Adenylate Deaminase Deficiency in a Mutant Murine T Cell Lymphoma Cell Line*

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From a population of wild type 549 cells, a clone, DTB6, was isolated in a single step from selective medium containing thymidine and dibutyryl cyclic AMP that exhibited a 60% deficiency in AMP deaminase (AMP-D) activity. The AMP-D deficiency conferred to the DTB6 cells a striking susceptibility to killing by low concentrations of either adenine or adenosine, the latter in the presence of an inhibitor of adenosine deaminase activity. This growth supersensitivity of DTB6 cells toward adenine could be ameliorated by the addition of hypoxanthine to the culture medium. Immunoprecipitation of AMP-D from wild type and mutant cells revealed that the DTB6 cell line contained markedly diminished amounts of the AMP-D isozyme which reacts with antisera to the predominant isoform expressed in adult kidney. The quantities of the AMP-D isozyme immunoprecipitated by antisera raised to the predominant isoform prepared from adult heart were equivalent in the two cell lines. Although Northern blot analyses revealed no alterations in mRNA sizes or levels encoded by either of the AMP-D genes, Southern blots of genomic DNA hybridized to a cDNA specific for the ampd2 gene revealed the presence of a new BamHI restriction fragment in the DNA of DTB6 cells. These data suggested that a point mutation has occurred in the ampd2 gene of DTB6 cells which encodes the AMP-D isozyme recognized by the kidney antisera.

The DTB6 cells also possessed a virtual complete deficiency in thymidine kinase activity. The two enzyme deficiencies were distinguishable. The ability to isolate single step mutants with two seemingly independent biochemical abnormalities raises the speculation that there may be some link between cellular functions responsible for purine nucleotide and thymidine metabolism.

Adenine and adenosine have a variety of physiological and pharmacological effects (1), and both are growth-inhibitory and cytotoxic toward mammalian cells (2-6). Most, but not all, of the effects of these purines require metabolism to the nucleotide level. Adenine and adenosine are converted to AMP by the enzymes adenine phosphoribosyltransferase and adenosine kinase, respectively. Subsequent metabolism of AMP in animal cells depends on the relative intracellular activities of two enzymes, AMP kinase and AMP deaminase (AMP aminohydrolase, EC 3.5.4.6). The latter catalyzes the effectively irreversible deamination of AMP to IMP and ammonia. Several important regulatory functions for AMP deaminase (AMP-D)1 have been proposed. These include ammonia production in muscle (7, 8), the maintenance of adenylate pool sizes and overall energy charge (9, 10), and the balance between adenylyl and guanylate nucleotides in cells (11, 12). The enzyme is also an important component of the purine nucleotide cycle (13, 14). Moreover, AMP is an important enzyme modulator, and, therefore, AMP-D plays an indirect regulatory role for many pathways of intermediary metabolism (15).

AMP-D is a ubiquitous enzyme that is expressed in a tissue and developmental stage specific manner (16 18). Recent studies have demonstrated that this enzyme activity is encoded by two syntenic genes in human, rat, and mouse (19).1 One gene, referred to as ampd1, is expressed at high levels in skeletal muscle, and, in the rat, this gene encodes two 2.5-kb transcripts that differ by alternative splicing of the second exon (21).1 4 The other gene, called ampd2, is expressed predominantly in non-muscle tissues of the adult animal and embryonic muscle or undifferentiated myoblasts (19, 21). The ampd2 gene encodes a 3.4-kb transcript in the rat. Assignment of the various protein isoforms of AMP-D that have been identified by antisera and gel chromatography procedures (23, 24) to these respective genes is not completely defined presently, but it appears that antisera raised to the predominant isoform expressed in adult skeletal muscle recognizes a product of the ampd1 gene (21), and the antisera raised to the predominant isoform expressed in adult kidney recognizes a product of the ampd2 gene (19). A third antisera raised to the predominant isoform in adult cardiac muscle probably recognizes a product of the ampd1 gene that may have been posttranslationally modified.1 Thus, it appears that the specific

1. The abbreviations used are: AMP-D, AMP deaminase, kb, kilobase pair(s); HGPRTase, hypoxanthine-guanine phosphoribosyl-transferase; APRTase, adenine phosphoribosyltransferase; SDS, sodium dodecyl sulfate; EHNA, ethythro-9-(2-hydroxy-3-nonyl) adenine; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; HAT, hypoxanthine/aminopterine/thymidine.

