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PURINE NUCLEOSIDE PHOSPHORYLASE DEFICIENCY

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Purine nucleoside phosphorylase (PNP) deficiency is a rare inherited disease accounting for approximately 4% of patients with severe combined immunodeficiency. Thirty-three patients have been reported. PNP-deficient patients suffer from recurrent infections, usually beginning in the first year of life. Two thirds of patients have evidence of neurologic disorders. Findings range from spasticity to developmental delay, to mental retardation. One third of patients develop autoimmune disease. The most common manifestation of this is autoimmune hemolytic anemia. Idiopathic thrombocytopenic purpura and systemic lupus erythematosis have also been reported. Patients usually present with infections but approximately one fourth have come to medical care initially for neurological problems. In PNP deficiency, T- and B-cell immunity are affected. T-cell function may be profoundly deficient, may be normal at birth and then decrease with time, or may fluctuate repeatedly between low and normal. B-cell function can be normal but is deficient in approximately one third of patients. PNP protein is a trimer of approximately 90,000 daltons. It is found in most tissues of the body but is at highest levels in lymphoid tissues. This tissue distribution explains why the lymphoid system is predominantly affected in PNP deficiency. Many mechanisms have been proposed to explain the metabolic toxicity in PNP deficiency. The elevated dGTP found in PNP deficiency is thought to inhibit ribonucleotide reductase and, thus, impede cell division. Depressed GTP levels may correlate with neurologic dysfunction. The gene for PNP has been cloned; it is located on the long arm of chromosome 14. Studies of a mutant PNP gene isolated from one patient showed that a point mutation resulting in an amino acid substitution was responsible for PNP deficiency. PNP deficiency has a grave prognosis. No patient has reached the third decade of life. Twenty-nine of the 33 reported patients have died from their disease. Prenatal diagnosis is currently available. Many different therapies have been utilized for PNP deficiency including bone marrow transplantation, red cell transfusions, and supplementation of the diet with purines and pyrimidines. None of these therapies has been consistently successful. In light of the poor prognosis for PNP deficiency, bone marrow transplantation should be considered for all patients. In the future, improved forms of therapy such as gene therapy may become available.

KEY WORDS: Purine nucleoside phosphorylase, combined immunodeficiency, bone marrow transplantation, mutations, gene therapy.

PNP deficiency is a rare inherited disease characterized by profound T-cell immunodeficiency. Infants with this disorder resemble patients with severe combined immunodeficiency (SCID). They suffer from recurrent infections and failure to thrive. Thirty-three patients in 20 families have been identified to date. Despite the small number of patients, this disease has held the attention of investigators in diverse fields such as clinical immunology, metabolism, genetics, and molecular biology. This article reviews our current understanding of PNP deficiency and will indicate areas of future research.

HISTORY

Deficiency of PNP (EC 2.4.2.1), an enzyme of purine metabolism, was first described by Dr Eloise Giblett in 1975[1]. Dr. Giblett had earlier (in 1972)[2] discovered that SCID could be caused by lack of adenosine deaminase (ADA), another enzyme of purine metabolism[2]. Dr. Giblett identified the first PNP-deficient patient by screening other SCID patients for errors in purine metabolism. The first PNP-deficient patient had deficient T-cell function and normal B-cell function[1]. Subsequently, 32 additional patients have been identified with PNP deficiency (Table 1).

CLINICAL FINDINGS

PNP deficiency is a heterogeneous disorder which can be considered a subset of severe combined immunodeficiency (SCID). Approximately 4% of patients with SCID will be found to be PNP deficient[41].

The 33 patients who have been identified to date come from 20 kindreds (Table 1). All have suffered from recurrent infections. Usually infections begin in the first year of life. Some patients, however, have been asymptomatic until several years of age (for example, patient #7 who was well until age 6 years). The infections are similar to those found in SCID. They include sinusitis, pneumonia, urinary tract infections, pharyngitis, otitis, mastoditis, thrush, and diarrhea. Specific pathogens have included *Bordetella pertussis*, Pseudomonas, Legionella, cytomegalovirus, varicella, vaccinia, adenovirus, parainfluenza virus type 2, 3, and 4, Herpes, Epstein–Barr virus, Candida, and *Pneumocystis carinii*, in addition to the usual respiratory pathogens, *Haemophilus influenzae* and *Streptococcus pneumoniae*. One patient (#22) had chronic enteroviral meningoencephalitis (ECHO 6) a problem usually associated with X-linked agammaglobulinemia[48]. One patient (#24) has extensive verrucae plantaris.

If a patient seems to be relatively free of problems in the first few years of life, the physician must be aware that significant problems may well arise later. Patient #7, for example, was relatively well until he experienced a severe case of varicella at age 6. After this time, the patient experienced multiple problems with recurrent respiratory infections including pneumonia, pansinusitis and purulent otitis media[20]. He developed ANA-positive lupus with arthritis, pericarditis, pericardial effusion and hemolytic anemia[20]. Treatment consisted of steroids. Before his death at age 12, he developed Herpes encephalitis (with symptoms including transient hemiparesis, hemiparesthesia, headaches, seizures, and coma)[20]. At age 6, this patient appeared to be well. It is not yet clear whether other patients who do well early in life (for example #21 and #22) will develop similar life-threatening problems.

Most patients experience no difficulties with routine childhood immunizations. Most patients, when tested, have been found to make antibodies to tetanus and polio. No cases of paralytic polio have been reported. In the past, smallpox vaccination has led to disseminated vaccinia (#2, #3) leading to death (#2). Several patients have received BCG without any dissemination (#2, #31, #32).

Neurologic problems (not thought to be secondary to infection) are common in PNP-deficient patients. These problems have been variously characterized as

Table 1 PNP deficiency: clinical presentation and outcome

Patient	*Clinical infections*	*Neurologic problems*	*Therapy*	*Outcome*	*Refs*
1. San Francisco[a] LM	starting at 4 months recurrent otitis, diarrhea, pneumonia, disseminated varicella		uridine, thymosin fraction 5, irradiated red cell transfusions×3	death: varicella	1, 3,4
2. Paris	no problem with BCG,[b] at 18 months, generalized vaccinia from vaccine		transfer factor, thymus transplant, irradiated red cell transfusions×2	death: 20 months vaccinia	5,6
3. Utrecht HV	at 8 months, smallpox vaccine spread over body		thymus transplants 3 times	death: 3 years lymphosarcoma	7–15
4. Utrecht FV	UTI 14 months, bilateral otitis, mastoidectomy			death: 1.5 years GVHD after unirradiated red cell transfusion	8–16
5. Utrecht RV	otitis and respiratory infections from 15 months, colitis, very severe varicella treated with acyclovir	spastic tetraparesis	adenine, uridine, hypoxanthine, allopurinol, irradiated red cell transfusions, deoxycytidine and tetrahydrouridine	death: 9 years renal failure and generalized acyclovir resistant Herpes Zoster	8–12 14–17
6. Toronto MB	1st year of life developed sinus infections and pulmonary infections, severe bronchiectasis, chorioretinitis (probably toxoplasmosis) leading to blindness, CMV excretion in urine, pertussis, recurrent ringworm	borderline mental retardation	hypoxanthine, uridine, irradiated red cell transfusions	death: 17 years chronic pulmonary disease	18–20

Table 1 (continued)

Patient	*Clinical infections*	*Neurologic problems*	*Therapy*	*Outcome*	*Refs*
7. Toronto DB	severe varicella age 6, then recurrent respiratory infections, pansinusitis, otitis media, Herpes encephalitis (seizures, transient hemiparesis)		uridine and hypoxanthine	death at 12 years Herpes, encephalitis, lupus, needed immunosuppression	18–20
8. Rome CS	well until 18 months, pneumonia, CMV in urine, otitis		irradiated red cell transfusions	death: pneumonia	21
9. Chicago KK	pharyngitis, cough, pneumonia	delay walking, tremor, spasticity	irradiated red cell transfusions	death: pneumonia	22–25
10. London family Irish	UTI from 3 months, aseptic meningitis	developmental delay	lymphoma	death: age 2	26–28
11. London SB Irish	parainfluenza virus type III, urinary tract infection, thrush, respiratory syncytial virus	developmental delay noted at 3 months	deoxycytidine and tetrahydrouridine, guanine, po then IV	death: age2 parainfluenza type 3 pneumonia	26–29
12. Madrid Older brother	urinary tract infections, pseudomonas, sepsis		irradiated red cell transfusions	death: 21 months pseudomonas pneumonia	30, 31
13. Madrid MC, sister	respiratory infections, diarrhea, Pseudomonas pneumonia		irradiated red cell transfusions	death: 2 years pseudomonas, respiratory failure	31, 32
14. Madrid Younger brother	respiratory infections		irradiated red cell transfusions bone marrow transplant	death: herpes encephalitis	30, 32

Table 1 (continued)

Patient	*Clinical infections*	*Neurologic problems*	*Therapy*	*Outcome*	*Refs*
15. Bethesda H family sister		CNS vasculitis		death: 22 months	33
16. Bethesda H Family, brother	pneumonia			death: 4 months pharyngeal tumor, pneumonia	33
17. Bethesda H Family, JH	Herpes from 2 1/2 years, Legionella, PCP	developmental delay, encephalitis at age 2, spastic tetraparesis		death: PCP and Legionella	33
18. London family #2 sister Arab	chickenpox (pneumonia, carditis), recurrent pneumonia, otitis	developmental delay		death: age 4 chickenpox	26 34,35
19. London family #2 6th sibling Arab	after 4 years, pneumonia, viral infections, thrush	spastic tetraparesis, developmental delay, hypertonia, increased DTR		death: 4 1/2 years viral infection	26 34, 35
20. London family #2 brother Arab	ear infections, oral thrush, mouth ulcers	CNS delay hypotonia		death: age 3 cause of death unknown	26,29 34, 35
21. Durham KF	near fatal varicella	post infections encephalopathy	IVIG, Haploidentical bone marrow transplant, thymosin fraction 5	alive on IVIG 12/90 10 years old	36, 37
22. Durham TP	recurrent infection from 22 months, severe varicella, severe eczema, chronic enteroviral meningoencephalitis (ECHO 6)	late onset ataxia, behavioral problems	IMIG, thymosin fraction 5	alive 9 years old 12/90	37, 38

