Table 1. Clinical profiles of patients with ulcerative colitis

Case	Age	Sex	Clinical Course	Extent of Disease	Sulfapyridine (g)	Prednisolone (mg)	UC-DAI	Plasma Cells*
Y.S.	36	F	Chronic	Entire	3.0	0	12	−
K.M.	30	F	Chronic	Left	3.0	40	5	n.d.
F.I.	49	F	Chronic	Entire	0	20	5	+
M.T.	45	F	Chronic	Left	2	20	10	+
J.I.	36	F	Chronic	Left	4.0	17.5	8	+
K.O.	39	M	Chronic	Entire	3.0	0	6	+
S.K.	29	F	Chronic	Entire	4.0	5	7	−
I.E.	51	M	Relapse	Entire	3.0	0	5	n.d.
K.Y.	22	F	Relapse	Entire	4.0	7.5	9	n.d.
K.K.	40	F	Relapse	Left	3.0	0	9	n.d.
J.Y.	39	F	Relapse	Entire	3.0	15	6	−
K.W.	52	F	Relapse	Proctitis	3.0	0	4	+
Y.O.	47	M	Relapse	Left	3.0	0	7	+
K.N.	20	F	Relapse	Entire	3.0	40	8	−
N.K.	39	F	Relapse	Entire	3.0	55	6	n.d.
M.A.	44	F	Relapse	Entire	4.0	0	6	+
N.K.	25	M	Relapse	Entire	3.0	55	10	+
H.O.	70	F	Initial	Entire	3.0	0	0	−
A.N.	30	F	Initial	Entire	1.0	0	1	+
T.I.	47	M	Initial	Entire	2.0	0	1	−
F.Y.	60	F	Initial	Proctitis	4.0	0	3	−

Sex: F, female; M, male; Disease course: chronic, chronic continuous type; relapse, relapse/remittent type; Initial, initial attack type; Extent of Disease: Entire, entire colotis; Left, left sided colitis; *Basal plasma cell infiltration, n.d.: not done.

the sera of patients with CD[10]. For these reasons, ANCA and the anti-Saccharomyces cerevisiae antibody can serve as clinical tools for making a differential diagnosis between UC and CD. Recently, ANCA has come to be considered a serological indicator of a higher relapse rate in UC[11]. However, determination of ANCA positivity by indirect immunofluorescence microscopy (IIF)--which is clinically available--seems to present problems for judging the positivity, and in some cases may depend on the objectivity of the observers. Recently, the flow cytometry method has come to be available in general hospitals. The present study was performed to clarify the clinical usefulness of flow cytometry for determining the positivity of ANCA, comparing this with the IIF method.

MATERIALS AND METHODS

Subjects

Twenty-one patients with UC in both active and inactive stages and seven patients with CD at the Niigata University Hospital were enrolled in this study. The diagnosis of UC and CD was based on clinical, endoscopic and histopathologic features which were consistent with the criteria established for UC and CD. All subjects gave informed consent to take part in the study. The clinical characteristics of the patients examined in this study were analyzed according to age, gender, diseased sites, clinical course, and ulcerative colitis-disease activity index (UC-DAI) at the endoscopic examination, and basal plasmacytosis in the colon biopsy specimens[12]. Diseased sites of patients with UC were divided into three groups: entire colitis, left sided colitis, and proctitis. The clinical course of UC was divided into three groups: chronic continuous, relapse/remittent, and initial attack types (Table 1). Basal plasmacytosis was determined to be positive when it was found in more than one of the specimens obtained during the endoscopy or surgery.

Methods

a) Flow cytometry

We used the modified method by Yang and et al. for detecting ANCA by flow cytometry (FCM)[13]. This method provides quantitative measurment of the intracellular fluorescence and objective judgement of ANCA positivity. We have applied this technique with some modification for the detection and quantification of ANCA in UC.

Cell preparation: Polymorphonuclear indicator cells (PMN) were prepared from heparinized blood obtained from healthy controls. First, 5 ml fresh blood was layered onto 3 ml Ficoll-Paque (Amersham Pharmacia Biotech, Little Chalfont) and centrifuged at 450 × g for 20 min. The mononuclear cellular interface and supernatants were removed and the cell pellet was resuspended in 10 ml MEM medium, and 2 ml of 6% dextran/PBS was added to this suspension. Erythrocyte sedimentation was performed for 30 min. The buffy coat was collected and washed twice with PBS supplemented with 2% NCS and 0.1% NaN_3 (PBS/NCS/Az).

