tions. 5. These observations are discussed in terms of present knowledge of the metabolism of epiphyseal cartilage.

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## Inhibition of Plaque Formation by Myxoma and Fibroma Viruses with Pyrimidine Analogues.* (30571)

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Several pyrimidine analogues have been reported to interfere with the synthesis of infective viral DNA(1). Our studies reveal that infection of tissue culture with myxoma or fibroma viruses is a good model system for detection of such viral DNA antimetabolites.

*Materials and methods. A. Tissue cultures. 1. Rabbit testicular continuous cell line ($RT_2$).* $RT_2$ cells, a continuous cell line initiated in our laboratories, were grown with Eagle's medium in Hanks' BSS + 10% fetal bovine serum in 16-oz prescription bottles. Roller tube cultures were prepared from these cultures.

*2. Primary rabbit kidney cultures (PRK).* Kidneys from 3-week-old rabbits were trypsinized and planted in 2-liter Povitsky bottles, using Eagle's medium in Hanks' BSS supplemented with 5% calf serum. The Povitsky cultures were expanded into 3-oz prescription bottles employing 10 ml Eagle's + 5% calf serum.

*3. Rabbit kidney continuous cell line ($RK_{13}$) -glaxo.* The growth medium consisted of medium 199 + 5% fetal bovine serum which was reduced to 2% in the maintenance medium. The same procedure was employed as with the PRK cells.

*B. Test systems.* In $RT_2$ cells the Sanarelli myxoma virus was added to the roller tubes in 0.2 ml amounts, and the cultures immediately fed with 2 ml of maintenance medium containing the desired concentration of compound. The cultures were examined daily for CPE.

In PRK and $RK_{13}$ cultures the myxoma virus was added to the monolayer for 2 hours to allow adsorption, the cultures washed twice, then 10 ml of the maintenance medium containing compounds was added. After incubation at 37°C for 4 days the fluids were removed and plaques counted under a stereoscopic microscope after staining with carbol fuchsin. The Kilham fibroma virus did not

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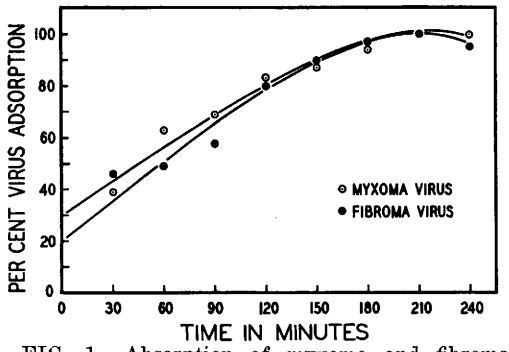


FIG. 1. Absorption of myxoma and fibroma viruses to monolayers of $RK_{13}$ cells.

produce sufficiently well defined plaques in the $RT_2$ and PRK cells to warrant testing compounds.

Replicate cultures of 7 to 10 bottles were performed in several tests in both the PRK and $RK_{13}$ cell lines to determine assay variation.

Serial 3-fold dilutions of myxoma and fibroma viruses were titrated in duplicate in $RK_{13}$ cells, and the PFU's were plotted against relative virus concentration. In addition, virus was added to monolayers and allowed to adsorb for varying periods of time, after which unadsorbed virus was removed by 2 to 3 fluid changes to determine time of maximum adsorption.

To determine the effect of compounds on the free virus, and/or adsorption of virus to cells, dilutions of compounds were added to myxoma and fibroma viruses, and incubated at 37°C for one hour, then added to monolayers for 2 to 3 hours. The fluids were removed and after washing of the cell sheets 2 to 3 times with phosphate buffered saline (PBS) the cultures were reconstituted with maintenance medium.

The compounds† were tested for toxicity by addition of varying concentrations in maintenance medium to monolayers which were observed macro- and microscopically for cellular degeneration.

*Results.* The per cent adsorption of myxoma virus to $RK_{13}$ monolayers after 30, 60, 120, and 180 minutes is 39, 63, 83, and 94, and for fibroma virus 46, 49, 80, and 97, respectively (Fig. 1).

When the number of PFU's was plotted against relative virus concentrations (Fig. 2) a slope of approximately one was obtained, thus confirming Schwerdt's(2) data.

A series of tests in replicate showed a variation of 2 standard deviations (Table I) ranging from 21% to 44% for myxoma virus in 8 tests, averaging 31%; and 29% to 41%, averaging 34% for fibroma virus in 3 different tests using $RK_{13}$ cells. In earlier tests the $RT_2$ line proved to be very unstable, and the variance in PRK cells averaged 87%.

