Proc. Natl. Acad. Sci. USA
Vol. 78, No. 2, pp. 1219–1223, February 1981
Medical Sciences

Selective killing of human malignant cell lines deficient in methylthioadenosine phosphorylase, a purine metabolic enzyme

(enzyme deficiency/polyamines/methotrexate/cancer chemotherapy)

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Communicated by Ernest Beutler, November 7, 1980

ABSTRACT **Seven out of 31 (23%) human malignant tumor cell lines had no detectable methylthioadenosine phosphorylase activity (<0.001 nmol/min per mg of protein), assayed with 5′-chloroadenosine as substrate. The enzyme-deficient cell lines were derived from five leukemias, one melanoma, and one breast cancer. None of 16 cell lines of nonmalignant origin, derived from lymphocytes, fibroblasts, and epithelial cells, lacked the enzyme (range, 0.156–1.447 nmol/min per mg of protein). As detected by autoradiography, intact enzyme-positive cell lines, normal immature bone marrow cells, and four specimens of malignant tumor cells incorporated the adenine moiety of 5′-chloroadenosine into nucleic acids; however, no enzyme-deficient cell lines used 5′-chloroadenosine. When both types of cell lines were cultured in a medium containing 0.4 μM methotrexate, 16 μM uridine, and 16 μM thymidine (or 10 μM azaserine alone), no cells grew. If methylthioadenosine was added to the same medium, only enzyme-positive cells increased in number; most enzyme-deficient cells were dead after 3 days. Thus, human malignant tumor cell lines naturally deficient in methylthioadenosine phosphorylase could be selectively killed when *de novo* purine synthesis was inhibited and methylthioadenosine was the only exogenous source of purines.**

Successful cancer chemotherapy ideally exploits defined metabolic differences between malignant and normal cells. Unfortunately, no clean-cut enzymatic differences have been discovered. In 1977, however, Toohey (1) described four mouse tumors that were deficient in the polyamine-related enzyme 5′-methylthioadenosine phosphorylase (MeSAdo phosphorylase). A preliminary survey of established human leukemic cell lines also disclosed that 5 of 13 were deficient in MeSAdo phosphorylase. The enzyme-negative cell lines excreted large amounts of 5′-methylthioadenosine (MeSAdo) into the culture medium (2).

In mammalian cells, MeSAdo is produced during synthesis of the aliphatic polyamines spermidine and spermine from decarboxylated *S*-adenosylmethionine (3) (Fig. 1). MeSAdo does not accumulate in normal tissues (4) but is cleaved rapidly to adenine and 5-methylthioribose-1-phosphate by MeSAdo phosphorylase (5), which is abundant in all organs of the rat examined (6). The adenine presumably is reconverted to purine nucleotides via adenine phosphoribosyltransferase (EC 2.4.2.7). The loss of MeSAdo phosphorylase, by decreasing adenine formation, would be expected to interfere with this salvage pathway.

The purposes of the present experiments were (*i*) to determine the distribution of MeSAdo phosphorylase levels and the incidence of enzyme deficiency among a large number of human cell lines derived from normal and malignant tissues of diverse origin, (*ii*) to develop a method for assessment of the phosphorylase in individual cells from normal and cancerous tissue specimens, and (*iii*) to devise a chemotherapeutic strategy that could selectively kill enzyme-deficient cells. The results show that MeSAdo phosphorylase was undetectable in 7 out of 31 (23%) cell lines derived from leukemia, malignant melanoma, and breast cancer, but in none of 16 cell lines derived from normal tissues. The enzyme was also identified by autoradiography in 98% of normal immature bone marrow cells and in four malignant tumor specimens. Enzyme-deficient malignant cell lines could be selectively killed by inhibiting *de novo* purine synthesis with methotrexate or azaserine in the presence of exogenous MeSAdo.

