CONTROL OF ADENOSINE DEAMINASE LEVELS IN HUMAN LYMPHOBLASTS

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INTRODUCTION

Adenosine deaminase (adenosine aminohydrolase, ADA, EC 3.5.4.4) is a purine catabolic enzyme which catalyzes the irreversible hydrolytic deamination of adenosine to produce inosine and equally as well catalyzes the conversion of deoxyadenosine to deoxyinosine (1). While ADA activity is widely distributed in human tissues, its activity is notably highest in lymphoid tissues, such as thymus, spleen, lymph nodes as well as circulating peripheral blood lymphocytes (2–6).

A genetic deficiency of ADA has been causally associated with an autosomal recessive form of severe combined immunodeficiency disease, resulting in profound T cell dysfunction and variable, but usually mild, B cell immunosuppression (7). In an attempt to define the molecular mechanisms in ADA deficiency disease, studies *in vitro* have revealed that the maturation of precursor T lymphocytes is inhibited by an ADA inhibitor while immature B lymphocytes are unaffected (8, 9). Also, the combination of deoxyadenosine and an ADA inhibitor leads to the selective accumulation of dATP and cytotoxicity in cultured T lymphoblasts but not in B lymphoblasts (10), thereby mimicking the ADA deficiency disease. A high level of deoxynucleotide degrading enzyme(s) in B lymphoblasts appears to protect these cells from accumulating toxic levels of dATP (11–13).

An associated, but less well understood, observation is that T cells or their precursors appear to be highly dependent on ADA activity for protection against deoxyadenosine toxicity and for normal cell maturation. Indeed, several studies have shown the level of ADA activity to be markedly elevated specifically in these T cells For example, ADA activity is 3- to 10-fold elevated in normal thymocytes as compared to immature B lymphocytes (4–6, 14, 15) and is 2- to 10-fold higher in T lymphoblasts cultured from patients with acute lymphoblastic leukemia than in cultured B lymphoblasts (5, 14, 16–21).

Possible causes for the elevated levels of ADA activity in T lymphoblasts

might include an alteration in the catalytic or physical properties of the enzyme or might involve induction or stabilization of the enzyme In this study, we have sought an explanation for the different levels of ADA expressed in cultured human T and B lymphoblast cell lines by examining the physical and kinetic properties of the enzyme and measuring its rate of synthesis and degradation in these two cell types

MATERIALS AND METHODS

Materials

[8-^{14}C] Adenosine (59 mCi/mmol) and [U-^{14}C] deoxyadenosine (500 mCi/mmol) were purchased from Amersham/Searle. Protosol, L-[^{35}S] methionine (1000–1100 Ci/mmol) and ^{125}I-labeled goat anti-rabbit IgG (1330 Ci/mmol) were obtained from New England Nuclear. Human erythrocyte ADA and human kidney ADA binding protein were purified to homogeneity and rabbit antiserum was prepared against each protein by previously described methods (22–25) The ADA binding protein was iodinated with carrier-free ^{125}I as previously described (25). All other reagents used were of the highest quality commercially available

Lymphoblast Cell Lines

The T lymphoblast cell lines, MOLT-4, RPMI-8402, CCRF-CEM and CCRF-HSB-2, were obtained from Hem Research (Rockville, MD). B lymphoblast cell lines, GM-130, GM-131, GM-333, GM-621 and GM-1078, were purchased from the Human Genetic Mutant Cell Repository (Camden, NJ), while the cell line, MGL-8, has been previously described (10).

Assays

Determinations of ADA enzyme activity, ADA immunoreactive protein, and total lymphoblast protein as well as assays for the K_m for substrates, K_i for the product, inosine, S_{20w}, and heat stability of ADA have been described (26).

Labeling Procedures for Lymphoblasts

T and B lymphoblasts were harvested during mid-log phase growth and washed in growth medium (RPMI containing 10% horse serum and 2mM glutamine). Cells were resuspended at 1×10^6cells/ml in growth medium containing 10–40 μCi/ml L-[^{35}S]methionine (1 μM) (depending on the cell line and the experiment) and incubated at 37° in an atmosphere of 5% CO_2 and air Cells were labeled for varying periods up to 12 hr, then harvested. Cell suspensions were freeze-thawed 5 times, dialyzed 1/1000 v/v in 10 mM

Tris/HCl, pH 7.4 overnight, and centrifuged at $100,000 \times g$ for 20 min (Beckman Airfuge) to yield a soluble protein cell extract.

