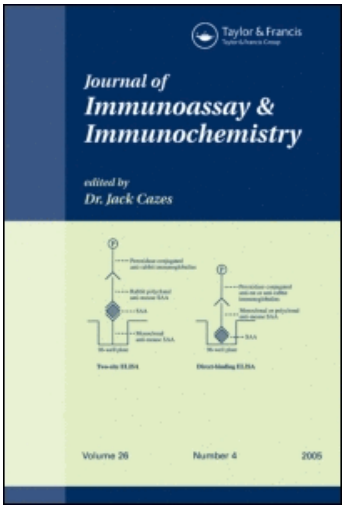

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Cystatin C Levels in Sera of Patients with Human Immunodeficiency Virus Infection. A New Avidin-Biotin ELISA Assay for Its Measurement

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Cystatin C levels in sera of patients with human immunodeficiency virus infection

A new avidin-biotin ELISA assay for its measurement

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KEY WORDS: Cystatin C. Cysteine proteinases inhibitor. Beta$_2$-microglobulin. ELISA. HIV.

ABSTRACT

A solid-phase enzyme-linked immunosorbent assay (ELISA) for determining human serum cystatin C is described. In 50 normal samples, cystatin C concentration was 1247 ± 224 µg / L (mean ± SD) which is in agreement with previously reported levels. Serum levels of cystatin C and ß$_2$-microglobulin (ß$_2$-M) were investigated in a time-course study during the development of human immunodeficiency virus (HIV) infection. We found a persistent and uniform increase in the ß$_2$-M concentration (2762 ± 1239 µg / L). In contrast to ß$_2$-M, on the basis of cystatin C levels, we found two distinct populations, one of which demonstrated an increased concentration (1620 ± 618 µg / L). Interestingly a second group (21% of patients) exhibited an initial significant decrease in cystatin

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C concentration with a mean value of 377 (range 55-850) μg / L, followed by an increase. The biphasic pattern of cystatin C serum, a major cysteine proteinase inhibitor, during the course of HIV infection suggests a possible role for these proteinases (or proteinase inhibitors) in the development of this syndrome.

INTRODUCTION

The cystatin superfamily is a group of cysteine proteinase inhibitors. Human cystatin C previously known as gamma-trace or post-gamma globulin, is a low molecular weight protein (13 kDa) described for the first time in 1961, in cerebrospinal fluid and isolated from urine of patients with tubular disorders. Under normal physiological conditions, cystatin C is present in all tested human biological fluids and is particularly abundant in seminal plasma and cerebrospinal fluid. Immunohistochemical studies of human cystatin C have shown that this protein is predominantly present in neuroendocrine cells. Large amounts of cystatin C are found in urine of patients with tubular disorders and in sera of patients with autoimmune diseases, due to a reduced glomerular filtration rate. Deposits of a cystatin C variant are observed during the course of hereditary cystatin C amyloid angiopathy with reduced levels of cystatin C in cerebrospinal fluid (1).

Secretion of cystatin C by monocytes or macrophages in culture medium (2, 3) and the specific interaction between cystatin C and the fourth component of complement may be of particular interest at tissue inflammation sites (4). Cysteine proteinases participate in numerous cellular processes including protein catabolism, proteolytic processing of prohormones and penetration of normal tissues by malignant cells (5). There are various examples of potentially pathogenic microorganisms that synthesize cysteine proteinases: *Entamoeba histolytica* (6), *Leishmania amazonensis* (7), *Schistosoma mansoni* (8) and certain viruses (9). Thus, cysteine proteinase inhibitors could also protect cells against exogenous proteinases.

$ß_2$-microglobulin ($ß_2$-M) is also a low molecular weight protein of 11.6 kDa. It was first isolated from urine of patients with tubular proteinuria (10). The urinary level of excreted $ß_2$-M has since been used as a sensitive marker of the functional integrity of the proximal tubule cells (11, 12, 13). $ß_2$-M is present on the surface of all nucleated cells, forming the light chain of their HLA antigen (14, 15). Interest in this protein has been focused on its role in immune recognition and immune regulation. In normal conditions, the concentration of $ß_2$-M in serum is relatively constant but rises in various lymphoid malignancies and inflammatory diseases as a result of increased synthesis and/or cell turnover (16). High concentrations of serum $ß_2$-M have been reported in patients with viral infections (including HIV infection) (17, 18), where it may be a potentially useful adjunct in monitoring disease activity (19, 20).

