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Seroprevalence and Disease Association of Antineutrophil Cytoplasmic Autoantibodies and Antigens in HIV Infection

Summary: This prospective study was designed to determine the role of antineutrophil cytoplasmic autoantibodies (ANCA) in HIV-infected patients. Immunofluorescence tests (IFT) and enzyme-linked immunosorbent assays (ELISA) were applied to sera of 199 consecutive outpatients. In the IFT 20% were positive. An atypical ANCA pattern was demonstrated in 67% of these, 33% revealed a perinuclear staining (pANCA). Specific ELISA revealed proteinase 3 (n = 2), myeloperoxidase (n = 1), lysozyme (n = 2), lactoferrin (n = 1), cathepsin G (n = 1), and human leukocyte elastase (HLE, n = 6). The target antigen remained unidentified in 26 patients. Perinuclear ANCA-positive patients showed atypical antigens in eight of 13 cases; all six patients with anti-HLE revealed a pANCA pattern. The antigens of atypical ANCA-positive patients remained unidentified in 21 of 26 (81%) cases. No signs of vasculitis were present in the ANCA-positive patients. ANCA are frequently found in the sera of HIV-positive patients. They bind to a variety of antigens. No correlation was found between ANCA positivity and autoimmune or opportunistic diseases.

Introduction

Antineutrophil cytoplasmic autoantibodies (ANCA) react with proteins inside cytoplasmic granules of neutrophils [1]. They have been described as markers of disease activity in autoimmune disorders, specifically small vessel vasculitis [2]. Several studies reported the occurrence of ANCA in HIV-infected patients [3–6] and autoimmune phenomena have even been suggested as playing a role in the pathogenesis of HIV infection and AIDS [7]. A pilot study performed at our outpatient unit suggested an association between ANCA and opportunistic diseases in HIV patients. We therefore prospectively evaluated the prevalence of ANCA, their antigen specificity, and possible disease associations in a cohort of HIV-positive patients.

Patients and Methods

In March and April 1995 serum samples of HIV outpatients were collected. Patients participating had given written informed consent prior to inclusion.

Clinical and laboratory tests: At the time of the study the patients' history was taken and the patients underwent a physical examination. Within 6 weeks of the study visit, a full white blood cell count and lymphocyte differentiation were done. Further laboratory parameters determined were erythrocyte sedimentation rate (ESR), serum levels of C-reactive protein (CRP), absolute type IgG gammaglobulin concentration, serum creatinine and urine sediment. If necessary, further diagnostic procedures were performed.

Indirect immunofluorescence technique in ANCA detection: Sera were screened for ANCA by the indirect immunofluorescence technique (IFT) according to standardized European guidelines [8]. In brief, cytospin preparations of granulocytes fixed on dried ethanol were incubated with several dilutions of patient sera (1:2 to 1:1,024 in phosphate-buffered saline). Antibody binding was detected with FITC-conjugated $F(ab)_2$ fragments of rabbit antihuman IgG (Dako, Copenhagen, Denmark).

Fluorescence patterns were classified as classic cytoplasmic (cANCA) when a cytoplasmic fluorescence pattern with accentuation within the nuclear lobes was observed (Figure 1a), as perinuclear (pANCA) when a perinuclear to nuclear fluorescence was observed (Figure 1b), and as atypical (aANCA) when more diffuse cytoplasmic staining patterns were present (Figure 1c). Samples with a pANCA pattern on ethanol-fixed neutrophils were also tested for differentiation of pANCA from antinuclear antibodies (ANA) on formalin-fixed neutrophils and Hep-2 cells.

Enzyme-linked immunosorbent assay for ANCA specificity: The presence of PR3-ANCA, CG-ANCA, LF-ANCA, HLE-ANCA, MPO-ANCA, and LZ-ANCA was detected by ELISA using direct coating with highly purified antigens (MPO at a concentration of 0.5 µg/ml (Calbiochem, Bad Soden, Germany); HLE, PR3, CG, LF, LZ, 2 µg/ml) in 0.1 M carbonate buffer, pH 9.6, overnight at 4°C. Control cells were incubated with coating buffer without antigen, and positive and negative reference sera. After washing with PBS and 0.05% Tween 20, the sera were incubated with blocking buffer (2% casein in PBS).

