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## Storage of Pyrimethamine in Cells *in vitro*. (23987)

M. KATHERINE COOK* AND LEON JACOBS (Introduced by J. Warren)

*U. S. Department of Health, P.H.S., N.I.H., Nat. Inst. of Allergy and Infectious Diseases,† Bethesda, Md.*

Residual pharmacological activity of certain insoluble drugs is maintained by injection of these substances, intramuscularly, to form a "depot" from which they are slowly and continuously released. Other drugs manifest prolonged activity without the need for such means of injection, and it is known that in these cases the substances are differentially distributed in the body. The binding of pharmacologically active substances in tissues or cells is not clearly understood and there is little information on occurrence of this phenomenon *in vitro*. Pyrimethamine (Daraprim) manifests prolonged residual activity following oral administration(1). Schmidt *et al.*(2) determined the concentrations of pyrimethamine in plasma, erythrocytes, and other tissues of rats and monkeys. It was shown that all fixed tissues examined contained significantly higher concentrations of drug than blood plasma. Smith and Ihrig(3) have also demonstrated that pyrimethamine persists for considerable periods in the body of both man and monkey. We observed previously(4) that in monkey kidney tissue cultures infected with *Toxoplasma gondii*, treated with pyrimethamine for 7 days, and then incubated for 11 days without drug, cellular degeneration due to proliferation of the parasite did not occur. Nevertheless, by inoculation of mice with these cultures immediately after removal of drug, the presence of toxoplasmas could frequently be demonstrated. These results were explained by possible storage of pyrimethamine, at a level inhibitory to the parasite, within cells growing in tissue culture. Experiments reported here were designed to demonstrate this storage of pyrimethamine in cells *in vitro* and to define the conditions under which it can occur.

*Materials and methods. Tissue culture*: Monkey kidney tissue cultures were prepared and maintained by the method of Melnick (5). Mouse mince cultures were prepared by the method of Chernin and Weller(6). Chick embryo heart explant cultures were prepared by standard plasma clot technics. HeLa cells, KB cells, conjunctival epithelium (Chang), and human intestinal cells (Henle) were purchased from Microbiological Associates, and maintained in Eagle's medium(7) with 10% calf serum, 100 units penicillin, and 100 $\mu$g streptomycin/ml. (HeLa maintenance medium). *Drug Solutions*. Daraprim, a brand of pyrimethamine, was supplied through the courtesy of Dr. G. H. Hitchings of Burroughs & Wellcome Research Laboratories, Tuckahoe, N. Y. Stock solutions of 100 mg % pyrimethamine were prepared by bringing 100 mg of pyrimethamine dissolved in 2 ml lactic acid, U.S.P.-85%, to 100 ml with maintenance medium. This drug solution was sterilized by 02 Selas filtration and stored at 4°C. All other solutions of pyrimethamine were prepared from such stock solutions. *Mice*. NIH general purpose albino female mice of 14-20 g were used. *Protozoa*. The RH strain of *Toxoplasma gondii* was used in all tests. Methods of handling the parasite have been described elsewhere (Cook and Jacobs, in press).

*Results*. Preliminary experiments to test storage of pyrimethamine in cells growing in tissue culture were performed as follows:

* Present address: Nat. Inst. of Allergy.

† Laboratory of Tropical Diseases.

TABLE I. Duration of Storage of Pyrimethamine in Monkey Kidney Epithelial Cells after Treatment for 5 Days with Various Levels of Drug. Lysis times of cultures and survival days of mice inoculated from them following challenge* with *Toxoplasma* on designated days after removal of drug.†

| | Day of challenge of cultures with *Toxoplasma* | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Conc. | 0 | | 1 | | 3 | | 4 | | 5 | | 7 | | 14 | |
| of drug | LT‡ | M‡ | LT | M | LT | M | LT | M | LT | M | LT | M | LT | M |
| .00 | 3 | 5.0 | 3 | 5.0 | 3 | 7.5 | 3 | 6.0 | 3 | 5.0 | 3 | 6.0 | 3 | 6.0 |
| .05 | 7 | 6.0 | 6 | 5.0 | 4 | 6.0 | | | | | | | | |
| .1 | N§ | 5.5 | 8 | 6.0 | 10 | 6.5 | 3 | 6.5 | 3 | 5.0 | 3 | 6.5 | 3 | 6.0 |
| .15 | N | 7.5 | N | 7.5 | N | 7.0 | N | 6.5 | 8 | 5.5 | 7 | 7.0 | 4 | 6.0 |
| .30 | N | 9.0 | | | | | N | 9.0 | N | 6.5 | N | 9.5 | | |
| .50 | N | 10.5 | | | | | | | | | N | S | N | S |

* Challenge was with $2 \times 10^6$ toxoplasmas. † Day of removal of drug = day 0.
‡ LT = lysis time of tissue cultures; M = avg survival days of mice inoculated from cultures.
§ N = No lysis was observed in 13 days of observation of cultures.

