Mitochondrial Basis for Immune Deficiency: Evidence from Purine Nucleoside Phosphorylase–deficient Mice

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Abstract

We generated purine nucleoside phosphorylase (PNP)-deficient mice to gain insight into the mechanism of immune deficiency disease associated with PNP deficiency in humans. Similar to the human disease, PNP deficiency in mice causes an immunodeficiency that affects T lymphocytes more severely than B lymphocytes. PNP knockout mice exhibit impaired thymocyte differentiation, reduced mitogenic and allogeneic responses, and decreased numbers of maturing thymocytes and peripheral T cells. T lymphocytes of PNP-deficient mice exhibit increased apoptosis in vivo and higher sensitivity to gamma irradiation in vitro. We propose that the immune deficiency in PNP deficiency is a result of inhibition of mitochondrial DNA repair due to the accumulation of dGTP in the mitochondria. The end result is increased sensitivity of T cells to spontaneous mitochondrial DNA damage, leading to T cell depletion by apoptosis.

Key words: immune deficiency • apoptosis • mitochondria • purine metabolism • T lymphocyte

Introduction

An inherited deficiency in either one of the two purine salvage enzymes adenosine deaminase (ADA)[1] or purine nucleoside phosphorylase (PNP) causes SCID disease (1, 2). Studies with mouse lymphoma T cell lines suggested that the impaired degradation of deoxyribonucleoside substrates of ADA and PNP leads respectively to accumulation of dATP or dGTP, which can inhibit ribonucleotide reductase activity, abrogating DNA synthesis or DNA repair (Fig. 1; references 3–6). dATP, but not dGTP, has also been found to form a complex with apoptotic protease-activating factor 1 (Apaf-1) and cytochrome C in the cytoplasm to activate caspases and induce apoptosis (7). Other mechanisms may also contribute to immunodeficiency associated with ADA deficiency, the more actively studied of the two disorders (8). However, the basis for immunodeficiency in PNP deficiency is still uncertain. Mice with PNP deficiency due to missense mutations showed a decline with age in numbers of immature and peripheral T cells and in T cell proliferation (9). These mice exhibited delayed immune deficiency characterized by a reduction in the numbers of immature and peripheral T cells and reduced T cell proliferation. In addition, mutations in PNP caused a secondary loss of deoxyguanosine kinase activity. The mechanism of the partial immune deficiency in these mice was not addressed.

To better address the mechanism of immunodeficiency, we generated totally PNP-deficient mice by homologous recombination. At an early age, PNP knockout mice exhibit T cell lymphopenia and abnormalities in T cell function. We propose that the abnormalities are due to selective accumulation of dGTP in the mitochondria of T cells,

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[1]*Abbreviations used in this paper:* ADA, adenosine deaminase; $\Delta\psi_m$, mitochondrial membrane potential; DN, double negative; DP, double positive; MNGIE, neurogastrointestinal encephalomyopathy; PNP, purine nucleoside phosphorylase; SP, single positive; TP, thymidine phosphorylase.
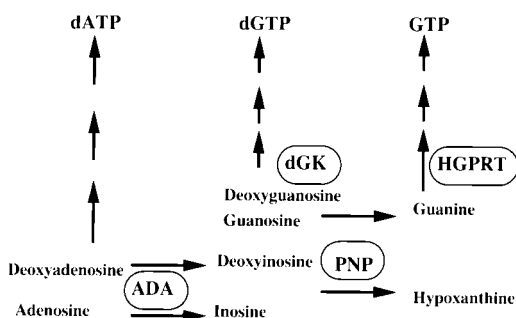
Figure 1. A schematic presentation of the role of PNP in the degradation and salvage pathways of purine nucleosides is depicted. PNP catalyzes the phosphorolysis of the products of the ADA reaction, inosine and deoxyinosine, to yield hypoxanthine and ribose-1-phosphate. Deficiency in PNP enzymatic activity leads to the accumulation of its substrates: inosine, deoxyinosine, guanosine, and deoxyguanosine. Of the four PNP substrates, only deoxyguanosine is phosphorylated by the mitochondrial deoxyguanosine kinase (dGK). Further phosphorylation of dGMP leads to the accumulation of dGTP, which may interfere with DNA synthesis or repair directly or by inhibition of ribonucleotide reductase activity. The PNP product guanine is salvaged back to the guanine nucleotide pools by hypoxanthine guanine phosphorybosyl transferase (HGPRT) activity.

leading to defective mitochondrial DNA repair and T cell apoptosis.

Materials and Methods

Generation of PNP⁻/⁻ Mutant Mice. Murine cDNA for PNP was used for screening genomic library of 129/Cj in λDASH phage vectors. Genomic clones were mapped and partially sequenced. A 7.0-kb genomic fragment containing the PNP catalytic sites within exons 3 and 4 was used to construct the targeting vector. A 1.2-kb genomic HindIII fragment containing exons 3 and 4 was replaced with a PGK*neo*-polA G418 resistance gene cassette (10) and a thymidine kinase (PGK-TK) expression cassette (11). E129/Cj embryonic stem cells (5×10^6) were electroporated with 20 μg of linearized targeting vector DNA. The embryonic stem cells were cultured onto G418 resistant murine fibroblasts and selection, in the presence of 300 μg/ml G418 and 2 μM Gancylovir, was initiated 48 h after electroporation. Double-resistant colonies were isolated after 10 d. PCR screening for homologous recombination was carried out using the diagnostic primers spanning the PGKneo and the short arm of the construct (see Fig. 2 A). Homologous recombination was subsequently confirmed by EcoRI digestion of genomic DNA and hybridization with probe A (see Fig. 2). Chimeric mice were produced by injection of embryonic cells into 3.5-d-old blastocysts (10). The contribution of embryonic stem cells to the germline of chimeric mice was assessed by breeding with C57BL/6 mice, and germline transmission of the PNP mutation was confirmed by Southern blot analysis of tail DNA. Mice heterozygous for the mutant gene were interbred to homozygosity.

Enzyme Assays. PNP enzymatic activity was assayed in cell lysates using cellulose TLC with [8-¹⁴C]inosine (50 mCi/mmol; Moravek Biochemicals, Inc.) as substrate, as described previously (3).

Deoxyguanosine kinase activity was assayed with [8-³H]2′-deoxyguanosine (4 mCi/mmol, Moravek Biochemicals, Inc.) as described (12).