MATERIALS AND METHODS

Cell Lines. Forty-seven different human cell lines were analyzed. Among 31 cell lines of malignant origin, CCRF-CEM (7), derived from acute lymphoblastic leukemia (ALL), was obtained from the American Type Culture Collection. Ian Trowbridge (Salk Institute, La Jolla, CA) kindly provided the following: RPMI 8402 (8), HPB-ALL (9), NALL-1 (10), and Reh (11), all derived from ALL; TALL-1 (12), derived from lymphosarcoma; U937 (13), derived from histiocytic leukemia; and Daudi, derived from African Burkitt's lymphoma (14). MOLT 4 (15), derived from ALL, came from J. Minowada (Roswell Park Memorial Institute, Buffalo, NY). Raji (16), derived from African Burkitt's lymphoma, K562 (17), from the pleural effusion of a patient with chronic myelocytic leukemia in blast crisis, and BJA-B, from Burkitt's lymphoma (18), were obtained from A. Theofilopoulos and F. Jensen (Scripps Clinic and Research Foundation). Louckes, from American Burkitt's lymphoma, was obtained from E. Kieff (University of Chicago). The 18 other cell lines of malignant origin, as follows, were all sent from Nelson-Rees' laboratory to the Scripps Clinic and Research Foundation for the present study: HT1417 (19) and Hs602T (19), derived from lymphoma; Hs324T (19), from reticulum cell sarcoma; Hs445T (20), from Hodgkin's disease; Hs427T (19), from lymphosarcoma; BT20 (21), MDA-MB-157 (22), SKBR-3 (23), and 734B (24), all from breast cancer; A375 (25), Hs294T (26, 27), Hs695T (27), Hs852T (27), Hs936T/Cl (28), Hs939T (29), and Hs944T, all from melanoma; Hs578T (30), from breast carcinosarcoma; and HBL-100 (31),§ from a milk sample. Among 16 cell lines of nonmalignant origin, WI-L2 (32), derived from a spleen sample of a patient with spherocytosis, came from R.

Abbreviations: MeSAdo, 5′-methylthioadenosine; ClAdo, 5′-chloroadenosine; ALL, acute lymphocytic leukemia; AdoMet, *S*-adenosylmethionine.

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§ Although this cell line is purported to be from normal tissue, it shows the same characteristics as tumor cells (e.g., altered morphology and karyotype).

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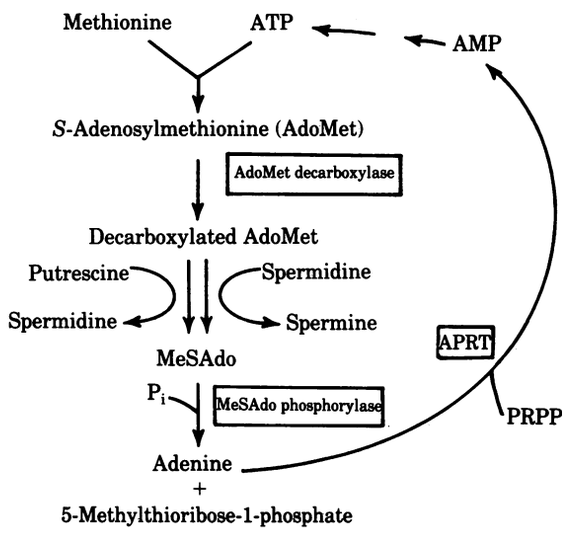

FIG. 1. Role of MeSAdo phosphorylase in adenine recycling by mammalian cells. APRT, adenine phosphoribosyltransferase; PRPP, phosphoribosyl pyrophosphate.

Lerner of Scripps. HCL-19E, HCL-20E, HCL-21E, and HCL-160E were established in N. Kamatani and D. Carson's laboratory by infection of human cord blood lymphocytes with Epstein–Barr virus; 70C, San, Kar, Lau, Ing, and Nel were similarly established from adult human peripheral blood lymphocytes. R7000, derived from foreskin, came from J. E. Seegmiller's laboratory (University of California, San Diego). Hs578Bst (30), Hs939Sk, Hs767Bl (33), and Hs888Lu, derived from normal breast, skin, urinary bladder, and lung, respectively, were sent from Nelson-Rees' laboratory.

The anchorage-dependent cell lines were routinely maintained either in Eagle's minimal essential medium (Eagle's medium) supplemented with 2 mM glutamine, nonessential amino acids, 10 mM sodium pyruvate, 100 units of penicillin per ml, and 100 μg of streptomycin per ml (all from GIBCO) and 10% (vol/vol) fetal bovine serum (Flow Laboratories, Rockville, MD) or in Dulbecco's modified Eagle's medium (original formula containing 4.5 g of glucose and 3.7 g of $NaHCO_3$ per liter) supplemented with 10 μg of bovine insulin per ml (Calbiochem), nonessential amino acids, antibiotics, and 10% fetal bovine serum. Cells growing in suspension were maintained in RPMI-1640 medium (Flow Laboratories) supplemented with antibiotics and 10% fetal bovine serum. All cultures were kept in logarithmic growth at 37°C in a humidified atmosphere of 95% air/5% CO_2.

