Biochemical Pharmacology, Vol. 28, pp. 189–192.

0006-2952/79/0115-0189 $02.00/0

[^{14}C]-D-PENICILLAMINE: UPTAKE AND DISTRIBUTION IN RAT LYMPHOCYTES AND MACROPHAGES

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(*Received* 13 *March* 1978; *accepted* 14 *June* 1978)

Abstract—[^{14}C]-D-Penicillamine rapidly enters the lymphocytes and freely diffuses out of the cells. Peak levels of radioactivity were found in lymphocytes after 15 min of incubation with [^{14}C]-D-penicillamine and no cell associated label was found after 2 hr of incubation. The macrophages take up [^{14}C]-D-penicillamine in a manner dependent on the drug concentration (range from 50 to 500 μg/ml) and on the duration of incubation (up to 24 hr). The drug is firmly bound to macrophages as 40 per cent of the label is still cell associated after 24 hr culture in drug-free medium. The bulk of radioactivity is membrane associated. The uptake of [^{14}C]-D-penicillamine is significantly increased in $NaIO_4$ pretreated macrophages.

The mode of action of D-penicillamine in rheumatoid arthritis still remains obscure. Several investigations have been focused on its possible action on immunological mechanisms [1–3] and numerous reports have described the effects of D-penicillamine on lymphocyte functions *in vitro* [4–8]. We have recently shown that macrophages preincubated with D-penicillamine are able to modulate the response of purified lymphocytes to the non-specific mitogen Concanavalin A [9, 10]. This finding might suggest that the macrophages are target cells for D-penicillamine.

We have now investigated the uptake of [^{14}C]-D-penicillamine both in purified lymphocytes and macrophages. In addition we have studied the subcellular distribution of D-penicillamine in macrophages and assessed its uptake in macrophages whose membranes were modified by previous treatment with sodium periodate [11].

MATERIALS AND METHODS

Suspensions of lymph node cells and peritoneal macrophages were prepared from inbred, female Lewis rats as described previously [7, 9]. Lymph node cells were depleted of adherent cells (macrophages and monocytes) by culture on glass Petri dishes overnight and peritoneal macrophages were purified by adhesion to 3 cm plastic Petri dishes for 2 hr. The purity of the cell populations was checked by morphological examination of Giemsa-stained preparations and by staining for non-specific esterase of macrophages [12]; less than 1 per cent contaminating cells were found in the purified lymphocyte- and macrophage preparations.

Purified lymphocyte suspensions were adjusted to 2×10^6 cells/ml in RPMI 1640 (25 mM Hepes), containing 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 1 per cent fetal calf serum (Grand Island Biological Co). After equilibration to 37°, [^{14}C]-D-penicillamine in the indicated doses was added to 1 ml aliquots of the cell suspension. After incubation the cells were rapidly filtered off on Celotate filter (Millipore type EAWP) and washed on the filters with 3 × 10 ml saline containing a large excess of unlabelled D-penicillamine (10 mg/ml). The material on the filters was digested overnight with 0.5 N NaOH and counted in 10 ml Dimilume ® (Packard Instruments) in a liquid scintillation counter. Purified macrophages adhering to 3 cm Petri dishes (Nunc No. 1420, Denmark) were incubated at 2×10^6 cells/ml as described for the lymphocytes. After incubation the adherent cells were washed with 3 × 1 ml saline, containing unlabeled D-penicillamine (10 mg/ml) and digested with 0.5 N NaOH.