Table 1 (continued)

Patient	*Clinical infections*	*Neurologic problems*	*Therapy*	*Outcome*	*Refs*
23. London family #3	pertussis recurrent infections	left hemiparesis neurological delay noted at 14 months		death: 4 1/2 years GVHD secondary to platelet transfusion	26, 39
24. Utrecht family #2	well until age 4, then rhinorrhea, parinfluenza recurrent respiratory infections, severe verrucae plantaris on feet	spastic tetraparesis from age 3, behavior disorder	antibiotic prophylaxzis	alive 9 years old 12/90	17, 40
25. Durham VS	recurrent infections, sinusitis, pneumonia, otitis	spasticity	matched bone marrow transplant with suppression	death: 7 years GVHD and CMV	41
26. Boston 1st sibling	recurrent otitis, pneumonia, severe thrush, failure to thrive	developmental delay	irradiated red cell transfusions	death: 1 year sepsis secondary to UTI	42, 43
27. Boston 2nd sibling	diagnosed in utero at 8 months, fevers	developmental delay, spastic diplegia	half-matched bone marrow transplant with suppression×2	alive 12/90, age 5 after bone marrow transplant.	42
28. Tokyo 1st sibling	repeated chest infections deaf, blind	developmental delay		death: 5 years lymphoma	44
29. Tokyo 2nd sibling	recurrent infections, since age 3 years, atypical pneumonia chickenpox	developmental delay	irradiated red cell transfusions	death: secondary to chickenpox	44
30. London family #4 Egyptian	recurrent chest infections 20 months to 5 years, neutropenia, adenoviral bronchiolitis	spastic tetraparesis noted at 18 months, late walking	HLA-identical bone marrow transplant with immunosuppression	death: age 6 pulmonary hemorrhage after transplant	29, 45

Table 1 (continued)

Patient	*Clinical infections*	*Neurologic problems*	*Therapy*	*Outcome*	*Refs*
31. Glasgow Family #1 1st sibling	no problem with BCG, oral thrush, refractory pneumonia	unable to sit		death: 23 months pneumonia	146, 46, 47
32. Glasgow Family #1 3rd sibling	no problem with BCG, loose stools in 3rd year, recurrent otitis, cough, appendix abscess	developmental delay	fetal thymus transplant	death: 7 years appendix abscess	146, 46, 47
33. Glasgow Family #2	recurrent urinary tract infections	ataxic diplegia		death: age 3 lymphoma	147

[a]Patients are identified by the city from which they were first reported and by their initials.
[b]Abbreviations: BCG, *Bacille bilie* de Calmette-Guerin; UTI, urinary tract infection; CMV, cytomegalovirus; PCP, Pneumocystis carinii pneumonia; CNS, central nervous sytem; DTR, deep tendon reflexes; GVHD, graft versus host disease; IVIG, intravenous immunoglobulin; IMIG, intramuscular immunoglobulin.

spastic tetraparesis (#5[10], #10[26], #19[26], #30[29,45]), spasticity (#9[22], #25[41]), spastic diplegia (#24[40], #27[42]), hemiparesis (#23[26]), retardation of motor development (#5[10], #9[22], #23[26], #31[46]), tremor (#9[22], #19[26]), ataxia (#22[37]), hypertonia (#19[26]), hypotonia, (#11[26], #20[26]), developmental delay (#10[26], #11[26], #18–20[26], #27[42], #26[42,43], #28–29[44], #32[46], #33[29]), hyperactivity (#6[20]), behavior problems (#22[38], #24[40]), and mild to severe mental retardation (#6[20], #20[26]). From the varied descriptions, one can see that there is no single characteristic finding in PNP-deficient patients. The neurologic problems have been found in 20 of the 33 patients. In patients #11, #23, #24, and #30–33 the neurologic symptoms became evident before the infectious problems. When neurologic problems are present in conjunction with profound T-cell deficiency, PNP deficiency is a likely diagnosis. One could argue that certain cases reported in the literature represent PNP deficiency. Perhaps the best example is the patient of Hagberg *et al.*[49] Reported in 1970, before the discovery of PNP deficiency, this patient had developmental delay, autoimmune hemolytic anemia, and recurrent infections[49].

Autoimmune problems are relatively common in PNP-deficient patients. Autoantibodies were found in 4 patients (#5[17], #7[20], #13[31], #24[17]). One patient had lupus with arthritis and pericarditis (#7[20]). One patient had central nervous system vasculitis (#15[33]). Seven of the 33 patients have had autoimmune hemolytic anemia (#7[20], #8[21], #9[22], #15[33], #17[33], #19[26,35], #23[26]). In two of four patients tested, the antibody has been determined to be directed against the Pr_a antigen[33]. Two patients have had idiopathic thrombocytopenic purpura (ITP) (#21[37], #23[26]). One patient possibly had autoimmune neutropenia (147). Therapy has included steroids and IVIG.

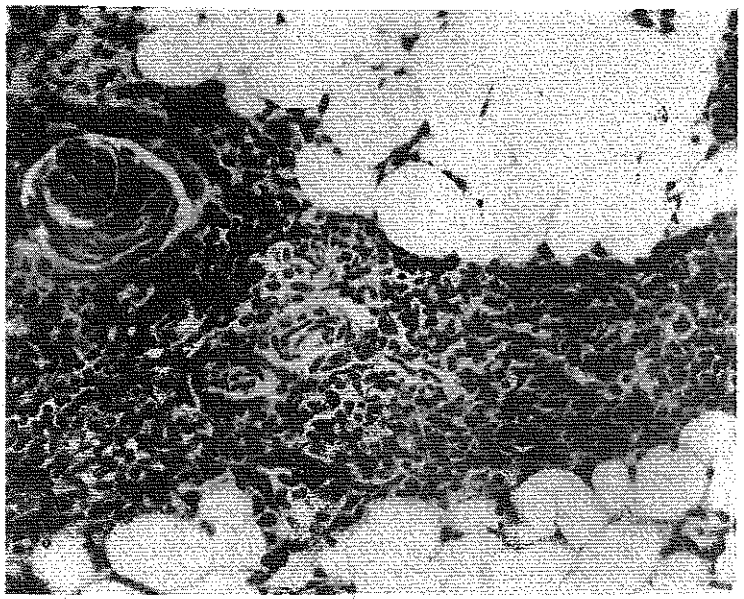
On history, an additional clinical problem, besides recurrent infections, autoimmune disease, and neurologic impairment, is failure to thrive. On physical examination, lymph nodes may be palpable but are small. The liver and spleen have been enlarged in patients with accompanying problems of hemolytic anemia, mononucleosis, and lymphoma. No thymus can be seen on chest X-ray.

Findings on autopsy have consistently shown depletion of lymphoid tissues. The thymus is small (Figure 1A). Usually Hassal's corpuscles are seen, but they are poorly formed. Tonsils are difficult to identify. They contain no germinal centers. In most lymph nodes, the paracortical fields are absent (Figure 1B). Plasma cells can usually be seen in the spleen, lymph nodes and lamina propria of the intestines. The red pulp of the spleen is small (Figure 1C).

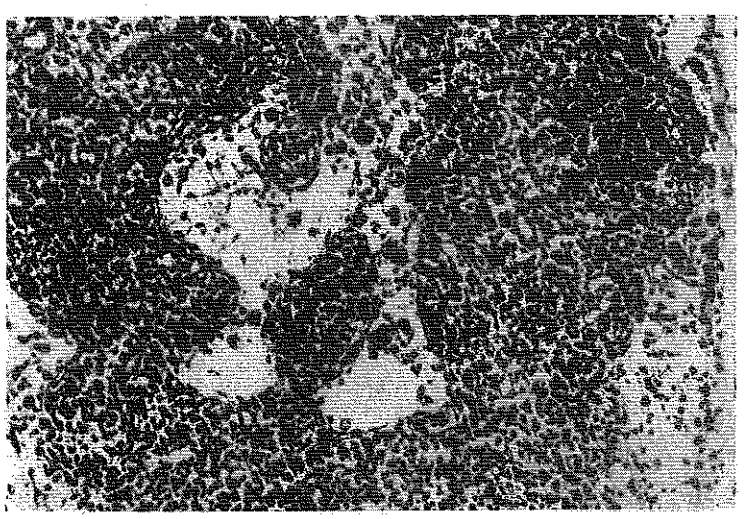
In addition to the history, the clinician may be led to the diagnosis by laboratory abnormalities. Uric acid is always very low. White blood cell (WBC) counts are usually within the normal range. The percentage of lymphocytes is usually very low (less than 10%). However, some patients can have totally normal lymphocyte counts. For example, the older brother in the Madrid family (#12) had a WBC of 10.2×10^9/l with 3% lymphocytes[30]. The younger brother (#14) had 10.9×10^9/l WBC with 32% lymphocytes[30].

In PNP deficiency, parents are often related (8/20 families in Table 1). In no reports to date, have any affected relatives been found other than siblings. This is consistent with an autosomal recessive mode of inheritance.

It is important to realize that there is a spectrum of disease in PNP deficiency. There can be heterogeneity even in the same family. For example, patient #6 from Toronto became sick in the first year of life. His brother (#7) was well until 6 years of life. This heterogeneity in siblings who carry the same genetic defect shows the importance of the genetic background and environmental influences on any given patient.

