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A rat model of purine nucleoside phosphorylase deficiency

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SUMMARY

Purine nucleoside phosphorylase (NP; EC 2.4.2.1) deficiency is associated with selective T-cell dysfunction and normal B-cell immunity. In order to create an *in vivo* model of this immune deficiency, we administered 8-aminoguanosine to rats. This water-soluble nucleoside was rapidly converted by NP to the more potent inhibitor 8-aminoguanine, which has a K_i of 0·19 μM. The accumulation of inosine in plasma showed that administration of 8-aminoguanosine was effectively inhibiting NP activity. The administration of 8-aminoguanosine with deoxyguanosine produced increased levels of dGTP only in thymus cells, and increased levels of GTP in cells from thymus, spleen and lymph node and in red cells. This correlated with assays of deoxyguanosine kinase, which showed significantly higher activity in thymus cells than in cells from spleen and lymph node. The intraperitoneal injection of 8-aminoguanosine alone or with deoxyguanosine for 8 consecutive days caused significant decreases in the number of thymus cells ($P < 0{\cdot}001$) and in lymph node and spleen lymphocytes ($P < 0{\cdot}01$). These data showed that the administration of 8-aminoguanosine to rats provided an animal model of NP deficiency that will allow studies of the specific regulation of T-cell function.

INTRODUCTION

Purine nucleoside phosphorylase (NP; EC 2.4.2.1) deficiency is associated with severely defective T-cell function and normal B-cell immunity (Giblett *et al.*, 1975). NP catalyses the conversion of purine nucleosides to their respective bases (Parks & Agarwal, 1972). In patients with NP deficiency, its substrates accumulate in plasma, and dGTP has been detected in a patient's erythrocytes (Cohen *et al.*, 1978). The biochemical mechanisms underlying this immune defect have been extensively investigated, mainly using human and mouse cells in culture (Kredich & Heishfield, 1983; Giblett, Chen & Osborne, 1981). It is generally accepted that in the absence of NP activity deoxyguanosine accumulates and is preferentially phosphorylated in T cells to produce dGTP, which is cytotoxic (Kredich & Hershfield, 1983; Giblett *et al.*, 1981; Osborne, 1981). A mechanism for the selectivity of this immune defect has implicated differences in deoxynucleoside kinase activity towards deoxyguanosine in T and B cells (Osborne & Scott, 1983). In order to provide an animal model of NP deficiency, we have administered 8-aminoguanosine alone and in combination with deoxyguanosine to rats. We administered the water-soluble NP inhibitor 8-aminoguanosine knowing that it was rapidly converted by NP to the more potent product inhibitor 8-aminoguanine (Stoeckler *et al.*, 1982; Osborne, Slichter & Deeg, 1985). Previous studies have used 8-aminoguanosine to inhibit NP in lymphoblastoid cells (Stoeckler *et al.*, 1982; Kazmers *et al.*, 1981; Osborne & Scott, 1983). The development of a rat model of NP deficiency may enable study of the selective control of T-cell lymphopoiesis, and be of benefit in tissue transplantation and cancer therapy.

Abbreviations: ADA, adenosine deaminase; HPLC, high-pressure liquid chromatography; HPRT, hypoxanthine phosphoribosyltransferase; i.p. intraperitoneal; NP, purine nucleoside phosphorylase.

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MATERIALS AND METHODS

Materials

8-Aminoguanosine was synthesized by the method of Holmes & Robins (1965) and was >99% pure by high-pressure liquid chromatography (HPLC). 8-Aminoguanine was produced by the hydrolysis of 8-aminoguanosine. Deoxy[8-^{3}H]guanosine (2·1 Ci/mM) was from Amersham International, Arlington Heights, IL. Purine nucleoside phosphorylase was purified from rat red cells by affinity chromatography (Osborne, 1980). All other reagents were from Sigma, St Louis, MO.

Animals, tissues and cell suspensions

Male and female Lewis rats, 4–8 weeks old, were used throughout the experiments. Thymus (free of adherent lymph nodes), spleen, and cervical lymph nodes were removed from etherized rats. Single cell suspensions from each tissue were obtained by teasing and washing in a balanced salt solution (Barton, 1982). Counts for cell viability were performed by Trypan blue dye exclusion. Blood specimens were collected in heparinized

syringes, and plasma and red cells were separated by centrifugation. Red cells were lysed 1:1 with water and the haemoglobin determined. Protein-free extracts of plasma and red cells were prepared either within 20 min of drawing or after rapid freezing and storage at −70° (Garrett & Santi, 1979). Red cell nucleotides were standardized using the mean haemolysate haemoglobin. Lymphoid tissues for nucleotide analysis were frozen in liquid nitrogen within 10 min of kill and were stored at −70° until extraction.