Analysis of Intracellular Nucleosides, GTP, and dGTP Pools. Intracellular nucleotides were extracted with 0.4 M ice-cold perchloric acid as described previously (13). After 5 min on ice, the cell extract was neutralized with 0.5 M tri-*n*-octylamine dissolved in 1,1,2-trichlorotrifluoroethane in the presence of 0.1% bromophenol blue until the solution changed to blue. Samples were centrifuged at 15,000 *g* for 1 min and frozen at −70°C until analyzed (14). Mitochondria were isolated by differential centrifugation (15). Nucleotides were separated on a Hewlett-Packard model 10848 chromatograph using a Partisil-5-SAX column (Whatman, Inc.; reference 3). Urinary nucleosides and deoxynucleosides were analyzed by reverse phase HPLC as described previously (3). Intracellular dGTP analysis was performed by the DNA polymerase method described by Sherman and Fyfe (16). Urinary nucleosides were determined using a C-18 reverse phase HPLC column (17).

Flow Cytometric Analysis. Flow cytometry was performed using a dual laser FACScan™ (Becton Dickinson). Single cell suspensions (10^6 cells) of either thymi, spleen, lymph node, or bone marrow were stained for three-color fluorescence analysis. Fluorescein-conjugated antibodies included CD3, TCR, IgM, and CD45 or CD34. Phycoerythrin-conjugated antibodies included CD4, CD11b, CD14, CD43, and Sca-1. Biotin-conjugated antibodies included CD8, IgM, NK1.1, CD19, B220, CD25, and CD24, and were developed with CyChrome-strepavidin. All antibodies were purchased from BD PharMingen. Control antibodies were FITC–Leu-4, PE–Leu-4, and biotin–Leu-1 (Becton Dickinson).

Anti–Fc receptor (CD16) antibody was used in all populations except thymus. Cells were washed in phosphate buffer containing 0.1% bovine albumin with 0.01% sodium azide (staining buffer) at 4°C. Pellets were then stained with the FITC-conjugated antibody at 4°C for 15 min, after which PE-conjugated antibody was added, and cells were further incubated for 30 min at 4°C. After washing twice in staining buffer, cells were stained with biotin-conjugated antibodies and incubated for 20 min at 4°C. Cy-Chrome strepavidin was added after washing, and after a further incubation of 15 min, cells were washed and resuspended in 0.5 ml of staining buffer, and were analyzed after filtering through a 0.8-μm filter and the addition of propidium iodide.

All fluorescence data were collected using logarithmic amplification on 10–50 K viable cells as determined by forward/side scatter and propidium iodide exclusion.

Determinations of Apoptosis. Annexin V binding was performed according to the manufacturer's instructions (Boehringer). In some experiments, as indicated, thymocytes were stained as described above with a combination of PE- and biotin–CyChrome-conjugated antibodies before annexin V staining. Previous experiments showed that the antibodies did not interfere with the annexin V stain. The PNP inhibitor, CI-1000 (2-amino 3,5-dihydro-7-[3-thienylmethyl]-4H-pyrrolo[3,2-d]-pyrimidin-4-one HCl), used in the apoptosis experiments were a gift of Dr. R.B. Gilbertsen (Parke-Davis). Mice overexpressing Bcl2 in thymocytes (described by Sentman et al. [18]) were obtained from The Jackson Laboratory.

Dissipation of mitochondrial membrane potential ($\Delta\psi_m$) was determined using potentiometric sensitive fuorochrome, 3,3′ dihexyloxacarbocyanine iodide (DiOC$_{(6)3}$; 20 nM) (19). Caspase activity was inhibited by *N*-benzoyloxycarbonyl-Val-Ala-Asp fluoromethylketone (Z-VAD.fmk; 50 μM).

Apoptotic nuclear DNA fragmentation was measured by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) technique using an FITC-conju-

gated dUTP kit (Boehringer) according to the manufacturer's instructions. The frequency of apoptotic cells as detected by fragmented nuclear DNA was determined by flow cytometry.

Cell Isolations and Cultures. Thymocytes were cultured for the amounts of time indicated, ranging from 2–12 h in complete medium (RPMI with 10% FCS, 0.1 M glutamine, and 0.05 M Hepes with 2×10^{-5} M 2-ME at 5×10^6 cells/ml in 24-well plates; Costar). In some experiments, TCR was cross-linked using purified anti-TCR mAb (20 μg/ml) and anti-CD3 mAb (145-2C11 mAb; 20μg/ml), anti-CD4 mAb 1:4 vol of supernatant (RL172 [20]), and anti-CD8 mAb supernatant (3-155 [20]) for the time of culture. Cells were then washed and tested for apoptosis using the annexin V staining. Evidence for cross-linking was performed in these cultured cells by staining for the antigen being cross-linked with a specific mAb directed to this antigen.

Purified T cells derived from both lymph nodes and spleen of PNP-deficient as well as control mice were obtained by nylon wool depleting these populations. In brief, 5×10^7 cells nylon wool–packed (Robbins Scientific) were poured into 10- or 20-ml syringes. Columns were preincubated for 45 min with the syringe volume of 1% BSA-PBS, and cells were loaded in 1–3 ml and incubated for another 45 min. Cells were then eluted with two column volumes, washed, and stained for CD3, CD11, and/or IgM mAb. Nylon wool lymph node T cells were on average >90% pure (range 86–98% in $n = 16$ experiments).

Cytotoxic T Cell Assay. Purified T cells derived from the spleens of PNP-deficient (H-2b) or wild-type controls were co-cultured with 20 cGy gamma-irradiated splenocytes derived from either CBA (H-2k) or DBA/2 (H-2d) at 10×10^6 responder with 10×10^6 irradiated stimulators in 20 ml final volume in flasks.

Half of the cultures were supplemented with a previously tested optimal dose of rIL-2 (provided by Dr. R. Miller, University of Toronto, Toronto, Ontario). After 5 d, recovered cells were counted and used in a 4-h chromium assay at E/T ratios ranging from 3–100:1 in u-shaped 96-well plates. Each culture was assayed against the haplotype of the stimulator used, third party stimulator or syngeneic H-2b. The tumor cell lines (3,000 cells per well) P815 (H-2d) and BW5467 (H-2k) were used as targets.

Percentage cytotoxicity was calculated as (experimental release − spontaneous release)/(total release − spontaneous release) × 100. Spontaneous releases were <12%.

