Brief Definitive Report

METABOLISM OF PURINES IN MACROPHAGES

Effect of Functional State of the Cells*

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Cell populations taken from the peritoneums of mice may be categorized in three groups: resident cells washed from the peritoneums of normal mice; elicited cells washed from the peritoneums of animals that have received a prior injection of a sterile inflammatory agent, and activated cells from animals previously infected with *Listeria monocytogenes* or Calmette-Guérin bacillus (1–3). Of the biochemical criteria that define functionally different cell populations, the plasmalemmal enzyme ecto-5′-nucleotidase is the most discriminatory (1, 4). This enzyme cleaves external 5′-AMP to adenosine, which may then be transported intracellularly by the macrophage purine nucleoside transport system (5, 6). Modulation of ecto-5′-nucleotidase activity has been studied in detail (7–10). The enzyme exhibited higher titers in cells cultured in the presence of substrate (AMP), other nucleotides, and adenosine (10). Of more direct relevance to modulation of ecto-5′-nucleotidase titers during activation was the finding that the difference in ecto-5′-nucleotidase activities in different macrophage populations depends upon the rate of endocytosis and resulting breakdown of the enzyme (7–9).

The goal of our study was to examine the activity of cytoplasmic enzymes that metabolize adenosine and to determine whether they vary in a manner that might be linked to the ecto-5′-nucleotidase activity and, thus, to the generation of substrate adenosine. These enzymes are: adenosine deaminase (EC 3.5.4.4), purine nucleoside phosphorylase (EC 2.4.2.1), and adenine phosphoribosyltransferase (EC 2.4.2.7). We selected the former two enzymes because they have been shown to be of crucial importance for a normal cellular immune response in vivo (11–13) and in vitro (14), and because adenosine deaminase has been implicated in the transformation of monocytes to macrophages in vitro (15). Purine nucleoside phosphorylase may act catabolically or as a salvage enzyme (16). Adenine phosphoribosyltransferase is an important salvage enzyme.

Materials and Methods

Animals. Male CD-1 mice (Charles River Breeding Laboratories, Wilmington, Mass.) 28–35 d old were used in all the experiments.

Reagents. Adenine, adenosine, adenosine-5′-monophosphoric acid, inosine, inosine-5′-monophosphoric acid, hypoxanthine, *p*-nitrophenylphosphate (disodium salt; Sigma 104 phosphate substrate), and bovine serum albumin (essentially fatty-acid free) were all purchased from Sigma Chemical Co., St. Louis, Mo. [^{32}P]adenosine monophosphate triethylammonium salt,

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[^{14}C]adenine, [^{14}C]adenosine, and [^{14}C]inosine were purchased from New England Nuclear, Boston, Mass. Cellulose thin-layer chromotography sheets (No. 13255) with fluorescent indicator were purchased from Eastman Kodak Co., Rochester, N. Y.

Activation of Macrophages. Macrophages were activated by injecting 1–4 × 10^5 *L. monocytogenes* intravenously into the tail veins of mice. 7 d later, the mice were killed by cervical dislocation, and peritoneal exudate cells were harvested as described below.

Caseinate-elicited Macrophages. The mice were injected with 3.0 ml of 1.2% sterile sodium caseinate. 4 d later, they were killed, and the cells harvested as described below (10).

Harvesting of Cells. Peritoneal macrophages were harvested by injecting 10 ml of Krebs-Ringer phosphate medium (KRP) intraperitoneally, aspirating, and then repeating the process. Cells were pooled, washed twice in KRP, and then adjusted to 1 × 10^7 cells/ml. All manipulations were performed at 4°C. Resident cells were defined as peritoneal macrophages washed from uninjected animals.

Preparation of Monolayers and Cell Sonicates. 1 ml of cell suspension was dispensed to each plastic Petri dish (35 × 10 mm; No. 3001; Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.). The dishes were then incubated at 37°C under a 5% CO_2 atmosphere for 2 h. At the end of the period, each dish was washed three times in isotonic saline, which was then aspirated. 1 or 2 ml of 10 mM Tris, pH 7.4 at 4°C, was then added to each dish. After 5 min, the cells were scraped off with a rubber policeman. They were then sonicated for two 30-s bursts using a Measuring & Scientific Equipment Ltd. (London, England) 100-W ultrasonic disintegrator (No. 7100). The sonicate was then spun at 800 *g* for 10 min. The supernatant fluids were aspirated and either assayed directly or frozen at −20°C for up to 2 wk. Enzyme activities of cell sonicates were stable up to 3 wk after being frozen at this temperature. Sonicates were used immediately after thawing (i.e., they were not repeatedly frozen and thawed.)

