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Inhibition of Chemotaxis by *S*-3-Deazaadenosylhomocysteine in a Mouse Macrophage Cell Line*

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Chemotaxis by a macrophage cell line, RAW264, is specifically inhibited by the intracellular accumulation of 3-deazaadenosylhomocysteine (3-deaza-AdoHcy). Intracellular accumulation of 3-deaza-AdoHcy is the result of incubation of the cells with 3-deazaadenosine, a compound that can function both as a substrate and as an inhibitor of adenosylhomocysteine (AdoHcy) hydrolase. Accumulation of AdoHcy *per se*, brought about in chemotactic cell lines by incubation with either 3-deazaadenosine or with 3-deazaaristeromycin, does not affect chemotaxis.

The specific role of 3-deazaadenosine as an inhibitor of macrophage cell line chemotaxis is supported by the following findings. Another macrophage cell line, RAW309CR, is resistant to the inhibition of chemotaxis by 3-deazaadenosine, and the resistance can be ascribed to the failure to accumulate 3-deaza-AdoHcy in this cell line. It is noteworthy that both RAW264 and RAW309CR accumulate similar amounts of AdoHcy after incubation with 3-deazaadenosine. The difference in the accumulation of 3-deaza-AdoHcy in RAW264 and RAW309CR is explained by the finding that sonicates of RAW264 rapidly form 3-deaza-AdoHcy but sonicates of RAW309CR do not. Both hydrolysis of AdoHcy and the inhibition of the hydrolysis of AdoHcy by 3-deazaadenosine are the same in sonicates of the two macrophage cell lines.

The discovery by Adler and Dahl (1) that methionine is required for bacterial chemotaxis led to a series of important studies on the role of methylation in chemotaxis. *In vivo* studies in *Escherichia coli* and *Salmonella typhimurium* established that the normal response to chemotactic stimuli depends on carboxymethylation of methyl-accepting chemotaxis proteins (2-9). Attempts to establish whether methylation reactions are analogously involved in eukaryotic chemotaxis have produced conflicting results. Protein carboxymethylation was stimulated by attractants in rabbit neutrophils (10), but carboxymethylation in guinea pig macrophages was not affected (11). On the other hand, incorporation of the methyl group of methionine into phosphatidylcholine was inhibited by attractant in both guinea pig macrophages (11) and rabbit neutrophils (12). These studies employed cells that were heterogeneous both in regard to cell type and cellular behavior. For example, only a fraction of human monocytes (20–40%) normally exhibit chemotaxis (13). Since the biochemical studies have used heterogeneous cell populations, precise correlation of biochemical events with the migrating cells remains undetermined.

In eukaryotes, intracellular methylations can be inhibited by decreasing the *S*-adenosylmethionine/*S*-adenosylhomocysteine ratio (14). AdoHcy[1] is a product inhibitor of all reactions that utilize AdoMet as methyl donor. The principal reaction for which AdoHcy is a substrate is catalyzed by AdoHcy hydrolase (EC 3.3.1.1), an enzyme first discovered by de la Haba and Cantoni (15). The reaction is reversible and equilibrium favors the direction of synthesis. 3-Deazaadenosine, an analog of adenosine, is both a potent inhibitor of AdoHcy hydrolase and a substrate of this enzyme, yielding *in vitro* a congener of AdoHcy, namely 3-deaza-AdoHcy (16). As a consequence of the intracellular accumulation of AdoHcy and 3-deaza-AdoHcy, the AdoMet/AdoHcy (or AdoMet/AdoHcy + 3-deaza-AdoHcy) ratio decreases and *in vivo* methylation can be inhibited (14, 16–20).

In this paper, we show for the first time that inhibition of chemotaxis by a macrophage cell line (21) is due specifically to the intracellular accumulation of 3-deaza-AdoHcy.