The ELISA double-antibody sandwich method is a useful and reproducible technique for quantitation of nanogram amounts proteins (21). The avidin-biotin interaction has been used in different enzyme immunoassays (22).

Because cystatin C can be a potential protector against exogenous cysteine proteinases, we studied the evolution of cystatin C levels during the development of human immunodeficiency virus (HIV) infection. We compared the contents of cystatin C and of $ß_2$-M, another potential marker of infectious diseases, in human serum from HIV seropositive patients.

MATERIALS AND METHODS

Subjects

Between 1984 and 1989 we performed a time course study on 70 HIV-1 seropositive patients, 4 females and 66 males (age 9-58 years, mean 36 ± 8.8 years) with a total of 246 samples. Each sample was obtained for medical results at approximately 6-month intervals. At first presentation, 34 individuals had CD4+

lymphocyte counts within the normal range (570-1575 / µl, mean 935 ± 308 / µl) with no major clinical disease and only minor symptoms, and 36 individuals had a major HIV-related disease or lymphopenia and CD4+ lymphocyte counts below 500 / µl. All subjects were repeatedly tested positive for HIV-1 antibodies in a recombinant HIV-1 enzyme immunoassay (Abbott) and confirmed by immunofluorescence and Western blot analysis (23). All subjects had normal serum creatinine concentration.

Materials

Polystyrene microtitration plates 96 F, Nunc (Roskilde, Denmark). DEAE Trisacryl M and activated biotin, IBF (Villeneuve-la-Garenne, France). HRP-avidin, Miles Laboratories (Naperville, Ill.). Tween 20, BSA (bovine serum albumin) RIA grade and o-Phenylenediamine (OPD) (Sigma Chemical Company,St Louis, MO).

$ß_2$-microglobulin assay

$ß_2$-M levels in serum were determined by the ELISA double sandwich technique developed in our laboratory according to Lambré (24) or inhibition enzyme immunoassay (25). The $ß_2$-M level in normal serum is 1270 ± 250 µg / L.

Purification of human cystatin C and anti-human cystatin C IgG

Protein estimation of cystatin C and anti-human cystatin C were performed as described by Lowry et al. (26). Human urinary cystatin C was purified from urine of patients with renal failure, according to a protocol previously described (27, 28) and stored at - 80° C. One goat was immunized against purified human cystatin C. Immunization was performed according to the dorsal multiple site protocol (29, 27). Anti-human cystatin C IgG was purified by salt precipitation using 50% saturated ammonium sulfate, followed by DEAE Trisacryl M anion exchange chromatography in Tris-HCl buffer, 100 mmol / L, pH 7.5. Electrophoresis was used to determine that the preparation was free of contaminants. Anti-human cystatin C was conjugated with activated biotin according to the manufacturer's instructions.

Cystatin C quantitation

The buffers used in this assay, including coating buffer, rinse buffer, serum diluent buffer, antibody diluent buffer, HRP-diluent buffer and enzyme buffer were prepared as follows: coating buffer: sodium carbonate-bicarbonate, 100 mmol / L, pH 9.6; washing buffer: phosphate buffer, 10 mmol / L, pH 7.4 containing NaCl, 140 mmol / L (PBS) and 1% Tween 20; serum diluent buffer / antibody diluent buffer / HRP-avidin diluent buffer: PBS, pH 7.4, containing BSA, 10 g / L; enzyme buffer: potassium phosphate buffer, 100 mmol / L, pH 6.0. Anti-human cystatin C IgG solution was diluted with coating buffer to give a protein concentration of 20 mg / L. Wells of a microtiter plate were filled with 200 µl of this solution and the plate was incubated overnight at 4° C. The solution was then discarded and the wells rinsed 5 times with washing buffer. Duplicate wells of a microtiter plate were filled with 200 µl of cystatin C standard solutions (0.195 to 25 µg / L) or samples appropriately diluted with serum diluent buffer. The plate was incubated for 30 min at 37° C. Wells, rinsed 5 times with rinse buffer, were filled with 200 µl of biotinylated anti-human cystatin C (1.2 mg / L) with antibody diluent buffer. The plate was incubated for 30 min at 37° C. Wells were emptied and washed 5 times with rinse buffer. A stock solution of HRP-avidin (2 g / L) was diluted 1 / 5,000 with HRP-avidin diluent buffer, 200 µl aliquots were distributed between the wells and the plate was incubated at room temperature in the dark for 15 min. The solution was discarded, wells were rinsed 5 times with rinse buffer and then 200 µl of freshly prepared peroxidase substrate solution (0.5 g / L o-phenylenediamine and 0.015 % hydrogen peroxide in potassium phosphate buffer, 100 mmol / L, pH 6.0) was added to each microtiter well. The plate was allowed to react at room temperature in the dark for 15 min. The enzyme reaction was then stopped by adding 50 µl of 5 M H_2SO_4 and the absorbance read at 492 nm with a Titertek Multiskan Photometer (Flow Laboratories). The cystatin C standard stock (0.5 g / L) was diluted 1 / 20,000 giving a cystatin C concentration