Membrane fixation: Cell membrane stabilization was performed by paraformaldehyde (PFA) treatment in 1.5 ml micro test-tube at room temperature. First, 1.0×10^6 PMN were suspended and fixed in 250 μl of 4% PFA/PBS, pH 7.2, for 10 min. One volume of PBS/NCS/Az was then added and the suspension was spun for 2 min at 800 × g, then washed again with PBS/NCS/Az.

Permeabilization and staining: The fixed cell pellet was resuspended in 250 μl of 0.2% Tween 20 in PBS and incubated for 15 min at 37℃. One volume of PBS/NCS/Az was added, and then the suspension was spun for 2 min at 800 × g. The fixed and permeabilized 1.0×10^6 indicator cells were resuspended in 50 μl PBS/NCS/Az and 1 μl of 0.1% saponin/PBS. Then 10 μl sera from the patient samples under five grades of doubling dilution (1/4～1/64) were added to the cells and incubated for 20 min at 4℃. Duplicates for each serum tested were used. Sera from healthy volunteers were used as normal controls. To determine the effect of the fixation and permeabilization on neutrophil cytoplasmic antigens, FITC labeled monoclonal anti-myeloperoxidase (MPO) antibodies (Caltag, San Francisco, CA, USA), isotype control serum, and patient sera known to contain antibodies to proteinase 3 (PR3) and MPO (The Binding Site, Birmingham, England) were used. After two washes in PBS/NCS/Az, the pellets were resuspended in 50 μl PBS/NCS/Az and 1 μl of 0.1% saponin/PBS and 10 μl of FITC-labeled rabbit F $(ab')_2$ anti-human immunoglobulin (DAKO, Kyoto, Japan) or isotype control serum were added and incubated for 20 min at 4℃.

Flow cytometry: Indicator cells were washed twice and resuspended in 500 μl PBS/NCS/Az for testing on the FACScan flow cytometer (Becton Dickinson, USA). The population of fixed and permeabilized in dicator cells was gated using the forward and side scatter signal and FL-1 signal intensity of FITC-fluorescence of anti MPO monoclonal antibody.

Each analysis was performed in comparison with normal control serum. When a significant difference in mean fluorescence intensity (MFI) from the normal control was observed, the maximum final dilution of the samples was identified as the titer of ANCA (Fig. 1).

b) Indirect immunofluorescence method

The method is described in the directions of the commercial kit (The Binding Site, Birmingham, England). Sera from the patients and controls, which were diluted 1 : 24 on PBS at pH 7.4, were applied on a well-defined area of the slides in which there were formalin-fixed neutrophil substrates, and incubated for 30 min in a moist chamber at the room temperature. After washing with PBS, the slides were incubated with a droplet of FITC-conjugated anti-human immunoglobulin for 30 min in a humid chamber at room temperature. The cells were examined under a fluorescence microscope. The immunofluorescence pattern was evaluated by three independent observers who were unaware of the clinical diagnosis. In each series of test, positive and negative control sera which were contained in the kits were simultaneously examined. The ANCA (p-ANCA) test was considered positive when a distinct perinuclear fluorescence pattern was observed. A sample was considered negative if the specific neutrophil staining was equivalent to or less than the negative control well. A sample was considered ANCA positive if generalized granular staining was present.

c) Statistical analysis

Percentages of ANCA positivity among the different groups were determined by use of a chi-squared test. Sensitivity was defined as the ratio of true positives to true positives plus false negatives, and specificity as the ratio of true negatives to true negatives plus false positives.