MATERIALS AND METHODS

Cells—Mouse macrophage cell lines RAW264 and RAW309CR were obtained from the Cell Distribution Center, Salk Institute, San Diego, CA and cultured in MEM containing 10% heat-inactivated (56 °C for 30 min) fetal calf serum, 100 international units/ml of penicillin and 100 μg/ml of streptomycin as described previously (21). The day before an experiment, the cells were scraped into the medium and centrifuged at 150 × *g* for 10 min. The cell pellet was resuspended in MEM containing 10% heat-inactivated fetal calf serum at a concentration of 7.5×10^5 viable cells/ml, and 4 ml were added to 6-cm dishes. Viable cells were determined by trypan blue exclusion.

Chemotaxis Assay—Migration of macrophage cell lines across a 10-μm thick Nucleopore polycarbonate filter containing 5-μm holes was measured in a 48-well chemotaxis chamber as described previously (21, 22). The top wells contained 50,000 cells in MEM with 10% heat-inactivated fetal calf serum, and the bottom wells contained a 1:100 dilution of endotoxin-activated mouse serum (23) in RPMI 1640 medium. After incubation for 4 h at 37 °C in an atmosphere of 5% CO_2, 95% air, the migrated cells were counted. Assays were performed in duplicate and the standard error was less than 20% when more than 100 cells were counted. In most experiments, less than 1% of the input cells migrated in the absence of attractant. Because of the error in counting low numbers of cells, the effect of 3-deazaadenosine on the migration in the absence of the attractant was not studied.

Determination of AdoMet, AdoHcy, and 3-deaza-AdoHcy—Cells groing in 6-cm dishes were incubated in 3 ml of MEM containing 5 μCi/ml of [^{35}S]methionine (Amersham/Searle). The specific activity of the methionine was adjusted so that the methionine concentration was the same as that in MEM (0.1 mM). At the end of the labeling period, the cells were rapidly washed once with 10 ml of methionine-free MEM. In some experiments, MEM which contained 0.1 mM nonradiactive methionine and 10% heat-inactivated fetal calf serum was then added for various times. The incubation was terminated by

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[1] The abbreviations used are: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; MEM, modified Eagle's medium.

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removal of the medium and addition of 2 ml of cold 5% sulfosalicylic acid to the cells in the dish. The sulfosalicylic acid suspension was removed and the dish was rinsed twice with 1 ml each of 5% sulfosalicylic acid. The sulfosalicylic acid suspension and the two rinses were combined and centrifuged at 3,000 × *g* for 20 min at 4 °C. To determine the amount of ^{35}S in AdoMet, AdoHcy, and 3-deaza-AdoHcy, the supernatant was filtered through either a glass fiber or Millex (0.22 μm) filter and applied to a VYDAC (Applied Sciences Inc.) cation exchange column as described previously (20, 24).

AdoHcy Hydrolase Assays in Sonicates—Cells were harvested and centrifuged at 150 × *g* for 10 min at 25 °C. The pellet was washed twice with 10 ml each of Dulbecco's phosphate-buffered saline without magnesium or calcium and suspended to approximately 1 × 10^8 cells/ml in H_2O at 25 °C. The suspension was cooled to 0 °C in an ice bath and sonicated in a water bath sonicator (Beckman model CC-25 ultrasonic cleaner) for a total of 2 min in 30-s pulses. No intact cells remained after sonication. The sonicate was centrifuged at 1500 × *g* for 20 min at 4 °C. The supernatant fluid was removed, adjusted to 10 mM potassium phosphate (pH 7.6), 0.07 mM sodium EDTA, and 2 mM dithiothreitol, and stored at −20 °C.

