Anticytokeratins Are a Potential Source of False-Positive Indirect Immunofluorescence Assays for C-ANCA

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Antibodies to neutrophil cytoplasmic antigens (ANCA) targeted toward granule enzymes have been recognized as a valuable diagnostic tool in the detection of Wegener's granulomatosis and systemic vasculitides. However, the most commonly used method of detection, the indirect immunofluorescence assay, is prone to false-positive results due to antibodies of different pathological significance either targeted to, or cross-reacting with, similarly distributed epitopes. Using double immunofluorescence, the present study demonstrates that anticytokeratin antibodies are able to produce false-positive C-ANCA immunofluorescence assays. In addition, a case of natural appearance of cytokeratin-reactive antibodies causing a false-positive "pseudo-ANCA" staining pattern in a patient presenting with sepsis is

reported. Since the expression of cytokeratins is almost exclusively confined to epithelial cells, the most plausible explanation for both phenomena is a crossreaction of anticytokeratin antibodies with granule associated epitopes. Due to the natural appearance of anticytokeratin antibodies in association with a variety of other pathologic entities, it is of crucial importance for the diagnostic significance of the C-ANCA immunofluorescence assay to exclude anticytokeratin caused false-positive results. It is shown that supplementary indirect immunofluorescence tests performed on cultured human epithelial cells readily distinguish anticytokeratin caused "pseudo-ANCA" from true C-ANCA. J. Clin. Lab. Anal. 12:54–59, 1998. © 1998 Wiley-Liss, Inc.

Key words: anticytokeratin antibodies; ANCA; ANCA detection; streptococcal sepsis; Wegener's granulomatosis

INTRODUCTION

Autoantibodies to neutrophil cytoplasmic antigens (ANCA) were first described by Davis et al. (1) in patients with segmental necrotizing glomerulonephritis. Subsequently it was demonstrated that ANCAs are targeted toward neutrophil granule enzymes, appear with a high frequency in patients with Wegener's granulomatosis (2) and systemic vasculitis (3), and represent valuable diagnostic and monitoring parameters (2, 3).

ANCAs are detected primarily by visualization via an indirect immunofluorescence assay (IIF), where three different staining patterns are apparent: a diffuse granular cytoplasmic pattern with a central accentuation (C-ANCA), a perinuclear pattern (P-ANCA), and a pattern possessing characteristics of both, C- and P-ANCA, designated as X-ANCA (4), or atypical ANCA (5). Employing Western blot tests and sequence analysis Proteinase 3 (PR3) (5–7) and Myeloperoxidase (MPO) (3) were characterized as C-ANCA

and P-ANCA target antigens and identified as the major autoantigen in Wegener's granulomatosis (8, 9) and systemic vasculitis (3), respectively. Other lysosomal proteins (5, 10–12) and most recently the bactericidal/permeability-increasing protein (13, 14) were also identified to act as target antigens of ANCAs (although at lower frequencies). Subsequently different patterns of appearance of C- and/or P-ANCAs were found to distinguish various forms of systemic vasculitis, and new classifications integrating serological data were developed (15–17). However, ANCAs were also detected regularly in other groups of diseases such as rheumatic diseases (18) and inflammatory bowel diseases (19, 20). Although a number of supplementary solid phase as-

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says for anti-PR3 and anti-MPO antibodies have been developed to increase test specificity (21–23), the IIF assay is still the most commonly used tool of ANCA detection and the “golden standard” of sensitivity (24, 25).

However, because any IIF assay only reflects the presence of some antibody and the distribution of the corresponding antigen regardless of its molecular identity, it is likely that numerous other antibodies that are not directed toward the yet characterized epitopes on neutrophil granule enzymes may mimic C-ANCA as well as P-ANCA staining patterns in the IIF assay. Thus similarly distributed molecules acting or cross-reacting as targets for antibodies of different pathologic significance should be taken into account, since they may yield false-positive ANCA IIFs. Since the first report about such false-positive results due to “pseudo-ANCA” have been presented at the 1st International Workshop on ANCA (26), an increasing body of evidence has accumulated demonstrating that under various pathological conditions misleading, and—in the original sense of antigranule enzyme targeted antibodies—false-positive ANCA IIF staining patterns may occur. For example, in the presence of neutrophil alloantibodies to the NB1 and the Mart-antigen false-positive ANCA IIFs have been observed (27). Positive C-ANCA IIFs of different pathologic significance were observed in association with amoebic liver abscess (28), thyroid diseases treated with propylthiouracil (29), and infection (30). In some cases, uncritical interpretation of C-ANCA IIF proved even disastrous for the patients (31). Consequently, it is of crucial importance for the diagnostic value of ANCA IIF further to identify and characterize antibodies and antigenic sites that yield ambiguous cytoplasmic staining, as well as to develop supplementary tests, that allow for identification of false-positive IIFs.

