Proc. Natl. Acad. Sci. USA
Vol. 76, No. 2, pp. 925-929, February 1979
Immunology

Purine excretion by mouse peritoneal macrophages lacking adenosine deaminase activity

(immunodeficiency/deoxyadenosine/adenosine/uric acid/purine nucleoside kinases)

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Communicated by Howard Green, November 30, 1978

ABSTRACT **Deoxyadenosine, a cytotoxic purine nucleoside, is excreted in large amounts by patients with severe combined immunodeficiency disease associated with deficiency of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4). To identify the source of the purine nucleoside, purine excretion by macrophages was studied by using mouse peritoneal macrophages as an experimental model system. Normally, macrophages excrete a large quantity of uric acid into the culture medium. However, in the presence of deoxycoformycin, a potent inhibitor of adenosine deaminase, these macrophages also excreted deoxyadenosine. Furthermore, phagocytosis of nucleated erythrocytes augmented the excretion of deoxyadenosine. Macrophages are involved in the phagocytosis of nuclei that are extruded from normoblasts during erythropoiesis and also of senescent cells in lymphoid organs. A hypothesis is proposed that macrophages of the reticuloendothelial system are a source of deoxyadenosine, which is one of the two cytotoxic purine nucleosides (the other is adenosine) apparently responsible for the suppression of immune functions in patients with adenosine deaminase deficiency.**

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) catalyzes the deamination of adenosine and deoxyadenosine. An association between a deficiency in this enzyme and two cases of severe combined B and T cell immunodeficiency, a rare hereditary disease in which both humoral and cell-mediated immunity is impaired, was reported in 1972 by Giblett *et al.* (1). Increasing clinical evidence has accumulated that suggests a causal relationship between the enzyme deficiency and this specific form of immunodeficiency disease (2, 3). Also, recent reports have shown that inhibition of adenosine deaminase by drugs results in the suppression of immune functions in mice (4, 5). Although the specific mechanism by which the enzyme deficiency leads to the immunodeficiency state is not yet understood, it is likely that adenosine and deoxyadenosine are involved, because both of these substrates of adenosine deaminase have been found to be highly toxic to mammalian cells in culture. Green and Chan (6) have shown that adenosine is lethal to human lymphoblastoid cells at micromolar ranges. However, adenosine deaminase, by converting adenosine to nontoxic inosine, protects lymphoid cells against the cytotoxic effects of adenosine (7–10). More recently, Chan (11), Carson *et al.* (12), and Simmonds *et al.* (13) have reported that deoxyadenosine prevents blastic transformation of mitogen-stimulated human lymphocytes that lack adenosine deaminase activity. Clearly, lymphoid cells deficient in this enzyme are more susceptible to the toxic effects of both adenine nucleosides.

The normal plasma levels of adenosine are low: i.e., on the order of 0.1 μM (14). However, in a patient with adenosine deaminase deficiency, the plasma concentration of adenosine is raised to 3.6 μM (14). The patient also excretes large amounts of deoxyadenosine (13, 15), suggesting an accumulation of this deoxynucleoside in the extracellular fluid. It appears then that the adenosine deaminase deficiency condition, aside from rendering the patients' lymphoid cells more sensitive to cytotoxic effects of adenosine and deoxyadenosine, leads to an accumulation of these toxic nucleosides in the patients' body fluids. These combined effects of the enzyme deficiency state are probably the cause of the suppression of lymphoid cell proliferation in these patients.

The source(s) of these toxic purine nucleosides in patients has not yet been identified. It is likely that the nucleosides originate from nucleic acids as a result of tissue degradation. In this paper, evidence will be presented that macrophages of the reticuloendothelial system could be a source of the toxic purine nucleosides. Specifically, using an experimental model that involves the phagocytosis of chicken erythrocytes by mouse peritoneal macrophages, we will demonstrate that macrophages do digest the nucleic acid contents of phagocytized cells and subsequently excrete purine and pyrimidine components. In untreated macrophages, the excreted purine is predominantly in the form of uric acid. In macrophages lacking adenosine deaminase activity, however, a large amount of deoxyadenosine is also excreted. Thus, macrophages, by virtue of excreting the cytotoxic purine nucleoside, could play a crucial role in the pathogenesis of the severe combined immunodeficiency disease that is associated with adenosine deaminase deficiency.