Gamma Irradiation and T Lymphocyte Proliferation Assay. For gamma irradiation experiments, T cells from the thymus or spleen were suspended in RPMI 1640 with 10% FCS at 10×10^6 cells/ml, and were exposed to varying doses of gamma irradiation from a ^{137}Cs irradiator. Total spleen or lymph node cells (3×10^5 cells) or nylon wool–purified T cells (3×10^5 cells) were cultured in complete medium in 96 flat-bottomed plates (Costar) in the presence or absence of Con A (2 μg/ml) and IL-2 (10 U/ml). Lymphocyte proliferation was measured after 72 h. 1 μCi [³H]thymidine was added 4 h before the termination of the cultures.

Results

Generation of PNP-deficient Mice. We used homologous recombination to generate a mouse line that lacks PNP activity by replacing a 1.2-kb PNP gene fragment containing the catalytic site (nucleotides 324–870 spanning exons 3 and 4) with a neomycin (PGK*neo*) gene cassette (reference 11; Fig. 2). The selected PNP-deficient stem cell clones were injected into blastocysts of C57BL/6 mice to generate 129/C57BL/6 chimeric mice. After germline transmission
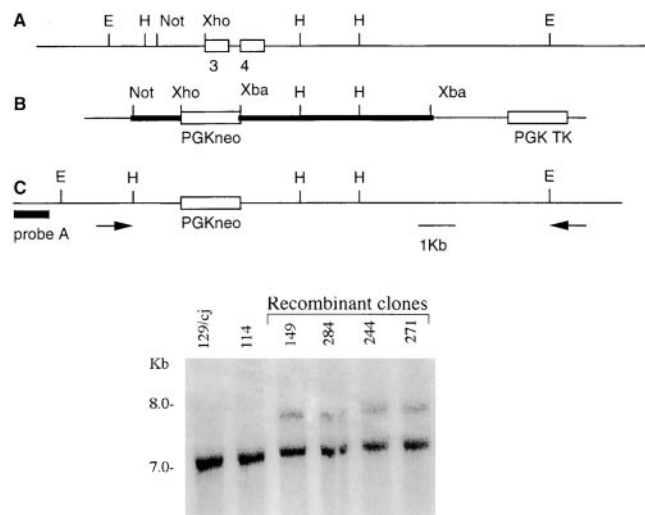

Figure 2. PNP targeting. Partial map of the PNP locus (A) and the targeting construct (B), whereby the 1.8-Kb PGKneo cassette (bold) replaces a 1.2 genomic fragment and the targeted locus (C). Diagnostic 1, oligodeoxynucleotide-TATGCAGTTCTGAATCCTCCCTGTAGCAGG; diagnostic 2, deoxyoligonucleotide-TCTACTGCTAGGATCTAAGAA-CAGAGACTAGTG. Diagnostic 1/diagnostic 2 oligodeoxynucleotides synthesize a PCR fragment of 3.0 Kb with wild-type DNA as substrate and two bands of 3.0 Kb (wild-type allele) and 3.8 Kb (recombinant allele). Bottom: Southern blot analysis of representative clones. Genomic DNA was digested with EcoRI and hybridized with probe A. Genomic 129 DNA and the DNA of nonrecombinant clone contain a nonrecombinant band of 7.0 Kb. Four clones (149, 284, 244, and 271) contain the nonrecombinant and recombinant bands of 7.0 Kb and 7.8 Kb, respectively.

of the targeted DNA, PNP$^{-/-}$ homozygosity was diagnosed using PCR of tail DNA and PNP enzyme assays (<0.2% of PNP$^{+/+}$).

Metabolic Abnormalities in PNP-deficient Mice. To understand the metabolic consequences of PNP deficiency, we first analyzed the levels of PNP substrates excreted in their urine. The urine of PNP-deficient mice contains large amounts of the four PNP substrates (inosine, deoxyinosine, guanosine, and deoxyguanosine), similar to PNP-deficient patients (reference 17; Table I A). The intracellular concentration of GTP is reduced in PNP$^{-/-}$ cells, reflecting the lack of a guanosine kinase and inability to form (and hence salvage) guanine (Table I; Fig. 1; references 21, 22). In contrast, dGTP pools were elevated by about eightfold in PNP$^{-/-}$ cells. As was proposed previously (14), phosphorylation of deoxyguanosine, the rate-limiting step in its conversion to dGTP, may be limited by an unexplained secondary loss of deoxyguanosine kinase activity found in cells of PNP-deficient mice (Table I B; Fig. 1; reference 23). In preliminary studies, we observed a substantial increase in whole blood PNP activity (20% of wild-type level) in each of three PNP knockout mice treated with polyethylene glycol (PEG)-modified PNP, which also eliminated the excretion of PNP substrates and alleviated the secondary loss of deoxyguanosine kinase activity (data not shown).

Because deoxyguanosine kinase localizes to the mitochondria (24–26), we have analyzed the distribution of in-

Table I. *Urinary Levels of PNP Substrates, Intracellular Levels of Guanine Nucleotides, and Deoxyguanosine Kinase Activity in Thymocytes from PNP-deficient Mice*

	Urinary purine nucleosides			
Mice	Inosine	Guanosine	dInosine	dGuanosine
	nmol/g creatinine	*nmol/g creatinine*	*nmol/g creatinine*	*nmol/g creatinine*
PNP$^{-/-}$	46.8 (9.7)	34.5 (7.3)	20.7 (2.7)	16.5 (1.5)
PNP$^{+/-}$	<0.5	<0.5	<0.5	<0.5

	Intracellular guanine nucleotides			
Thymocytes	dGTP	GTP	Deoxyguanosine kinase	Mitochondrial dGTP
	pmol/10^6 cells	*pmol/10^6 cells*	*pmol/mg/min*	*pmol/μg protein*
PNP$^{-/-}$	17.3 (2.8)	695.7 (25.4)	6.3 (0.9)	0.45 (0.08)
PNP$^{+/-}$	2.2 (0.74)	971.4 (32.8)	130.2 (9.8)	<0.05

Nucleoside, deoxynucleoside, nucleotide, and deoxynucleotide levels and enzymatic activities were determined as described in Materials and Methods. Top: urinary nucleoside levels normalized to creatinine levels are averages of five individual daily measurements, with SD given in parentheses. Bottom: thymocyte dGTP, GTP, and deoxyguanosine levels represent averages of four individual mice, with SD given in parentheses.

tracellular dGTP pools (Table I). We found a marked increase in dGTP associated with washed mitochondria from PNP-deficient thymocytes compared with mitochondria from PNP-expressing cells. Consistent with this observation, incubation of PNP-expressing thymocytes with deoxyguanosine in the presence of PNP inhibitor led to a large accumulation of dGTP in the mitochondria (data not shown). In contrast, incubation of thymocytes with the ADA inhibitor deoxycoformycin and deoxyadenosine resulted in a substantial increase of intracellular dATP pools, but no increase in mitochondrial dATP levels (data not shown). Thus, the localization of the respective deoxynucleoside kinases determines the intracellular location of dNTP pools because deoxycytidine kinase, the enzyme partially responsible for deoxyadenosine phosphorylation and with low affinity towards deoxyguanosine, is a nuclear enzyme (27).

