Increased concentrations of serum pentosidine in rheumatoid arthritis

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Advanced glycosylation end products (AGEs) are thought to play an important role in the development of diabetic complications. Oxidative reactions are essential for the formation of some AGEs, termed glycoxidation products. Increased concentrations of pentosidine, one of such products, are found in tissue and serum in diabetes mellitus and in end-stage renal disease, suggesting that hyperglycemia and impaired renal function are important factors in AGE accumulation. We hypothesized that increased concentrations of pentosidine would also be found in pathological conditions associated with increased oxidative stress. We measured pentosidine in sera of patients with rheumatoid arthritis (RA), systemic lupus erythematosus, and diabetes. Increased serum pentosidine was found in RA (108.4 ± 146.5 nmol/L, \( P < 0.002 \)) and in diabetes (69.6 ± 42.4 nmol/L, \( P < 0.001 \)) as compared with healthy subjects (48.3 ± 12.0 nmol/L). These results prove that AGEs may accumulate in the absence of hyperglycemia or impaired kidney function.

Pentosidine is one of the few chemically characterized advanced glycosylation end products (AGEs).\(^1\) It is a highly fluorescent cross-link of Lys and Arg, bridged in an imidazopyridinium structure. Initially described as a product of the reaction of pentoses with proteins [1], it was later shown to be derived as well from glucose under physiological conditions of pH and temperature, provided that the reaction occurred in the presence of oxygen and traces of transition metals (oxidative conditions) [2]. Since these findings, pentosidine has been described as a glycoxidation product [3]. Pentosidine exists in human tissues such as skin and kidney [1, 4, 5], lens crystallines [6], and in plasma [7], serum [8, 9], and urine [10]. Concentrations of tissue pentosidine increase with age [11], and are increased in diabetes [1, 3] and, more overtly, in end-stage renal disease (ESRD) [11]. Serum pentosidine is increased in both conditions [7–9]. In diabetes, skin collagen pentosidine correlates positively with the presence of complications [4, 5, 12], and this provides pivotal evidence to support the Maillard hypothesis of diabetic complications [3, 13, 14]. According to this hypothesis, the accumulation of AGEs on tissue proteins, especially those with low turnover rates, causes structural damage; as these proteins undergo increased cross-linking, they become stiff, resistant to proteases and ultimately lose function. At the same time, a family of receptors expressed on the surface of macrophages and endothelial cells recognizes and binds to AGE structures, triggering the release of cytokines and initiating the production of oxidative stress. Taken together, the net result is a thickening of basal membranes and an increase in their permeability and procoagulant status. It should also be noted that, according to some authors, AGEs circulating in serum are toxic because of their putative capacity to react covalently with tissue proteins, depositing on them and exacerbating the damage caused by AGEs formed in situ [15, 16].

The increased concentrations of pentosidine found in diabetes are generally accepted to be a consequence of hyperglycemia. In fact, concentrations of fructose–lysine and fructose–valine, early glycation products, correlate with the mean glycemia over the last weeks before analysis; this is the rationale for the hemoglobin \( A_1c \) and fructosamine measurements. In ESRD, which is characterized by normoglycemia, the increase of pentosidine concentrations in tissue and serum is clearly associated with renal function impairment. Thus, kidney transplantation lowers pentosidine concentrations to nearly normal [17]; this suggests that some pentosidine precursor, perhaps a pentose or dicarbonyl sugar, is not efficiently excreted and accumulates in serum.

Given the intrinsic importance of oxidative reactions...
for the formation of pentosidine and other glycoxidation products, we hypothesized an increased glycoxidation in vivo as a consequence of conditions associated with increased oxidative stress. We chose to measure serum pentosidine in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), two conditions in which increased oxidative stress has been documented. Here we report the finding of increased serum pentosidine in RA.

**Materials and Methods**

**REAGENTS**

Acetonitrile (HPLC grade) was obtained from Romil Chemicals. Heptafluorobutyric acid (HFBA) and sodium borohydride were purchased from Sigma. All other chemicals used were from Merck, of the highest purity available.