MATERIALS AND METHODS

Culture Medium and Reagents. Macrophage cultures were routinely maintained in Eagle's essential medium without phenol red (Flow Laboratories, Rockville, MD, 1A-021C) supplemented with 25 mM Hepes (Sigma), 1% nonessential amino acid solution (GIBCO), and 15% heat-inactivated dialyzed fetal calf serum (Flow Laboratories). Cultures were incubated at 37°C in 5% CO_2/95% air. Brewer's thioglycollate medium (Difco) was prepared as in ref. 16.

Deoxycoformycin was a gift from J. Douros of the National Cancer Institute. All purine and pyrimidine bases and their nucleosides were purchased from Sigma. Methanol for high-pressure liquid chromatography was obtained from Burdick and Jackson Laboratories, Muskegon, MI. All other chemicals were reagent grade.

Peritoneal Macrophages. Cells were harvested from C57BL/6 mice of either sex (Charles River Breeding Laboratories) weighing 25–30 g, 4 days after intraperitoneal injection of Brewer's thioglycollate medium, according to the method of Michl *et al.* (16). The cells were plated in 35-mm dishes at a density of 2–2.5 $\times$ 10^6 per dish containing 2 ml of medium. The yield of macrophages averaged about 15 $\times$ 10^6 per animal. In some experiments, unstimulated macrophages were used.

Erythrocytes and Antiserum. Chicken erythrocytes and

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rabbit anti-chicken erythrocyte antiserum were gifts from L. Chan of the Department of Physiology, University of Connecticut Health Center. These erythrocytes were collected from 16- to 20-day White Leghorn chicken embryos (Spafas, Norwich, CT) as a 25% suspension in Alsever's solution. They were stored at 4°C for no more than 4 weeks before use. The rabbit anti-chicken erythrocyte antiserum was prepared by injecting chicken erythrocytes of 20-day embryos into rabbits. The serum was heated at 56°C for 30 min to inactivate complement and then stored in lyophilized form at −15°C. When reconstituted, it had an agglutination titer of greater than 1:10,000.

Phagocytosis Experiments. Antibody-coated chicken erythrocytes were prepared according to the method described by Michl *et al.* (16). A 0.1-ml sample of the erythrocyte suspension was added to each macrophage culture, at a ratio of 3–5 erythrocytes per macrophage. Where indicated, deoxycoformycin was added to the culture medium to the final concentration of 1 μg/ml 1 hr before addition of erythrocytes.

High-Pressure Liquid Chromatography. Two milliliters of medium that was harvested from the macrophage culture was prepared for high-pressure liquid chromatography by using the perchloric acid/KOH method (17). The neutralized extract was lyophilized and redissolved in 0.5 ml of water.

Purine and pyrimidine bases and their nucleosides in extracts were assayed by reverse-phase chromatography (Partisil PXS column, 10/25 octadecylsilane, Whatman) on a Micrometeric 8000 machine according to the method of Hartwick and Brown (18). The eluent was monitored at 254 nm. Material in each absorbance peak was identified by cochromatography with authentic compounds. For quantitation of deoxyadenosine and adenine, areas under the peaks were measured with a digitizer (Hewlett-Packard calculator, Model 10). Standard curves were obtained from adding known quantities of deoxyadenosine or adenine to the unspent culture medium after the acid extraction procedure.

Cell Extract Preparation. Macrophages were harvested from mice and cultured for 24 hr. After washing of monolayers, the cells were scraped off the plates and washed three times with phosphate-buffered saline (19). They were resuspended in 0.05 M Tris·HCl, pH 8/1 mM EDTA/0.1 mM dithiothreitol and then broken by sonication. The extract was centrifuged at 45,000 × *g* and the supernatant was stored in aliquots at −70°C until use. Protein concentrations were determined by the Lowry method (20), with bovine albumin (Sigma) as the standard.

