0031-6997/92/4404-0459$03.00/0
PHARMACOLOGICAL REVIEWS

Vol. 44, No. 4
Printed in U.S.A.

Pentostatin: Future Directions

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I. Introduction

PENTOSTATIN (2′-deoxycoformycin) is an anticancer agent that has just been approved by the United States Food and Drug Administration for the treatment of interferon refractory hairy cell leukemia. The drug's response rate in this disease has been reported to be >90% in some studies, and in a large clinical trial in which pentostatin was compared with interferon, based on previous smaller clinical studies, it is anticipated that pentostatin will be significantly superior to interferon in inducing remissions. In addition, the clinical responses to pentostatin are long lasting, and although little information exists, the current data suggest that, even when patients do relapse, they can be reinduced into remission with another course of pentostatin treatment. Thus, the discovery and development of pentostatin represents a major advance in hairy cell leukemia treatment and has provided patients with this disease the first real hope for long-term survival.

The approval of pentostatin for treatment of hairy cell leukemia may not, however, be the only use for this drug but, rather, may represent the infancy for this drug not only in the treatment of cancer but also as a therapeutic agent for organ and bone marrow transplantation, juvenile diabetes, and stroke-induced brain damage.

Pentostatin was initially isolated from *Streptomyces antibioticus* >17 years ago (Woo et al., 1974) and was found to be an extremely potent inhibitor of ADA* (EC

* Abbreviations used: ADA, adenosine deaminase; Ado, adenosine; dAdo, deoxyadenosine; SAH, S-adenosylhomocysteine; ara-A, 9-β-D-arabinofuranosyladenine; dCyt, deoxycytidine; ara-C, 9-β-D-arabinofuranosylcytosine; IL-2, interleukin; % T/C, 100 × (treated/control); EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; ara-AMP, vidarabine-5-phosphate; ddAdo, 2′,3′-diadenosine.

3.5.4.4.), an enzyme that participates in purine salvage metabolic pathways (Agarwal and Parks, 1977; Agarwal et al., 1977; Franco and Centelles, 1989). This enzyme is found in many mammalian tissues, and its highest activity is in the lymphoid system. The discovery that patients with severe combined immunodeficiency syndrome lacked ADA (Giblett et al., 1972; Meuwissen et al., 1975) suggested that this enzyme was essential for normal lymphocyte function. The progress of this drug as a therapeutic agent was extremely slow, because in spite of its in vivo equivalent activity with cyclosporin A in transplantation models (Ruers et al., 1985b), no clinical trials were ever conducted with pentostatin to determine its immunosuppressive potential. In initial clinical trials, pentostatin was extremely neurotoxic. Also, progress with pentostatin was hindered by the lack of preclinical models in which pentostatin alone was active as a single agent for cancer therapy or immunotherapy. The failure to develop drug-sensitive preclinical models curtailed combination chemotherapy and hampered the clinical testing of this drug. Even the mechanism of antileukemic activity of pentostatin was not precisely elucidated, and despite numerous biochemical studies, the mechanism by which pentostatin elicits its clinical activity still remains to be resolved.

Pentostatin has received "orphan" drug status because of its limited use in hairy cell leukemia. Results of biochemical and pharmacological studies reviewed here indicate future directions for this drug's expanded use for the treatment of cancer and immunological and perhaps cardiovascular diseases as well.

II. Pentostatin Effects on the Biochemistry of Adenosine Deaminase

A. Inhibition of Adenosine Deaminase

Pentostatin {(*R*)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol} was isolated from a fermentation broth of *S. antibioticus* by Woo et al. (1974) and was found to be an inhibitor of ADA, an enzyme that participates in purine salvage metabolic pathways (Agarwal and Parks, 1977; Agarwal et al., 1977; Franco and Centelles, 1989). The structure of pentostatin, compared with Ado, is shown in figure 1.

As shown in figure 2, ADA catalyzes the hydrolytic deamination of Ado to inosine, by direct water attack,

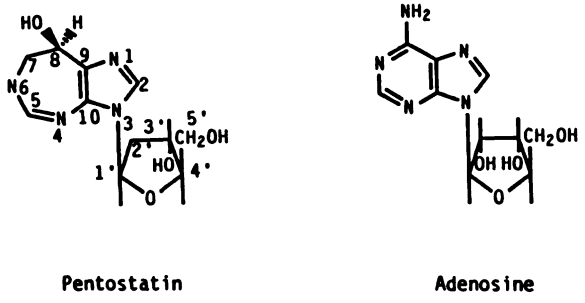

FIG. 1. Structure of pentostatin and Ado.

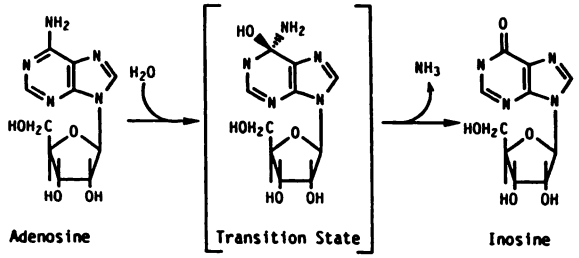

FIG. 2. Deamination of Ado.

with the enzyme acting as a general base catalyst (Frick et al., 1986).

The tetrahedral carbon at position 8 of pentostatin mimics the purported tetrahedral carbon in the transition state of the deamination reaction of Ado to inosine, thus suggesting that pentostatin is a transition state analog inhibitor of the enzyme. Pentostatin is a stable molecule that has enzyme-binding properties of the transition state intermediate of the deamination reaction and, therefore, might be expected to bind to and inhibit the enzyme.

Agarwal et al. (1977) determined the parameters of ADA inhibition using partially purified human erythrocyte ADA with Ado as the substrate. Because pentostatin was found to be a tightly bound inhibitor of ADA, they used nontraditional steady-state kinetic measurements, and the K_i was determined by the ratio of k_2/k_1, where E is enzyme, I is

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI$$

inhibitor, k_1 is the enzyme inhibitor association rate constant, and k_2 is the complex dissociation rate constant. From their studies, they determined a K_i of 2.5×10^{-12} M. Other investigators have also determined the K_i to be in the 10^{-12} M range using partially purified P388 ADA with ara-A as substrate (Lee et al., 1981). Jackson et al. (1986) determined the K_i values for pentostatin inhibition of ADA derived from rat liver, rat intestine, a rat hepatoma, and a human B cell line (WI-L2), with Ado as substrate, to be between 4.8×10^{-13} M and 9.1×10^{-12} M. In addition, the apparent dissociation rate constant, k_2, corresponded to an enzyme inhibitor complex half-life of 68 hours. All of these data demonstrate that pentostatin is an extremely tightly bound inhibitor of ADA.

B. Pentostatin Inhibition of Adenosine Deaminase Isoenzymes

Two isoenzymes of ADA exist that have different molecular weights, kinetic properties, and tissue distributions. The most extensively studied isoenzyme is termed ADA_1. Its molecular weight can range from 33 to 45 kDa and can exist in two major forms: as a monomer and as a dimer with a complexing protein with a combined molecular weight of 270 to 280 kDa (Akedo et al.,

1972; Dadonna and Kelly, 1977, 1978; Hunt and Hoffee, 1982; Nishihara et al., 1973). The genetic absence of ADA results in severe combined immunodeficiency syndrome (Giblett et al., 1972; Meuwissen et al., 1975). Ratech and his coworkers (1981) were the first to describe another human ADA isoenzyme referred to as ADA_2 with a molecular weight of 100 kDa. ADA_2 is distinct from ADA_1 and can only exist as a monomer. Although ADA_1 has been extensively studied and characterized, relatively little is known about ADA_2 in spite of the fact that it makes up the majority of ADA activity in human plasma (Niedzwicki et al., 1991; Ratech and Hirschorn, 1981).

Most kinetic studies of pentostatin have not appreciated the potential importance of ADA isoenzymes. This is in spite of a report by Ratech et al. (1981) demonstrating that ADA_2 was relatively resistant to inhibition by pentostatin and other inhibitors of ADA. Recently, however, Niedzwicki and Abernethy (1991) determined K_i values for a number of ADA inhibitors using partially purified ADA_2 from human plasma. They reported a K_i value for pentostatin of 19×10^{-12} M using ADA_2. Unfortunately, they did not determine the K_i value for ADA_1 using the identical assay conditions. Unless under their assay conditions the K_i for pentostatin against ADA_1 is significantly lower, this finding suggests that ADA_2 is not particularly resistant to pentostatin, but this conclusion requires further investigation. In addition, in spite of the report of Ratech et al., as early as 1981, there were still no clinical studies of pentostatin that examined the ADA isoenzyme patterns in pentostatin-responsive and -unresponsive tumors.

C. Physical Biochemistry

The conformational changes of ADA that occur as a result of pentostatin binding to the enzyme have been examined by fluorescence spectroscopy using human thymus enzyme (Philips et al., 1987, 1989). Treatment of isolated ADA with pentostatin causes a slow conformational change in the transition state inhibitor complex resulting in a decrease in fluorescence of tryptophan residues that appear to be near the active site; these data suggest that structural changes in the enzyme are induced as a result of its interaction with pentostatin.

D. Stereochemistry of Inhibitors

A number of structural analogs of pentostatin have been prepared to probe the stereochemistry of pentostatin binding and inhibition of ADA. The seven-membered ring of pentostatin was found to be essential for ADA inhibition (Montgomery et al., 1985). The asymmetric carbon at position 8 in the ring was also found to be required because the dissociation constant of the 8-keto compound was 10^7 lower than that for pentostatin (Schramm and Baker, 1985). Furthermore, the *R* configuration at carbon 8 was essential for tight binding of the compound to ADA (Schramm and Baker, 1985).