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AMP-D isozymes expressed in different tissues and at different stages of development arise from differential expression of distinct genes, alternative splicing of the primary transcript of one of the genes, possibly post-translational modification of one or more of the peptides, and the incorporation of heterologous subunits into the polymeric native enzyme. Genetic approaches have proven invaluable in elucidating the functional role of key enzymes in cellular metabolism. In this investigation, we report the isolation of a mouse S49 T cell line which is functionally and biochemically deficient in AMP-D activity. The mutant cell line, DTB6, contained much lower levels of the isofrom AMP-D which reacts with antisera raised to the predominant isofrom expressed in adult rat kidney. Further, Southern blot analysis indicated that the DNA of mutant cells contained a point mutation in the amp2 gene which encodes the isoenzyme immunoprecipitated with the kidney antisera. Interestingly, the DTB6 cells were also completely deficient in thymidine kinase activity, and the potential relationship between the two enzyme deficiencies has been explored.

**Materials and Methods**

**Chemicals and Reagents**—[CH3-14C]thymidine (56 mCi/mmol) and [3H]thymidine (77 Ci/mmol) were purchased from Moravek Biochemicals. [14C]AMP (390 mCi/mmol) was bought from Du Pont-New England Nuclear. Methylxanthine was isolated from Lederle Parenterals. cytochrome 9 (2-Hydroxy-9-nonyladenine (ENHA) was acquired from the Burroughs Wellcome Co. N-Methyl-N-nitro-N-nitroso- guanidine (MNNG) was procured from Sigma. All nonradioactive nucleotides, nucleosides, and nucleobases, including the purine base guanidine (MNNG) was procured from Sigma. All nonradioactive nucleotides, nucleosides, and nucleobases, including the purine base guanidine (MNNG) was procured from Sigma. All nonradioactive nucleotides, nucleosides, and nucleobases, including the purine base guanidine (MNNG) was procured from Sigma.

**Cell Culture and Maintenance**—The wild type S49 cell line is a T cell lymphocytic lymphoma that was originally derived from a BALB/c mouse injected with mineral oil (25). The growth and lymphocytic properties of wild type S49 cells have been described previously in detail (5, 25). Wild type and mutant S49 cells were cultured in Costar multiwell (24-well) tissue culture plates. 105 exponentially growing cells in 1.0 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum increased their cell density 16-30-fold, approximately four to five cell doublings.

**Isolation of the DTB6 Cell Line**—A clonal wild type S49 cell line was mutagenized by a 3-h exposure to 3 µg/ml MNNG. Cells were separated from the mutant by centrifugation and resuspended in fresh medium lacking MNNG. The mutagenized cells were allowed to proliferate for seven cell divisions to ensure expression of the mutant phenotype. The cells were then plated in semisolid (0.3%) agarose (Seakem-ME) overlaying mouse embryo fibroblasts as described by Coffino et al. (20). The DTB6 cell line was isolated from a mutagenized wild type population that was plated in selective medium containing 1 mM thymidine and 1 mM dibutyryl cyclic AMP. Thymidine is cytotoxic to cells by altering the substrate specificity of ribonucleotide reductase and consequently depleting intracellular deoxy-CTP pools (27), while dibutyryl cyclic AMP kills S49 cells by an independent mechanism, activation of the cAMP-dependent protein kinase (28, 29).

**Isolation of Secondary Derivatives of DTB6 Cells**—To generate secondary derivatives of the DTB6 cells deficient in purine base phosphoribosylation, the DTB6 cells were mutagenized and single cell cloned in semisolid medium overlaying mouse embryo fibroblast feeder layers as described above for the isolation of the DTB6 cell line. A cell line deficient in hypoxanthine-guanine phosphoribosyltransferase (HGPRTase), DTB6-Tg20, was isolated by virtue of its transferase (APRTase)-deficient derivative of the DTB6 cell line, DTB6-DAP20, was isolated from a plate containing 100 µM 2,6-diaminopurine.

**Phosphoribosylation**—The DTB6 cell line which is functionally and biochemically deficient in HGPRTase, DTB6-Tg20, was isolated by virtue of its transferase (APRTase)-deficient derivative of the DTB6 cell line, DTB6-DAP20, was isolated from a plate containing 100 µM 2,6-diaminopurine.

