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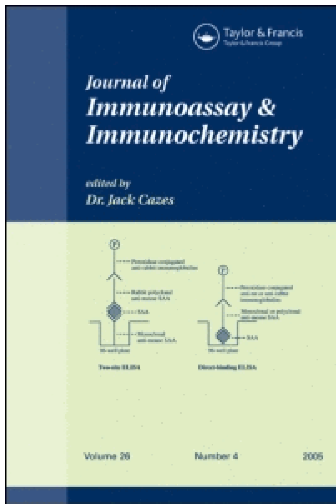

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ADVANCES IN THE DEVELOPMENT OF A RELIABLE ASSAY FOR THE MEASUREMENT OF STOOL DECAY-ACCELERATING FACTOR IN THE DETECTION OF COLORECTAL CANCER

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ADVANCES IN THE DEVELOPMENT OF A RELIABLE ASSAY FOR THE MEASUREMENT OF STOOL DECAY-ACCELERATING FACTOR IN THE DETECTION OF COLORECTAL CANCER

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ABSTRACT

We have previously shown that stool concentrations of decay-accelerating factor (DAF; CD55), a membrane-bound complement-regulatory protein, are significantly elevated in patients with colorectal cancer and that the measurement of

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stool DAF may be a valuable test for the detection of colorectal cancer. Accordingly, we are working to develop a clinically useful immunoassay for fecal DAF. A requirement for such assay is a plentiful and reliable supply of anti-DAF antibodies. We developed a sandwich enzyme-linked immunosorbent assay (ELISA) for DAF in stool specimens, using two monoclonal anti-DAF antibodies recognizing different epitopes on the DAF molecule. When we first used a biotin-labeled antibody and enzyme-linked streptavidin method, we often observed stool interference, probably due to the presence of a substance(s) with biotin activity which non-specifically bound to the Fc portion of IgG of the first anti-DAF antibody on the ELISA wells. By the use of inorganic salts in the sample-dilution buffer and HRP-labeled anti-DAF as second antibody, we circumvented the stool interference and established that the new ELISA system could reliably measure DAF at low concentrations in stool specimens. Because the new assay system uses only monoclonal antibodies, we can now consistently supply ample amounts of antibodies for routine measurement of stool DAF.

Key Words: Colorectal cancer; CD55; Immunoassay; Stool

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INTRODUCTION

Decay-accelerating factor (DAF; CD55) is a membrane-bound glycoprotein that regulates the activation of the autologous complement cascade by inhibiting the formation of, and promoting the catabolism of, C3 and C5 convertases.[1,2] In ours and others' previous work,[3,4] we showed enhanced expression of DAF in human colorectal cancer. That finding led us to ask whether the stools of patients with colorectal cancer contain increased amounts of DAF, and we developed an enzyme-linked immunosorbent assay (ELISA) to measure DAF in stools.[5] We found that stool DAF concentrations were significantly elevated in patients with colorectal cancer and concluded that the measurement of stool DAF may be a valuable test for the detection of colorectal cancer.[5]

In the original ELISA for stool DAF, we used a combination of mouse monoclonal and rabbit polyclonal anti-DAF antibodies.[5] In the present study, in order to achieve a stable DAF assay using a uniform source of antibodies, we refined the ELISA system by using only monoclonal anti-DAF antibodies. Other workers[6] had developed a sensitive ELISA utilizing biotin-streptavidin for the detection of adenovirus in feces. Thus, we first

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used a biotin-labeled antibody and enzyme-linked streptavidin method for the measurement of DAF. However, we often observed non-specific reactions. Therefore, we investigated the explanation for these non-specific reactions and we developed a reliable ELISA system for measurement of fecal DAF.

EXPERIMENTAL

Stool Specimens

One to three spontaneous stool samples (1–5 g) were obtained from each of 58 patients with colorectal cancer (25 women and 33 men, 40–90 years old; mean age 66 years) and 30 control subjects who underwent total colonoscopic examination because of abdominal symptoms or for screening for colorectal cancer and were found to have no colorectal disease (8 women and 22 men; 17–79 years old; mean age 53 years). Informed consent was obtained from each patient. The stools were weighed, suspended in an equal volume of phosphate-buffered saline containing 1% bovine serum albumin, 0.05% Tween 20, and 1 mM phenylmethylsulfonylfluoride, and centrifuged at 8000 g for a few seconds. Supernatants were collected and kept frozen at −80°C until use.

