Serum Adenosine Deaminase: Isoenzymes and Diagnostic Application

J. P. J. Ungerer, H. M. Oosthuizen, S. H. Bissbort, and W. J. H. Vermaak

Human adenosine deaminase (ADA; EC 3.5.4.4) consists of three isoenzymes: ADA₁, ADA₁-CP, and ADA₂. We developed an electrophoretic technique to distinguish between these three isoenzymes. The isoenzyme pattern was studied in tissue and cell homogenates, as well as in serum from normal subjects and from patients with increased serum ADA who had either hepatitis, infectious mononucleosis, tuberculosis, pneumonia, rheumatoid arthritis, or acute lymphoblastic leukemia (ALL). The highest ADA activity was found in lymphocytes and monocytes. ADA₂ could be detected only in monocytes (18% of total ADA activity). It was also the predominant isoenzyme in the sera of controls and all disease groups, except for ALL—the only condition evaluated that is not of an inflammatory nature. We conclude that serum ADA reflects monocyte/macrophage activity or turnover in most diseases studied. The exception is ALL, where serum ADA most probably originates from lymphocyte precursors.

Additional Keyphrases: enzyme activity · electrophoresis, polyacrylamide gel electrophoresis · lymphocytes · monocytes · leukemia · inflammation

Adenosine deaminase (ADA; adenosine aminohydrolase, EC 3.5.4.4) catalyzes the deamination of adenosine and deoxyadenosine to inosine and deoxyinosine (1). Human ADA exists in at least three molecular forms (2). ADA₁ is a monomeric protein with a molecular mass of ~35 kDa (gene assignment, chromosome 20). ADA₁-CP (molecular mass ~280 kDa) is composed of two ADA₁ molecules connected via a combining protein (CP; binding protein) (gene assignment, chromosomes 2 and 6). The third isoenzyme, ADA₂, appears to be coded by a separate gene locus of unknown chromosomal position (2).

Serum ADA activity is increased in various diseases such as liver diseases, tuberculosis, typhoid, infective mononucleosis, and certain malignancies, especially those of hemopoietic origin (3–5). The origin of serum ADA and the mechanism by which serum activities are increased have not been fully elucidated. This knowledge could enhance the diagnostic utility of serum ADA determinations. Usually, total ADA activity is measured without determining the contribution of each isoenzyme. We endeavored to gain a better understanding of why serum ADA increases by studying its isoenzymes. To accomplish this, we also had to develop an electrophoretic technique that could reliably separate the three isoenzymes. We investigated various tissues and cell types, as well as sera from normal subjects and from patients with various diseases showing increased ADA activity. Only diseases known to have a propensity towards increased activities of serum ADA (3–5), and for which a suitable number of patients could be found to form homogeneous groups, were included in the study.

Materials and Methods

Procedures

ADA activity. ADA activity was determined at 37 °C, according to the method of Giusti and Galanti (4). The substrate in this method is adenosine, and the ammonia liberated is determined by Berthelot’s reaction. We used a control sample (mean ADA, 14 U/L) to determine the precision of this assay; the CV was 10%. (The CV was found to decrease with higher ADA values (4).) One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 µmol of ammonia per minute from adenosine at standard assay conditions. A 50-µL sample is required. Venous blood specimens were collected; only sera free from hemolysis were used, because erythrocytes contain ADA activity and can falsely increase the ADA results for serum (4). Enzyme stability posed no problem: the enzyme is stable in serum for at least 24 h at 25 °C, 7 days at 4 °C, and 3 months at −20 °C (6, 7).

Electrophoresis. We used the method of Buel and MacQuarrie (8), with modifications. For 5% polyacrylamide gel electrophoresis (PAGE; LKB Instruments, Inc., Rockville, MD) we used a phosphate buffer system (0.1 mol/L bridge buffer and 0.05 mol/L gel buffer) at pH 6.7. We applied 10 µL of sample to the gel and carried out the electrophoresis horizontally at 200 V for 5 h at ~4 °C. The isoenzymes were made visible by using an ADA enzyme-staining reaction (9, 10) with a cellulose acetate sheet overlay. The staining reaction mixture was as follows: 24 mg of adenosine, 1 U of nucleoside phosphorylase, 0.5 U of xanthine oxidase (Boehringer Mannheim, Mannheim, FRG), 12 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium salt, <1 mg of phenazine methosulfate (Sigma Diagnostics, St. Louis, MO), and 16 mg of sodium phosphate in 8 mL of staining buffer (0.3 mol/L Tris · 0.2 mol/L histidine · HCl, pH 7.8). A cellulose acetate sheet was soaked with this mixture and applied to the gel, followed by incubation at 37 °C for 1 h. The staining took place almost exclusively in the cellulose acetate sheet. The relative activity of each isoenzyme was determined by densitometrically scanning (Cliniscan II; Helena Labs., Beaumont, TX) the
cellulose acetate sheet without delay after the incubation period. To determine the between-run precision of this technique, we used results from duplicate samples in different runs. The SD of the determination of the percentage of each isoenzyme's contribution to total ADA activity was 6%. The within-run SD was 4%. The sheets can be stored air-dried but the color intensity decreases slowly within hours to days. To verify the positions of the various isoenzymes, we incorporated erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; a gift from Burroughs Wellcome Co., Research Triangle Park, NC), 100 μmol/L, in the staining solution. This substance specifically and completely inhibited only the ADA and ADA1+CP isoenzyme fractions (2, 11, 12).