Test and control sera were applied at a dilution of 1:50. Bound antibodies were detected by incubation with alkaline phosphatase conjugated goat antihuman IgG (Sigma Chemical, Munich, Germany). After a final wash, the cells were developed with *p*-nitrophenylphosphate (Sigma). The optical density was read at 405 nm. Results were considered positive when the value

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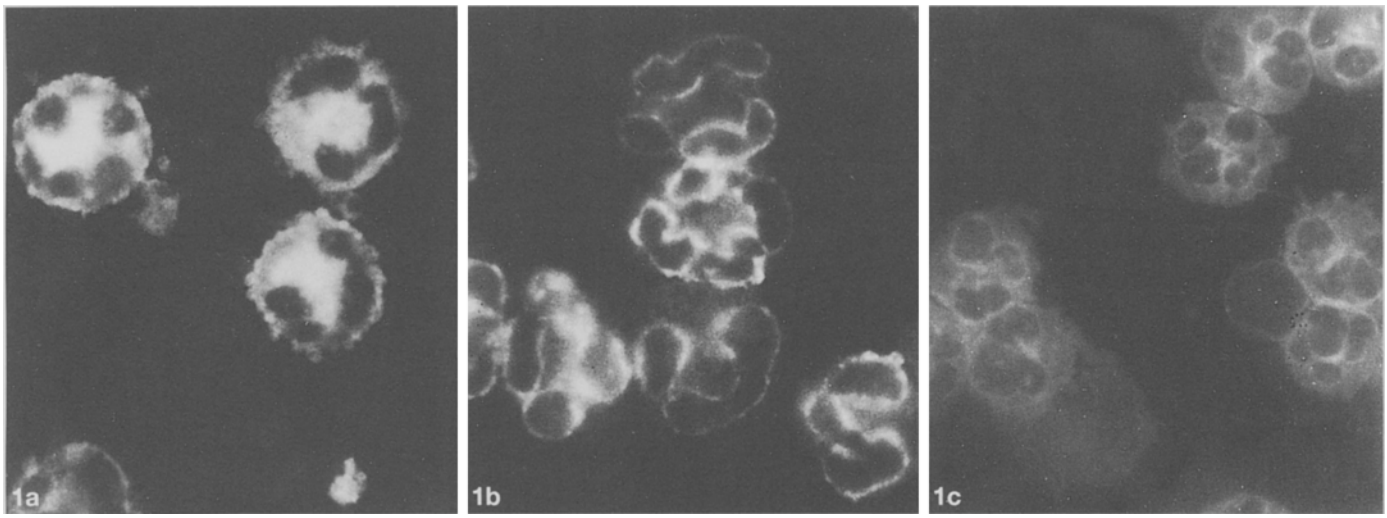

Figure 1: a) Fine granular cytoplasmic ANCA pattern as seen by IFT (cANCA). b) Perinuclear ANCA pattern (pANCA). c) Homogeneous cytoplasmic pattern with staining of perinuclear zone of PMN (aANCA).

obtained was more than three standard deviations above the mean value in 250 normal control sera. All ANCA-positive and ANCA-negative sera were analyzed by ELISA [9].
For both tests sera were not heated. Previous precipitation of immune complexes by the polyethylene glycol method did not change the results of ELISA and IFT.
Statistical analysis: Fisher's exact test was used to compare differences between groups of patients. P-values below 0.05 were considered statistically significant.

Results

Patient Characteristics

One hundred ninety-nine patients participated in the study. The 46 of 199 (23.1%) patients positive for either or both ANA and ANCA did not differ significantly from the 153 of 199 (76.9%) antibody-negative patients, as far as demographical data and the stage of HIV disease are concerned.
One hundred eighty-one male and 18 female patients with a median age of 39 years were included. The time since diagnosis of HIV infection ranged from 0.1 to 11.7 years, with a median of 3.3 years. The median CD4 cell count was 80/µl or 8% of the total lymphocyte count. At the time of serum sampling 77% of all patients had developed AIDS, and 76% were on current antiretroviral therapy.