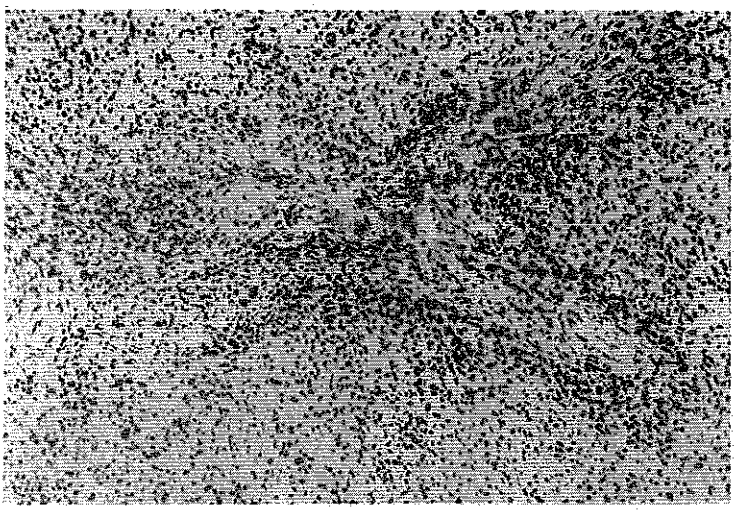


1 (A)



1 (B)

One paper has appeared describing a kindred with immune deficiency in which there was a combined ADA and PNP deficiency[50]. The family is different from the cases reported in Table 1. All family members have normal PNP levels in their white blood cells. The two immunodeficient siblings have erythrocyte PNP levels that are 50% and 20% of normal. The significance of the PNP abnormalities is unclear. PNP levels in this range are not thought to cause immune deficiency. (Certainly, parents of PNP-deficient children have reduced levels of PNP and are immunologically normal.) Understanding this family will have to await further



1 (C)

Figure 1 Histopathology. (A) The thymus is severely reduced in size. Considerable fatty tissue is present. The stromal portion consists of epithelial cells with numerous Hassal's corpuscles varying in size and maturity (patient #1[3]). (B) Mesenteric lymph node showing gross lymphocytic depletion (patient #31[46]). (C) Spleen showing lymphocytic depletion in the perivascular sheath (patient #31[39]). The photomicrograph in (A) was reproduced with permission from *Clinical Immunology and Immunopathology*, vol. 10, A.J. Ammann *et al.*, Immunotherapy and immunopathologic studies in a patient with nucleoside phosphorylase deficiency, ©1978 by Academic Press, Inc.[3] The photomicrographs in (B) and (C) were reproduced with permission from the *Archives of Disease in Childhood*, vol. 50, J. Graham-Pole *et al.*, Familial dysequilibrium-diplegia with T-lymphocyte deficiency, ©1975 by Archives of Disease in Childhood[46].

characterization of the genetic defects in the family members. Since the PNP levels in this family are only moderately depressed, it is not included in Table 1.

In summary, PNP deficiency is a disorder with a spectrum of clinical findings the most important of which are recurrent infections, neurological impairment, and autoimmune hemolytic anemia. Recurrent infection is consistent with the T-cell deficiency. Often the infectious problems increase with age as T-cell function declines (see below). The clinical findings found more frequently in patients with PNP deficiency than in other patients with SCID are hemolytic anemia and neurologic problems.

IMMUNOLOGY

T-Cell Function

PNP deficiency is classified as a disease of T-cell dysfunction. T-cell numbers are profoundly low. In most patients the T-cell percentage is less than 20%, often as low as 1–3% (usually with an absolute lymphocyte count of less than 500/μl). T-cell responses to mitogens such as a phytohemagglutinin (PHA), concanavalin A (ConA), and poke weed mitogen (PWM) are very poor. Most patients have very

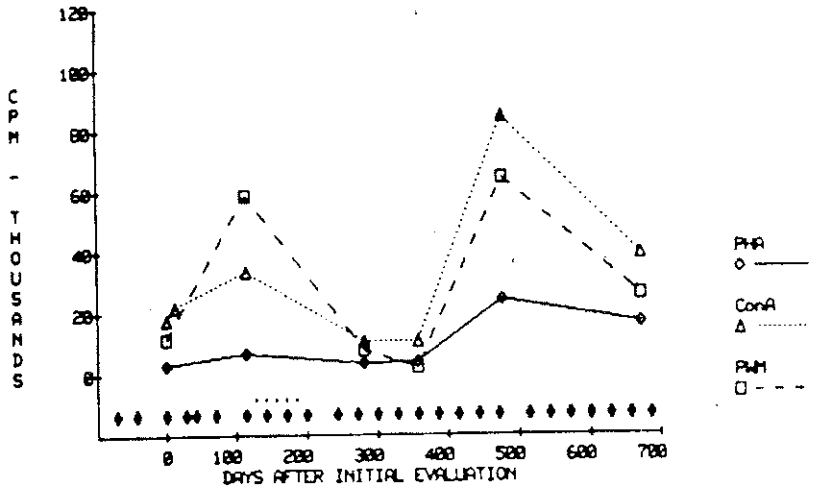


Figure 2 Variation of T-cell function in PNP deficiency. Lymphocyte responses to mitogens in PNP-deficient patient #22. #, intramuscular immunoglobulin; . . ., TP5 injections. Reproduced with permission from the *Journal of Clinical Immunology*, vol. 7, M.L. Markert *et al.*, Adenosine deaminase and purine nucleoside phosphorylase deficiencies: Evaluation of therapeutic interventions in eight patients, ©1987 by Plenum Publishing Corporation[37].

low responses in mixed lymphocyte cultures (MLC). Candida skin tests are unreactive.

Although PNP-deficiency is predominantly characterized by T-cell deficiency, there are two exceptions to this generalization. First, T-cell function can be normal at birth and decrease with age. A good example is patient #5. At birth, PHA and MLC responses were normal. By 3 months, this function had decreased somewhat and by 15 months only 5% of normal PHA responsiveness was observed[10]. The same pattern was found in the Paris patient (#2). This patient had no difficulty with a BCG immunization given early in life. He had a positive PPD at 5 months. This patient's T-cell response was absent at 15 months[5,6]. The T-cell function of patients #4[9], #11[28], #24[40], and #27[42] was similarly shown to decrease with time. Thus, one cannot rule out PNP deficiency when early T-cell function is normal. The second exception is the finding that in some PNP-deficient patients, T-cell function fluctuates through time without any therapeutic interventions. The best example is Durham patient #22 who showed a dramatic waxing and waning of T-cell function without any predictable pattern[37] (Figure 2). Patient #23 has exhibited a similar pattern of T-cell acitivity[37].

B-Cell Function

Early reports of PNP-deficient patients stressed that B-cell function seemed to be normal or increased. B-cell percentages were normal in most patients. Most patients had normal levels of immunoglobulins and had specific antibodies against the immunogens which the child had received (e.g. tetanus, diphtheria).

Isohemagglutinins were normal. Four patients had monoclonal gammopathies (#3[9], #5[10], #9[23] #13[31]). Many had slightly elevated immunoglobulins (#12[30], #13[31], #24[40], #29[44]). Autoantibodies including those causing autoimmune hemolytic anemia and ITP were found in 10 patients (#5, #7, #8, #9, #15, #17, #19, #21, #23, #24) (see references in discussion of autoimmune disease above).

In contrast to the data showing good B-cell function are other data showing poor B-cell function. Patient #2 had specific antibodies early in life. However, at the time of his last infection, his ability to make antibodies had decreased[6]. Patient #4 had low levels of specific antibodies[9]. Patient #8 had decreasing antibody levels with time[21]. Patient #10 had weak isohemagglutinins suggesting poor B-cell function[27]. Patient #17 had no specific antibodies and was IgA and IgG deficient[33]. Patient #21 had low immunoglobulin levels and poor response to øX174[37]. Patient #22 was initially diagnosed as B-cell deficient because of low immunoglobulins and absent titers to specific antigens[37]. In patient #24, although specific antibodies were found early in life, at age three, the patient was unable to mount an antibody response against a new antigen[40]. Patient #31 had decreasing immunoglobulin levels with time[46]. Thus, 9 of 33 patients showed evidence of B-cell dysfunction (and the B-cell function of 7 of the 33 patients has not been evaluated or is not published). In some of the cases, the etiology of B-cell dysfunction might have been poor T-cell help. It is difficult to make this argument for all the patients, however, since patient #22 presented with abnormal B-cell function but normal T-cell function[37].

It is unclear why some patients appear to have normal B-cell function, why others have abnormal B-cell function, and why the T-cell function of some patients fluctuates with time. Molecular biology may give us some clues. However, the unique genetic background of each patient probably affects the immune phenotype.

Because of the heterogeneity in clinical findings and immune function, some patients with PNP deficiency have been classified as having Nezelof syndrome[51]. Nezelof's initial patient suffered from recurrent infections from the age of 4 months. He had absent T-cell function and normal B-cell function. On autopsy, lymphoid tissues were atrophic. The thymus was tiny with absent Hassel's corpuscles[45]. This characterization is consistent with that of many PNP-deficient patients. However, many PNP-deficient patients do not fit this description because of defects in B-cell function or because the T-cell dysfunction appears later in life. The more general term "severe combined immunodeficiency" will be used in this review because both T and B-cell function can be affected in PNP deficiency.

The level of PNP needed in man to be phenotypically (and immunologically) normal is not known. Both of the Toronto brothers (#6, #7) had 0.5% of normal activity[19]. Since most patients have undetectable levels of PNP activity, this level of 0.5% was thought to be significant. The patients were thought to have mild disease. Both brothers, however, died in the second decade of life from complications of PNP deficiency[20]. The Japanese patient (#29) had approximately 4.8% of normal PNP activity in erythrocytes and was severely affected[44].