III. Cell Biology of Pentostatin

A. Adenosine and Deoxyadenosine Metabolism

The mechanism by which inhibition of ADA leads to cytotoxicity in certain cells is best understood by a review of the metabolic pathways in which Ado or dAdo participate (Cohen and Barankiewicz, 1985; Franco and Centelles, 1989; Glazer, 1980a,b; Ho et al., 1989). Figure 3 shows some of the routes by which Ado and dAdo may be metabolized. Both are deaminated by ADA (fig. 2) or, through the action of the appropriate kinase, can also be phosphorylated to (d)AMP and then to (d)ATP. Ado and dAdo can serve as substrates for the enzyme, SAH hydrolase, which forms SAH from (d)Ado and homocysteine. SAH is involved in transmethylation reactions that are important for DNA and RNA processing and metabolism. The product of the ADA reaction, inosine, can be further metabolized to (d)ATP or (d)GTP with subsequent incorporation into RNA and DNA. Thus, the inhibition of Ado and dAdo deamination by pentostatin can dramatically alter key pathways in purine metabolism and thereby have profound effects on cell growth, function, and regulation.

B. Tissue Specificity of Adenosine Deaminase and Enzymes in Purine Metabolism

ADA activity can differ by as much as 2 orders of magnitude, depending on the animal and tissue type (Ho et al., 1980; Tedde et al., 1979). Ho et al. (1980) observed that, for both human and rat tissue, the highest ADA activity was found in the spleen, followed by the small intestines. In mice, however, intestinal tissue possessed the highest ADA activity, followed by the thymus (Ho et al., 1980; Tedde et al., 1979).

Other enzymes in the purine metabolic pathway also differ in their tissue distribution and activity (Yin et al., 1980). Mouse phosphoribosyl pyrophosphate synthetase, the source of phosphoribosyl pyrophosphate, which participates in both de novo and salvage purine pathways (fig. 3), was most active in thymocytes, followed by spleen, splenocytes, and erythrocytes, and was much less active in colon, jejunum, lung, kidney, and liver. Therefore, the combined effects of ADA, 5′-nucleotidase, purine nucleoside phosphorylase, AMP deaminase, hypoxanthine guanine ribosyl transferase, and other enzymes contribute to metabolite fluxes on which pentostatin exerts some action; this effect is likely to differ depending on the cell type and activities (and localization) of each enzyme that participate in this pathway.

This complexity has been examined in a number of cell types of both normal tissue and leukemic or tumor tissue (Deibel et al., 1981; Fishbein et al., 1981; Hall et al., 1979; Ho et al., 1980, 1986, 1987, 1989; Ho and Ganeshaguru, 1988; Murray et al., 1986; Smyth and Harrap, 1975; Sylwestrowicz et al., 1982).

The enzyme activity expressed as the V_{max} for a number of enzymes involved in purine metabolism is pre-

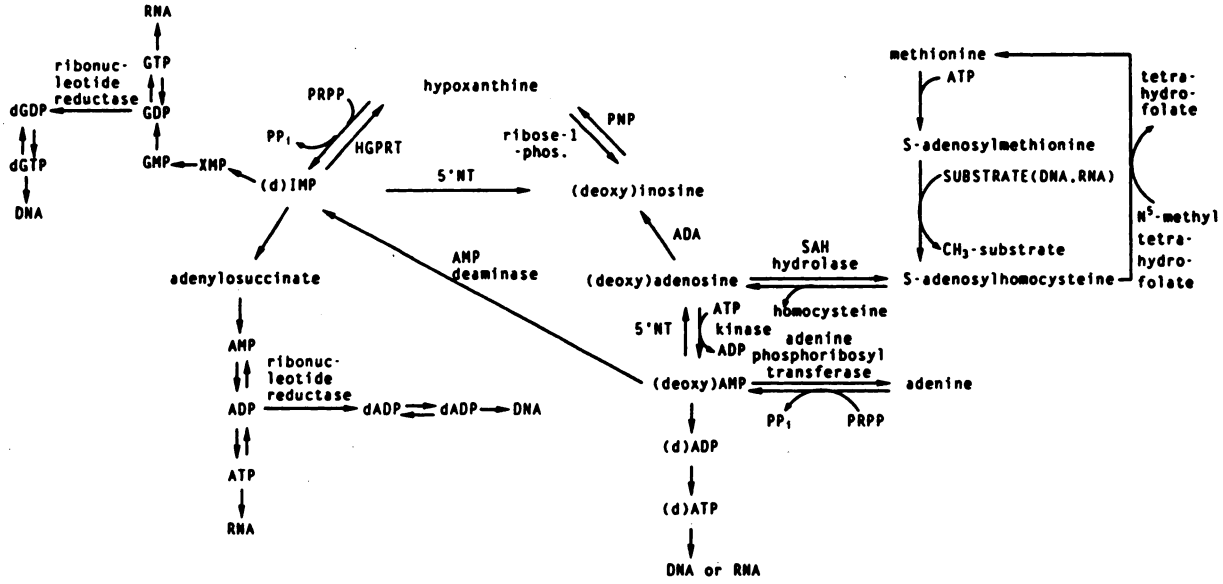

FIG. 3. Diagram of purine metabolism. Abbreviations: PNP, purine nucleoside phosphorylase; 5'NT, 5'-nucleotidase; (d)IMP, (d)inosine 5'-monophosphate; HGPRT, hypoxanthine guanine phosphoribosyl transferase.; PRPP, phosphoribosylpyrophosphate; PPi, pyrophosphate. Compiled from: Cohen and Barankiewicz, 1985; Franco and Centelles, 1989; Glazer, 1980a,b; Henderson, 1985; Ho et al., 1989.

TABLE 1

*Enzyme activities of key enzymes in purine metabolism**

	nmol/min/mg Protein							Reference
	AMP-DA	AMP 5'NT	Ado Kinase	ADA	IMP 5'NT	PNP	HGPRT	
Primary rat culture myotubes	599.5	4.13	1.75	18.6	1.36	24.4	3.0	Zoref-Shani et al., 1987
Unseparated mouse thymocytes	2.9	0.63	3.5		0.83			Dornand et al., 1985
Cortical mouse thymocytes	2.1	0.55	3.2		0.75			Dornand et al., 1985
Medullary mouse thymocytes	14.6	0.38	3.1		0.7			Dornand et al., 1985
Unseparated mouse splenocytes	44.2	0.75	2.3		0.91			Dornand et al., 1985
Mouse T-splenocytes	41.3	0.87	2.3		0.83			Dornand et al., 1985
Mouse B-splenocytes	34.2	0.7	2.3		1.1			Dornand et al., 1985
Normal human T-cells		7.48		16.9		88.4		Ho et al., 1987
Normal human B-cells		11.6		8.05		63		Ho et al., 1987
Human acute lymphocytic leukemia		6.3		82.5		29.3		Ho et al., 1987
Human B-chronic lymphocytic leukemia		0.47		6.3		29.6		Ho et al., 1987
Human centrocytic lymphoma		2.26		14.5		29.1		Ho et al., 1987
Human hairy cell leukemia		2.15		3.7		115.3		Ho et al., 1987
MOLT-4	1.0			860				Fishbein et al., 1981
CEM	1.0			222				Fishbein et al., 1981
WI-L2	17.3			31				Fishbein et al., 1981
K562	3.4			28				Fishbein et al., 1981
Human lymphocyte	14.8			17.7				Fishbein et al., 1981
Acute megalocytic leukemia				6.83				Ho et al., 1980
Chronic megalocytic leukemia				1.95				Ho et al., 1980
Chronic megalocytic leukemia (blast)				6.18				Ho et al., 1980
Acute lymphocytic leukemia				8.05				Ho et al., 1980
Chronic lymphocytic leukemia				1.12				Ho et al., 1980
Normal lymphocytes				1.0				Ho et al., 1980
CCRF-CEM			1.03	230		27		Carson et al., 1978
WI-L2			1.69	32		74		Carson et al., 1978

* Values shown were, in some cases, calculated from data provided within the references to allow expression in common units. Abbreviations used: AMP-DA, AMP deaminase; AMP 5'NT, 5'-nucleotidase with AMP as substrate; IMP 5'NT, 5'-nucleotide with inosine 5'-monophosphate as substrate; PNP, purine nucleoside phosphorylase; HGPRT, hypoxanthine guanine phosphoriboxyl transferase.

sented in table 1. Normal cells showed ADA levels from 1.0 to 18.6 nmol/min/mg protein with substantial elevation in human leukemia T-cell lines (MOLT-4, CEM) and human acute lymphocytic leukemia (Ho et al., 1987). Although the absolute values were somewhat different, Ho et al. (1980) also observed elevated ADA levels in patients with acute lymphocytic leukemia. In a subsequent study, Ho et al. (1987) compared hairy cell leukemia cells with normal peripheral T- or B-lymphocytes and in hairy cell leukemia cells observed reduced ADA activity, elevated purine nucleoside phosphorylase activity, and lower 5′-nucleotidase activity. As the data in Table 1 demonstrate, the activities of AMP deaminase and 5′-nucleotidase also vary considerably depending on cell type. In general, however, ADA activity is highest in lymphoid tissue, mitogen-stimulated lymphocytes, and blast cells of acute lymphocytic leukemia (Ho and Ganeshaguru, 1988). Circulating T-lymphocytes possess greater ADA activity than do B-lymphocytes (Ho et al., 1986).