Table 1: Frequency of autoantibodies detected by IFT.

Autoantibodies	No.	(%)
Any autoantibody	46	(23.1)
aANCA	25	(12.6)
aANCA + ANA	1	(0.5)
pANCA	9	(4.5)
pANCA + ANA	4	(2.0)
ANA alone	3	(1.5)
"Irregular"	4	(2.0)
No autoantibody	153	(76.9)
Total	199	(100.0)

IFT

Of the 46 of 199 (23.1%) patients with positive results in the immunofluorescence tests, 39 of 46 (84.8%) IFT results were classified as ANCA. These were pANCA in 13 of 39 (33.3%) and atypical ANCA in 26 of 39 (66.7%) patients. Of the ANCA-positive sera, five of 39 (12.8%) revealed antinuclear antibodies at the same time, while three of 46 (6.5%) sera showed ANA alone. In total, eight of 46 (17.4%) sera exhibited ANA.
Four of 46 (8.7%) immunofluorescent tests detected a cytoplasmic staining on formalin-fixed granulocytes with no reaction in the ethanol fixation. These "irregular" sera could not be assigned to one of the established staining patterns and should be addressed separately. In 153 of 199 (76.9%) patient sera, no autoantibodies were detected in the IFT performed. Results are displayed in Table 1.

Target Antigens

The target antigen was identified in 13 of 39 (33.3%) ANCA-positive sera. The antigens recognized by ELISA were PR3 (n = 2), MPO (n = 1), LZ (n = 2), LF (n = 1), CG (n = 1) and HLE (n = 6). In 26 of 39 (66.7%) ANCA-positive cases, the three ANA-positive and all four sera with "irregular" IFT staining, no target antigen was identified. No sera were positive in more than one assay and no ANCA-negative patient tested positive in the ELISA.

Table 2: Current diagnoses in cases with identified antigens and their corresponding IFT results.

Antigen		IFT	Diagnoses
Proteinase 3	(n = 2)	aANCA	Hepatitis B*
		aANCA	Progressive multifocal leukencephalopathy*
		aANCA	No current disease diagnosed
Myeloperoxidase	(n = 1)	aANCA	Non-Hodgkin's lymphoma
Lysozyme	(n = 2)	aANCA	Kaposi's sarcoma
		pANCA	Cytomegalovirus disease
Lactoferrin	(n = 1)	aANCA	Herpes simplex virus type II*
			Kaposi's sarcoma*
Cathepsin G	(n = 1)	pANCA	Kaposi's sarcoma
Elastase	(n = 6)	pANCA	Kaposi's sarcoma
		pANCA	Kaposi's sarcoma*
		pANCA	Non-Hodgkin's lymphoma
		pANCA	Cytomegalovirus disease
		pANCA	Cytomegalovirus disease*
		pANCA	Hepatitis B*
		pANCA	*Mycobacterium kansasii* pneumonia*
		pANCA	Intestinal microsporidiosis*
		pANCA	Sudden loss of hearing†

* more than one opportunistic disease at the time of serum sampling; † no apparent association with immunodeficiency.

Clinical and Laboratory Abnormalities

Both ANCA-positive and ANCA-negative groups did not differ significantly concerning fever, ESR, CRP, serum IgG levels or absolute CD4-positive cell count, leukocyte and neutrophil counts (data not shown).

We found no difference between both groups in clinical signs, i.e. loss of weight, night sweats, myalgia, joint or abdominal pain, neuropathy, skin lesions or unexplained upper or lower respiratory symptoms, so that no biopsies were done. None of our patients had ever been diagnosed with vasculitis or autoimmune disease. Renal function, as indicated by normal urine analysis results and serum creatinine levels, was not impaired in any ANCA- or ANA-positive nor in 151 of 153 (98.7%) negative patients.

Current Opportunistic Disease

Opportunistic diseases were diagnosed according to CDC recommendations [10]. Overall, 98 of 153 (64.1%) patients without and 31 of 39 (79.5%) patients with ANCA were treated for opportunistic diseases at the time of serum sampling. 12 of 39 (30.8%) patients with and 20 of 153 (13.1%) without ANCA had Kaposi's sarcoma. Non-Hodgkin's lymphoma had been diagnosed in four of 39 (10.3%) cases of ANCA-positive and in 13 of 153 (8.5%) ANCA-negative patients. Five of 39 (12.8%) patients with and 21 of 153 (13.7%) without ANCA had cytomegalovirus disease.