Crude plasma membrane fractions were prepared from macrophages incubated with [^{14}C]-D-penicillamine for 2 and 24 hr by the method described by Werb and Cohn [13]. Uptake of [^{14}C]-D-penicillamine was determined after digestion with 0.5 N NaOH. Aldehyde groups were induced on purified macrophages by treatment for 10 min at 22° with 10^{-3} M $NaIO_4$ in phosphate-buffered saline at pH 7.2 (PBS), as described by Remold [11]. After washing of the cells with 3 × 1 ml culture medium, the cells were incubated with [^{14}C]-D-penicillamine (100 μg/ml) for 4 hr. Control macrophages were treated in parallel with PBS and the percentage of viable cells was estimated by the eosin exclusion method immediately after $NaIO_4$ treatment and at the end of the experiment. Both control and treated macrophages were more than 90 per cent viable under these conditions. The [^{14}C]-D-penicillamine used in the experiments was a generous gift from Dr. Maul, Isotopenlabor Chemie, Bayer AG. It was supplied as the acetone adduct (D-isopropyliden-penicillamine·HCl) at a specific activity of 3.2 μCi/mg. Just prior to use the adduct was cleaved by heating in distilled water at 100° for 30 min under nitrogen. The purity of the obtained D-penicillamine·HCl was checked by thin layer chromatography in butanol:100 per cent acetic acid:H_2O (4:1:1) [14]. No difference was found compared to unlabeled D-penicillamine (Sigma). The radiochemical purity was assessed by autoradiography, using Agfa Osray M-3 X-ray film. [^{14}C]-D-Penicillamine was adjusted to the desired concentration in RPMI 1640 pH 7.4, calculated on the basis of

the weight of free D-penicillamine obtained from the original acetone adduct. Uptake of [^{14}C]-D-penicillamine in lymphocytes, macrophages and macrophage plasma membrane fractions was expressed as c.p.m./10^6 cells ± standard error of the mean (S.E.M.). Significance of the increase in uptake in $NaIO_4$-treated macrophages as compared to controls was assessed by Student's '*t*' test. All results were corrected for blank values, resulting from adsorption of [^{14}C]-D-penicillamine to Celotate filters or plastic Petri dishes.

RESULTS

Uptake of [^{14}C]-D-*penicillamine in lymphocytes and macrophages.* When purified lymphocytes, depleted of glass-adherent macrophages and monocytes, were incubated with 10 μg/ml of [^{14}C]-D-penicillamine at 37°, a rapid initial uptake of label was seen (Table 1). Maximum uptake was observed after 15 min of incubation. Longer periods of incubation resulted in decreased uptake and after 2 hr of incubation no [^{14}C]-D-penicillamine could be found in association with the lymphocytes. Similar kinetics were found for lymphocytes incubated at 4° or with higher concentrations of [^{14}C]-D-penicillamine (200 μg/ml) (results not shown).

When purified peritoneal macrophages adhering to plastic Petri dishes were incubated with 10 μg/ml of [^{14}C]-D-penicillamine a very low uptake of label was observed during the initial 2 hr of incubation. The uptake showed a 4-fold increase after 24 hr of incubation (Table 1).

In order to study the mechanism of uptake of [^{14}C]-D-penicillamine by purified macrophages, increasing concentrations of [^{14}C]-D-penicillamine were added to macrophage cultures incubated for 2 and 24 hr (Fig. 1). A linear uptake of label was found in macrophages incubated with 10–500 μg/ml of [^{14}C]-D-penicillamine for 2 hr. Incubation for 24 hr resulted in linear uptake with concentrations from 10 to 200 μg/ml; at higher concentrations of [^{14}C]-D-penicillamine the rate of uptake was lower, indicating saturation of the cell and/or excretion of label. No decrease in cell viability of lymphocytes or macrophages was found after incubation with [^{14}C]-D-penicillamine (200 μg/ml) for 24 hr as assayed by the cosin exclusion test.

Table 1. Uptake of [^{14}C]-D-penicillamine in lymph node lymphocytes and peritoneal macrophages from Lewis rats

Time of incubation with [^{14}C]-DPA, min	Uptake of [^{14}C]-DPA in lymphocytes, C.p.m./10^6 lymphocytes, Mean ± S.E.M.	Uptake of [^{14}C]-DPA in macrophages, C.p.m./10^6 macrophages. Mean ± S.E.M.
1	32 ± 10	n.d.
5	496 ± 156	12 ± 1
15	514 ± 97	n.d.
30	254 ± 108	15 ± 3
60	94 ± 21	16 ± 3
120	0	16 ± 1
24 hr	0	62 ± 5

Lymphocytes, depleted of glass-adherent cells, and purified macrophages were incubated at 2 × 10^6 cells/ml with [^{14}C]-D-penicillamine ([^{14}C]-DPA, 10 μg/ml) for various lengths of time, at 37°. Uptake of radioactivity was determined in the lymphocytes by collection of the cells on Celotate filters and in the macrophages by digestion of the cells with 0.5 N NaOH. Each value represents the mean of 3 separate determinations.

