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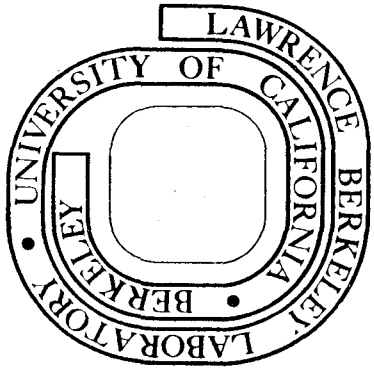

# Preferential Inhibition of the Growth of Virus-transformed Cells in Culture by Rifazone-$8_2$, a new Rifamycin Derivative

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ABSTRACT Rifazone-$8_2$ (R-$8_2$), a new rifamycin derivative, is shown to preferentially inhibit the growth of virus-transformed chick cells in culture. Macromolecular synthesis and glucose uptake of transformed cells are also appreciably decreased in the presence of low concentrations of R-$8_2$ where the normal cells appear unaffected. While R-$8_2$ is shown to be a selective inhibitor of RNA-directed DNA polymerase *in vitro*, its action on the growth of transformed cells may involve some other mechanism.

Introduction

Rifamycin and its derivatives have been shown to inhibit focus formation by RNA tumor viruses in various cell types in culture (1-5). The *in vitro* demonstration that RNA-directed DNA polymerase (RDP) is inhibited by the action of some of these derivatives (6-13) has raised the possibility that the drugs may inhibit focus formation by inhibiting RDP activity. Smith *et al.* have shown recently that those rifamycin derivatives that are toxic to leukemic human leucocytes are also those that inhibit RDP best *in vitro* (14).

Rifampicin, a well known derivative of rifamycin has been shown to inhibit the replication of vaccinia virus in tissue culture when added at very high levels (> 100 μg/ml) (15,16) and to reduce the incidence of Adenovirus-induced tumors in male hamsters (16). In chick cells it has been argued that rifampicin a) inhibits focus formation by Rous sarcoma virus (1), b) has no effect on transformation, as it is toxic to both normal and transformed cells (17), and c) is preferentially toxic to transformed cells (20). Variation in culture conditions, serum concentration, and cell density undoubtedly play a role in such contradictory findings. For example, whether or not amphotericin B is present in culture medium, may drastically change the result of focus inhibition (4,19). Furthermore, the high concentration of rifampicin used in these experiments (20-80 μg/ml) under some conditions is quite toxic to normal cells and makes interpretation of these data very difficult. Rifampicin itself has little or no effect on RDP activity *in vitro* at concentrations used in tissue culture studies (7,17,20). We have tested several new rifamycin derivatives synthesized in this laboratory which have been shown to be

inhibitors of RDP *in vitro* and inhibitors of focus formation *in vivo* in other cell systems (4-6). In addition, we have tested Rifazone-$8_2$ (R-$8_2$), a new rifamycin which is to date the most selective inhibitor of viral RDP *in vitro* (6,21). We find that at low concentrations (3-10 μg/ml), R-$8_2$ can selectively inhibit the growth of transformed cells and prevent focus formation while allowing the normal cell growth and function to continue.

## Materials and Methods

Growth of Cell Cultures. Primary cultures were prepared from 10-day old C/O or C/B type SPF chick embryos as described previously (23,24), except that Amphotericin B (Fungizone) was eliminated at this point (25). The cells were seeded in Medium 199, which was supplemented with tryptose phosphate broth (2%), calf serum (1%), and heated chicken serum (1%). The medium was changed on day 3. Secondary cultures were prepared 4 days after the primary seeding by trypsinization of primary cultures and were seeded at the desired cell concentration in 60 or 35 mm petri dishes. An additional 1 mg/ml of glucose was added to the medium at this time, bringing the final concentration of glucose to 11.00 mM, and calf serum concentration was raised to 2%. For studies with transformed cultures, half the cells of a single embryo were infected 4 hours after primary seeding with $4 \times 10^5$ focus forming units of SR-RSV or B-RSV. Secondary cultures were prepared as above.

Focus Assay. Assays were performed essentially as described (22), with slight modifications. We found that gentle removal of the agar on day 4 or 5 and addition of either liquid medium or another agar overlay enhances the visibility of foci. Four hours after seeding secondary normal cells at

$2.5 \times 10^6$ per 60 mm dishes, medium was removed and cells were exposed to the appropriate dilution of virus in 0.5 ml for 1 hr. The virus was then removed, the monolayer rinsed, and appropriate concentrations of drugs were added together with 1 µg/ml Fungizone in 0.5 ml of medium 199. Rifamycins were dissolved in dimethyl sulfoxide (DMSO) so that all cultures had a final concentration of 0.1% DMSO. Thirty minutes later the derivatives were removed and cells were overlaid with agar-medium containing the same concentration of the derivatives. In experiments where the rifamycins were added at a later time, the agar layers of control cultures were removed and replaced with agar containing DMSO at the same time. A known titre of SR virus stock accompanied all assays.

Measurements of the Rate of DNA and Protein Synthesis and Glucose Uptake. Radioactive assays were performed as previously described (25,26). All radioactive compounds were purchased from New England Nuclear.

Rifamycin Derivatives. These were synthesized as previously described (20,21).

## Results

### Focus Formation

When foci were scored on day 8 and 6 for SR-RSV or B-RSV respectively, there was a marked inhibition of the number of foci in the presence of rifazone-$8_2$ (R-$8_2$) (Table I). Ten µg/ml dimethylbenzyl rifampicin (DMB) also caused appreciable inhibition with SR-RSV. Rifazacyclo-16 (RC-16), previously shown to be an inhibitor of MSV focus formation on UC1-B cells (4), was not very effective in chick cells. Rifampicin, at 20 µg/ml, had no effect on focus formation. The few remaining foci in the presence of the effective rifamycins were usually much smaller than the control foci (Fig. 1).

