THE JOURNAL OF INVESTIGATIVE DERMATOLOGY

Vol. 58, No. 5
Printed in U.S.A.

PERCUTANEOUS ABSORPTION OF METHOTREXATE*

P. C. H. NEWBOLD, M.R.C.P.† AND R. B. STOUGHTON, M.D.

ABSTRACT

The *in vitro* penetration of methotrexate (MTX) through human and hairless mouse skin was determined by two techniques. One followed tritiated MTX by scintillation counting and the other followed cold MTX by determining the inhibition of dihydrofolate reductase. Both techniques gave ample evidence for penetration of MTX through the skin of hairless mice and man.

There is no longer any doubt about the efficacy of methotrexate (MTX) in dermatology (1). Toxicity has limited its use in the treatment of psoriasis and other benign dermatoses. As the route of administration and other factors appear to be relevant to the incidence of toxic effects, a number of different regimens have been suggested on theoretical grounds and are now being evaluated. Ideally perhaps, topical application of the drug would reduce the incidence of side effects, and increase the speed of response. The primary biochemical target for the drug, dihydrofolate reductase, is present in the skin, and the level of the enzyme is known to be increased in psoriasis (2). However, clinical studies with topical MTX have been few and the results are conflicting. Fry and McMinn discussed the earlier work and reported some clearing of psoriasis with the use of 0.2% MTX in aqueous cream under polythene occlusion (3). Other studies, using 1% MTX in aqueous dimethylsulfoxide under polythene, produced local pain and ulceration, but no benefit (4). These authors showed that less than 0.55% of topically applied MTX was excreted in the urine over 72 hours, while most of the MTX remained in the stratum corneum. Van Scott and Reinertsen also reported the presence of MTX in the urine after topical application in psoriasis (5). We used two methods to study the penetration of MTX through human skin *in vitro*. One was to apply tritium-labeled MTX and assess penetration on the basis of recovery as measured by liquid scintillation counting. The other method was to assay the recovered MTX on the basis of its ability to inhibit dihydrofolate reductase activity.

METHODS AND MATERIALS

Human leg skin was obtained from amputated limbs. Only skin appearing grossly normal was used, and 16–20 separate specimens could be obtained from each leg. The subcutaneous fat was carefully removed with a scalpel after thawing, and the dermal surface was sponged with ethanol. Separate specimens were cut from each large piece and applied to the mouth of a glass cup with the dermal side facing inwards. The glass cup is open and continuous with a U-tube which is plugged with a glass stopper. This allows filling of the cup with saline or buffer so that the dermis is fully bathed (diagram in ref. 6). A plastic ring 1.5 cm in diameter is cemented to the epidermal side with Duco® cement. Then 0.01 ml of labeled or cold MTX is applied to the epidermal surface, within the plastic ring. The specimens are incubated in a humidity chamber, at 30 °C wet bulb and 33°C dry bulb for 20 hours. Aliquots are then removed for scintillation counting or for biochemical assay of MTX.

The amount of MTX recovered from the saline or buffer on the dermal side is calculated and the percentage penetration can be easily determined, knowing the total amount applied to the epidermal surface.

H^3 labeled methotrexate. The 3′-5′-T sodium salt (250 mCi/mM, Amersham/Searle) was supplied by the generosity of Lederle Laboratories. One millicurie was dissolved in 0.5 ml of dimethylacetamide (7). Four-fold dilutions of this solution were made so that 0.01 ml applied to the skin contained 32, 8, 2, or 0.5 micrograms of MTX per 1.24 cm^2. Standard precautions and corrections were made for quenching. For each experiment, one large skin specimen was divided into 16 separate units so that four penetration determinations could be made for each amount applied. The same procedure was used to study penetration of H^3-MTX through hairless mouse skin. The mice were 3–4 months old, and the skin of the back of one mouse was used for each determination, using the same amounts of labeled MTX. With applications of 0.5 microgram H^3-MTX per 1.24 cm^2 (the lowest amount of radioactivity applied in these experiments) we recovered activity 3–6 times background. One ml aliquots were added to 12 ml of standard toluene based counting fluid, and external standardization was used. Samples were counted with around 16% efficiency.