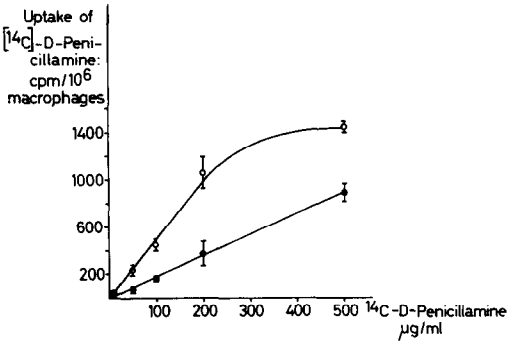

Fig. 1. Rate of uptake of [^{14}C]-D-penicillamine in peritoneal macrophages. Purified macrophages (2 × 10^6 cells/ml) were incubated with various concentrations of [^{14}C]-D-penicillamine (10–500 μg/ml) and the uptake of radioactivity was determined after 2 hr (●——●) and 24 hr (○——○) of incubation. Each point represents the mean of 3 separate determinations (± S.E.M.).

Release of [^{14}C]-D-*penicillamine from macrophages.* When purified peritoneal macrophages were incubated with [^{14}C]-D-penicillamine for 24 hr, washed extensively and reincubated in fresh medium without drug, the label was released at a rapid initial rate during the first 4 hr in drug-free medium (Fig. 2). This rate of release paralleled the initial rate of uptake of [^{14}C]-D-penicillamine. During the next 20 hr the label was released at a much slower rate and after 24 hr of incubation 35 per cent of the total label was still cell-associated. This material was not released from the cells by a 30 min chase with unlabeled D-penicillamine (10 mg/ml). No decrease in the number of viable adherent cells was found during the 48 hr of incubation, as assayed by the eosin exclusion test.

Binding of [^{14}C]-D-*penicillamine to macrophage plasma membrane fraction.* Several mechanisms could account for the persistence of label in macrophages after incubation in drug-free medium: uptake by

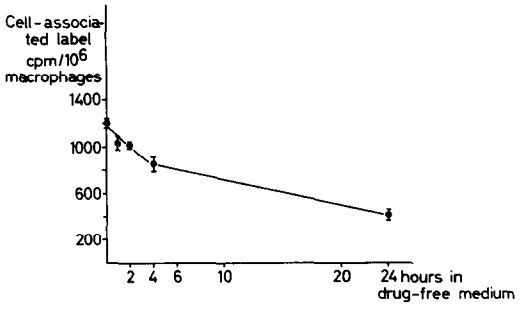

Fig. 2. Release of label from peritoneal macrophages. Purified macrophages (2 × 10^6 cells/ml) were incubated for 24 hr with [^{14}C]-D-penicillamine (200 μg/ml). The macrophages were washed and incubated in drug-free medium for various lengths of time (0, 1, 2, 4 and 24 hr) prior to assessment of the cell-associated label. Each point represents the mean of 3 separate determinations (± S.E.M.).

Table 2. Uptake of [^{14}C]-D-penicillamine in plasma membrane fraction from peritoneal macrophages

Time of incubation with [^{14}C]-DPA, (hr)	Uptake in unfractionated macrophages C.p.m./10^6 cells ± S.E.M.	Uptake in macrophage membrane fraction C.p.m./10^6 cells ± S.E.M.	% radioactivity in membrane fraction
2	705 ± 56	355 ± 27	50
24	1176 ± 252	870 ± 160	74

Purified macrophages (2 × 10^6 cells/ml) were incubated with [^{14}C]-D-penicillamine (200 μg/ml) for 2 or 24 hr. The uptake of radioactivity was determined in whole, unfractionated macrophages and in the plasma membrane fraction. The uptake in the plasma membrane fraction was calculated as percentage uptake, compared to the uptake in unfractionated cells. Each value represents the mean ± S.E.M. of 4 separate determinations.

diffusion and/or endocytosis followed by binding to intracellular structures, and/or binding to binding sites or reactive groups on the cell surface membrane. Table 2 shows the amount of label associated with a crude plasma membrane fraction from macrophages incubated with 200 μg/ml of [^{14}C]-D-penicillamine for 2 and 24 hr. The membrane fractions were extensively washed with excess of unlabeled D-penicillamine before determination of incorporated label. After 2 hr of incubation 50 per cent of the label was found in the membrane fraction, after 24 hr of incubation 74 per cent of the label was membrane-associated. More than 50 per cent of the membrane associated label could be precipitated with trichloroacetic acid, indicating association with membrane protein-fractions.