TABLE I. Precision of Plaque Assay of Myxoma Virus in $RK_{13}$ Cultures.

| Experiment No. | No. bottles | Range ($M \pm 2\sigma$)* | $\frac{200\sigma}{M}$ (%) | Avg |
|---|---|---|---|---|
| 1 | 10 | $68 \pm 30$ | 44 | |
| 2 | 10 | $27 \pm 12$ | 44 | |
| 3 | 10 | $63 \pm 20$ | 32 | |
| 4 | 10 | $67 \pm 16$ | 24 | 31 |
| 5 | 7 | $92 \pm 20$ | 21 | |
| 6 | 7 | $74 \pm 18$ | 23 | |
| 7 | 6 | $43 \pm 10$ | 23 | |
| 8 | 7 | $35 \pm 14$ | 40 | |
| Precision of Plaque Assay of Fibroma Virus in $RK_{13}$ Cultures | | | | |
| 1 | 10 | $34 \pm 14$ | 41 | |
| 2 | 10 | $46 \pm 13$ | 29 | 34 |
| 3 | 10 | $45 \pm 15$ | 33 | |

* M = mean; $\sigma$ = standard deviation.

The earliest testing of compounds was car-

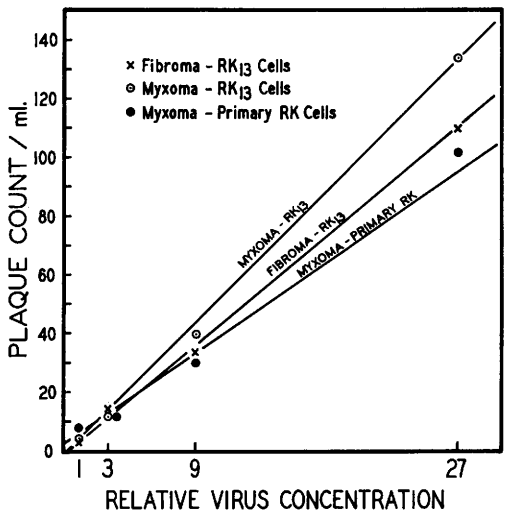


FIG. 2. Relationship between plaque counts and relative virus concentration.

† Supplied by the CCNSC through the courtesy of Dr. Michael A. Chirigos and Dr. Randall Thompson.

TABLE II. Inhibition of Myxoma Infection in $RT_2$ Cells.

| Compound | Conc, μg/ml | Logs of virus inhibited | | | |
|---|---|---|---|---|---|
| | | Exp 1 | Exp 2 | Exp 3 | Avg |
| CA | 1 | 2.5 | 1.0 | 3.0 | 2.6 |
| IUDR | 500 | 3.0 | 5.7 | 4.0 | 5.2 |
| BUDR | 1000 | 3.5 | 5.7 | 4.5 | 5.1 |
| TA | 100 | 3.0 | — | 4.0 | 3.7 |

ried out in $RT_2$ cells. Of the 14 tested 4 were found to inhibit CPE of myxoma virus. 5-Iodo-2′-deoxyuridine (IUDR) and 5-bromo-2′-deoxyuridine (BUDR) were non-toxic in concentrations greater than 1000 μg/ml and were inhibitory to virus in concentrations as low as 10 μg/ml. 1-β-D arabinofuranosyl cytosine (CA) was slightly toxic at 1 μg/ml, but inhibitory at a concentration of 0.1 μg/ml. Thymine 1-β-D arabinofuranosyl (TA) was toxic at concentrations greater than 100 μg/ml and inhibitory at 1 μg/ml. The following compounds were non-inhibitory: 6-mercaptopurine (6-MP); N-methyl isatin thiosemicarbazone (NMITC); 5,6 dichlorobenzimidazole, 1-β-D ribofuranosyl (DRB); 5-bromouracil (BU); pseudourea, 2-(3-(2-benzimidazolyl) propyl -2- thio); 2,6-diamino purine hydrate; 4,4′-biphenyl-diglyoxyl aldehyde dihydrate; 1-H pyrazolo (3,4-d) pyrimidine; 2-benzimidazole methanol, α phenyl (HBB); pryrazolo (3,4-d) pyrimidine 4-01.

In further tests the myxoma virus was titrated to determine the actual amount of virus inhibited (Table II). CA at a concentration of 1 μg/ml reduced the infectivity by an average of 2.6 logs. IUDR, BUDR, and TA at concentrations of 500, 1000, and 100 μg/ml reduced infectivity by 5.2, 5.1, and 3.7 logs, respectively.

