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Effect of immunosuppressive drugs on the release of metalloproteinases from human polymorphonuclear leukocytes

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Abstract. The concentration of the metalloproteinases type I collagenase and gelatinase was measured in isolated polymorphonuclear leukocytes (PMNLs) of renal transplant recipients treated either with cyclosporin A (CyA) and prednisolone (Pr) ($n = 8$) or azathioprine (Aza) and Pr ($n = 8$), and of healthy subjects ($n = 12$). PMNLs of CyA- and Aza-treated transplant patients displayed markedly higher gelatinase content (2427 ± 489 and 3284 ± 357 ng/10^7 cells) than PMNLs of controls (528 ± 83 ng/10^7 cells). There was also a higher content of type I collagenase in PMNLs (3374 ± 292 ng/10^7 cells) of Aza-treated patients and significantly elevated levels in PMNLs of patients receiving CyA (3625 ± 229 ng/10^7 cells) compared with healthy subjects (2878 ± 151 ng/10^7 cells). In contrast, neutrophil lactoferrin content was lower in transplant patients. Thus, immunosuppressive drugs may reduce the release of leukocyte proteinases, which are known for their deleterious role in proteolytic tissue and matrix breakdown. In vitro, the effects of different immunosuppressive drugs on the release of lactoferrin, collagenase and gelatinase were investigated on FMLPNTL-stimulated PMNLs isolated from healthy subjects. CyA but not Aza or Pr caused inhibition of gelatinase, collagenase and lactoferrin release.

Key words: Immunosuppression and metalloproteinases – Metalloproteinases and immunosuppression

Recent studies have demonstrated that cyclosporin A (CyA) reduces degranulation of polymorphonuclear leukocytes (PMNLs) in patients following cadaveric renal transplantation by measuring neutrophil elastase, myeloperoxidase and lactoferrin [10]. The neutral proteinases, elastase and chymotrypsin-like proteinase (Cathepsin G) are present in primary (azurophil) granules of human PMNLs, probably together with myeloperoxidase [3, 15], whereas collagenase, lactoferrin and vitamin B12 binding protein are localized in specific granules [8]. A small vesicular organelle (C particle) has been described by Dewald et al. [6] containing the metalloproteinase gelatinase. Recent data suggest that gelatinase is a compound of specific granules [9]. This enzyme was first purified by Sopata and coworkers [19, 20]. The molecular weight of the latent human PMNL gelatinase was found to be approximately 98 kD (SDS-Page, reducing condition) [12]. The enzyme was shown to be identical with type IV/V collagenase described by Tschesche et al. [21]. PMNL procollagenase was purified to homogeneity and showed an apparent molecular weight of 85 kD [22]. Proteolytic processing of the procollagenase by PMNL elastase resulted in an additional latent form with an apparent molecular weight of 70 kD [11].

Increasing evidence suggests that proteolytic enzymes of PMNLs are not only involved in intracellular breakdown of ingested phagocytized material, but are also released into surrounding tissue and interstitial fluid as well as into circulating blood.

The present study was designed to investigate whether different immunosuppressive drugs affect main granulocyte components in vitro and in vivo with particular reference to the metalloproteinases gelatinase and type I collagenase.

Patients and methods

Informed consent was obtained from all patients prior to the study. Three groups of patients were investigated. Group 1 consisted of eight renal transplant patients treated with CyA and prednisolone (Pr). The mean age of the group was 41.2 ± 3.9 years, and mean duration of transplantation was 39.1 ± 7.1 months. Group 2 consisted of eight renal transplant patients treated with azathioprine (Aza) and Pr. The mean age of this group was 46.0 ± 2.6 years and mean duration of transplantation was 102.6 ± 9.6 months. Twelve healthy persons with a mean age of 30.8 ± 3.2 years acted as controls.

PMNLs were prepared from 10 ml whole blood anticoagulated with 1.5% Na-EDTA in phosphate buffered saline (PBS) as described by Harbeck et al. [7]. Five mililiters 63% Percoll was placed in 15 ml conical tubes and underlayered with 5 ml 72% Percoll. A 5 ml sample of EDTA-blood was introduced into this gradient and the mixture was centrifuged at 500 *g* for 25 min at room temperature.

