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BIPHASIC TOXICITY OF DIETHYLDITHIOCARBAMATE, A METAL CHELATOR, TO T LYMPHOCYTES AND POLYMORPHONUCLEAR GRANULOCYTES: REVERSAL BY ZINC AND COPPER

Demetrios A. Rigas, Chrysiis Eginitis-Rigas and Charlotte Head

Department of Biochemistry and Division of Medical Genetics

School of Medicine

University of Oregon Health Sciences Center

Portland, Oregon 97201

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Summary: A new type of toxicity biphasically dependent on concentration was observed with diethyldithiocarbamate, a metal chelator utilized in medicine. As judged by cell survival and [^{3}H]Urd incorporation, diethyldithiocarbamate was maximally toxic to T lymphocytes and polymorphonuclears at 2.5×10^{-5} M (first phase) and at higher than 2.5×10^{-3} M (second phase), but was not toxic at intermediate concentrations around 2.5×10^{-4} M. The response of chelator treated T lymphocytes to phytohemagglutinin was also biphasic. The first toxic phase was partially reversed by 2.5×10^{-5} M $ZnCl_2$, while the second phase was partially reversed by 10^{-2} M $CuCl_2$. This suggests that inhibition of Zn-metalloenzymes in the first phase and of Cu-metalloenzymes in the second may play a crucial role in the mechanism of toxicity. The second toxic phase may be in part due to the observed inhibition of superoxide dismutase rendering the cells susceptible to oxygen toxicity, like obligate anaerobes.

INTRODUCTION. Sodium diethyldithiocarbamate (DDC) is a sulfhydryl metal chelator with high affinity for Cu (II), Ni and Zn. It is used in the determination of these metals (1-3), and in nickel carbonyl poisoning (4,5). Antabuse®, used in aversion therapy of alcoholism (6,7), is the oxidized disulfide form of DDC, and is interconvertible with it *in vivo* (8). DDC inhibits the cytosolic Cu-Zn-superoxide dismutase (SOD) *in vitro* (9-12) and *in vivo* (11,12), as well as the F_e- and Mn-SOD *in vitro* (13). While studying the effects of DDC on the SOD and radiosensitivity of lymphocytes

ABBREVIATIONS: DDC, diethyldithiocarbamate; SOD, superoxide dismutase; PPHA, protein phytohemagglutinin; [^{3}H]Urd, [^{3}H]uridine; PMN, ploymorphonuclear granulocyte; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)-benzene.

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(14), we observed a new type of toxicity with biphasic dependence on the DDC concentration. This biphasic toxicity and its reversal by Zn^{++} and Cu^{++} is the subject of this report.

MATERIALS AND METHODS. Aliquots of normal human defibrinated, platelet depleted, blood (15) were preincubated for 2 hours at 37° C with 10^{-9} M to $2.5x10^{-3}$ M freshly dissolved DDC (Sigma Chemical Co., St. Louis, Mo.) in medium RPMI 1640 (Grand Island Biological Co., New York, N.Y.). To reverse the DDC toxicity, preincubation was continued for one more hour following addition of $ZnCl_2$ and/or $CuCl_2$.

Cultures were set up in 12x75 mm polystyrene culture tubes (16). To each tube 50 µl of preincubated blood and 100 µl of RPMI 1640 (containing 10 units penicillin, 10 µg streptomycin and 25 ng fungizone) were added. They were gassed with 5% CO_2 in air, capped tightly and incubated at 37°C completely submerged in a shaking water bath for 24 hours. Viable cells were identified by their ability to exclude trypan blue (17). Differential counts of mononuclears (lymphocytes and monocytes) and polymorphonuclears (PMN) were done with a hemocytometer. RNA synthesis was determined from the incorporation of [5-^{3}H]uridine ([^{3}H]Urd) (New England Nuclear, Boston, Mass.), 1 µCi of which with or without 2.5 µg of phytohemagglutinin, PPHA, (18) in 100 µl RPMI 1640 was added after 20 hours of incubation. Four hours later the cells were transferred to glass tubes and washed by centrifugation 3 times with 0.15 M NaCl, once with acid-acetone (1 vol. 1 N HCl to 100 vol. acetone), once with 5% trichloroacetic acid, and twice with amhydrous methanol. They were solubilized with NCS® (Amersham Corp., Arlington Heights, Ill.) and counted by liquid scintillation in 0.5% PPO-0.03% POPOP-toluene after decay of chemiluminescence. Counting times necessary to give 99% confidence that the error is not over 5% were obtained from our previously described nomogram (19).