AdoHcy hydrolysis was measured by conversion of [^{14}C]AdoHcy to [^{14}C]inosine in the presence of excess adenosine deaminase as described previously (16). The reaction mixture (0.5 ml) was composed of 150 mM potassium phosphate (pH 7.6), 1.0 mM EDTA, 0.1 mM [^{14}C]AdoHcy, 2 units of adenosine deaminase (Type I; Sigma Chemical Co.), and sonicate. Synthesis of [^{3}H]3-deaza-AdoHcy was measured after incubation of 20 μM [^{3}H]3-deazaadenosine, 1 mM homocysteine, 11 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.6), 1 mM EDTA, and sonicate at 37 °C in a total volume of 90 μl. Sonicate and homocysteine were preincubated in buffer for 10 min at 37 °C and the reaction was started by the addition of [^{3}H]3-deazaadenosine. After 20 min, the reaction was stopped by addition of 1 ml of 50 mM HCl at 0 °C. [^{3}H]3-deaza-AdoHcy and [^{3}H]3-deazaadenosine were separated on SP-Sephadex columns by modification of previously published methods (16, 25). The reaction mixture was applied to an SP-Sephadex C-25 column (0.8 × 5 cm) equilibrated in 0.01 M HCl. [^{3}H]3-Deazaadenosine was washed from the column with 35 ml of 50 mM HCl and [^{3}H]deaza-AdoHcy was subsequently eluted into two scintillation vials with 10 ml of 1 N HCl. Hydrofluor (12 ml) was added to each vial and the vials were counted in a scintillation counter.

Chemicals—3-Deazaadenosine, 3-deazaaristeromycin, 5′-deoxy-5′-(isobutylthio)-3-deazaadenosine, and [^{3}H]3-deazaadenosine were obtained from the Southern Research Institute, Birmingham, AL. [^{14}C]AdoHcy was prepared according to the method of de la Haba and Cantoni (15). Sinefungin was obtained from Lilly Research Laboratories, Indianapolis, IN.

RESULTS

A number of compounds known to inhibit AdoHcy hydrolase, AdoMet synthetase, or methyltransferases were tested for the capacity to inhibit chemotaxis by a mouse macrophage cell line, RAW264 (Table I). 3-Deazaadenosine, a potent inhibitor of and substrate for AdoHcy hydrolase (16), and cycloleucine or ethionine, AdoMet synthetase inhibitors (26), inhibited chemotaxis in a dose-dependent fashion (Fig. 1). The most potent inhibitor was 3-deazaadenosine, which inhibited chemotaxis at concentrations as low as 30 μM. Structurally related compounds such as 5′-deoxy-5′-(isobutylthio)-3-deazaadenosine (27) and 3-deazaaristeromycin had no effect on chemotaxis. It is especially noteworthy that *in vitro*, 3-deazaaristeromycin is a more potent inhibitor of beef liver AdoHcy hydrolase than is 3-deazaadenosine.[2] Sinefungin, a compound that structurally may be considered an analog of either AdoMet or AdoHcy and that *in vitro* is a potent inhibitor of a number of methyltransferases (28), did not affect chemotaxis at nontoxic concentrations.

Experiments were performed to determine whether these compounds interacted with AdoHcy hydrolase *in vivo* and thereby caused changes in the intracellular levels of AdoHcy and AdoMet. Cells were prelabeled for 3 h with [^{35}S]methionine to avoid any effects the various inhibitors may have on

[2] P. K. Chiang, personal communication.

TABLE I
Inhibition of chemotaxis

RAW264 cells (3 × 10^6/6-cm dish) were plated 18 h before the addition of inhibitor. The cells were incubated with inhibitor for 3–4 h, harvested by scraping, and centrifuged. The cell pellet was resuspended to 1.25 × 10^6 cells/ml in MEM containing 10% heat-inactivated fetal calf serum and inhibitor, and 40 μl were added to the top wells of a multiwell chemotaxis chamber. The bottom wells contained inhibitors at the same concentration as in the top wells and a 1:100 dilution of endotoxin-activated mouse serum.

