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THE ACTION OF THE ADENOSYLHOMOCYSTEINE HYDROLASE INHIBITOR, 3-DEAZAADENOSINE, ON PHAGOCYTIC FUNCTION OF MOUSE MACROPHAGES AND HUMAN MONOCYTES

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SUMMARY. An inhibitor of adenosylhomocysteine hydrolase, 3-deazaadenosine, caused profound inhibition of phagocytosis of opsonized erythrocytes by mouse resident peritoneal macrophages in vitro. The inhibition was evident at concentrations as low as $2x10^{-7}$M, and increased with increasing concentration and time of exposure to the analogue. It was not associated with detachment of the macrophage monolayers or with loss of cell viability. Although the inhibition was not reversible, progression of the functional impairment was interrupted by washing out the analogue. In striking contrast, phagocytic function of human blood monocytes was unaffected by 3-deazaadenosine.

INTRODUCTION

Adenosylmethionine (AdoMet) has been identified as the methyl donor to more than 60 different methyl acceptors, including biogenic amines, RNA and DNA, and proteins (1). The role of methylation in control of cellular motility has been well established for the bacterial swimming-tumbling cycle, in which carboxymethylation of glutamic acid residues in bacterial proteins has been shown (2,3,4). Methylation may also be involved in chemotactic responses of mammalian leukocytes (5). Transmethylation is sensitive to inhibition by the product adenosylhomocysteine (AdoHcy); the Ki's for inhibition of the different methylases in mammalian cells vary over a wide range. It was found recently that 3-deazaadenosine inhibits hydrolysis of AdoHcy to adenosine

Abbreviations: AdoHcy, adenosylhomocysteine; 3-deaza-AdoHcy, 3-deazaadenosylhomocysteine; EHNA, 9-erythro-(2-hydroxyl-3-nonyl)adenine hydrochloride; ^{51}CrEA, ^{51}Cr-labeled sheep erythrocytes with bound rabbit IgG anti-Forssman antibody; MSP, macrophage stimulating protein.

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and homocysteine(6). The reaction catalyzed by AdoHcy hydrolase is reversible and the equilibrium is strongly in the direction of synthesis (7); 3-deazaadenosine can also function as a substrate in the synthetic reaction (6, 8). Therefore administration of 3-deazaadenosine in a variety of tissues leads to increases in the cellular concentration of AdoMet, AdoHcy and the appearance of 3-deaza-AdoHcy (6, 9). It was expected that these changes would inhibit certain transmethylation reactions and cause alterations in cellular functions. Recently it was shown that 3-deazaadenosine inhibited Rous sarcoma virus replication and malignant transformation in chick embryo cells (10).

In view of the importance of transmethylation for bacterial motility, the present studies were undertaken to determine whether inhibition of methylation affected phagocytosis by mammalian leukocytes. Phagocytosis can be considered a motility function since it requires organized translational movement of a portion of the cell during engulfment of the particle and it lends itself to study since it can readily be quantified.

MATERIALS AND METHODS

Reagents. Macrophage stimulating protein (MSP) was partially purified from human serum by G-200 Sephadex gel filtration, DEAE-cellulose chromatography and isoelectric focusing (11). 3-Deazaadenosine was synthesized by Dr. John A. Montgomery of Southern Research Institute, Birmingham, Alabama. The adenosine deaminase inhibitor, 9-erythro-(2-hydroxyl-3-nonyl)adenine hydrochloride (EHNA) (12), was obtained from Dr. H. J. Schaeffer, Burroughs Wellcome Co, Research Triangle Park, N.C. Adenosine and l-homocysteine thiolactone were obtained from Calbiochem., La Jolla, Calif. All of the above reagents were dissolved in water or phosphate buffered saline (0.15M NaCl, 20 mM, pH 7.1 potassium phosphate), stored at -20°C, and thawed and diluted for each experiment. Ethidium bromide and fluorescein diacetate were obtained from Calbiochem. Stock solutions of ethidium bromide (0.2 mg/ml phosphate buffered saline) and fluorescein diacetate (5 mg/ml acetone) were stored at -20°C. For use in vital dye staining, 1 volume of ethidium bromide stock was mixed with 1 volume of diluted fluorescein diacetate stock (25 ul stock + 10 ml tissue culture medium).