Two theories have been put forth regarding the role of PNP in immune development. The first hypothesis concerns the role of PNP in N-region additions[52]. N-regions are nucleotides inserted by the enzyme terminal deoxynucleotidyl transferase (TdT) between the V, D, and J gene segments of immunoglobulins and T-cell receptors. The insertion of N nucleotides must relate

in some way to the relative levels of nucleotides in the cells. The enzymes ADA and PNP play a critical role in regulation of the levels of these nucleotides. Abnormalities of expression of PNP could theoretically affect N regions in B or T cells. The Km of TdT for purine nucleotides is 5×10^{-5} to 2×10^{-4} M depending on the cation present[53]. Normal intracellular concentrations of deoxynucleotides are 10^{-6} to 10^{-5} M[54]. Although there are no reports of dGTP levels in peripheral lymphocytes from patients with PNP deficiency, it is thought that dGTP is elevated (see below). Possibly N regions from patients with PNP deficiency will be found to contain an increased percentage of guanine residues.

The second hypothesis deals with the role of PNP in negative or positive selection in the thymus[55]. To date the mechanism by which most thymocytes are killed while in the thymus is not known. Nor is the mechanism known by which certain T cells are selected to expand and exit from the thymus. Perhaps variation in the levels of PNP during thymic development (see below) are involved in these processes.

There are no data to support either the "N region" or "thymic selection" hypotheses at this time. To test these hypotheses experimentally, animal studies may be helpful. A PNP-deficient mouse has recently been created by mutagenesis[56]. This mouse has not yet been characterized immunologically. One cannot predict whether or not a PNP-deficient mouse will be immunodeficient because the level of PNP in murine lymphocytes is much less than that in human lymphocytes[57].

PROTEIN STRUCTURE

PNP in human erythrocytes is a trimer of molecular weight 84,000 to 94,000 daltons[58–60]. The subunit molecular weight is approximately 30,000 daltons[59]. Native PNP in isoelectric focusing reveals a smear of proteins in the range of pH 5.0–6.1[60]. When PNP is subjected to isoelectric focusing in urea (denaturing isoelectric focusing), four subunits with different isoelectric points (pIs) are detected (pI of 6.63, 6.41, 6.29, and 6.2)[60]. The latter three subunits probably have been modified by deamidation, phosphorylation, or acetylation to become more negatively charged[60]. The differently modified subunits may combine to form trimers with many different pIs, this explaining the smear seen on native isoelectric focusing. There are no inter-molecular disulfide bonds in PNP[61].

X-ray crystallography has been used to study human PNP[62,63]. At the 3.2 Å level of resolution[63], the human trimer is symmetric, the three subunits are identical. Each subunit is roughly spherical with a diameter of 40 Å. There is a high solvent volume in the crystals. Each subunit contains both a mixed β-sheet composed of eight strands and a mixed β-sheet composed of 5 strands. The sheets are flanked by seven α-helices. The substrate binding is located near the subunit–subunit boundary and comprises seven segments from one subunit and one segment from the adjacent subunit.

ENZYMATIC ACTIVITY

The enzyme PNP catalyzes the phosphorolysis of inosine and deoxyinosine to hypoxanthine and phosphorolysis of guanosine and deoxyguanosine to guanine

(for review, see Ref. 64). The units of activity of PNP are usually expressed as nmoles or μmoles of substrate converted to product per hour (or minute) per mg protein (or per 10^6 cells) at 37°C. The units of PNP activity vary in different publications.

In the human PNP trimer, each subunit contains a binding site for substrate[65]. The Km for inosine ranges from 2.2×10^{-5} to 10×10^{-5} M[66-68]. Similar values have been reported for guanosine in studies of PNP from several species[66,69,70]. At high inosine concentrations (0.5 mM and higher) there is substrate activation of PNP[66], however, this has no clinical significance as plasma inosine levels have not been found to be this high.

TISSUE DISTRIBUTION OF PNP

PNP is found in many tissues of the body. Although PNP has been considered a housekeeping gene, the level of PNP activity varies in different tissues. For example, PNP activity in kidney is 100 nmol/min/mg protein; in small intestine, 64; in liver, 36; in lung, 38; in heart, 32; in brain, 10; and in peripheral granulocytes, 121[71].

Lymphoid tissues have a wide range of PNP activity. For example, PNP activity in the thymus has been measured at 23 nmol/min/mg protein; spleen, 54; and peripheral blood lymphocytes, 115[71–73]. Cortical thymocytes have lower PNP activity than medullary thymocytes[73,74].

Very few studies have been done examining the levels of PNP in lymphocyte subsets. Cowan *et al.*[75] showed that null cells [probably mostly natural killer (NK) cells] had a relatively high PNP activity relative to mature T cells or B cells when expressed per cell. However, since null cells contain more protein, when expressed per mg protein, T, B, and null cells have approximately the same levels[75].

PURINE AND PYRIMIDINES IN PNP DEFICIENCY

To understand the metabolic imbalance in PNP deficiency, it is helpful to review the relevant biochemical pathways. During this discussion, the rationale will be given for many of the therapies which will be mentioned later.

Purine Metabolic and Salvage Pathways

Figure 3 shows guanine, guanosine, and guanylic acid (GMP). Deoxyguanosine and dGMP differ from the ribose forms only by having an "H" instead of of "OH" at the 2' position in the ribose moiety.

Figure 4 shows the relevant purine pathways. In normal lymphoid cells, levels of ATP and GTP are high. Both of these molecules are used as sources of energy. They contribute to the formation of cAMP and cGMP, respectively, which act as second messengers. dGTP and dATP are normally present at low levels. They are mainly used in DNA synthesis.

The salvage pathway is active in lymphoid tissues[77]. Normally, deoxyguanosine, which forms secondary to DNA breakdown, is converted by PNP to guanine, and

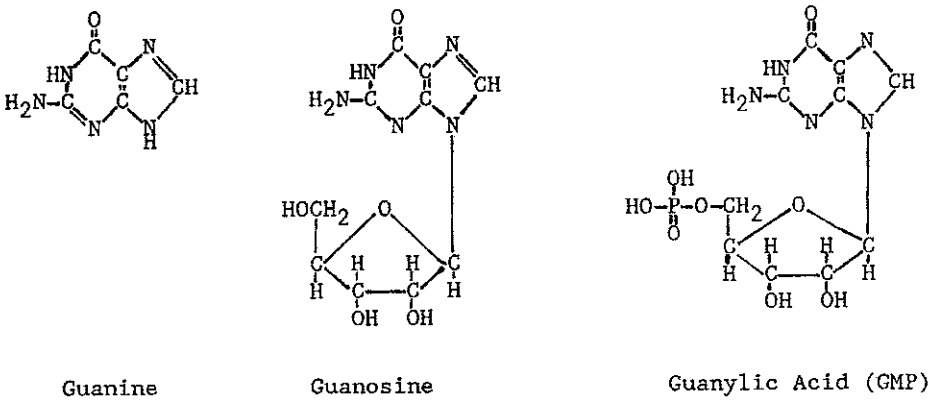


Figure 3 Biochemical structures for guanine, guanosine and guanylic acid (GMP).

then is salvaged by the enzyme HGPRT to GMP. Likewise, any guanosine which forms from breakdown of GMP is degraded by PNP to guanine and then is salvaged to GMP. Although deoxyguanosine, guanosine, and guanine can diffuse across cell membranes, these are not found in the plasma in appreciable levels because of the active salvage pathway.

In PNP deficiency, the salvage pathway is not active because there is no substrate. To maintain purine levels, *de novo* purine synthesis must take place. As a result, total levels of excreted purines increase dramatically[78].

Biochemical Abnormalities

Table 2 shows examples of the biochemical abnormalities in patients with PNP deficiency. There is variation among patients in the plasma and erythrocyte levels of nucleotides. ATP is low in some patients[76]. GTP also can be lower than shown in Table 2[76].

Low uric acid levels

In PNP deficiency, neither guanine nor hypoxanthine (precursors of uric acid) can be formed from their corresponding nucleosides. Thus, in PNP deficient patients, uric acid is very low in the plasma and urine. The highest values reported were those of the Toronto brothers (with plasma urate of 154 μmol/l)[79].

Elevated dGTP—inhibition of ribonucleotide reductase (EC 1.17.4.1)

In patients with PNP deficiency, levels of dGTP are elevated. In normal cells, deoxyguanosine (dGR) can undergo phosphorolysis to guanine (by PNP) or dGR can be converted to dGMP by deoxycytidine kinase[80]. When PNP enzyme is present, dGR predominantly undergoes phosphorolysis because the Km for dGR of PNP is approximately 50 μM whereas the Km of deoxycytidine kinase for dGR is 180 μM[81]. In PNP deficiency, dGR is phosphorylated to dGMP. The activity of the enzyme deoxycytidine kinase is restricted to thymus and peripheral blood lymphocytes[71,82]. Thus, the tissue which accumulates dGTP is lymphoid tissue.