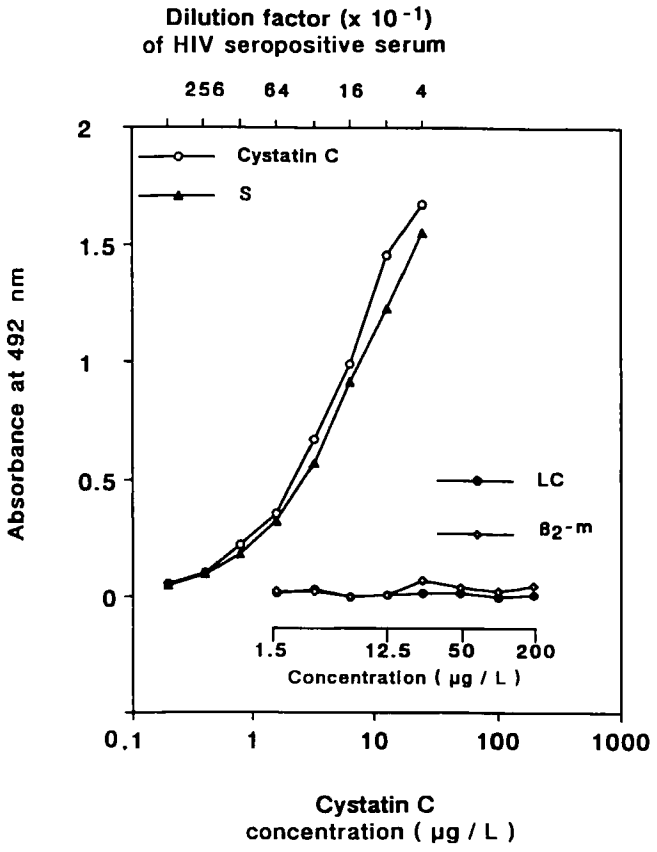

Fig. 1. Cystatin C standard curve, specificity and serum interference.
The cystatin C standard (0.5 g / L) was diluted 1 / 20,000 to give a cystatin C concentration of 25 µg / L. Serial dilutions (1 / 2) were performed beginning with this concentration to construct the standard curve (o) and with the serum of an HIV seropositive patient (▲). Addition of purified human light chains of IgG (●) and human β_2-M (◊) did not induce significant displacement of the standard curve.

Table 1. Cystatin C and β_2-M concentrations in HIV seropositive patients and correlation between these two proteins as a function of sampling. Comparison with normal levels. All data are expressed as mean ± SD. Student's *t* test was used for statistical analysis.

	Normal level n=50	Total samples n=246	First samples n=70	Cyst. C < 850 µg/L n=15
Cystatin C (µg/L)	1247 ± 224	1620 ± 618 $P< 0.001$	1487 ± 781 $P< 0.05$	377 ± 216 $P< 0.001$
β_2-M (µg/L)	1270 ± 250	2762 ± 1239 $P< 0.001$	2645 ± 1428 $P< 0.001$	1522 ± 516 NS
coefficient of correlation	0.20	0.54	0.65	0.10

of 25 µg / L. Beginning with this concentration, serial dilutions were prepared and corresponding optical density values were plotted against the known concentrations of cystatin C to give the standard curve (Fig. 1). The cystatin C concentration in samples was obtained by interpolation from the standard curve. Assays were performed in the same run in duplicate. Low values of cystatin C concentration were confirmed in a second assay run.