**Measurement of Cellular Purine Nucleoside Triphosphate Pools**—Wild type and DTB6 cells were suspended at a concentration of 5 X 106 cells/ml in medium and grown overnight to ensure exponential and asynchronous growth. After exposure to 0.1 mM adenine for varying lengths of time, the cells were extracted for nucleotide determinations by the method of Khym (34). The ATP and GTP contents of the neutralized extracts were determined by high performance liquid chromatography on a Partisil-SAX column using 0.4 m ammonium phosphate, pH 5.4, as the mobile phase (34). The preparation of Cell Extracts for Enzyme Assays—Cells were harvested by centrifugation and washed once with 50 ml of cold phosphate-buffered saline as described (35). The cells were then resuspended in a small volume of 50 mM Tris, pH 7.4, 5 mM MgCl2, and 1 mM dithiothreitol and lysed by three successive 10-s bursts on a Biosonic III sonicator. The extracts were centrifuged at 30,000 X g for 30 min, and the supernatant was used. The supernatant mixture contained: 0.4 M imidazole/HCl, pH 7.0, 0.1 M KCl, 2 mM ATP, and 2 mM AMP. The AMP concentration in the assay mixture was varied when the affinities of the enzymes from wild type and DTB6 cells for substrate were determined. The reaction was initiated with a small volume of cell
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extract to make the final volume 0.4 ml and incubated at 37 °C. At various time intervals up to 60 min, the reactions were terminated by the rapid addition of 0.85 ml of a solution containing 0.53 M phenol and 0.84 mM sodium nitroprusside. Color development was initiated by the addition of 0.55 ml of a solution containing 0.022 N NaOH and 0.62% sodium hypochlorite and incubation at 53 °C for 40 min. After cooling, the absorbancies were measured at 520 nm. The assay was linear with the amount of cell extract and for 40 min.

The second method for quantitating AMPS-D activity was radio-
metric (24). Briefly, the assay mixtures contained 50 mM imidazole/HCl, pH 7.0, 150 mM KCl, 1 mM mercaptoethanol, and 10 mM [3H]AMP (330 mCi/mmol). Aliquots were spotted onto polyethylene-
mine-cellulose sheets and [3H]AMP-separated from [3H]AMPD using thin layer chromatography using saturated (NH4)2SO4/100 mM KPi, pH 6.0; isopropanol alcohol, 75:18:2 as the mobile phase. The product was localized by ultraviolet irradiation, excised, and counted by liquid scintillation. Assays were linear with time and extract amount for all values reported.

Thymidine Kinase Assay—Thymidine kinase activity was mea-
sured in 50 mM Tris, pH 7.4, 5 mM MgCl2, 1 mM dithiothreitol, 5 mM ATP and either 12 μM or 30 μM [3H]thymidine (56 mCi/mmol) in a volume of 500 μl at 37 °C. The reaction was initiated by the addition of a small volume of extract, and 50-μl portions were removed at 10-
min intervals and applied to DEAE-Whatman filter papers. Radi-
activity associated with product formation was quantitated as de-
scribed above for the adenine and thymidine incorporation measure-
ments.

APRTase and HGPRTase Assays—APRTase and HGPRTase levels in wild type and mutant cells were quantitated by the method described by Iovannisci et al. (38).

APRTase Assay—The concentration of protein in the cell extracts was measured by the method of Bradford (39).

Immunological Analyses of AMP-D Isomers—Antisera directed against the predominant AMP-D isomers expressed in the adult rat kidney and in the adult rat heart were prepared and immunopurified as described by Ogasawara et al. (23). These antisera were kindly provided by Dr. Ogasawara (Dept. of Biochemistry, Institute for Developmental Research, Aichi, Japan). The immunoprecipitation experiments were carried out as described previously (24).

Isolation and Analysis of DNA and RNA—The characterization of the cDNA probes for the ampdl and ampd2 genes is described in detail by Morikita et al. (19). Genomic DNA was isolated from 349 cells by the method of Blin and Stafford (40). Fractions of wild type and mutant genomic DNA restricted with either BamHI, EcoRI, or HindIII were resolved on 0.8% agarose gels and transferred to Nytran filters (Schleicher & Schuell) as described by Southern (41). The filters were hybridized to 107 dpm of [32P]DNA at 42 °C in 5 X SSC, 0.1% SDS, followed by two washes at 55 °C in 0.1 X SSC, 0.1% SDS, and 0.05 M sodium phosphate, and 0.1% SDS for 15-20 h. Filters were washed sequentially at 58-60 °C in 5 X SSC, 3 X SSC, 1 X SSC, and 0.1 X SSC, each wash containing 0.5% SDS, and subjected to autoradiography as described by Sabina et al. (42).

Total cellular RNA was extracted in guanidinium isothiocyanate, isolated by CsCl gradient centrifugation, and the poly(A)+ RNA was fractioned from 200 μg of total RNA on oligo(dT) columns as described by Maniatis et al. (43). The poly(A)+ RNA was fractionated on 1.2% agarose-formaldehyde gels, transferred to Nytran filters (Schleicher & Schuell) as described by Southern (41). The filters were hybridized to 107 dpm of [32P]DNA at 42 °C in 5 X SSC, 0.1% SDS, followed by two washes at 55 °C in 0.1 X SSC, 0.1% SDS, and exposed to x-ray film for autoradiography as described (42).