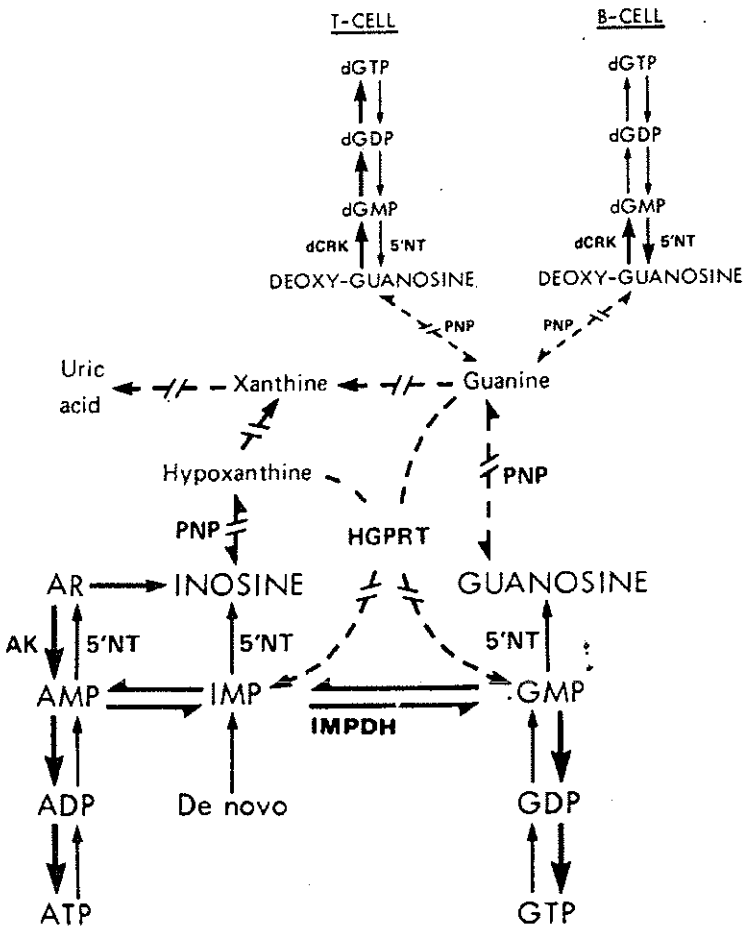


Figure 4 Purine metabolic pathways, including hypothetical mechanisms of toxicity in PNP deficiency, based on dGTP accumulation. The solid line indicates normal pathways of purine metabolism. The dashed lines are pathways inhibited or inoperative in PNP deficiency. In this defect inosine, guanosine, deoxyinosine and deoxyguanosine replace uric acid as the end product of purine metabolism. Abbreviations: dCRK, deoxycytidine kinase; AK, adenosine kinase; 5'NT, 5' nucleotidase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; AR, adenosine; IMPDH, IMP dehydrogenase. Reproduced with permission from *Biochemical Pharmacology*, vol. 31, H.A. Simmonds *et al.*, GTP depletion and other eruthrocyte abnormalities in inherited PNP deficiency, ©1982 by Pergamon Press plc[76].

The phosphorylated nucleotides cannot diffuse out of the cell. Little dGMP is converted by cytoplasmic nucleotidase to deoxyguanosine which could diffuse out of the cell[54]. The dGTP, thus, builds up and inhibits ribonucleotide reductase[80,83].

The enzyme ribonucleotide reductase, which is inhibited by dGTP, is needed for synthesis of deoxynucleotides[84,85]. The activities of ribonucleotide reductase are shown in Figure 5. This enzyme is subject to feedback inhibition and activation as

Table 2 Biochemical abnormalties in patients with PNP deficiency[a]

	Patient #11	*Patient #19*	*Patient #20*	*Patient #23*	*Control*
Plasma (μmol/l)					
uric acid	7	T[b]	5	T	(130–230)
inosine	46	41	49	62	—
guanosine	9	11	13	23	—
deoxyinosine	2	7	6	19	—
deoxyguanosine	2	6	5	14	—
Urine (mmol/mmol)					
uric acid	T	T	T	T	(0.2–1.0)
inosine	1.68	1.9	1.44	1.1	—
guanosine	0.57	0.87	0.74	0.36	—
deoxyinosine	0.32	0.38	0.41	0.36	—
deoxyguanosine	0.29	0.41	0.33	0.29	—
Red cell nucleotides (μmol/l packed cells)					
ATP	1044	1012	1569	1784	1549±72
ADP	130	132	149	134	144±47
AMP	10	39	12	13	10±8
GTP	5	6	5	9	68±11
GDP	5	4	4	7	15±3
dGTP	3	5	4	7	—
dGDP	3	4	5	4	—
NAD	276	307	213	319	72±15

[a]Modified with permission from the *Archives of Disease in Childhood*, vol. 62, H.A. Simmonds *et al.*, Central nervous dysfunction and erythrocyte guanosine triphosphate depletion in purine nucleoside phosphorylase deficiency, ©1987 by Archives of Disease in Childhood[26].

[b]Abbreviations: T, trace, —, not normally detectable; NAD, nicotinamide adenine dinucleotide.

indicated in the figure. In PNP deficiency, dGTP levels increase, leading to inhibition of synthesis of dCTP and dTTP but activation of synthesis of dATP. One might predict that the relevant site of ribonucleotide reductase toxicity in PNP deficiency is the thymus because in the thymus there is much cell death. Perhaps 90% of thymocytes normally die. With the cell death, there is breakdown of DNA and deoxyguanosine accumulates. This contributes to an accumulation of dGTP which will be toxic for the T cells. Inhibition of ribonucleotide reductase is not likely to be the sole mechanism of toxicity in PNP deficiency because ribonucleotide reductase is not normally active in resting T cells[86–88]. The lymphopenia of PNP deficiency implies that even resting T and B cells are affected. The precise mechanisms involved for the toxicity to resting cells are unclear. *In vitro* studies on normal resting T and B cells suggest that dGR is not toxic to these cells[89]. However, normal resting T and B cells differ from PNP-deficient T and B cells; thus the role of dGR remains undefined in resting lymphocytes.

Because ribonucleotide reductase inhibition theoretically could lead to a starvation for dCTP and dTTP, deoxycytidine and thymidine have been given as therapy in some patients. Figure 6 shows how these compounds could relieve a deficiency of dCTP and dTTP. This therapy has not been effective (see below). Ribonucleotide reductase inhibition may not significantly affect dCTP and dTTP

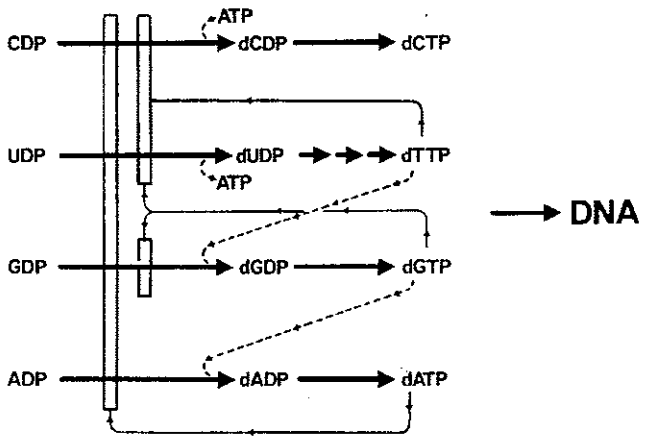


Figure 5 Ribonucleotide reductase. Scheme of the physiological regulation of deoxyribonucleotide synthesis. The broken arrows stand for positive effects, the open bars for negative effects. Reproduced with permission from the *Annual Review of Biochemistry*, vol. 48, L. Thelander and P. Reichard, Reduction of ribonucleotides, ©1979 by Annual Reviews Inc.[84].

levels. Cohen *et al.*[77] showed that the pyrimidine dNTPs are primarily formed by salvage. Here "salvage" refers to uptake of extracellular deoxyribonucleosides which are then phosphorylated in the cell by deoxycytidine kinase.

To prevent accumulation of dGTP, investigators have tried to prevent the initial phosphorylation of deoxyguanosine to dGMP by the enzyme deoxycytidine kinase. The conversion of dGR to dGMP by this enzyme can be inhibited by deoxycytidine (dCR). The Km for dCR is 2 μM, for dGR is 180 μM[81]. Patients have been given dCR orally and intravenously. Most administered dCR is rapidly inactivated because of the presence of the enzyme deoxycytidine deaminase in the liver and in the gut[11]. Because the deaminase can be inhibited by tetrahydrouridine (THU), combination therapy with THU and dCR has been given to patients relatively unsuccessfully (see below).

Much early work on PNP deficiency stressed that B-cell function in patients was normal. The authors proposed a variety of biochemical mechanisms to account for the selective toxicity to T cells and sparing of B cells in PNP deficiency[91]. The mechanisms have included a higher level of dGR phosphorylation by T cells than B cells[92] and conversely, greater dephosphorylation of dGMP by B cells than T cells by a cytoplasmic nucleotidase[93].

In contrast to these early studies, the clinical data presented above show that many PNP-deficient patients have B-cell dysfunction. The question arises as to how the earlier studies showed sparing of B cells in PNP deficiency when B cells are not always spared *in vivo*. The inconsistency may be secondary to the fact that the cells used as the basis for the experimental work have been human Epstein–Barr-virus-transformed B-cell lines, malignant T-cell lines, and a variety of murine cell lines. None of these is identical to human peripheral blood cells. As noted above, murine and human lymphoid cells have quite different PNP levels. Secondly, there is no effective PNP inhibitor. Many studies have assumed

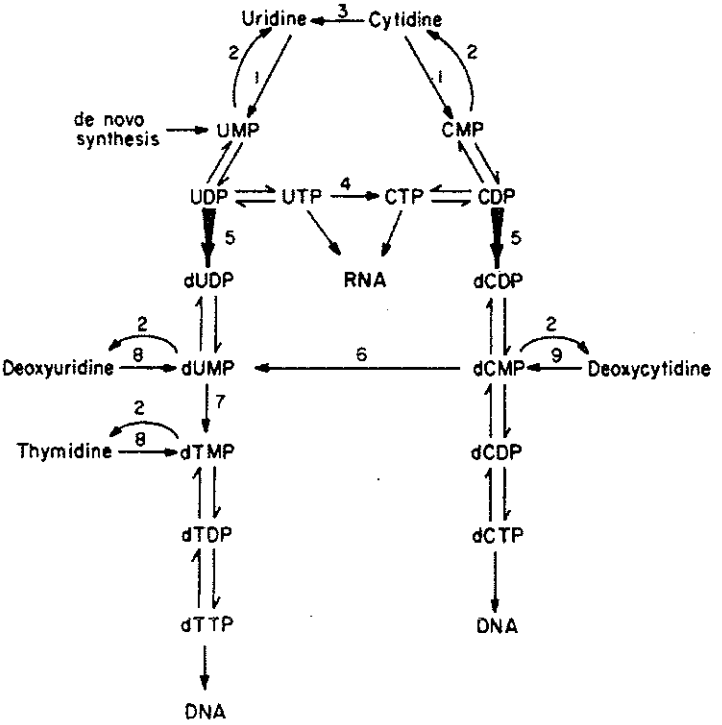


Figure 6 Pathways of cytidine, uridine, deoxyuridine, and thymidine incorporation into nucleic acids. 1, uridine kinase; 2, 5'-nucleotidase; 3, cytidine deaminase; 4, CTP synthetase; 5, ribonucleotide reductase; 6, dCMP deaminase; 7, thymidylate synthetase; 8, thymidine kinase. Reproduced with permission from the *Journal of Clinical Investigation*, vol. 64, J.M. Wilson *et al.*, Purinogenic immunodeficiency diseases: differential effects of deoxyadenosine and deoxyguanosine on DNA synthesis in human T lymphoblasts, ©1979 by the American Society for Clinical Investigation, Inc.[90].

inhibition of intracellular PNP by 8-aminoguanine when, in fact, the PNP enzyme was not inhibited[94]. Many studies have treated normal cells with dGR and observed toxicity. Sidi and Mitchell[95] showed that this toxicity was PNP-dependent and related to accumulation of GTP. Thus, studies in normal cells treated with 8-aminoguanine are suspect.