Statistical analysis

All data are expressed as mean ± SD. Student's *t* test was used for statistical analysis (*** $P< 0.001$, ** $P< 0.01$, * $P< 0.05$).

RESULTS

Quantitation of human cystatin C by ELISA

The standard curve (Fig. 1) was obtained with various amounts of pure human cystatin C. The assay detectability limit was 0.195 µg / L, equivalent to 14.7 pmol / L. The detection range was between 0.195 and 25 µg / L. The intra-assay and inter-assay coefficients of variation were estimated from 20 determinations of the same normal serum run in the same assay and in 20 different assays respectively. The mean values and standard deviation were 1288 ± 56 µg / L (coefficient of variation 4.3 %) in intra-assay and 1221 ± 94 µg / L (coefficient of variation 7.7 %) in inter-assay. Serial dilutions of serum of HIV seropositive patients give a displacement curve parallel with the standard curve consistent with immunological identity (Fig. 1). No cross-reaction was found when human cystatin C antiserum was tested with up to 200 ng human light chains of IgG and human $ß_2$-M purified in the laboratory (Fig. 1). The assay appears to measure cystatin C but not other cysteine proteinase inhibitors or other serum factors.

Cystatin C and $ß_2$-M levels in serum of HIV seropositive patients

Using this assay, the mean values and standard deviation for cystatin C concentrations in 50 normal adults were 1247 ± 224 (range 856-1695) µg / L. Cystatin C and $ß_2$-M levels in 246 serum samples of 70 HIV seropositive patients were determined (Table 1). Cystatin C and $ß_2$-M mean values (M ± SD) were 1620 ± 618 (range 55-4250) µg / L and 2762 ± 1239 (range 660-8100) µg / L respectively, with a correlation coefficient between cystatin C and $ß_2$-M of 0.54. Cystatin C and $ß_2$-M mean values in the first serum samples were 1487 ± 781 (range 55-4250) and 2645 ± 1428 (range 660-7900) µg / L respectively, with a correlation coefficient of 0.65.

Figure 2 shows the distribution of patients as a function of cystatin C level or of $ß_2$-M level in the first sample. In 15 of the 70 patients (21%), the first sample presents a reduced level of cystatin C (< 850 µg / L) with a mean value of 377

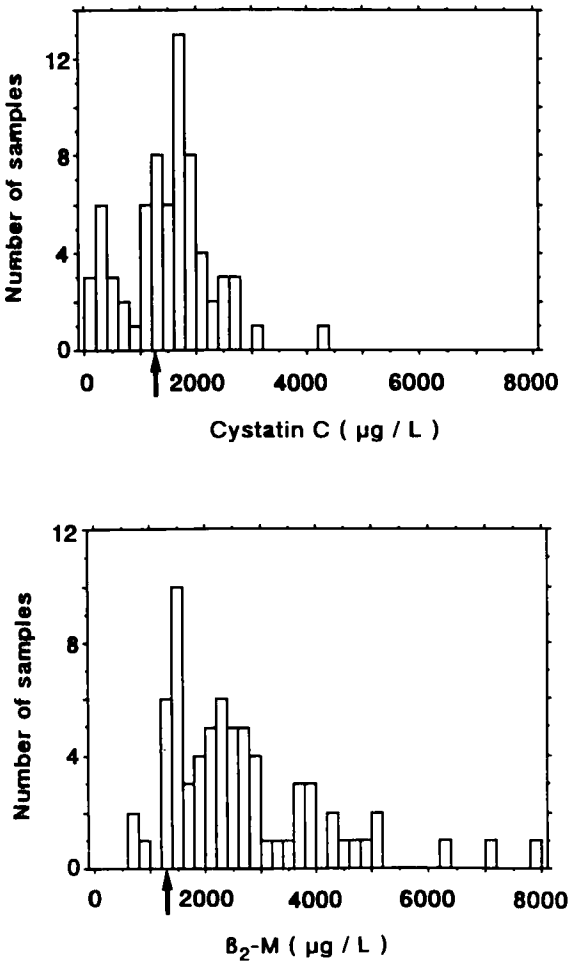

Fig. 2. Frequency distribution of cystatin C and $ß_2$-M concentrations for initial samples (n = 70, width = 200 µg / L). Cystatin C and $ß_2$-M were assayed using ELISA methods as described above. An arrow indicates cystatin C or $ß_2$-M concentration mean value in 50 normal sera.

