Abstract. Infections are one of the most important complications of hemodialysis (HD). The high concentrations of adenosine (Ado) and of its metabolites during HD may contribute to the dialysis-induced immune deficiency through their known ability to alter lymphocyte function. The influence of HD on Ado metabolism was assessed in mononuclear cells through the measurement of (1) the concentrations of nucleosides in mononuclear cells and (2) the activities of mononuclear cell Ado deaminase (MCADA) and Ado kinase, two enzymes involved in Ado concentration regulation. Nine end-stage renal failure hemo- dialyzed patients (five men and four women; mean age, 69 ± 10 yr) and eight healthy volunteers (four men and four women; mean age, 53 ± 19 yr) were included in the study. Before HD, Ado, deoxyadenosine, and inosine concentrations were respectively 2.9-, 2.5-, and 2.5-fold higher in mononuclear cells of patients than in healthy volunteers. During HD, Ado concentration decreased by 34%, whereas inosine concentration increased by 27%. Before HD, MCADA activity increased by nearly 50% but remained lower than in control subjects. After HD, MCADA activity level of patients did not differ from that of control subjects and was unchanged by HD. The influence of Ado on in vitro mononuclear cell proliferation and interferon-γ production also was evaluated. Ado inhibited cell proliferation and interferon-γ production in a dose-dependent manner, and these inhibitions were stronger for patients than for healthy volunteers. The high concentrations of Ado and deoxyadenosine in mononuclear cells and the low MCADA activity level likely are involved in the immune defect of patients who are undergoing HD.

Despite improvements in dialysis and medical therapies, infections account for a significant proportion (12 to 15%) of deaths in the chronic dialysis population (1). The immune defect observed in patients with end-stage renal disease could explain partly the frequency of infections. However, hemodialysis (HD) actually worsens this immunodeficiency because of recurrent predialytic activation of mononuclear cells induced by blood contact with the dialysis membrane and dialysate, which in turn results in a deactivation state (2). Many humoral and cellular factors likely are implicated in this immune defect (3). Among them, adenosine (Ado) and deoxyadenosine (d-Ado), both strong immunosuppressive agents (4–6), might be implicated because plasma concentrations of Ado (7) and metabolites (8) are high in patients who are undergoing HD.

Ado is released by endothelial cells and by several tissues, more particularly during ischemia (9). Intracellular Ado comes from the hydrolysis of nucleotides through a 5’nucleotidase. d-Ado is formed from the hydrolysis of deoxyadenosine mono- phosphate. The extracellular metabolism of Ado and d-Ado is mediated by two mechanisms. First, Ado and d-Ado are taken up quickly and efficiently by red blood cells, via an equilibrative facilitated diffusion system (10) (Figure 1A). Nucleosides also are taken up efficiently by mononuclear cells via a sodium-dependent concentration system (11). Because of its rapid uptake, the plasma half-life of Ado is very short (a few seconds). Second, Ado and d-Ado are deaminated rapidly into inosine and deoxyinosine, respectively, by adenosine deaminase (ADA; Figure 1A).

ADA is found in large amounts particularly in mononuclear cells (mononuclear cell adenosine deaminase [MCADA]) (12,13), where it plays a major role in Ado concentration regulation in both extracellular (13) and intracellular spaces (14,15). It also is implicated in T-cell activation via noncovalent binding to the T-cell antigen CD26 (16). Intracellular Ado concentration also is regulated strongly by adenosine kinase (AKA) activity (17,18). Indeed, AKA phosphorylates Ado and nucleosides into nucleotides (19). Thus, low MCADA (15) or AKA activity level (20,21) results in an increased intra- and extracellular Ado concentration. A normal MCADA activity level prevents adenosine accumulation and thus ensures normal lymphocyte development and function (22). Inherited ADA deficiency, which induces high Ado and d-Ado concentrations in body fluids, causes severe combined immunodeficiency...
syndrome (5). The immune system defect occurs because of the accumulation of toxic purine metabolites, particularly d-Ado and deoxyadenosine triphosphates (d-ATP), both of which inhibit the ribonucleotide reductase activity of T cells (23) (Figure 1B). Furthermore, lymphocytes are particularly sensitive to Ado and d-Ado because of their ability to accumulate d-ATP (24).