One can show some differential effects of PNP deficiency on peripheral T and B cells. However, in light of the B-cell immunologic defects documented above, we need better understanding of how the metabolic abnormalities affect both T and B cells. To understand the disease fully, we must also learn why immune function in PNP deficiency can vary with time. As noted above, many patients have normal T- and B-cell function early in life and have poorer function later.

Low GTP levels

In PNP deficiency, the enzyme HGPRT (of the salvage pathway) is not active because no substrate (guanine or hypoxanthine) is present. With no salvage, levels

of GTP fall[86,96,97]. Contributing to the fall of GTP is the fact that GMP is converted to IMP and then inosine which is not toxic. Simmonds *et al.*[26] have proposed that low GTP levels correlate with the severity of neurologic deficits in patients. Some investigators have given guanine and hypoxanthine to patients because of the deficiency of these compounds and the thought that treatment with guanine might help elevate GTP levels.

Another mechanism contributes to the low GTP levels. In PNP deficiency, nicotinamide adenine dinucleotide (NAD) increases[26,27], probably because 5-phosphoribosyl-pyrophosphate (PRPP) is present at high levels[35,98]. Elevated PRPP increases the activity of the enzyme nicotinamide phosphoribosyltransferase (EC 2.4.2.23), which makes NAD. NAD is a cofactor for IMP dehydrogenase, however, high levels of NAD (over 250 μM) inhibit the enzyme IMP dehydrogenase[99] which converts IMP to GMP. This inhibition decreases formation of GTP by both the *de novo* and salvage pathways[76].

The mechanism by which low GTP levels may be detrimental is unclear. GTP is important in G protein activation required for many pathways of cell signaling. GTP regulates the assembly of microtubules which is also important in cell activation[100]. Levels of cGMP, an important second messenger, may also be affected in PNP deficiency. Naylor *et al.*[101] showed that addition of the thymic hormone, thymosin fraction 5, to murine thymocytes led to an increase in intracellular cGMP. One could speculate that if GTP levels are decreased in PNP deficiency, then cGMP production may be decreased. Alteration of this signaling pathway could possibly affect T- and B-cell activation or differentiation.

The hypothesis that low GTP levels are related to nervous system disease leads to a worrisome corollary: PNP inhibitors may harm the brain. PNP inhibitors are being developed for cancer and AIDS therapy. The potential for neurotoxicity should be investigated.

Increased de novo purine synthesis

There are two pathways used in the synthesis of purines, the salvage pathway and the *de novo* pathway. As mentioned above, the salvage pathway is inactive in PNP deficiency because of lack of substrate. The lack of activity of the salvage pathways leads to elevation of PRPP (which has been found in some patients[35,98]). The elevation of PRPP will increase activity of the *de novo* purine pathway since PRPP is the first compound utilized in the *de novo* pathway). The *de novo* pathway leads to the formation of IMP. IMP enters into the purine pathways as shown in Figure 4. In PNP deficiency, *de novo* synthesis of purines is increased as evidenced by the high level of excretion of purines. (Note that there is not an increase in *de novo* purine synthesis in red blood cells, because red cells do not have the enzymes of the *de novo* pathway[102,103]).

Investigators have tried to turn off the overproduction of purines by giving adenine[14]. Adenine is converted to AMP by the enzyme adenine phosphoribosyltransferase. This reaction should deplete the PRPP needed for purine *de novo* synthesis. This treatment has not been effective in decreasing purine overproduction[14].

In spite of the elevated excretion of purines in patients with PNP deficiency, some investigators have proposed that *de novo* purine synthesis is inhibited by PNP deficiency[104]. The mechanism may be inhibition by high levels of dGDP of PRPP amidotransferase, the first enzyme of *de novo* purine synthesis. Such a

decrease in purine synthesis should result in a decrease in ADP and ATP levels. A low ATP level was found in patient #11[76]. This mechanism of toxicity is not felt to be significant in PNP deficiency.

Inhibited synthesis of pyrimidines

The pathways by which cytidine, uridine, deoxyuridine, and thymidine are incorporated into DNA and RNA are shown in Figure 6. *De novo* synthesis requires that PRPP eventually form UMP. In contrast to the hypotheses above (proposing elevation of PRPP and increased *de novo* purine synthesis), some investigators have hypothesized that PRPP is low in PNP deficiency causing an inhibition in *de novo* pyrimidine synthesis. This hypothesis was proposed after finding 2 patients (#1, #5) with orotic aciduria[105]. An early step in *de novo* pyrimidine synthesis (not shown in Figure 6) requires PRPP to convert orotic acid to orotidine 5' phosphate. Orotic aciduria implies a defect in this step involving the enzyme orotidine 5' phosphate pyrophosphorylase. One possible problem could be lack of PRPP. Although it is known that PRPP levels are normal or high in PNP deficiency (normal in the Toronto patients #6, #7[106] elevated in patients #5[98], #19 and #20[35]) perhaps in some cellular compartment, PRPP is low. Treatment with uridine to bypass this problem (inhibition of *de novo* pyrimidine synthesis) has been tried[105]. This therapy has been ineffective, and indeed most patients have not had orotic aciduria[106].

There is a second proposed mechanism (which has not received much support) to account for inhibition of pyrimidine synthesis. The high inosine levels in PNP deficiency might inhibit ADA (Ki=116 μM)[107]. High adenosine levels in cells (as occurs in ADA deficiency) has been shown to lead to pyrimidine starvation[108]. Inosine levels in PNP deficiency do not approach 116 μM, however, and therapy with uridine to relieve the pyrimidine starvation, has been ineffective in reconstituting the immune deficits[14].

Interaction of methionine with purines

Figure 7 shows the interaction of methionine biochemistry with purines. Four mechanisms have been proposed involving this pathway and PNP deficiency. In the first case, S-adenosylhomocysteine hydrolase (SAHH) converts S-adenosylhomocysteine (SAH) to adenosine and homocysteine. High levels of inosine can inhibit this enzyme. Inhibition of SAHH has been reported in PNP deficiency[110,111]. However, the Ki, 5.85 mM[111], is so high, that this inhibition is probably only significant in long lived erythrocytes. Inhibition of SAHH results in elevation of SAH levels which inhibit methylation reactions in which the methyl group is donated by S-adenosylmethionine (SAM)[112,113].

In the second mechanism, inhibition of SAHH leads to depletion of homocysteine which in turn inhibits methionine production[109]. This could interfere with protein synthesis in the cell[114].

Thirdly, the reaction catalyzed by methionine synthetase is coupled to the production of tetrahydrofolate from the storage form 5-methyltetrahydrofolate (see Figure 7). Thus, the active form of folate may be depressed in PNP deficiency. Many patients have been reported to have megaloblastic bone marrows (patients #1[1], #5[8], #9[22]). This finding has led to speculation that folate metabolism may be affected in PNP deficiency.

The fourth additional abnormality which could be caused by SAHH inhibition is

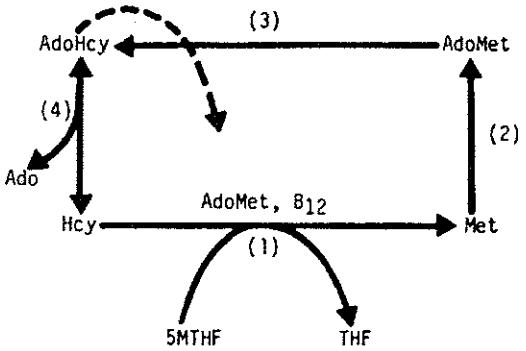


Figure 7 Methionine synthesis and conversion to homocysteine. Reactions: (1) methionine synthetase; (2) methionine adenosyltransferase; (3) methyltransferase reactions; and (4) S-adenosylhomocysteine hydrolase. Hcy, homocysteine; AdoHcy, S-adenosylhomocysteine; 5MTHF, 5-methyltetrahydrofolate; THF, tetrahydrofolate; and B_{12}, cobalamin. The broken arrow indicates that S-adenosylmethionine inhibits its own formation, thereby inhibiting most intracellular methylation reactions. Reproduced with permission from the *Journal of Clinical Investigation*, vol. 74, G.R. Boss and R.B. Pilz, Decreased methionine synthesis in purine nucleoside-treated T and B lymphoblasts and reversal by homocysteine, ©1984 by The American Society for Clinical Investigation, Inc.[109].

ATP depletion. Red blood cells need SAHH to generate adenosine for ATP pools. This pathway is not available in PNP deficiency[76]. Patient #11 was shown to have low ATP levels. However, other patients[26] have had normal ATP levels.