Measurement of MeSAdo Phosphorylase in Cell Extracts. Cells in late logarithmic growth phase were washed three times in isotonic phosphate-buffered saline (pH 7.4) and then frozen and thawed three times at a density of 5×10^7 cells per ml in distilled water. Insoluble material was removed by sedimentation at 20,000 × *g* for 30 min at 4°C, and the resulting clear supernatants were frozen at −70°C for up to 90 days without loss of enzyme activity. For routine assay of MeSAdo phosphorylase, 5′-chloro[2-^{3}H]adenosine (ClAdo), an alternative substrate for the enzyme, was used (34). ClAdo had been prepared from [2-^{3}H]adenosine (specific activity 13.6 Ci/mmol, 1 Ci = 3.7×10^{10} becquerels; New England Nuclear) as described (35) and then purified further on a C_{18} μBondapak column (Waters Associates) eluted with 8% (vol/vol) acetonitrile in water at a flow rate of 1 ml/min. The incubation mixture for MeSAdo phosphorylase assay contained 10–100 μg of protein, 50 μM potassium phosphate (pH 7.4), and 20 μM Cl[2-^{3}H]Ado, 0.4 μCi in a total volume of 100 μl. The reaction was initiated by addition of radioactive substrate, and the mixture was incubated at 37°C for 10–60 min. The reaction was then terminated by immersion in a boiling water bath for 2 min. Insoluble material was sedimented at 2500 × *g* for 10 min, and 10 μl of the supernatants was applied to polyethyleneimine-cellulose sheets (no. 5504, Merck, Darmstadt, Federal Republic of Germany). Radioactive adenine was separated from ClAdo in 1 M ammonium acetate developed in the presence of unlabeled standards. Adenine was visualized under ultraviolet light and cut out, and radioactivity was measured in a liquid scintillation spectrometer at an efficiency of 23%. The formation of product was linear with respect to both protein concentration and time for each of the values reported.

Autoradiography. For measurement of MeSAdo phosphorylase by autoradiography, horse serum, which lacks the enzyme, was substituted for fetal calf serum, which had abundant enzyme activity (0.59 nmol/min per ml). Monolayer cultures on Lab-Tek microslides (four-chamber slides, Lab-Tek Products, Naperville, IL) were incubated in Eagle's medium containing 10% horse serum and 0.25 μCi of Cl[2-^{3}H]Ado in a volume of 0.2 ml. After 12 hr at 37°C, cells were fixed and processed for autoradiography by standard methods (36). Cell lines growing in suspension were similarly incubated in RPMI-1640 medium with 10% horse serum at a cell density of 4–10 × 10^5 ml in a volume of 0.2 ml containing 0.25 μCi of Cl[2-^{3}H]Ado in Falcon 2058 plastic tubes. Twelve hours later, the cells were washed three times in ice-cold isotonic phosphate-buffered saline (pH 7.4) and pelleted onto microscope slides with a cytocentrifuge (Shandon Southern Instruments, Sewickley, PA). Fixation of the cells and autoradiography were as described above. In each case the exposure period was 3–5 days. All slides examined for autoradiography were also stained with Giesma solution (Eastman Kodak), except those containing both ^{3}H and ^{14}C. In some experiments, [2-^{3}H]adenine (33.7 Ci/mmol) was substituted for Cl[2-^{3}H]Ado.

Normal and leukemic bone marrow cells, obtained by aspiration, were separated from erythrocytes by dextran sedimentation (37). After the cells were washed twice in RPMI-1640 medium, they were suspended at a density of 10^7/ml in the same medium supplemented with 20% fetal bovine serum plus 10% (vol/vol) dimethyl sulfoxide and then frozen at −80°C. Adenocarcinoma cells were obtained by thoracentesis and were washed and processed similarly. Subsequently, all samples were thawed, washed, and resuspended at a density of 10^6 cells per ml in RPMI-1640 medium with 10% horse serum and Cl[2-^{3}H]Ado exactly as for cell lines. After 12 hr of incubation, the cells were harvested and processed for autoradiography. Normal and leukemic peripheral blood lymphocytes were also isolated from venous blood by Ficoll/Hypaque sedimentation (37). The normal cells were incubated at a density of 1×10^6/ml in RPMI-1640 medium with 10% fetal calf serum and 5 μg of phytohemagglutinin-M per ml (Sigma). After 64 hr, the cells were washed and suspended in RPMI-1640 medium with 10% horse serum and Cl[2-^{3}H]Ado for assay of MeSAdo phosphorylase. The leukemic cells were processed directly without preculturing with phytohemmagglutin. In one experiment, [2-^{3}H]adenine and Cl[8-^{14}C]Ado (55.7 Ci/mol) were incubated simultaneously with normal bone marrow cells and then were processed for autoradiography by the thick emulsion method of Perdue *et al.* (38), which permits discrimination between ^{14}C and ^{3}H grains in single cells.