We showed previously that plasma Ado concentration is increased in patients who are undergoing HD (7). Taking into account the facilitated diffusion system and the concentrative sodium-dependent transport system of nucleosides across the cell membrane, we hypothesized that Ado and d-Ado concentrations are high in mononuclear cells of patients who are undergoing HD. Because ADA and AKA are thought to play a major role in the regulation of intracellular nucleoside concentration, we evaluated their activities in mononuclear cells. Finally, we also evaluated the influence of Ado on the T-cell proliferation and interferon-γ (IFN-γ) production in patients who are undergoing HD. We chose IFN-γ rather than interleukin-2 (IL-2) because IFN-γ is a good marker of human peripheral blood lymphocytic activity (25) and because IL-2 has been studied carefully during HD in humans (26,27).

Materials and Methods

Patients

For patients and control subjects, the ingestion of coffee or tea was suspended 72 h before samples were taken. Patients who had been treated with papaverine, diprydamole, or immunosuppressive agents during the preceding 6 mo were excluded from the study. All participants gave their consent according to the Helsinki convention. Nine adults (five men and four women) on maintenance HD, who were randomly chosen among 128 patients with end-stage renal failure in the same center, constituted the group of patients who were undergoing HD. Their mean age ± SD was 69 ± 11 yr (range, 49 to 85 yr), and their mean duration of maintenance HD was 47 ± 34 mo (range, 17 to 132 mo). Causes of end-stage renal failure were diabetic nephropathy (n = 2), chronic glomerulonephritis (n = 1), interstitial nephritis (n = 1), polycystic kidney disease (n = 1), nephroangiosclerosis (n = 3), and cholesterol embolism (n = 1). No patient had anti–human immunodeficiency virus or hepatitis B surface antigen or hepatitis C antibodies. The patients underwent HD three times per week for 5 ± 1 h with cellulose diacetate low-flux membranes (A15, A18; Althin, Miami, FL). The hemodialyzers were never reutilized, with a diode array detector. Lyophilized samples (500 µg) were used, with a diode array detector. Lyophilized samples (500 µg) were assayed for ADA and AKA activity levels.

Blood Samples for Ado, d-Ado, and Inosine Assays

Sample collection has been described elsewhere (28,29). Briefly, blood collected from the brachial vein (8 ml/sample) was drawn into vacuum tubes containing a stopping solution (0.2 mM dipyridamole, 4.2 mM Na₂-ethylenediaminetetraacetic acid, 5 mM 9-erythro (2-hydroxy-3-nonyl) adenine, 79 mM 2'-deoxyadenosine-5'-diphosphate, 1 IU/ml heparin sulfate, 200 µg/ml dcf, and 0.9% NaCl), which prevents degradation and uptake of Ado. The samples were transferred to special Vacutainer tubes (Ficoll-based CPT system; Becton Dickinson, Le Pont de Clai, France) and centrifuged at 500 × g for 30 min. Then 1 ml of interphase cells was pipetted off and washed three times with 3 ml of the stopping solution before assessment of cell viability by trypan blue dye test exclusion. Granulocyte contamination and mononuclear cell number were measured (Coulter Beckman, Fullerton, CA). These techniques resulted in cell preparations that were >98% viable and that contained <3% of granulocytes. Lymphocyte proportion was always more than 95%. Cells were suspended in stopping solution (6.5 ± 1.5 × 10⁶ cell/ml) and frozen (−80°C). Samples were submitted to four freeze-thaw cycles (−80°C, +37°C) and centrifuged (2500 × g for 10 min) to obtain clear supernatant cell extracts. Supernatants were deproteinized by addition of 100 µl of perchloric acid (6N) and then centrifuged (1500 × g for 10 min). Samples were lyophilized before being chromatographed.