When the foci were scored again 3 days later, the apparent inhibition had decreased in almost all cases suggesting an inhibition of the growth of the focus once it had been formed (Table I).

a. Normal Cells at High Cell Density. Under the condition of agar assay (more than 1 x $10^5$ normal cells/$cm^2$), 10 μg/ml of R-$8_2$ had no effect on the growth properties of normal cells (Fig. 2). DMB, however, was toxic under these conditions. To avoid the complication of toxicity under assay conditions, we chose to work with R-$8_2$ alone. The pronounced inhibition of focus formation by R-$8_2$, therefore, is not due to toxicity to the normal cells in the monolayer. As a further control, the normal cells from the uninfected areas of drug treated cultures were removed from under agar and replated. They showed normal growth properties when compared to control cultures re-grown after agar removal and were morphologically normal as well.

b. Comparison of Normal and Transformed Cells at Low densities. When cells were seeded at lower cell densities (2 x $10^4$ cells/$cm^2$) they were more sensitive to R-$8_2$ than cells at the higher cell densities (compare Figs. 2 and 3a). Howwever, at comparable cell densities and growth rates, transformed cells were always much more sensitive than normal cells (Fig. 3). While normal cells were unaffected by 5 μg/ml of R-$8_2$, the growth of transformed cells was considerably inhibited by 3 μg/ml of R-$8_2$ after 48 hr (Fig. 3c). Three days after addition of 5 μg/ml R-$8_2$, the morphology and cell number were still the same in untreated and treated normal cultures (Fig. 4A, B). The R-$8_2$ treated transformed cells, however, were drastically reduced in number and the remaining cells were either vacuulated or had a normal morphology (Fig. 4D). DNA synthesis as measured by [$^3$H]-thymidine incorporation (26) and uptake of glucose as measured by [$^3$H]-2 desoxyglucose (26)

showed the same pattern of preferential sensitivity to R-$8_2$ (Table II). The soluble pool of [$^3$H]-thymidine was not affected by the presence of R-$8_2$. The rate of protein synthesis as measured by [$^3$H]-leucine incorporation into the acid insoluble pool was the least sensitive to the action of R-$8_2$, although transformed cells were still more affected (Table IIb).

Effect of R-$8_2$ on Previously Formed Foci

To understand to what degree this preferential toxicity to transformed cells could explain the focus inhibition, two kinds of experiments were performed. In one series of focus assays, 10 μg/ml R-$8_2$ was added 1 hr after infection, resulting in more than 90% inhibition of focus number after 8 days. If the action of R-$8_2$ was solely on inhibiting the initiation of transformation one would expect that addition of R-$8_2$ 2 days later would have no effect on the number of foci produced. In fact, when R-$8_2$ was added 2 days after infection there was still more than 40% inhibition of the foci if scored on day 8. Furthermore, the remaining foci were smaller than control (compare Fig. 1C and D), and the percent inhibition decreased to about 10% by day 11 when the foci became quite visible. This experiment indicated to us that a large proportion of the focus inhibition observed (Table I) may be attributed to the preferential toxicity of transformed cells to R-$8_2$ in addition to any inhibition of initiation of transformation.

In a second series of experiments B-RSV foci were allowed to develop until they were visible (5 days). The agar was then removed gently and complete medium was added with or without 10 μg/ml of R-$8_2$. The foci continued to grow with a measurable rate in control cultures (Fig. 5A, B, Fig. 6). The removal of the agar resulted in a "necrotic" focus as the

piled up transformed cells in the center of the focus were lost to the medium (Fig. 5B). The growth of the foci after the addition of 10 μg/ml R-$8_2$, however, was virtually arrested (Fig. 5C, D, Fig. 6). It has been shown previously that 48 hr after seeding, the normal chicken cells seeded at high density are no longer susceptible to transformation by B-RSV (22). The focus of B-RSV, therefore, is comprised essentially of the progenies derived from the initially transformed cell. Indeed, we observed no additional foci in control cultures, despite the fact that virus is released into the medium after agar removal. Thus the lack of focus growth in the presence of R-$8_2$ is due to inhibition of transformed cell growth rather than an inhibition of secondary infection.

Mixed Cultures

Two experiments were performed in which 50% normal and 50% transformed cells were plated and allowed to grow in the presence and absence of R-$8_2$ (5 μg/ml) for 3 days. In the first experiment, where the cells were plated at a low density ($1 \times 10^4$ cells/$cm^2$), cells grown in the absence of R-$8_2$ appeared to be completely transformed (Fig. 7A), while cultures grown in the presence of R-$8_2$ appeared to be largely normal by the end of the 3-day period (Fig. 7B). In the second experiment, where the cells were plated at a higher density ($5 \times 10^4$ cells/$cm^2$), cells grown without R-$8_2$ appeared to be completely transformed as expected. The cells with R-$8_2$ present, however, were still in a monolayer and the majority of cells were normal, although dispersed transformed cells could be seen in the culture. Consistent with previously described results, these mixed culture experiments indicate a preferential inhibition and/or killing of transformed cells. Furthermore, they might suggest an additional role of R-$8_2$ in preventing

secondary infection by inactivating the virus itself. Whether the presence of R-$8_2$ in these experiments selects for normal cells by killing transformed cells, or whether it actually causes a reversion toward a normal morphology, is a question currently under investigation.

## DISCUSSION

The selective inhibition and/or destruction of neoplastic cells while the normal cell growth and function continues, is a general aim of cancer chemotherapy. We have shown that rifazone-$8_2$, a specific inhibitor of RDP (21) also inhibits the growth of virus-transformed cells in tissue culture without appreciable side effects to normal cell growth. Preferential inhibition of transformed cells has been reported for rifampicin previously (18). However, rifamipicin does not inhibit RDP appreciably (9) and the dosage needed for focus inhibition (more than 20 μg/ml) is toxic to normal chick cells in our hands.