RESULTS

A representative fluorescence histogram of untreated neutrophils, Tween 20-treated neutrophils, and

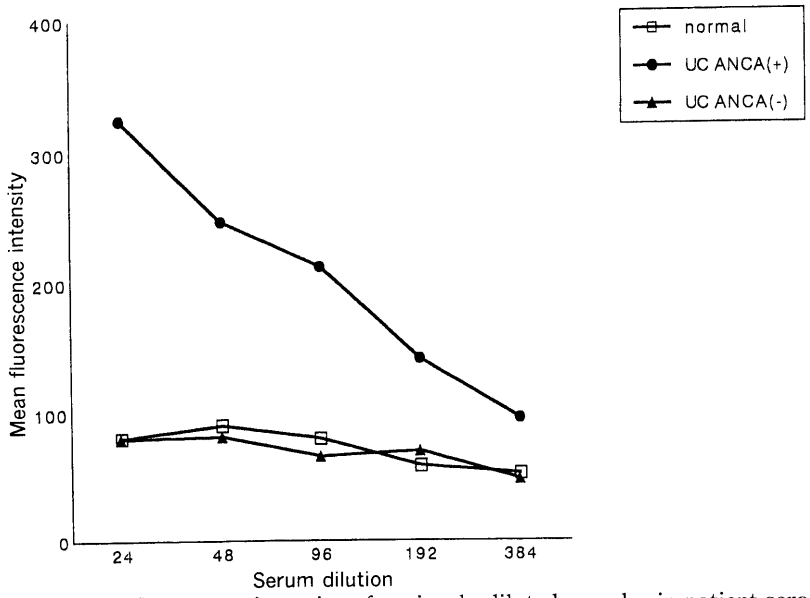

Fig. 1. Mean fluorescent intensity of variously diluted samples in patient sera and normal control sera. Confirmation of a significant difference in MFI from the normal control identified the maximum dilution of the sample as the titers of ANCA.

Tween 20 and Saponin-treated neutrophils which were incubated and stained with FITC labeled monoclonal anti-myeloperoxidase (MPO) antibody are shown in Fig. 2. The treatment of neutrophils with Saponin clearly showed the presence of an intracellular MPO antigen. A representative fluorescence histogram of Saponin-treated neutrophils incubated with ANCA positive and negative sera tested and stained with FITC-labeled rabbit F (ab')$_2$ anti-human immunoglobulin is shown in Fig. 3, demonstrating that the evaluation ot positivity by flow cytometry was made easily and objectively.

Forty-two sera from 21 patients with UC were examined for the detection of ANCA by flow cytometry and IIF, and seven sera from 7 patients with CD were examined by flow cytometry. Flow cytometry showed ANCA positivity in UC in thirty-five of 42 sera tested, with IIF showing this in twenty-eight of 42 sera tested, indicating that flow cytometry detected a higher rate of ANCA positivity than did IIF ($P<0.004$). Thirty-three of 42 sera yielded the same results by flow cytometry and IIF (Table 2). Eight IIF ANCA negative sera were found to be positive when they were examined by flow cytometry. When compared with IIF, the flow cytometry method for the detection of ANCA had a sensitivity of 96.5% and a specificity of 63.6%. ANCA positivity by flow cytometry and by IIF was 23 and 19 of 28 sera tested in entire colitis, 10 and 9 of 10 sera tested in left sided colitis, and 2 and 0 of 4 sera tested in proctitis,

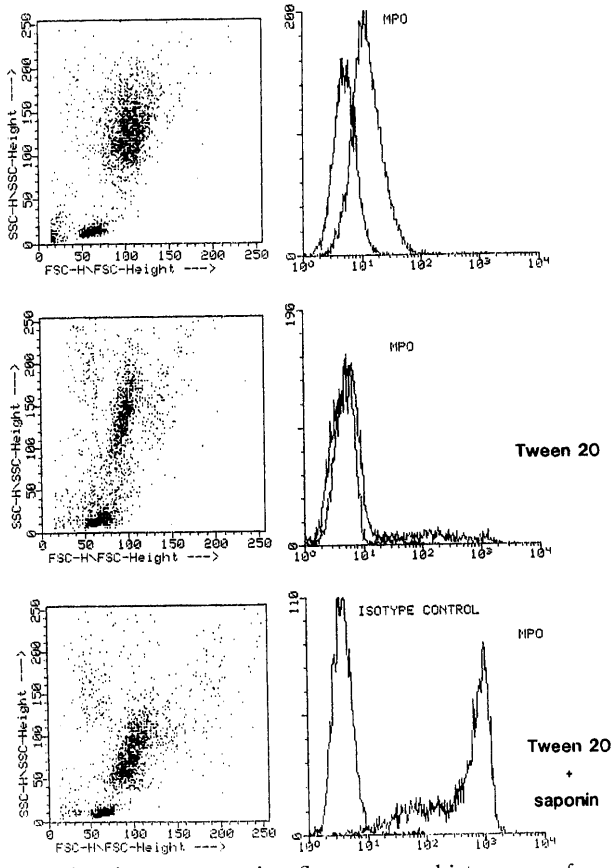

Fig. 2. A representative fluorescence histogram of untreated neutropils, Tween 20 treated neutropils, and Tween 20 and Saponin treated neutrophils in using an FITC labeled monoclonal anti-MPO antibody.