Enzyme assays

Purine nucleoside phosphorylase was measured spectrophotometrically at 293 nm with inosine as substrate (Osborne, 1980) and at 258 nm with guanosine and deoxyguanosine as substrates (Stoeckler *et al.*, 1982) using 0·1 M Na phosphate buffer, pH 7·5, at 30°. Deoxyguanosine kinase was determined by a DE disc assay as previously described except for the addition of 25 μM 8-aminoguanine to the assay to prevent nucleotide formation via NP (Osborne & Scott, 1983).

Plasma nucleoside and base analysis

Plasma extracts were analysed with a Waters (Waters Associates Inc., Milford, MA) high-pressure liquid chromatograph (Osborne, Hammond & Dale, 1983).

Nucleotide analysis

Red cell nucleotides were determined by high-pressure liquid chromatography using a Whatman SAX column and gradient elution (Osborne *et al.*, 1983). Guanine nucleotides in lymphoid tissue were measured using an isocratic procedure with a Whatman SAX column as previously described (Osborne & Scott, 1983).

RESULTS

Deoxyguanosine kinase assays

The deoxyguanosine kinase activities of cell extracts from thymus, lymph node and spleen were 84·7, 33·9, and 27·2 nmol/hr/mg protein, respectively. This decreasing order of activity correlates with the decreasing proportion of T cells in these tissues as the lymph node contains 70–80% T cells and the spleen 60–65% T cells (Barton, 1982). These results compare with those from human lymphoblastoid cells where T cells have a four-fold greater deoxyguanosine kinase activity than B cells (Osborne & Scott, 1983).

Kinetic constants

The inhibition constant of 8-aminoguanine for the purified red cell enzyme was determined using inosine concentrations over the range 12–200 μM, with and without the addition of 0·5 μM inhibitor. The double-reciprocal plot was linear over this substrate range, and the intercept on the ordinate indicates competitive inhibition (Fig. 1). A K_i value of 0·19 ± 0·04 μM and a K_m of 88·5 ± 15·8 μM were calculated using the Wilkinson (1961) procedure. The K_m value for inosine is in agreement with a previous report (Agarwal *et al.*, 1975). At higher substrate levels the plots curved downwards indicating apparent substrate activation. Similar results have been reported with human NP (Stoeckler *et al.*, 1982). K_m values obtained for guanosine and deoxyguanosine were 34·2 ± 6·6 μM and 103·5 ± 27·6 μM, respectively, and were calculated from assays monitoring guanine formation. Similar values have been reported for bovine NP (Agarwal *et al.*, 1975).

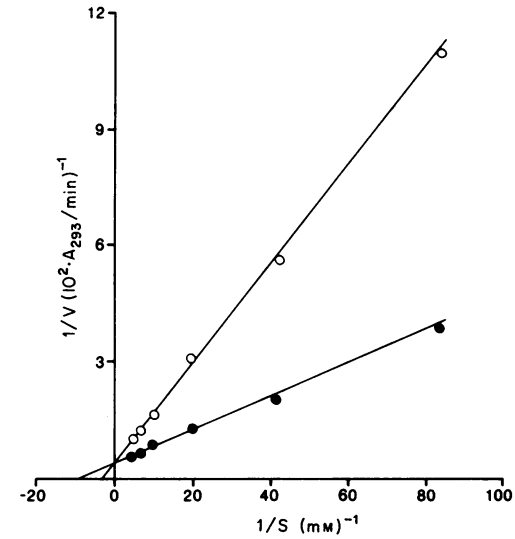

Figure 1. Inhibition of rat erythrocyte NP by 8-aminoguanine. Double-reciprocal plots of initial velocity and substrate: (●) inosine; (○) inosine plus 0·5 μM 8-aminoguanine.