The present study demonstrates the occurrence of false-positive C-ANCA IIF due to anticytokeratin antibodies. In addition, the natural appearance of a false-positive C-ANCA IIF due to antibodies reactive with cytokeratins in a patient presenting with sepsis due to infection with β -hemolytic streptococcus type A is reported. In conclusion, the spectrum of antigen-antibody-pairs capable of producing false-positive C-ANCA IIFs has to be extended to include cytokeratins and anticytokeratin antibodies. Consequently, before certain clinical backgrounds, the diagnosis of C-ANCA from IIFs must not solely be based on cultured granulocytes but should also comprise antigenic substrates that enable distinction between cytokeratin-specific antibodies and true C-ANCA.

MATERIALS AND METHODS

Patient History

A 53-year-old female patient presented with symptoms of a systemic inflammatory response due to a superficial crural thrombophlebitis. According to the definition of the ACCP/SCCM Consensus Conference Committee (32), the clinical diagnosis of sepsis was stated. Blood samples for culture and

screening for a potential concomitant autoimmune process were taken and antibiotic treatment with flucloxacillin and amoxicillin plus clavulanate was initiated. Because the presence of C-ANCA-like autoantibodies could be detected in the IIF, but not in the ELISA, the findings did not permit a diagnostic conclusion, thus prompting the supplementary tests as described below. Blood culture, however, revealed the presence of a systemic infection due to β -hemolytic streptococcus type A, susceptible to both antibiotics. Under antibiotic treatment septic symptoms receded during the following 2 weeks, and the patient had fully recovered and was dismissed 4 weeks later. In order to check for later appearance of true C-ANCA or vasculitic symptoms, the patient was clinically and serologically postexamined in 4–6-week intervals until 18 months postdismissal. During this period no indication to a later development of vasculitis or any other disease was stated.

Antibodies and Antigenic Substrates

Blood samples from the patient for IIF and ELISA testing were taken on the second day after admission to hospital. Serum was obtained by standard centrifugation, aliquoted, and frozen at -30°C until analysis. Positive and negative C-ANCA control sera as well as FITC-conjugated affinity purified anti-human IgG (F:P ratio = 1,5), PBS, and mounting medium were taken from NOVALITE® IFA-Autoimmune Disease Diagnostic Kits (Inova Diagnostics, San Diego, CA). For double immunostaining, the FITC-conjugated secondary antibody to human IgG provided with the kit was replaced by affinity purified goat-antihuman IgG (Calbiochem, product no. 401456) labeled with Rhodamine (F:P ratio = 3,28). Mouse monoclonal antipan-cytokeratin antibodies, recognizing human cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, and 19, were purchased from Sigma (St. Louis, MO; product no. C-2562). Affinity isolated goat-antimouse IgG (Sigma, product no. F-5262) conjugated to FITC (F:P ratio = 4) was used to detect mouse-anticytokeratin antibodies. Indirect immunofluorescence tests were performed on cultured human epithelial cells (HEp2-cells), ethanol-fixed human neutrophil granulocytes, and formaldehyde-fixed human neutrophil granulocytes on commercially available standard substrate slides (Inova) as well as on fresh smears of isolated neutrophils, which were prepared following the standard procedure delineated by Wiik (33).

Immunofluorescence Staining

Prepared substrate slides were incubated with appropriately diluted test sera (patient or C-ANCA positive and negative controls) for 30 minutes at room temperature in a humid chamber. Slides were then briefly flushed with an excess of PBS and rinsed for 7 minutes under permanent stirring in a coplin jar filled with PBS. Excess PBS was then blotted off and the secondary antibody was applied for 30 minutes. Finally, slides were washed again as described above. For single immunofluorescence staining, slides were immediately mounted and