Based on the differences in enzyme activity in the purine pathway in dissimilar tissues, pentostatin inhibition of ADA should be expected to have distinct consequences for various cell types. If, for example, dAdo kinase is relatively active in a particular cell type, then pentostatin inhibition of ADA would probably result in increased dATP levels. However, if AMP deaminase is relatively active, then the dAMP formed would be deaminated to (d)inosine 5′-monophosphate and further metabolized to hypoxanthine, which abrogates the effect. Thus, one might expect that the ability to predict how pentostatin will affect cell proliferation in a given tissue, cell type, or leukemia could be deduced by the elucidation of the activities of the other enzymes that participate in purine metabolism. However, Ho and his coworkers (1989) found that determination of the activities of ADA, 5′-nucleotidase, 2′-dAdo kinase, and SAH hydrolase in leukemias failed to provide any insight into the pentostatin responsiveness in these leukemias. Moreover, incubation of leukemic cells in vitro with pentostatin caused inhibition of ADA, accumulation of dATP, a reduction in ATP and NAD pools, a suppression of SAH hydrolase activity, and an increase in DNA strand breaks in all leukemic samples irrespective of their clinical response to pentostatin. Grever and his coworkers (personal communication) also observed a similar lack of correlation between clinical responses to pentostatin and numerous biochemical parameters including ADA activity, dAdo and dATP levels, and cytidine kinase activity.

As we will discuss in subsequent sections, numerous other mechanisms may also be involved in pentostatin's cytotoxicity, and depending on the cell or leukemia type, there may be more than one effect on purine metabolism by pentostatin inhibition of ADA activity that leads to cell death.

C. Cell Cycle Effect

The effect of pentostatin in combination with dAdo or ara-A on cell cycle perturbations has not been extensively studied, and in fact, there are no reports of cell cycle effects with pentostatin alone. ara-A plus pentostatin inhibited CCRF-CEM cells in S phase of the cell cycle (Dow et al., 1980). Addition of dCyt to the treated cells had no effect on the ara-A/pentostatin DNA synthesis inhibition but did relieve the DNA synthesis inhibition brought about by 2-fluoro-ara-A alone. A dCyt kinase-deficient CCRF-CEM cell line resistant to 2-fluoro-ana-A was sensitive to ara-A/pentostatin. These data suggest that at least ara-A/pentostatin combination treatment of cells in vitro results in inhibition of DNA synthesis brought about by changes in the nucleotide pools. The effect of pentostatin and dAdo on entry of activated peripheral blood lymphocytes into the cell cycle has also been characterized (Redelman et al., 1984). The synthesis of RNA in the G_0-G_1 phase was found to be inhibited to some extent by pentostatin alone and substantially inhibited in the presence of pentostatin and dAdo. This RNA synthesis inhibition precluded initiation of DNA synthesis and progression through the cell cycle resulting in a G_0-G_1 block.

IV. Molecular Pharmacology

A. In Vitro Cytotoxicity

The important role of ADA in normal lymphocyte function was indicated by the finding that, in lymphocytes from patients with severe combined immunodeficiency syndrome, there were low or undetectable levels of the enzyme present (Carson et al., 1978, 1979, 1981; Cohen et al., 1978; Giblett et al., 1979; Kefford and Fox, 1983). The erythrocytes of immunodeficient, ADA-deficient children were found to contain >50-fold amounts of dATP; the latter compound was proposed as the toxic metabolite responsible for immune deficiency because immunocompetent, ADA-deficient erythrocytes did not contain elevated levels of dATP (Cohen et al., 1978). Carson et al. (1978) examined the relationship between ADA, Ado, and dAdo and growth delay in T and B leukemia lymphoblasts because the T-cells possess greater levels of ADA than do B-cells (Ho et al., 1987). They found that T-cells were substantially more sensitive to dAdo than to Ado and were more susceptible to dAdo than were B-cells. Carson et al. (1978) observed that dAdo-treated T-cells had elevated dATP, whereas dAdo-treated B-cells did not. They concluded, therefore, that dATP was responsible for enhanced cytotoxicity in T-cells. The reason for the elevated dATP in dAdo-treated leukemia T-cells compared to B-cells appears to be due to the reduced catabolism of deoxyribonucleotides that occurs in T-cells compared to B-cells as well as to reduced levels of 5′-nucleotidase (Carson et al., 1979; Ho et al., 1987). The catabolism of dATP appears to proceed exclusively through the deamination of dAdo, whereas

adenine ribonucleotides are deaminated to inosine 5′-monophosphate (Barankiewicz and Cohen, 1984; Cohen and Barankiewicz, 1985). Thus, pentostatin inhibition of ADA precludes the salvage of the deoxyribonucleotides, resulting in an accumulation of dATP.

In spite of the biochemical rationale as to why pentostatin should be cytotoxic to hemopoietic cells in vitro, the drug alone has little effect on cell growth in vitro. Thymidine incorporation into leukemic T-cells was not appreciably affected until pentostatin concentrations exceeded 10^{-5} M for 48 hours (Jurjus et al., 1984). Thymidine incorporation into B-cell leukemic lines was inhibited at 10^{-6} M pentostatin, whereas non-T, non-B leukemic lines were of intermediate sensitivity; peripheral blood lymphocytes were the most sensitive to pentostatin. However, most investigators have reported that pentostatin alone has no appreciable effect on the proliferation of a wide variety of in vitro cell types, including T- and B-cell lymphoblasts (Cohen, 1977; Jackson et al., 1986; Lee et al., 1981; Shewach and Plunkett, 1982).

Although pentostatin alone lacks appreciable antiproliferative activity in most in vitro test systems, numerous investigators have reported that pentostatin can significantly potentiate the activity of Ado and dAdo and their analogs in many different cell types in vitro (Aye et al., 1982; Crabtree, 1978; Jackson et al., 1986; Kazmers et al., 1983; Lapi and Cohen, 1977). Aye et al. (1982), for example, examined the effects of pentostatin alone and in combination with dAdo on erythroid, granulocyte, and T-lymphocyte colony formation from human bone marrow or peripheral blood. Concentrations of pentostatin as high as 1.0 mM had no effect on colony formation, but even at 1 μM, pentostatin potentiated the growth inhibitory effect of dAdo by as much as 10-fold in all three cell types. Perhaps because of the biochemical differences between T- and B-cells, discussed previously, it is not surprising that T-cells appear to be more sensitive in vitro to the growth inhibitory effects of pentostatin in combination with Ado or dAdo. Jackson et al. (1986) reported that the T-cell leukemia, CCRF-CEM, cells were significantly more sensitive to the cytotoxic effects of pentostatin plus dAdo than were the B-cell leukemia cells, WI-L2. Kazmers et al. (1983) reported similar results with other cultured T- and B-cell leukemias and found that, in T-cell leukemias, the cytotoxicity correlated with increased dATP levels.

Because of its antiviral activity and potential as an anticancer agent, ara-A has been studied extensively in combination with pentostatin (Crabtree, 1978). Like many other Ado analogs, the six-amino acid group of ara-A is subject to deamination by ADA.

Pentostatin inhibition of ADA increases the half-life of ara-A, which can then be phosphorylated to ara-ATP intracellularly and thus interfere with DNA synthesis (Lepage et al., 1976; Shewach and Plunkett, 1979, 1982). As with dAdo, the combination of ara-A and pentostatin has been studied in T- and B-cell lines in vitro. Jackson et al. (1986) demonstrated that 2 μM pentostatin potentiated ara-A activity in vitro by 5-fold in B-cell leukemia and 10-fold in T-cell leukemia. Kuroki et al. (1989) found that pentostatin potentiated the activity of ara-A in all 30 of the human cultured cell lines of various origins that they studied. Several other laboratories (Adamson et al., 1977; Spremulli et al., 1982; Verhoef and Fridland, 1983) examined the combination of pentostatin and ara-A, as well as other Ado analogs, in a variety of non-T, non-B acute lymphocytic leukemia cell lines and compared their sensitivity to the drug combination with T-, B-, and myeloid cell lines. In general, the non-T, non-B acute lymphocytic leukemia cell lines were more sensitive to ara-A alone and to the combination of pentostatin/ara-A than were the other cell types.

One caveat to the combination studies with pentostatin and ara-A and their effect on cytotoxicity and dATP levels was raised by Plunkett et al. (1982). They measured the intracellular concentrations of dATP and ara-ATP in CCRF-CEM cells treated with increasing concentrations of pentostatin and ara-A. They found that with increasing cytotoxic concentrations of drugs there was an increase in the intracellular levels of both dATP and ara-ATP. Because ara-ATP competes for the same substrate site on DNA polymerase as does dATP (Dicioccio and Srivastava, 1977), they concluded that the synergy between pentostatin (which would increase dATP levels) and ara-A (which would increase ara-ATP) might not be as striking as perhaps expected.

B. Deoxycytidine Reversal of Pentostatin-Deoxyadenosine-induced Cytotoxicity

Carson et al. (1978) and Mitchell and her coworkers (1978) demonstrated that dCyt could reverse the cytotoxicity of pentostatin and dAdo in human T-cell lymphoblasts such as CCRF-CEM and, to a lesser extent, in a B-cell line, WI-L2. This paradoxical effect of dCyt on dAdo activity can be explained by the findings that two enzymes can potentially phosphorylate dAdo, Ado kinase, and dCyt kinase (Carson et al., 1980; Thuillier et al., 1981). In contrast to murine lymphocytes in human lymphoid tissue, dAdo appears to be a better substrate for dCyt kinase than for Ado kinase. Human T-cells generally have higher levels of dCyt kinase than do human B-cells and, hence, the greater ability of dCyt to reverse the cytotoxicity of pentostatin/dAdo in CCRF-CEM than in WI-L2 cells.