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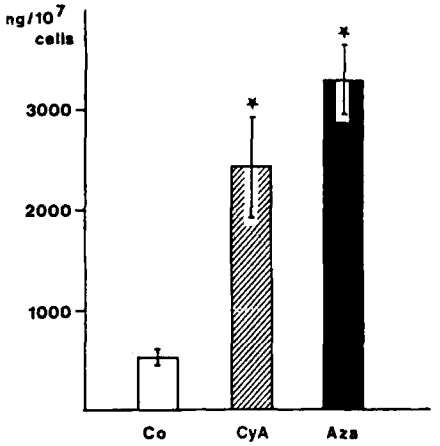

Fig. 1. Gelatinase concentration in PMNLs of healthy controls *(Co)* ($n = 12$) and renal transplant patients treated with cyclosporin A and prednisolone *(CyA)* ($n = 8$) or azathioprine and prednisolone *(Aza)* ($n = 8$). Mean values ± SEM. * $P < 0.05$ controls versus renal transplant patients

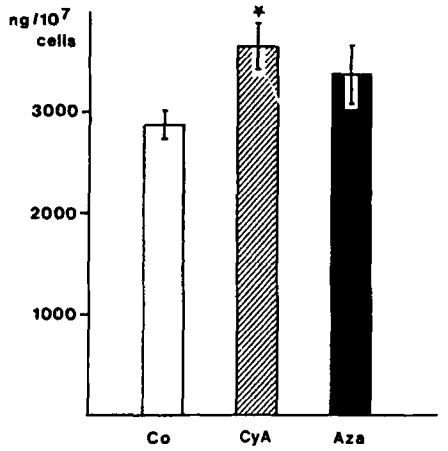

Fig. 2. Type I collagenase concentration in PMNLs of healthy controls *(Co)* ($n = 12$) and renal transplant patients treated with cyclosporin A and prednisolone *(CyA)* ($n = 8$) or azathioprine and prednisolone *(Aza)* ($n = 8$). Mean values ± SEM. * $P < 0.05$ controls versus renal transplant patients

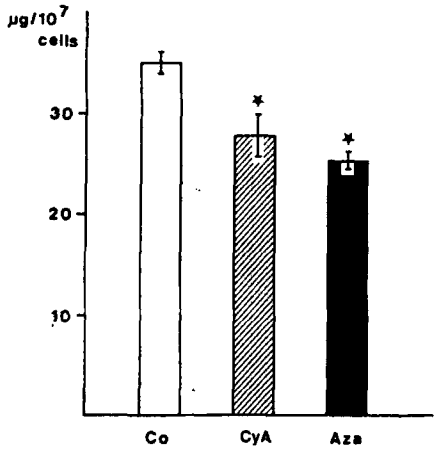

Fig. 3. Lactoferrin concentration in PMNLs of healthy controls *(Co)* ($n = 12$) and renal transplant patients treated with cyclosporin A and prednisolone *(CyA)* ($n = 8$) or azathioprine and prednisolone *(Aza)* ($n = 8$). Mean values ± SEM. * $P < 0.05$ controls versus renal transplant patients

Table 1. *Clinical data and blood chemistry of controls and renal transplant patients under immunosuppression with cyclosporin A (CyA) and prednisolone or azathioprine (Aza) and prednisolone*

Patients	Age (years)	Creatinine (mg/dl)	Urea (mg/dl)	Leukocytes (cells/µl)
Controls	30.8 ± 3.2	1.0 ± 0.03	28.4 ± 3.4	6600 ± 448
CyA	41.0 ± 3.9	1.5 ± 0.20***	54.3 ± 5.9***	7000 ± 1198
Aza	46.0 ± 2.6	1.1 ± 0.08	30.7 ± 4.2	6500 ± 544

Mean values ± SEM
* $P < 0.05$ controls versus CyA and Aza
** $P < 0.05$ CyA versus Aza

PMNLs were harvested in the supernatant and were washed twice with PBS free from Ca^{2+} and Mg^{2+}. The cells were then resuspended in PBS.