Sample Preparation for Determining MCADA and AKA Activities

We used the procedure previously described (12) with some modifications. Briefly, samples (8 ml) of whole blood were collected from each patient and healthy volunteers in special Vacutainer tubes (see above) and then centrifuged at 500 × g for 30 min. A 1-ml sample of interphase cells (7.6 ± 1.10⁶) was pipetted off and washed four times with 3 ml NaCl 0.9%, to eliminate plasmatic ADA. Aliquots of 1 ml were submitted to four freeze-thaw cycles before centrifugation (2500 × g for 10 min) to obtain clear supernatant cell extracts. The cell extracts were assayed for ADA and AKA activity levels.

Ado, d-Ado, and Inosine Assays

The technique has been described elsewhere (28,29). Briefly, a Hewlett Packard HP 1100 modular system (Lesulils, France) was used, with a diode array detector. Lyophilized samples (500 µl) were mixed with 1 ml of phosphate buffer (NaH₂PO₄/Na₂HPO₄ [pH 4]), injected in a 1-ml loop, and then eluted with a methanol gradient on
a Merck LIChrophor C18 column (0% for 3 min, then 10 to 25% methanol for 15 min). The intra- and interassay coefficients of variations for nucleosides ranged between 1 and 3%. The limit of detection at 254 nm was 1 pmol in 1 ml of plasma matrix injected.

**Identification and Quantification**

Retention times and spectra were compared with those of exogenous adenosine and metabolites. Quantifications were made by comparing areas obtained for samples with areas of known quantities of nucleosides.

**MCADA Activity**

We used the technique previously described (30), with some modifications. Briefly, 750 µl of 28 mM Ado was mixed with 125 µl of cell extracts and with 125 µl of bovine serum albumin 7% in NaCl 0.9%. Aliquots then were incubated for 1 h at 37°C. The reaction was started by adding the substrate and was stopped by cold immersion. The Johnson and Johnson Clinical Chemistry colorimeter test was used to quantify the ammonia concentrations. The intra- and interassay coefficients of variation ranged between 3 and 5%.

**AKA Activity**

We used the procedure previously described (31), with some modifications. Briefly, 125 µl of cell extracts was incubated (37°C for 20 min) with 875 µl of a solution (NaH₂PO₄/Na₂HPO₄ [pH 5.5]), supplemented with 1 mM 6-MMPR (substrate), 1 mM ATP, 1 mM MgCl₂, and 1 mM dithiothreitol. The reaction was started by adding the substrate and stopped by adding 100 µl of perchloric acid (6N). Samples were deproteinized by centrifugation (1500 × g for 10 min), and the supernatant was lyophilized before chromatography analysis. Lyophilized samples were dissolved in 1 ml of phosphate buffer and then chromatographed (Merck RP8 column) at 300 nm. The samples were eluted in a 20 to 65% methanol gradient for 30 min. 6-MMPR-5-phosphate was identified by elution time and spectra comparison and quantified, as were the nucleosides. The intra- and interassay coefficients of variation ranged between 1 and 4%.

**Mononuclear Cell Proliferation**

Mononuclear cells of patients or healthy volunteers were obtained as described above. Cells (4.5 ± 1 × 10⁶/0.5 ml) were cultured in a CO₂ incubator at 37°C, for 48 h in 1.5 ml of RPMI 1640, with 4% of fetal bovine serum. Cells were preactivated with Con A (1 ng/ml). Aliquots obtained from patients or healthy volunteers were separated into four groups: one with dcf (an adenosine deaminase inhibitor), 1 nM/ml; one with dcf + Ado (1 nM/ml); one with dcf (1 nM/ml) + Ado (3 nM/ml); and one control. Aliquots (50 µl) were pipetted off and examined for cell viability (trypan blue dye exclusion test) and count per milliliter with Coulter Epics XL) at time 0, then after 24 and 48 h of incubation.