Hershfield et al. (1982) reported that the sensitivity of dCyt kinase-deficient CCRF-CEM to dAdo was 3-fold less than the parent line. dAdo had little effect on an Ado kinase-deficient CEM line, but the loss of both activities completely eliminated dAdo phosphorylation and decreased dAdo cytotoxicity by approximately 100-fold. However, the difference in kinase levels between the CCRF-CEM and WI-L2 lines could not account for

the much lower rates of dAdo phosphate formation in the WI-L2 cell line.

The findings that both Ado kinase and dCyt kinase can phosphorylate dAdo raises the possibility that leukemias and lymphomas resistant to ara-C by virtue of the loss of dCyt kinase might still retain some sensitivity to pentostatin. Consistent with this hypothesis, Brockman et al. (1979) demonstrated that 2-fluoro-ara-A was active in ara-C-resistant L1210 tumors. Perhaps, pentostatin treatment should be considered as a potential clinical strategy in ara-C-resistant leukemias.

C. Involvement of dATP in Pentostatin-induced Cytotoxicity

Seventeen years after the discovery of pentostatin, it seems clear that no one mechanism can completely account for the antineoplastic activity of pentostatin. The inhibition of ribonucleotide reductase by elevated levels of dATP has been suggested as an explanation for the activity of the pentostatin-dAdo combination (Burgess et al., 1985; Kefford and Fox, 1983; Lee et al., 1984; Matsumoto et al., 1982; Seegmiller, 1985). Although this proposal may apply to cells undergoing division (leukemic lymphocytes, mitogenically stimulated lymphocytes), slow-growing or quiescent cells, which are also sensitive to this combination, should not be affected by inhibition of ribonucleotide reductase. Moreover, even when dATP levels increased in cultured leukemic T-cells in response to treatment with pentostatin and dAdo, the other deoxynucleotide pools did not measurably decrease (Tanaka and Kimura, 1985), casting doubt on the allosteric inhibition of ribonucleotide reductase by dATP as the primary or only mechanism of action in all cell types. The study by Brox et al. (1982) also cast doubt on inhibition of ribonucleotide reductase by dATP as the sole mechanism of action of pentostatin. In their colony-forming assays of human T-lymphocytes, B-lymphocytes, and granulocytes, they found no significant differences in cytotoxicity when cells were treated with pentostatin combined with dAdo rather than Ado. Whereas dATP formed from dAdo can inhibit ribonucleotide reductase, the metabolism of Ado to ATP would not, yet both were similarly active against these human lymphoid cells.

The time course of pentostatin effects in lymphocytes, as a function of their activation state, was examined by Thuillier et al. (1981) in an attempt to characterize the critical targets of ADA inhibition. Rat thymocytes were stimulated by concanavalin A, and DNA and RNA synthesis was evaluated. They observed that, as the time after activation increased, the inhibition of thymidine incorporation by pentostatin and dAdo decreased. That is, the longer cells were activated, the more resistant they were to pentostatin and dAdo. They also examined blastogenesis of the thymocytes as a function of time of treatment after concanavalin A stimulation. Simultaneous treatment with concanavalin A and pentostatin/dAdo resulted in inhibition of blast cell number, DNA, RNA, and protein synthesis. A 12-hour gap between concanavalin A and pentostatin/dAdo treatments had no effect on those parameters, and a 24-hour gap between treatments actually enhanced blastogenesis and precursor incorporation. Thuillier and his coworkers (1981) concluded that the maximum cell sensitivity to pentostatin/dAdo occurred in the inactivated state before the proliferation phase and before DNA synthesis was initiated. The mitogen recognition phase, which takes place before DNA synthesis and includes methylation processes, may be disrupted in some fashion by pentostatin/dAdo or, at the least, the disruption in thymocyte metabolism takes place in the same time frame as mitogen recognition.

Colledge et al. (1982) also examined the time course of pentostatin effects on T-cell colonies seeded from peripheral blood mononuclear cells. Treatment of phytohemagluttinin-stimulated T-cell colonies with pentostatin showed maximal inhibition with pentostatin 2 hours after treatment. Longer intervals had no effect on the number of colonies formed compared with untreated controls. The maximum effect of pentostatin occurred 20 hours prior to onset of DNA synthesis in the phytohemagluttinin-stimulated cells. The authors, therefore, concluded that ribonucleotide reductase inhibition was not the mechanism of pentostatin's action.

D. Inhibition of Interleukin 2

Cohen and Glazer (1985) reported that pentostatin either alone or in combination with Ado or dAdo inhibited IL-2 mRNA synthesis in treated splenocytes and Jurkett T-cell leukemia cells. Their results with mycophenolic acid suggested that inhibition of IL-2 mRNA synthesis was specific and not due to a more general reduction in RNA synthesis. Because this laboratory previously demonstrated that pentostatin inhibited lymphoblastogenesis only when it was present at the beginning of mitogen treatment (Earle and Glazer, 1983), and IL-2 message expression is also an early event in the mitogenic process, the authors speculated that perhaps pentostatin in some way could induce impairment of lymphocyte function and immunosuppression through an IL-2-mediated process. Ruers and his group (1985a) also examined the effect of pentostatin and dAdo on human T-cell activation and the involvement of IL-2 and its receptor in this process. They, too, found that pentostatin caused an inhibition of IL-2 production as well as a reduction in IL-2 receptors. The causal relationship among pentostatin-induced ADA inhibition, IL-2 inhibition, and pentostatin's inhibition of lymphoblastogenesis is, however, far from clear.

E. Inhibition of RNA Synthesis

Redelman et al. (1984) also used the lectin-stimulated activation of T-cells as a model to examine which path-

ways in the activation process were affected by pentostatin/dAdo. T-cell growth factor-dependent events leading to increased RNA synthesis at the G_0-G_1 boundary of the cell cycle were most sensitive to drug treatment. Surface marker expression (T-cell growth factor receptor measured by anti-Tac monoclonal antibody) was not affected, suggesting new RNA was not required for such expression or that the effects on RNA took place after marker induction.

Matsumoto et al. (Matsumoto et al., 1983, 1984; Yu et al., 1984) determined the rate of RNA synthesis in resting peripheral blood lymphocytes and CCRF-CEM cells treated with dAdo and pentostatin. They found that in both cell types, in the presence of pentostatin, dAdo reduced uridine incorporation in a time- and dose-dependent fashion. The inhibition of RNA synthesis coincided with increased dATP in the resting peripheral blood lymphocytes. The ATP levels and leucine incorporation both decreased but not until well after inhibition of RNA synthesis was observed. To eliminate the complication of cytoplasmic effects on RNA synthesis, they also examined nuclear transcription in CCRF-CEM nuclei and peripheral blood lymphocyte nuclei treated with pentostatin and dAdo. RNA transcription was inhibited in both cell types in a time-dependent manner, and in cells treated with dAdo, RNA transcription was inhibited in a concentration-dependent manner. These data suggest that, in addition to ribonucleotide reductase, another possible mechanism for pentostatin activity is through the inhibition of RNA synthesis.

1. Pentostatin-induced inhibition of methylation reactions. Based on an initial study by Stern and Glazer (1980), who showed that RNA methylation was inhibited in L1210 cells by pentostatin plus Ado analogs, the effect of pentostatin on SAH hydrolase has been investigated as a potential mechanism of pentostatin activity (Boss and Pilz, 1984; Helland and Ueland, 1983; Hershfield, 1984). SAH is, in many instances, a competitive inhibitor of all methyltransferases with K_i values less than the K_m for S-adenosylmethionine (substrate for methyltransferases). Normally, the S-adenosylmethionine/SAH ratio is high, thereby allowing methylation of acceptor molecules. A product of that reaction is SAH, which is hydrolyzed by SAH hydrolase to maintain the S-adenosylmethionine/SAH ratio.

SAH hydrolysis is a highly reversible reaction which, in the presence of elevated Ado or dAdo concentrations, is inhibited. ADA inhibition by pentostatin in some cell types can result in elevated levels of dAdo and Ado, which can cause a reduction in nucleic acid methylation in mouse and human lymphocyte cell lines and in stimulated human peripheral blood lymphocytes (Johnston and Kredich, 1979; Kredich and Hershfield, 1979; Kredich and Martin, 1977). Hershfield (1979) and Ueland (1982) observed that the addition of dAdo or ara-A to human lymphocyte cultures resulted in the loss of SAH hydrolase activity and an accompanying reduction of NAD to NADH with subsequent hydrolysis of the bound nucleoside to adenine. Therefore, a divergence of effects can occur between Ado (end product inhibition of SAH hydrolase) and dAdo or ara-A ("suicide inhibition") on SAH hydrolase. Furthermore, as opposed to nucleotides, the nucleosides (or deoxynucleosides) can bind to SAH hydrolase.

The possibility that pentostatin/dAdo acts through inhibition of methylation as opposed to inhibition of ribonucleotide reductase was also examined by Lee et al. (1984). They studied the mechanism of action of pentostatin/dAdo in both resting and rapidly dividing human lymphoid cells. They found that, in exponentially growing lymphoblasts, pentostatin/dAdo produced significant cytotoxicity without inhibition of SAH hydrolase but with a substantial increase in dATP concentrations. However, in resting lymphocytes in vitro, pentostatin and dAdo caused reduced cell viability that was accompanied by inhibition of SAH hydrolase. Although they also observed elevated dATP in these treated cells, hydroxyurea, which is a known inhibitor of ribonucleotide reductase, did not produce cytotoxicity. Lee et al. (1984) concluded that, in rapidly dividing lymphoid cells, inhibition of ribonucleotide reductase by elevated dATP may be the more likely mechanism for pentostatin/dAdo cytotoxicity. In contrast, in quiescent lymphocytes, pentostatin/dAdo-induced cytotoxicity may be due to inhibition of SAH hydrolase by Ado or dAdo.