Inhibitor	Concentration range	Inhibition of chemotaxis[a]
	μM	
AdoHcy hydrolase		
3-Deazaadenosine	—[b]	+
3-Deazaaristeromycin	0.01–1000	−
5′-Deoxy-5′-(isobutylthio)-3-deazaadenosine[c]	0.001–300	−
AdoMet synthetase		
Cycloleucine	—[b]	+
Ethionine	—[b]	+
Methylases		
Sinefungin	40–400	−

[a] A compound was considered an inhibitor of chemotaxis only if inhibition increased with increasing concentration of inhibitor and if inhibition was more than 50% at the highest concentration tested.
[b] See Fig. 1.
[c] 1 × 10^{-3} M 5′-deoxy-5′-(isobutylthio)-3-deazaadenosine was toxic to the cells.

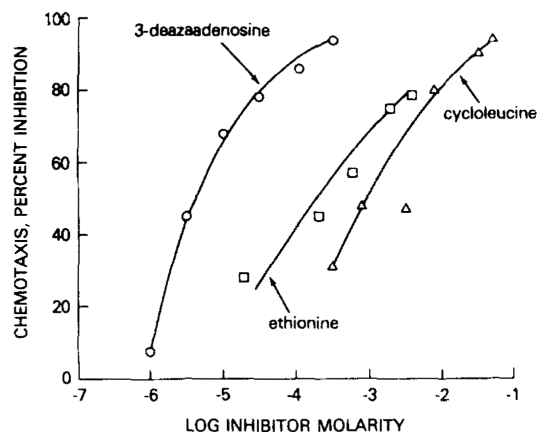

FIG. 1. **Inhibition of RAW264 macrophage cell line chemotaxis by 3-deazaadenosine, ethionine, and cycloleucine.** Cells were incubated with the inhibitor for 3–4 h before the chemotaxis assay. The figure shows the dose-response curves from two different experiments. In the control for the *3-deazaadenosine curve*, 20,700 cells (41%) migrated in response to the attractant and 400 (<1%) migrated to diluent. In the control for the *ethionine* and *cycloleucine curves*, 29,300 (59%) migrated in response to the attractant and 1,800 (4%) migrated to diluent.

the uptake of [^{35}S]methionine. It was determined that at 3 h, [^{35}S]methionine was in isotopic steady state with [^{35}S]AdoMet (not shown). After labeling, the cells were washed and incubated with medium containing nonradioactive methionine with or without addition of various inhibitors. In the absence of inhibitor, [^{35}S]AdoMet decreased with a half-life of 26 min (Table II) due to the utilization of [^{35}S]AdoMet for intracellular methylations. The formation of [^{35}S]AdoHcy was barely detectable, since the products of the hydrolysis of AdoHcy are rapidly metabolized. Partial inhibition of AdoHcy hydrolase would be expected to result: (*a*) in an accumulation of [^{35}S]AdoHcy; and (*b*) in a slower decline in [^{35}S]AdoMet if the levels of AdoHcy are sufficient to inhibit transmethylation reactions. Moreover, in the case of 3-deazaadenosine, accu-

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TABLE II

Effect of inhibitors on AdoMet and AdoHcy

RAW264 cells (3×10^6/6-cm dish) were labeled with 5 µCi/ml of [^{35}S]methionine in MEM without fetal calf serum for 3 h, washed, and incubated for the times indicated with MEM containing nonradioactive methionine, inhibitors, and 10% heat-inactivated fetal calf serum.

Inhibitor[a]	AdoMet				AdoHcy				3-Deaza-AdoHcy	
	0 min	15 min	60 min	120 min	0 min	15 min	60 min	120 min	15 min	60 min
	dpm/10[6] cells									
None	43,600	33,500	9,100	2,200	360	160	ND[b]	ND		
3-Deazaadenosine		33,400	15,300			2,500	2,100		700	1,200
3-Deazaaristeromycin		33,100	18,900			3,300	8,300			
5′-Deoxy-5′-(isobutyl-thio)-3-deazaadenosine		33,800	13,200			260	130			

[a] The concentrations of the inhibitors were: 3-deazaadenosine, 30 µM; 5′-deoxy-5′-(isobutylthio)-3-deazaadenosine, 0.2 mM; and 3-deazaaristeromycin, 0.5 mM.