Abnormalities in Lymphocyte Subpopulations in PNP-deficient Mice. Analysis of thymocyte subpopulations in PNP$^{-/-}$ 3-mo-old mice reveals a twofold increase in the frequency of immature CD4$^-$CD8$^-$ double negative (DN) cells and a decrease in the total cell numbers of CD4$^+$CD8$^+$ double positive (DP) and CD4$^+$ and CD8$^+$ single positive (SP) thymocytes (Fig. 3 A and Fig. 4 A).

Similar to the thymus, both the spleen and lymph nodes of PNP-deficient mice exhibit decreased numbers of CD4$^+$ and CD8$^+$ T cells (Fig. 3 A and Fig. 4 A). The spleen and lymph nodes of PNP-deficient mice also show an increase in the frequency of immature CD19$^+$IgM$^-$ pre-B cells with no concomitant change in the frequency of IgM$^+$ mature B cells (Fig. 3 B and Fig. 4 B). The spleens of PNP$^{-/-}$ mice also contained increased numbers of CD11$^+$ myeloid cells (Fig. 4 B).
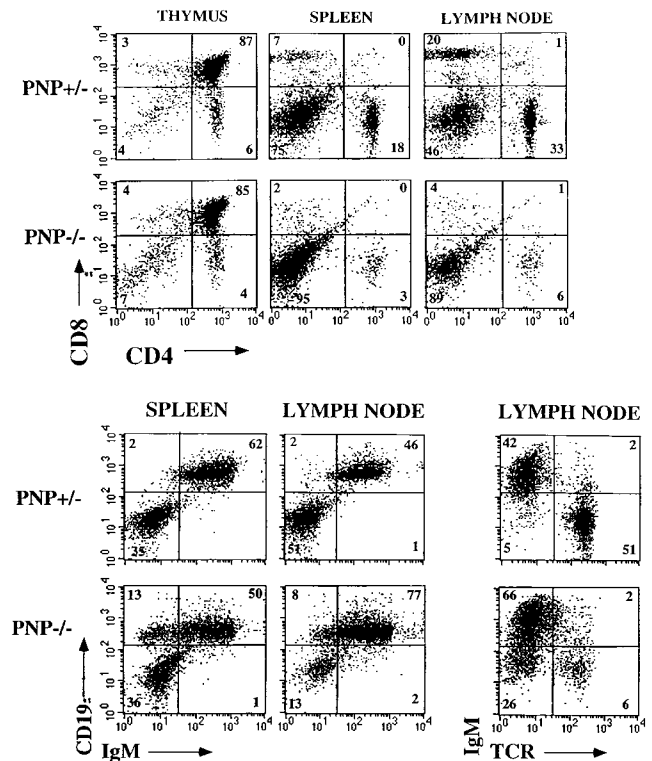

Figure 3. T and B lymphocyte subpopulations in lymphoid organs of PNP-deficient mice. Single-cell suspensions of thymocytes, splenocytes, and lymph node cells from 9-wk-old PNP-deficient mice and their heterozygous littermates were stained for three-color fluorescence analysis. Cells were first reacted with CD3 mAb (2C11-145 FITC conjugated) and CD4 mAb (RM4.5 PE conjugated), then with CD8 mAb (53-6.7 biotin conjugated), and finally with CyChrome-streptavidin. Top: percentage of cells in the individual subpopulation are shown in each quadrant.
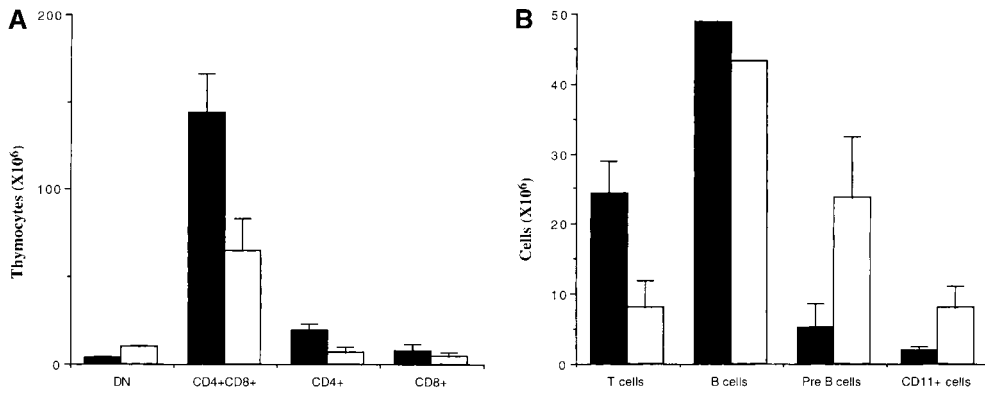
Figure 4. Total numbers of lymphocyte subpopulations in the thymus, spleen, and lymph nodes of PNP-deficient mice. (A) Total numbers of cells per thymus of thymocyte subpopulations recovered from thymi of PNP$^{-/-}$ (white bars) and PNP$^{+/-}$ (black bars) 9–12-wk-old mice were analyzed by three-color flow cytometry for DN (CD4$^-$CD8$^-$), DP (CD4$^+$CD8$^+$), and SP CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ thymocytes. Results are means of eight independent experiments with one to three mice each. Vertical bars represent the SD. (B) Total numbers of T cells (CD3$^+$), B cells (IgM$^+$), pre-B cells (CD19$^+$IgM$^-$), and myeloid cells (CD11$^+$) are averages of eight experiments.