When 50 μM 8-aminoguanine was incubated for 24 hr at 23° with rat red cell lysate, no significant degradation was observed by HPLC analysis. Previous data have shown no reaction of 8-aminoguanine with human HPRT or rat liver guanase (Stoeckler *et al.*, 1982).

Plasma and RBC analysis

In order to follow the metabolism of 8-aminoguanosine and deoxyguanosine, plasma and RBC samples were analysed following i.p. injections into rats at doses of each nucleoside of 24 mg/kg and 48 mg/kg. The nucleosides were administered in 0·15 M NaCl, pH 9·3, at concentrations of 3·5 mg/ml. Chromatograms of plasma extracts obtained pre- and 0·5 hr post-injection showed a rapid accumulation of 8-aminoguanine, the product of the reaction between 8-aminoguanosine and NP (Fig. 2). The NP activity of rat red cells was 38·5 ± 6·8 ($n = 4$) μmol/min/g Hb. The accumulation of the NP substrate inosine was evident. A large peak of guanine, derived from the action of NP on deoxyguanosine, was observed. The data from these experiments showed that NP was effectively inhibited as inosine (its major physiological substrate) accumulated (Table 1). Similar results were obtained from two other animals. At neither of the two concentrations did deoxyguanosine accumulate in plasma. Adenosine showed elevations for reasons that are not obvious. The levels of inosine observed were not sufficient to cause product inhibition of adenosine deaminase, and hence accumulation of adenosine (Osborne, Chen & Scott, 1979). Deoxyadenosine did not accumulate. Plasma tryptophan and deoxycytidine did not vary abnormally. The dexoycytidine levels were in agreement with previously published values (Cha, Lakhchaura & Hsu, 1983).

HPLC analysis of red cell nucleotides showed large increases

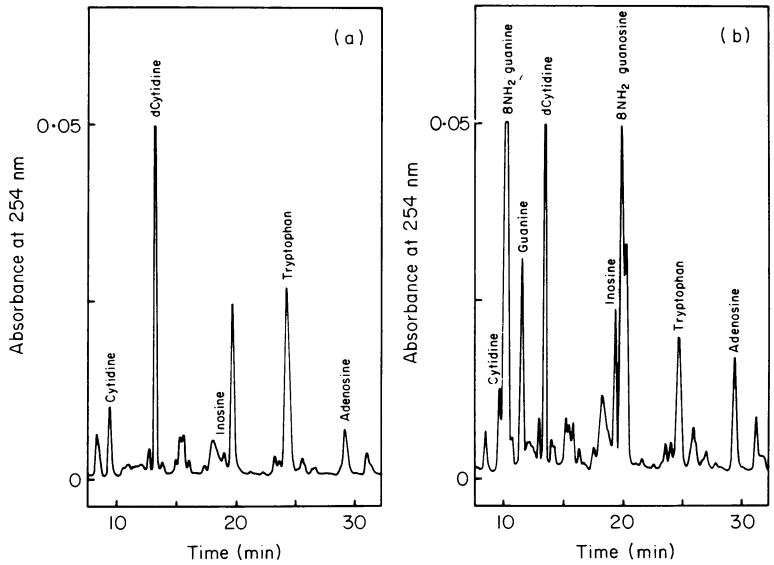

Figure 2. Chromatograms of rat plasma extracts. Samples were obtained (a) before and (b) 0·5 hr after the intraperitoneal administration of 8-aminoguanosine plus deoxyguanosine at 24 mg/kg each nucleoside. The detector was set at 0·05 full scale.

in GTP over the 24-hr period post-injection. From a control GTP level of 0·060 mmol/l RBC, the 24 mg/kg injection caused an increase to 0·105 mmol/l RBC, and the 48 mg/kg dose resulted in a 24 hr GTP level of 0·095 mmol/l RBC. Also, GDP increased from a control level of 0·025 mmol/l RBC to 0·032 mmol/l RBC and 0·037 mmol/l RBC for the lower and higher levels, respectively.

DeoxyGTP was not detectable in RBC from either animal. The limit of detection of the HPLC method is $<0{\cdot}002$ mmol/l RBC. These elevations of RBC guanine nucleotides are in accord with the accumulation of guanine in plasma, as the intracellular action of HPRT on guanine would initiate the synthesis of GTP.