RESULTS

Growth Rate Experiments—The DTB6 cell line was the only clone isolated from over 3 × 106 mutagenized wild type cells plated in selective medium containing 1 mM concentrations of both thymidine and dibutyryl cyclic AMP that expressed the unique growth phenotype described below. As shown in Fig. 1, the DTB6 clone was much less sensitive to the cytotoxic effects of thymidine than wild type cells. Whereas the effective concentration of thymidine that inhibited the growth of wild type cells by 50% (EC50) value was 30 μM, the EC50 value of the DTB6 cells was approximately 500 μM. Comparison of the abilities of the two cell lines to grow

FIG. 1. Sensitivity of wild type and DTB6 cells to thymi-
dine. The growth-inhibitory and cytotoxic effects of increasing concentrations of thymidine on wild type (○) and DTB6 (●) cells were determined as described under "Materials and Methods." The results are plotted as a percentage of growth in the absence of thymidine as a function of thymidine concentration.

FIG. 2. Adenine toxicity in wild type and mutant cells in the absence and presence of hypoxanthine. The effects of increasing concentrations of adenine on the growth rate of wild type (○, ■) and DTB6 (C, □), as shown in Panel A, and of DTB6-Tg20 (▲, ▽), and DTB6-DAP100 (●, △) cells, as displayed in panel B, in the absence (○, ■, ▲, ▽) or presence (■, □, △, ●) of 0.5 mM hypoxanthine were determined as described under "Materials and Methods." 0.5 mM hypoxanthine alone did not influence the growth rate of either cell line.

in HAT medium indicated that DTB6 cells, unlike wild type parental cells, could not salvage thymidine. DTB6 cells were also much less sensitive toward the growth-inhibitory effects of two analogs of thymidine, 5-bromodeoxyuridine and 5-
fluorodeoxyuridine, but were just as sensitive as wild type cells to a spectrum of other structurally related compounds, including guanosine, deoxyguanosine, deoxyadenosine, 5-fluo-
rouridine, 5-azacytidine, and, surprisingly, dibutyryl cyclic AMP (data not shown). Unexpectedly however, DTB6 cells were considerably more sensitive to the toxicity of adenosine (data not shown) in the presence of 10 μM EHNA (44), a potent inhibitor of adenosine deaminase activity, and to ade-
nine (Fig. 2A). Whereas the EC50 value of wild type cells for adenosine was about 400 μM, that of the DTB6 mutant cells was approximately 10 μM. To determine whether the exquisite
growth susceptibility of DTB6 cells to adenine toxicity required the metabolism of the purine base to the nucleotide level, the sensitivity of the APRTase-deficient (DTB6-DAP100) subclone of the DTB6 cell line, as well as the HGPRtase-deficient derivative (DTB6-Tg20), to growth inhibition by adenine were evaluated. As demonstrated clearly in Fig. 2B, the DTB6-DAP100 cells exhibited a greatly decreased growth sensitivity toward adenine, whereas the DTB6-Tg20 cell line was just as sensitive as the parental DTB6 strain to growth inhibition by the purine base. Thus, the supersensitivity of DTB6 cells to adenine required conversion of the nucleobase to AMP.

The EC50 value of DTB6 cells for adenine could be markedly increased to that of wild type cells by the addition of 0.5 mM hypoxanthine to the culture medium, a concentration that did not alter the sensitivity of wild type cells to adenine (Fig. 2A). Although the HGPRtase-deficient mutant was just as sensitive as the DTB6 parental cell line to adenine toxicity, the addition of hypoxanthine at a concentration of 0.5 mM did not ameliorate the adenine supersensitivity (Fig. 2B). Thus, rescue of DTB6 cells from adenine toxicity by hypoxanthine required the latter’s salvage to the nucleotide level.

Adenine Incorporation Measurements—One mechanism that could explain the increased sensitivity of DTB6 cells to adenine toxicity might be an escalated capability to assimilate adenine from the culture medium. Therefore, the abilities of wild type and DTB6 cells to incorporate [14C]adenine into phosphorylated derivatives were compared. As displayed in Fig. 3, both wild type and mutant cells took up adenine at roughly equivalent rates. Thus, increased incorporation of adenine into nucleotide pools could not account for the drastic sensitivity of the DTB6 cells to adenine toxicity.