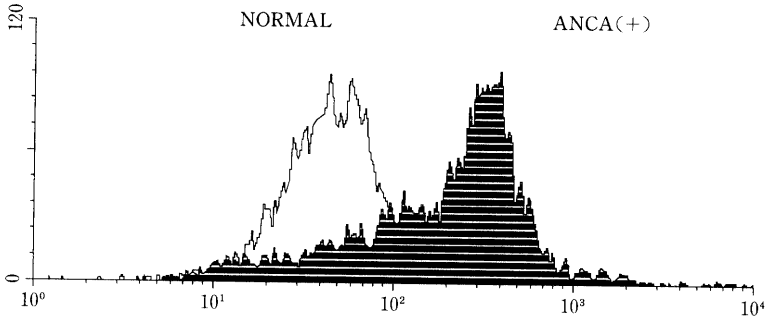

Fig. 3. A representative fluorescence histogram of neutrophils incubated with ANCA positive and negative sera and stained with FITC-labeled rabbit F (ab')$_2$ anti-human immunoglobulin.

Table 2. ANCA positivity in ulcerative colitis analysed by flow cytometry and IIF

	IIF	
	Positive	Negative
Flow cytometry positive	27	8
negative	1	6

Table 3. Positivity of ANCA shown by flow cytometry and IIF among diseased areas and clinical courses

		ANCA-positive	
		Flow cytometry	IIF
Entire colitis	28 sera	23	19
Left-sided colitis	10 sera	10	9
Proctitis	4 sera	2	0
Chronic continuous	14 sera	14	12
Relapse/remittent	20 sera	19	15
Initial attack	8 sera	2	1

Table 4. Positivity of ANCA by flow cytometry among clinical courses

	Flow cytometry		IIF	
	Positive	Negative	Positive	Negative
Continuous and relapse/remittent	33	1	27	7
Initial attack	2	6	1	7

respectively (Table 3). This became a respective 14 and 12 of 14 sera tested in the chronic continuous type, 19 and 15 of 20 sera tested in the relapse/remittent type, and 2 and 1 of 8 sera tested in the initial attack type. These results showed that ANCA positive cases increased with the flow cytometry method more than by IIF methods. The positive rate of ANCA shown by flow cytometry and IIF methods in patients with the three clinical course types of UC are given in Table 4, and the titers of ANCA in UC and CD are given in Fig. 4. The positive rates of ANCA in the continuous and relapse/remittent types of UC (intractable type) were significantly higher than for the initial attack type of UC (non-intractable) ($P<0.01$) in the flow cytometry method as well as IIF. However, some of the intractable cases showed negative results by the IIF method as compared with those by flow cytometry. Titers of ANCA in intractable cases were also higher than in the initial attack type of UC and CD.

However, they had no significant correlation with the presence of basal plasmacytosis in the colonic mucosa.

DISCUSSION

There are many kinds of antibodies and autoantibodies in the serum of patients with UC. However, the roles of these antibodies in the pathophysiology of UC have not yet been clarified. Despite this, ANCA is available as a tool for the diagnosis of several diseases such as ANCA positive glomerone-