Cold methotrexate. Commercial MTX (Lederle Laboratories) was made up to a 2.5% solution in 50 mM tris-chloride with 1 M KCl, pH 7.2. The buffer was warmed to 40°C before use, and then cooled. The penetration wells were filled with buffer (10 ml) and all air bubbles were removed. MTX concentrations of 0.5% and 2.5% were used, and 0.01 ml was applied to the epidermal side of each well. A control group, using 0.03 ml of buffer alone was always run in parallel. Light exposure was kept to a minimum during these experiments, because of the known photo-decomposition of MTX. After 20 hours, aliquots were removed, checked for pH,

Received January 14, 1972; accepted for publication February 1, 1972.
This work was supported by Grant No. AM 11649 from the National Institutes of Health. P. C. H. Newbold was the recipient of a travel grant from the Wellcome Foundation.
* From the Division of Dermatology, Scripps Clinic and Research Foundation, 476 Prospect Street, La Jolla, California 92037.
† Present address: Postgraduate Medical School, Hills Road, Cambridge CB2 1QT, England.

and then assayed. Dimethylacetamide (DMAC) was also used for making up the MTX as indicated. In these experiments pure MTX powder was used, which was a generous gift of Dr. Harriet Kiltie of Lederle Laboratories.

Preparation and purification of dihydrofolate reductase. Dihydrofolate reductase was purified from *Lactobacillus casei* strain MTX/R as reported elsewhere (8). The enzyme assay is based upon the decrease in optical density at 340 nonometres, using a multiple sample absorbance recorder, Gilford model 2000. The method of Mathews and Huennekens (9) was used except that the buffer was tris-chloride, 50 mM, pH 7.5, and there was no mercaptoethanol present. All incubations and assays were carried out at 37° C, and under these conditions one unit of enzyme activity will reduce one micromole of dihydrofolate. In all cases, blanks were subtracted from the results.

Biochemical assay of methotrexate. We used the principle of Werkheiser *et al.* (10) based upon the standard assay procedure. The stock *L. casei* dihydrofolate reductase was adjusted to 0.1 units/ml and stabilized with albumin (0.1 mg/ml). The standard assay contained in 1.0 ml, 50 μmoles tris-chloride, pH 7.5, 0.01 unit enzyme, the sample, and the usual amount of dihydrofolate. The cuvette was pre-incubated for one minute at 37° C, and the residual activity assayed following the addition of the standard amount of NADPH. Methotrexate was deemed to be absent from a solution when the absorbance of the control and sample cuvettes was similar. All assays were performed in triplicate, and the average results compared with a graph of the inhibition of the enzyme produced by known concentrations of MTX. The sensitivity of the isotopic method is slightly greater than the assay procedure, but the counting method could include compounds derived from the MTX by degradation or other alteration such that the enzyme might no longer be inhibited. This could explain the uniform presence of radioactivity in samples as compared to frequent absence of activity as measured by the biochemical technique.

RESULTS

H^3 methotrexate. Table I shows the results of the assays using tritiated MTX in human and hairless mouse skin. The amount penetrating varied greatly between specimens, which is usual with this technique. However, it is clear that MTX can penetrate the skin of both man and mouse when applied topically. With the varying amounts of MTX used (0.5–32 μg/1.24 cm^2), there were no significant differences in percentage penetration. However, with hairless mouse skin there seemed to be a greater fraction absorbed with the lower amounts used. Thus, at 32 μg/1.5 cm^2 there was an average of 4.38% penetration compared with 16.3% at 0.5 μg/1.24 cm^2.

Cold methotrexate. The results using human leg skin are summarized in Table II. The solutions were prepared as described above and each set of four results refers to one group of adjacent wells, each on a small portion taken from a large specimen. Individual variations are again extremely wide and it is clear that the absorption from weaker solutions is relatively more efficient. There is a suggestion that DMAC helps absorption, as it reduced the number of zeros, but it did not change overall percentages penetrating at each dose level.

COMMENT

These results show clearly that MTX can be absorbed through the dermis and epidermis, but to a widely variable extent, even between pieces of skin immediately adjoining one another. Some of this discrepancy may reflect variable adherence to the stratum corneum (4). As the enzymic assay for MTX is an optical one, the presence of other compounds with an absorbance at 340 nanometres, whether these are of natural origin, derived from bacteria, or foreign material, could interfer with the assay. It is possible that some drug taken by the patient could influence the assay as well (11), as we had no records for the sources of the skin used. Tissue health, bacterial action, the presence of drugs or their metabolites could all influence the uptake of pteridines in the