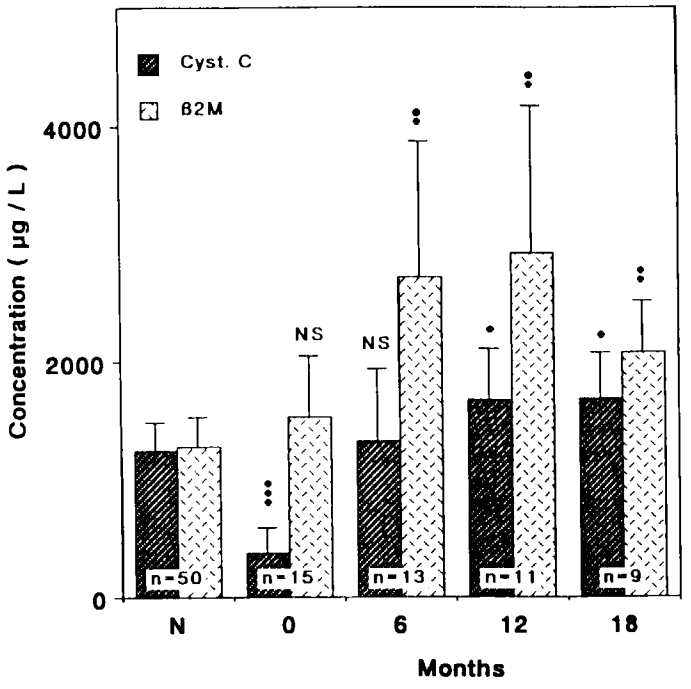

Fig. 3. Time-dependent evolution of cystatin C and β_2-M levels in the 15 patients presenting a lowered cystatin C concentration in the first sample compared with normal concentrations (represented by the first 2 blocks named: N). Blocks are mean values ± SD. Time interval between samplings was about 6 months. An asterisk indicates a statistically significant difference (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$) between cystatin C and β_2-M levels in samples of HIV-1 seropositive patients and normal concentrations.

(range 55-850) µg / L. The corresponding mean value for β_2-M level was 1522 ± 516 µg / L with a correlation coefficient of 0.10, indicating no correlation between cystatin C and β_2-M levels (Table 1). Only 3% of patients had an initial β_2-M level below 1000 µg / L.

Significantly lowered cystatin C levels ($P < 0.001$) were only found in first samples as shown in Figure 3. The evolution of cystatin C and β_2-M levels in the

15 patients with lowered cystatin C concentrations were followed for the first two years. In the second sample (n = 13), cystatin C levels returned to normal values: 1320 ± 609 µg / L, whereas $ß_2$-M levels increased: 2718 ± 1144 µg / L ($P <$ 0.01). Cystatin C and $ß_2$-M levels also increased in the third sample: 1661± 451 µg / L ($P < 0.05$) and 3218 ± 1928 µg / L ($P < 0.01$) respectively, and remained stable in the fourth sample at a high level at 1682 ± 388 µg / L, ($P < 0.05$) and 2075 ± 449 µg / L, ($P < 0.01$) respectively.

DISCUSSION

The human cystatin C ELISA we have developed has a higher sensitivity when compared with similar assays described in man (30, 31), with a detection limit of 0.195 µg / L and it is reproducible. Our results, with avidin-biotin ELISA, show that the cystatin C concentration in normal human serum is 1247± 224 µg / L (mean ± SD, n = 50). This result is in agreement with those previously reported (30, 31) using either polyclonal or monoclonal antibodies.