**Protein determination.** We used a Coomassie Brilliant Blue G250 dye-binding method (Bio-Rad Protein Assay; Bio-Rad Labs., Richmond, CA) to quantify protein.

**Serum enzyme determinations.** Alanine aminotransferase (ALT) and lactate dehydrogenase (LD) were determined with routine, automated methods, with SMAC and RA-XT analyzers (Technicon Instruments Corp., Tarrytown, NY).

**Tissue and Cell Preparations**

The tissue samples were obtained during autopsy within 3 h after an accidental death. Thus, no adverse effects on the enzyme studies should be expected (7). Tissue samples were sonicated in 500 μL of a 10 mL/L aqueous solution of Triton X-100 with a probe sonicator (Soniprep; MSE, UK). We used three 10-s bursts while keeping the specimens cool in ice water. Debris was removed by centrifugation at ~6000 x g for 5 min at 4°C and the supernatant was used directly for analysis. Mononuclear cells were separated from EDTA-treated blood from a normal donor by a Hypaque/Ficoll method (Histopaque-1077; Sigma Diagnostics). Monocytes were separated from the lymphocytes through adherence to Falcon plastic culture flasks (13). Neutrophils were isolated according to Ras et al. (14). The harvested cells were lysed by freezing and thawing at least three times before use.

**Patients**

We used sera from 16 control subjects. The patients included in this study were grouped according to diagnosis: acute viral hepatitis A (21 cases), infectious mononucleosis (15), tuberculosis (12), acute lymphoblastic leukemia (ALL) (8), rheumatoid arthritis (8), and pneumonia (5). The viral infection groups consisted of cases consecutively diagnosed, all having been confirmed with serological tests; serum ADA activities were almost invariably increased in these patients. The patients in the other disease groups were selected on the basis of having serum ADA activity above the normal reference range (14–22 mmol/L), the purpose of this study being directed mostly towards the cause and nature of increases in serum ADA. Patients with these latter diseases often have normal values for serum ADA activity, depending on the stage and activity of the disease. All the patients with ALL in whom serum ADA was increased had blast cell crises.

**Results**

**Electrophoretic Studies**

ADA in erythrocytes showed the typical ADA1 banding pattern (2, 10), which migrated the most anodally. Sera contained two ADA bands, one situated close to the application point and the other in an intermediate position (Figure 1). We used two methods to identify these bands. First, the most-cathodal band was identified as ADA2, because it was the only enzyme band not inhibited by EHNA. EHNA selectively inhibits only ADA1 forms (ADA1 and ADA1+CP) (2, 11, 12). Second, we incubated erythrocyte lysate, which contains only ADA1 (15), with normal serum, which contains excess CP (16, 17). ADA1 (from erythrocytes) and free CP combined to form the ADA1+CP isoenzyme, which occupied the intermediate position (Figure 1), as well described by others (14, 15). The electrophoretic pattern of ADA is schematically represented in Figure 2. In normal sera, ADA2 predominated. By densitometric scanning, the mean ratio of ADA2 to ADA1 was 70/30.

ADA1+CP was the dominant ADA form in liver, lung, muscle, and pancreas tissue; kidney tissue showed only
ADA1+CP (Figures 3 and 4, and Table 1). In contrast, ADA1 was most abundant in the spleen, lymphocytes, monocytes, and neutrophils. ADA2 could be detected only in monocytes, which showed 82% ADA1 and 18% ADA2; no ADA1+CP was discernible. The presence of ADA2 was confirmed with EHNA inhibition studies.

In all the sera with increased ADA, the ADA2 form predominated, except in ALL patients (Figures 1 and 5, and Table 2).

**ADA Activity Studies**

The total ADA activity and isoenzyme activity found in the various tissues are shown in Table 1. The individual isoenzyme activity was determined by using the percentage of total activity as determined by PAGE and densitometric scanning. The highest activity per gram of protein was found in lymphocytes and monocytes. The individual ADA, ALT, and LD values found in each category are shown in Figures 5 and 6. The median enzyme values in each category are summarized in Table 2. The mean serum ADA activity in the control group was 15 U/L, consisting of ADA2 (11 U/L) and ADA1+CP (4 U/L).

**Discussion**

The greatest ADA activity was found in lymphocytes and monocytes. Their activity was ~10-fold that found in the other tissues examined. Only ADA1 and ADA1+CP could be detected in tissue samples, the exception being monocytes, which contained 18% ADA2 (Table 1). This indicates that ADA2 is an enzyme unique to monocyte/macrophage cell lineage. Gakis et al. (18) came to the same conclusion, using different substrates, as well as Kₘ determinations, to distinguish between the isoenzymes.