There are a number of possible explanations for this increased toxicity of R-$8_2$ to the transformed cells. 1) There is abundant evidence that cancer cells in general have altered permeability (27) and al'ered membrane properties (28), factors which could account for the observations described here. Once the nature of these differences is understood they may further be exploited to synthesize new derivatives which can cross the membrane of specific tumor cells more readily. 2) The drug may act partially by preventing transformation through inhibition of reverse transcriptase (12). If a continuing involvement of this enzyme in the growth of transformed cells is postulated, the additional inhibitory action of R-$8_2$ on the growth of transformed cells may be explained. 3) Alternatively, R-$8_2$ may be inhibiting an as yet unknown enzyme function(s) which might be essential

to the growth of transformed cells. These possibilities are not mutually exclusive. The additional action of R-$8_2$ on the infectivity of the virus itself should also be investigated (12). To what extent a change in permeability of transformed cell membrane is responsible for the observed effects will be studied by use of radioactive derivatives. The effect of R-$8_2$ and other rifamycin derivatives in various cell systems and animals is under investigation.

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Abbreviations used: R-$8_2$ or Rifazone $8_2$, rifaldehyde-N,N-di-n-octyl-hydrazine-hydrazone; RDP, RNA-directed DNA polymerase; RSV, Rous sarcoma virus; B-RSV, Bryan high titre strain of RSV; SR-RSV, Schmidt-Ruppin sub-group A strain of RSV; Rif, Rifampicin; DMB, 2',6'-dimethyl-4'-benzyl-4'-desmethylrifampicin; DMSO, dimethyl sulfoxide.

Table I

Focus Inhibition - % of Control

| | | | Schmidt-Ruppin* | | Bryan+ | |
|---|---|---|---|---|---|---|
| | | | Day 8 | Day 11 | Day 6 | Day 9 |
| Cont (DMSO) | | | 100 ($80\pm20$)† | 100 ($85\pm20$) | 100 ($70\pm20$) | 100 ($70\pm20$) |
| Rif | 20 | /ml | $100 \pm 10$ | $100 \pm 10$ | $100 \pm 10$ | $100 \pm 8$ |
| RC-16 | 10 | /ml | $75 \pm 10$ | $85 \pm 10$ | - | - |
| DMB | 1 | /ml | $50 \pm 7$ | $85 \pm 8$ | - | - |
| DMB | 10 | /ml | 0-2 | $10 \pm 6$ | - | - |
| R-$8_2$ | 1 | /ml | $90 \pm 10$ | $90 \pm 10$ | $90 \pm 10$ | $95 \pm 10$ |
| R-$8_2$ | 5 | /ml | $40 \pm 10$ | $70 \pm 15$ | $45 \pm 10$ | $70 \pm 15$ |
| R-$8_2$ | 10 | /ml | 0-1 | $15 \pm 10$ | $10 \pm 8$ | $20 \pm 10$ |

50-100 focus forming units of Schmidt-Ruppin (SR) subgroup A or Bryan strain of Rous sarcoma virus were assayed under agar as described in Methods. The foci were scored on day 8 and 11 for SR and on days 6 and 9 for the Bryan strains. Amphotericin B (1 μg/ml) was present throughout the focus assay, even though at best it improved the inhibition by only 10%.

* Average of 6 experiments

+ Average of 4 experiments

† The number in parenthesis indicates the actual number of foci which was set equal to 100.

Table IIa

[$^3$H]-Thymidine Incorporation

| | % of control after 48 hr µg of drug per ml | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 3 | 5 | 10 | 15 | 20 |
| N | 100 (38600)* | 100 | 100 | 74 | 39 | 20 |
| T | 100 (44600) | 70 | 64 | 51 | 28 | 4 |

* DPM/mg protein. Average of 3 experiments.

Procedure was as described in Fig. 3 and Methods.

Table IIb

[$^3$H]-Leucine Incorporation and [$^3$H] 2-Deoxyglucose Uptake

| | Leucine | | 2-DG | |
|---|---|---|---|---|
| | N | T | N | T |
| | % of control after 48 hr | | | |
| Control | 100 (32100)* | 100 (42900) | 100 (43900) | 100 (98100) |
| 3 g/ml | 100 | 100 | 108 | 72 |
| 5 g/ml | 100 | 88 | 105 | 53 |
| 10 g/ml | 85 | - † | 85 | 42 |

* The numbers in parentheses represent DPM/mg protein.

† Too few cells left for determination. Average of triplicate samples of one experiment.

Figure Legends

Fig. 1. Focus formation in the presence and absence of R-$8_2$. Focus of SR-RSV on day 8. A) in 0.1% DMSO, B) in the presence of R-$8_2$ (10 μg/ml) added 1 hr after virus infection, C) in 0.1% DMSO, the agar overlay was replaced after 2 days. D) in the presence of R-$8_2$ (10 μg/ml) added 2 days after virus infection.

Fig. 2. Growth of high density normal cells in the presence of rifamycin derivatives. Normal cells were plated at 1 x $10^5$/$cm^2$ in the presence or absence of the chemicals as described in Methods. Average of 4 measurements.

Fig. 3. Growth of normal and RSV-transformed cells in the presence of R-$8_2$. Normal and SR-transformed cells were seeded at 2 x $10^4$ cells/$cm^2$ 4 days after primary seedings. The medium contained either DMSO or R-$8_2$ (3, 5, 10 and 20 μg/ml). Cells were counted on successive days in triplicate.