For pulse-chase experiments, T and B cells were pulse labeled for 8 to 12 hr in growth medium containing [^{35}S]methionine as described above, washed twice in growth medium and resuspended at $0.7–0.8 \times 10^6$ cells/ml in growth medium containing 2 mM non-radioactive methionine. Cells were harvested at various time intervals and extracts were prepared as above.

Isolation and Analysis of Labeled Lymphoblast ADA

[^{35}S]-Labeled cell extract (either T or B cell extract) was incubated for 15 min at 37° with a 5-fold molar excess of ^{125}I-binding protein to produce a tight binding complex with the labeled lymphoblast ADA. The [^{35}S]ADA-^{125}I-binding protein complex was isolated after native polyacrylamide gel electrophoresis (7.5%, pH 8.9) and subsequently immunoprecipitated with either anti-ADA serum or anti-binding protein serum as described (26). The immunoprecipitated [^{35}S]ADA-^{125}I-binding protein complex was subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis; the gel was then sliced, extracted with a Protosol solution and analyzed for radioactivity in a toluene-based scintillation fluid (26).

RESULTS AND DISCUSSION

Properties of ADA in Cultured T and B Lymphoblasts

Several commercially available human T and B lymphoblast cell lines were harvested during mid-log phase of growth and analyzed for ADA activity. As shown in Table 1, the level of ADA activity was similar in cell extracts of 6 unrelated normal B cell lines with a mean enzyme specific activity of 72 nmol/min/mg (range 66–81). In the 4 different T cell lines studied, the level of ADA activity differed by 2-fold, ranging in specific activity from 475 to 1064 nmol/min/mg. The level of ADA activity in these T cell lines was elevated by approximately 7- and 14-fold over that in the B cell lines, comparable with previous reports (14, 19–21).

In order to determine whether the elevated ADA activity in T cells might be due to enzyme activation or an altered molecular form of the enzyme, the kinetic and physical properties of the enzyme in T cells were compared to that in B cells. In all T and B cell lines, the enzyme exhibited a similar K_m for adenosine (55–70 μM), and deoxyadenosine (50–70 μM), K_i for inosine (600–700 μM) and V_{max} using adenosine. Enzyme assay of mixtures of T and B lymphoblast cell extracts indicated that the quantity of ADA activity expressed was additive, suggesting the absence of any endogenous inhibitors or enzyme activators in these cell extracts. Analysis of all T and B lymphoblast cell extracts by sucrose density gradient centrifugation showed that all of the

TABLE 1 ADENOSINE DEAMINASE IN CULTURED HUMAN LYMPHOBLAST CELL LINES

Lymphoblast Cell Lines*	Sp Act nmol/min / mg cell prot	CRM[††] ng ADA CRM / mg cell prot	Abs Sp Acts[§] μmol/min / mg ADA CRM
B cell			
normal (6)	72 ± 5[†] (66–81)	132 ± 11 (122–149)	545 ± 15
T cell			
MOLT-4	1064 ± 89	1940 ± 132	548 ±22
RPMI-8402	965 ± 52	1803 ± 150	535 ± 14
CCRF-CEM	501 ± 32	969 ± 100	520 ± 23
CCRF-HSB-2	475 ± 12	890 ± 27	534 ± 15

*Protein concentration of lymphoblast extracts ranged from 6–12 mg/ml Number of normal B lymphoblast cell lines examined indicated in parentheses

[†]Mean ± S D (six determinations and 2 harvests of each cell line) Range given in parenthesis

[††]The abbreviation CRM means immunologically cross reactive material

[§]Absolute specific activity of ADA was calculated by dividing the specific activity (nmol/min/mg) by the ng CRM/mg (From Ref 26)

ADA activity was present at an S_{20w} ranging from 3.6 to 3.9. These values were consistent with an S_{20w} of 3 8, previously calculated for purified human erythrocyte ADA ($M_r = 38{,}000$) (22)

The charge properties of T and B cell ADA were examined using urea isoelectric focusing gel electrophoresis. As shown in Figure 1, purified human erythrocyte ADA, added as a standard, exhibits 3 prominent immunoreactive bands detectable by autoradiography after binding with anti-ADA rabbit IgG and ^{125}I-labeled goat anti-rabbit IgG. These immunoreactive bands are similar in distribution and relative intensity to the isozymes previously described for the erythrocyte enzyme using either non-denaturing isoelectric focusing or polyacrylamide gel electrophoresis (2, 22) Figure 1 also shows that under this assay condition, the representative T cell extracts, RPMI-8402 and CCRF-CEM, and the B cell extracts, MGL-8 and GM-130, exhibit only one apparently identical ADA immunoreactive band corresponding to the most basic erythrocyte ADA isozyme