Uptake of [^{14}C]-D-*penicillamine by* $NaIO_4$*-treated macrophages.* Treatment of purified peritoneal macrophages with 10^{-3} M $NaIO_4$ in PBS for 10 min at 22° increased the uptake of [^{14}C]-D-penicillamine (100 μg/ml) during a 4-hr incubation by 129 per cent, compared to macrophages treated with PBS alone (Table 3). No decrease in viable adherent cells was seen under these conditions, as assayed by the eosin exclusion test.

Table 3. Uptake of [^{14}C]-D-penicillamine in $NaIO_4$-treated macrophages

$NaIO_4$-treatment of macrophages	Uptake of [^{14}C]-DPA in macrophages C.p.m./10^6 cells ± S.E.M.	% Increase of uptake in $NaIO_4$-treated macrophages
0	244 ± 23	
10^{-3} M	558 ± 25	129 ($P < 0.001$)

Purified macrophages (2 × 10^6 cells/ml) were pretreated with phosphate buffered saline (PBS) or with 10^{-3} M $NaIO_4$ in PBS for 10 min at 22°. After washing, the cells were incubated with [^{14}C]-D-penicillamine (100 μg/ml) for 4 hr and the uptake of label was determined after digestion of the cells with 0.5 N NaOH. Each value represents the mean ± S.E.M. of 4 separate determinations.

DISCUSSION

[^{14}C]-D-Penicillamine is rapidly taken up by purified lymphocytes during the first 15 min of incubation, both at 37° and at 4°. These results are consistent with an uptake by diffusion, a mechanism which may also account for the uptake of D-penicillamine by erythrocytes [15]. No label was found in lymphocytes after 2 hr of incubation. D-Penicillamine entering the lymphocytes may be kept in the reduced state by the intracellular glutathione system and may also be able to freely diffuse out of the cells where it would be trapped as disulphide. Jellum and Skrede [16] have reported that oxidation of D-penicillamine to the corresponding disulphide occurs in aqueous solution at pH 7.4 slowly. As our experiments have been performed in presence of fetal calf serum no conclusion can be drawn regarding the permeability of the lymphocytes to penicillamine disulphide, as disulphide formation may also have occurred between D-penicillamine and serum protein SH-groups.

At a concentration where [^{14}C]-D-penicillamine (10 μg/ml) was readily taken up by the lymphocytes, virtually no uptake was seen in purified peritoneal macrophages. At higher concentrations (> 50 μg/ml) a slow uptake was evident, dependent on the concentration of [^{14}C]-D-penicillamine and the length of incubation. After 24 hr of incubation with 200 μg/ml of [^{14}C]-D-penicillamine, 10^6 macrophages contained approximately 100 ng of D-penicillamine. The profile of uptake of [^{14}C]-D-penicillamine by macrophages seems to be clearly different from that observed in the lymphocytes.

When the labeled macrophages were exposed to medium without D-penicillamine the label was initially released at a rapid rate comparable to the initial rate of uptake, then the release occurred at a slow rate and after 24 hr 40 per cent of [^{14}C]-D-penicillamine was still cell bound. The phagocytic activity is not impaired in cells cultured in the presence of D-penicillamine [17] and it may be suggested that D-penicillamine, as disulphide or bound to serum protein, is engulfed by peritoneal macrophages. The high levels of radioactivity we have found associated with macrophage membrane fractions suggest that D-penicillamine may also bind to reactive groups or binding sites at the level of cell membranes. This hypothesis is consistent with the finding that 40 per cent of the label is still cell-bound after 24 hr of culture in drug-free medium.