Fig. 3C is the percent cells left on the dish after 48 hr (taken from 3A and 3B; the cell number in control cultures was set equal to 100).

Fig. 4. Morphology of normal and transformed cells in the presence of R-$8_2$. Legend as in Fig. 3, except that cells were seeded at 1.5 x $10^4$ cells/$cm^2$ and medium was changed on day 2. Control and treated cultures (5 μg/ml R-$8_2$) were photographed 3 days after seeding. A and B, normal cells in the absence and presence of R-$8_2$. C and D, transformed cells in the absence and presence of R-$8_2$.

Fig. 5. Arrest of focus growth after addition of R-$8_2$.
Two sets of focus assay plates of B-RSV were allowed to develop until foci were visible (5 days). The agar overlay was then removed and replaced with regular medium containing 0.1% DMSO or R-$8_2$ (10 μg/ml in 0.1% DMSO). Visible foci were encircled with black pen and numbered and they were then photographed on successive days. Arrows indicate the boundary of focus in each case. A, control focus 1 hr after addition of liquid medium; B, the same focus as in A, 82 hr later; C, focus 1 hr after addition of liquid medium and R-$8_2$ (10 μg/ml); D, the same focus as in C, 82 hr later.

Fig. 6. The rate of growth of previously formed foci in the presence or absence of R-$8_2$.
Experiment was performed as described in Fig. 5. The area under the foci was estimated by use of a planimeter. Each curve is the average of 3 foci.

Fig. 7. Morphology of mixed cultures in the presence and absence of R-$8_2$.
50% normal and 50% transformed cells were seeded at a total population of $1 \times 10^4$ cell/$cm^2$. The pictures were taken 3 days after seeding. A), culture with DMSO. B), culture with R-$8_2$ (5 μg/ml).