Measurement of phagocytosis. The assay was based on previously published methods from this laboratory (13). Leukocytes were obtained by lavage of the peritoneal cavity of normal C3H/HeN mice with Dulbecco's modified Eagle's tissue culture medium containing 10% heat-inactivated fetal calf serum. The cell population comprised approximately equal numbers of macrophages and lymphocytes, a few mast cells and practically no neutrophils. The cells were centrifuged, resuspended in medium with fetal calf serum at a concentration of 600,000 macrophages per ml, and 0.5 ml aliquots were distributed in the polystyrene wells of Costar 24 Cluster Dishes (Costar, Cambridge, Mass.). After a 1 hour incubation in 5% carbon dioxide - 95% humidified air, non-adherent cells were washed off. A monolayer of adherent macrophages and lymphocytes remained. Fresh medium without fetal calf serum, containing a 1/200 dilution of MSP, was added to the wells. Drugs to be tested were also added at this point, which was designated time zero for all experiments. Erythrocytes for

measurement of phagocytosis (^{51}CrEA) were prepared by incubating sheep erythrocytes with rabbit IgG anti-Forssman antibody and ^{51}Cr for 60 minutes, washing, and adjusting to a concentration of 1.5×10^7 ^{51}CrEA/ml. They were added to culture wells at various times. After 1 hour, non-adherent erythrocytes were washed away, adherent but non-ingested erythrocytes were lysed with a buffer that did not damage the macrophage monolayer; after washing, the macrophages with their ingested erythrocytes were solubilized by addition of a 0.5% solution of sodium lauryl sulfate. ^{51}Cr in the solution was counted in a Packard autogamma scintillation spectrometer.

Estimation of macrophage number and viability. The number of macrophages in a randomly selected central field in culture wells was determined by phase microscopy with a 16x phase objective and an eyepiece grid. For studies on cell viability, glass cover slips were placed in culture wells before cells were added. Cell viability was determined by removing cover slips with adherent cells, inverting on a 5 ul drop of fluoresceine diacetate - ethidium bromide solution, and examining by fluorescence microscopy. Viable cells were green, since they took up dye and converted it to fluorescein. Dead cells were red, since they were incapable of excluding ethidium bromide (14).

Phagocytosis by human peripheral blood monocytes. A monocyte-rich cell suspension was prepared from heparinized human venous blood by centrifugation through a Ficoll-Hypaque solution (LSM Solution, Bionetics, Kensington, Md.) (15). After the cells were added to culture wells, the remainder of the procedure was the same as for mouse macrophages.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of a wide range of concentrations of 3-deazaadenosine on the phagocytic uptake of opsonized erythrocytes (^{51}CrEA) by monolayers of mouse resident peritoneal macrophages. Uptake of ^{51}CrEA was determined at various times after the addition of 3-deazaadenosine. The Figure shows that inhibition of phagocytosis was both dose and time dependent. The slopes of the curves suggest that the effect of even the lowest concentration of 3-deazaadenosine continues to progress beyond 3 hours. This was born out in other experiments (see Fig. 3). Significant inhibition of phagocytosis by 3-deazaadenosine has been observed at concentrations as low as 2×10^{-7}M, tested after 5 hours of incubation. In monolayers, the phagocytic rates of which were not elevated by addition of macrophage stimulating protein, 3-deazaadenosine also inhibited phagocytosis.

As noted above, 3-deazaadenosine is a potent inhibitor of adenosylhomocyteine hydrolase, and can also be utilized in the direction of synthesis to form 3-deazaAdoHcy. Since the limiting factor in the formation of 3-deaza-AdoHcy may be cellular Hcy concentration, we determined the effect of Hcy thiolactone on the inhibition of phagocytosis by 3-deazaadenosine. Fig. 2 shows that addition of this compound caused