[b] ND, was not accurately quantifiable. In some cases a trace amount was found.

mulation of 3-deaza-AdoHcy would be expected as a result of the utilization of this compound by AdoHcy hydrolase in the biosynthetic direction. Table II shows that treatment of the cells with 3-deazaadenosine resulted in the accumulation of [^{35}S]AdoHcy and [^{35}S]3-deaza-AdoHcy as well as a decrease in the rate of utilization of [^{35}S]AdoMet. In contrast, since 3-deazaaristeromycin is not a substrate for AdoHcy hydrolase, only AdoHcy accumulated after treatment with this compound. For example, at 60 min, 4 times more [^{35}S]AdoHcy was found in cells treated with 3-deazaaristeromycin than in cells treated with 3-deazaadenosine. If total nucleosidylhomocysteine (AdoHcy + 3-deaza-AdoHcy) is considered, cells treated with 3-deazaaristeromycin showed 2.5 times more radioactivity in AdoHcy than was observed in total nucleosidylhomocysteine in cells treated with 3-deazaadenosine.

Since 0.03 mM 3-deazaadenosine inhibited chemotaxis and 0.5 mM 3-deazaaristeromycin did not, and since both compounds caused an accumulation of AdoHcy but only 3-deazaadenosine caused the intracellular formation of 3-deaza-AdoHcy, the results reported in Tables I and II suggest that inhibition of chemotaxis in RAW264 cells is mediated by 3-deaza-AdoHcy and not by AdoHcy.

TABLE III

Effect of 3-deazaadenosine on chemotaxis

Cells were incubated with 100 µM 3-deazaadenosine at 37 °C in 75-cm^2 T-flasks. Cells were harvested and chemotaxis to a 1:100 dilution of endotoxin-activated mouse serum was measured in a multiwell chemotaxis chamber for 4 h. 3-Deazaadenosine (100 µM) was included in both the top and bottom wells of the chemotaxis chamber. In Experiment 1, the cells were preincubated with 3-deazaadenosine for 3 h at 37 °C and for 2 h at 25 °C during preparation for the chemotaxis assay. In Experiment 2, the cells were preincubated with 3-deazaadenosine at 37 °C for 5 h and for 2.5 h at 25 °C during preparation for the chemotaxis assay.

Cell line	Inhibition	
	Experiment 1	Experiment 2
	%	
RAW264	57	90
RAW309CR	0	0

Evidence in support of this conclusion was obtained when we examined the effects of 3-deazadenosine on chemotaxis by another mouse macrophage cell line, RAW309CR. The attractant specificity, time course of chemotaxis, and cell density dependence of chemotaxis for RAW264 and RAW309CR are similar (21). However, RAW264 was inhibited by 100 µM 3-deazaadenosine, whereas RAW309CR was not inhibited (Table III). Viability of either cell line was not significantly affected by 100 µM 3-deazaadenosine as judged by trypan blue exclusion, and there was little or no effect on phagocytosis of IgG-coated sheep red blood cells.

The effect of 3-deazaadenosine on the accumulation of AdoHcy and 3-deaza-AdoHcy in the two macrophage cell lines is shown in Table IV. In this experiment, the cells were labeled with [^{35}S]methionine in the absence and presence of 30 µM 3-deazaadenosine. The uptake of [^{35}S]methionine was not inhibited in either cell line, and both cell lines accumulated similar levels of AdoHcy; however, the amount of 3-deaza-AdoHcy synthesized in RAW264 cells was 7 times greater than in RAW309CR cells. These results support the conclusion that 3-deaza-AdoHcy is the inhibitor of chemotaxis in RAW264, and that RAW309CR does not accumulate sufficient 3-deaza-AdoHcy to inhibit its chemotactic response. The results suggest that AdoHcy hydrolase from RAW264 is inhibited by 3-deazaadenosine and, like the AdoHcy hydrolase from beef liver (16), utilizes 3-deazaadenosine as a substrate for the synthesis of 3-deaza-AdoHcy, whereas the enzyme from RAW309CR is subject to inhibition by 3-deazaadenosine but utilizes this adenosine analog as a substrate less efficiently.