**SUBJECTS**

Serum samples were obtained from 60 patients with RA, 37 patients with SLE, and 61 diabetic patients (11 with insulin-dependent diabetes mellitus and 50 with non-insulin-dependent diabetes mellitus) treated at outpatient clinics at the Santiago University Hospital. RA and SLE were diagnosed according to the criteria of the American Rheumatism Association [18, 19]. Serum was also obtained from 57 healthy volunteers. The guidelines of the Human Studies Committee of our institution, in agreement with those of the Helsinki Declaration, were followed. Urea, creatinine, glucose, total serum proteins, and albumin were measured immediately in serum by conventional methods with a Hitachi 747 autoanalyzer and commercial kits (Boehringer Mannheim). Normal ranges for these analytes in our laboratory are: total protein, 65–83 g/L; albumin, 40–52 g/L; glucose, 4.1–5.8 mmol/L; urea, 2.0–7.3 mmol/L; creatinine, 35.4–114.9 μmol/L. Fructosamine was measured with the same instrument with a commercial kit (Boehringer Mannheim). Sera were stored at −30 °C until used for pentosidine analysis.

**PENTOSIDINE MEASUREMENTS**

Serum pentosidine was measured by a modification of the method of Takahashi et al. [8]. Two hundred microliters of serum, equivalent to ~15 mg of protein, were transferred to borosilicate tubes with Teflon screw caps, and protein was precipitated with 2 mL of 100 g/L trichloroacetic acid (TCA). After centrifugation, pellets were washed with 2 mL of 100 g/L TCA and spun again. Washed pellets were reduced at room temperature with 3 mL of a 10 mmol/L NaBH₄ solution in 0.1 mol/L NaOH for 4 h. Precipitation and reduction of samples are not strictly necessary for pentosidine measurement, but this treatment results in cleaner chromatograms [2]. Reduced samples were hydrolyzed by addition of 3 mL of concentrated (12 mol/L) HCl at 130 °C for 18 h. Tubes were purged with nitrogen before hydrolysis. Hydrolysates were evaporated under reduced pressure in a Speed-Vac concentrator (Savant) and the resulting residues rehydrated in 250 μL of deionized water and filtered through a 45-μm Durapore membrane (Millipore). Two hundred microliters of the filtrate were diluted with 10 mL of deionized water and applied to a 0.7 × 1.5 cm SP-Sephadex C-25 cation exchange column (Pharmacia) equilibrated with water. The column was washed with 20 mL of 0.1 mol/L HCl and eluted with 5 mL of 1 mol/L HCl. The eluate was evaporated to dryness under reduced pressure and reconstituted in 500 μL of 1 mL/L HFBA: acetonitrile, 95:5. One hundred microliters of sample, corresponding to about 2 mg of protein hydrolysate, were injected into an HPLC system (Gilson) consisting of models 305 and 306 pumps, a model 811C dynamic mixer, a model 805 manometric module, and an Aspec XL autosampler. Separation was achieved on a 15 × 0.46 cm Spherisorb C-18 column (Teknokroma) with a linear gradient of 90 mL/L to 180 mL/L acetonitrile over 1 mL/L HFBA in 35 min, followed by a column cleaning step of 15 min with 900 mL/L acetonitrile in 1 mL/L HFBA. The effluent was monitored with a model F2000 fluorescence spectrophotometer (Hitachi) equipped with a flow cell; the excitation and emission wavelengths were set at 335 and 378 nm, respectively. The photomultiplier voltage was 700 mV and excitation and emission bandpasses were 10 nm. Pentosidine was synthesized and purified according to the procedure of Dyer et al. [2]. A calibrator of pure pentosidine, generously provided by J.W. Baynes (University of South Carolina), was used for calibration purposes. The interassay CV of the technique was 6.1% at a concentration of 38 ± 1.7 nmol/L, and the smallest amount of quantifiable analyte was 200 fmol per injection.

**STATISTICAL ANALYSIS**

Statistical significance was determined nonparametrically by the Mann–Whitney tests between two groups. The F-test was used to determine the statistical significance of linear regression. Sigma-Stat statistical analysis software was used (Jandel Scientific).