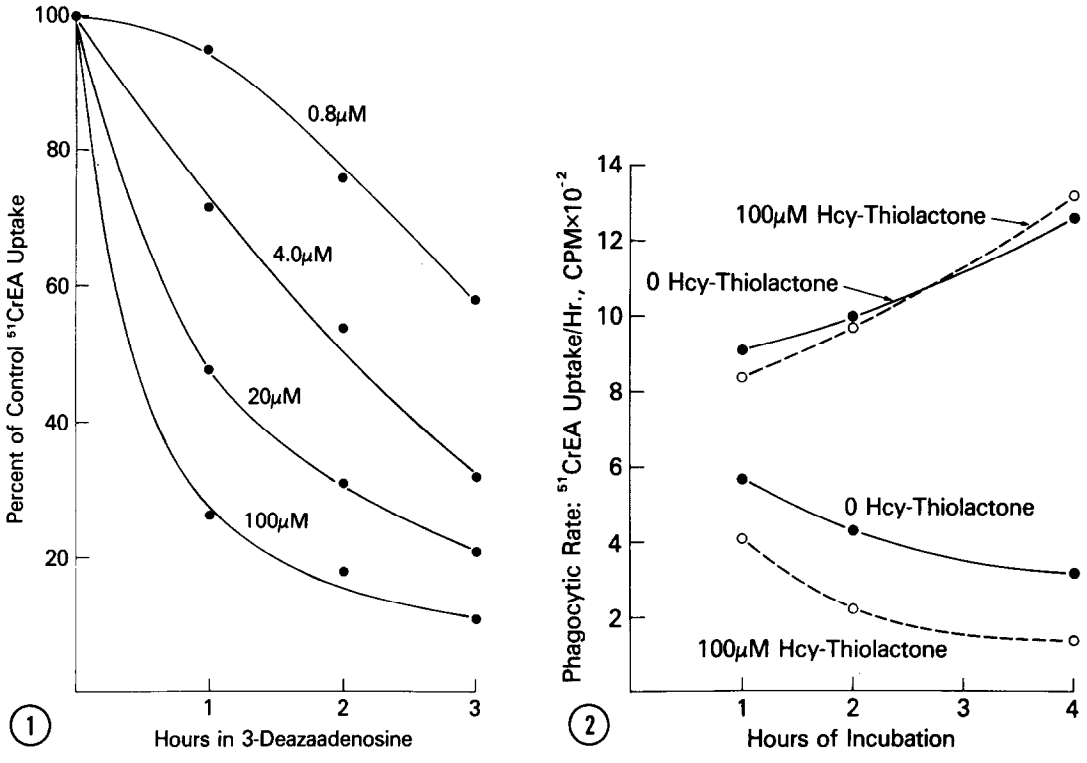

Fig. 1. Effect of 3-deazaadenosine on phagocytic uptake of ^{51}CrEA. Phagocytic rates were determined for triplicate culture well macrophage monolayers at 1, 2 and 3 hours after addition of the inhibitor.

Fig. 2. Effect of Hcy-thiolactone on inhibition of phagocytosis by 3-deazaadenosine. Upper curves: no 3-deazaadenosine. Lower curves: 10 uM 3-deazaadenosine, added at time zero. ^{51}CrEA were added to triplicate macrophage monolayer wells at 1, 2 and 4 hours for determination of phagocytic rate.

a moderate increase in the inhibitory action of 3-deazaadenosine. The results are consistent with previous suggestions that cellular actions of 3-deazaadenosine are due to accumulation of AdoHcy, 3-deazaAdoHcy or both.

The results shown in Fig. 3 are from an experiment on reversibility in which monolayers in multiple culture wells were incubated with 1 uM 3-deazaadenosine. At various times, all wells were washed and culture fluid with or without 3-deazaadenosine was added back. Phagocytic uptake was determined at the times indicated. Continuous incubation with 3-deazaadenosine caused inhibition of phagocytosis that was progressive with time, so that by 9 hours the rate was less than 10% of the control. After 3-

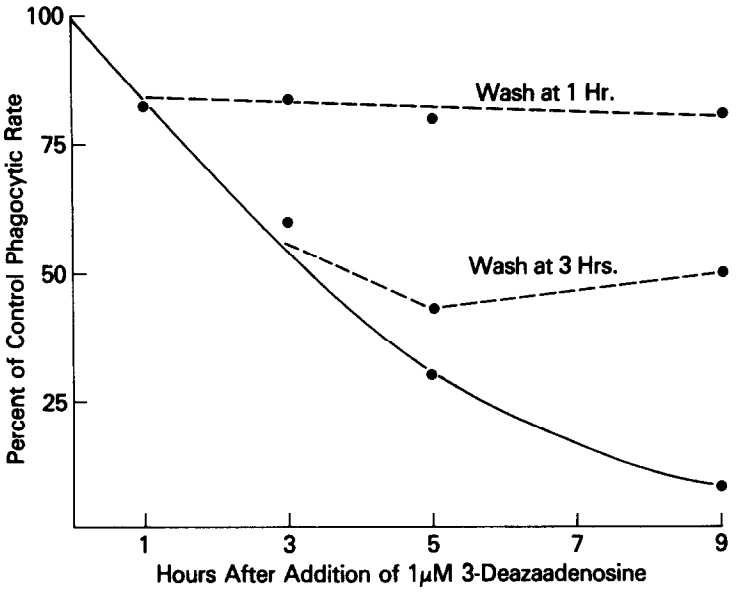

Fig. 3. Effect of removal of 3-deazaadenosine. At time zero, 1 uM 3-deazaadenosine was added to macrophage monolayers. Solid line: 3-deazaadenosine throughout. Dashed lines: monolayers washed and medium without 3-deazaadenosine was added.

deazaadenosine was removed, we observed little or no recovery. However the progressive decline in phagocytic rate was interrupted.