To test this interpretation, AdoHcy hydrolase activity was measured in sonicates. The results (Fig. 2) show that sonicates from both cell lines hydrolyzed AdoHcy equally well and that

TABLE IV

Effect of 3-deazaadenosine on AdoMet and AdoHcy

Cells (3×10^6/6-cm dish) were plated 18 h before the addition of 5 µCi/ml of [^{35}S]methionine in MEM with and without 30 µM 3-deazaadenosine. The cells were labeled for 100 min and the radioactivity in AdoMet, AdoHcy, and 3-deaza-AdoHcy was determined.

Cell line	3-Deaza-adenosine	Methionine uptake[a]	AdoMet	AdoHcy	3-Deaza-AdoHcy	Total nucleosidyl homocysteine[b]
			dpm/10[6] cells			
RAW264	−	287,800	30,500	100		
RAW264	+	367,500	43,600	3,700	9,800	13,500
RAW309CR	−	193,300	26,100	<100		
RAW309CR	+	229,700	35,400	4,100	1,400	5,500

[a] Uptake of [^{35}S]methionine into whole cells. This was measured by counting an aliquot of the sulfosalicylic acid suspension before centrifugation.

[b] Calculated by addition of the disintegrations per min in AdoHcy and 3-deaza-AdoHcy.

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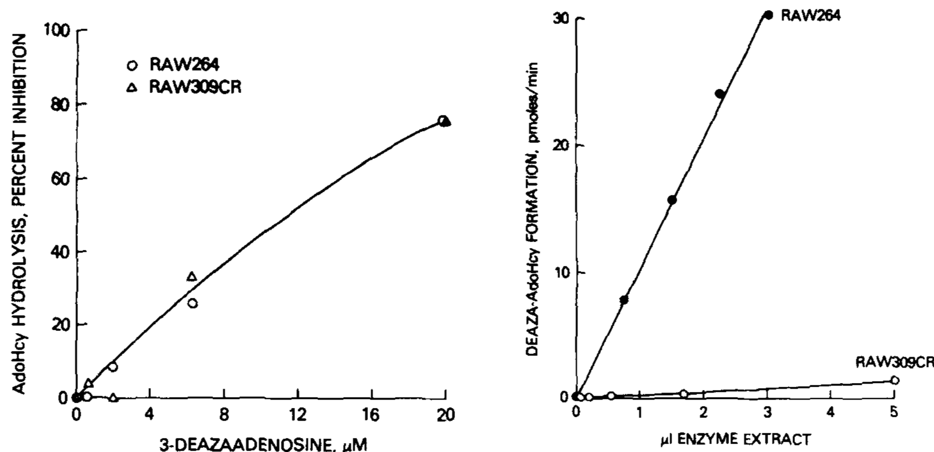

FIG. 2 (*left*). **Inhibition of AdoHcy hydrolase activity in sonicates of RAW264 and RAW309CR cells.** The activity in sonicates without inhibitors was 27.1 pmol/min/10^6 cells for RAW264 and 25.2 pmol/min/10^6 cells for RAW309CR.