Fig. 1. Double-immunostaining of ethanol-fixed neutrophil granulocytes, obtained with (a) mouse-antihuman pan-cytokeratin antibodies and (b) C-ANCA positive control serum (final magnification = 860 $\times$). Double immunostaining of formaldehyde-fixed neutrophil granulocytes, obtained with (c) mouse-antihuman pan-cytokeratin antibodies and (d) serum of the pa-

tient with septic inflammation (final magnification = 650 $\times$). Double immunostaining of HEp2 cells, obtained with (e) mouse-antihuman pan-cytokeratin antibodies and (f) serum of the patient with septic inflammation (final magnification = 750 $\times$).

covered, whereas for double immunofluorescence staining, the same protocol was repeated applying mouse-antihuman-pan-cytokeratin antibodies as the first layer and FITC-conjugated goat antimouse IgG as the second layer. Positive and negative C-ANCA control sera were used in dilutions recommended in the kit protocol. The optimum working dilutions for anticytokeratin antibodies (1:80) as well as of the secondary, fluorescence-conjugated antibodies (FITC-conjugated antibodies 1:150, rhodamine-conjugated antibodies 1:120) were determined previously by serial dilutions. With the patient's serum, additional dilution series were performed to determine the titer

of autoantibodies. All staining patterns obtained by double immunofluorescence were confirmed by separate single immunofluorescence staining of the respective cells with the low-F:P-ratio secondary antibodies listed above, and by negative controls with only the conjugates applied.

The cytokeratin specificity of the patient's antibodies responsible for the C-ANCA staining pattern was counterchecked by preabsorbing the patient's serum with graded concentrations of cytokeratin 18 fragments (30, 120, 600, and 1,200 U/L) 'provided as standards with the TPSTM EIA kit (Beki Diagnostics AB, Bromma, Sweden). Subsequently the same stain-

ing procedures as described above for the untreated patients serum were performed with the preabsorbed sera.

Inspection of immunostained slides and photography were done at 40× and 60× magnification on a Nikon Microphot-FXA microscope.

ELISA

Besides the IIF assay, the patient's serum was repeatedly tested for PR3 and MPO specific antibodies using the quantitative SYN-ELISA kit (ELIAS Medizintechnik GmbH, Germany).

RESULTS

Double Immunofluorescence Staining on Neutrophil Granulocytes

Applying the mouse antipan-cytokeratin antibodies to ethanol-fixed and to formaldehyde-fixed human neutrophil granulocytes resulted in regular staining patterns typical for C-ANCA. On ethanol-fixed cells, a diffuse granular cytoplasmic staining pattern with a typical accentuation of fluorescence intensity between the nuclear lobes was apparent (Fig. 1a). On formaldehyde-fixed granulocytes, the cytoplasmic staining pattern with an epinuclear accentuation, characteristically pronounced in granularity, appeared again (Fig. 1c). Comparing these patterns with the positive C-ANCA control, no distinct differences were recognizable on ethanol-fixed (Fig. 1b) or on formaldehyde-fixed granulocytes. Applying the patient's serum up to a dilution of 1:80 on ethanol- and formaldehyde-fixed cell preparation (Fig. 1d) yielded identical cytoplasmic staining patterns, indistinguishable from the positive C-ANCA controls and the antipan-cytokeratin pattern (Fig. 1c).

Double Immunofluorescence Staining on HEp2 Cells

Application of antipan-cytokeratin to HEp2 cells reveals the cytokeratin-specific three-dimensional network of intermediate filaments (Fig. 1e). With the patient's serum an almost identical, colocalized, definite cytoskeletal staining pattern appeared (Fig. 1f). The stained structures formed a basketlike cage around the nucleus and extended as a three-dimensional network of thin threads from the nuclear membrane to the cortex zone (Fig. 1f). Although the staining pattern obtained with the patient's serum was less prominent than that achieved with the monoclonal antibodies, the target filaments detected were unequivocally identical.

Application of the positive C-ANCA control serum to HEp2 cells resulted in a faint, almost homogeneous staining of the entire cytoplasm with no intracytoplasmic structures distinguished.

Negative controls with either the negative patients serum from the NOVA LITE® kit or only the individual secondary antibody applied exhibited no staining on either of the above cell types.

Applying the patients serum samples preabsorbed with graded concentrations of cytokeratin 18 fragments to the same cell substrates revealed a progressive reduction of the fluorescence signal at 30, 120, and 600 U/L and full elimination at 1,200 U/L on all cell substrates, thereby confirming the responsibility of the patients cytokeratin-reactive antibodies for the "pseudo C-ANCA" staining.

ELISA

The results of the quantitative ELISA assays for PR3- and MPO-specific antibodies in the patient's serum were repeatedly within the normal range (<7 U/ml).