Miscellaneous hypotheses

Other hypotheses have been put forth to account for toxicities in PNP deficiency. For example, Mann and Fox[115] proposed a role for increased dATP. Increased dATP has not been found in patient samples, however. A recent study by Duan *et al.*[116] suggests that RNA synthesis may be inhibited in PNP deficiency. IL2 receptors may be involved as well. Noma *et al.*[117] showed that treatment of normal IL-2-depleted T-cell lines with dGR upregulated expression of IL2 receptors. The significance of these hypotheses is not known at this time.

MOLECULAR BIOLOGY

The human PNP mRNA is 1700 nucleotides long[118]. It was cloned by Williams *et al.*[119]. The cDNA sequence predicts a protein of 289 amino acids corresponding to a molecular weight of 32,153 daltons. The structural gene is composed of 6 exons spanning 9 kb of DNA[120]. The gene has been expressed *in vitro* in eucaryotic cells and produces active PNP[120]. It will be interesting to determine the regulation of PNP expression during T- and B-cell development and differentiation. Upon mitogen stimulation of peripheral blood lymphocytes, levels of intracellular ribonucleotides increase dramatically[121]. Phorbol ester stimulation of T- and B-cell lines result in a 200% increase in PNP mRNA levels by 12 hr[122]. This suggests a role for protein kinase C in regulation of PNP gene expression[122].

CHROMOSOMAL LOCATION AND MODE OF INHERITANCE

The PNP gene has been assigned to human chromosome 14q13 by somatic cell hybridization techniques[123] and by gene dosage studies[124–126]. In the mouse, PNP maps to chromsome 14[127].

Because the structural gene for PNP is located on an autosome, the pattern of inheritance for PNP deficiency is autosomal recessive. Every patient carries mutations in both PNP alleles—one mutation inherited from the father, one from the mother. The two mutations present in a single patient will most likely be different if the parents are unrelated. Apparently, carrying one mutant allele is not detrimental. Heterozygous parents have all been free of clinical disease, despite having half-normal levels of PNP enzyme. Since the PNP enzyme functions as a trimer, it is possible that a mutation will be discovered in which a mutant PNP monomer will combine with normal monomers to produce a totally inactive enzyme. This has not yet been described for PNP although this mechanism is known to be responsible for certain collagen disorders.

PATIENTS ENZYMES: PROTEIN AND MOLECULAR ANALYSES

Mutant PNP enzymes have been studied in patients from 5 families. To date, the findings suggest that several different mutations cause PNP deficiency. From studies of other human diseases, one would expect that the most common type of mutation will be point mutations leading to amino acid substitutions. The mutation in patient #1 has been elucidated at the molecular level. The protein and DNA data for that patient and available studies on four other patients are summarized below.

Patient #1

Gudas *et al.*[7] studied the PNP protein of patient #1. The patient's cells contained no detectable PNP protein nor PNP activity. The crude hemolysate from the parents had an activity of 0.007 or 0.005 units/mg protein compared to 0.025 for normals[7]. This was consistent with the parents' having a single functional PNP allele. The Km of parental PNP for inosine was normal at 34 μM[7]. Native isoelectric focusing of the parents' erythrocyte lysates showed more basic PNP protein than normal (pI 5.0–6.4, instead of the normal 5.0–6.1[7]). The PNP protein purified from the parents included two new subunits on denaturing two-dimensional gels (in addition to the normal PNP subunits). The molecular weight of the new subunits was 500 daltons greater than normal.

Williams *et al.*[120] studied the molecular defect in this patient. They cloned the PNP gene from liver DNA and sequenced the exons. A G to A point mutation in the third exon changed amino acid 89 from glutamic acid to lysine. Thus, the mutant protein was more alkaline, consistent with the isoelectric focusing data. It was thought this this substitution was also responsible for the abnormal migration on SDS-PAGE.

Both parents (who were second cousins) were shown to be heterozygous for this mutation by hybridization of oligomers to parental DNA[120]. As would be expected in a family in which the parents are related, the patient is homozygous for the mutation.

To prove that the mutation identified in this patient was indeed responsible for the lack of PNP activity, the mutant protein was synthesized in murine 3T3 cells. The resulting 3T3 cell lysates were found to have immunoreactive protein that migrated in a more alkaline position in isoelectric focusing[120]. The mutant protein had no enzyme activity. Expression of the mutant protein lowered the basal activity of PNP in the mouse cell, presumably by combining with normal mouse subunits and inactivating them. It is interesting that immunoreactive PNP could be detected in the transfected cells, whereas none is detected in the patient. This finding could be secondary to increased expression of the PNP in the transfected cells. The level of RNA in the transfected cell was not studied, however, so the reason for the presence of immunoreactive PNP in this experiment is unknown.

Ealick[58,63,128] has proposed that the role of glutamic acid 89 is to stabilize the protonated form of a histidine residue that serves as a reversible proton source in the phosphorolysis reaction. Replacing glutamic acid 89 with a positively charged lysine could prevent the proton transfer and might explain the inactivity of the mutant enzyme[128].

Patients #6, #7

Fox *et al.*[19] and Osborne *et al.*[129] described two brothers who expressed 0.5% of normal PNP activity in hemolysates. The brothers appear to have inherited 2 different mutant alleles from their parents. Both alleles result in inactive proteins. The patients had approximately half-normal levels of crossreactive material (though to be paternal) by Ouchterlomy diffusion and immuno-electrophoresis[130]. Radioimmunoassay and isoelectric focusing were used to determine that the mother's allele results in unstable PNP; the father's allele results in stable PNP. Enzyme analyses revealed that the paternal mutant enzyme had a Michaelis–Menton constant tenfold higher than normal and was heat unstable. By isoelectric focusing the mutant enzyme was shown to have a more positive charge than normal (pI was 5.08–5.26 compared to a normal value of 5.5–5.8)[19,131].

Patients #5, #9, #29

The mutant PNP proteins in three other patients have been studied. Patient #5 has PNP with no enzymatic activity nor immunoreactivity. In this family, parental PNP has a slightly elevated Km and slightly decreased heat stability[13,130]. Patient #9 had 0.07% of normal activity. This patient's PNP protein was not immunoreactive. The Km was three times normal[130,131]. Patient #29 had the highest PNP activity of all the patients (4.8% of normal in erythrocytes and 14% of normal in liver). The Km was normal. This patient had immunoreactive material by Western blotting. Molecular studies showed normal-sized PNP mRNA. It is thought that a point mutation is the most likely problem[44].

DIAGNOSIS

Enzymatic Testing

PNP deficiency is diagnosed by PNP enzymatic testing in erythrocyte lysates[19].

The red cell lysate is incubated with ^{14}C-inosine at 37°C for varying times. Substrate and product (^{14}C-hypoxanthine) are separated by thin layer chromatography. The conversion to product is quantified by counting the radioactivity in both substrate and product. Other methods including using high-performance liquid chromatography (HPLC) have been reported[132]. The abnormal nucleoside concentrations in plasma, urine and red cells can also be determined by HPLC[133]. One family in Table 1 (patients #31 and #32) were diagnosed by enzymatic testing of frozen fibroblasts which had been stored for many years[29,47].

Prenatal Diagnosis

Carapella de Luca *et al.*[134] and Kleijer *et al.*[135] have reported prenatal testing for PNP deficiency. The testing was done in women who had previously had affected offspring. Amniotic cells obtained at 16 weeks were grown in tissue culture. PNP activity was assayed in the amniotic fluid[19,132]. The PNP activity in normal amniotic fluid (n=22) was 4.4–30.2 nmol/h/mg protein. One fetus identified as normal or heterozygous had amniotic fluid with an activity of 4.8 nmol/h/mg. For cultured amniocytes, the control range of PNP activity was 562–3678; the unaffected fetal amniocytes had levels of 2229 and 1014 nmol/h/mg. The affected fetal amniocytes had undetectable activity. The purine profile of the amniotic fluid from a normal fetus showed a uric acid of 200 μmol/l (nl 160–280) and undetectable hypoxanthine, xanthine, guanine, inosine, deoxyinosine, guanosine, and deoxyguanosine. In the affected fetal amniotic fluid, inosine was 1.45 μmol/l, guanosine 0.5 μmol/l, deoxyinosine 0.3 μmol/l, and deoxyguanosine 0.24 μ mol/l[135]. Recently, corionic villus sampling has also been used successfully to diagnose PNP deficiency[136].

TREATMENT

PNP deficiency is associated with significant morbidity. Twenty-nine of the 33 reported patients have died. Infection has been a prominent factor in 16 deaths (3 secondary to varicella), tumor in 5 patients, graft versus host disease (GVHD) in 3 (two after receiving unirradiated blood products), and autoimmune disease in two patients.

These mortality statistics show that most therapies used to date have not been effective. The four patients who are alive include one patient cured by bone marrow transplantation (#27), and 3 that have not been cured and are being treated long-term with IVIG or antibiotics (#21, #22, #24). In view of the significant morbidity and mortality associated with this disease, one must be aggressive in management of these patients. In the section that follows, various therapies will be discussed that have been used in PNP deficiency.

It is important when reviewing the original data, to realize that the treatment studies have not been controlled. Interpretation of the efficacy of therapy is difficult because the baseline of immunologic function can vary even without any intervention (see Figure 2). Thus, it can be difficult to determine whether function improves secondary to the treatment or due to chance alone.

Bone Marrow Transplantation

Five patients have been treated with bone marrow transplantation (#14, #21, #25, #27, and #30). Two are alive (#21, #27), only one with successful immune reconstitution (#27), and that patient only after 2 transplants. Patient #27 received antithymocyte serum and cyclophosphamide prior to his first T-cell-depleted bone marrow transplant. Because this transplant never engrafted, a second T-cell-depleted transplant was done with more aggressive suppression, busulfan, cyclophosphamide, and anti-thymocyte serum. Anti T-12 antibody was used for the T-cell depletion. The patient developed mild GVHD and was treated with steroids. This transplant was successful in reconstituting the patient's immune system. In spite of this therapy, the neurological problems in this child (see Table 1) did not improve after bone marrow engraftment[42].