F. Pentostatin Effects on DNA Strand Breakage

A number of investigators have studied the effect of pentostatin on DNA damage and repair. Brox et al. (1984b) and Cohen and Thompson (1986) observed that unstimulated human T-lymphocytes treated with pentostatin and dAdo showed DNA single-strand breaks that were dose and time dependent. The formation of these single-strand breaks was potentiated when the cells were incubated with pentostatin and aphidicolin, an inhibitor of DNA polymerase α. The results from these studies suggested that elevated dATP inhibited DNA polymerase α and thus caused enhanced DNA damage (Brox et al., 1984a). Another study of dAdo-induced DNA damage showed that dAdo caused a gradual increase in DNA strand breaks in nonactivated human peripheral blood lymphocytes (Seto et al., 1986). Human peripheral blood lymphocytes were treated with pentostatin, dAdo, and radiation. The rejoining of DNA strands was inhibited in a time- and dose-dependent fashion, which was also dependent on phosphorylation of dAdo. These data suggest that nondividing peripheral blood lymphocytes break and rejoin DNA in an equilibrium, which dATP accumulation progressively destroys, resulting in accumulation of strand breaks.

The combination of radiation and pentostatin/dAdo was found to be synergistic in L5178Y lymphoblasts that

were not dividing. The rate and extent of DNA single-strand breaks was reduced concomitantly with an increase in dATP (Cicurel and Schmid, 1988). Only a small increase in enhancement of DNA strand breakage was seen in proliferating lymphoblasts; repair was more rapid than in quiescent cells and was not affected by pentostatin/dAdo in spite of a 2-fold increase in dATP compared to quiescent cells. Chronic lymphocytic leukemia cells repaired DNA damage as rapidly as proliferating L5178Y lymphoblasts, but this repair was inhibited by pentostatin/dAdo. These results also suggest that pentostatin has effects on DNA repair that is induced by radiation damage.

Matsumoto et al. (1988) examined the effect of pentostatin/dAdo on the repair of DNA damage in the CCRF-CEM T-cell line. DNA strand breaks accumulated and were not completely repaired after removal of the drugs. Characterization of the DNA synthesis that did take place showed inhibition of replicative, not repair, DNA synthesis. A CCRF-CEM mutant with no dAdo kinase or dCyt kinase activity did not demonstrate the replicative DNA synthesis inhibition, indicating that the deoxynucleoside was necessary for activity. Because strand breaks increased and replicative DNA synthesis was inhibited, but not repair synthesis, Matsumoto et al. (1988) proposed that pentostatin treatment of cells caused perturbation of DNA ligation.

Lamballe et al. (1989) purified DNA ligase from human B- or T-cell acute leukemias (Cohen and Thompson, 1986). The combination of pentostatin and dATP preferentially inhibited the ligase from the T-cell acute lymphocytic leukemia cell line but had no significant effect on B-cell ligase. AMP-ligase is the active form of the enzyme under normal circumstances. In the presence of elevated dATP, the dAMP-ligase complex is formed with an attendant decrease in the pentostatin K_i from 10^{-6} M for AMP-ligase to 10^{-8} M for dAMP-ligase. Thus, pentostatin stabilized the dAMP-ligase complex and inhibited its activity.

The effect of pentostatin on DNA repair may also involve NAD^+. Koya et al. (1985) found that, in CCRF cells treated with pentostatin and dAdo, both NAD^+ and ATP decreased and dATP increased in a time- and concentration-dependent fashion and, in the same time period, that cytotoxicity was apparent. The activity of poly(ADP-ribose)synthetase did not change appreciably, suggesting that NAD^+ reduction was a result of ATP depletion. In chronic lymphocytic leukemia, NAD^+ levels decreased along with an increase in DNA strand breaks (Carrera et al., 1986). The involvement of NAD^+ in pentostatin cytotoxicity was also suggested by the reduction in DNA strand breaks in lymphocytes from peripheral blood exposed to pentostatin/dAdo and pretreated with niacin (Weitberg and Corvese, 1990). Strand breaks were proposed as a stimulator of repair that occurs through poly(ADP-ribosylation) utilizing NAD^+ as a co-factor (in contrast to what was reported by Koya et al., 1985).

All of these studies suggest an involvement of DNA strand breakage and/or repair in the activity of pentostatin, but they do not clearly elucidate the mechanism. Indeed, Ganeshaguru et al. (1987) observed that in T-, B-, and hairy cell leukemia cells, although levels of dATP, ATP, and NAD^+ were similarly affected by pentostatin treatment, hairy cell leukemia was the most responsive to the drug. It should also be noted that there are differences in pentostatin-induced DNA repair between proliferating and quiescent cells, between different cell types, and between radiation-induced DNA damage and pentostatin/dAdo-induced damage. The role of DNA repair in pentostatin's antileukemic action is, therefore, still uncertain.

G. Resistance to Pentostatin

Resistance to pentostatin in vitro has been produced but only through heroic attempts. Stable resistance arising from culturing cells in subtoxic concentrations of pentostatin alone has not been reported. Rat hepatoma cells, deficient in Ado kinase, were grown in high concentrations of Ado and in pentostatin, mutagenized with ethyl methane sulfonate, and selected for resistance (Hoffee et al., 1982). Hoffee and her coworkers isolated cell clones that were 6- to 20-fold more resistant to pentostatin/Ado than was the parental line. These cells contained up to 20 times greater ADA activity than did the parental line. The K_m for Ado and the K_i for pentostatin of the isolated enzyme from all the resistant lines did not vary from the parental values, suggesting that the enzyme in the resistant lines was identical with the parental ADA. After the level of resistance was further increased, physical-biochemical and immunological analysis showed an increase in the ADA activity in the resistant cells (Hunt and Hoffee, 1982); this increase was caused by an increase in the rate of enzyme synthesis resulting from increased ADA mRNA without an increase in ADA degradation (Hunt and Hoffee, 1983b). The increase in mRNA was due to ADA gene amplification (Hunt and Hoffee, 1983a). In Chinese hamster ovary cells made resistant to pentostatin by the same mechanism described above, the ADA was not amplified, but, rather, the ADA mRNA was more stable, thus resulting in an elevated amount of ADA being produced (Rowland et al., 1985).

Kubota et al. (1983) observed that the human histocytic lymphoma cell line, DHL-9, was relatively resistant to pentostatin/dAdo compared to CCRF-CEM cells (concentration that inhibits by 50% for dAdo of 90 versus 5 μM for CCRF-CEM in the presence of pentostatin). In contrast to the rat hepatoma cell line reported by Hoffee et al. (1982), which was shown to have elevated ADA levels, the DHL-9 cell line possessed virtually no ADA

activity. Not surprisingly, pentostatin did not potentiate the activity of Ado in these DHL-9 cells.

Hershfield et al. (Hershfield et al., 1982; Kurtzberg and Hershfield, 1983, 1985), produced a pentostatin/dAdo-resistant cell line whose primary mechanism of resistance appeared to be due to an increased catabolism of deoxynucleotides. In their experiments, they fused a T-cell line (CCRF-CEM) with a B-cell line (WI-L2) and found that the resultant T × B hybrid was about 50-fold more resistant to pentostatin/dAdo than was the parent T-cell. However, the hybrid cells were still significantly less resistant to pentostatin/dAdo than were the parent B-cells. The rate of deoxynucleotide catabolism in the hybrid was more than 25 times greater than found in the T-cell line. Because of the demonstration of resistance to pentostatin and dAdo by this fusion cell line, the possibility was raised of in vivo T-cell/B-cell fusions giving rise to resistant clones, but no evidence has been presented to document such a phenomenon in vivo.

Finally, it should be pointed out that, to date, there is no evidence that altered drug transport contributes to pentostatin resistance (Agarwal et al., 1979; Chen et al., 1986; Rogler-Brown et al., 1978; Rogler-Brown and Parks, 1980; Siaw and Coleman, 1984). Siaw and Coleman (1984) examined the uptake of pentostatin in in vitro leukemic cell lines, CCRF-CEM and MOLT-4. Both the T-cell and the B-cell lines accumulated pentostatin with similar kinetics. Uptake was linear for the first hour and reached a steady state after 10 hours. At 1 μM extracellular pentostatin, intracellular levels were 1.5 pmol/10^6 cells (0.4 μM) and at 10 μM of extracellular drug, the intracellular level was about 4 μM.

V. Activity against Mouse Tumors and Human Tumor Xenografts

A. Antitumor Screening Results for Pentostatin Alone

A summary of the National Cancer Institute in vivo screening data is shown in table 2. Pentostatin showed slight activity against subcutaneous B16 melanoma in a life span assay (% T/C = 130) using a drug schedule of every 3 hours for 3 days but failed to meet National Cancer Institute criteria for activity with the same dosage and schedule when B16 tumor weight was determined (% T/C = 72; National Cancer Institute criteria for activity is % T/C ≤ 42 for subcutaneous tumor (Goldin et al., 1981)). Against all other murine and human xenograft tumors, pentostatin failed to meet National Cancer Institute activity criteria. Pentostatin was completely without effect against P388 murine leukemia, LOX human amelanotic melanoma, L1210 murine leukemia, L1210 tumors resistant to ara-C and L-alanosine, ependymoblastoma, C38 and C26 murine colon tumors, and HT-29 human colon adenocarcinoma. In murine mammary tumor CD8F1, Lewis lung, and MX-1 human mammary carcinoma, some slowing of tumor growth versus the control was apparent, but this effect was not sufficient to meet National Cancer Institute standards for activity [subcutaneous tumor, % T/C ≤ 42; subrenal capsule tumor, % T/C ≤ 20 (Goldin et al., 1981)].