DISCUSSION

In summary, the results of the present investigation enable two conclusions: (1) antibodies to cytokeratins can produce a false-positive C-ANCA on the standard IIF set-up (ethanol- and formaldehyde-fixed human neutrophil granulocytes), and (2) naturally occurring antibodies to cytokeratin will cause misleading false-positive C-ANCA IIF and must be taken into account for interpretation.

The first of these conclusions presents a dilemma regarding the molecular basis of the false-positive C-ANCA IIF obtained with mouse antipan-cytokeratin. On one hand, the expression of cytokeratins has so far been exclusively detected in embryonic cells and cells of epithelial origin (34), with the exception of some mesenchymal cells in lower vertebrate species (35). On the other hand, the antipan-cytokeratin antibodies used are definitely specific for human cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, and 19. Thus cross-reactivity based on epitopes in common with some cytoplasmic constituent of neutrophil granulocytes is the most plausible explanation for the false-positive C-ANCA IIF. The staining pattern on neutrophil granulocytes indicates that the epitope likely resides either on the granule membrane or on any of the granule enzymes. Further, this epitope appears to be independent from the fixation mode, in contrast with some of the established target epitopes of true ANCA, that are subject to changes in availability and relocation due to different fixatives (36).

The second conclusion has two aspects: the natural occurrence of anticytokeratin antibodies in principle, and the appearance in conjunction with sepsis. Whereas the occurrence of anticytokeratin autoantibodies is a well-known phenomenon (e.g., in anti-SLA positive autoimmune hepatitis (37), Type 1 diabetes (38), psoriatic arthropathy (39), Crohn's disease (40), after long-term therapy with leukocyte-derived interferon-alpha (41), and long-term hemodialysis (42)), the appearance in conjunction with streptococcal sepsis is a novel observation. However, there are several reports in the literature stating the appearance of positive C-ANCA IIF tests associated with infectious diseases (43, 44). Most interestingly, Mezzano et al. (45) report the appearance to autoantibodies

producing positive C-ANCA IIF tests in several patients with post-streptococcal glomerulonephritis. Although the specificity of these antibodies remains unexplored, the results suggest that an interrelationship between streptococcal infection and the appearance of false-positive C-ANCA is more than coincidental. Moreover, the plausibility of a pathophysiological connection between infection and the appearance of autoantibodies receives additional support from the work of Shoenfeld (46), who, based on his results in an experimental model for induction of autoimmunity, developed a concept of infection-triggered, idiopathic induction of autoantibody production.

With respect to the pathogenetic origin of the patient's antibodies, several mechanisms have to be considered. 1. Antibodies may be primarily targeted to a streptococcal component and secondarily cross-react with both cytokeratins and a neutrophil cytoplasmic antigen, due to common epitopes in all three targets. Similar multiple cross-reactions have been previously observed for antibodies to a glycylalanine repeat sequence of Epstein-Barr virus nuclear antigen-1 with collagen, cytokeratin, and actin (47). 2. Antibodies may be directed against a neutrophil granule component that is surface expressed and possibly modified due to streptococcal infection and secondarily crossreact with cytokeratin. This explanation is at least partially refuted by the negative results of the MPO- and PR3-ELISA. 3. Antibodies may be targeted to cytokeratins exposed due to cell injury during the inflammatory process, and secondarily cross-react with some neutrophil cytoplasmic antigen. Since the staining pattern obtained with the antibody on HEp2 cells was unequivocally identified as cytokeratin-specific and is much less ambiguous than the C-ANCA-like pattern on neutrophils, the third explanation appears most plausible. Furthermore, the two former explanations cannot account for the C-ANCA like pattern on neutrophils obtained with the monoclonal mouse antipan-cytokeratin antibody and would necessitate the assumption of an additional, coinciding cross reaction between the monoclonal mouse antibody and the granule component. However, this line of reasoning is not conclusive, and further investigation is required.

For the clinical interpretation of positive C-ANCA IIFs, the present findings mean that the possibility of autoantibodies to cytokeratins (due to streptococcal infection or other pathologic entities) and associated diseases must be taken into account. Especially in cases with a potential infectious origin of these antibodies, it is crucially important to distinguish them from true C-ANCA due to autoimmune diseases, since consequent therapeutic concepts are mutually contraindicated. For these cases supplementary IIFs on HEp2 cells provide a useful, reasonable means of differential diagnosis.

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