Selective Killing of MeSAdo Phosphorylase-Negative Cells. Phosphorylase-positive and -negative cell lines were cultured in RPMI-1640 medium containing 10% horse serum, 0.4 μM methotrexate, 16 μM thymidine, and 16 μM uridine (all from

Calbiochem), with or without 16 μM MeSAdo (Sigma). In some experiment, 10 μM azaserine (Calbiochem) was substituted for the methotrexate/pyrimidine mixture. Initial cell density was 1.5×10^5 ml. After 3 days, the number of viable cells was counted in a hemocytometer with 0.1% trypan blue.

RESULTS

ClAdo-Cleaving Activity in Human Cell Lines. Of the 47 human cell lines analyzed, 7 (15%) had undetectable MeSAdo phosphorylase activity, as analyzed by radioassay of ClAdo-cleaving activity (Fig. 2). Considering the sensitivity of the radioenzyme assay, the activity of each enzyme-negative cell line was at most 0.001 nmol/min per mg of protein (i.e., <1% of the lowest activity of enzyme-positive cell lines). Among the cell lines containing MeSAdo phosphorylase, the logarithmic enzyme activity was normally distributed with a mean of 0.57 nmol/min per mg of protein. Two of the enzyme-deficient malignant cell lines (CCRF-CEM and RPMI 8402) were derived from T-cell leukemias, two (NALL-1 and Reh) from non-T-, non-B-cell leukemias, one (K562) from chronic myelogenous leukemia in blast crisis, one (Hs294T) from malignant melanoma, and one (734B) from breast cancer. Thus, among 31 cell lines of malignant origin, 7 (23%) were enzyme deficient. On the other hand, none of 16 cell lines of nonmalignant origin lacked MeSAdo phosphorylase, including anchorage-dependent cell lines with fibroblastic or epithelioid structure and Epstein–Barr virus-transformed peripheral blood B lymphocytes growing in suspension. Two of the enzyme-deficient malignant cell lines and five of the enzyme-containing cell lines have been maintained continuously in our laboratory for more than 1 year without detectable alteration in enzyme activity.

In 16 of the 47 cell lines, including 5 enzyme-deficient and 11 normal cell lines, MeSAdo phosphorylase was also assayed by the radiochemical method of Pegg and Williams-Ashman (5), with [*methyl*-^{3}H]MeSAdo as substrate, with separation of the products and reactants by column chromatography. Each cell line unable to cleave ClAdo was similarly deficient in MeSAdo-cleaving activity. Among the MeSAdo phosphorylase-positive lines, there was excellent correlation between logarithmic enzyme activities assayed with the alternative substrates ($r = 0.961$, $P < 0.001$).

Detection of ClAdo-Cleaving Activity by Autoradiography. Ten individual cell lines containing MeSAdo phosphorylase were incubated with Cl[2-^{3}H]Ado and analyzed by autoradiography. In each case, every intact cell incorporated radioactivity into cytoplasm or nucleus (or both) (>200 grains per cell, Fig. 3*A*). When six MeSAdo phosphorylase-deficient cell lines were analyzed similarly, no cells were found that incorporated grains over background levels (<30 grains per cell, Fig. 3*B*) although all incorporated [^{3}H]adenine as well as enzyme-positive cell lines (data not shown).