Cultures of monkey kidney epithelium were exposed for one week to 0.5 mg % pyrimethamine before being infected with *T. gondii*. Pyrimethamine-treated cells were washed 3 times and renewed with 1.5 ml of maintenance medium. These cultures were challenged with 200,000 toxoplasmas immediately after washing. No lysis occurred within 7-day incubation period while similarly infected control cultures showed complete cellular degeneration in 4 days. In another experiment, cultures were infected with 200,000 toxoplasmas for one or 2 days prior to exposure to pyrimethamine at concentration of 0.5 mg % in nutrient medium and kept on drug for an additional day after infection, after which they were renewed with maintenance medium. These cultures showed no lysis during observation period of 17 days while control cultures lysed in 5 days.

In additional experiments, monkey kidney tissue cultures were treated with 0.5 mg % pyrimethamine for one week and then were challenged with 2,000,000 toxoplasmas. After 9 days' additional incubation, none of these cultures showed cytopathic effects and mouse inoculations with cell suspensions from these tubes were negative. Other cultures treated with 0.05 mg % pyrimethamine for 5 days before receiving the same challenge showed 4+ lysis on day 4. Control tubes of untreated *Toxoplasma*-infected cells showed 4+ lysis in 3 days and mice inoculated with cell suspensions from these tubes died in 6 days.

Further experiments to define drug storage in monkey kidney cells relative to concentration of drug in nutrient medium were performed. The results of one of these experiments are presented in Table I.

At concentrations of 0.15 mg %, pyrimethamine was apparently stored for 4 or more days in the cells. Cultures infected 4 days after removal of the drug did not lyse during 13 day observation period; cultures infected 5 and 7 days after removal of drug showed some delay in lysis time. However, concentration of stored drug was obviously not high enough to cause death of parasites, since mice inoculated from these cultures died of toxoplasmosis. At 0.30 mg %, storage was similarly evident for periods to one week, and at 0.50 mg % for as long as 2 weeks. With the latter concentration, enough drug was stored to kill the parasites. In other experiments, some storage of drug for 3 weeks, but not for 4 weeks, was seen in cultures exposed for 5 days to 0.50 mg % pyrimethamine, as evidenced by a delay in lysis of cultures following challenge with *Toxoplasma;* mice inoculated from these cultures died of toxoplasmosis, but there was an increase in their survival time.

Replicate experiments, performed with different cell lots, showed that these results were not always consistent. To determine the cause of these discrepancies, other experiments were performed to ascertain whether extent of drug storage could be correlated with age of cells.

Cultures one to 4 weeks old were treated with pyrimethamine at 0.5 mg % for 5 days and then were challenged with 2,000,000 toxoplasmas after additional intervals of one to 4 weeks. Mouse inoculations were performed to

demonstrate presence of parasites. A difference was apparent between 4-week-old cells and younger cells, in that mice inoculated with the latter survived the longest, while the oldest cells showed no difference from non-treated control tubes. Drug storage apparently was at a maximum in 14-day tissue culture cells although storage was also demonstrated at moderate levels in 7- and 21-day-old cells. These differences possibly are related to variations observed in previous storage experiments. The supernatant fluid of 7-day tissue cultures did not show the rapid drop in pH characteristic of older cultures. This may have been due in part to smaller number of cells present in cultures. Possibly there is a correlation between this difference in the ability of these cells to lower the pH and their ability to store pyrimethamine, especially if the drug is activated in host cells in some manner. Twenty-one and 28-day cells had passed their peak as regards cell division and necrotic areas could be seen in some of the cell sheaths in these cultures but rapid drops in pH of medium continued to occur. The failure to demonstrate storage or the lower rates of storage in older cultures may be due in part to presence of necrotic areas in the cell sheaths.

Additional tissue culture experiments designed to compare abilities of other cells to store pyrimethamine were performed. In every case 0.5 mg % pyrimethamine in HeLa maintenance medium and a 7-day drug exposure time were used. At end of exposure period the cultures were washed 3 times with maintenance medium, were replaced with HeLa maintenance medium and were reincubated for 2 additional days. At the end of this 2-day "rest" period the cultures received another medium change, were challenged with 200,000 RH strain toxoplasmas and were reincubated for one week. Control tubes were non-drugged cells receiving a similar inoculation of toxoplasmas. Inoculations into mice of pools of these cell cultures were performed at end of this incubation period to check for presence of viable parasites. All tissue cultures used in these experiments were incubated in stationary racks. The results of one of these experiments may be seen in Table II.