TABLE 2

*Screening results with pentostatin from the drug evaluation branch at the National Cancer Institute**

Tumor	Implant route	Optimal dose (mg/kg/day)	Treatment schedule†	Measured parameter	% T/C
B16	IP	1.0	Q01D×09	Life span	98
	SC	0.4	Q03H×24	Life span	130
	SC	0.4	Q03H×24	Tumor wt	72
Mammary CD8F1	SC	8.0	Q07D×05	Tumor wt	60
HT-29	Subrenal	2.0	Q04D×03	Tumor wt	176
C26	IP	10.0	Q04D×02	Life span	116
C38	SC	5.0	Q07D×02	Tumor wt	90
Ependymoblastoma	SC	0.5	Q#A×10‡	Life span	122
L1210	IP	0.4	Q03H×24	Life span	103
	IP	0.5	Q04D×02	Life span	95
	IP	0.5	Q03D×03	Life span	93
	IP	3.0	Q01D×09	Life span	101
L1210/ara-C	IP	0.4	Q03H×24	Life span	98
L1210/L-alanosine	IP	3.0	Q01D×09	Life span	102
LX-1	Subrenal	4.0	Q01D×10	Tumor wt	69
Lewis lung	IV	2.0	Q01D×09	Life span	99
	SC	0.4	Q03H×24	Tumor wt	68
LOX	ip	16.0	Q04D×03	Life span	100
MX-1	Subrenal	2.0	Q01D×10	Tumor wt	41
	Subrenal	8.0	Q04D×03	Tumor wt	81
P388	IP	4.0	Q01D×01	Life span	100
	IP	0.5	Q01D×05	Life span	109
	IP	3.0	Q01D×09	Life span	100
	IP	0.2	Q03H×24	Life span	100

* Selected from Screening Data Summaries, Drug Evaluation Branch, Drug Development Program, Division of Cancer Treatment. Abbreviations; IP, intraperitoneal; SC, subcutaneous; IV, intravenous; wt, weight.

† Schedule abbreviations are frequency of treatment × total number of doses administered.

‡ Q#A×10, twice per day for a total of 10 injections.

Perhaps of more relevance to the clinical data, Ratech et al. (1984b) examined the effect of pentostatin on T- and B-cell murine lymphomas in vivo. They found that BAL 9, a lymphoma of the Lyt-1^+,2^+T-cell phenotype, was the most sensitive tumor to pentostatin. Two lymphomas of the Lyt-1^-,2^+ T-cell phenotype, BAL 5 and AKT-lt, as well as two B-cell phenotype lymphomas, were moderately inhibited by pentostatin in vivo, whereas BAL 13, a lymphoma of the Lyt-1^+,2^- phenotype, was completely resistant to pentostatin. The response of BAL 9 and the resistance of BAL 13 correlated with dATP accumulation, but the moderate sensitivity of the other T- and B-cell lymphomas could not be explained by dATP levels, suggesting that pentostatin's cytotoxic action in these lymphomas was by another mechanism.

B. Effect of Pentostatin on the Antitumor Activities of Adenosine Analogs in Vivo

ADA is known to play an important role in the antitumor activity of many adenine nucleosides. Various Ado

analogs can serve as substrates for ADA (Agarwal et al., 1978), and the corresponding hypoxanthine nucleoside deamination products have markedly less biological activity than do the parent compounds (Plunkett, 1985). A number of studies have demonstrated that the sensitivity of tumors or tumor cells in culture to Ado analogs is inversely related to the specific activity of the tumor ADA (Adamson et al., 1977; Cass and Au-Yeung, 1976; Fernandez-Mejia et al., 1984; Johns and Adamson, 1976; Lepage et al., 1976).

Plunkett and his coworkers (1979b) compared pentostatin with EHNA in inhibiting P388 tumor deamination of ara-A in vivo. They found that in vivo pentostatin was a more potent inhibitor of P388 ADA activity than was EHNA. EHNA-induced enzyme inhibition appeared to be immediate after injection, but this inhibitory activity was transient in duration. In contrast, pentostatin inhibition of ara-A deamination was initially low but was maximized after 15 minutes and sustained for almost 10 hours. Thus, in vivo results were consistent with a rapid dissociation of the enzyme-EHNA complex and a much slower dissociation of the enzyme-pentostatin complex. Similar findings were reported by Brockman et al. (1977). They observed that treatment with pentostatin (0.2 mg/kg) of mice bearing L1210 tumors resulted in the persistent inhibition of ADA in vivo, whereas treatment with EHNA (2 mg/kg) caused only a transient inhibition of ADA activity. Tedde et al. (1979) reported that continuous intravenous infusion of pentostatin into C57BL mice for 5 days resulted in significant inhibition of ADA activity in thymus tissue, marginal inhibition of enzyme activity in jejunum, ileum, and spleen, and no inhibition in stomach activity. The presence of transplantable colon tumor resulted in greater inhibition of tissue ADA, particularly in the jejunum. In this same study, colon tumor ADA activity was also significantly inhibited. Lee et al. (1977) found that a single intraperitoneal injection of pentostatin in mice at doses that ranged from 0.001 to 0.004 mmol/kg inhibited ara-A deamination for up to 24 hours. Indeed, several studies have demonstrated that pentostatin inhibition of ADA leads to increased and prolonged plasma levels of Ado analogs such as ara-A (Borondy et al., 1977; Suling et al., 1978). That the sparing effect of pentostatin on ara-A does not result in increased toxicity but rather increased antitumor activity may be related to the ability of host tissue to recover faster from ara-A-induced damage than tumor tissue. Plunkett et al. (1979a) observed that the inhibition of DNA synthesis by ara-A was potentiated by pentostatin in P388 leukemia, murine bone marrow, and gastrointestinal mucosa, but recovery was faster in host tissues than in P388, in which DNA synthesis remained inhibited for >9 hours. In addition, tumor cells had higher ara-ATP levels and a slower turnover of nucleotides than did host tissues.

The synergistic antitumor effects of Ado analogs and pentostatin have now been observed in a number of experimental systems in vivo. Pentostatin has been shown to potentiate the antitumor activity of ara-AMP in L1210. When a drug schedule of every 3 hours for a 24-hour period repeated every 4 days was used, ara-AMP alone resulted in moderate activity (% T/C of 150), whereas pentostatin alone had no activity. When the two drugs were combined, pentostatin significantly potentiated the activity of ara-A with a % T/C of 287 and one of six 60-day survivors. In a subsequent study in which the same drug schedule was used, pentostatin was compared with EHNA for their abilities to potentiate either ara-A or ara-AMP in L1210 leukemia (Elliott and Leopold, 1991). As in the study by Jackson et al. (1986), pentostatin alone had no antitumor effect, and EHNA was also without activity. Pentostatin was significantly better than EHNA in potentiating both ara-A and ara-AMP in L1210. The optimal doses of pentostatin plus ara-A produced a % T/C of 261 compared with a % T/C of 177 for EHNA plus ara-A. For pentostatin plus ara-AMP, the optimal doses resulted in a % T/C of 288 versus 188 for EHNA plus ara-AMP. Pentostatin has also been shown to enhance the antitumor activity of ara-A-formate and ara-A against murine L1210 leukemia (Cass and Au-Yeung, 1976).

The inclusion of pentostatin with cordycepin increased the mean survival times of mice bearing P388 ascites by about 100% (% T/C = 200), whereas cordycepin alone resulted in a % T/C of 130. Inclusion of pentostatin resulted in marked therapeutic potentiation of ara-A in P388 or P388/ara-C-bearing mice (Schabel et al., 1976) as well as against intracerebral implants of L1210 tumor (Lee et al., 1977). When mice bearing P388 ascites leukemia were treated with xylosyladenine in combination with pentostatin, there was an increase in both median survival time of mice and the number of long-term survivors (Adamson et al., 1977). The simultaneous administration of 3′-dAdo-N′-oxide and pentostatin significantly increased the survival time of Ehrlich ascites tumor-bearing mice over 3′-dAdo-N′-oxide alone (Svendsen et al., 1988). Pentostatin also increased the long-term survivors of P388 leukemia in mice treated with 9-β-(2′-azido-2′-deoxy-D-arabinofuranosyl)adenine over mice treated with 9-β-(2′-azido-2′-deoxy-D-arabinofuranosyl)adenine alone (Lee et al., 1981).

Pentostatin has also been shown to somewhat enhance the activity of ara-A and 8-azaAdo against solid tumors, causing a 60% partial response in the Ridgeway osteogenic sarcoma (Schabel et al., 1979). Ado analogs such as ara-A, however, are generally considered to be antileukemic; pentostatin itself has no significant solid tumor activity. Thus, very little attention has been focused on the combination of ara-A and pentostatin in in vivo solid tumor models.

VI. Other Biological Activities

A. Immunosuppressive Actions of Pentostatin

Pentostatin alone or in combination with Ado analogs has been extensively examined for immunosuppressive

activity both in vitro and in vivo. Pentostatin alone or in combination with Ado or Ado analogs causes a number of immunological responses, some of which are clearly interrelated, such as selective cytotoxicity to competent T-cells, suppression of lymphoproliferative responses to T-cell mitogens, and inhibition of lymphocyto-mediated cytolysis.