Effects of Adenine on Purine Nucleoside Triphosphate Pools—The ability of hypoxanthine to ameliorate adenine toxicity in DTB6 cells suggested that low concentrations of adenine were inducing a cellular starvation for guanylate nucleotides. Therefore, the effects of exogenous adenine on the cellular ATP and GTP levels were determined as a function of time. As shown in Fig. 4, incubation of the wild type and DTB6 cells with 0.1 mM adenine, a concentration which killed mutant but not wild type cells, affected the nucleoside triphosphate pools differently in the two cell lines. Whereas the GTP/ATP ratio of wild type cells was relatively constant during a 24-h incubation with adenine, that of mutant cells was markedly diminished at 4 h and remained low throughout the experiment.

Measurements of AMP-D Activity—The increased sensitivity of DTB6 cells to growth inhibition by either adenosine-EHNA or adenine, the ability of hypoxanthine to ameliorate the toxic effects of adenine, and the decrease in the GTP pools relative to the ATP concentrations as a consequence of adenine incubation were all properties of a previously described mutant S49 cell line, 100-10, that possessed a genetic alteration in AMP-D activity (45). Therefore, the levels of AMP-D in wild type and DTB6 cell extracts were compared. The data presented in Fig. 5 indicated that the DTB6 cells possessed only 40% of the levels of AMP-D activity of wild type cells. In four completely independent determinations, the specific activity of these four determinations was 33.7 ± 0.4 and 14.4 ± 1.2 nmol/min/mg of protein for parental and mutant cells, respectively. However, unlike the enzyme from the 100-10 cell line (45), the residual 40% enzyme activity in DTB6 cells had a similar apparent \( K_m \) value for AMP (1 mM) and similar sensitivities to inhibitors, such as inorganic phosphate and GTP, as the wild type enzyme (data not shown). This decreased AMP-D activity of DTB6 cells could account for their greater sensitivity to inhibition by adenine and adenosine EHNA.

FIG. 3. Adenine incorporation by wild type and DTB6 cells. The abilities of wild type and mutant cells to take up 2 \( \mu \)M [14C]adenine from the culture medium were assessed by the protocol described under "Materials and Methods."

FIG. 4. ATP and GTP pools in wild type and DTB6 cells exposed to adenine. Wild type and DTB6 cells were exposed to 0.1 mM adenine for varying lengths of time, the cells were harvested and extracted, and their nucleoside triphosphate content was determined as described under "Materials and Methods."

FIG. 5. AMP-D assays. The levels of AMP-D activity in soluble extracts of wild type (0) and DTB6 (0) were quantitated as a function of time. This is one of four completely independent determinations. The average specific activity of these four determinations was 33.7 ± 0.4 and 14.4 ± 1.2 nmol/min/mg of protein for parental and mutant cells, respectively.


**Immunological Identification of AMP-D Isoforms in Wild Type and Mutant Cells**—Exploiting polyclonal antisera directed toward the principal AMP-D isoforms in kidney and heart of the adult rat, the relative proportions of the two isoforms in extracts of wild type, DTB6, and DTB6-Tg20 cells were determined by immunoprecipitation. In wild type S49 cells, the percentages of total AMP-D immunoprecipitated by the antisera generated against the predominant isoforms from kidney and heart, respectively, were 82% and 64%, Table I. This is consistent with the observations of Sabina et al. (21) that most adult non-muscle tissues in the rat express both isoforms of the enzyme. Also compatible with the findings of Sabina et al. (21) is the observation that more than 100% of AMP-D activity could be accounted for by immunoprecipitation with the two antibodies. This suggests that heteropolymers of the native enzyme contain both AMP-D isoforms, resulting in a cross-reaction of the antibodies used in the immunoprecipitation assay. Although both isoforms were also detected in DTB6 and DTB6-Tg20 cells, the isoform which is recognized by the heart antisera is clearly the predominant form expressed in both AMP-D-deficient cell lines. In the DTB6 and DTB6-Tg20 cells, the antisera raised against the kidney AMP-D isofrom immunoprecipitated 26% and 23% of the total AMP-D activity, respectively, whereas the antisera raised against the heart isoform immunoprecipitated about 89% of the total cellular AMP-D activity in the mutant cells. Therefore, the AMP-D deficiency in DTB6 cells could be attributed to a specific diminution of the AMP-D enzyme which is precipitated by the antisera raised to the kidney isoform.