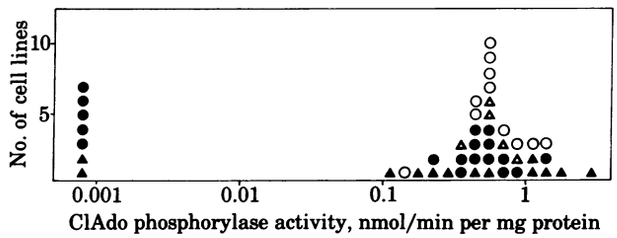

FIG. 2. ClAdo phosphorylase activity in extracts of human cell lines. ○, Anchorage-independent cell lines of nonmalignant origin; ●, anchorage-independent cell lines of malignant origin; △, anchorage-dependent cell lines of nonmalignant origin; ▲, anchorage-dependent cell lines of malignant origin.

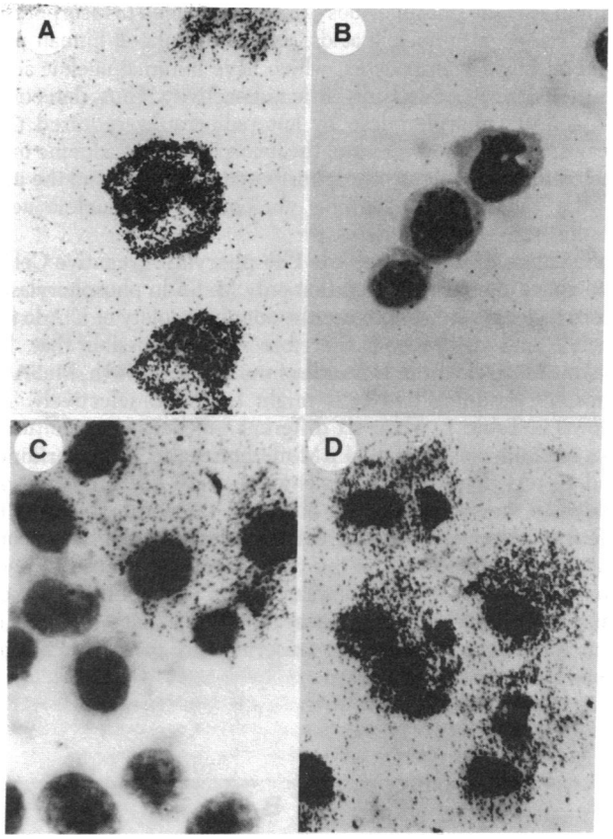

FIG. 3. Autoradiographic detection of MeSAdo phosphorylase in cell lines and normal bone marrow. Cells were cultured with 0.25 μCi of Cl[2-^{3}H]Ado in medium containing 10% horse serum. After 12 hr, they were fixed and autoradiography was performed. (*A*) Enzyme-positive cell line (Raji). (×330.) (*B*) Enzyme-negative cell line (NALL-1). (×330.) (*C*) Enzyme-positive cell line (SRBR-3) plus enzyme-negative cell line (734B). (×210.) (*D*) Normal bone marrow cells. (×330.)

More than 98% of nucleated cells in human bone marrow, excluding mature granulocytes, lymphocytes, and erythrocytes, incorporated Cl[2-^{3}H]Ado into nucleic acids, as detected by autoradiography (>200 grains per cell, Fig. 3*D*). When analyzed by the method of Perdue *et al.* (38), all the bone marrow cells incorporating [^{3}H]adenine also incorporated Cl[^{14}C]Ado. One hundred percent of human peripheral blood lymphoblasts stimulated with phytohemagglutinin were positive for MeSAdo phosphorylase, as detected by autoradiography (data not shown).

In order to determine whether or not the adenine moiety obtained by cleavage of ClAdo by MeSAdo phosphorylase-positive cells could be transferred to enzyme-negative cells, mixed cultures were prepared containing two cell lines, one of which was enzyme positive and the other enzyme negative. In six separate experiments, the enzyme-negative cells did not incorporate significant amounts of ClAdo, as detected by autoradiography, even when cocultured with enzyme-positive cells (e.g., Fig. 3*C*). Thus, there was no evidence for metabolic cooperation between enzyme-positive and enzyme-negative cells under these conditions.

Cells from two bone marrow specimens and one peripheral blood specimen from patients with ALL, and cells from an adenocarcinomatous pleural effusion of unknown origin were also incubated with Cl[2-^{3}H]Ado. In each case, more than 70% of the nucleated cells incorportated radioactivity into nucleic acid (Fig. 4).