After addition of 0.5% Triton X100 to the cell suspension to facilitate destruction of PMNL granules, total release was assured by pulsed sonification for 30 s with a Branson sonifier (B-15, duty cycle 50%, output control 5) while cooling on ice. Cell membrane fragments were removed from the sample by centrifugation at 20000 *g* for 30 min. Human leukocyte collagenase and gelatinase were determined in the supernatant using a competitive- and sandwich-enzyme-linked immunosorbent assay [1]. PMNL lactoferrin was also determined with an enzyme-linked immunosorbent assay using commercially available antibodies and antigen. To prevent destruction of antibodies by simultaneously released serine proteinases, this class of enzymes was inhibited by the addition of 0.4 mmol/l diisopropylfluorophosphate to the sample before examination.

In vitro studies were performed to evaluate the effects of different immunosuppressive drugs on the release of lactoferrin, collagenase and gelatinase. PMNLs were isolated from ten healthy subjects (mean age 26.0 ± 5.0 years) as described above. After in vitro incubation of 2×10^6 cells/ml at 37°C for 5 min, neutrophils were stimulated with 10^{-8} and 10^{-9} mol/l of FMLPNTL (Formyl-Norleucyl-Leucyl-Phenylalanyl-Norleucyl-Tyrosyl-Leucin). After 5 min of incubation, lactoferrin, collagenase and gelatinase were determined as described above. Unstimulated PMNLs acted as controls. CyA, Pr and/or Aza were also added in final concentrations of 400 ng/ml, 100 ng/ml and 3 µg/ml, respectively. The vehicle for CyA had no effect on neutrophil degranulation.

Statistical methods

Data are given as mean values ± SEM. For analysis of significance, the paired and unpaired Wilcoxon tests were used.

Results

Table 1 summarizes the clinical and laboratory findings from eight renal transplant patients under immunosuppression with CyA and Pr, eight patients treated with Aza and Pr and twelve healthy subjects as controls.

Figure 1 represents the gelatinase content of PMNLs of transplant patients and controls. PMNL gelatinase concentration was significantly lower in controls (528 ± 83 ng/10^7 cells) compared with transplant patients treated with Aza (3284 ± 357 ng/10^7 cells) and with CyA (2427 ± 489 ng/10^7 cells). The results obtained with type I collagenase are shown in Fig. 2. PMNLs of controls contained lower levels of type I collagenase (2878 ± 151 ng/10^7 cells) compared with patients treated with Aza (3374 ± 292 ng/10^7 cells) and CyA

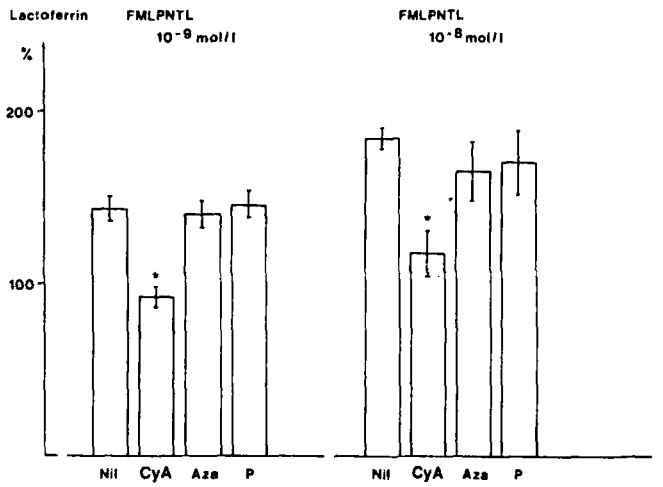

Fig. 4. Effect of cyclosporin A *(CyA)*, azathioprine *(Aza)* and prednisolone *(Pr)* on the release of lactoferrin from FMLPNTL-stimulated PMNLs isolated from healthy subjects. Lactoferrin release without stimulation was 100%. Mean values ± SEM. * $P < 0.05$ *nil* versus *CyA, Aza, Pr*