Enzyme Assays. *Kinases.* Adenosine kinase (EC 2.7.1.20) and deoxyadenosine kinase (EC 2.7.1.76) assays have been published (17, 21, 22). Deoxycoformycin, 1 μg/ml, was added to the assay mixtures to inhibit adenosine deaminase (23).

Deaminase. Adenosine deaminase was assayed by using [^{14}C]adenosine or [^{14}C]deoxyadenosine (New England Nuclear) as substrates and thin-layer chromatography (17). The reaction products of adenosine deaminase assays of crude, unfractionated macrophage extracts included inosine (or deoxyinosine), hypoxanthine, xanthine, and uric acid. The combined radioactivities in these compounds were taken as the adenosine deaminase activity in the crude extract.

Phosphorylases. Inosine phosphorylase (EC 2.4.2.1) reaction mixture contained 0.06 M Tris·HCl, at pH 7.4, 1.3 mM ribose 1-phosphate, 0.34 mM [^{14}C]hypoxanthine at a specific activity of 10 μCi/μmol (1 Ci = 3.7 × 10^{10} becquerels), and cell extracts at approximately 50 μg per ml. The reaction product, inosine, was separated from hypoxanthine by thin-layer chromatography on cellulose plates (Brinkmann, MN300), with distilled water as solvent. For adenosine phosphorylase (EC 2.4.2.-) we followed the method of Hatanaka *et al.* (24). Deoxycoformycin was also included in the assay mixture to prevent deamination of adenosine, the reaction product.

Phosphoribosyltransferases. Assays for adenine phosphoribosyltransferase (EC 2.4.2.7) and hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) have been published (25).

RESULTS

Effect of Deoxycoformycin on Phagocytosis. It has been reported (26) that, during the course of maturation of human monocytes, the adenosine deaminase level was induced by as much as 9-fold, and that inhibition of adenosine deaminase by *erythro*-9-(2-hydroxy-3-nonyl)adenine resulted in the inhibition of the maturation of monocytes to macrophages. We therefore monitored the effects of deoxycoformycin, a potent inhibitor of adenosine deaminase, on activated mouse peritoneal macrophages by phase-contrast microscopy. This drug, when added to the macrophage culture at 1 μg/ml, showed no effect on the morphology of the macrophages up to 7 days. However, addition of deoxyadenosine (but not adenosine) at 25 μM with deoxycoformycin to the culture medium caused cell detachment and cell death in 2–3 days. Deoxycoformycin at 1 μg/ml did not affect the phagocytosis process microscopically. In the presence of the drug, internalization of antibody-coated chicken erythrocytes, disappearance of phase-dense granules within macrophages, and clearing of erythrocytes all proceeded as normal. However, the absorbance by the culture medium at 468 nm, the absorbance maximum of bilirubin (27), at the end of the 2-day incubation period was 5–10% less than that of the normal culture to which no drug was added. This result indicates that digestion of macromolecules by macrophages might be slightly impaired in the presence of the drug.

Purine and Pyrimidine Excretion by Activated Mouse Peritoneal Macrophages. We examined the excretion of purines and pyrimidines by activated peritoneal macrophages by using high-pressure liquid chromatography. Fig. 1 shows the UV absorbance patterns of materials that were eluted from the reverse-phase column, from 2-day culture medium (Fig. 1*B*) and unspent medium itself (Fig. 1*A*). Major peaks are numbered sequentially. The medium itself contained about 10 UV-absorbing substances (Fig. 1*A*). They included vitamins

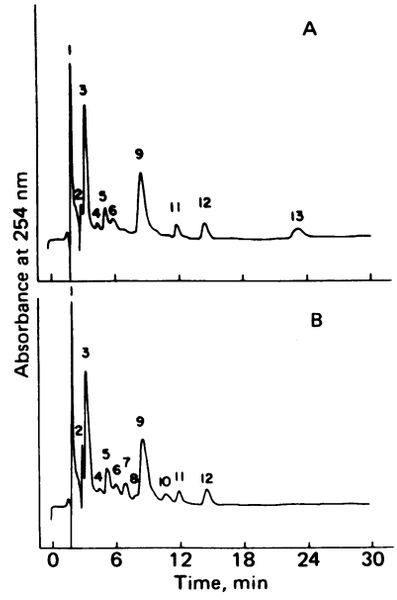

FIG. 1. Elution patterns of materials in unspent culture medium (*A*) and in 2-day activated mouse peritoneal macrophage culture medium (*B*). The medium was extracted by the acid extraction method, concentrated 4-fold, and then assayed by reverse-phase chromatography. Each assay used 100 μl of the processed sample. Numbering of the eluted materials is explained in the text.