**Results**

Serum pentosidine was increased in RA patients when compared with control subjects. Values were 108.4 ± 146.5 and 48.3 ± 11.5 nmol/L, respectively (P <0.0001, Fig. 1). Diabetic patients showed the expected increase in serum pentosidine: 69.6 ± 42.4 nmol/L (P <0.002). Serum pentosidine in SLE patients was 63.2 ± 59.6 nmol/L, not statistically different from the control group. It is noteworthy that the distribution of pentosidine values in diabetic, RA, and SLE patients is not parametric, showing a dense cluster of low values similar to those of the control group, and a “smear” of high values: i.e., many patients have normal pentosidinemia and only a certain number of cases in each population shows increased serum pentosidine values. Therefore, though as a group the SLE patients do not have increased pentosidinemia, many patients do show increased serum pentosidine values, some of them
overtly increased (more than three times the mean of the control group). This smearing pattern is consistent with previously reported data of diabetic serum pentosidine [7, 9]. Of interest, three young RA patients diagnosed with juvenile arthritis, a subtype of RA, had overtly increased serum pentosidine values (106, 108, and 311 nmol/L). Serum fructosamine values for the same samples are shown in Fig. 2. As anticipated, diabetic patients had increased serum fructosamine concentrations, nearly doubling those of healthy controls (441 ± 82 mmol/L and 258 ± 23 mmol/L, respectively, P <0.0001). Both RA and SLE patients showed marginally increased serum fructosamine values (278 ± 43, mmol/L, P <0.01 and 278 ± 47 mmol/L, P <0.01, respectively). Such marginal increases confirm that increased pentosidinemia in RA and some SLE cases is not a consequence of a generalized increase of glycation. Table 1 summarizes additional analytical characteristics of the subjects. All had normal serum creatinine and urea values, consistent with their normal kidney function. Serum pentosidine correlated with age in the control group, in agreement with Takahashi et al. [8] (Fig. 3A). However, serum pentosidine did not show a statistically significant correlation with age in the RA group (Fig. 3B) or in the SLE group (data not shown). Additionally, synovial fluid from three patients with RA, obtained during routine puncture and extraction procedures, was also analyzed. After hydrolysis, these samples revealed the presence of pentosidine (Fig. 4), which was further confirmed by mixing experiments with an authentic pentosidine calibrator. The concentration of pentosidine in synovial fluid was 1.13 ± 0.58 nmol/g protein.

Discussion
The presence of increased concentrations of serum pentosidine in RA and in some cases of SLE confirms the complexity of AGE accumulation in vivo. All RA and SLE patients in this study were normoglycemic and had intact kidney function, as documented by their clinical records and confirmed by their serum creatinine and urea values (Table 1). Consequently, an alternative mechanism to those operating in diabetes and ESRD must be invoked to explain their increased pentosidinemia. A likely explanation for the formation of pentosidine in these patients involves increased oxidative stress. Both RA and SLE are autoimmune diseases associated with increased oxidative stress (for a review, see ref. 20). In RA, the synovial fluid of the inflamed joint contains numerous neutrophils, many of which are activated and produce O$_2^-$ and H$_2$O$_2$, which, in the presence of iron, are converted to OH$^-$. Hypochlorite (HOCl) is also produced by activated neutrophils through the action of myeloperoxidase. HOCl is able to oxidize free amino acids to the corresponding aldehydes [21]. Anderson et al. have recently shown that HOCl converts serine to glycolaldehyde, which, in the presence of protein, generates carboxymethyllysine (CML), another glycoxidation product [22]. Similar reactions, initiated by hypochlorite, superoxide, or hydroxyl radicals, may lead to the generation of reactive carbohydrate intermediates, such as dicarbonyls or pentoses, which would readily react with proteins to generate pentosidine. In fact, oxidative degradation of lipids seems to occur in the synovial fluid from RA patients, as documented by increased concentrations of thiobarbituric acid-reactive substances [20]. Moreover, the synovial fluid and the serum of these patients contain decreased con-
centrations of ascorbate and increased dehydroascorbate/ascorbate ratios [23]; dehydroascorbate is an active precursor of pentosidine [6], and a recent report suggests that it might be a precursor of the increased serum pentosidine found in ESRD [24]. Taken together, this prooxidant milieu offers a reasonable explanation for increased pentosidine formation. Pentosidine is, in fact, present in synovial fluid (Fig. 4), where it is mostly protein-bound (data not shown). The presence of increased pentosidine concentrations in serum, however, where it is also mostly protein-bound [25 and our own unpublished data], indicates that the precursor molecule(s) generated by oxidative reactions diffuses from the sites of inflammation. This could lead to a generalized increased of tissue pentosidine.

