibiotics or their access to the intracellular site where they interfere with viral replication, and some step of this replication is blocked selectively.

Since in transformed cells RNA-dependent DNA polymerase is, in principle, no more required for virus replication [19], and since AF/013 is active on DNA-dependent RNA polymerase [14, 15] and RSV replication depends on DNA-dependent RNA synthesis [20, 21], the inhibition of RSV production by the rifamycins could be at the stage of transcription

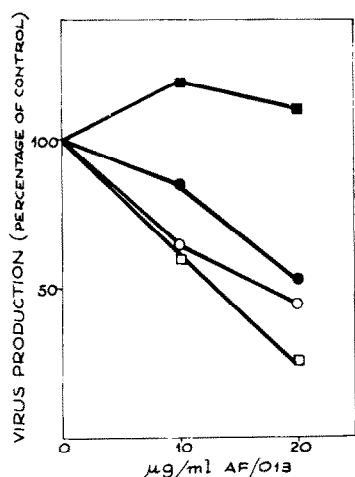


Fig. 5. Inhibition of virus production in cells transformed by SR4 (wild type virus; open symbols) and by FU-19 (*ts* mutant of SR4) (filled symbols) after 24 hr incubation with AF/013. ○, ●: cells incubated at 37°; □, ■: cells incubated at 41°.

of the viral DNA template required for the synthesis of copies of viral RNA. Furthermore, since overall cellular RNA synthesis is much less affected by the antibiotics than virus production, it may be that transcription of the viral DNA template requires a special DNA-dependent RNA polymerase which could be virus-coded.

The results presented also show that the rifamycin derivatives studied do not inhibit the process of cell transformation. Hence, the reduction of the size of foci of Rous cells observed in the experiments is presumably due to reduction of recruiting of new cells by infection following the reduction of virus production in the transformed cells.

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