Pentostatin/dAdo-mediated inhibition of phytohemagluttinin-stimulated proliferation of human peripheral blood lymphocytes is also accompanied by the accumulation of dATP (Albert et al., 1984; Bluestein et al., 1978). Human T-cell activation appears to be more sensitive to pentostatin/dAdo than is non-T-cell activation (Cohen et al., 1984). High levels of dATP, however, are not always associated with pentostatin/dAdo inhibition of T-cell function. The combination of pentostatin and low concentrations of dAdo inhibited concanavalin A-stimulated mouse T-cells in the absence of significant dATP levels (Albert et al., 1981) and blocked newly activated human T-cells in the G_0-G_1 interphase 15 hours before the initiation of DNA synthesis (Redelman et al., 1984).

Grever et al. (1983) observed that human lymphocytes, preincubated in vitro for 72 hours in culture medium containing 10^{-6} M pentostatin plus 10^{-6} M dAdo, had significantly reduced lymphocyte antibody and non-antibody-dependent (natural killer cell) cellular cytotoxicity as measured by ^{51}Cr release microcytotoxicity assay. Lymphocytes preincubated with either agent alone under the same conditions had no impairment in cytotoxicity. In this study, although pentostatin completely inhibited lymphocyte ADA activity, the combination of pentostatin/dAdo again produced no significant alterations in intracellular nucleotide pools, suggesting the possibility of alternative mechanisms for impairment of immune effector cells. Wolberg and Zimmerman (1985) also observed that the combination of pentostatin and either Ado or dAdo inhibited lymphocyte-mediated cytolysis. They found that pentostatin/Ado treatment of lymphocytes inhibited lymphocyte-mediated cytolysis of target cells and increased cAMP to that level observed with other lymphocyte-mediated cytolysis inhibitors, such as prostaglandins, histamine, and cholera enterotoxin. However, the mechanism by which pentostatin/dAdo inhibited lymphocyte-mediated cytolysis could not be explained on the basis of elevated cAMP and is still unclear.

Gray and Grever (1982) observed that the combination of pentostatin and dAdo could also inhibit macrophage phagocytosis. At concentrations of pentostatin and dAdo that did not impair macrophage viability (1×10^{-6} M for both), they observed that the phagocytosis of red blood cells sensitized with human IgG anti-D antibody was reduced by as much as 63%.

In in vivo mouse studies with pentostatin, Luebke et al. (1987) reported that, after treating mice with 2 to 4 mg pentostatin/kg body weight intraperitoneally daily for 5 days, the number and relative percentage of circulating lymphocytes decreased 24 and 72 hours after the last pentostatin injection. Lymphoproliferative responses to T-cell mitogens also were suppressed for at least 72 hours after the final injection, but the mixed lymphocyte response was normal after 24 hours but reduced at 72 hours posttreatment. In contrast to the study of Grever et al. (1983), natural killer cell activity was greater in treated mice than in controls. In addition, the antibody responses of mice treated with pentostatin prior to immunization with sheep erythrocytes were suppressed but were enhanced if pentostatin was given after immunization.

A number of investigators have also reported that the timing of pentostatin administration in relation to immunization was critical to the immune response (Ratech et al., 1982, Romo et al., 1988, Thuillier et al., 1981, Uberti et al., 1979a,b). Ratech et al. (1984a) reported similar findings in two different strains of mice (AKR and BALB/c) with three different antigens (sheep erythrocytes, trinitrophenyl-Ficoll and trinitrophenyl-*Brucella abortus*). However, treatment of athymic mice with the same pentostatin protocol (10 mg pentostatin/kg body weight subcutaneously 4 days prior to immunization or 10 mg/kg injected 1 day after immunization followed by 1 mg/kg injected subcutaneously for the next 2 days) caused suppression of antigen plaque-forming cell response regardless of whether pentostatin was given before or after immunization. Based on these results, the authors speculated that pentostatin can have a suppressive effect on B-cells and on suppressor T-cells but appeared to lack an effect on helper T-cells.

Romo et al. (1988) also observed a differential effect of pentostatin on T-cell subpopulations. They found that the addition of pentostatin simultaneously with phytohemagluttinin or concanavalin A to in vitro mononuclear lymphocytes isolated from either infant blood withdrawn from the placental cord or from normal adult volunteers did not affect cell proliferation in either neonates or adult lymphocytes. If pentostatin was added before the mitogens, there was a significant inhibition of adult lymphocyte proliferation but a stimulatory effect in neonate lymphocytes. Veit et al. (1984) carried out somewhat similar experiments with rat splenic T-cells using pentostatin in combination with dAdo and found, like the previous studies, that the timing of pentostatin addition could alter the proliferative response of lymphocytes to mitogens.

Barton (1985) also demonstrated a selective effect of pentostatin on T-cell subpopulations. In normal rats, only cortical thymocytes were significantly affected by a 9-day treatment with 0.25 mg pentostatin/kg body weight. Thymocyte precursors, medullary thymocytes, and peripheral T-cells were unaffected. Ballow and Pantschenko (1981) reported somewhat similar results

from their study in mice. They found that, with different stages of maturation, the susceptibility of thymocyte subpopulations to pentostatin changed, with less mature cortical thymocytes being more sensitive to pentostatin and the more mature medullary thymocytes less responsive to pentostatin.

Immunosuppressive activity of pentostatin, and to a lesser extent by EHNA, was demonstrated by allograft acceptance of LSTRA tumor cells in BALB/c mice across the H-2 histocompatibility locus (Adamson et al., 1978; Chassin et al., 1977). A single dose of 7.5 to 15 mg pentostatin/kg body weight administered 24 hours before the transplant resulted in 53 to 68% allograft acceptance versus 19% acceptance using a similar regimen with 200 mg EHNA/kg. Using the Fisher to Lewis rat islet allograft model (pancreas), Lum et al. (1980) tested the immunosuppressive effect of 2 mg pentostatin/kg body weight (with and without 10 to 20 mg ara-A/kg) in rats and observed that neither ara-A nor pentostatin alone inhibited the immune response to the allograft, but in combination, both compounds delayed allograft rejection.

Ruers et al. (1985a) compared the effect of pentostatin with cyclosporin A on graft survival in a rat skin transplantation model. Pentostatin, given by continuous infusion (0.5 or 0.75 mg/kg, days −1 to 12), was as effective as cyclosporin A using similar conditions in preventing skin graft rejection. At 0.75 mg pentostatin/kg body weight and 40 mg/kg for cyclosporin A, the median skin graft survival time for both drugs was approximately 20 days. In a subsequent study in which the same pentostatin protocol and rat skin transplantation model were used, Ruers et al. (1985b) reported that lymphocytes isolated from animals treated with pentostatin were also inhibited in their response to concanavalin A.

In view of the mild clinical side effects of pentostatin at the lower doses presently used for the treatment of hairy cell leukemia, it is surprising that there have been no clinical studies to examine pentostatin as an immunosuppressive drug. Pentostatin could play a role in organ transplantation, juvenile diabetes, or in other autoimmune diseases such as multiple sclerosis. These areas would offer significant potential for pentostatin therapy. There are also no reports, to date, of the immunosuppressive activity of pentostatin and cyclosporin A together. It would be extremely provocative to combine these drugs and determine whether there is a synergistic effect on the immune system, considering the different mechanisms by which these drugs act.

The immunosuppressive action of pentostatin appears also to have utility in the removal of T-lymphocytes and T-cell neoplasms from bone marrow. A number of laboratories have demonstrated experimentally that pentostatin can selectively purge bone marrow of T-cells, and this elimination may have important implications for graft versus host disease and for allogeneic bone marrow transplantation in patients with leukemia and non-Hodgkins lymphoma (Copelan et al., 1986, 1987, 1988; Fabian and Williams, 1988; Glazer, 1988; Haleem et al., 1987; Johnston et al., 1986; Montgomery et al., 1986, 1990; Russell et al., 1986; Schwartz et al., 1987; Sheridan et al., 1989; Sheridan and Gordon, 1986; Wiedl et al., 1985).

Two major problems of bone marrow transplantation are graft versus host disease and infection. Graft versus host disease can be prevented by the elimination of competent T-lymphocytes from the bone marrow graft. The current methods for this elimination include E-rosetting and monoclonal antibodies (Reinherz et al., 1982) or lectin treatment (Reisner et al., 1981). These methods are not, however, completely effective and present the risk of infection. Fabian and Williams (1988) observed that, in in vitro culture of human bone marrow cells, the combination of pentostatin and Ado eliminated all detectable T-cells, but hemopoietic progenitor cells were unaffected.

In patients with leukemia and non-Hodgkins lymphoma, ablative therapy followed by syngeneic or allogeneic bone marrow transplantation often provide the only hope for long-term survival. Donor suitability, however, restricts this procedure to only 40% of potential recipients. Because the vast majority of remission marrows in patients with leukemia contain undetectable tumor cells, autologous marrow cannot be used as a source of stem cells for marrow rescue unless the remaining malignant cells are eliminated.

The use of immunological and pharmacological purging techniques has met with limited success. Several laboratories have now demonstrated that the combination of pentostatin and dAdo in conjunction with immunoseparation can eliminate T-cell neoplasms from human bone marrow (Copelan et al., 1987; Haleem et al., 1987; Montgomery et al., 1986, 1990; Russell et al., 1986). For example, Haleem et al. (1987) developed an assay that permits the detection of 5 log of T-lymphoma cells in the presence of a 20-fold excess of human bone marrow. Using this method, Haleem and his coworkers combined the use of monoclonal antibodies with pentostatin and dAdo to completely eliminate added malignant T-cells such as Jurkett, HSB, H-9, or T-cell neoplasms from bone marrow of patients with T-cell leukemia. The combined treatment did not affect normal human bone marrow precursors, and one concludes from this study and others that pentostatin might be an important addition to bone marrow purging methodology.