TABLE I

Tritium-labeled methotrexate

	MTX applied		% recovered from each sample	Average recovered
Man	0.5 μg	20 hours	2.74, 2.83, 3.43, 5.77	3.69%
	2.0 μg	20 hours	1.26, 1.54, 3.48, 4.19	2.74%
	8.0 μg	20 hours	0.92, 1.98, 3.50, 8.24	3.88%
	32.0 μg	20 hours	0.94, 2.45, 2.74, 6.95	3.27%
Mouse	0.5 μg	20 hours	11.83, 12.46, 14.36, 26.94	16.39%
	2.0 μg	20 hours	8.25, 8.57, 11.07, 14.98	10.72%
	8.0 μg	20 hours	2.47, 2.81, 4.42, 6.00	3.92%
		4 hours*	0.62, 1.98, 5.39, 5.56	3.39%
		20 hours*	2.34, 5.61, 11.02, 19.96	9.73%
	32.0 μg	20 hours	3.12, 3.60, 3.94, 6.85	4.38%
		4 hours*	1.09, 2.15, 2.36, 4.51	2.53%
		20 hours*	2.89, 7.38, 11.77, 19.02	9.52%

* 4 hour and 20 hour samples were from same specimen.

TABLE II

Penetration of cold methotrexate

All incubations were for 20 hours.

Solvent	MTX applied	% MTX recovered from each sample	Average
Buffer	0.1 mg	0, 0, 10.0, 24.0	8.5%
		0, 0, 8.0, 12.0	5.0%
		0, 0, 4.0, 8.0	3.0%
		Above series combined:	5.5%
Buffer	0.5 mg	0, 0, 0, 0	0
		0, 0, 4.8, 5.4	2.55%
		0, 1.3, 1.3, 1.3	0.95%
		Above series combined:	1.15%
DMAC	0.1 mg	0, 4, 12.0, 24.0	10.0%
		4.0, 4.0, 18.0, 20.0	11.5%
		0, 0, 0, 12.0	3.0%
		0, 0, 4.0, 8.0	3.0%
		Above series combined:	7.15%
	0.5 mg	0, 1.9, 2.7, 4.1	2.15%
		1.0, 2.2, 5.0, 7.0	3.8%
		0, 0, 0, 2.2	0.55%
		0, 0, 1.0, 1.0	0.50%
		Above series combined:	1.75%

skin, and perhaps also the level of activity of dihydrofolate reductase. It is known that folate depletion predisposes to MTX toxicity (12), and in laboratory animals MTX uptake is increased by folate depletion (Newbold, P. C. H., unpublished data). High levels of folate can reduce uptake (13), and the rapidly growing cells of the psoriatic population may be protected from inhibition until systemic stores of folinic acid are exhausted (14). Leukemic cells differ widely in their ability to transport antimetabolites, and this favors the selection of resistant lines (15). The same phenomenon may be seen with the relatively immature cells of psoriatic plaques, although they may also gain resistance by altering transport as do L 1210 leukemia cells (16). Rapidly growing cells seem to be more vulnerable to killing by MTX (17) so psoriatic tissue should respond, as indeed it does after parenteral administration. Liver alteration of the drug remains a possibility, although an unlikely one in view of the known fact that the bulk of a dose is rapidly eliminated unchanged in the urine (18). It has been shown that a number of ions considerably influence the activity of dihydrofolate reductase (19), so that passage of MTX through the tissues, especially if they have been altered by death and disease, may alter the assay. Nevertheless, our data show that activity inhibitory to the enzyme is recovered from the dermal side of the absorption wells. Recent work has shown that there are non-specific binding proteins in a number of tissues, which may be important in the uptake, storage and toxicity of MTX (8). Some work in progress suggests that patients with psoriasis have a different pattern, with fewer binding proteins than normal (20). This could explain tissue sensitivity, and uptake patterns, as other evidence suggests that an animal species has one dihydrofolate reductase in all organs and tissues (21). We have confirmed saturability of the uptake mechanism as shown in other tissues (22). This may explain the disparity of the results quoted above (3, 4). Evidence to date suggests that a therapeutic effect might be seen in psoriatic lesions by the application of dilute solutions of MTX under occlusion, protected from light, and following treatment of the lesions with sodium azide (23). Another possibility, actively under investigation, is that some other inhibitor such as a quinazoline may be more effective (24). Antimetabolites can be of great value topically (14) and it may be only a matter of time until psoriasis also can be treated in this way.

We acknowledge helpful advice from and discussions with colleagues, especially Dr. Nigel Harding, Dr. Louis Forman, and Dr. Arthur Rook. We also acknowledge the technical assistance of Mrs. Carolyn Worth.