(peaks 1, 2, 11, 12), amino acids (peaks 1, 3, 4), and as-yet-unidentified compounds (peaks 5, 6, 9, 13).

Several purines and pyrimidines and their nucleosides were excreted into the medium by macrophages after two days of incubation (Fig. 1*B*). Some of these appeared in the chromatographic pattern as new peaks and others cochromatographed with compounds that were present in the unspent medium itself. The major purine that was excreted by the macrophages was uric acid (coeluted with a vitamin in peak 2). Hypoxanthine and xanthine (coeluted as peak 8) were also excreted, but in very small amounts. No adenine or purine nucleosides could be detected. The pyrimidines and their nucleosides in the macrophage culture medium included uracil (coeluted with an amino acid in peak 3), thymidine (peak 10), and probably deoxycytidine (peak 7). In the chromatograph shown, uracil was not well resolved from an amino acid that was present in medium itself, namely peak 3 in Fig. 1*A*, although sometimes it could be (see Fig. 3*A*). The identity of the material that eluted as peak 7 has not been confidently established; it could be deoxycytidine, deoxyuridine, or both. The excretion of uracil is predictable from our earlier results with mouse fibroblasts (28). The excretion of thymidine is a confirmation of observations by Opitz *et al.* (29) and Stadecker *et al.* (30). Peak 13, which was present in medium prior to incubation, was sometimes, but not always, found missing in the medium harvested from the 2-day macrophage culture. Apparently, it could be metabolized into some other form and disappear from the culture medium.

Purine and Pyrimidine Excretion by Activated Macrophages after Phagocytosis of Chicken Erythrocytes. We then examined the purine and pyrimidine excretion pattern of macrophages that had phagocytized nucleated chicken erythrocytes. The results are shown in Fig. 2*A*. Comparison to Fig. 1*B* indicates that more uric acid (peak 2) and uracil (peak 3) were excreted by these macrophages. Also, the thymidine excretion (peak 10) became very substantial; so did excretion of deoxycytidine or deoxyuridine (peak 7). Notably absent in Fig. 2*A* are deoxyadenosine and deoxyguanosine, which are the two other residues in DNA. Our explanation for their absence is that these purine nucleosides had been converted to some other form, most likely to uric acid, and then excreted.

Purine Excretion by Activated Macrophages Deficient in Adenosine Deaminase Activity. We next examined the effects of adenosine deaminase deficiency on the purine excretion pattern. Fig. 2*B* shows the excretion pattern given by macrophages that have phagocytized chicken erythrocytes in the presence of deoxycoformycin, a potent inhibitor of adenosine deaminase. This pattern is quite different from that in the absence of the drug (Fig. 2*A*). Uric acid (peak 2) excretion, though still substantial, became less. The most striking differences were the appearance of two new peaks: adenine (peak 14) and deoxyadenosine (peak 15). Deoxycoformycin itself absorbs UV light and would have appeared between peaks 9 and 10. However, the amount of the drug added to the medium was so small that it could not be detected in the chromatograph.

Identification of adenosine by this chromatographic method was problematic because it was not well separated from an unidentified UV-absorbing material (peak 13) present in the medium. In one experiment, we replaced, after 24 hr of incubation, the complete culture medium with serum-free medium and then performed the phagocytosis experiment. We found that the chromatographic pattern (not shown) of the culture medium was cleaner and very little material was eluted in the peak 13 area. Under these experimental conditions, adenosine was not detectable in the culture medium to which deoxycoformycin had been added. We conclude, therefore, that adenosine was not excreted by macrophages after the phagocytosis of chicken erythrocytes.