Diseases present in those patients whose target antigens were identified are given in Table 2. No association between antigens and underlying opportunistic diseases could be shown.

Discussion

The association of autoimmune phenomena and HIV infection has been discussed for nearly as long as AIDS itself [11]. In 1990 *Koderisch* et al. reported the occurrence of antineutrophil cytoplasmic antibodies in HIV-infected patients [3]. Based upon incidental [6, 12] and systematic [4, 5] investigation, 87 HIV-infected patients have been reported to test positive for ANCA so far. Several different IFT and ELISA techniques have been applied in these studies. We report on a prospective series of 199 unselected consecutive HIV patients.

In our series no correlation was found between stage of disease and the occurrence of ANCA. These results are consistent with previous analyses [3–5]. Patients treated with nucleoside analogues tended to be ANCA-positive more frequently than patients without current antiretroviral therapy, but this difference was not statistically significant.

So far, the staining patterns reported include 16 cytoplasmic, seven perinuclear, and ten atypical ANCA [4, 5, 13]. ANCA occur in HIV patients with a frequency varying from 18% [4] to 41.9% [5]. In our series the prevalence was 19.6% (39 of 199). We found no cANCA, but pANCA in 13 of 39 (33.3%) and the atypical staining pattern in 26 of 39 (66.7%) sera. However, in 8.7% of the sera we found a cytoplasmic staining by IFT on formalin fixed granulocytes and no reaction in the ethanol fixation. The antigen related to this type of ANCA remained unidentified. Recently, it has been shown that the bactericidal/permeability increasing protein (BPI) may be destroyed in ethanol fixation [14]. Unfortunately, there was not enough serum available to focus on this new aspect when the

BPI-ELISA became available. Antinuclear antibodies, with or without matching clinical symptoms, have frequently been reported in HIV patients [15, 16]. We found them in 4% of our series.

The ELISA findings depicted previously are seven anti-PR 3, 26 anti-MPO, and two anti-HLE antibodies, plus 11 IFT-positive but ELISA-negative serum samples. We identified the target antigen in 13 of 39 (33.3%) cases by specific ELISA and detected antibodies against all six proteins we tested for. To our knowledge, this is the first report on specific LZ, LF, and CG ELISA in HIV patients. The antigen could not be determined in 26 of 39 (66.7%) sera with positive ANCA immunofluorescence tests. Whether or not these sera contain ANCA directed against one of the antigens recently identified, e.g. bactericidal/permeability increasing protein [17] and azurocidin [18], remains subject to future investigation.

Unspecific clinical and laboratory signs of inflammation and elevated gammaglobulin serum levels, lymphopenia and leukopenia are common in HIV-infected patients, especially with advanced immunodeficiency [19, 20]. In our analysis neither fever nor the laboratory markers showed any significant difference between ANCA-positive and ANCA-negative patients. Our results do not confirm the reported [3, 4] association between ANCA and elevated serum gammaglobulin concentrations. There have been contradictory reports concerning the association of altered full white blood counts and ANCA [4, 6, 21]. However, our data do not support any association of a low white blood cell count, neutropenia, lymphopenia or reduced CD4-positive cell counts and ANCA.

In accordance with the results of two large series published, none of our patients showed any evidence of cutaneous or systemic vasculitis [3, 5]. To date, only two ANCA-positive HIV patients have been reported to suffer from vasculitic disease [4, 6]. ANCA have been found in two of 11 (18.2%) patients in the presence of AIDS-associated malignancies [4]. In our institution the prevalence of ANCA in patients with an HIV-dependent malignancy was 43.6%, including 12 of 39 (30.8%) patients who had Kaposi's sarcoma. HIV-negative patients may also present with ANCA when suffering from myelodysplasia or lymphoma [22].