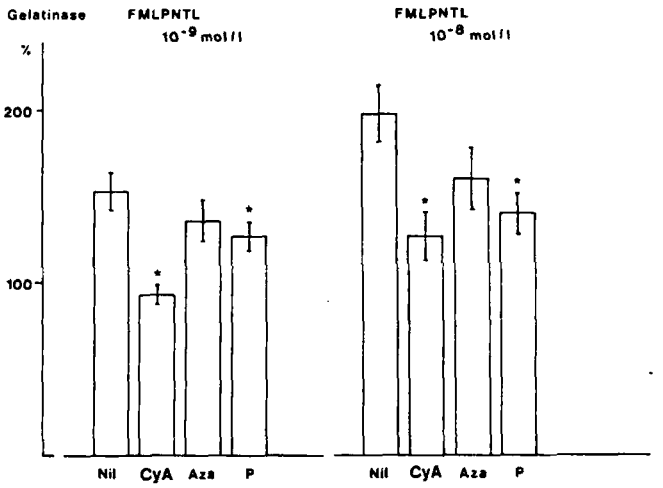

Fig. 5. Effect of cyclosporin A *(CyA)*, azathioprine *(Aza)* and prednisolone *(Pr)* on the release of collagenase from FMLPNTL-stimulated PMNLs isolated from healthy subjects. Release of collagenase without stimulation was 100%. Mean values ± SEM. * $P < 0.05$ nil versus CyA, Aza, Pr

(3652 ± 229 ng/10^7 cells). Figure 3 shows PMNL lactoferrin content of patients and controls. Patients treated with Aza displayed a lactoferrin concentration of 25.2 ± 1.2 µg/10^7 cells and those treated with CyA a concentration of 27.0 ± 2.1 µg/10^7 cells. Higher lactoferrin concentrations were found in PMNLs of controls (35.0 ± 0.9 µg/10^7 cells, $P < 0.05$).

Figure 4 displays the effect of immunosuppressive drugs on the release of lactoferrin. PMNLs were isolated from controls and stimulated with various concentrations of FMLPNTL. Concentrations of 10^{-9} and 10^{-8} mol/l stimulated lactoferrin release by 150% and 173%, respectively. CyA, but not Aza or prednisolone, caused significant inhibition of lactoferrin release (Fig. 4).

Figure 5 shows the effect of CyA, Aza and Pr on the release of collagenase from FMLPNTL-stimulated PMNLs obtained from controls. Again, CyA, but not Aza or Pr, caused significant inhibition of collagenase release after stimulation with FMLPNTL in concentrations of 10^{-9} and 10^{-8} mol/l (Fig. 5). Comparable observations were made concerning the release of gelatinase (Fig. 6). The addition of Pr to CyA or Aza had no additional effect (Table 2).

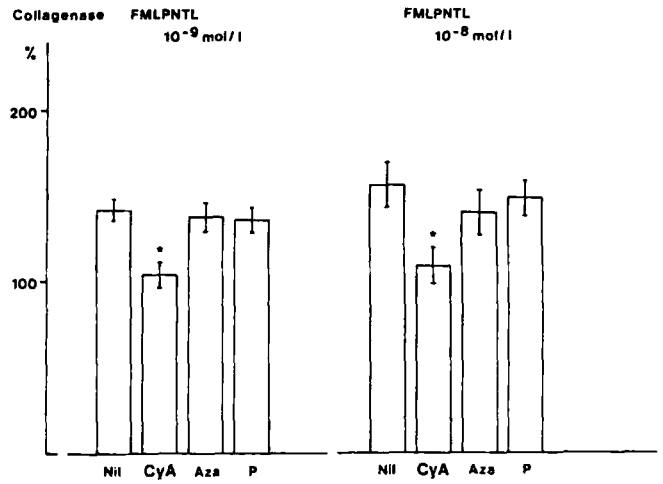

Fig. 6. Effect of cyclosporin A *(CyA)*, azathioprine *(Aza)* and prednisolone *(Pr)* on the release of gelatinase from FMLPNTL-stimulated PMNLs isolated from healthy subjects. Release of gelatinase without stimulation was 100%. Mean values ± SEM. * $P < 0.05$ nil versus CyA, Aza, Pr