Normal serum contains ADA1+CP and ADA2 with no ADA1. Because ADA2 could be demonstrated only in monocytes, one might assume that the serum ADA2 originates from this cell type. However, the reason why ADA2 is the predominant serum enzyme, in contrast to the intracellular monocyte ADA pattern (82% ADA1 and 18% ADA2), still has to be determined. Perhaps ADA2 is actively secreted by monocytes or the lifetime of ADA2 in serum is longer than that of ADA1. A third possibility is that certain subpopulations of monocytes might have a different isoenzyme profile. The absence of ADA2 in serum can be explained by the presence of excess CP, so that any ADA1 would be converted to ADA1+CP. This conversion was demonstrated by the incubation of erythrocyte ADA1 and normal serum (Figure 1). The ADA1+CP in serum may thus originate from tissues containing ADA1 or ADA1+CP. We detected ADA1 in a few cases of ALL with very high total serum ADA activity (results not shown), in which any free CP was probably exhausted.

Although both ADA1+CP and ADA2 contribute to the high ADA activity in the patients examined, ADA2 still predominated (Figure 5 and Table 2). The only exception was in patients with ALL, in whom ADA1+CP was relatively greater than ADA2. In all the clinical categories except the ALL group, one would expect cellular immunity, including the monocyte cell lineage, to be involved. This, and the indication that ADA2 originates exclusively from monocytes, would explain the isoenzyme pattern found in these diseases. LD is present in most tissues of the body, and damage to these tissues results in increases in serum LD activity. ALT is specifically present in high amounts in hepatocytes, so that increased activities of ALT in serum reflect hepatocyte damage with enzyme leakage (19). Two additional findings indicate that either the mechanism whereby a serum ADA increase occurs, or the origin of the enzyme, differs from that of ALT and LD. Comparing the enzyme activities shows that the increase of ADA activity relative to that of other enzymes is much greater in infectious mononucleosis than in hepatitis.

Median serum activities of ADA, ALT, and LD in the infectious mononucleosis group were 120, 216, and 261 U/L, respectively, vs 56, 2025, and 310 U/L, respectively, in the hepatitis group (Table 2). Moreover, analyzing the data in the hepatitis group by linear regression, we found no correlation between the serum ADA and serum ALT or LD values (r = 0.08 and 0.07, respectively). In contrast, serum activities of ALT and LD were correlated (r = 0.67). The same trend was observed in the other disease groups studied. Thus, in hepatitis, serum ADA activities seem to reflect monocyte activity or origin, whereas increases in the transaminases reflect hepatocyte damage.
The monocyte/macrophage plays an integral part in cell-mediated immunity. Perhaps human serum ADA, especially the ADA$_2$ isoenzyme, can be a convenient marker to evaluate cell-mediated immunity. Various other tests to measure products or constituents of macrophages have been used for this purpose: e.g., serum lysozyme and serum neopterin determinations (20, 21). Serum lysozyme has the disadvantage of being less specific, in that the enzyme is also present in neutrophils and renal tissue, and its clearance from plasma may vary with renal function (21). Neopterin seems to be produced specifically by activated macrophages in response to interferon-gamma. Diseases known to cause increases in serum neopterin concentrations (20) are also those that cause increases in ADA$_2$ (3-5). However, studies comparing serum ADA activities with other markers that originate from monocyte/macrophage cells are necessary to determine whether the same physiological processes are being reflected.

Only in ALL was ADA$_{1+CP}$ primarily responsible for the increase in total serum ADA (Figure 5 and Table 2). ADA$_1$ is the dominant isoenzyme in lymphocytes. Thus, leakage of this enzyme from lymphoblasts is probably the cause of increased serum ADA in this patient group, the ADA$_1$ being converted to ADA$_{1+CP}$ in plasma. Serum ADA is useful as a tumor marker in evaluations of ALL patients (22). It seems to provide a good indication of the total mass of leukemic cells in bone marrow (22) and may further be of help diagnostically in undifferentiated acute leukemias, as well as in detecting the early onset of blast crisis in chronic myeloid leukemia (23).

In conclusion, in most of the diseases studied the serum ADA seems to originate exclusively from the monocyte/macrophage lineage and therefore reflects the involvement of the cellular immune system. In ALL, on the other hand, lymphoblasts are apparently the source of increased serum ADA. Isoenzyme determinations could be used to differentiate between these two causes (macrophage vs lymphocyte/lymphoblast origin) of increases in serum ADA. The PAGE technique described is suitable for this purpose. We propose, in light of these results, that serum ADA, specifically ADA$_2$, may be an indicator of monocyte/macrophage activation or turnover and could thus be a useful adjunct to the present enzyme repertoire in clinical medicine.

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### References

Table 2. Median Serum Enzyme Activity in Clinical Groups

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Calculations for isoenzyme % and U/L as in Table 1.

Fig. 6. Activities of serum ALT and LD in the seven groups studied

Abbreviations as in Fig. 5