One obvious mechanism of binding is via —S—S-bridges, generating mixed disulphide with membrane thiol-groups. This does not seem to account for a substantial amount of membrane-associated D-penicillamine as extensive equilibration and washing with unlabeled D-penicillamine does not reduce the amount of bound label. Another possible binding mechanism is the interaction of D-penicillamine with aldehyde groups resulting in the formation of thiazolidine rings [18, 19]. Aldehyde groups may originate on macrophage membranes from the decarboxylation and the deamination of aminoacids by the peroxidase/H_2O_2 system [20, 21] and their number can be increased by treatment of the macrophage with sodium periodate as described by Remold [11]. The highly significant enhancement of [^{14}C]-D-penicilla-

mine uptake by macrophages pretreated with $NaIO_4$ may suggest that D-penicillamine interacts with aldehyde groups on macrophage membranes. It does not exclude, however, the possibility that other or additional mechanisms are involved.

Acknowledgements—The generous gift of [^{14}C]-D-penicillamine from Dr. Maul is gratefully acknowledged. We wish to thank M. S. Bente Hasselriis, M. S. Elisabeth Greve Petersen and M. S. Lisbeth Christiansen for their excellent technical assistance.

REFERENCES

1. I. A. Jaffe, *Ann. N.Y. Acad. Sci.* **256**, 330 (1975).
2. K. Altman and M. S. Tobin, *Proc. Soc. exp. Biol. Med.* **118**, 554 (1965).
3. R. Bluestone and L. S. Goldberg, *Ann. rheum. Dis.* **32**, 50 (1973).
4. S. Roath and R. Willis, *Postgrad. Med. J.* **50**, 56 (1974).
5. K. Schumacher, G. Marker-Alzer and R. Preuss. *Arzneimittel-Forsch.* **25**, 603 (1975).
6. P. A. Kendall and D. Hutchins, in *Penicillamine Research in Rheumatoid Disease* (Ed. E. Munthe), p. 198. Oslo (1976).
7. L. Binderup, E. Bramm and E. Arrigoni-Martelli. *Drugs Exp. Clin. Res.* **2**, 181 (1977).
8. S. S. Frøland, E. Munthe, T. Abrahamsen, Ø. Førre and J. Aaseth, in *Penicillamine Research in Rheumatoid Disease* (Ed. E. Munthe), p. 185. Oslo (1976).
9. E. Arrigoni-Martelli, L. Binderup and E. Bramm, in *Perspectives in Inflammation* (Eds D. A. Willoughby, J. P. Giroud and G. P. Velo), p. 295. London (1977).
10. L. Binderup, E. Bramm and E. Arrigoni-Martelli, *Scand. J. Immunol.* **7**, 259 (1978).
11. H. G. Remold, *J. Immunol.* **118**, 1 (1977).
12. L. T. Yam, C. Y. Li and W. H. Crosby, *Am. J. Clin. Path.* **55**, 283 (1971).
13. Z. Werb and Z. A. Cohn, *J. biol. Chem.* **247**, 2439 (1972).
14. D. Donnert and F. Planas-Bohne, *Arzneimittel-Forsch.* **22**, 1423 (1972).
15. J. Aaseth, *Acta Pharmac. Tox.* **39**, 289 (1976).
16. E. Jellum and S. Skrede, in *Penicillamine Research in Rheumatoid Disease* (Ed. E. Munthe), p. 68. Oslo (1976).
17. H. Chwalinska-Sadowska and J. Baum, *J. clin. Invest.* **58**, 871 (1976).
18. H. T. Nagasawa, D. J. W. Goon. E. G. DeMaster and C. S. Alexander, *Life Sci.* **20**, 187 (1977).
19. M. E. Nimni, *Proc. R. Soc. Med.* **70**, 65 (1977).
20. A. J. Sbarra, A. A. Jacobs, R. R. Strauss, B. B. Paul and G. W. Mitchell, *Am. J. clin. Nutr.* **24**, 272 (1971).
21. J. M. Zglizynski, T. Stelmaszyska, M. Ostrowski, J. Nashkalski and J. Sznajd, *Eur. J. Biochem.* **4**, 540 (1968).