Because of the sensitivity of the $RT_2$ cells to toxicity of the compounds we investigated PRK cells. Although the variation of the test in the PRK line was larger than desired, this line proved to be useful in screening of compounds for inhibition of myxoma virus (Table III). Usually from 2 to 6 tests were performed for each concentration of compound and results averaged. In the PRK cell lines IUDR, BUDR, 5-fluoro-2′-deoxyuridine (FUDR), CA, TA, and 6-azauridine (AZUR) were found to completely inhibit plaque formation by myxoma virus. CA proved to be the most active and most toxic compound, inhibiting at concentrations as low as 0.001 μg/ml. IUDR and BUDR were similar in activity, producing 50% and 34% inhibition, respectively, at levels of 0.1 μg/ml. TA and AZUR were next in order of activity whereas FUDR was least active.

Attention was next turned to the newly acquired $RK_{13}$ line for testing of antiviral compounds, using both myxoma and fibroma viruses. This was a stable line which propagated rapidly. The plaques produced by myxoma virus are clearly defined areas of degeneration surrounded by heavily stained cells. Fibroma virus produces heavily stained areas of cells which appear to be clumps of aggregated and fused cells.

After determination of toxic levels, varying concentrations of compounds were tested at least twice, or more frequently if the results were inconsistent. The following compounds strongly suppressed plaque formation by both viruses: namely, IUDR, BUDR, AZUR, CA, TA, 5-fluorouracil (FU), NMITC, and 4-amino-N-methylfolic acid (Methotrexate). A partial inhibition of both viruses was noted with HBB and FUDR. The inhibition of 6-MP was weak for myxoma, and questionable for fibroma (Table IV). No significant inhibition was obtained with BU, DRB, 1-H pyrazolo (3,4-d) pyrimidine; pseudourea, 2-(3-(2-benzimidazolyl) propyl -2- thio); 2,6-diamino purine hydrate; 4,4′-biphenyl-diglyoxyl aldehyde dihydrate.

None of the active compounds exhibited virucidal activity, nor prevented adsorption of myxoma and fibroma viruses to the $RK_{13}$ cells.

TABLE III. Inhibition of Myxoma Virus in Primary Rabbit Kidney Cells.

| Conc, μg/ml | Compounds | | | | | |
|---|---|---|---|---|---|---|
| | IUDR | BUDR | FUDR | CA | TA | AZUR |
| 100 | — | — | 100* | — | 100 | — |
| 5 | 100 | — | 91 | — | 83 | 100 |
| 1 | 95 | 100 | 0 | T† | 64 | 57 |
| .5 | — | 47 | | 100 | 42 | — |
| .1 | 50 | 34 | 0 | 100 | 5 | — |
| .001 | — | — | | 62 | 0 | — |

* % Inhibition of plaques. Avg of 2-10 tests.
† T = toxicity. — = not tested.

TABLE IV. Inhibition of Myxoma and Fibroma Viruses in $RK_{13}$ Cells.

| Compound | μg/ml | % Inhibition of plaques* Myxoma | Fibroma |
|---|---|---|---|
| FU | 100 | 67 | 100 |
| | 10 | 28 | 11 |
| | 1 | 0 | 18 |
| HBB | 100 | 52 | 37 |
| | 10 | 6 | 34 |
| | 1 | 12 | 30 |
| Methotrexate | 100 | 55† | 87 |
| | 10 | 69† | 46 |
| | 1 | 69† | 50 |
| IUDR | 10 | 100 | 100 |
| | 1 | 47 | 99 |
| | .1 | 2 | 30 |
| FUDR | 10 | † | 14 |
| | 1 | 95 | 57 |
| | .1 | 62 | — |
| BUDR | 10 | 100 | 100 |
| | 1 | 47 | 100 |
| | .1 | 10 | 17 |
| AZUR | 10 | 93 | 100 |
| | 1 | 85 | 98 |
| | .1 | 40 | 66 |
| CA | 1 | 100 | 83 |
| | .1 | 56 | 39 |
| | .001 | 23 | 20 |
| TA | 100 | 100 | 97 |
| | 10 | — | 57 |
| | 1 | 0 | 20 |
| 6-MP | 12.5 | | 31 |
| | 5 | 66 | 4 |
| | 1 | 18 | — |
| NMITC | 50 | 100 | 92 |
| | 25-12.5 | 44 | 52 |
| | 10 | 50 | — |

* Avg of 2 to 4 tests. — = not tested.
† Some cell toxicity.

For the most part the dosage responses were of 2 types, differing in steepness. BUDR showed the typical 2 types of response to myxoma and fibroma viruses which are seen in Fig. 3.

*Discussion.* Our results confirm and extend those of previous investigators in that these inhibitory compounds do not exert a direct virucidal activity, nor do they interfere with adsorption of the virus to the cells. In general, the mechanism of action appears to be an intracellular interference with enzyme systems necessary for synthesis of infective viral DNA(3,4).