In other experiments, human B lymphoblastoid cells defi-

cient in adenine phosphoribosyltransferase failed to incorporate Cl[2-^{3}H]Ado into nucleic acids, and unstimulated human peripheral blood lymphocytes, which have minimal nucleic acid synthesis, incorporated only little radioactivity. Thus, detection of MeSAdo phosphorylase by autoradiography required the cleavage of ClAdo to adenine, the incorporation of adenine into nucleotides via adenine phosphoribosyltransferase, and the use of the radiolabeled adenine nucleotides for nucleic acid synthesis.

Selective Killing of MeSAdo Phosphorylase-Negative Cells. The above results indicated that only MeSAdo phosphorylase-positive cells were able to use the adenine moiety of ClAdo for nucleic acid synthesis. It therefore seemed possible that, by making MeSAdo the only source of purines for growth, MeSAdo phosphorylase-negative cells might be killed selectively. As shown in Table 1, when six different cell lines were cultured in a medium containing 0.4 μM methotrexate, 16 μM uridine, and 16 μM thymidine, no cells grew. If 16 μM MeSAdo was added to the same medium, however, the number of cells in the four enzyme-containing cell lines increased. On the contrary, most of the cells in the two enzyme-deficient cell lines were dead after 3 days. Similar results were obtained when enzyme-positive and enzyme-negative cells were cultured in a medium containing 10 μM azaserine with or without exogenous MeSAdo (Table 1).

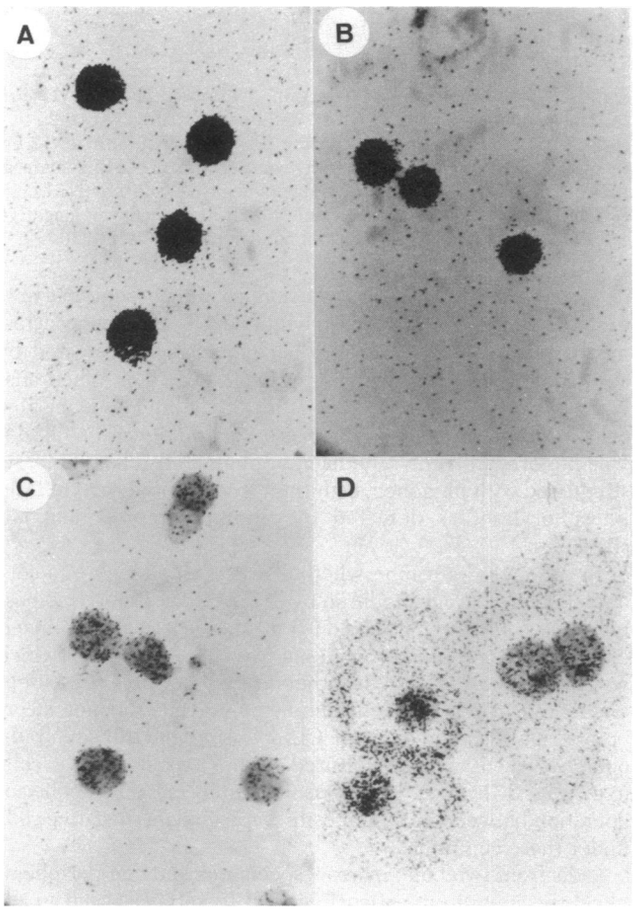

FIG. 4. Autoradiographic detection of MeSAdo phosphorylase in tumor cell specimens. (×330.) The assay was performed as described in Fig. 3. (*A* and *B*) Bone marrow aspirates from ALL. (*C*) Peripheral blood specimen from ALL in relapse. (*D*) Pleural fluid aspirate, adenocarcinoma of unknown origin.

Table 1. Selective killing of MeSAdo phosphorylase-negative cells

		Growth, % of control*			
		Azaserine		Methotrexate	
Cell line	MeSAdo phosphorylase	Without MeSAdo	With MeSAdo	Without MeSAdo	With MeSAdo
CEM	–	12	18	9	9
K562	–	10	12	13	12
Raji	+	14	100	8	103
U937	+	NT	NT	13	72
WI-L2	+	9	76	3	79
70C	+	NT	NT	13	98

Cells were seeded at a density of 1.5×10^5 ml in medium supplemented with 10% horse serum; 16 μM adenine, 16 μM MeSAdo, or no purines; and 0.4 μM methotrexate or 10 μM azaserine. The methotrexate-supplemented cultures also contained 16 μM thymidine and uridine. After 3 days, the cells were counted in a hemocytometer with 0.1% trypan blue. In adenine-supplemented cultures, the number of cells increased 3- to 10-fold. NT, not tested.