ANCA have been found in one AIDS patient with pulmonary tuberculosis [12]; compared to our series with four patients suffering from tuberculosis, all of them ANCA-negative, this might be a coincidental finding. Infectious diseases in HIV-negative patients are reportedly associated with ANCA, e.g. pulmonary aspergillosis [23] and invasive amoebiasis [24]. These diseases were present in none of our patients.

Some ANCA antigens are enzymes essential to host defense mechanisms [25, 26]. If ANCA bind to and thereby inhibit the antiinfective properties of these antimicrobial enzymes, this could result in a decreased host defense against fungal [25], and perhaps against bacterial and viral pathogens. All three types of infection are frequently found in HIV patients. Until now ANCA has not been described in association with viral infection, but recent results elucidated the etiology of Kaposi's sarcoma as dependent on latent HHV8 infection [27, 28]. Whether ANCA play a role in chronically active herpesvirus infection or are a mere epiphenomenon in HIV infection remains to be investigated in future studies.

References

1. **Gross, W. L., Schmitt, W. H., Csernok, E.:** ANCA and associated diseases: immunodiagnostic and pathogenetic aspects. Clin. Exp. Immunol. 91 (1993) 1–12.
2. **Jennette, J. C., Falk, R. J., Andrassy, K., Bacon, P. A., Churg, J., Gross, W. L., Hagen, E. C., Hoffman, G. S., Hunder, G. G., Kallenberg, C. G., McCluskey, R. T., Sinico, A., Rees, A. J., van Es, L. A., Waldherr, R., Wiik, A.:** Nomenclature of systemic vasculitides. Proposal of an international consensus conference. Arthritis Rheum. 37 (1994) 187–192.
3. **Koderisch, J., Andrassy, K., Rasmussen, N., Hartmann, N., Tilgen, W.:** "False-positive" anti-neutrophil cytoplasmic antibodies in HIV infection. Lancet 335 (1990) 1227–1228.
4. **Klaassen, R. J., Goldschmeding, R., Dolman, K. M., Vlekke, A. B., Weigel, H. M., Eeftinck Schattenkerk, J. K., Mulder, J. W., Westedt, M. L., von dem Borne, A. E.:** Anti-neutrophil cytoplasmic autoantibodies in patients with symptomatic HIV infection. Clin. Exp. Immunol. 87 (1992) 24–30.
5. **Savige, J. A., Chang, L., Horn, S., Crowe, S. M.:** Anti-nuclear, anti-neutrophil cytoplasmic and anti-glomerular basement membrane antibodies in HIV-infected individuals. Autoimmunity 18 (1994) 205–211.
6. **Davenport, A., Grant, P.:** False positive auto-antibodies in HIV infection. Nephron 59 (1991) 515–516.
7. **Martinez-A., C., Marcos, M., de la Hera, A., Marquez, C., Alonso, J., Toribio, M., Coutinho, A.:** Immunological consequences of HIV infection: advantage of being low responder casts doubts on vaccine development. Lancet ii (1988) 454–457.
8. **Wiik, A.:** Delineation of a standard procedure for indirect immunofluorescence detection of ANCA. APMIS (Suppl. 6) (1989) 12–13.
9. **Hagen, E. C., Andrassy, K., Chernok, E., Daha, M. R., Gaskin, G., Gross, W., Lesavre, P., Ludemann, J., Pusey, C. D., Rasmussen, N., Savage, C. O. S., Sinico, A., Wiik, A., van der Woude, F. J.:** The value of indirect immunofluorescence and solid phase techniques for ANCA detection. A report on the first phase of an international cooperative study on the standardization of ANCA assays. EEC/BCR Group for ANCA Assay Standardization. J. Immunol. Methods 159 (1993) 1–16.
10. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. MMWR Morb. Mortal. Wkly. Rep. 41 (1992) 1–19.
11. **Rubinstein, A., Butkus Small, C., Bernstein, L.:** Autoantibodies to T-cells in adult and pediatric AIDS. Ann. N. Y. Acad. Sci. 437 (1984) 508–512.
12. **Li, P., Leung, J., Lai, F., Wang, A., Lui, S., Leung, C., Lai, K.:** Use of antineutrophil cytoplasmic antibodies in diagnosing vasculitis in a Chinese patient population. Am. J. Nephrol. 14 (1994) 99–105.
13. **Sakkas, L., Kistis, C., Akritidis, N.:** Antineutrophil cytoplasmic autoantibodies in human immunodeficiency virus infection. Am. J. Kidney Dis. 24 (1994) 731.
14. **Stoffel, M. P., Csernok, E., Herzberg, C., Johnson, T., Carroll, S. F., Gross, W. L.:** Anti-neutrophil cytoplasmic antibodies (ANCA) directed against bactericidal/permeability increasing protein (BPI): a new seromarker for inflammatory bowel disease and associated disorders. Clin. Exp. Immunol. 104 (1996) 54–59.
15. **Kopelman, R. G., Zolla-Pazner, S.:** Association of human immunodeficiency virus infection and autoimmune phenomena. Am. J. Med. 84 (1988) 82–88.