As a control, purine excretion by activated macrophages themselves in the presence of deoxycoformycin was studied. The results are shown in Fig 3. Deoxyadenosine and, perhaps, adenine were also excreted by these macrophages. Adenosine was not detectable. The excretion of deoxyadenosine as shown

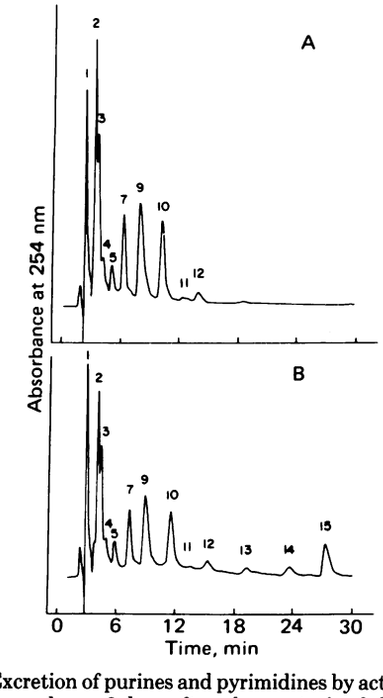

FIG. 2. Excretion of purines and pyrimidines by activated mouse peritoneal macrophages 2 days after phagocytosis of chicken erythrocytes in the absence (*A*) and in the presence (*B*) of deoxycoformycin. The method was as described in the legend to Fig. 1. The same number of chicken erythrocytes was added to each culture. The acid extraction procedure caused very little hydrolysis of deoxyadenosine. See text for the numbering of peaks.

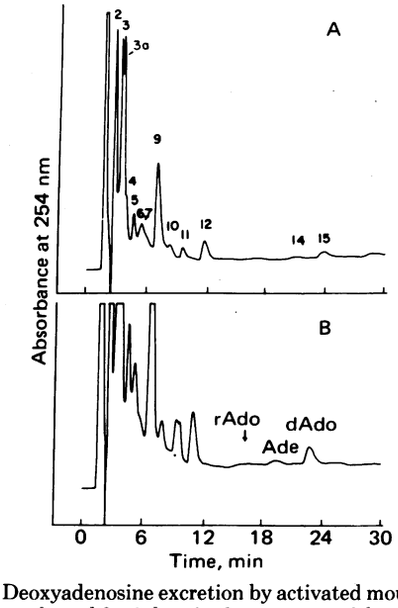

FIG. 3. Deoxyadenosine excretion by activated mouse peritoneal macrophages cultured for 2 days in the presence of deoxycoformycin. The method was as described in the legend to Fig. 1. *B* is the chromatographic pattern obtained from using 2.5 times as much material as in *A*. The numbering of peaks in *A* is identical to that in Fig. 1. Peak 3 became two peaks, one of which is uracil and the other, an amino acid (see text). The arrow in *B* indicates the elution position of adenosine (rAdo). Ade, adenine; dAdo, deoxyadenosine.

in Fig. 2*B* could be attributed to both phagocytized chicken erythrocytes and to excretion by macrophages themselves, with the former accounting for approximately 80% of total deoxyadenosine excreted.

Purine Excretion by Normal Macrophages Deficient in Adenosine Deaminase. The experiments described thus far involved the use of macrophages that were obtained from thioglycollate-injected mice. These macrophages are abnormal inflammatory cells. Compared to normal peritoneal macrophages, they have higher amounts of lysosomal enzymes (31) and a lower content of 5′-nucleotidase (32). Therefore, we examined purine excretion by normal, resident macrophages by a similar technique, i.e., after phagocytosis of chicken erythrocytes. The results are presented in Fig. 4). Once again, deoxyadenosine and adenine were found to be excreted into the culture medium when deoxycoformycin was added (Fig. 4*B*) but were absent in medium devoid of the adenosine deaminase inhibitor (Fig. 4*A*).

Purine Salvage Enzymes in Macrophages. The purine excretion pattern of a cell is the net result of the overall activities of enzymes, the so-called salvage enzymes, that are involved in the interconversion and reutilization of purine bases and nucleosides. To understand the mechanism of purine excretion by macrophages, the purine salvage enzyme activities in activated macrophage extracts were determined. The results are summarized in Table 1.