Assay of Ecto-5′-Nucleotidase. The 5′-nucleotidase assay was performed exactly as described by Lazdins and Karnovsky (10) based on the method of DePierre and Karnovsky (17).

Assays of Adenosine Deaminase and Purine Nucleoside Phosphorylase. The enzymes were assayed according to the method of Van der Weyden and Bailey (18). 50 μl of cell sonicates was mixed with 50 μl of 1.2 mM [^{14}C]adenosine in 100 mM Tris, pH 7.4, in test tubes (10 × 70 mm) for measurement of adenosine deaminase. The reaction mixture was incubated for 10 min at 37°C. The reaction was stopped by the addition of 50 μl of absolute ethanol. The test tubes were then placed for 2 min in a sand bath that had been heated to 95°C. They were cooled and spun at 250 *g* for 10 min. 20 μl of the clarified mixture was then spotted on cellulose thin-layer chromatography plates. A mixture of cold carrier adenosine and inosine was then applied, and the plates were developed for 1.5–2.0 h in a solution of 0.100 M sodium phosphate, pH 6.8; saturated $(NH_4)_2SO_4$; and *n*-propanol (100:60:2 vol/vol/vol). The R_f values in this system were: adenosine, 0.23, inosine, 0.53; and hypoxanthine, 0.38. The plates were dried, cut into 2- × 2-cm squares, and the radioactivity associated with each square was determined by liquid scintillation counting in a toluene:ethanol scintillation mixture to measure the conversion of [^{14}C]adenosine to [^{14}C]inosine. The results were corrected for quenching. The assay was linear with time for 60 min; and it was also linear with protein concentrations between 25 and 120 μg/ml in the final reaction mixture. All assays were performed within these ranges.

In the assay of purine nucleoside phosphorylase, the final reaction mixture contained 50 μl 1 mM inosine in 0.1 M potassium phosphate buffer, pH 7.5. The reaction was allowed to proceed for 20 min before being stopped. Chromatography was performed as for the adenosine deaminase assay. A cold-carrier mixture of hypoxanthine and inosine was used to detect the product. The hypoxanthine spot was identified by fluorescence, cut out, and counted. The conversion of [^{14}C]inosine to [^{14}C]hypoxanthine was measured. The reaction was linear between 23.4 and 140 μg/ml protein in the final reaction mixture; and it was also linear with time for 45 min. All reactions were run within these ranges.

Assay of Adenine Phosphoribosyl Transferase. The assays of adenine phosphoribosyltransferase was performed by the method of Arnold and Kelley (19). All sonicates were first dialyzed against 10 mM Tris, pH 7.4, as described by Hochstadt (20) to remove nucleotides that are known to be inhibitory. The concentration of [^{14}C]adenine was 400 μm in the final incubation mixture. The concentration of 5′-phosphoribosyl-1-pyrophosphate (PRPP) as the Mg^{++} salt was 5 μM. The reaction was linear with time for 35 min; and it was also linear with protein

concentrations between 25 and 135 μg/ml. All assays were performed within these ranges. After 30 min, the reaction was stopped, and 20 μl of the reaction mixture was chromatographed on cellulose plates. The solvent system was the same as for the other two assays, and the conversion of [^{14}C]adenine to [^{14}C]AMP was measured.

Results

To establish that the peritoneal macrophages washed from mice infected with *L. monocytogenes* were activated, the level of the enzyme ecto-5′-nucleotidase was measured and compared to that of resident cells. Activated cells were found to have 8.3 ± 2.8 nmol/min per mg protein (sp act) in two experiments. Resident cells had 26.7 ± 2.2 nmol/min per mg protein (sp act). The activities are similar to those previously found for these cell populations (4).