16. **Kyriakis, K., Tosca, A., Katsantonis, J., Hatzivasiliou, M., Eliopoulos, G., Stratigos, J.:** Detection of autoimmunity parameters in the acquired immunodeficiency syndrome (AIDS). Int. J. Dermatol. 31 (1992) 113–116.
17. **Zhao, M. H., Jones, S. J., Lockwood, C. M.:** Bactericidal/permeability-increasing protein (BPI) is an important antigen for anti-neutrophil cytoplasmic autoantibodies (ANCA) in vasculitis. Clin. Exp. Immunol. 99 (1995) 49–56.
18. **Zhao, M., Lockwood, C.:** Azurocidin is a novel ANCA antigen in vasculitis. Clin. Exp. Immunol. 101 (1995) 41.
19. **Chess, Q., Daniels, J., North, E., Macris, N.:** Serum immunoglobulin elevations in the acquired immunodeficiency syndrome (AIDS): IgG, IgA, IgM, and IgD. Diagn. Immunol. 2 (1984) 148–153.
20. **Lane, H., Masur, H., Edgar, L., Whalen, G., Rook, A., Fauci, A.:** Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. N. Engl. J. Med. 309 (1983) 453–458.
21. **McCance-Katz, E., Hoecker, J., Vitale, N.:** Severe neutropenia associated with anti-neutrophil antibody in a patient with acquired immunodeficiency syndrome. Pediatr. Infect. Dis. J. 6 (1987) 417–418.
22. **Savige, J. A., Chang, L., Smith, C. L., Duggan, J. C.:** Anti-neutrophil cytoplasmic antibodies (ANCA) in myelodysplasia and other haematological disorders. Aust. N. Z. J. Med. 24 (1994) 282–287.
23. **Cho, C., Asuncion, A., Tatum, A.:** False-positive antineutrophil cytoplasmic antibody in aspergillosis with oxalosis. Arch. Pathol. Lab. Med. 119 (1995) 558–561.
24. **Pudifin, D. J., Duursma, J., Gathiram, V., Jackson, T. F.:** Invasive amoebiasis is associated with the development of anti-neutrophil cytoplasmic antibody. Clin. Exp. Immunol. 97 (1994) 48–51.
25. **Bartunkova, J., Fucikova, T., Tesar, V., Janatkova, I., Rychlik, I., Sulkova, S.:** Antineutrophil cytoplasmic antibody-positive sera inhibit candidacidal activity of granulocytes. Exp. Nephrol. 3 (1995) 58–60.
26. **Weiss, J., Victor, M., Kao, L., Elsbach, P.:** Killing of gram-negative bacteria by neutrophils: role of O_2-independent system in intracellular killing and evidence of O_2-dependent extracellular killing. In: *Herkart, P., Martz, E.* (eds.): Mechanisms of cell-mediated toxicity II. Plenum Press, New York 1985, pp. 35–50.
27. **Chang, Y., Cesarman, E., Pessin, M., Lee, F., Culpepper, J., Knowles, D., Moore, P.:** Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science 266 (1994) 1865–1869.
28. **Gao, S., Kingsley, L., Hoover, D.:** Seroconversion to antibodies against Kaposi's sarcoma-associated herpesvirus-related latent nuclear antigens before the development of Kaposi's sarcoma. N. Engl. J. Med. 335 (1996) 233–241.