**Southern Analysis of AMP-D Isoforms in Wild Type and DTB6 Cells**—Further support for a specific functional alteration of the AMP-D isozyme which is recognized by the kidney antisera in DTB6 cells was obtained from Southern blot analyses. Southern blotting of genomic DNA from wild type cells digested with BamHI and probed with the 32P-labeled rat cDNA for the ampd2 gene, the gene which encodes the chief AMP-D isofrom expressed in adult rat kidney (19), revealed a single hybridizing DNA fragment around 9.0 kb, Fig. 6. However, BamHI digests of restricted DNA prepared from DTB6 and DTB6-Tg20 cells contained, in addition to the 9.0-kb band, a second DNA fragment around 6.7 kb that hybridized to the ampd2 cDNA probe. In contrast, the restriction patterns of BamHI-digested genomic DNA probed with the rat ampd1 cDNA were identical in the three cell lines (Fig. 6). Normalization of the signal intensities to ampd2 in Fig. 6 demonstrated that each of the individual BamHI-restricted fragments recognized by the ampd2 probe in the

**Table I**

| Cell line      | Anti-kidney antisera | Anti-heart antisera | %Immuneactivity |
|----------------|----------------------|---------------------|-----------------|
| Wild Type      | 82 ± 7               | 64 ± 3              |                 |
| DTB6           | 26 ± 14              | 89 ± 5              |                 |
| DTB6-Tg20      | 23 ± 16              | 89 ± 5              |                 |
| DTB6-Tg20-ade2 | 6 (4,9)              | 93 (90,95)          |                 |

mutant cell lines exhibited one-half the intensity of the single band observed in the wild type cell line. No differences among the three cell lines were observed when the ampd2 cDNA probe was hybridized to HindIII- or EcoR1-restricted genomic DNA.

**Northern Blots of AMP-D Transcripts in Wild Type and Mutant Cells**—Northern analyses of poly(A)+ RNA revealed that the transcript sizes and amounts of ampd2 gene product were equivalent in wild type, DTB6, and DTB6-Tg20 cells (Fig. 7). The mutant cell lines expressed 80–95% of the wild type levels of the ampd2 transcript when normalized to the abundance of the a-tubulin transcript (Fig. 7).

**Measurements of Thymidine Kinase Activity**—The growth resistance of DTB6 cells to thymidine and its analogs and the inability of the mutant cells to proliferate in HAT medium suggested that this mutant cell line lacked thymidine kinase.
activity. Indeed, comparison of the levels of thymidine kinase activity in wild type and DTB6 cell extracts indicated that the latter was virtually completely deficient in thymidine kinase activity (Fig. 8). Moreover, the mutant cells were also incapable of incorporating exogenous [3H]thymidine at a concentration of 10 μM into phosphorylated metabolites (data not shown), indicating a functional deficiency in thymidine kinase activity in situ.

Characterization of Intraspecies Tetraploid Hybrids—In order to assess whether some unusual dominant regulatory mutation was responsible for both the deficiency in AMP-D and thus in thymidine kinase, a DTB6 × wild type hybrid cell line was constructed. However, the DTB6 × wild type and the wild type × wild type cell lines possessed similar growth sensitivities to both adenine and 5-fluorodeoxyuridine, implying that both enzyme deficiencies behaved in recessive fashions (data not shown).

Characterization of DTB6 Derivatives Selected for Reversion of Either AMP-D or Thymidine Kinase Activities—The isolation of a mutant cell line in a single step that possessed two biochemically defined enzyme deficiencies contributing to its genetically altered phenotype raised the possibility that the two deficiencies were controlled by a single genetic function. To analyze the genetic relationship between the AMP-D and thymidine kinase deficiencies, the DTB6 cells were selected for reversion of one enzyme defect in order to determine whether the other enzyme deficiency also reverted to the parental phenotype. As demonstrated in Table II, restoration of the AMP-D activity in the DTB6-Tg20-Ade2 and DTB6-Tg20-Ade3 cell lines to wild type levels did not result in any increase in thymidine kinase activity as measured by direct enzyme assay and by their insensitivity to growth inhibition by 5-fluorodeoxyuridine. Interestingly, the antisera raised against the anti-kidney AMP-D isoform only immunoprecipitated 6% of the total cellular AMP-D activity in DTB6-Tg20-Ade2 cells. Table I. As the amounts of AMP-D activity in the adenine-resistant revertant and wild type cells are roughly equivalent, these data imply that the DTB6-Tg20-Ade2 cell line expresses augmented levels of the AMP-D isoform that is recognized by the antibodies prepared against the heart isoform. Moreover, since the total AMP-D activity precipitated by both antibodies was about 100%, this suggested that heteropolymers of the two isoforms are not formed in the DTB6-Tg20-ade2 cells. Southern blots of BamH1-restricted genomic DNA made from DTB6-Tg20-Ade2 cells reveal the same polymorphism when probed with the ampD2 cDNA as the DTB6 and DTB6-Tg20 DNA (data not shown). The isolation of the DTB6-Tg20-Ade2 and DTB6-Tg20-Ade3 cell lines was performed using the HGPRTase-derivative of the DTB6 cell line as a parent in order to prevent salvage of purines released into the adenine-containing selective medium by cell lysis. DTB6 cells could not be directly selected for adenine resistance, even in semisolid agarose containing adenine at concentrations as high as 1.5 mM, presumably due to circumvention of the adenine-mediated guanylate starvation. The HGPRTase deficiency (6-thioguanine resistance) of the DTB6-Tg20-Ade2 and DTB6-Tg20-Ade3 cell lines also served as an insurance marker to circumvent the possibility that the adenine resistant revertants were merely contaminants of wild type S49 cells inadvertently introduced into the DTB6 cell culture.