The other nucleotides analysed remained reasonably constant throughout the 24-hr period, with mean values for the two animals as follows: ATP $0{\cdot}567 \pm 0{\cdot}045$; ADP $0{\cdot}189 \pm 0{\cdot}021$; AMP $0{\cdot}113 \pm 0{\cdot}015$ (mmol/l RBC, $n=8$). These values are in agreement with previously published data for normal rats (Dean, Perrett & Sensi, 1978). Similar results were obtained from two different animals.

Guanosine nucleotides in lymphoid tissues

In order to determine if dGTP was accumulating in lymphocytes, we administered deoxyguanosine and 8-aminoguanosine to three test rats (24 mg/kg of each nucleoside i.p.) and 0·15 M

Table 1. Serial plasma analysis after the administration of 8-aminoguanosine and deoxyguanosine*

	Rat 1†				Rat 2‡			
	Time (hr)				Time (hr)			
	Pre	0·5	4	24	Pre	0·5	4	24
$8NH_2$guanine	—	48·1	31·9	1·6	—	104·1	133·5	8·2
Guanine	ND§	9·1	ND	ND	ND	39·5	1·2	ND
dCytidine	20·2	17·4	23·7	22·7	17·4	17·6	22·4	20·4
Inosine	1·0	5·9	5·0	5·7	0·8	54·2	10·7	4·7
$8NH_2$guanosine	—	14·8	4·2	ND	—	20·6	14·9	ND
Tryptophan	59·8	43·2	31·7	41·8	57·6	33·0	36·3	41·3
Adenosine	2·4	4·9	1·7	2·2	2·2	9·7	3·7	0·5

* Units are given as μmol/l plasma.

† I.p. injection of 8-aminoguanosine plus deoxyguanosine at 24 mg/kg each nucleoside.

‡ I.p. injection of 8-aminoguanosine plus deoxyguanosine at 48 mg/kg each nucleoside.

§ ND, Not detected. Limit of detection $<0{\cdot}1$ μmol/l.

Table 2. Guanine nucleoside triphosphates in lymphoid tissue* 90 min post-injection (24 mg/kg i.p.)

	Thymus		Lymph node		Spleen	
Nucleotide†	GTP	dGTP	GTP	dGTP	GTP	dGTP
Saline	35·0	5·9	30·9	ND‡	20·1	ND
8NH_2guanosine + deoxyguanosine	45·0	10·8	38·8	ND	24·0	ND

* Tissue pooled from three animals.
† nmol/g tissue.
‡ ND, Not detected. Limit of detection <0·1 nmol/g.

saline to three control animals. Ninety minutes post-injection, the animals were killed and the lymphoid tissues removed, rapidly frozen in liquid nitrogen, and stored at −70° until analysis. GTP and dGTP were measured in cell extracts by a simple isocratic procedure, as previous experience with human cells showed that >90% of the nucleotides were present as triphosphates (Osborne & Scott, 1983). The test rats, in comparison to controls, showed increased levels of GTP in thymus, spleen and lymph node, but only thymus showed an accumulation of dGTP (Table 2). These data agreed with the deoxyguanosine kinase activities, which showed levels in thymus cells three-fold higher than lymph node and spleen cells.

Effect of long-term NP inhibition on rat lymphoid tissues

Rats were injected for 8 consecutive days with either 0·15 M NaCl, deoxyguanosine, 8-aminoguanosine, or both of the nucleosides, and then killed and the cells in thymus, lymph node and spleen counted (Table 3). Six rats were tested in each group. For treatment with 8-aminoguanosine alone, or 8-aminoguanosine plus deoxyguanosine, cell counts were significantly decreased for thymus ($P<0·001$) and for spleen and lymph node ($P<0·01$). The greatest effect was seen in thymus after the co-administration of both purines, where an 86% decrease in cell number was observed. No significant differences were found for any of the tissues after treatment with deoxyguanosine alone. From observations in plasma, where guanine was formed from deoxyguanosine even in the presence of 8-aminoguanine (Fig. 2, Table 1), it is likely that deoxyguanosine administered alone would be rapidly metabolized by NP and not result in the intracellular accumulation of dGTP.