**IFN-γ Assay**

IFN-γ was assayed on cell supernatant after 24 and 48 h of activation and culture (concannavalin 1 ng/ml). The IFN-γ concentrations were measured by use of the quantitative sandwich enzyme immunoassay, as recommended by the manufacturer (Immunotech, Marseille, France). This immunoassay uses an immobilized monoclonal antibody specific for human IFN-γ and a second monoclonal anti–IFN-γ antibody that is biotinylated. The binding of streptavidin-horseradish peroxidase conjugate to the human complex is followed by the addi-
tion of a chromogenic substrate of the peroxidase. The sensitivity of the assay was 0.08 IU/ml. The intra- and interassay coefficients of variation ranged between 5 and 10%.

**Statistical Analyses**

ANOVA one-way analysis was used to compare nucleoside concentrations and enzyme activity levels between patients and control subjects. The Wilcoxon test was used for intragroup comparisons (cell number and IFN-γ production as a function of time in the same group). The Spearman coefficient of correlation was used for correlation studies. *P* > 0.05 was considered significant.

**Results**

**Intra–Mononuclear Cell Nucleoside Concentrations**

Before HD, Ado, d-Ado, and inosine concentrations (in pmol for 10^7 cells) were, respectively, 2.9-, 2.5-, and 2.5-fold higher in patients than in control subjects (32.6 ± 10 pmol versus 11 ± 2 pmol; 30 ± 17 pmol versus 12 ± 2 pmol; 48 ± 19 pmol versus 19 ± 4.7 pmol; Figure 2A). After HD, Ado concentration decreased (34% on average, 32.6 ± 10 versus 21.6 ± 7.9; *P* < 0.03). d-Ado concentration did not decrease significantly (30 ± 17 versus 26 ± 13), but inosine concentration increased significantly by 27% (48 ± 19 versus 61 ± 12.7; *P* < 0.02).

**MCADA and AKA Activities**

Before HD, MCADA activity level was 2.07-fold lower in patients than in control subjects (80 ± 18.6 versus 166 ± 33 IU; ANOVA *P* < 0.001; Figure 2B). After HD, MCADA activity level increased significantly (80 ± 18.6 versus 118 ± 24 IU; *P* < 0.01) but remained lower than that in control subjects (118 ± 24 versus 166 ± 33 IU; ANOVA *P* < 0.005). Before and after HD, AKA activity level was not significantly different in patients and in healthy volunteers (49 ± 9 versus 51 ± 17 and 49 ± 9 versus 47 ± 16 IU, respectively; *P* > 0.05).

**Correlations between Nucleoside Concentrations and MCADA Activity Levels**

There was an inverse correlation between intramonicellular cell nucleoside concentration and MCADA activity level in healthy volunteers (Spearman’s *r* = −0.8; *P* < 0.05 for both Ado and d-Ado). Before HD, there was an inverse correlation between Ado or d-Ado concentration and MCADA activity level (*r* = −0.73 and *r* = −0.55, respectively; *P* < 0.05). After HD, the correlations persisted (*r* = −0.8 and *r* = −0.67; *P* < 0.05). Finally, there was an inverse correlation between the MCADA activity increase during HD and the duration of the dialysis treatment (*r* = −0.85; *P* < 0.005; Figure 3).

**Effects of Ado on Mononuclear Cell Number and IFN-γ Production**

In control conditions (Figure 4A), cell proliferation at 48 h was greater in healthy volunteers (+17% compared with 24 h) than in patients (+12%; *P* < 0.05).

With dcf alone (Figure 4B), cell proliferation was slightly inhibited after 48 h of incubation but less so in healthy volunteers than in patients (*P* < 0.05). With dcf and Ado 1 μM (Figure 4C), cell number at 48 h was lower than that with dcf alone (Figure 4B). With dcf and Ado (3 μM; Figure 4D), cell number decreased significantly at 48 h (*P* < 0.05 compared with 24 h); however, this decrease was larger in patients (−22%) than in healthy volunteers (−16%; *P* < 0.05).