The level of ADA immunoreactive protein termed CRM was determined in all lymphoblast cell extracts by radioimmunoassay. As shown in Table 1, the level of ADA immunoreactive protein in T lymphoblast cell lines was markedly elevated by approximately 7- and 14-fold over that in the B cell lines However, in all cell lines, the amount of enzyme protein was found to be directly proportional to the enzyme activity as noted from the calculated absolute specific activity of the enzyme (Table 1). These data then suggest that the high steady-state level of ADA in T cells probably arises from changes in its rate of synthesis and/or degradation relative to B cells.

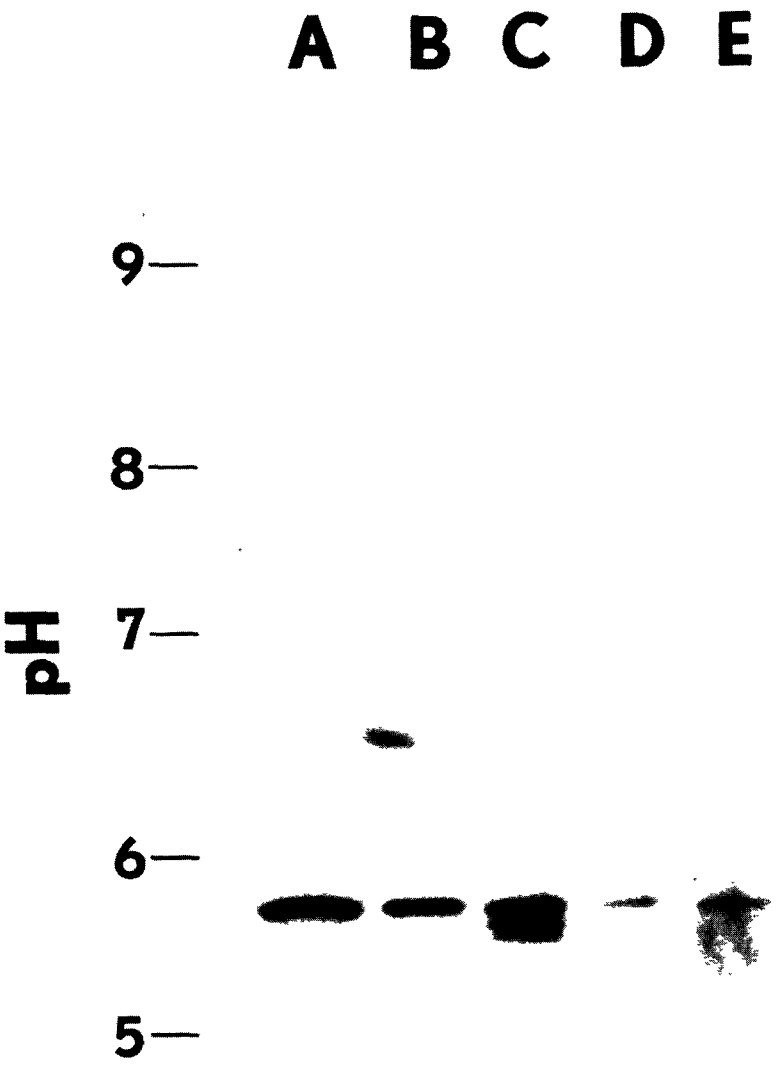

FIG. 1 Urea isoelectric focusing gel of ADA Crude T and B lymphoblast cell extracts and purified erythrocyte ADA were isoelectric focused under denaturing conditions and electrophoretically transferred to nitrocellulose paper Immunoreactive ADA was detected after binding rabbit anti-ADA IgG and ^{125}I-goat-anti-rabbit IgG by autoradiography. A T-cell, RPMI-8402, B T-cell, CCRF-CEM, C purified erythrocyte ADA, D B-cell, MGL-8, E. B-cell, GM-130. (From Ref. 26).