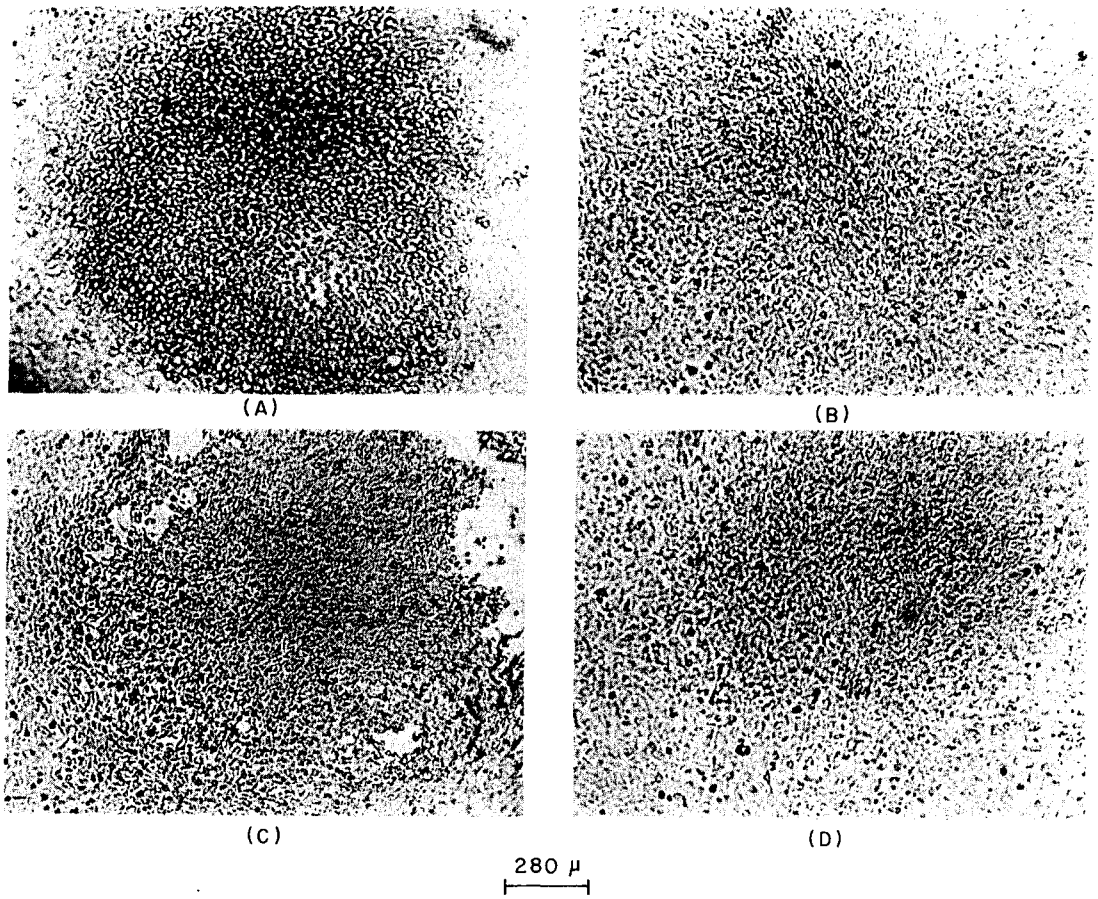


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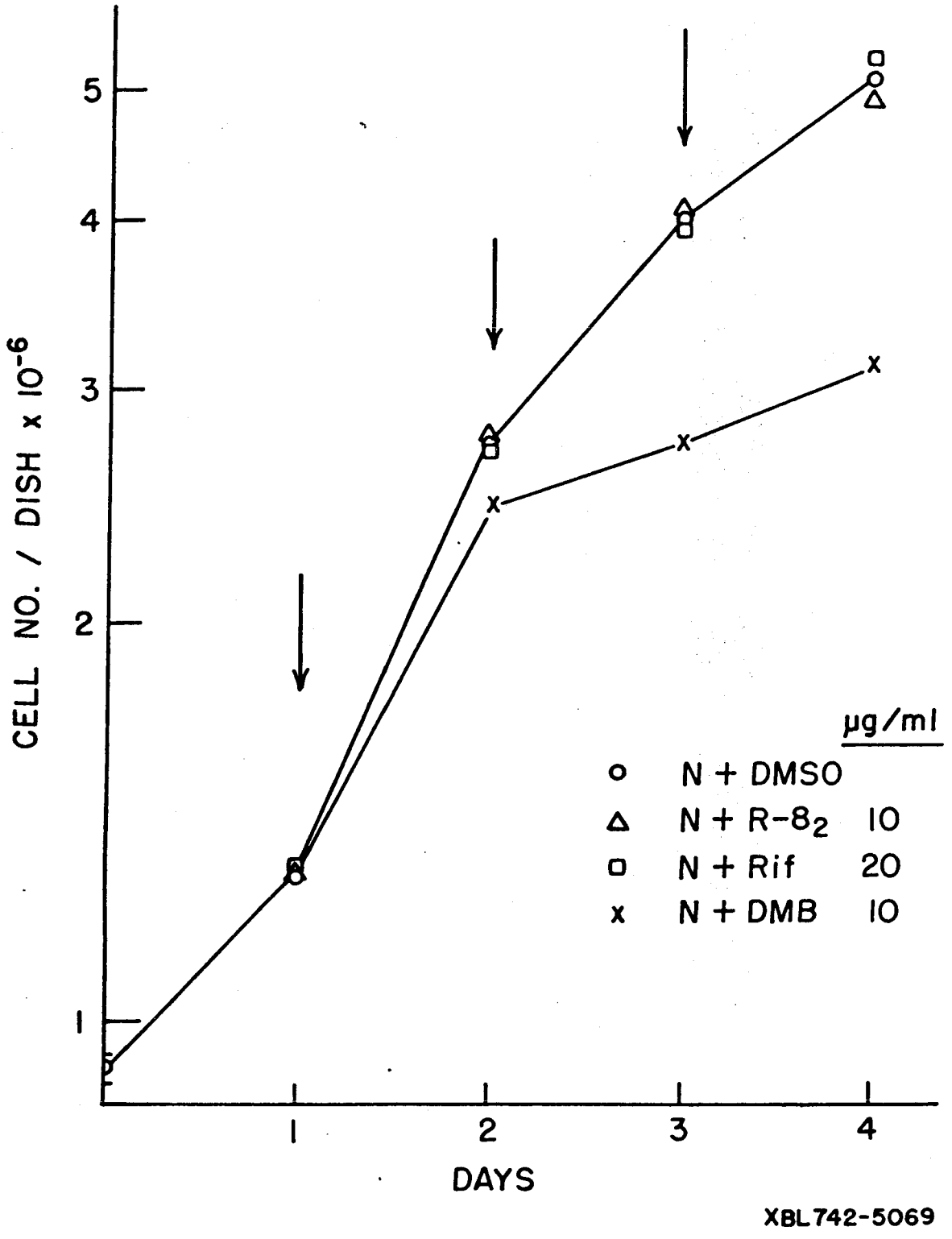
Fig. 1.


CELL NO. / DISH x $10^{-6}$
5
4
3
2
1
1
2
3
4
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µg/ml
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N + R-$8_2$ 10
N + Rif 20
N + DMB 10
XBL742-5069

Fig. 2.