RESULTS. Figure 1A shows the survival of mononuclears and PMN after 24 hours of culture without phytohemagglutinin. Low DDC concentrations (10^{-9} M to $2.5x10^{-6}$ M) appeared to improve cell survival, probably through chelation of

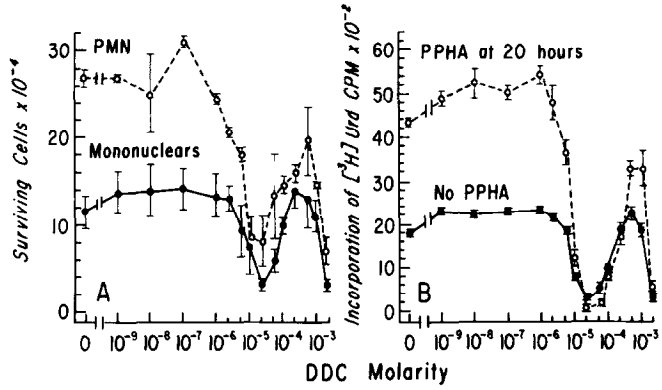

Figure 1. Effect of DDC on cell survival and incorporation of [^{3}H]Urd. Points represent the mean from 3 experiments. Five replicate cultures in each per point (1 for counting the surviving cells, 4 for [^{3}H]Urd incorporation). Vertical bars indicate the standard error of the mean. A. Cell survival after 24 hours culture without PPHA: ●——●, mononuclear leukocytes; o- - -o, PMN. B. Incorporation of [^{3}H]Urd during the last 4 of 24 hours in culture: ●——●, without PPHA; o- - -o, PPHA added at 20 hours of culture.

toxic trace metals. Higher concentrations became abruptly toxic, the cell survival reaching a minimum at 2.5×10^{-5} M (first phase of toxicity). However, as the concentration increased further, toxicity decreased and the cell survival reached a maximum around 2.5×10^{-4} M. At even higher concentrations, DDC became toxic again and the cell survival decreased rapidly. The incorporation of [^{3}H]Urd during the last 4 hours followed a similar course (Fig. 1B). In the abscence of PPHA, the incorporation followed closely the cell survival curve, indicating that it can be used as a measure of cell survival. The ability of the surviving cells to respond to PPHA, as measured by the [^{3}H]Urd incorporation, varied with the molarity of DDC, being highly positive (increased incorporation) at non-toxic concentrations and decreasing, becoming even negative (decreased incorporation), at toxic concentrations. The reported reversal of the DDC inhibition of SOD by Cu^{++} (10), and of the *o*-phenanthroline inhibition of DNA replication by Zn^{++} (20), prompted us to investigate the possible reversal of toxicity of 2.5×10^{-5} M (first phase) and 2.5×10^{-3} M (second phase) DDC by 2.5×10^{-5} M or 10^{-2} M $ZnCl_2$ and/or $CuCl_2$. The results (Table 1) indicate that while preincubation with 2.5×10^{-5} M DDC reduced the

TABLE 1. Reversal of Toxicity of DDC by $ZnCl_2$ and $CuCl_2$

Preincubation of Blood with:			Incorporation of [^{3}H]Uridine
DDC*	$ZnCl_2$**	$CuCl_2$**	
	Molarity		*Net CPM/culture*
0	0	0	2,600±54†
$2.5x10^{-5}$	0	0	224±14
$2.5x10^{-5}$	$2.5x10^{-5}$	0	1,241±33
$2.5x10^{-5}$	0	$2.5x10^{-5}$	118±8
$2.5x10^{-5}$	0	10^{-2}	68±7
$2.5x10^{-5}$	$2.5x10^{-5}$	$2.5x10^{-5}$	814±23
$2.5x10^{-3}$	0	0	279±19
$2.5x10^{-3}$	$2.5x10^{-5}$	0	265±21
$2.5x10^{-3}$	10^{-2}	0	75±8
$2.5x10^{-3}$	0	10^{-2}	1,112±29
$2.5x10^{-3}$	10^{-2}	10^{-2}	1,763±11
0	$2.5x10^{-5}$	0	2,556±76
0	0	$2.5x10^{-5}$	2,261±33
0	$2.5x10^{-5}$	$2.5x10^{-5}$	2,420±30
0	10^{-2}	0	365±15
0	0	10^{-2}	561±43