IFN-γ concentration in the supernatant of Con A–treated cells was lower in patients than in healthy volunteers (*P* < 0.03 at 48 h; Figure 5A). With dcf alone (Figure 5A), IFN-γ concentration was slightly inhibited as early as 24 h only in patients (*P* < 0.02 compared with without dcf) and in both patients and healthy volunteers at 48 h (healthy volunteers with dcf versus healthy volunteers without dcf, *P* < 0.02). With Ado (1 μM) and dcf (Figure 5B), IFN-γ concentration decreased both in healthy volunteers (mean, −85% at 24 h and...
–95% at 48 h) and in patients (mean, –77% at 24 h and –96% at 48 h), compared with dcf alone. With Ado (3 \mu M) and dcf, IFN-\gamma concentration decreased by 92% at 24 h and by 98% at 48 h in patients and by 92% at 24 h and 98% at 48 h in healthy volunteers.

Discussion
Abnormal Levels of Ado an Its Metabolites and Enzymes in Patients Who Were Undergoing HD

We found high concentrations of Ado and d-Ado in mononuclear cells before HD. These high intracytoplasmic concentrations are due partly to the low MCADA activity, because AKA activity was normal. Indeed, Ado and d-Ado concentrations were highly correlated with MCADA activity levels. Alternatively, the high intracellular nucleoside concentrations can be explained by the high plasmatic Ado and metabolite concentrations in patients who were undergoing HD (7,8). This hypothesis is supported by the equilibrative facilitated diffusion system and the concentrative sodium-dependent nucleoside transports in mononuclear cells. During HD, Ado and d-Ado concentrations decreased, whereas MCADA activity levels increased by nearly 50%, but the latter remained lower than in control subjects. The decrease in Ado and d-Ado may be due to the rise in MCADA level, because inosine, the product of Ado deamination, increased concomitantly. This increase in inosine level may participate in the activation of lymphocytes that is induced by HD. Indeed, inosine contained in dialyzable leukocyte extracts activates lymphocytes (32).

Why is MCADA activity low at the basal state in patients who are undergoing HD? One hypothesis is that uremic toxins depress MCADA activity. This has been suggested by studies that showed improved erythrocyte ADA activity during HD sessions (33). This also is suggested by our results that showed increased MCADA activity during HD. However, we did not retain this hypothesis because in previous studies, we demonstrated that undialyzed patients with chronic renal failure had normal MCADA activity levels (7) and normal plasmatic Ado concentrations (34). Another hypothesis is that the decrease in MCADA activity results from the deactivation of lymphocytes. During HD sessions, lymphocytes are activated by contact with the extracorporeal circuit (26,27,35). This activation also is shown by the predialytic increase in MCADA activity that we observed, yet MCADA expression markedly increases when T cells are activated (36,37). The repeated activation of lymphocytes during HD sessions may in turn lead to cell deactivation. Such a phenomenon has been reported for IL-2 receptors, whose number decreases with time during long-term HD treatment (27).

Mononuclear Cell Abnormalities

Many studies have demonstrated the negative impact of Ado and d-Ado on lymphocyte functions. Indeed, T cells exposed to nucleosides, in a medium with low ADA activity, accumulate

**Figure 3.** Correlation curve between the increase in MCADA activity during the HD session and the duration of the HD expressed in months. Spearman’s \( r = -0.85; \ P < 0.005 \).