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ELISA Protocol

Human DAF was purified from pooled human erythrocyte stroma, and mouse monoclonal and rabbit polyclonal antibodies to DAF were prepared as described.[5,7] In the new system, we used two of these mouse monoclonal antibodies (IgG1) 1C6 and 4F11 which recognize different epitopes on the DAF molecule. The 1C6 antibody is directed to the active site on the DAF molecule, i.e., short consensus repeat (SCR) 3, while the 4F11 antibody recognizes SCR 4.[8] Monoclonal antibody 1C6 was labeled with biotin as described.[9,10] Horseradish peroxidase (HRP)-labeled 1C6 antibody was also prepared as described.[11]

The wells of microtiter plates were coated with 4F11 monoclonal anti-DAF antibody, and serially diluted stool supernatants were added to the wells. After washing, either biotin-labeled or HRP-labeled 1C6 anti-DAF antibody was added. After washing, bound 1C6 antibody was detected with HRP-labeled streptavidin (Chemicon International, Inc., Temecula, CA, U.S.A.) and 2,2′-azino-di-3-ethylbenzo-thiazoline-6-sulphonic acid (ABTS) as substrate when biotin-labeled 1C6 antibody was used, and only with ABTS when HRP-labeled 1C6 antibody was used. Optical densities

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(OD) at 415 nm were measured on an automated ELISA plate reader. A calibration curve was obtained from several dilutions of known quantities of purified DAF, and the concentrations of stool DAF were calculated. Samples were analyzed in duplicate.

As a reference system, the previous ELISA system was prepared as described.[5] Briefly, the wells of microtiter plates were coated with 1C6 mouse monoclonal anti-DAF antibody. Stool supernatants were added to the wells, and then rabbit polyclonal anti-DAF IgG was added. After washing, bound rabbit antibody was detected with HRP-labeled goat F(ab′)2 anti-rabbit IgG (Tago, Inc., CA, U.S.A.) and ABTS. Amounts of DAF were determined as described above. The relationship between DAF, determined by the new and the previous ELISA, was evaluated by Pearson's correlation coefficient.

For the analysis of non-specific reactions, IgG antibodies against unrelated antigens; anti-CD62E (ELAM-1) (Becton Dickinson, San Jose, CA, U.S.A.), anti-CD59/homologous restriction factor 20 (HRF20)(a gift from Prof. Hidechika Okada, Nagoya City University School of Medicine, Nagoya, Japan),[12] and anti-CD50 (DAKO, Glostrup, Denmark) were used instead of 4F11 anti-DAF antibody. F(ab′)2 fragments of anti-CD50 (Caltag Laboratories, Burlingame, CA, U.S.A.) and Fc portion of normal mouse IgG (Rockland, Gilbertsville, PA, U.S.A.) were also used. To examine the avidin–biotin interaction in non-specific reactions in the DAF ELISA, serially diluted stool samples were added to the wells coated with 4F11 anti-DAF antibody. After washing, wells were incubated with free avidin and biotin (Avidin/Biotin Blocking Kit, Vector Laboratories, Inc., Burlingame, CA, U.S.A.), sequentially. Then, biotin-labeled 1C6 anti-DAF antibody, HRP-labeled streptavidin and ABTS were added sequentially.

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RESULTS

When we measured DAF amounts in serially diluted stool samples using biotin-labeled 1C6 anti-DAF antibody as a second antibody, unusual curves of OD with two peaks at low and high dilutions of stool specimens were often observed (173 of 287 specimens); the curves seemed different from those of a prozone phenomenon (Fig. 1). To analyze the cause of the unusual OD curves, the following experiments were conducted. First, we omitted a single reagent in each step of the ELISA. When we omitted the 4F11 first antibody, or HRP-labeled streptavidin, the unusual curve disappeared but, when we omitted biotin-labeled 1C6 antibody, it was still observed (Fig. 1). With stool specimen omitted, no binding was observed (data not shown). These findings suggested that HRP-streptavidin directly

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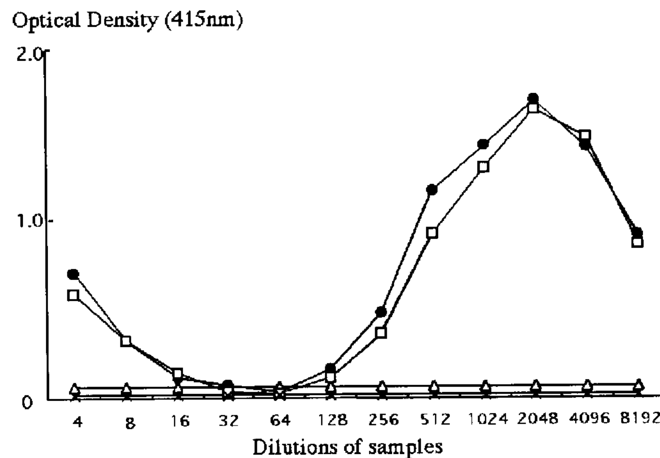

Figure 1. ELISA for stool DAF using biotin-labeled second antibody. Serially diluted stool samples were added to microtiter wells coated with 4F11 anti-DAF antibody. After washing, biotin-labeled 1C6 anti-DAF antibody, HRP-labeled streptavidin and ABTS were added sequentially. An OD curve with two peaks at low and high dilutions of stool specimens is seen (●). This non-specific binding is not observed without addition of 4F11 anti-DAF antibody (△) or HRP-labeled streptavidin (×) but is still present without addition of biotin-labeled 1C6 antibody (□).

bound to a substance(s) in stool specimens trapped by the 4F11 first antibody on the wells. Also, blocking with free avidin and biotin abrogated this binding (Fig. 2), suggesting that the binding occurred via the biotin-streptavidin interaction.