TABLE II. Comparison of Ability of Various Tissue Culture Cells to Store Pyrimethamine.

| Tissue culture system | No. of mice dead of 5 inoculated from tissue cultures challenged with 200,000 RH toxoplasmas | |
|---|---|---|
| | Cells treated with .5 mg % pyrimethamine | Untreated cells |
| Monkey kidney epithelium | 0 | 5 |
| Mouse mince cultures | 5 ( 9 )* | 5 ( 8 ) |
| Chick embryo heart explants | 0 | 5 ( 6 ) |
| HeLa cells | 0 | 5 ( 6 ) |
| KB cells | 4 (10 ) | 5 ( 7.5) |
| Conjunctival epithelium (Chang) | 5 (10.5) | 5 (10.5) |
| Human intestinal cells (Henle) | 0 | 5 (11.0) |

* Figures in parentheses are avg survival days of mice.

It can be seen that when 0.5 mg % pyrimethamine is present in the nutrient medium, monkey kidney cells, chick embryo heart explants, HeLa cells and human intestinal cells are able to store pyrimethamine in levels inhibitory to the parasite. Slight increases in survival times of mice inoculated from treated versus non-treated cultures indicate that mouse mince cultures and KB cells are not as efficient as the other types of cells in storing pyrimethamine, while conjunctival epithelium (Chang) showed no evidence of drug storage under the conditions used. This may be due to differences in metabolic pathways and rates of metabolism in the cells, as well as to possible differences in permeability of cell membranes and/or the affinity of protoplasmic granules, vacuoles and colloids of the various cells for the drug.

In addition to the above evidence, storage of pyrimethamine in monkey kidney cells was confirmed by microbiological assay technic using inhibition of growth of *Streptococcus faecalis* R as indicator of the presence of antifolic acid activity(3). These assay procedures indicated that pyrimethamine or antifolic acid metabolites of pyrimethamine were present in sonic extracts prepared from monkey kidney cells at least one week after ter-

mination of drug treatment with 0.5 mg % pyrimethamine. Sonic disruption of cells was accomplished by exposing the cellular suspensions to oscillations of a 9 kc Raytheon oscillator for 20 minutes at 0°C. The cell extracts were then centrifuged for 20 minutes at 2,500 rpm to remove gross flocculent material. The supernatant fluid was further cleared by high speed centrifugation at 15,000 rpm for one hour in a Spinco Model L centrifuge. Antifolic activity was not demonstrated in cell extracts prepared by rapid freezing-and-thawing technics.

*Discussion.* Ability of a cell to store a drug or active products of the drug *in vitro* suggests an explanation for the prolonged activity of pyrimethamine. The drug or its active product is probably bound to the proteins in the cell and thus prevented from rapid elution from the cell. The failure of conjunctival epithelium to store pyrimethamine must be confirmed; at present there is no obvious explanation for this difference. The demonstration of anti-folic activity in sonic extracts of cells pretreated with pyrimethamine but not in extracts prepared by freezing and thawing is probably an indication of how tightly the drug is bound to the cellular proteins.

Schmidt *et al.*(2) have already shown prolonged activity of pyrimethamine against *Plasmodium cynomolgi* and localization of the drug and or metabolic products indistinguishable therefrom in tissues of monkeys treated with pyrimethamine. The present report shows storage of pyrimethamine at a cellular level in a tissue culture system and defines the conditions necessary to demonstrate such storage.

*Summary.* 1. Storage of pyrimethamine or active products of pyrimethamine occurs in tissue cultures of monkey kidney treated with the drug. This has been demonstrated by parasite challenges of treated cultures and by microbiological assay with *Streptococcus faecalis R.* 2. The limits of this storage phenomenon have been defined. 3. Some differences in storage of pyrimethamine were noted in monkey kidney cells of various ages. 4. Chick embryo heart explants, HeLa cells and human intestinal cells are able to store pyrimethamine in levels inhibitory to parasite. 5. Mouse mince cultures and KB cells possess a slight ability to store pyrimethamine. 6. Conjunctival epithelium (Chang) shows no evidence of drug storage.

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## Reversal of Oxythiamin Toxicity in Neurospora. (23988)

D. V. SIVA SANKAR* (Introduced by Joseph Reilly)
*University Biochemical Laboratory, Madras, India*

Oxythiamin acts as an antimetabolite of thiamin in the rat and in other organisms(1-5). De Caro *et al.*(5, also 3,4,6) investigated the original finding that oxythiamin increased urinary excretion of thiamin and level of blood pyruvate in the rat, but did not produce the characteristic neuromuscular syndrome produced by administration of pyrithiamin. While both oxythiamin and pyrithiamin could lower the level of thiamin in various tissues, pyrithiamin was much more potent in this respect than oxythiamin. These authors con-

* Present address: Dept. of Chemistry, Adelphi College, Garden City, N. Y.