Similarly, a HAT-resistant derivative of DTB6 cells, DTB6-HAT+, was also analyzed for its biochemical and growth phenotypes (Table II). DTB6-HAT+ cells possessed about 11% of the wild type thymidine kinase activity, which could account for its survival in HAT medium. This 11% restoration of wild type thymidine kinase activity did not quite confer wild type growth sensitivity to 5-fluorodeoxyuridine. DTB6-HAT+ cells were almost as sensitive to growth inhibition by adenine (Table II) and adenosine-EHNA (data not shown) and contained about as much AMP-D activity as the DTB6 parental cells (Table II). An HGPRTase-deficient DTB6 cell line could not be used in the derivation of the HAT-resistant DTB6 clone, since the selective pressure would require the parental cell line to revert both the HGPRTase and thymidine kinase deficiencies.

**DISCUSSION**

By direct enzymatic assay, which measures all isoforms, DTB6 cells contained approximately 40% of the levels of AMP-D activity as wild type cell extracts. The deficiency in AMP-D activity in situ could account for the extraordinary growth sensitivity of the DTB6 cells to both adenine and adenosine (+10 μM EHNA). Since insertion of a secondary mutation conferring APRTase deficiency into DTB6 cells ameliorated their growth supersensitivity toward adenine, the toxic effects of low concentrations of adenine on the mutant...
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cells must require phosphoribosylation of the purine base. Therefore, the supersensitivity of DTB6 cells to adenosine and adenine is likely to be mediated by increased accumulation of adenylate nucleotides which are known to inhibit the two rate-limiting enzymes governing the de novo purine biosynthetic pathway, phosphoribosylpyrophosphate synthetase (46) and phosphoribosylpyrophosphate-glutamine amidotransferase (47). Accumulation of adenylate nucleotides and decreased production of purine nucleotides would diminish the cellular content of guanine nucleotides (Fig. 4) and could explain the observation that hypoxanthine, a source of salvageable guanylate nucleotides, circumvented adenine toxicity in the DTB6 cells (Fig. 3). These data, therefore, provide powerful genetic evidence that an intact AMP-D activity plays a critical role both in regulating a balance between adenylate and guanylate nucleotide pools in somatic cells and in the detoxification of adenylate nucleotides.

S49 cells contain two different AMP-D activities, one which is precipitated by an antisera raised to the predominant isoform found in adult rat heart and one which is recognized by antisera generated to the predominant isoform detected in adult rat kidney. Both AMP-D isoymes are also co-expressed in most adult non-muscle tissues in the rat (19). Immunologic data suggested that the levels of the AMP-D isoform detected by the kidney antisera were diminished in the DTB6 cells, since a specific decrease in immunoreactivity with the anti-kidney AMP-D isoform antibody was observed (Table 1). Interestingly, the total amounts of AMP-D accounted for by immunoprecipitation by the anti-kidney and anti-heart isoform antibodies in both wild type and mutant S49 cells were greater than 100%. Similar data have also been observed in the rat (21). These data are consistent with the premise that heteropolymers are being formed between the two peptides, resulting in a “cross-reaction” of the antibodies in the immunoprecipitation assay.

That one of the two AMP D genes has been mutated in the DTB6 cells is supported by the Southern blot data in Fig. 6. Specifically, an additional BamHI fragment that recognizes the ampD2 cDNA probe has been created within the genomic DNA of the mutant cells. Whether this new BamHI site is located within the coding sequence for the ampD2 gene remains to be determined. In contrast, the restriction patterns of wild type, DTB6, and DTB6-Tg20 DNA were identical when probed with the ampD1 cDNA. That a mutant allele has been created in one of the two ampD2 loci of the DTB6 cells is further supported by normalization of the ampD2 signals on Southern blots to those of ampD1. It is likely that the genetic alteration in the mutant cells affecting AMP-D activity was the result of a point mutation in an ampD2 gene, rather than a deletion, insertion, or rearrangement, as no differences were observed in the hybridization patterns of wild type and mutant genomic DNA restricted with HindIII or EcoRI when probed with the ampD2 cDNA (data not shown) and due to the similarities in both the amounts and size of the ampD2 encoded transcripts in wild type and AMP-D-deficient cells (Fig. 7).