Next, we characterized the binding of substances with biotin-like activity to the first antibody. When we substituted IgG antibodies to other antigens unrelated to DAF (anti-CD62E, anti-CD59, or anti-CD50) for the 4F11 anti-DAF antibody, the unusual curve was still observed (Figs. 3 and 4). When we used $F(ab')^2$ fragments of anti-CD50 antibody, there was no binding, but there was binding when the Fc portion of normal mouse IgG was used (Fig. 4). Thus, the substance(s) with biotin activity seemed to bind non-specifically to the Fc portion of the first antibody on the wells.

When we increased the NaCl concentration of the buffer suspending stool specimens from 0.14 M to 0.4 M, the non-specific reactions were eliminated (Fig. 5). However, because we feared that this measure would not consistently eliminate the non-specific reactions, which occurred frequently, we abandoned the use of the biotin-labeled antibody and decided, instead, to use HRP-labeled 1C6 anti-DAF antibody as the second antibody, as well

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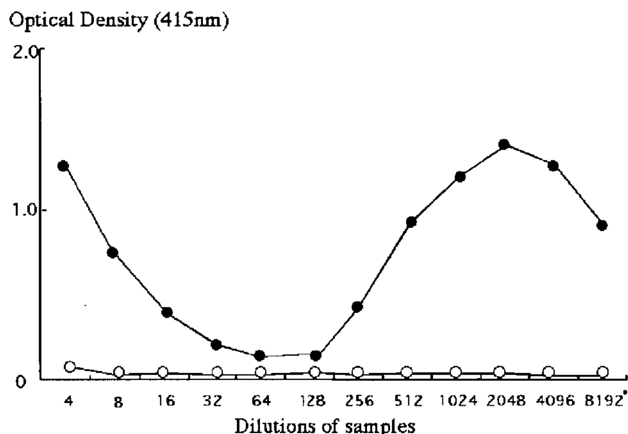

Figure 2. Blocking with free avidin and biotin. Serially diluted stool samples were added to the wells coated with 4F11 anti-DAF antibody. After washing, wells were incubated sequentially with free avidin and biotin. Then, biotin-labeled 1C6 anti-DAF antibody, HRP-labeled streptavidin and ABTS were added sequentially. The non-specific binding at low and high dilutions of this stool specimen (●) is eliminated by incubation with free avidin and biotin (○).

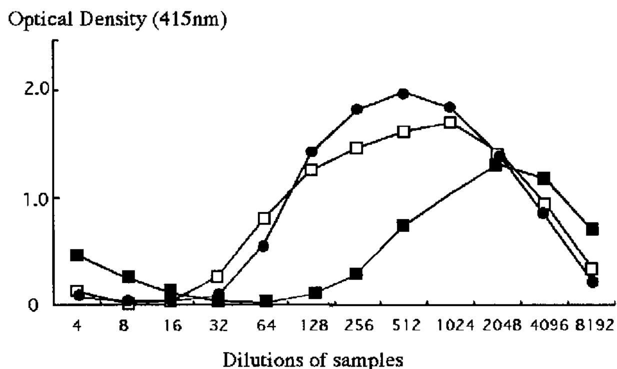

Figure 3. ELISA for stool DAF using biotin-labeled second antibody with various first antibodies. Microtiter wells were coated with 4F11 anti-DAF antibody or IgG antibodies to other antigens unrelated to DAF (anti-CD62E and anti-CD59). Serially diluted stool samples were added to the wells. After washing, biotin-labeled 1C6 anti-DAF antibody, HRP-labeled streptavidin and ABTS were added sequentially. Non-specific bindings seen with 4F11 anti-DAF antibody (●) are still present with anti-CD62E (□) and anti-CD59 antibodies (■).