SLE shares with RA an autoimmune origin, and it is equally associated with increased activation of neutrophils. A recent study showed that anticardiolipin antibodies, often present in SLE, are directed against epitopes formed during lipid peroxidation in the presence of protein [26]; their detection with cardiolipin-based ELISA is dependent on artifactual oxidation of cardiolipin during the assay. CML has recently been shown to form during the oxidation of polyunsaturated fatty acids in the presence of protein [27]. This process probably involves short-chain carbonyl compounds such as glyoxal. It is conceivable that in autoimmune diseases, a basal increase of oxidative stress leads to the generation of active carbonyl compounds (carbohydrate or lipid-derived) that react with protein and phospholipid-free amino groups, generating CML, pentosidine, and other structures. Some of these structures may be immunogenic and elicit the generation of autoantibodies.

Our results do not support the concept of serum AGE toxicity. A necessary consequence of the proposed pathogenicity of serum AGEs is that common complications should arise in all conditions associated with increased serum AGE concentrations. Therefore, the characteristic complications of diabetes mellitus should be clearly present in nondiabetic ESRD patients, who exhibit increased serum AGEs and pentosidine, and, in accordance with our results, in RA patients. Although both diabetic and ESRD patients share an increased risk of atherosclerosis [16], diabetic-like retinopathy is not prevalent in

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DM, diabetes mellitus.

Fig. 3. Correlation of serum pentosidine vs age.

(A) Controls (linear regression equation: y = 0.206x + 37.9; r = 0.342, P < 0.01). (B) RA patients (linear regression equation: y = 0.198x + 99.2; r = 0.0218, P = 0.87, not significant).

Fig. 4. Representative chromatograms of (A) serum from a RA patient, (B) synovial fluid from a RA patient, and (C) same sample as in B supplemented with authentic pentosidine.
ESRD of nondiabetic origin. RA is free from diabetic-like complications, and the presence of AGEs is extremely unlikely to contribute to the observed pathological manifestations of this disease, which are caused by the inflammatory process itself. Here AGEs are clearly an effect of the basic pathological process (inflammation and, perhaps, increased oxidative stress), and not its cause. If, as has been proposed, circulating serum AGEs cause pathology by reacting with tissue proteins or binding to specific receptors on endothelial cells and macrophages, eventually contributing to the development of microangiopathy and macrovascular disease, they should produce the same effects in RA. It is therefore conceivable that circulating AGEs may be irrelevant by-products. An improbable alternative explanation of our results would be that pentosidine is increased in certain instances without a concomitant increase in other AGEs. Increases in pentosidine, however, have always been associated with global increases in AGEs in diabetes, ESRD, and in experiments performed in vitro with a variety of glycating agents [2, 3, 5]. Caution about a lack of toxicity of circulating AGEs might be also extended to AGEs in general; i.e., it is possible that AGEs formed on tissues are just innocuous consequences of increased hyperglycemia and (or) oxidative stress, without a major role as sources of pathology; this case would be greatly strengthened if pentosidine (or other AGE) concentrations were demonstrated to be increased in tissues in RA and SLE patients. In this respect, Takahashi et al. have described increased concentrations of pentosidine in articular cartilage in RA compared with patients with osteoarthritis [28]. Additional studies to investigate the possible presence of increased concentrations of pentosidine in tissue from RA patients would be of great interest; in the meantime, the possibility that tissue AGEs are, in fact, pathogenic cannot be dismissed.

To conclude, the presence of increased concentrations of serum pentosidine in RA and in several SLE patients indicates that this compound is not a simple marker of glycoxidation in diabetes, but can be used as a more general marker of oxidative stress in different pathologies. It remains to be seen if pentosidine measurements in RA, SLE, and other autoimmune or inflammatory diseases are clinically useful. It is, for example, unclear why only some patients show increased pentosidinemia. Studies to determine the relation between serum pentosidine and active periods of RA are in progress. In any case, the study of pentosidine and other AGEs in diseases other than diabetes may provide valuable information regarding the validity of the Maillard hypothesis of diabetic complications.

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