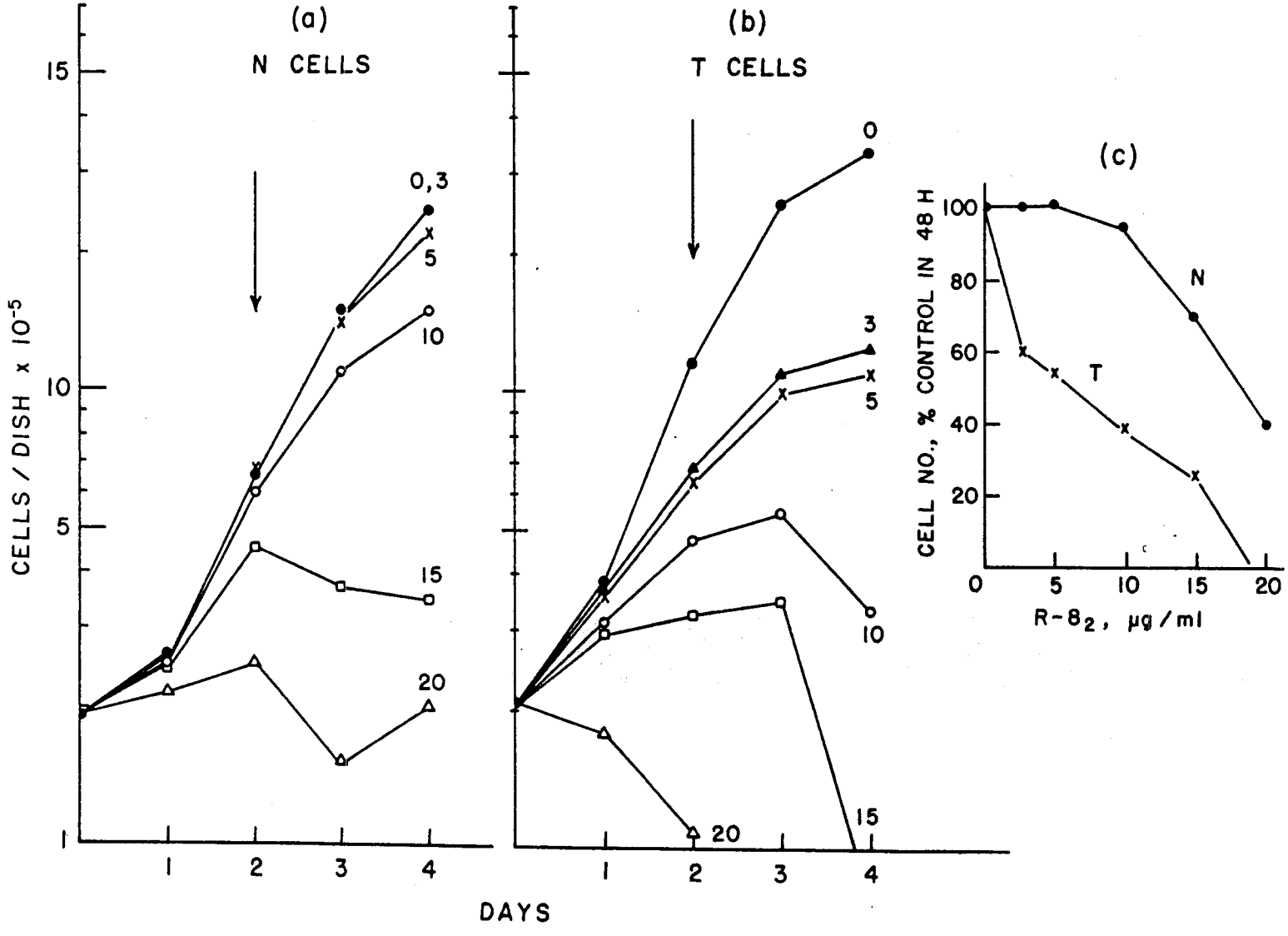


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Fig. 3.

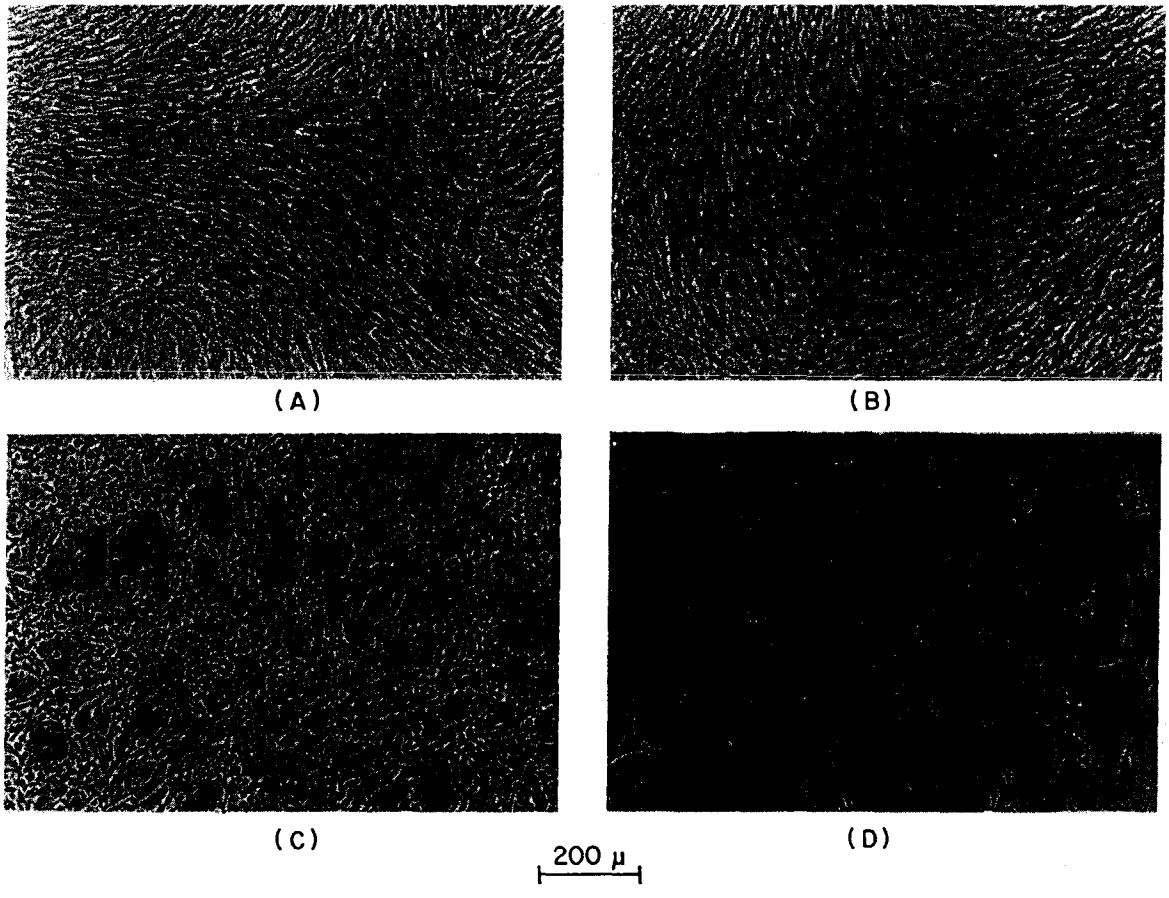


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Fig. 4.