Macrophage extracts contained high levels of adenosine deaminase activity. This enzyme activity was completely inhibitable by deoxycoformycin. When the drug was added to the assay mixture at 1 μg/ml, more than 99% of the original adenosine deaminase activity was inhibited. Macrophages also contained high adenosine kinase activity; its specific activity was about one-eighth that of adenosine deaminase. On the other hand, deoxyadenosine kinase activity was very low, amounting to only about 1% of adenosine kinase activity, and it could be detected only after prolonged incubation.

Inosine phosphorylase catalyzes the reversible phosphorolysis of inosine and deoxyinosine. Its activity in peritoneal macrophage extracts was the highest of all purine salvage enzymes that have been assayed thus far: about 4 times the specific activity of adenosine deaminase (Table 1). The extracts also contained low but detectable adenosine phosphorylase activity, equivalent to only 0.6% of inosine phosphorylase activity, which is consistent with another report (33). The presence of the adenosine phosphorylase activity in the crude macrophage extracts could account for the formation and excretion into medium of adenine by cells in which adenosine deaminase was inhibited (Fig. 2*B*).

Table 1. Specific activities of purine salvage enzymes in extracts of stimulated mouse peritoneal macrophages

Enzyme	Sp. act.*
Adenosine kinase	44
Deoxyadenosine kinase	0.5
Adenosine deaminase	330†, 410‡
Adenosine deaminase + deoxycoformycin§	<2†¶
Inosine phosphorylase	1300
Adenosine phosphorylase	8.3
Adenine phosphoribosyltransferase	19
Hypoxanthine phosphoribosyltransferase	16
Xanthine oxidase	3.4‖

* nmol per hr per mg protein at 37°C (except for xanthine oxidase).
† Adenosine as substrate.
‡ Deoxyadenosine as substrate.
§ One microgram per ml.
¶ Calculated from assays counting less than twice background.
‖ nmol per hr per 10^6 macrophages at 37°C (T.-s. Chan and V. Bilanchone, unpublished data).

The macrophage extracts also contained both phosphoribosyltransferases capable of acting on hypoxanthine and adenine (Table 1). Although macrophages had adenine phosphoribosyltransferase activity, they apparently could not adequately convert adenine into adenine nucleotides, thus resulting in excretion of adenine. Xanthine oxidase activity was also found to be present in the macrophages (Table 1 and unpublished data). This finding explains the fact that macrophages excrete mainly uric acid, rather than hypoxanthine and xanthine, both of which are substrates for xanthine oxidase, as in the case for fibroblasts (21).

The results of enzyme assays, presented in Table 1, were from experiments using extracts from stimulated microphages. Whether normal macrophages contain these purine salvage enzymes has not yet been determined. However, judging from the purine excretion patterns shown in Figs. 2 and 4, the enzymes that are involved in adenosine and deoxyadenosine metabolism should be similar in both types of macrophages.

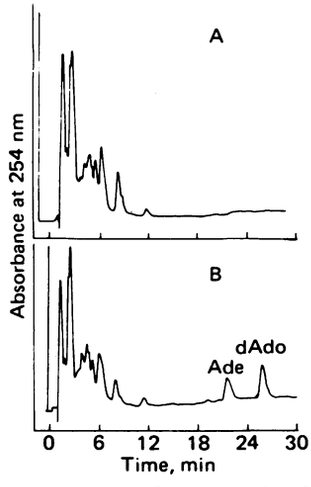

FIG. 4. Excretion of purines by unstimulated mouse peritoneal macrophages 2 days after phagocytosis of chicken erythrocytes in the absence (*A*) and in the presence (*B*) of deoxycoformycin. The method was as described in the legend to Fig. 3. The new peaks in *B* are adenine (Ade) and deoxyadenosine (dAdo). This chromatography was performed on a Waters (M. L.) high-pressure liquid chromatography machine in J. S. Kittredge's laboratory, the Marine Biomedical Institute, the University of Texas Medical Branch, Galveston, TX.