The DTB6 cell line is the second mutant S49 cell line that has been isolated that possesses a genetically induced functional deficiency in AMP-D activity. The biochemical alteration in the previously described S49 cell line, 100-10, however, is very different from that of the DTB6 clone (45). The AMP-D activity of 100-10 cells has a normal Vmax value for AMP, increased sensitivity to inhibition by inorganic phosphate, and an increased Km for GTP compared to the wild type enzyme. The AMP-D of DTB6 cells possesses none of these kinetic aberrations, but rather a 60% deficiency in enzyme level. The in situ phenotype of the 100-10 and DTB6 cells are similar, however, in that both cell lines are supersensitive to adenine and adenosine-EHNA toxicity and can be rescued by the provision of an exogenous source of guanlylate nucleotides, i.e. hypoxanthine, to the culture medium.

The phenotype of the 100-10 cells is complicated by the presence of a second mutation, one that encodes a genetically altered protein. One subunit of ribonucleotide reductase that renders the enzyme insensitive to the negative allosteric effector, deoxy-ATP. This insensitivity to inhibition by deoxy-ATP can account for the resistance of 100-10 cells to thymidine. The resistance of the DTB6 cells to thymidine cannot be attributed to a genetic alteration in a gene for ribonucleotide reductase, but rather to a complete deficiency in the enzyme thymidine kinase. That the two independently derived 100-10 and DTB6 cell lines, isolated by distinct selective strategies and methods, contain different genetic alterations in their AMP-D activities, as well as second mutations in a pyrimidine metabolizing function, raises the intriguing possibility that different genetic linkage or genetic regulatory mechanisms between elements of the purine and pyrimidine nucleotide pathways. It must be noted, however, that the DTB6 cells were isolated after mutagenesis with MNNG, a process that could have conceivably introduced multiple mutations. It is also conceivable that the selective strategy selected for a thymidine kinase deficiency in a pre-existing frequently occurring AMP-deficient variant within the wild type population. However, this laboratory has screened literally hundreds of S49 clones over the last 15 years, and adenine growth sensitivity, a screen for a functional AMP-D deficiency, has been observed previously only in the 100-10 cell line (45). Moreover, it is apparent that it is possible to uncouple the genetically induced deficiencies in AMP-D and thymidine kinase, since the DTB6-Tg20-Ad2 and DTB6-Tg20-Ade3 cells retained their thymidine kinase deficiency. Whether the restoration of 11% of the wild type levels of thymidine kinase in the DTB6-HAT+ cells resulted in an equal increment in AMP-D activity is difficult to assess accurately, since the DTB6 cells were only 60% deficient in the latter enzyme. Nevertheless, the generation in a single step of a second mutant S49 cell line possessing a genetically induced lesion in AMP-D and a somewhat similar phenotype attributed to a second enzyme alteration is unexpected and noteworthy.

The DTB6 cell line was cloned derived in a single step in selective medium containing 1 mM thymidine and 1 mM dibutyryl cyclic AMP. The rationale for the selective strategy containing two drugs whose intracellular cytotoxic effects and metabolic activation pathways are seemingly independent was based on the observations that a percentage of mutant S49 cell lines with genetic alterations in ribonucleotide reductase activity that conferred cellular growth resistance to thymidine had also acquired growth resistance to dibutyryl cyclic AMP (20, 22). The deficiency in thymidine kinase could, therefore, account for the ability of the DTB6 cells to survive in thymidine. However, growth experiments revealed, somewhat surprisingly, that the mutant cells were just as sensitive as wild type cells to the other selective agent, dibutyryl cyclic AMP. This suggests that the partial deficiency in adenylate deaminase activity conferred some selective growth advantage for the mutant cells during the prolonged selection conditions. Since dibutyryl cyclic AMP induces a cell cycle-dependent diminution of purine biosynthetic capacity, one could conjecture that the AMP-D deficiency permitted the DTB6 cells to efficiently salvage adenine and adenosine released from the
multitude of lysed cells. This would not be expected to induce a guanylate starvation, since released guanylate nucleotides would serve as a source of guanylate nucleotides for the DTB6 cells.

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