Lymphocyte Function in PNP-deficient Mice. We assessed the function of T cells from PNP-deficient mice and tested the ability of cytotoxic T cells to specifically kill in a mixed lymphocyte reaction against H-2^k– and H-2^d–bearing stimulator cells. PNP-deficient spleen cells (H-2^b) were cocultured for 5 d in the presence of irradiated spleen cells derived from either DBA/2 mice (H-2^d; data not shown) or CBA mice (H-2^k; Fig. 5). The cultures were set up in the presence or absence of IL-2. Recovered cells were then tested in a 4-h ^{51}Cr-release assay for their ability to lyse allogeneic H-2^k targets or third party H-2^b targets. In the absence of exogenous IL-2, H-2^k–stimulated PNP-deficient T cells were unable to kill either H-2^k–bearing or H-2^d–bearing targets. However, in the presence of IL-2, the ability of PNP-deficient T cells to kill allogeneic H-2^k cells was restored to a level comparable to that of their heterozygous littermates (Fig. 5). This observation suggests that T cells from PNP-deficient mice have an impaired ability to mount an immune response in the absence of exogenously added IL-2.

Enhanced Thymocyte Apoptosis in PNP Deficiency Is Initiated in the Mitochondria. To examine whether the depletion of DP thymocytes is due to enhanced apoptosis, we analyzed the frequency of apoptotic cells in thymocyte subpopulations from PNP$^{-/-}$ mice and their heterozygous littermates (Fig. 6). Freshly isolated PNP-deficient thymocytes show a twofold increase in the frequency of apoptotic thymocytes, measured by annexin V binding (28), compared with their heterozygous littermates. The frequency of apoptotic cells in the PNP$^{-/-}$ thymocyte subpopulation is inversely related to Bcl2 expression: it is highest at the DP stage (8.7% of total PNP$^{-/-}$ DP cells), lower in SP CD4 and CD8 cells, and absent in immature DN cells.

To examine whether Bcl2 expression in the thymus may offer protection from apoptosis caused by PNP deficiency, we incubated thymocytes from C57BL/6 and Bcl2 transgenic mice (overexpressing Bcl2 in the thymus [18]) in the presence of deoxyguanosine and a PNP inhibitor (29). The frequency of apoptotic cells was monitored by annexin V binding. PNP inhibition in the presence of deoxygua-
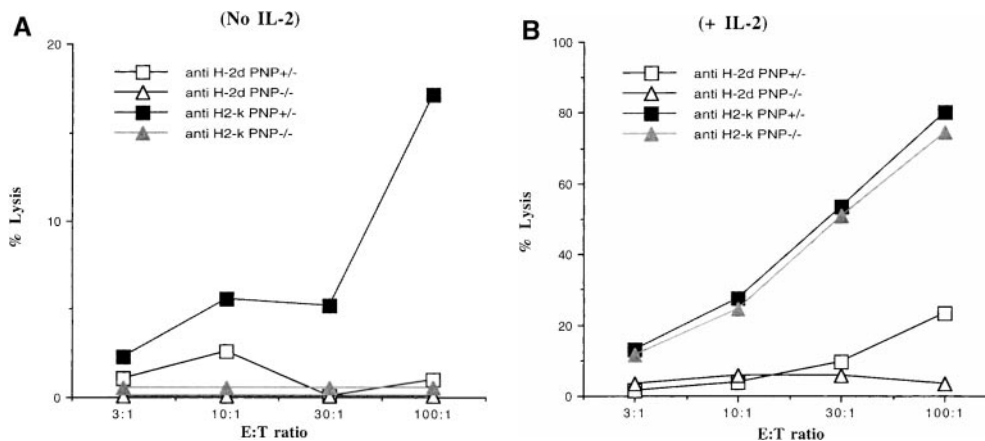

Figure 5. Analysis of allogeneic cytotoxic T cell response of PNP$^{-/-}$ lymphocytes. Splenocytes derived from PNP$^{-/-}$ (triangles) or PNP$^{+/-}$ (squares) mice were cultured for 5 d with allogeneic irradiated (20 cGy) CBA (H-2^k) splenocytes in the presence (B) or absence (A) of IL-2. On day 5, cytotoxic T cell function was assayed at the indicated E/T ratios in a 4-h chromium-release assay against BW5437 (H-2k) targets (filled symbols) or third-party P815 (H-2d) targets (open symbols).
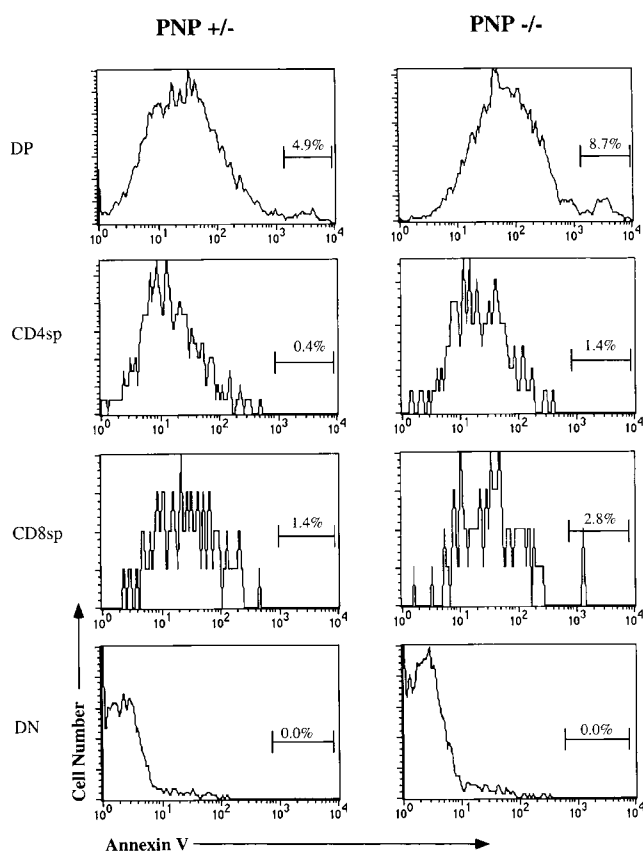
Figure 6. Increased frequency of apoptotic cells in freshly isolated thymocytes in PNP-deficient mice. Three-color analysis of apoptotic thymocyte subpopulations was carried out in freshly isolated thymocytes stained with conjugated fluorescent antibodies against CD4 and CD8, and then stained with conjugated annexin V as described in Materials and Methods. The frequencies of annexin V–positive apoptotic cells in DP, CD4$^+$ (CD4sp), CD8$^+$ (CD8sp), and DN thymocyte subpopulations of PNP$^{-/-}$ and heterozygous littermates are presented. Data from a single experiment representative of five additional experiments with similar results.
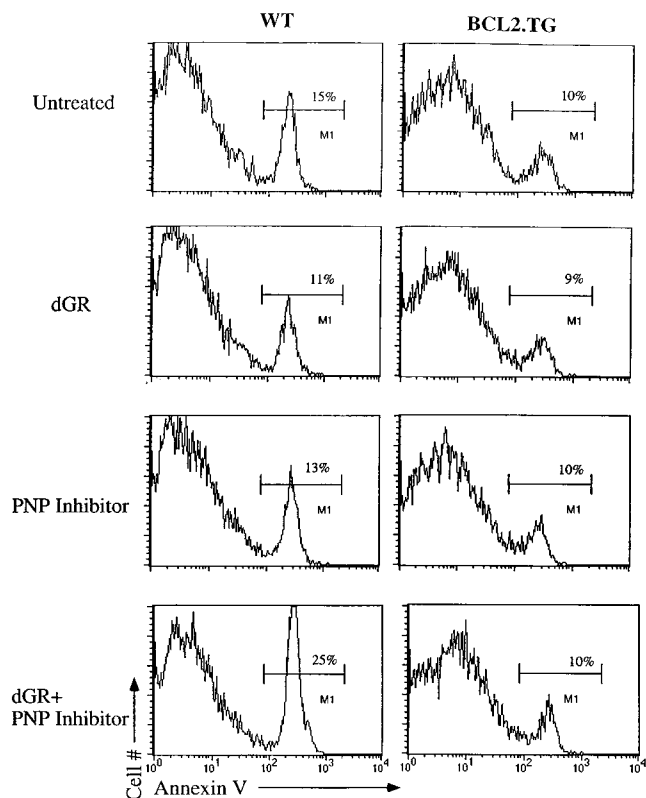