These results taken together -- increasing inhibition with time, enhancement of the effect by Hcy, and interruption of the progressive decline without reversibility when 3-deazaadenosine was removed -- suggests that the effects on phagocytosis are due to accumulation of a reaction product (AdoHcy or 3-deaza-AdoHcy), and that in this particular cell the product is removed extremely slowly, if at all.

Since the macrophage monolayers were washed many times in the course of the experiments, it was important to determine if the observed inhibition of ^{51}Cr uptake was simply due to 3-deazaadenosine-induced detachment of cells from the polystyrene surface. This did not occur, since cell numbers in control and 3-deazaadenosine-treated culture wells were the same. We also found that despite the inhibition of phagocytic function (to 50% of the control after 5 hours in 1 uM 3-deazaadenosine) there were fewer than 5% dead macrophages by criteria of vital dye staining. Despite this high viability, there is no reason to believe that impairment by 3-deazaadenosine is confined

Table I

Effect of 3-deazaadenosine on phagocytosis by monolayers of human peripheral blood monocytes

	^{51}CrEA uptake, cpm/monolayer/hr, at	
	2 hrs*	6 hrs
No Additions	3960 ± 70	3830 ± 110
3-Deazaadenosine		
20 uM	4530 ± 70	4240 ± 160
100 uM	4610 ± 100	4390 ± 100
100 uM + 50 uM Hcy-thiolactone	4930 ± 210	4470 ± 130

*Additions to monolayers were at time zero. ^{51}CrEA were added at 2 or 6 hrs. Results are means ± S.E.M. for triplicate culture wells.

to phagocytosis. We have observed decreased chemotactic responses in these cells. It would be of interest to determine whether functional derangements caused by 3-deazaadenosine are restricted or widespread. It should also be emphasized that there appears to be a wide spectrum of cell sensitivity to 3-deazaadenosine. The concentration of 3-deazaadenosine used to inhibit replication of Rous sarcoma virus in chick embryo fibroblasts was 100 uM; despite these levels, the cells divided (showing that many subcellular motility functions were intact) and there was no impairment of DNA or protein synthesis (10). Table I shows that in contrast to the effects on mouse peritoneal macrophages, 3-deazaadenosine did not inhibit phagocytic activity of human peripheral blood monocytes. This striking result may be a reflection of maturational differences between blood monocyte and tissue macrophage, or it may represent a species difference. Alterations during maturation of the mouse mononuclear phagocyte include peroxidase staining (16), response to chemotactic factors (17) and 5'-nucleotidase activity (18). It would be of interest if 3-deazaadenosine sensitivity were also a marker of macrophage maturation.

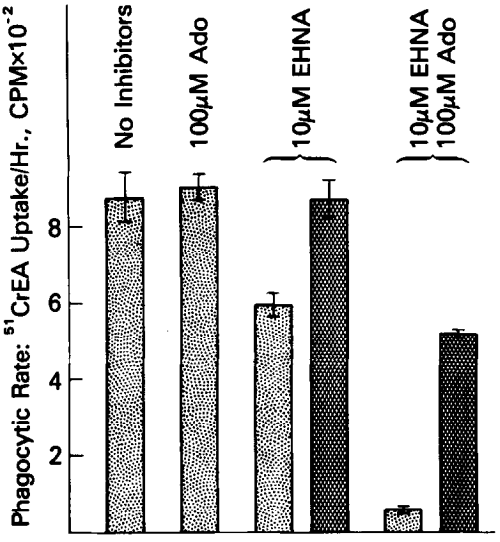

Fig. 4. Effect of EHNA on phagocytosis. Agents were added at time zero. At 1 hour, wells were aspirated and fresh medium with (stipple) or without (cross-hatch) agents were added and ^{51}CrEA uptake was determined. Error bars are S.E.M. for triplicate culture wells.

Another intervention that leads to an increase in cellular concentration of AdoHcy is addition of an inhibitor of adenosine deaminase, which increases the amount of cellular adenosine available to react with Hcy (19). Fig. 4 shows the effect of EHNA, a competetive inhibitor of adenosine deaminase, on phagocytosis. EHNA alone caused measurable inhibition of phagocytosis. The inhibition was enhanced by addition of adenosine, which had no effect in the absence of EHNA. Partial reversal of the inhibition occurred after removal of EHNA and adenosine. These results fit with the biochemical schema proposed above, but bring into sharp focus the question why the EHNA effect is reversible and the 3-deazaadenosine inhibition is not. The difference may be due to the formation and metabolic effects of 3-deaza-AdoHcy.

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