* Added at the beginning of the three hour preincubation.
** Added after two hours preincubation.
† Mean ± Standard Error of the Mean, obtained from 4 replicate cultures.

incorporation of [^{3}H]Urd to about 10% of the controls, further preincubation with $2.5x10^{-5}$ M $ZnCl_2$ partially reversed it, raising it to around 50% of the controls. On the other hand, $2.5x10^{-5}$ M or 10^{-2} M $CuCl_2$ did not reverse the inhibition, but appeared to increase it and to impair the ability of $ZnCl_2$ to reverse it. Neither $2.5x10^{-5}$ M $ZnCl_2$ and/or $2.5x10^{-5}$ M $CuCl_2$ had any effect on the [^{3}H]Urd incorporation in the absence of DDC. In contrast, the inhibition by $2.5x10^{-3}$ M DDC was not reversed by $2.5x10^{-5}$ M or 10^{-2} M $ZnCl_2$,

but was partially reversed by 10^{-2} M $CuCl_2$. However, 10^{-2} M $ZnCl_2$ augmented the reversal by 10^{-2} M $CuCl_2$. Either metal alone at 10^{-2} M without DDC was toxic. Thus, partial reversal of the first phase of toxicity was accomplished by $ZnCl_2$ and of the second phase by $CuCl_2$.

DISCUSSION. No previous report of a biphasically toxic substance was found, but a biphasic effect of γ-rays on uracil incorporation by *E. coli* (21), and of $HgCl_2$ on formamidase (22) has been observed. The biphasic toxicity of DDC does not appear to be cell specific, as the differential counts of the surviving cells and the response to T lymphocyte mitogen PPHA demonstrate that it is exerted towards at least two types of cells, PMN and T lymphocytes. Large doses of DDC (1.5 g per kg) have been given to mice without ill effects (11,26), while lower doses (0.5 to 0.6 g per kg) were found by others to be toxic (27). These observations suggest that DDC may be biphasically toxic also *in vivo*. In view of the use of DDC and Antabuse® in medicine (4,6,7), its biphasic toxicity assumes high significance, as a good understanding is essential to avoid potential hazards in its clinical use. It is possible that other metal chelators are also biphasically toxic. Others have studied the inhibition of [^{3}H]thymidine incorporation in phytohemagglutinin lymphocytes caused by the chelators α-phenanthroline (20) and EDTA (23), which is also reversible by Zn^{++}, but did not study them over a wide enough concentration range to ascertain if they are biphasically toxic or not.

The mechanism of biphasic toxicity of DDC is not known, but the reversal of its toxicity by Zn^{++} and Cu^{++} suggests chelation of these metals. The ability of Zn^{++} to reverse the first phase of toxicity and of Cu^{++} the second suggests Zn^{++} chelation at a lower DDC concentration than Cu^{++}. This is in reverse order of the stability of metal chelates (24). A plausible explanation is that the chelated Zn^{++} and Cu^{++} are not free, but bound to metalloenzymes, where through interactions with neighboring groups Cu^{++} may be rendered less accessible to DDC than Zn^{++}. Thus it appears that inhibition of Zn-metalloenzymes by DDC plays a crucial role in the first toxic phase, and of Cu-

metalloenzymes in the second. Our observation of inhibition of SOD by DDC in the second phase of toxicity (14) offers a plausible explanation of its mechanism. It is likely that inhibition of SOD renders the cells as susceptible to oxygen toxicity as obligate anaerobes (25).

Many cellular substances bind metals reversibly (28), their specificity ranging from relatively low as in metallothionein, which binds many metals and particularly Zn^{++} (29), to very high as in copper-chelatin which is highly specific for Cu^{++} (30). Our observations support the view that such metal binding substances play an important role in metabolic control and viability of cells. Metal chelators are being used in medicine (4,31) and may be important in cancer chemotherapy (32), while metal ions appear to be involved in carcinogenesis (33). These considerations argue in favor of further studies in this area.