Table 3. Cell counts from thymus, spleen and lymph node after injection of six rats per treatment group for 8 consecutive days*

	Thymus (cells × 10^{-8})	Spleen (cells × 10^{-7})	Lymph node (cells × 10^{-7})
Saline	4·4±0·6	6·0±2·0	2·9±0·9
8NH_2guanosine	1·3±0·7†	2·7±0·6‡	1·0±0·6‡
Deoxyguanosine	3·2±1·1	8·6±2·6	3·3±1·2
8NH_2guanosine + deoxyguanosine	0·6±0·2†	3·6±0·7‡	1·2±0·6‡

* The dose of each purine was 24 mg/kg i.p. per day.
† $P<0·001$ (comparison with saline control).
‡ $P<0·01$ (comparison with saline control).

At 24 hr after the final nucleoside injections, the rats receiving 8-aminoguanosine either alone or with deoxyguanosine showed a mean 8-aminoguanine level of 2·8±1·9 μmol/l plasma with a range of 0·9–6·5 μmol/l. At the same sample times, the two groups of rats receiving deoxyguanosine showed a mean increase of 42% in red cell GTP compared to the saline-treated controls.

DISCUSSION

The advances made in the study of ADA deficiency by the availability of specific powerful inhibitors have highlighted the lack of similar inhibitors of NP. In this study the use of the NP inhibitor 8-aminoguanosine to create an animal model of NP deficiency was investigated. The soluble nucleoside was rapidly converted by rat NP to the relatively insoluble purine base, 8-aminoguanine, which was not further metabolized by rat red cells. It was shown previously that 8-aminoguanine is not a substrate of human HPRT, rat liver guanase or xanthine oxidase (Stoeckler *et al.*, 1982). Thus, 8-aminoguanine appears to be a specific inhibitor of NP and can be administered as 8-aminoguanosine, its nucleoside precursor. The kinetic data showed that 8-aminoguanine was a potent inhibitor of rat erythrocyte NP with a K_i of 0·19 μM. This compares with the inhibition constants of 0·2–1·2 μM reported for human red cell NP (Stoeckler *et al.*, 1982). From the K_i value, it can be estimated that free inhibitor concentrations of 48 μM and 2 μM 8-aminoguanine would give 99·6% and 90·5% inhibition of NP, respectively. These inhibitor levels represent the maximum and minimum observed in plasma and provide kinetic evidence to suggest that an enzyme-deficient state was produced by 8-aminoguanosine administration. In humans the complete absence of NP activity is not necessary to produce immune dysfunction as two affected children had 0·5% of normal activity in their red cells (Osborne *et al.*, 1977). The rapid accumulation of inosine in plasma was further indication that NP activity was functionally inhibited. One of the most evident metabolic consequences of NP deficiency in humans is the accumulation of inosine in plasma (Kredich & Hershfield, 1983; Giblett *et al.*, 1981).

Exogenous deoxyguanosine did not accumulate in plasma but its metabolite guanine was observed and its anabolism was documented as an increase in red cell GTP. Similarly, increases in GTP were observed in thymus, lymph node and spleen. The accumulation of cytotoxic dGTP was only observed in thymus, and this tissue showed the greatest decrease in cell count from the co-administration of 8-aminoguanosine and deoxyguanosine. Cell counts from thymus, lymph node and spleen showed that 8-aminoguanosine, either alone or with deoxyguanosine, resulted in significant lymphopenia in all three lymphoid tissues. To produce lymphopenia, inhibition of NP was required as deoxyguanosine administered without inhibitor did not significantly decrease cell counts. However, it has been reported that deoxyguanosine alone abrogated T-cell suppressor function in mice (Dosch *et al.*, 1980).

The deoxyguanosine kinase assays showed highest activities in lymphoid tissues, with the greatest proportion of cells in the T-cell lineage, i.e. thymus > lymph node > spleen. This is in

agreement with the distribution of deoxyguanosine kinase activity in human T and B cells (Osborne & Scott, 1983). The decreases in cell numbers produced by 8-aminoguanosine plus deoxyguanosine treatment were positively correlated with deoxyguanosine kinase activity as lymphocytes were reduced 86%, 60% and 40% in thymus, lymph node and spleen, respectively. Previous studies have shown high deoxynucleoside kinase activities in thymus and T cells (Durham & Ives, 1970; Krenitsky *et al.*, 1976).

This study has shown that the *in vivo* simulation of NP deficiency can be achieved by the administration of 8-aminoguanosine. It enables the rat to be used as a model of this inherited defect and permits *in vivo* studies of a means to control T-cell function selectively.

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