REFERENCES

1. Burrows, D., Shanks, R. B. and Stevenson, C. J.: Methotrexate in dermatology. Brit. J. Derm., *80:* 348, 1968.
2. Grignani, F., Martelli, M. F., Tonato, M. and Finzi, A.: Folate-dependent enzymes in human epidermis. Arch. Derm., *96:* 577, 1967.
3. Fry, C. and McMinn, R. M. H.: Topical methotrexate in psoriasis. Arch. Derm., *96:* 483, 1967.
4. Comaish, S. and Juhlin, L.: Site of action of methotrexate in psoriasis. Arch. Derm., *100:* 99, 1969.
5. Van Scott, E. and Reinertsen, R.: Morphologic and physiologic effects of chemotherapeutic agents in psoriasis. J. Invest. Derm., *33:* 357, 1959.
6. Stoughton, R. B. and Fritsch, W.: Influence of dimethylsulfoxide on human percutaneous absorption. Arch. Derm., *90:* 512, 1964.
7. Munro, D. D. and Stoughton, R. B.: Dimethylacetamide and dimethylformamide effect on percutaneous absorption. Arch. Derm., *92:* 585, 1965.
8. Newbold, P. C. H. and Harding, N. G. L.: Affinity chromatography of dihydrofolate reductase. Biochem. J., *124:* 1, 1971.
9. Mathews, C. K. and Huennekens, F. M.: Further studies on dihydrofolate reductase. J. Biol. Chem., *238:* 3436, 1963.
10. Werkheiser, W. C., Zakrzewski, S. F. and Nichol, C. A.: Assay for 4-amino folic acid analogs by inhibition of folic acid reductase. J. Pharmacol. Exp. Ther., *137:* 162, 1962.
11. Waxman, S., Corcino, J. J. and Herbert, V.: Drugs, toxins and dietary amino acids affecting vitamin B12 or folic acid absorption or utilization. Amer. J. Med., *48:* 599, 1970.
12. Hellman, S., Iannotti, A. T. and Bertino, J. R.: Determinations of the levels of serum folate in patients with carcinoma of the head and neck treated with methotrexate. Cancer Res., *24:* 105, 1964.
13. Divekar, A. Y., Vaidya, N. R. and Braganca, B. M.: Active transport of aminopterin in Yoshida sarcoma cells. Biochim. Biophys. Acta, *135:* 927, 1967.
14. Nurse, D. S.: Effect of antimetabolites on epidermal structures. Arch. Derm., *87:* 258, 1963.

15. Kessel, D., Hall, T. C. and Roberts, DeW.: Modes of uptake of methotrexate by normal and leukemic human leukocytes in vitro and their relation to drug response. Cancer Res., *28:* 564, 1963.
16. Sirotnak, F. M., Kurita, S. and Hutchison, D. J.: On the nature of a transport alteration determining resistance to amethopterin in the L1210 leukemia. Cancer Res., *28:* 75, 1968.
17. Hryniuk, W. M., Fischer, G. A. and Bertino, J. R.: S-phase cells of rapidly growing and resting populations. Differences in response to methotrexate. Molec. Pharmacol., *5:* 557, 1969.
18. Henderson, E. S., Adamson, R. H., Denham, C. and Oliverio, V. T.: The metabolic fate of tritiated methotrexate. 1. Absorption, excretion, and distribution in mice, rats, dogs, and monkeys. Cancer Res., *25:* 1008, 1965.
19. Reyes, P. and Huennekens, F. M.: Ion-dependent activation of dihydrofolate reductase from L1210 cells. Biochemistry, *6:* 3519, 1967.
20. Newbold, P. C. H. and Harding, N. G. L.: To be published.
21. Bertino, J. R., Iannotti, A. T., Perkins, J. P. and Johns, D. G.: Dihydrofolate reductase from guinea pig liver and small intestine. Biochem. Pharmacol., *15:* 563, 1966.
22. Rubin, R., Owens, E. and Rall, D.: Transport of methotrexate by the choroid plexus. Cancer Res., *28:* 689, 1968.
23. Goldman, I. D.: Transport energetics of the folic acid analogue methotrexate in L1210 cells-enhanced accumulation by metabolic inhibitors. J. Biol. Chem., *244:* 3779, 1969.
24. Hutchison, D. J.: Quinazoline antifolates: Biologic activities. Cancer Chemotherapy Reports, *52:* 697, 1968.