FIG. 3 (*right*). **Formation of 3-deaza-AdoHcy in sonicates of RAW264 and RAW309CR cells.** One microliter of sonicate was prepared from 8.1 × 10^4 RAW264 cells or 5.2 × 10^4 RAW309CR cells. On a per cell basis, hydrolysis of AdoHcy was essentially the same in both sonicates.

this activity was inhibited to the same degree by 3-deazaadenosine. On the other hand, the sonicates from RAW309CR utilized 3-deazaadenosine as a substrate much less efficiently than the enzyme from RAW264 (Fig. 3). When sonicates were mixed and AdoHcy hydrolase activity was measured with 3-deazaadenosine as a substrate, the activity was additive, suggesting that the RAW309CR sonicate did not contain inhibitors of 3-deaza-AdoHcy synthesis.

DISCUSSION

It is established that AdoHcy inhibits essentially all the reactions in which AdoMet participates as a substrate; therefore a very large number of AdoHcy analogs have been synthesized in different laboratories (29) in the hope of discovering analogs that have greater specificity and that are capable of inhibiting only one or a limited number of AdoMet-dependent reactions. Indeed, when tested *in vitro* against different enzymes, the ratios of the inhibition constants for AdoHcy and AdoHcy analogs varied over a wide range (28). *In vivo*, however, the limited permeability of AdoHcy and its analogs represents a serious obstacle to the pharmacological utilization of these compounds. Here we show for the first time that the intracellular accumulation of an AdoHcy analog, 3-deaza-AdoHcy, can have biological effects that are different from those that can be demonstrated when intracellular levels of AdoHcy are experimentally increased. Therefore, it should be possible to take advantage of the relatively broad specificity of AdoHcy hydrolase toward its purine substrate to generate inside the cells a variety of analogs of AdoHcy with inhibitory specificities that differ from those of the natural compound and with potentially valuable pharmacological activities.

The present experiments provide additional evidence for the hypothesis that in eukaryotes, as in prokaryotes, biological methylation is involved in the chemotactic response. Both the inhibitory effects of 3-deazaadenosine and those of cycloleucine and ethionine are best explained by the single assumption that one (or more) enzymatic reaction(s) in which the methyl group of AdoMet is transferred to a suitable acceptor is necessary for chemotaxis by the RAW264 macrophage cell line. It can also be concluded that this reaction(s) is more sensitive to inhibition by 3-deaza-AdoHcy than by AdoHcy. It should be noted that Borchardt *et al.* (30) have shown that sinefungin and A9145C, compounds structurally related to AdoHcy, have inhibition constants for calf thymus protein carboxymethylase that are 5–40 times lower than the inhibition constants for AdoHcy. A recent report by Zimmerman *et al.* (31) indicates that accumulation of AdoHcy or 3-deaza-AdoHcy may perturb cyclic AMP metabolism. However, since inhibition of chemotaxis was specifically related to accumulation of 3-deaza-AdoHcy, their observations are probably not relevant to our studies.

As to the nature of the transmethylation reaction that is critically involved in the chemotactic response in the cell line studied, only conjecture is possible at this time. Carboxymethylation has been clearly identified as an essential feature of the mechanism of chemotaxis in bacteria (4–9). *In vivo* carboxymethylation of membrane proteins in human erythrocytes has been recently reported (32), but no specific biological function can as yet be ascribed to this carboxymethylation reaction.

A relationship of phospholipid methylation to eukaryotic chemotaxis has been postulated and it has been suggested that calmodulin, a protein that is a substrate for protein methylase, may modulate this reaction (33). Axelrod and his collaborators have advanced the hypothesis that phosphatidylethanolamine methylation is essential to a variety of physiological responses such as lymphocyte mitogenesis (34), histamine release by basophils and mast cells (35–37), and leucocyte chemotaxis (12). However, as we will show elsewhere, both 3-deazaadenosine and 3-deazaaristeromycin inhibit incorporation of the methyl group of methionine into phosphatidylcholine by more than 90% in RAW264 cells, whereas we show in this communication that administration of 3-deazaadenosine, but not that of 3-deazaaristeromycin, causes inhibition of chemotaxis.

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