Patient #21 received a half-matched T-cell-depleted transplant without any peritransplant conditioning. Engraftment never occurred[37]. Because the patient has done well (her major problem is asthma not infection), she has not been given a second transplant with immunosuppression.

Three patients have died secondary to bone marrow transplantation. The details from patient #14 are unavailable. After bone marrow transplant, the patient developed Herpes encephalitis which left him in a vegetative state. His family took him from the hospital to die at home[32]. Patient #25 received an HLA-identical transplant after immunosuppression with cytarabine and cyclophosphamide. The patient developed problems with CMV and GVHD post transplant. (The bone marrow donor was secreting CMV at the time of transplant.) The patient died 10 months post-transplant[41]. Patient #30 was 6 years old and in poor clinical condition at the time of HLA-identical bone marrow transplant. The patient had chronic pulmonary disease secondary to recurrent respiratory infections including adenovirus pneumonia requiring ventilation. The patient was given mild immunosuppression with busalfan and cyclophosphamide. He died of pulmonary hemorrhage while he was thrombocytopenic and neutropenic before engraftment occurred[45].

Red Cell Transfusions

Eleven patients (#1, #2, #5, #8, #9, #12, #13, #14, #26) have been treated with red cell transfusions. Partial and temporary immune reconstitution have been reported in 2 patients (#5, #9). This therapy did not help the neurologic problems of these patients and both died of infections despite this therapy. Of note, patient #5 became hepatitis B positive, probably secondary to this therapy[17].

Regarding enzyme replacement (with red blood cell transfusions), one should be aware of a similar approach to therapy for adenosine deaminase (ADA) deficiency, another form of SCID. In this disease, polyethylene glycol (PEG) modified bovine ADA (PEG-ADA) has been given by injections to ADA-deficient patients[137]. Approximtely 25 times more ADA is given as PEG-ADA than is given in an erythrocyte transfusion program. With PEG-ADA, immune effects are seen only at high doses, much higher than can be given with erythrocyte transfusions. It is likely that much greater doses of PNP than can be given by erythrocyte transfusion are needed to achieve substantial immunological effects. Possibly, PEG-PNP will be developed and tested in the future.

Deoxycytidine (dCR) Plus Tetrahydrouridine (THU)

There are two rationales for giving dCR plus THU: (1) dCT can bypass the ribonucleotide reductase inhibition caused by elevated dGTP (theoretically leading to a deficiency of dCTP), and (2) dCR can compete with dGR for phosphorylation by deoxycytidine kinase, thus decreasing levels of dGTP. Regarding the first mechanism, administration of dCR might bypass the pyrimidine block. However, there is a high activity of deoxycytidine deaminase in the gut mucosa and in the liver[11], thus, all dCR is deaminated soon after it is ingested or taken intravenously. THU is an inhibitor of deoxycitidine deaminase. Thus, the two compounds have been given together. dCR therapy was used by Stoop *et al.*[11] in patient #5 and by Watson *et al.*[138] in patient #11. In patient #5, the dGTP levels decreased in the patient's erythrocytes, from 5 to 1 μmol/l packed cells. Levels of dCR in the plasma were detectable, dCTP could not be measured while the patient was on therapy. While on a two month trial of dCR and THU, patient #5 experienced an increase in white blood cell numbers. *In vitro* T-cell responses increased somewhat. For patient #11[138], treatment with dCR alone or with THU led to an increase in GTP and ATP. NAD levels remained high. The absolute lymphocyte count did not change. T-cell percentages remained low. PHA responses continued a downward trend. Both of these patients died of infection.

Uridine Therapy

This therapy was given based on the rationale that in PNP deficiency there might be pyrimidine starvation. Oral therapy was given to patient #1[139] over 9 months. While on therapy the patient's T-cell percentages increased, PHA responsiveness increased to 10% of normal, mixed lymphocyte responsiveness did not improve. The patient contracted varicella and died while on therapy. Patient #5 was given uridine but only had a small increase in T-cell number[139]. Thus, results from the dCR and uridine trials suggest that pyrimidine starvation does not play a major role in the immunodeficiency seen in PNP deficiency.

Guanine

Patient #11 was treated with oral guanine. The rationale for this therapy was to provide a substrate for HGPRT to make GMP and then GTP. This therapy was followed by transient improvement in T-cell function, but this improvement was brief and could not be shown to be related to the therapy[138].

A caution must be given regarding treatments with purines. Patients with PNP deficiency are at a theoretical risk for renal complications, in particular, kidney stones. As was discussed above, PNP-deficient patient excrete large amounts of inosine, deoxyinosine, guanosine, and deoxyguanosine. The urine is almost saturated with these compounds. Red cells have been found in the urine of patients[15]. Although patients with PNP deficiency have not developed kidney stones nor renal failure, these are potential problems. This must be kept in mind when various therapies such as nucleosides or purine bases are considered for a patient. The clinician should follow the urinalysis and hydrate the patient if red blood cells are seen in the urine[15]. Two patients with HGPRT deficiency illustrate this problem. One patient with HGPRT deficiency developed renal failure

secondary to uric acid nephropathy[140]. A second patient, with Lesch-Nyhan syndrome, was given adenine as therapy to suppress *de novo* purine synthesis. This patient developed renal failure secondary to stone formation[141]. Simmonds feels that the stone formation was secondary to 2,8-dihydroxyadenine[15]. Use of bases to prevent *de novo* purine formation can work *in vitro* but not *in vivo*[15].

Thymic Factors

Thymosin fraction 5 (TP5) has been given to three patients, #1, #21, and #22. This therapy seemed to be quite helpful for patient #1[139]. After 9 months of therapy, T-cell percentages became normal; T-cell function as measured by PHA responsiveness and MLC was 50% of normal. However, the patient developed IgE-mediated reactions to the injections and treatment had to be discontinued. For patients #21 and #22, because of the variation in the baseline of T-cell function, the effect of TP5 could not be properly evaluated. At the time it was given, it was not thought to be effective[37,41].

Transplantation of Fetal Thymus

Fetal thymic transplants were used in patients #2, #3, and #31. None of these transplants was curative. Three transplants were done in patient #5[139]. After the first transplant, PHA responsiveness increased to 15% of normal, but then it returned to baseline. After the second transplant, PHA responsiveness increased to 10% of normal. After a third transplant, the patient was also given transfer factor. The patient died of lymphosarcoma before results from the third transplant could be evaluated. Part of the lack of success with these transplants may be the fact that each thymus came from a fetus of at least 16 weeks gestation. At this stage, mature T cells are present, which could cause GVHD. The mature T-cells of the donor thymus would never cooperate with host antigen-presenting cells. Patient #5 probably rejected these transplants. The thymic transplants might have boosted immune function in part by replacing thymic factors such as TP5.

Killed Vaccines

All patients with immunodeficiency should be given killed vaccines such as the Salk polio vaccine, not live or attenuated vaccines. With regards to the polio vaccine, family members should receive the Salk vaccine to prevent infection of the immunodeficient family member. Although no PNP-deficient patient has been reported with paralytic polio, a significant proportion of cases in the United States occur in immunodeficient patients who have been exposed to the Sabin vaccine[142].

Prophylactic Antibiotics and IVIG

For patients with specific antibody deficiency, IVIG should be given monthly. Antibiotics should be given as necessary. Trimethoprim/sulfamethoxazole is given as prophylaxis against *Pneumocystic carinii* pneumonia. Ketoconazole is given to control thrush. Inhaled antibiotics can be helpful for severe pulmonary disease.

Summary of Therapeutics

After reviewing the cases reported in this article, it is clear that we have not yet found the optimal therapy for PNP deficiency. My recommendation is bone marrow transplantation for all patients as early as possible. Bone marrow transplantation is not guaranteed to be successful, however. Only 2 of the first 5 patients treated with bone marrow transplantation are alive and only one is immunologically normal. The factors which contributed to unsuccessful bone marrow transplantation were preexisting infectious disease or chronic lung disease. Patients treated with alternative therapies (enzyme replacement, metabolic protocols) have invariably died before age 20. Thus, if the diagnosis of PNP deficiency can be made early, before the patient is clinically compromised, bone marrow transplantation may give the patient an opportunity for a normal life.

FUTURE PROSPECTS: GENE THERAPY

Gene therapy may become available as treatment for genetic diseases such as PNP deficiency. PNP is a logical candidate disease. The mortality and morbidity are high; no current therapies are consistently successful; the normal cDNA has been cloned; and the affected tissue (bone marrow) is accessible. Preliminary studies have been done in mice.

McIvor *et al.*[143] made a retroviral vector containing the PNP cDNA. Coculture of murine bone marrow with cells producing the retrovirus led to infection of murine bone marrow cells. In transfer experiments (bone marrow from gene-therapy recipient mouse into an irradiated mouse), the presence of the transferred gene could be detected by Southern analysis. Gene expression, however, was not detected.

Work on gene therapy at present focuses on three areas. First is infection of the immature stem cell. Retroviruses only infect replicating cells[144]. Stem cells normally are relatively quiescent. If retroviruses are used for gene insertion, then interleukins are used to force the stem cell to cycle. This issue is important because only infection of the stem cell will give lasting expression of the transduced gene. If mature T or B-cells are infected, the effect of the therapy will be short lived.

The second area of work in PNP gene therapy is the development of the optimal vector which will result in good expression. Work in ADA has shown that one cannot predict which vector will result in good expression[145]. Many vectors must be made and tested.

The third area of investigation in PNP deficiency centers on whether or not normal gene regulatory elements will need to be included in the vector. It is totally unknown whether PNP regulation affects immune development. Animal experiments may help answer these questions (if the PNP deficient mouse is immunodeficient)[56]. The correlation of murine findings with human disease may be problematic, however, since the distribution of PNP in humans and mice is quite different.

The final obstacles to PNP gene therapy will be the small number of patients. Will investigators invest the time and will granting institutions expend the

necessary funds to address the critical issues listed above before human experimentation begins?

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