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References

1. Cooper, C. W. (1961) in *Treatise on Analytical Chemistry*, eds. I. M. Kolthoff, P. J. Elving, and E. P. Sandell, Interscience, New York, Part II, Vol. 3, 1-41.
2. Kanzelmeyer, J. H. (1961) in *Treatise on Analytical Chemistry*, eds. I. M. Kolthoff, P. J. Elving, and E. P. Sandell, Interscience, New York, Part II, Vol. 3, 95-169.
3. Dale, J. M., and Banks, C. V. (1962) in *Treatise on Analytical Chemistry*, eds. I. M. Kolthoff, P. J. Elving, and E. P. Sandell, Interscience, New York, Part II, Vol. 2, 377-440.
4. West, B., and Sunderman, F. W. (1958) Am. J. Med. Sci. 236, 15-25.
5. Sunderman, F. W., and Sunderman, F. W., Jr. (1958) Am. J. Med. Sci. 236, 26-31.
6. Lundwall, L., and Baekeland, F. (1971) J. Nerv. Ment. Dis. 153, 381-394.
7. Mottin, J. L. (1973) Q. J. Stud. Alcohol 34, 444-472.
8. Fried, R. (1976) Ann. N.Y. Acad. Sci. 273, 212-218.
9. Asada, K., Takahashi, M., and Nagate, M. (1974) Agr. Biol. Chem. 38, 471-473.
10. Misra, H. P. (1975) Fed. Proc. 34, 624.
11. Heikkila, R. E., Cabbat, F. S., and Cohen, G. (1976) J. Biol. Chem. 251, 2182-2185.
12. Heikkila, R. E., and Cohen, G. (1977) in *Superoxide and Superoxide Dismutases*, eds. A. M. Michelson, J. M. McCord, and L. Fridovich, Academic Press, New York, 367-373.

13. Puget, K., Lavelle, F., and Michelson, A. M. (1977) in *Superoxide and Superoxide Dismutases*, eds. A. M. Michelson, J. M. McCord, and I. Fridovich, Academic Press, New York, 139-150.
14. Rigas, D. A., Eginitis-Rigas, C., Bigley, R. H., Stankova, L., and Head, C. Submitted for publication.
15. Schoepflin, G. S., Goetzl, E. J., and Austen, K. F. (1978) Cell. Immunol. 35, 330-339.
16. Park, B. H., and Good, R. A. (1972) Proc. Nat. Acad. Sci., U.S.A. 69, 371-373.
17. Rabinowitz, Y. (1964) Blood 23, 811-828.
18. Rigas, D. A., Head, C., and Eginitis-Rigas, C. (1972) Physiol. Chem. Phys. 4, 153-165.
19. Rigas, D. A. (1968) Intern. J. Appl. Radiat. Isot. 19, 453-457.
20. Williams, R. O., and Loeb, L. A. (1973) J. Cell Biol. 58, 594-601.
21. Boyle, J. M. (1976) in *Radiation and Cellular Control Processes*, ed. J. Kiefer, Springer-Verlag, New York, 5-17.
22. Schoenbeck, L. (1968) Ph. D. Dissertation, University of Oregon Health Sciences Center, School of Medicine, 134-138.
23. Chesters, J. K. (1972) Biochem. J. 13, 133-139.
24. May, I., and Schubert, L. (1961) in *Treatise on Analytical Chemistry*, eds. I. M. Kolthoff, P. J. Elving, and E. P. Sandell, Interscience, New York, Part I, Vol. 3, 847-850.
25. McCord, J. M., Keele, B. B., Jr., and Fridovich, I. (1971) Proc. Nat. Acad. Sci., U.S.A. 68, 1024-1027.
26. Deitrich, R. A., and Erwin, V. C. (1971) Mol. Pharmacol. 7, 301-307.
27. Christensen, H. E., Luginbyhl, T. T., and Carrol, B. S. (1974) *The Toxic Substances List*, U.S. Dept. HEW, Rockville, Md., 175-176.
28. Eichhorn, I. (1973) *Inorganic Biochemistry*, Elsevier, Amsterdam, 121-1210.
29. Kägi, J. H. R., Himmelhoch, S. R., Whanger, P. D., Bethune, J. L., and Vallee, B. L. (1974) J. Biol. Chem. 249, 3537-3542.
30. Winge, D. R., Premakumar, R., Wiley, R. D., and Rajagopalan, K. V. (1975) Arch. Biochem. Biophys. 170, 253-266.
31. Seven, M. J., and Johnson, L. A. (1960) *Metal-Binding in Medicine*, Lippincott, Philadelphia.
32. Furst, A. (1960) *Metal-Binding in Medicine*, eds. M. J. Seven and L. A. Johnson, Lippincott, Philadelphia, 336-344.
33. Furst, A., and Haro, R. T. (1969) Progr. Exp. Tumor Res. 12, 102-133.