The increase in serum $ß_2$-M concentration observed in our study is comparable with that already described (2762 ± 1239 µg / L). The cystatin C level increased in all HIV seropositive patients (1620 ± 618 µg / L) but less so than $ß_2$-M. The first samples presented a significantly lowered cystatin C level (< 850 µg / L) with a mean value of 377 µg / L in 21% of patients and there was no longer any correlation between cystatin C and $ß_2$-M levels.

No connection between lowered cystatin C level and other known clinical or biological parameters (CD4, CD4/CD8, anti-HIV antibodies) has been found.

In the group of 15 patients with lowered cystatin C levels in the first sample, only one sample was taken from 2 individuals, 11 showed a normal or increased cystatin C level at the second sample, and two at the third sample. No correlation was found with treatment or clinical evolution in these patients. Similar results have been published (2) showing a lowered cystatin C secretion for monocytes

stimulated by LPS. Increase in cystatin C observed later could be the result of an inflammatory state.

The ELISA described in this article is praticable, simple and reproducible. It allowed us to demonstrate that in HIV seropositive patients, 2 populations exist and are different for the serum cystatin C concentration at the beginning of the disease.

REFERENCES

1. Barrett, A.J., Rawlings, N.D., Davies, M.E., Machleidt, W., Salvesen, G. and Turk, V. Cysteine proteinase inhibitors of the cystatin superfamily. In: Barrett, A.J. and Salvesen, G. eds. Proteinase inhibitors. Amsterdam: Elsevier Scientific Publishing, 1986: 515-69.

2. Warfel, A.H., Zucker-Franklin, D., Frangione, B. and Ghiso, J. Constitutive secretion of cystatin C (γ-trace) by monocytes and macrophages and its downregulation after stimulation. J. Exp. Med. 1987; 166: 1912-17.

3. Chapman, H.A., Reilly, J.J., Yee, R. and Grubb, A. Identification of cystatin C, a cysteine proteinase inhibitor, as a major secretory product of human alveolar macrophages in vitro. Am. Rev. Respir. Dis. 1990; 141: 698-705.

4. Ghiso, J., Saball, E., Leoni, J., Rostagno, A. and Frangione, B. Binding of cystatin C to C4: The importance of sense-antisense peptides in their interaction. Proc. Natl. Acad. Sci. USA. 1990; 87: 1288-91.

5. Abrahamson, M. Human cysteine proteinase inhibitors. Isolation, physiological importance, inhibitory mechanism, gene structure and relation to hereditary cerebral hemorrhage. Scan. J. Clin. Lab. Invest.1988; 48: Suppl.191, 21-31.

6. Reed, S.L., Keene, W.E. and McKerrow, J.H. Thiol proteinase expression and pathogenicity of *Entamoeba histolytica*. J. Clin. Microbiol.1989; 27: 2772-7.

7. Prina, E., Antoine, J.C., Wiederanders, B. and Kirschke, H. Localization and activity of various lysosomal proteases in *Leishmania amazonensis*-infected macrophages. Infect. Immun. 1990; 58: 1730-7.

8. Klinkert, M.Q., Felleisen, R., Link, G., Ruppel, A. and Beck, E. Primary structures of Sm31/32 diagnostic proteins of *Schistosoma mansoni* and their identification as proteases. Mol. Biochem. Parasitol. 1989; 33: 113-22.

9. Korant, B.D., Brzin, J. and Turk, V. Cystatin, a protein inhibitor of cysteine proteinases alters viral protein cleavages in infected human cells. Biochem. Biophys. Res. Commun. 1985; 127: 1072-6.

10. Berggard, I. and Bearn, A.J. Isolation and properties of a low molecular weight ß2-microglobulin in human biological fluids. J. Biol. Chem. 1968; 243: 4095-103.

11. Peterson, P.A., Evrin, P.E. and Berggard, I. Differentiation of glomerular, tubular and normal proteinuria. Determination of urinary excretion of ß2-microglobulin, albumin and total protein. J. Clin. Invest. 1969; 48: 1189- 98.

12. Wibell, L., Evrin, P.E. and Berggard, I. Serum ß2-microglobulin in renal disease. Nephron. 1973; 10: 320-31.

13. Manuel, Y., Collé, A., Leclercq, M. and Tonnelle, C. Low molecular proteinuria. In: Berlyne, G.M. and Giovannetti, S. eds. Contribution to Nephrology vol 1 . Basel: Karger 1975: 156-62.