The activities of the enzymes of purine nucleoside metabolism of the three different cell populations are shown in Table I. Activation or elicitation was accompanied by apparent increases in enzyme titer. Although the activities of adenosine deaminase in the sonicates of elicited and resident cells were significantly different, those for activated and resident cells were not.

The activity of purine nucleoside phosphorylase was comparable in elicited and activated cells, and both populations showed markedly elevated titers with reference to resident cells. A statistically significant difference ($P < 0.005$) between activities in elicited and resident cell populations, and between activated and resident cell populations was found.

The pattern of activity of adenine phosphoribosyltransferase followed that of purine nucleoside phosphorylase. The activity of this enzyme was eight times higher in activated and elicited cells than in resident peritoneal macrophages ($P < 0.005$), and the titer in the two former cell populations appeared to be the same.

A low-molecular-weight substance, present in resident cells, might be responsible for the expression of the different levels of enzyme activity seen in the various peritoneal macrophage populations. When cell sonicates were dialyzed up to 12 h, no change in activity of adenosine deaminase, purine nucleoside phosphorylase, or adenine phosphoribosyltransferase was found. Further evidence for the lack of low-molecular-weight inhibitors or activators is given by the mixing experiments of Table II. When cell sonicates of resident, elicited, or activated cells were mixed, the activities were additive. This argues against the presence of inhibitors or activators in resident, elicited, or activated cells.

The K_m values for each enzyme are essentially the same in resident, activated, and elicited cell populations. The K_m values for adenosine deaminase were 68–140 μM. For purine nucleosidase, the K_m values (inosine) were 45–72 μM, and those for adenine

TABLE I

Activity of the Enzymes of Purine Metabolism of Mouse Peritoneal Macrophage Populations*

Macrophage	Adenosine deaminase	Purine nucleoside phosphorylase	Adenine phospho-ribosyltransferase	Ecto-5′-nucleotidase	
Resident	38.1 ± 8.1	6.5 ± 0.3	0.6 ± 0.3	26.7 ± 2.4	45.7 ± 2.1‡
Activated	49.8 ± 10.0	58.9 ± 11.6§	4.0 ± 1.0§	8.3 ± 2.8§	19.1 ± 1.1‡§
Elicited	70.6 ± 27.3§	48.9 ± 18.3§	4.4 ± 2.5§	—	2.7 ± 0.3‡§

* Activity expressed as nmol/min per mg protein; $n = 3$–5.

‡ Taken from Karnovsky et al. (4).

§ Statistical comparison with the activity of resident cell population shows $P < 0.005$ using Student's t test.

TABLE II

The Effect of Mixing Cell Sonicates of Different Macrophage Populations on the Activity of the Enzymes of Purine Metabolism*

Macrophages	Enzyme					
	Adenosine deaminase		Purine nucleoside phosphorylase		Adenine phosphoribosyl-transferase	
Resident	39.3		6.3		0.7	
Activated	29.9		61.9		3.4	
Elicited	72.5		57.9		3.7	
	Found	Expected	Found	Expected	Found	Expected
Activated and resident	32.0	34.6	34.0	34.1	2.1	2.1
Activated and elicited	55.1	55.9	58.3	60.0	3.9	3.6
Elicited and resident	59.4	56.1	37.1	32.1	2.0	2.2

* Activity expressed as nmol/min per mg protein.