Figure 7. Effect of Bcl2 overexpression on thymocyte apoptosis induced by deoxyguanosine in the presence of a PNP inhibitor. Thymocytes from C57BL/6 and BCl2TG mice were incubated in RPMI 1640 medium with 10% FCS in the presence or absence of 60 μM deoxyguanosine and 20 μM of the PNP inhibitor CI-1000 (2-amino 3,5-dihydro-7-(3-thienylmethyl)-4H-pyrrolo[3,2-d]-pyrimidin-4-one HCl) as indicated for 12 h, and the frequency of CD4$^+$CD8$^+$ DP apoptotic cells was analyzed by annexin V staining.

nosine caused increased thymocyte apoptosis in C57BL/6 (Fig. 7). Deoxyguanosine or PNP inhibitor added separately did not affect the frequency of apoptotic thymocytes. Overexpression of Bcl2 in thymocytes from transgenic mice completely protects against deoxyguanosine-induced apoptosis in the absence of PNP activity. No apoptosis was induced by any of the other three PNP substrates (inosine, deoxyinosine, or guanosine) in the presence of the PNP inhibitor (data not shown).

Dissipation of $\Delta\psi_m$ is an early apoptotic event that is independent of caspase activity for intramitochondrial apoptotic agents, but $\Delta\psi_m$ is dependent on caspase activity if the apoptotic signal is extramitochondrial (19). Thus, resistance of $\Delta\psi_m$ to caspase inhibition is indicative of apoptotic signals that originate within the mitochondria. Deoxyguanosine in the presence of PNP inhibitor caused rapid dissipation of $\Delta\psi_m$, similar to other apoptosis-inducing agents. This dissipation of $\Delta\psi_m$ was resistant to cytosolic caspase inhibition, indicating that deoxyguanosine initiates apoptosis within the mitochondria (Fig. 8). In contrast, the deoxyguanosine-induced fragmentation of nuclear DNA, a late

event in apoptosis, was inhibited by caspase inhibitor. These results are consistent with the hypothesis that accumulation of dGTP in the mitochondria is responsible for the apoptosis observed in PNP-deficient thymocytes.

Sensitivity of PNP-deficient T Cells to Gamma Irradiation. Imbalance of dNTP pools has been shown to interfere with DNA repair (5, 30–32). We have examined the possibility that mitochondrial dGTP accumulation may predispose PNP-deficient T cells to DNA damage. Thymocytes from PNP-deficient mice exhibit increased sensitivity to gamma irradiation compared with their heterozygous littermates. After 12 h of incubation after gamma irradiation, in PNP$^{-/-}$ thymocytes, 50% apoptosis was observed at about 200 cGy, whereas a dose of 1,000 cGy was needed to induce 50% apoptosis in thymocytes from heterozygous mice (Fig. 9).

To assess the sensitivity of peripheral T cells to gamma irradiation, splenocytes from PNP-deficient mice and their heterozygous littermates were gamma irradiated, and their proliferation was monitored after stimulation by Con A (Fig. 10). Exogenous IL-2 was added to all cultures, as PNP-deficient T cells have decreased mitogenic response
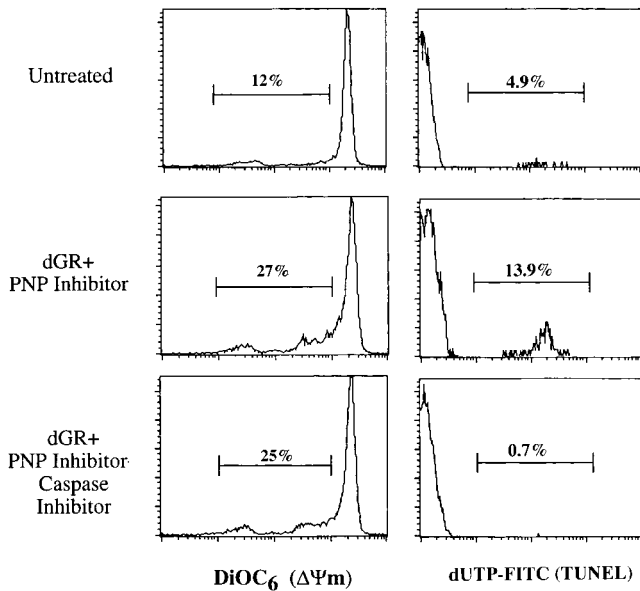
Figure 8. Effects of inhibition of caspase activity on deoxyguanosine-induced $\Delta\psi_m$ dissipation and nuclear DNA fragmentation. Thymocytes from C57BL/6 mice were incubated in RPMI 1640 medium with 10% FCS in the presence or absence of 60 μM deoxyguanosine and 20 μM of the PNP inhibitor CI-1000 (2-amino 3,5-dihydro-7-(3-thienylmethyl)-4H-pyrrolo[3,2-d]-pyrimidin-4-one HCl) as indicated for 12 h. $\Delta\psi_m$ was determined using potentiometric sensitive fuorochrome, $DiOC_{(6)3}$ (20 nM; reference 19). Percentages of cells with dissipated $\Delta\psi_m$ are indicated above the bar covering area of cells with lower $DiOC_6$ fluorescence intensity. Caspase activity was inhibited where indicated by Z-VAD.fmk (50 μM). Nuclear DNA fragmentation was measured by the TUNEL technique as described in Materials and Methods. Percentages of apoptotic cells are indicated above the bars.
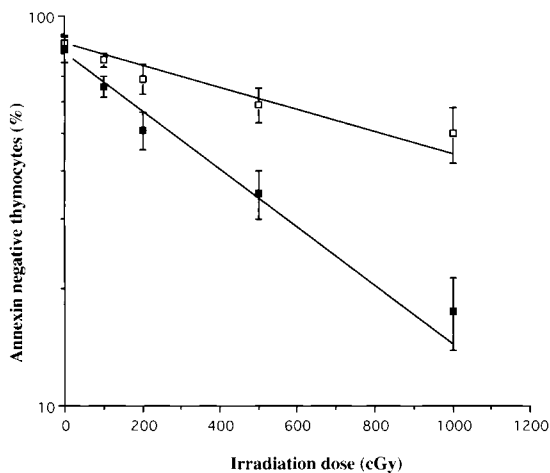