14. Grey, H.M., Kubo, R.T., Colon, S.M., et al. The small subunit of HL-A antigens is ß2-microglobulin. J. Exp. Med. 1973; 138: 1608-12.

15. Neauport-Sautes, C., Bismuth, A., Kourilsky, F.M. and Manuel, Y. Relationship between HL-A antigens and ß2-microglobulin as studied by immunofluorescence on the lymphocyte membrane. J. Exp. Med. 1974; 139: 957-68.

16. Creswell, P., Springer, T., Strominger, J.L., Turner, M.J., Grey, H.M. and Kubo, R.T. Immunological identity of the small subunit of HL-A antigens and ß2-microglobulin and its turnover on the cell membrane. Proc Natl. Acad. Sci. USA. 1974; 71: 2123-7.

17. Francioli, P. and Clément, F. Beta2-microglobulin and immunodeficiency in homosexual man. N. Engl. J. Med. 1982; 307: 1402-4.

18. Grieco, M.H., Reddy, M.M., Kothari, H.B., Lange, M., Buimovici- Klein, E. and William, D. Elevated ß2-microglobulin and lysosyme levels in patients with acquired immune deficiency syndrome. Clin. Immunol. Immunopathol. 1984; 32: 174-84.

19. Zolla-Pazner, S., William, D., El-Sadr, W., Marmor, M. and Stahl, R. Quantitation of ß2-microglobulin and other immune characteristics in a prospective study in men at risk for acquired immune deficiency syndrome. JAMA. 1984; 251: 2951-5.

20. Franzetti, F., Cavalli, G., Foppa, C.U., Amprimo, M.C., Gaido, P. and Lazzarin, A. Raised serum ß2 microglobulin levels in different stages of human immunodeficiency virus infection. J. Clin. Lab. Immunol. 1988; 27: 133-7.

21. Engvall, E. and Perlman, P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 1971; 8: 871-4.

22. Guesdon, J., Ternynck, T. and Avrameas, S. The use of avidin-biotine interaction in immunoenzymatic techniques. J. Histochem. Cytochem. 1979; 27: 1131-9.

23. Mathez, D., Paul, D., De Bélilovsky, C., et al. Productive human immunodeficiency virus infection levels correlate with AIDS-related manifestations in the patient. Proc. Natl. Acad. Sci. USA. 1990; 87: 7438-42.

24. Lambré, C. and Kasturi, L. A microplate enzyme immunoassay (ELISA) for anti-influenza antibodies. J. Immunol. Methods. 1979; 26: 61-9.

25. Carlier, Y., Collé, A., Tachon, P., Bout, D. and Capron, A. Quantification of ß2-microglobulin by inhibition enzyme immunoassay. J. Immunol. Methods. 1981; 40: 231-8.

26. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 1951; 193: 265-75.

27. Tonnelle, C., Collé, A., Leclercq, M., Sire, J. and Manuel, Y. The different electrophoretic forms of post γ-globulin their antigenic identity and their structural variability. Biochim. Biophys. Acta. 1977; 490: 35-43.

28. Leung-Tack, J., Tavéra, C., Gensac, M.C., Martinez, J. and Collé, A. Modulation of phagocytosis-associated respiratory burst by human cystatin C: role of the N-terminal tetrapeptide Lys-Pro-Pro-Arg. Exp. Cell. Res. 1990; 188: 16-22.

29. Vaitukaitis, J., Robbins, J.B., Niesschlag, E. and Ross, G.T.J. A method for producing specific antisera with small doses of immunogen. J. Clin. Endocrinol. Metab. 1971; 33: 988-91.

30. Olafsson, I., Löfberg, H., Abrahamson, M. and Grubb, A. Production, characterization and use of monoclonal antibodies against the major extracellular human cysteine proteinase inhibitors cystatin C and kininogen. Scand. J. Clin. Lab. Invest. 1988; 48: 573-82.

31. Ishiguro, H., Ohkubo, I., Mizokami, M., Titani, K. and Sasaki, M. The use of monoclonal antibodies to define levels of cystatin C in normal human serum. Hybridoma. 1989; 8: 303-13.