Isolation and Analysis of Labeled Lymphoblast ADA

Measuring the turnover of ADA in cultured T and B lymphoblasts using immunoprecipitation techniques was difficult due to the small amount of ADA protein present in these cells The enzyme was found to represent only 0 19 to 0 09% of total protein in the T cell lines studied and only 0.01% of total soluble cell protein in B lymphoblasts. Preliminary trials to directly immunoprecipitate ADA from a [^{35}S]methionine-labeled T or B lymphoblast extract were generally unsuccessful as judged by SDS-gel analysis. A major contaminant was demonstrated to co-immunoprecipitate and co-migrate with ADA after gel electrophoresis. Thus, a purification step for ADA isolation was designed for use prior to immunoprecipitation, utilizing the complexation of ADA with a specific ADA binding protein (25). This complex formation was found to stabilize ADA catalytic activity allowing for both a purification and good recovery (85–90%) of the enzyme after native polyacrylamide gel electrophoresis. The formation of this ADA complex with a ^{125}I-labeled binding protein also provided a convenient means of detecting the location of the enzyme complex in the polyacrylamide gel by gamma counting and further the ^{125}I-activity could serve as an internal standard for assessing recovery of the complex in the subsequent immunoprecipitation step and SDS-gel analysis (26).

Controls, demonstrating the specificity of lymphoblast ADA immunoprecipitation, are shown in Figure 2. The [^{35}S]ADA-^{125}I binding protein complex was purified from a representative T cell extract, CCRF-CEM, by native polyacrylamide gel electrophoresis, immunoprecipitated with antibody and then analyzed after SDS-gel electrophoresis as described (26). As shown in Figure 2A, when the isolated labeled ADA complex is immunoprecipitated with anti-ADA serum, only two radioactive peaks are apparent after SDS-gel electrophoresis (closed circles). The subunit molecular weights are approximately 94,000 and 40,000 corresponding to the dissociated subunit of ^{125}I-binding protein and [^{35}S]ADA, respectively. When a 100-fold excess of purified erythrocyte ADA is added to the labeled extract prior to immunoprecipitation, the SDS-gel profile reveals that both peaks of radioactivity are prevented from binding to the antibody (Fig 2A, open circles).

In an attempt to further identify the radioactive peak at R_f 0.60 as T cell ADA, immunoprecipitation of gel-purified [^{35}S]ADA-^{125}I-binding protein complex was performed using anti-ADA binding protein serum. As shown in Figure 2B, again only two peaks of radioactivity are detected with subunit molecular weights of 94,000 and 40,000 (closed circles). Note that the peak of radioactivity corresponding to the putative [^{35}S]ADA, R_f 0.60 is quantitatively the same as observed in Figure 2A. However, the peak size of the ^{125}I-binding protein subunit (R_f 0.2) is increased, presumably reflecting the ability of the antibody to quantitatively immunoprecipitate ^{125}I-binding protein

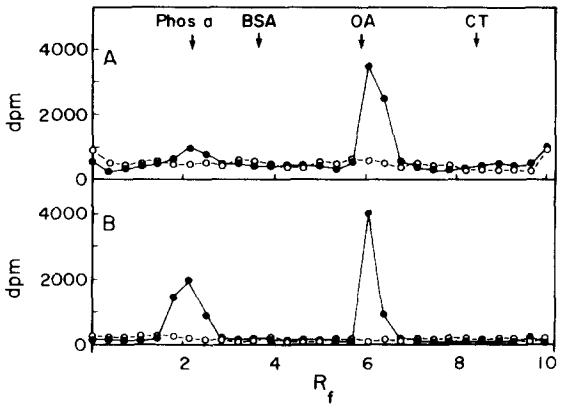

FIG 2 Controls for specificity of ADA immunoprecipitation CCRF-CEM, T-lymphoblasts (2×10^6 cells) were labeled for 12 hr in RPMI medium containing 10% horse serum and 10 μCi/ml [^{35}S]methionine (1 μM final concentration) The [^{35}S]ADA-^{125}I-binding protein complex was formed and isolated from the crude T-cell extract by native polyacrylamide gel electrophoresis The enzyme complex was then immunoprecipitated using antibody and analyzed after SDS-gel electrophoresis The figures show the radioactive SDS-gel profile after immunoprecipitation A with anti-ADA serum (● — ●) and antiserum in the presence of a 100-fold excess of unlabeled purified ADA (○ — ○), and B with anti-binding protein serum (● — ●)and antiserum in the presence of a 100-fold excess of unlabeled purified binding protein (○ — ○) Subunit molecular weight markers are phos a, phosphorylase a, BSA, bovine serum albumin, OA, ovalbumin and CT, chymotrypsinogen (From Ref 26)

whether free or associated with ADA. When a 100-fold excess of unlabeled purified human kidney ADA binding protein is added to the labeled extract prior to immunoprecipitation, both radioactive peaks are prevented from binding to the antibody (Fig 2B, open circles). In this case, the binding protein antibody only recognizes the ^{125}I-labeled binding protein portion of the [^{35}S]ADA-^{125}I-binding protein complex and does not cross-react with the ADA portion of the complex.