Figure 9. Effect of gamma irradiation on thymocyte apoptosis in PNP-deficient mice. Thymocytes from PNP-deficient mice (■) and their heterozygous littermates (□) were suspended in RPMI 1640 medium with 10% FCS, and were gamma irradiated with the indicated dose. Irradiated thymocytes (10^6 ml) were incubated in RPMI 1640 medium with 10% FCS for 12 h, and the frequency of apoptotic cells was monitored by annexin V binding as described in Materials and Methods. Averages of annexin V–negative cells of three different experiments using two to three mice each.
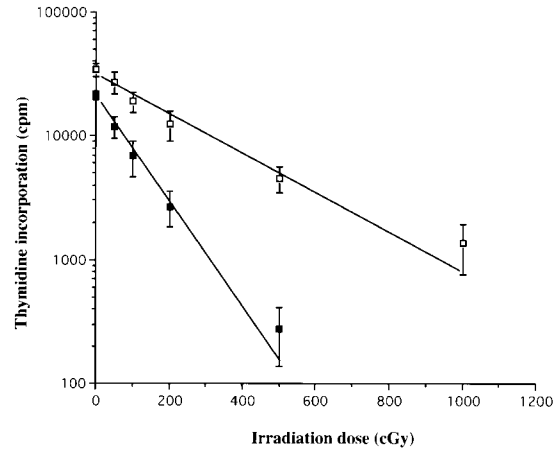
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Figure 10. Effect of gamma irradiation on T cell proliferation in PNP-deficient mice. Purified T cells from PNP-deficient mice (■) and their heterozygous littermates (□) were gamma irradiated at the indicated dosage, and equal numbers of T cells (3×10^5) were aliquated in quadruplicates onto microtiter plates containing 0.2 ml RPMI 1640 medium with 10% FCS in the presence of 2 μg/ml Con A and IL-2 (10 U/ml). T cell proliferation was measured after 4 h of [^{3}H]thymidine pulse. Results are averages of quadruplicate wells. Three independent experiments using two to three mice each gave similar results.

in the absence of IL-2 (data not shown; references 9, 33). Similar to PNP-deficient thymocytes, mature T cells from the spleens of PNP-deficient mice are more sensitive to gamma irradiation (90% reduction at 300 cGy) than their heterozygous littermates (90% reduction at 800 cGy). These observations are consistent with an impaired DNA repair in PNP-deficient thymocytes.

Discussion

In humans, PNP gene mutations that result in extensive loss of enzymatic activity cause severe T cell deficiency with variable abnormalities in humoral immunity (2, 34–37). Similar to the human disease, PNP deficiency in mice causes an immunodeficiency that affects T lymphocytes more severely than B lymphocytes. PNP knockout mice exhibit impaired thymocyte differentiation (Fig. 3), reduced mitogenic and allogeneic responses (Fig. 5; data not shown), and decreased numbers of maturing thymocytes and peripheral T cells (Fig. 3 and Fig. 4 A). Mice with less complete PNP deficiency because of missense mutations showed a more gradual postnatal decline in T cell numbers and in T cell function (9, 33). PNP knockout mice provide a good experimental model for studying the biochemical basis of immunodeficiency, and for testing potential therapies for the disease in humans. Preliminary studies suggest that a previously developed PEG-PNP that shows reduced immunogenicity in normal mice (38) corrects the metabolic abnormalities and prolongs the life of PNP knockout mice.

The immune deficiency disease caused by loss of PNP enzymatic activity could be due to either interference with purine salvage resulting in depletion of GTP or to the ac-

cumulation of one or more of the PNP substrates inosine, guanosine, deoxyinosine, and deoxyguanosine. Decreased intracellular levels of GTP observed in PNP deficiency (Table I) are unlikely to contribute to the immune dysfunction, as a similar decrease in intracellular levels of GTP in hypoxanthine guanine phosphorybosyl transferase (HG-PRT) deficiency has no effect on immune function in patients with Lesch-Nyan syndrome (39). The only other intracellular metabolic abnormality in PNP$^{-/-}$ mice, as well as in PNP$^{+/-}$ mice, is the expansion of intracellular dGTP pools (Table I), which are normally tightly regulated in mammalian cells (40, 41).

The observed increase in intracellular dGTP pools in PNP deficiency is modest (17 pmol/10^6 cells, or eightfold normal levels) compared with the 4–5 mM concentration of deoxyguanosine accumulated in the urine of these mice (Table I). This may be partly explained by end product inhibition of deoxyguanosine kinase activity by dGTP (42, 43). In addition, cells from PNP$^{-/-}$ mice exhibit a secondary loss of deoxyguanosine kinase activity in all tissues examined, further limiting the potential of deoxyguanosine accumulation (Table I B; reference 14). The underlying mechanisms have not been established, although partial restoration of deoxyguanosine kinase activity after treatment with PEG-PNP clearly indicates an effect of a PNP substrate or metabolite. It is not clear whether deoxyguanosine kinase activity is reduced in cells of human patients with PNP deficiency. However, in PNP-deficient mice, this effect is likely to moderate the immune dysfunction, and perhaps prevents the neurologic abnormalities often present in human patients.