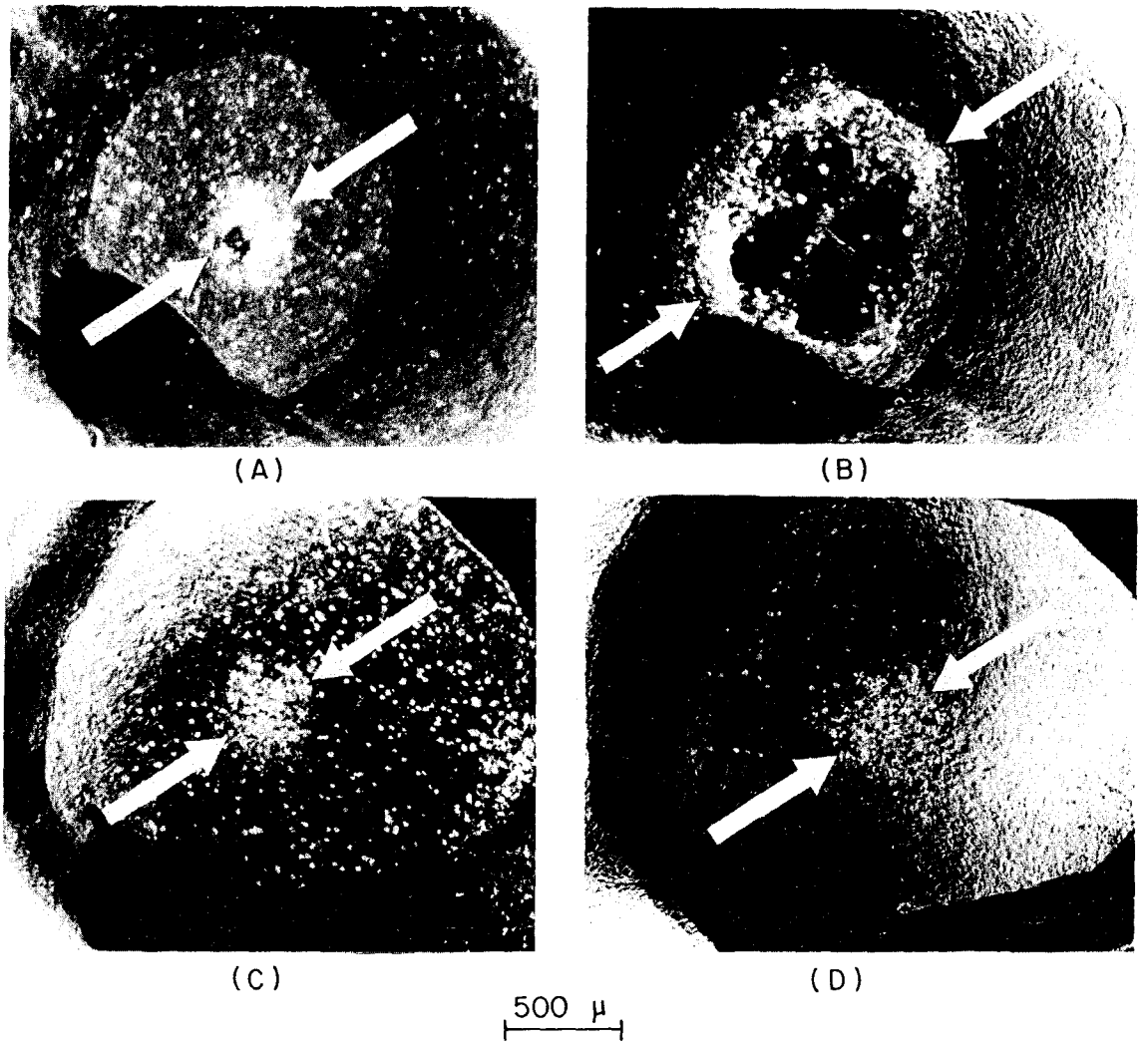


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Fig. 5.