Thus the polyacrylamide gel purification step in combination with antibody immunoprecipitation was completely effective in isolating apparently homogeneous lymphoblast ADA and allowed an unambiguous quantitation of [^{35}S]ADA from labeled T cell extract. Similar results were also obtained using labeled B cell extracts (data not shown).

Turnover of Lymphoblast ADA

Using the cell labeling and enzyme isolation techniques described, the apparent rates of ADA synthesis and degradation were determined in representative B and T cell lines By radioimmunoassay, the B cell lines studied, GM-130 and MGL-8, were each shown to have an equal quantity of ADA protein while the T cell lines, CCRF-CEM and RPMI-8402, had a level

of enzyme protein which was elevated by 7- and 14-fold, respectively, over that in the B cell lines

As noted in Figure 3A, the apparent relative rate of ADA synthesis was found to be similar in both B cell lines, GM-130 and MGL-8, (375 dpm/dpm $\times 10^6$ total protein/hr). However, while both T cell lines, CCRF-CEM and RPMI-8402, showed the same apparent relative rate of ADA synthesis (800 dpm/dpm $\times 10^6$ total protein/hr), this rate was found to be 2-fold elevated over that in the B cell lines. Since the rate of ADA synthesis was

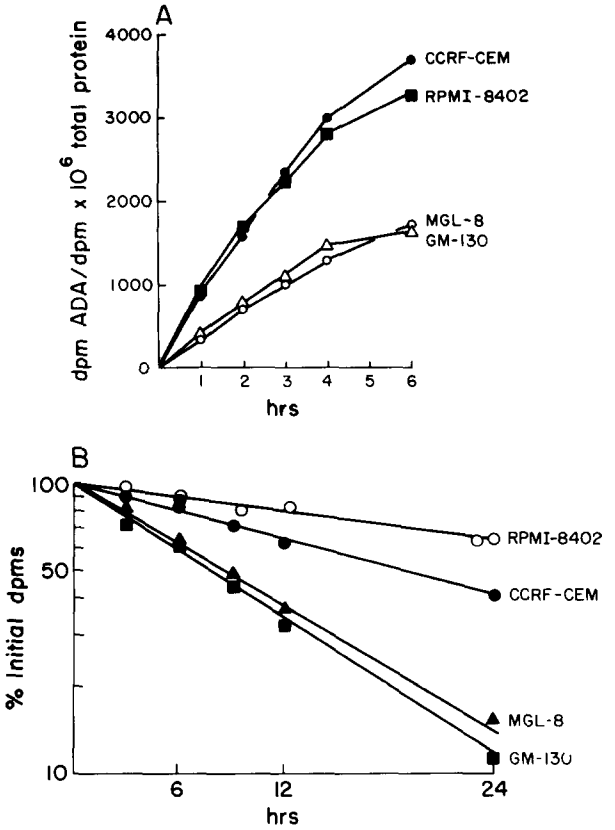

FIG 3 Apparent rate of lymphoblast ADA synthesis A Representative T-cell lines, RPMI-8402 and CCRF-CEM, and B-cell lines, MGL-8 and GM-130, were labeled with [^{35}S]methionine for various time periods and the [^{35}S]ADA isolated and quantified as described in Fig 2 Each time point represents the mean of 4 experiments per cell line performed in duplicate B Apparent rate of lymphoblast ADA degradation The T and B cell lines noted above were pulse labeled with [^{35}S]methionine for 12 hr, resuspended in chase medium and aliquots of cells were removed at specified time intervals The [^{35}S]ADA was isolated and quantified as described The initial dpm of [^{35}S]ADA were between 3000 and 5000 at the beginning of the degradation period for all cell lines studied Each time point represents the mean of 3 experiments per cell line performed in duplicate (From Ref 26)

being compared in different cell types, the apparent rate of [^{35}S]methionine isotope incorporation into ADA was normalized by expressing it as a percentage of the rate of incorporation into total soluble cell protein for each cell line examined. This normalization would correct for any differences between cell types in the rate of isotope uptake or in dilution of isotope specific radioactivity by endogenous unlabeled methionine