Discussion

The role of PMNLs in the immune response is of considerable importance. Neutrophils are present in the tissues during rejection and contribute to the destructive processes initiated by lymphocytes. The importance of PMNLs is clearly related to their ability to secrete substances such as collagenase and gelatinase. This study indicates that even in stable renal transplant patients the levels of these enzymes are affected by long-term immunosuppression. Since protein synthesis in PMNLs occurs during cell differentiation, raised levels of type I collagenase and gelatinase should represent increased storage due to inhibition of secretion. However, immunosuppressive drugs affect intracellular content and the release of lactoferrin, collagenase and gelatinase in a different manner. The differential release of proteinase from the same granule compartment has indeed been reported by Schmidt [18] but has not yet been verified. This seems rather to be an indication for storage of the enzymes in different granules. One could argue that the differences in intracellular enzyme levels in transplant patients may be due to different mechanisms, i.e. they might be related to the effects of Pr rather than CyA and Aza, or may even be secondary to the consequences of renal transplant. It has been shown that pulse steroid therapy (500–1000 mg methylprednisolone daily) is necessary to reduce PMN degranulation [2]. Furthermore, surgical trauma stimulates PMN degranulation with normalization within a couple of weeks, even after renal transplantation [10].

Table 2. Effect of the combination of cyclosporin A (CyA) and prednisolone and azathioprine (Aza) and prednisolone on the release of lactoferrin, collagenase and gelatinase from FMLPNTL-stimulated PMNLs isolated from healthy subjects. Resting release was set 100%

Conditions	Lactoferrin (%)	Collagenase (%)	Gelatinase (%)
FMLPNTL 10^{-9} mol/l			
PMNLs alone	143 ± 7.3	142 ± 6.2	153 ± 11.2
PMNLs + CyA	99 ± 7.7***	102 ± 7.7***	113 ± 13.5*
PMNLs + Aza	149 ± 13.6	138 ± 9.4	141 ± 14.1
FMLPNTL 10^{-8} mol/l			
PMNLs alone	184 ± 6.4	156 ± 13.4	198 ± 16.8
PMNLs + CyA	115 ± 18.3***	126 ± 15.6***	138 ± 16.7*
PMNLs + Aza	195 ± 12.6	154 ± 11.7	161 ± 12.2

Mean values ± SEM from 10 experiments
* $P < 0.05$ nil versus CyA and Aza
** $P < 0.05$ CyA versus Aza

Neutrophils can participate directly in the process of collagen breakdown by the secretion of collagenolytic enzymes. This collagenolysis occurs in vivo in a type-selective manner through the actions of at least three different enzymes reaching the extracellular space under different stimuli [14]. Elastase of the azurophilic granules degrades types III and IV collagens. It has been shown that CyA but not Aza or Pr inhibits the release of neutrophil elastase in vitro [10]. This study was performed to investigate whether immunosuppressive drugs affect collagenolytic enzymes of specific granules and/or C-particles. The interstitial collagenase [8] and gelatinase degrade native type IV and V collagen [6, 12, 21]. From several studies it is suggested that metalloproteinases from different sources are involved in the development of proteinuria by their action on the glomerular filtration barrier. Neutral proteinases generated by mesangial cells, monocytes and PMNLs may be implicated in the degradation of glomerular basement membrane [3, 13, 17]. Urinary excretion of metalloproteinases and serine-type proteinases in rats with nephrotic syndrome was significantly correlated with proteinuria and the urinary excretion of both laminin and type IV collagen [4].

In a previous study PMNL lactoferrin of CyA-treated patients was not different from that of healthy subjects [10]. In the present study, PMNL lactoferrin of CyA- or Aza-treated renal transplant recipients was lower compared with controls (Fig. 3). One explanation for this difference may be that patients in the previous study were investigated 3–5 months after transplantation, but in this study after 32–46.2 months for CyA and 93–112.2 months for Aza. There is evidence of impaired renal function in the CyA group (Table 1) but not in the Aza group. It appears that there are no significant differences between proteinase levels in the two groups of immunosuppressed patients, despite the differences in graft function. Studies are under consideration to investigate whether rejection triggers type I collagenase and gelatinase release into the tissues with a consequent effect on cellular levels.

In conclusion, immunosuppressive drugs may prevent or reduce the release of the metalloproteinases from PMNLs, thereby lowering the risk of tissue and organ destruction. On the other hand, a decrease in release of antimicrobial proteins [16] like myeloperoxidase may enhance the risk of bacterial, viral or fungal infections.

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