**Figure 4.** Effects of adenosine and deoxycoformycin (dcf) on mononuclear cell proliferation of nine hemodialyzed patients (■) and eight healthy volunteers (▲). dcf was added to prevent quick deamination of Ado into inosine. Cells were activated with concanavalin A (1 ng/ml) and were examined for cell viability (trypan blue dye test and count, Coulter Epics XL) 24 and 48 h after incubation. Results are expressed for \( 10^6 \) cells at the beginning of the incubation. (A) Spontaneous proliferation; (B) cells with dcf (1 \mu M); (C) cells with dcf (1 \mu M), and Ado 1 \mu M; and (D) cells with dcf (1 \mu M) and Ado 3 \mu M. a, ANOVA \( P < 0.05 \) compared with patients; b, Wilcoxon test \( P < 0.05 \) compared with 24 h; c, ANOVA \( P < 0.05 \) compared with dcf alone at the same time (in reference to B); d, ANOVA \( P < 0.05 \) compared with spontaneous proliferation (in reference to A). Statistical analysis was performed only in the case of an overlap between SD.
d-Ado and d-ATP (4,24), which inhibit both ribonucleotide reductase and, hence, DNA synthesis (4) (Figure 1B). d-Ado also is toxic for ADA-inhibited human peripheral blood lymphocytes (38). d-Ado also has been reported to block RNA synthesis (39) and to foster an accumulation of strand breaks in DNA (40). Moreover, in mixed lymphocyte culture, d-Ado decreases IL-2 production and inhibits IL-2 receptor expression (41). d-Ado at 1 to 3 μM blocks the transition of the stimulated lymphocytes from G0 to G1 via the inhibition of protein phosphorylation (6). Finally, an excess of nucleosides induces apoptosis in human peripheral blood mononuclear cells (42), and adenosine analogs induce apoptosis in normal and neoplastic lymphocytes (43,44). The sensitivity of T cells to Ado was attributed to their high nucleoside kinase activity (45).

The impact of Ado and d-Ado has never been studied in lymphocytes from patients who are undergoing HD. We found that Ado induced a dose-dependent decrease in cell number and IFN-γ production. Furthermore, activated lymphocytes from patients, in the absence of any drug except Con A, proliferated less than those from healthy volunteers and were more sensitive to Ado and/or dcf. Because the intracellular ADA level is lower in patients, we hypothesized that mononuclear cells of patients are more sensitive to dcf-induced ADA decrease and then Ado increase. This hypothesis is supported by the results of Dong et al. (46) showing that CD26-transfected Jurkat cells, which express high quantities of MCADA, are more resistant to the inhibitory action of Ado on cell proliferation and IL-2 production.

We also found that the activation of lymphocytes by Con A made IFN-γ production lower in patients than in healthy volunteers. Moreover, IFN-γ production decreased in the presence of Ado but more so for patients than for healthy volunteers.

In conclusion, we found high Ado and d-Ado concentrations and low MCADA activity levels in the mononuclear cells of patients who were undergoing HD. These may participate in the immune deficiency of these patients. Further investigations are needed to determine how HD induced MCADA deficiency.

References

Figure 5. IFN-γ concentration (in IU for 10^6 cells) in the supernatant of activated lymphocytes (concanavalin A 1 ng/ml) of nine patients (●) and eight healthy volunteers (□). IFN-γ was assayed for patients and healthy volunteers 24 and 48 h after activation with dcf alone (1 μM) or in association with Ado (1 or 3 μM) and finally without drug, except concanavalin A. dcf was added to prevent the quick deamination of Ado into inosine. (A) Effects of dcf in comparison with spontaneous proliferation. (B) Effects of Ado (1 or 3 μM) in comparison with dcf. A variance analysis (ANOVA, one-way analysis) was used for intergroup comparison. Wilcoxon test was used to compare IFN-γ production as a function of time. Statistical analysis was performed only in the case of an overlap between the values. A, ANOVA P < 0.05 compared with healthy volunteers; b, Wilcoxon test P < 0.05 compared with 24 h.


24. Mitchell BS, Mejias E, Daddona PE, Kelley WN: Purinogenic immunodeficiency diseases: Selective toxicity of deoxyribo-