When the apparent rate of ADA degradation was examined (Fig 3B), both B cell lines showed a similar apparent rate of enzyme degradation with a $t_{1/2}$ of approximately 7 and 9 hr, while between the T cell lines a 2-fold rate difference was observed with a $t_{1/2}$ of 19 hr for CCRF-CEM and 39 hr for RPMI-8402 Thus, respectively the T lymphoblast cell lines showed approximately a 3- and 6-fold slower rate of ADA degradation relative to that in the B cell lines Thus, from alterations in both the apparent rate of enzyme synthesis and degradation, the T cell lines studied exhibited a 6- and 12-fold difference in net turnover of ADA relative to B-lymphoblast cell lines consistent with the observed differences in enzyme activity

The apparent differences in the rate of enzyme degradation between the T cell lines and among the T and B cell lines could not be rationalized by any structural differences in the ADA, since the size of the protein and its isoelectric pH were identical in all cell lines. As well, there was no evidence of any difference in the stability of the enzyme in these cell extracts as measured by *in vitro* heat lability The possibility still exists that ADA may exhibit a different *in vivo* stability toward proteases in T than in B cells due to intracellular compartmentation or interaction with specific cellular ligands Alternatively, but less likely, different levels of a specific ADA protease in T and B cells may account for the altered rates of enzyme degradation These and other possibilities remain to be explored

We next sought to determine whether the expression of ADA in lymphoblasts could be altered by metabolic effectors T and B lymphoblast cell lines, at an initial cell density of 3×10^5 cells/ml, were individually cultured for 72 hr either alone or in the presence of an additive and cell aliquots were analyzed at 24-hr intervals Under the conditions of this experiment, the level of ADA activity and/or immunoreactive protein in both T and B cells was unchanged when these cells were cultured in the presence of either the ADA substrates, adenosine or deoxyadenosine (1 mM), the products of the ADA reaction, inosine or deoxyinosine (5 mM) or the ADA inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (0.01 mM). Further, a selected group of immunosuppressive drugs, including dexamethasone, hydrocortisone, and prednisolone (tested at a final concentration of 0 1 mM) also failed to change the steady-state level of ADA activity in these cells Thus, the different levels of ADA observed in T and B cells could not be experimentally controlled in our cell culture system suggesting that the enzyme was under strict genetic regulation

SUMMARY

High levels of adenosine deaminase (ADA) activity have been associated with normal T cell differentiation and T cell disease, such as acute lymphoblastic leukemia; however, possible mechanisms controlling the level of this enzyme have not been explored. In this study, the properties and rate of turnover of ADA are compared in cultured human T and B lymphoblast cell lines (1) Relative to B lymphoblasts, the level of ADA activity in extracts of T lymphoblast cell lines (MOLT-4, RPMI-8402, CCRF-CEM and CCRF-HSB-2) is elevated 7- to 14-fold and differs by 2-fold among the T-cell lines. (2) In T and B lymphoblast extracts, the enzyme is apparently identical based on K_m for adenosine and deoxyadenosine, K_i for inosine, V_{max} for adenosine, S_{20w}, isoelectric pH, and heat stability. Further, by radioimmunoassay the quantity of ADA immunoreactive protein is proportional to the level of enzyme activity in all cell lines studied. (3) Using a purification and selective immunoprecipitation technique, the enzyme turnover could be assessed in cell lines labeled with [^{35}S]methionine. The apparent rate of ADA synthesis, relative to total protein, is 2-fold faster in both T cell lines (RPMI-8402 and CCRF-CEM) than in the B cell lines (MGL-8 and GM-130) The apparent half-life ($t_{½}$) for the enzyme degradation is 19 and 39 hr, respectively, for CCRF-CEM and RPMI-8402, while the $t_{½}$ for both B cell lines is 7–9 hr. From the net rate of synthesis and degradation, the T cell lines exhibit a 6- and 12-fold difference in ADA turnover relative to B cells, consistent with the observed differences in enzyme activity. (4) The level of ADA (activity and/or protein) in cultured T or B lymphoblasts is not influenced by either substrates or products of the ADA reaction or an ADA inhibitor or a selected group of immunosuppressive drugs added to these cells in culture.

These studies indicate that while ADA is apparently identical in all T and B lymphoblasts, alterations in both the rate of ADA synthesis and degradation lead to its accumulation and high steady-state level in T cells.

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