B. Antiviral Activity of Pentostatin Alone and in Combination with 9-β-D-Arabinofuranosyladenine

Ara-A is a clinically useful antiviral agent with low toxicity that inhibits the replication of several viruses; it was the first antiviral agent licensed in the United States for systemic use against herpes infections. Ara-A is,

however, rapidly metabolized both in vivo and in culture by ADA to hypoxanthine arabinosine, which has significantly less antiviral activity than ara-A. A number of investigators have, therefore, combined ara-A with pentostatin and observed a significant increase in the antiviral activity of ara-A against a number of different viruses (Hooper and Woloschak, 1987; Shannon, 1977; Sloan et al., 1977; Wigand, 1979; Williams et al., 1977). For example, the activity of ara-A against Rauscher murine leukemia virus replication was potentiated in vitro by pentostatin (Shannon, 1977). Pentostatin (3.72 μM) itself had no effect against Rauscher murine leukemia virus in Swiss mouse embryo cells, whereas ara-A inhibited virus replication in a concentration-dependent manner. At a concentration of 0.4 μg/mL ara-A, no inhibition of virus replication was observed, but when coincubated with pentostatin, there was a 90% inhibition of replication.

Wigand (1979) examined the inhibition of adenovirus replication by ara-A in several different cell lines and reported that the potentiation of the antiviral effect of ara-A by pentostatin was directly related to the level of ADA activity found in each cell line. Thus, pentostatin appeared to potentiate ara-A activity by preventing its deamination to hypoxanthine arabinosine. Sloan et al. (1977) examined the effect of pentostatin on ara-A activity against HSV/1 and vaccinia virus in HEP-2 cells in vitro. They found that as little as 0.05 μg/mL pentostatin significantly potentiated the antiviral activity of ara-A against both viruses. In mice infected intracerebrally with HSV/1, pentostatin, compared with ara-A alone, dramatically increased the number of 21-day survivors.

One point that should be noted is that viral resistance to ara-A does not appear to be related to cellular ADA levels. Fleming and Coen (1984) isolated five HSV ara-A-resistant mutants and reported that, in plaque reduction assays, the addition of pentostatin to ara-A-treated KOS cells infected with these mutants had no effect on ara-A activity. It appears from their studies that ara-A resistance is the result of altered viral polymerase and not cellular ADA.

Among the dideoxynucleosides studied to date, the purine analog ddAdo shows more selectivity as an inhibitor of human immunodeficiency virus in vitro than do other dideoxynucleosides; it has a 5-fold higher therapeutic index than 3′-azidothymidine (Mitsuya and Broder, 1986). The major antiviral action of ddAdo is thought to be due to inhibition of viral DNA polymerase by dideoxyadenosine triphosphate. ddAdo is activated by cellular, and not viral, enzymes to dideoxyadenosine triphosphate, but ddAdo can also be deaminated by ADA to 2′,3′-dideoxyinosine, which also has activity against human immunodeficiency virus (Cooney et al., 1987). A number of investigators, therefore, carried out studies to determine whether anti-human immunodeficiency virus activity of ddAdo could be potentiated by pentostatin (Agarwal et al., 1989; Carson et al., 1988; Cooney et al., 1987; Johnson et al., 1988). Contrary to expectations, pentostatin did not have a significant effect in increasing ddAdo activity in any of these studies. Because ddADO is susceptible to deamination, this lack of ara-A modulation by pentostatin appeared paradoxical. Recent evidence from Johnson et al. (1988) using CEM cells, however, suggests that activation of ddAdo can occur directly by its phosphorylation to 2′,3′-dideoxy-AMP by either dCyt or Ado kinases or indirectly through deamination to 2′,3′-dideoxyinosine with subsequent phosphorylation to 2′,3′-dideoxyinosine 5′-monophosphate and reamination to 2′,3′-dideoxy-AMP via adenylsuccinate synthetase and lyase.

Finally, it should be pointed out that pentostatin alone has no anti-human immunodeficiency virus activity as determined in the antiviral drug screening assays conducted by the National Cancer Institute Developmental Therapeutic AIDS program.

C. Effect of Pentostatin on Adenosine's Involvement in Central Nervous System Functions

The role of Ado in the modulation of both peripheral and central neurotransmission is supported by a considerable amount of biochemical, electrophysiological, and pharmacological evidence (for reviews, see Franco and Centelles, 1989; Phillis and Wu, 1983; Williams, 1984). In electrophysiological studies, Ado causes depressant actions on spontaneous and evoked synaptic potentials (Kreutzberg et al., 1983). Ado interacts with specific Ado receptors, and the interaction with these receptors can also cause coronary and brain vessel vasodilation, relaxation of smooth muscle, and a lipolytic effect in fat cells (Burnstock, 1986). In the central nervous system, Ado can exert a continuous depression of the firing of some neurons (Phillis and Wu, 1983).

Pentostatin can play a role in neurotransmission and vasodilation by increasing the extracellular levels of Ado, thereby potentiating the physiological effects of this nucleoside (Franco and Centelles, 1989; Skolnick et al., 1978). Phillis and Edstrom (1976) reported that pentostatin, given alone, depressed the spontaneous firing of hippocampal neurons and potentiated the actions of Ado. Radulovacki and his coworkers (Radulovacki, 1985; Radulovacki et al., 1983, 1985) administered 0.5 or 2 mg/kg pentostatin intraperitoneally to rats implanted with electroencephalogram or electromyelogram electrodes. They found that, during 6 hours, pentostatin induced a sleep-like state. The 0.5-mg/kg dose increased REM sleep and reduced REM sleep latency, whereas the higher pentostatin dose increased the deep slow-wave sleep (S_2). The mechanism of this central nervous system depression is probably due to the increased accumulation of Ado in the brain because intracerebroventricular administration of Ado to rats, cats, dogs, and fowls was also found to increase deep slow-wave sleep and total sleep (Feldberg

and Sherwood, 1954; Haulica et al., 1973; Lekic, 1977; Radulovacki, 1985). These results are also consistent with electroencephalogram recordings of patients treated with pentostatin in whom slowing of brain electrical activity developed (Bono and Poster, 1980).

Ado is also a potent cerebral vasodilator (Morii et al., 1986) and is released into the interstitial space of the brain during hypoxia and ischemia (Phillis et al., 1987; Van Wylen et al., 1986; Zetterstrom et al., 1982). Inosine, the product of the deamination of Ado by ADA, is inactive as a vasodilator (Phillis et al., 1988). A number of laboratories have, therefore, examined the effect of pentostatin in cerebral ischemia models and have reported that pentostatin affords protection against cerebral damage elicited by hypoxia or ischemia (Phillis and DeLong, 1987; Phillis and O'Regan, 1988, 1989; Phillis et al., 1988). Pentostatin has been demonstrated to enhance the hypoxia/ischemia-induced release of Ado from rat cerebral cortex with a significant increase in the Ado levels in the interstitial fluid of the cerebral cortex (Phillis et al., 1988). Thus, pentostatin reduces ischemia-induced brain damage by preventing the deamination of Ado and effectively increasing its level in extracellular areas of the brain. Ado may, in turn, prevent brain damage by enhancing the resynthesis of ATP (Foker et al., 1980), inhibiting amino acid release in the ischemic brain (Fastbom and Fredholm, 1985), reducing membrane calcium permeability (Wu et al., 1982), or reducing radical formation (Cronstein et al., 1986), or any combination of these. To date, however, the cerebroprotective effect of pentostatin has not been examined after carotid occlusion. If this can be established, pentostatin may have utility as a therapeutic drug for cerebral ischemic damage.

D. Effect of Pentostatin on Malaria Parasites

The growth and maintenance of malaria parasites depends on a supply of purines from their hosts (Wiesmann et al., 1984). Both the parasites and the red blood cells lack the enzymes for de novo synthesis of purines, and, therefore, the parasites must depend on salvage pathways for synthesis of purine nucleotides (Konigk, 1977). Hypoxanthine has been shown to be the major purine utilized by malaria-infected red blood cells (Webster and Whaun, 1981). One source of hypoxanthine is through the catabolism of Ado, reactions that require ADA and purine nucleoside phosphorylase (Webster et al., 1982). One of the major contributing factors to the successful survival of the malaria parasite appears to be its ability to insert into the red blood cell certain enzymes such as an enhanced ADA (Wiesmann et al., 1984). Wiesmann et al. (1984) infected four adult rhesus monkeys with *Plasmodium knowlesi*. When the parasite levels reached a mean of 5.9% parasitized red blood cells, a single dose of 250 μg/kg pentostatin intravenously was administered. In all four monkeys, ADA levels increased with increasing levels of infection but decreased significantly in response to pentostatin treatment. ADA activity remained inhibited for 3 to 4 days following pentostatin treatment and gradually returned to control levels on day 7. Although levels of parasitized red blood cells also decreased to <1%, they did gradually return after a 2- to 3-day period.

VII. Conclusions

What does the future hold for pentostatin other than as a therapy for hairy cell leukemia? In cancer chemotherapy, pentostatin also has clinical activity in other leukemias. Elucidation of the mechanism of action of pentostatin in clinically responsive leukemias will facilitate the development of preclinical in vitro and in vivo models to study pentostatin action in other tumors. Model development and mechanism of action studies will hasten the rational design of combination chemotherapy for use in cancer treatment.

There also are other diseases in which pentostatin may prove useful as a therapeutic agent. Pentostatin might have efficacy in the prevention of graft versus host disease alone or in combination with cyclosporin A, juvenile diabetes, or other autoimmune diseases, such as multiple sclerosis, sarcoidosis, lupus, Grave's disease, and myasthenia gravis.

Thus, the future clinical use of pentostatin may not be restricted just to treatment of hairy cell leukemia but may have significant clinical efficacy in a number of immunopathological diseases.

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