The observations described here, particularly the evidence that dGTP accumulation occurs selectively in mitochondria (Table I) and that PNP-deficient thymocytes and splenic T cells show increased sensitivity to irradiation (Table I; Fig. 10), offer new insight into the biochemical basis for immunodeficiency. We postulate that accumulation of dGTP in the mitochondria of T lymphocytes initiates apoptosis by interfering with the repair of mitochondrial DNA damage. This hypothesis is supported by several observations. The mitochondrial and T cell specificity of dGTP accumulation are consistent with the subcellular localization (44) and tissue distribution (13, 45, 46) of deoxyguanosine kinase activity. Human deoxyguanosine kinase activity is localized exclusively in the mitochondria, and thus dGTP accumulates as expected in the mitochondria (47). That dGTP accumulation preferentially inhibits mitochondrial DNA synthesis or repair rather than nuclear DNA synthesis is suggested by the finding that PNP-deficient cells are sensitive to gamma irradiation, but are able to replicate their nuclear DNA in response to mitogen in the presence of IL-2 (Fig. 10).

Other observations provide additional evidence that the T cell damage in PNP deficiency originates in the mitochondria. Overexpression of deoxyguanosine kinase in the mitochondria leads to increased sensitivity to anticancer deoxyguanosine analogues (48). dGTP-mediated inhibition of mitochondrial DNA synthesis or repair might also initiate

apoptosis by inducing release of cytochrome C into the cytoplasm.

Recently, the mutations responsible for the human mitochondrial disease neurogastrointestinal encephalomyopathy (MNGIE) were localized to the thymidine phosphorylase (TP) gene. TP phosphorylates thymine to yield thymidine, and thus has parallel activity in the pyrimidine salvage pathway to that of PNP in the purine salvage pathway (49). Patients with MNGIE syndrome have multiple deletions in their mitochondrial DNA and develop a muscular neurological disorder starting in their twenties. It has been postulated that dTTP accumulation in the mitochondria of these patients may be responsible for abnormalities in mitochondrial DNA synthesis and repair (49). Although the tissue-specific phenotype in the two diseases may vary according to the specific tissue expression of the respective kinases, it is likely that the neurological defects, including cerebral ataxia, common to PNP-deficient and MNGIE patients (35, 50–52) may similarly be due to inhibition of mitochondrial DNA maintenance by either dGTP or dTTP (Fig. 11).
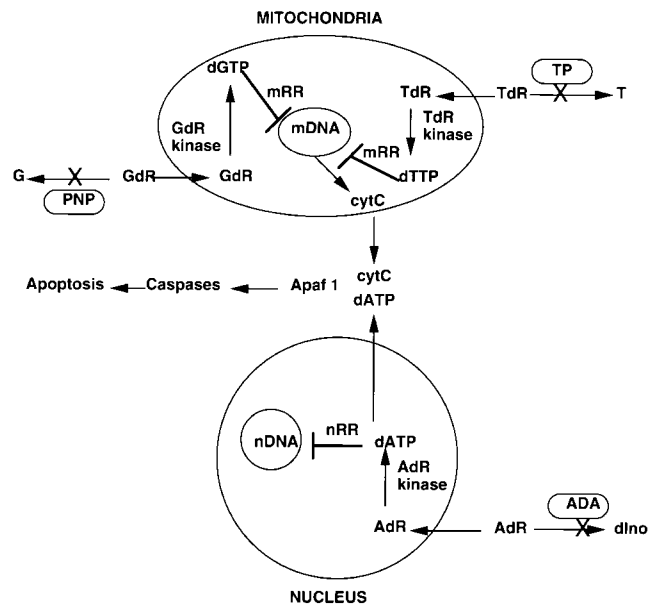

Figure 11. Proposed mechanism of PNP, ADA, and TP deficiencies. Mutations in the cytosolic enzymes PNP and TP result in accumulation of deoxyguanosine (GdR) and thymidine (TdR), respectively. The accumulated deoxynucleosides are phosphorylated by the mitochondrial enzymes deoxyguanosine kinase (GdR kinase; reference 47) or thymidine kinase (TdR kinase; reference 61), resulting in the accumulation of dGTP and dTTP, respectively. The abnormal accumulation of dNTP may interfere with mitochondrial DNA synthesis, or repair directly or by inhibition of mitochondrial ribonucleotide reductase (mRR; reference 62), leading to apoptosis. In contrast, ADA deficiency leads to the accumulation of dATP in the nucleus because of nuclear localization of deoxycytidine kinase, the enzyme responsible for deoxyguanosine phosphorylation (reference 27). Accumulation of dATP leads to apoptosis in a p53-dependent manner by inhibiting nuclear ribonucleotide reductase (nRR), interfering with DNA repair, and by directly participating in caspase activation (references 7, 31, 60).

The T lymphocyte specificity of PNP deficiency may be due in part to the increased capability of T cells and thymocytes to accumulate purine dNTPs (13, 53, 54). On the other hand, stimulation of T cell thymocytes through their antigen receptor often leads to activation-induced apoptosis, a process that requires activation threshold to recruit the mitochondrial apoptotic apparatus (55, 56). It is thus possible that mitochondrial dGTP accumulation in PNP deficiency will lower that apoptotic threshold.

Mitochondrial DNA repair is of critical importance in view of the increased frequency of mitochondrial DNA damage compared with nuclear DNA (20-fold higher; reference 57). The immediate effects of deoxyguanosine-induced apoptosis suggest the existence of an early detection mechanism of accumulation of mitochondrial DNA damage leading to the activation of mitochondrial apoptosis. The mechanisms that link nuclear DNA damage to apoptosis are under intensive investigation and include the participation of p53, ataxia telangiectesia mutated gene (ATm), and possibly dATP (7, 58–60). In contrast, little is known about the mechanisms that link mitochondrial DNA damage to apoptosis. Changes in mitochondrial dNTP levels such as dGTP and dTTP may participate in linking mitochondrial DNA damage to apoptosis, analogous to the role of dATP in apoptosis and in nuclear DNA repair (7, 60). The PNP-deficient mice provide an excellent model to test this hypothesis further.

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