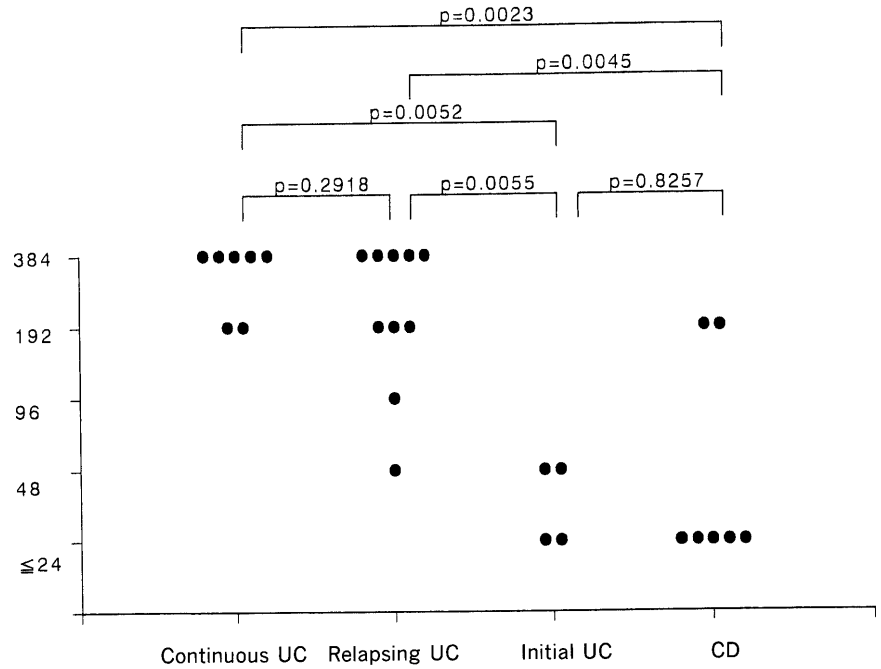

Fig. 4. Titers of ANCA by flow cytometry in the three clinical course types of UC and CD.

phritis, Wegener's granulomatosis (necrotizing vasculitis), UC, and CD [8,9,10]. Many antigens against ANCA have been found in the serum of patients, including proteinase 3 (PR3) in Wegener's granulomatosis, and myeloperoxidase (MPO) in Churg-Strauss syndrome and microscopic polyangitis[6]. On the other hand, several antigens against ANCA were found in the serum of patients with UC: lactoferrin, cathepsin, PR3, MPO-ANCA, HMG1, HMG2, and undetermined antigens which were proteins around 55-65 kDa in the azurophilic granules, 80-kDa protein, and 110-kDa cytosol component[14-18]. These antibodies may be produced by B and plasma cells infiltrating into the basal lamina propria of colonic mucosa in UC[2]. Recently, p-ANCA titer and total ANCA titer have been found to be good biological parameters as predictors of relapse in UC[11]. Several papers report that p-ANCA has no relationship to age at diagnosis, disease activity, and disease extension, and that p-ANCA positivity is associated with more aggressive forms of disease and p-ANCA negativity is associated with stable remission[19].

Since ANCA is frequently found in UC (30-60%) and less frequently in CD (9-10%), and the anti-saccharomyses antibody is frequently found in CD but hardly in UC, tests for measuring these antibodies are used for a differential diagnosis between UC and CD[20]. The prevalence of ANCA in the sera of patients with CD was slightly higher in the colonic type than the ileocolitis and ileitis types[21]. Staining patterns of ANCA are divided into perinuclear, cytoplasmic and nuclear types. Sera from patients with UC reacted to perinuclear and cytoplasmic components in most cases, and to nuclear components in a few cases[22]. However, evaluation of the positivity of ANCA in low titers by an IIF seems to depend on observer objectivity. A more objective determination of ANCA positivity is needed for clinical laboratories. We used flow cytometry to detect ANCA more objectively in the sera of patients with UC. The results obtained by flow cytometry were almost similar to those by IIF except for the low titers of ANCA. Yang and et al. reported that the flow cytometry method had a sensitivity of 93% and a specificity of 90% in autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and other illness, compared with IIF[13]. They concluded that the flow cytometry method had the advantage of providing an objective, reproducible, and quantitative measurement of ANCA. However, flow cytometry has drawbacks in being unable to allow discrimination between P-ANCA and C-ANCA and detection of the antinuclear factor (ANF). Still , the presence of ANF in the serum of patients with UC is reported to be rare except for the complication of primary sclerosing cholangitis[23]. The detection of ANCA is clinically important for making a differential diagnosis and predicting the relapse of UC.

CONCLUSION

Using the flow cytometry method to detect ANCA in the serum of patients with UC is clinically useful because it can detect ANCA objectively and quantitatively.

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