* Percent control growth = 100 × (cell growth in azaserine- or methotrexate-treated cultures with or without MeSAdo)/(cell growth in azaserine- or methotrexate-treated cultures with adenine).

DISCUSSION

Seven of 47 (15%) human cell lines were completely deficient in the purine metabolic enzyme MeSAdo phosphorylase. The deficiency was demonstrated by direct enzymatic assay with ClAdo as substrate, by autoradiography of single cells, and by the inability of the enzyme-negative cells, as compared to MeSAdo phosphorylase-positive cells, to use exogenous MeSAdo to supply purine requirements when *de novo* biosynthesis was inhibited.

All the MeSAdo phosphorylase-deficient cells were among the 31 cell lines of malignant origin. On the contrary, none of 16 cell lines established from normal tissues was deficient in the enzyme. One possible explanation for the enzyme deficiency among the malignant cell lines is that the normal cells from which the tumors were derived lacked MeSAdo phosphorylase. Several lines of evidence, however, argue against this possibility. First, the enzyme-negative tumor cell lines were derived from many different tissues, including T lymphocytes, non-T,non-B lymphocytes, myeloblasts, melanocytes, and mammary cells. Moreover, other cell lines of similar origin contained the enzyme. Finally, 98% of immature bone marrow cells, which should include members of the erythrocytic, granulocytic, and lymphocytic series, were enzyme positive by autoradiography. It thus appears most likely that the MeSAdo phosphorylase deficiency developed during growth of the tumors *in vivo* or during their prolonged passage *in vitro*. In this regard, it has been suggested that MeSAdo phosphorylase-deficient cells might have a selective growth advantage, because of a MeSAdo-induced acceleration in putrescine synthesis (2).

The 16 human cell lines established from normal tissues included anchorage-independent B lymphoblasts transformed by Epstein–Barr virus and anchorage-dependent cells from several different tissues with fibroblastic or epithelioid structure. Several of the B-lymphoblastoid cell lines have been maintained in continuous culture for several years. In this cell type, therefore, prolonged passage is not sufficient to select for MeSAdo phosphorylase deficiency.

It was possible to kill selectively the malignant cell lines deficient in MeSAdo phosphorylase by inhibiting *de novo* purine synthesis with methotrexate/pyrimidine (39) or with azaserine (40) in the presence of exogenous MeSAdo. Thus, in all cell lines listed in Table 1, the inhibition of growth induced by the combination of methotrexate and pyrimidine or by azaserine was

prevented by addition of exogenous adenine to the culture medium. Under these conditions, the conversion of adenine to nucleotides by adenine phosphoribosyltransferase provided a sufficient source of purines for nucleic acid synthesis. In the MeSAdo phosphorylase-positive cells incubated with methotrexate/pyrimidine or with azaserine, addition of MeSAdo to the culture medium also provided a sufficient source of purines for growth. Because the enzyme-deficient cells were unable to use the nucleoside, they failed to survive under such selective conditions.

By autoradiography, we were unable to demonstrate any transfer of the adenine moiety of MeSAdo from enzyme-positive to enzyme-negative cells cultured together. Thus, most of the adenine produced by MeSAdo cleavage was converted to adenine nucleotides rather than excreted. The selective killing of MeSAdo phosphorylase-deficient cells in a mixed cellular population would appear to be feasible.

In an initial screening of four human tumor specimens, all were MeSAdo phosphorylase positive. More extensive experiments are required to determine the true incidence of enzyme deficiency among human malignant neoplasms *in vivo*, both before and after chemotherapy. The demonstrated chemotherapeutic efficacy of methotrexate is limited by its toxicity to normal, rapidly proliferating tissues such as intestinal mucosa and bone marrow. Attempts have been made to rescue normal cells by infusing thymidine (41, 42). In a patient with malignant tumor deficient in the enzyme, the infusion of MeSAdo alone or in combination with thymidine might protect the normal tissues without affecting the toxicity of methotrexate for the malignant cells. In this way, a naturally occurring enzyme deficiency among human malignancies could be exploited successfully.

We thank Drs. M. Tavassoli, R. Longmire, and A. Yu for providing the clinical specimens. This work was supported by National Institutes of Health Grant GM 23200. W.A.N.-R. is supported by a contract from the Biological Carcinogenesis Branch, National Cancer Institute.

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