One question of interpretation is whether or not the reduced number of plaques in a

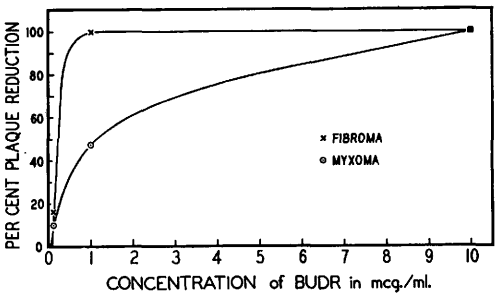


FIG. 3. Reduction of plaque counts of myxoma and fibroma viruses by BUDR.

"thin" or partially degenerated monolayer caused by toxicity of the compound should be interpreted as inhibitive due to the compound *per se*, or due to inability of such cells to support viral replication. We attempted to perform most tests using subtoxic concentrations. Some of the compounds such as IUDR, BUDR, TA, AZUR, and Methotrexate were inhibitory at concentrations 100 times or more lower than their toxic levels. Others were active at levels not too far removed from their toxic concentrations: *e.g.*, NMITC, CA, FU, and FUDR. The data obtained using these latter compounds were generally more inconsistent from test to test. Considerable inconsistency was noted in the testing of 6-MP and HBB for reasons not known. FUDR usually exerted greater inhibition at 10 μg than at 1 μg.

Although fibroma and virulent myxoma viruses produce a completely different clinical response in adult domestic rabbits and a different type of cell micropathology, the two viruses are closely related serologically. It was of interest that most of the active compounds inhibited both myxoma and fibroma viruses to a similar enough degree to warrant using only one for future screening.

NMITC was classified as inactive against myxoma virus in $RT_2$ cells but was found to be inhibitory in $RK_{13}$ cells. Rapp(4) reported that NMITC did not inhibit plaque formation of herpes zoster and herpes simplex viruses, although IUDR and CA were active; however, NMITC was employed at lower concentrations than in our tests. Since cell toxicity and antiviral effect do not appear to coincide one should consider using the most

stable line in screening compounds.

*Summary.* Of 3 tissue culture systems tested, namely, Rabbit Testicular, Primary Rabbit Kidney, and $RK_{13}$, the latter proved to be the most satisfactory for testing of inhibition of myxoma and fibroma viruses. Plaque formations by both viruses were completely or partially inhibited by compounds in the following order of effectiveness: CA, IUDR, AZUR, Methotrexate, BUDR, TA, NMITC, FU, FUDR. 6-MP and HBB exhibited moderate inhibition of myxoma virus, and weak variable inhibition of fibroma virus. The effective compounds were not virucidal and did not prevent adsorption of virus to cells.

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## Erythropoiesis During Pregnancy and Lactation. I. Effect of Various Hormones on Erythropoiesis During Lactation.* (30572)

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Because lactating rats have been found to have an increased red cell volume(1), and lactating mouse plasma and prolactin stimulate erythropoiesis(2), the red cell volume (RCV) and plasma erythropoietic activity were measured in postpartum lactating and nonlactating mice. The effect on the RCV of various hormones, known to be active in maintenance of lactation, also was studied in postpartum nonlactating mice and the erythropoietic activities of these hormones were assayed in polycythemic mice.

*Materials and methods.* Inbred virgin Swiss mice with a gestation of 20 days were used for breeding purposes in these experiments. Parturition and day 0 are used interchangeably to designate 0-12 hours after birth. Groups of nonlactating mice were obtained by removing the newborn during this period. The RCV was determined in parturition mice and in lactating and nonlactating mice on the days shown in Fig. 1. All RCVs were calculated from the total blood volume (TBV), determined with the $I^{131}$-tagged albumin dilution technique(3) and from the hematocrit corrected for the total body/venous hematocrit ratio(4). The RCV values of normal and prolactin treated mice obtained by this method closely agreed with those previously obtained by use of the Cr-51 *in vitro* tagged erythrocyte technique(2) in normal and prolactin injected mice.

Plasma was collected and pooled separately from each group of mice shown in Fig. 2 and then assayed as previously described in polycythemic mice(2), using a 72-hour incorporation of $Fe^{59}$ into erythrocytes as a measure of erythropoietic activity.

In a second group of experiments, non-lactating mice received subcutaneous injections of 250 or 500 $\mu$g of prolactin† for 15 days. Injection of 500 $\mu$g of prolactin was started at 0-12, 24-36 and 48-60 hours from parturition in order to determine if other factors present in the plasma of early postpartum mice enhance or inhibit the response to prolactin. Starting at 0-12 hours from birth, other groups of nonlactating mice were in-

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