DISCUSSION

This paper demonstrates that activated mouse peritoneal macrophages excrete substantial amounts of purines. Untreated macrophages excrete purines predominantly in the form of uric acid. However, in the presence of deoxycoformycin, a potent inhibitor of adenosine deaminase, these macrophages excrete deoxyadenosine as well. When they phagocytize nucleated chicken embryonic erythrocytes, this excretion of deoxyadenosine is greatly augmented. It has been shown that the macromolecular components of phagocytized bacterial or animal cells are degraded into acid-soluble materials (34, 35). Macrophages possess the enzymatic machinery capable of digesting nucleic acids into their constituents; some of these enzymes—such as acid ribonuclease, acid deoxyribonuclease, and acid phosphatase—are very active in lysosomal preparations of macrophages (36). These nucleic acid constituents are transported across the phagolysosomal membrane and enter into the cytoplasm, where reutilization and interconversion of purines take place. Eventually, some purines are also excreted.

We have proposed earlier that, in mammalian cells, activities of adenosine kinase and adenosine deaminase should be precisely regulated (6, 21). Excessive phosphorylation of adenosine would result in cell death due to lethal interruption of *de novo* pyrimidine synthesis (37). Adenosine deaminase activity in excess of adenosine kinase activity would lead to loss of purines derived from adenosine, a fact which has been proven experimentally (21). This hypothesis can now be extended to include deoxyadenosine such that the activities of deoxyadenosine kinase and adenosine deaminase should also be precisely balanced in a given cell. Macrophages, because they lack deoxyadenosine kinase activity, excrete a substantial amount of purines, mainly in the form of uric acid. When adenosine deaminase is inhibited by a specific inhibitor, deoxycoformycin (23), these macrophages excrete deoxyadenosine, accompanied by a reduction in uric acid excretion. These results support the contention that some uric acid is derived from deoxyadenosine *via* the adenosine deaminase pathway in wild-type macrophages.

The results from this experimental model could be used to explain the turnover of nucleic acids in bone marrow, spleen, lymph nodes, and other reticuloendothelial systems. In mammals, it has been shown that the extruded nucleus of a normoblast is immediately phagocytized by a macrophage that is situated nearby (38). The remainder of the cell, namely the mature erythrocyte, enters the blood circulation. Upon senescence, circulating erythrocytes are phagocytized by macrophages that line the sinusoids of the reticuloendothelial systems, a process known as "erythroclasia." According to our experimental results, the nucleic acids of the extruded nuclei, as well as the senescent erythrocytes, are digested inside the macrophages and their purine and pyrimidine components are then excreted. .

We can further extend our results to propose that, in patients with hereditary adenosine deaminase deficiency, macrophages are a source of deoxyadenosine in the extracellular fluid. The macrophages in the bone marrow are probably the main source of this nucleoside because the bulk of the DNA in normoblasts is phagocytized and digested by these macrophages. In man, the bone marrow could be the most significant site of DNA degradation as a result of erythropoiesis, although macrophages of other lymphoid organs might also contribute to deoxyadenosine excretion.

Macrophages have been known to play crucial roles in immune functions. The purpose of this paper is to point out that macrophages are also importantly involved in the turnover of nucleic acids in the reticuloendothelial systems. Some of the macrophages, called reticular cells, are strategically stationed along the pathways followed by circulating T and B lymphocytes (e.g., sinus spaces of lymph nodes and spleen). Thus, these lymphocytes are especially subjected to the effects of cytotoxic purines excreted by the reticular cells. Although this proposal remains speculative, this anatomical relationship could explain why lymphoid organs are uniquely susceptible to the deleterious effects of adenosine deaminase deficiency.

I thank Dr. Howard Green for helpful discussions, Dr. L. Chan for gifts of chicken embryonic erythrocytes and rabbit anti-chicken erythrocyte antiserum, and Dr. J. Douros of the National Cancer Institute for his gift of deoxycoformycin. Technical assistance by R. Laraia is also gratefully acknowledged. High-pressure liquid chromatography was performed using a facility supported by the Connecticut Research Foundation. This investigation was aided by grants from the National Institutes of Health.

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