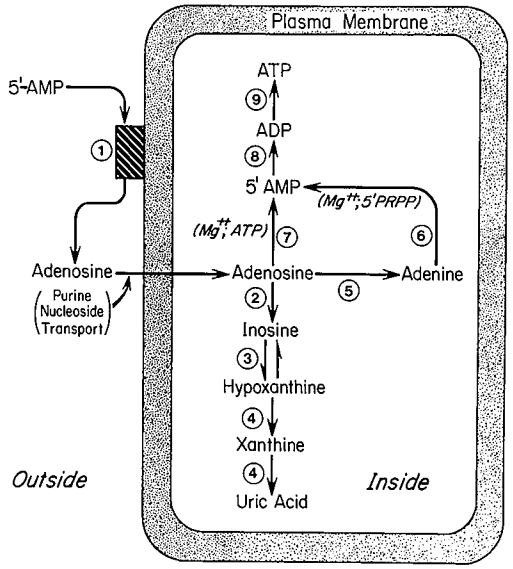

FIG. 1. Metabolism of adenine nucleotides and nucleosides in mouse peritoneal macrophages. Extracellular AMP (5'-AMP) is cleaved by the enzymes ecto-5'-nucleotidase (1) to adenosine and P_i. Adenosine is then transported intracellularly by the purine nucleoside transport system. It can then be metabolized by the pathways shown: (2) adenosine deaminase, (3) purine nucleoside phosphorylase, (4) xanthine oxidase, (5) adenosine phosphorylase (not established), (6) adenine phosphoribosyltransferase, (7) adenosine kinase, (8) adenylate kinase, and (9) nucleoside diphosphokinase. Reactions (1–3) and (6) have been examined in this paper.

phosphoribosyltransferase (adenine) were 60–83 μM. If there was a functionally different population of any of the enzymes, one might have seen a significantly different K_m for each of the enzymes studied in the sonicates of resident, activated, and elicited macrophages.

Discussion

The cellular relationships of some enzymes of purine nucleotide and nucleoside metabolism are shown in Fig. 1. Our findings indicated an intriguing reciprocal

relationship between the levels of the ectoenzyme (ecto-5′-nucleotidase) and the three intracellular enzymes.

It has been shown that the availability of adenine nucleotides and adenosine stimulated the appearance of the ectoenzyme in cultured macrophages (10). The reasons for this situation are not yet known, but in view of the effect of increased endocytosis, which diminishes the enzyme activity of the cells (8, 9), the obverse might apply in the above situation. It is possible that the increased titers of the three cytoplasmic enzymes might be an adaptation to diminished availability of substrate, controlled by ecto-5′-nucleotidase; i.e., efficiency of metabolism of adenine-containing substrates would thereby be enhanced. Such a situation would suggest that the cellular economy of adenosine metabolism depends in a real sense on intracellular substrate provided by the ecto-5′-nucleotidase from extracellular nucleotide.

Adenosine deaminase in human tissue has been shown to exist in three forms with different molecular weights. In tissues with high adenosine deaminase activity, the small form predominates. In tissues with low activity, the large form predominates. It is not known what functional significance this has, or whether such a situation exists in different peritoneal macrophage populations (21–23).

Fischer et. al. (15) argued for a role for the enzyme adenosine deaminase in the conversion of monocytes to macrophages in vitro. Their reasoning was based on the unique elevation of this enzyme's activity in cultured human monocytes which matured into macrophages over a period of 44 h. Furthermore, by culturing these cells with erythro-9-(2-hydroxy-3-nonyl) adenine, an inhibitor of adenosine deaminase, they were able to inhibit the conversion to macrophages. We felt that if adenosine deaminase were playing a cardinal role in the activation of macrophages, its activity might be specifically elevated in activated cell populations. As shown in Table I, this was not found to be the case.

It could be argued that the variations in enzyme levels we observed are reflections of well known variations in the composition of the cellular populations studied (24). This is probably a contributing factor, but it is unlikely to be the major cause of the phenomenon, because the changes in enzyme levels approach almost one order of magnitude in some cases. An intrinsic change in cellular enzyme titers is more probable.

Summary

Ecto-5′-nucleotidase is known to be diminished markedly in activated compared to control mouse macrophages. The level of three purine nucleoside metabolizing enzymes, adenosine deaminase (EC 3.5.4.4), purine nucleoside phosphorylase (EC 2.4.2.1), and adenine phosphoribosyltransferase (EC 2.4.2.7) were measured in the sonicates of different populations of mouse peritoneal macrophages. Levels of adenine phosphoribosyltransferase and purine nucleoside phosphorylase in macrophages that were elicited with sodium caseinate or activated in vivo by prior intravenous injection of *Listeria monocytogenes* were eight times higher than those in resident cells. Levels of adenosine deaminase also tended to increase and were two times higher in elicited cells than in resident cells. The K_m of each enzyme was the same in each cell population. The findings suggest that the levels of the ecto-5′-nucleotidase and of the intracellular enzymes are coordinated.

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