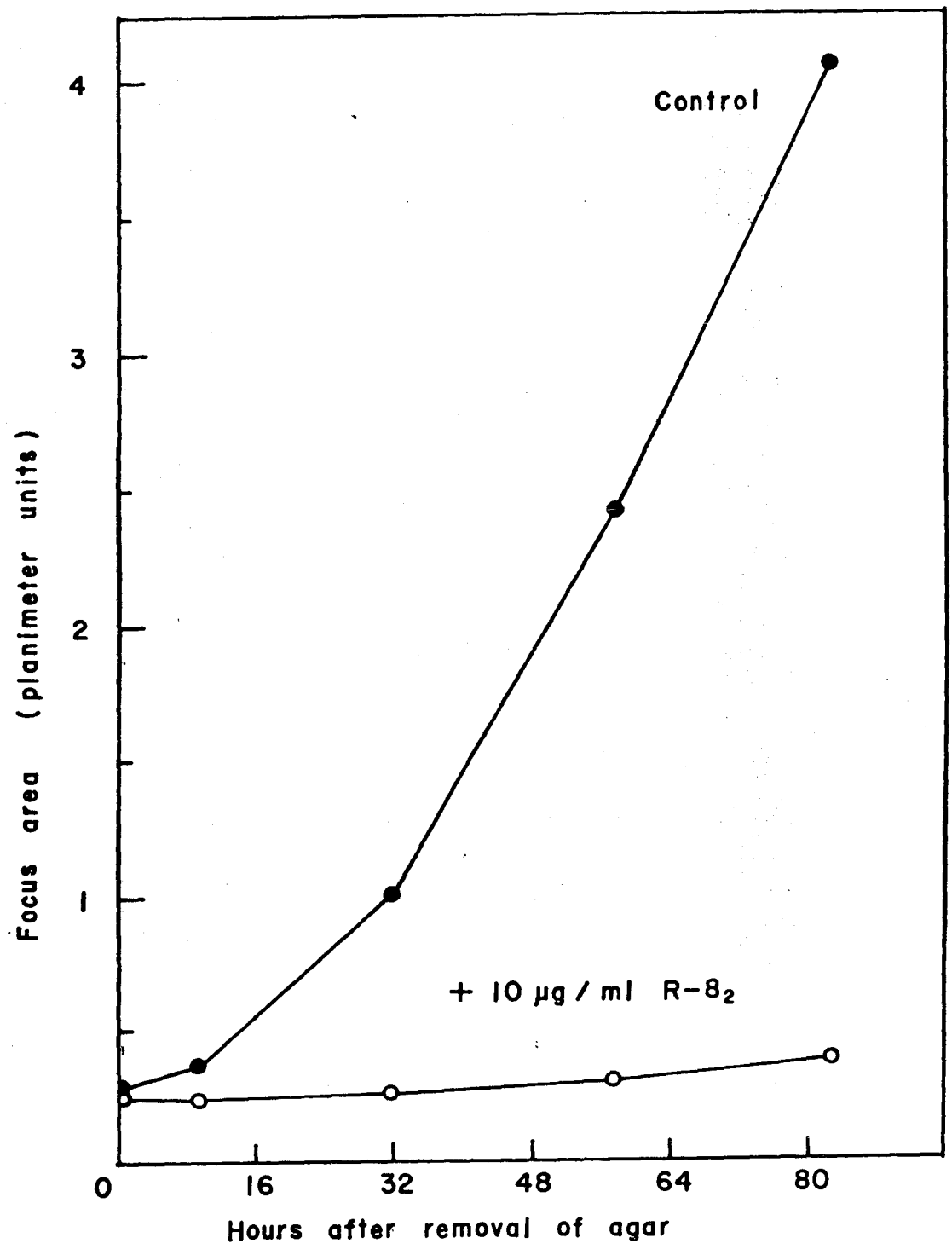
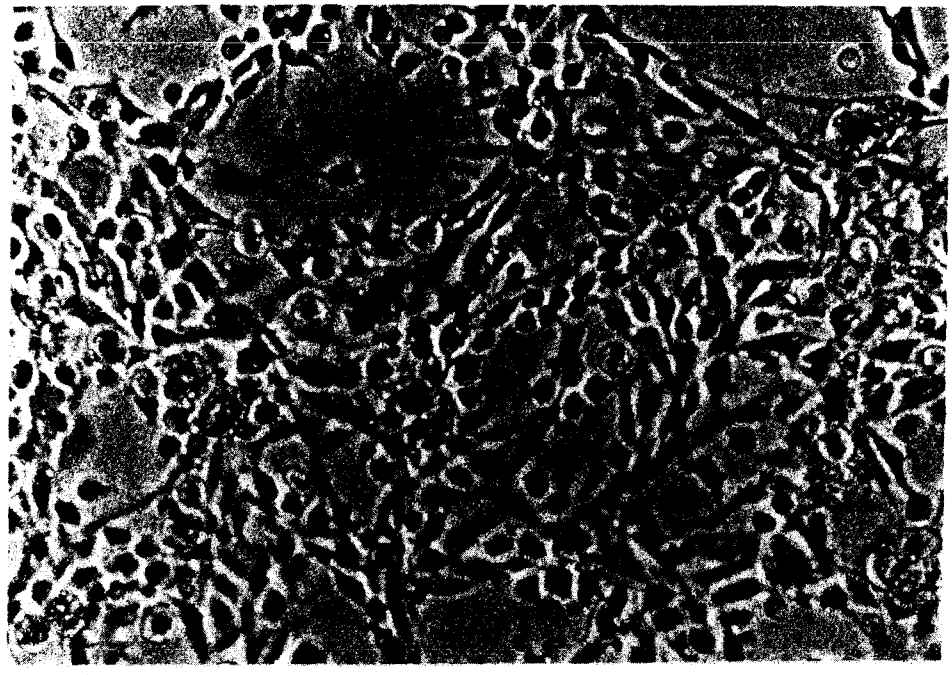
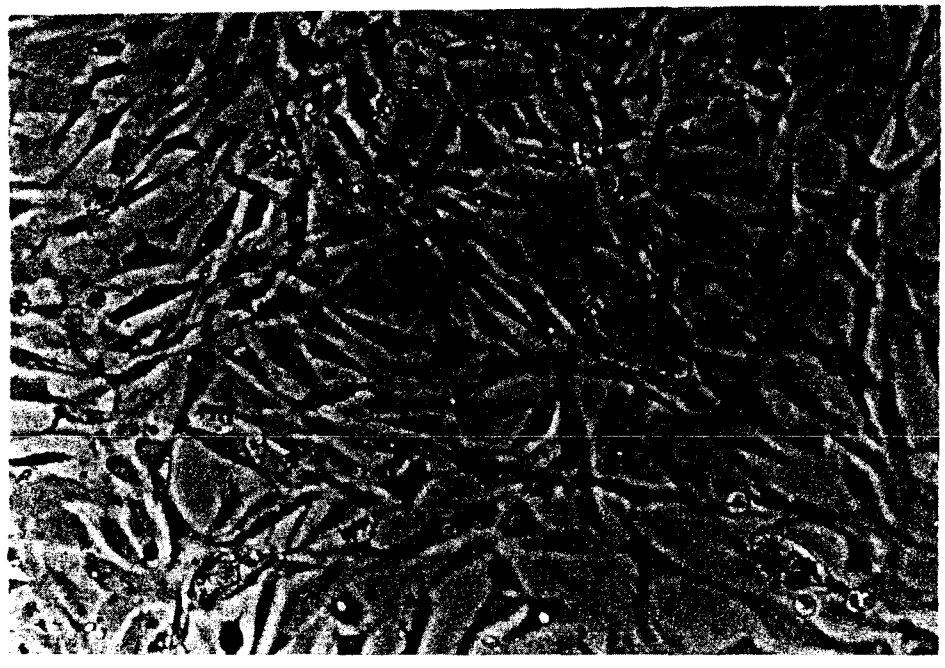

Control
+ 10 µg / ml R-8₂
Focus area (planimeter units)
0
1
2
3
4
16
32
48
64
80
Hours after removal of agar
XBL743-5080

Fig. 6.



(A)



(B)

80 μ

XBB 742-1092

Fig. 7.