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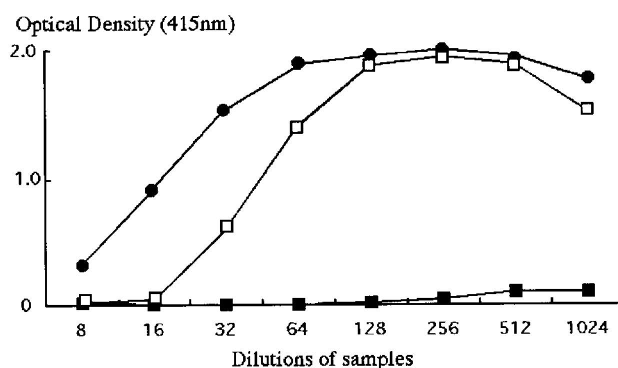

Figure 4. ELISA for stool DAF using biotin-labeled second antibody with F(ab′)^2 fragments or the Fc portion of IgG first antibody. Microtiter wells were coated with whole IgG anti-CD50 antibody, F(ab′)^2 fragments of anti-CD50 antibody, or the Fc portion of normal mouse IgG. Serially diluted stool samples were added to the wells. After washing, biotin-labeled 1C6 anti-DAF antibody, HRP-labeled streptavidin and ABTS were added sequentially. Non-specific binding seen with whole IgG anti-CD50 antibody (●) is not seen with F(ab′)^2 fragments of the anti-CD50 antibody (■) but is present with the Fc portion of normal mouse IgG (□).

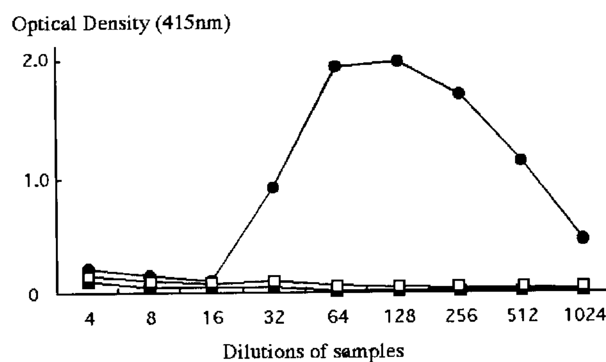

Figure 5. Elimination of non-specific binding with inorganic salts and HRP-labeled second antibody. Stool samples serially diluted with buffer containing NaCl at concentrations of either 0.14 M or 0.4 M were added to microtiter wells coated with 4F11 anti-DAF antibody. After washing, biotin-labeled 1C6 anti-DAF antibody, HRP-labeled streptavidin and ABTS were added sequentially. Increasing NaCl concentration of the buffer from 0.14 M (●) to 0.4 M (■) eliminated the non-specific binding. Use of HRP-labeled 1C6 anti-DAF antibody as a second antibody (□) also eliminated the non-specific binding seen with biotin-labeled antibody.

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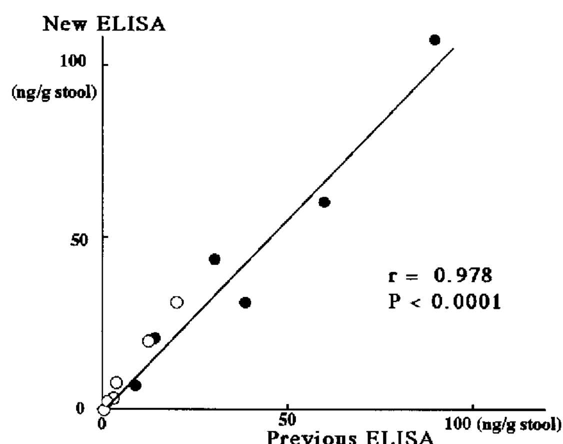

Figure 6. The relationship between DAF concentrations as determined by the new ELISA for stool DAF, using 4F11 and HRP-labeled 1C6 anti-DAF antibodies, and the concentrations obtained with the previous ELISA using the polyclonal second anti-DAF antibody. The concentrations obtained with the two assays correlate well in a linear manner ($r = 0.978$, $p < 0.0001$). ●, stools from patients with colorectal cancer ($n = 6$). ○, stools from control subjects ($n = 6$).

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as the buffer containing 0.4 M NaCl. Using the HRP-labeled 1C6 antibody, the non-specific reactions with biotin-labeled antibody were eliminated, as expected (Fig. 5). The new ELISA, using HRP-labeled 1C6 antibody and the buffer containing 0.4 M NaCl, was sensitive to 0.4 ng/mL and accurate to 12 ng/mL. DAF concentrations determined with the new ELISA correlated well with the concentrations obtained using the previous ELISA in a linear manner ($r = 0.978$, $p < 0.0001$) (Fig. 6).

DISCUSSION

In this study, we developed a new ELISA for measurement of DAF in stool specimens. Because the system uses only monoclonal antibodies, we can now consistently supply ample amounts of the needed antibodies for measurement of stool DAF. We also overcame a troublesome artifactual problem which occurred when we used a method employing a biotin-labeled antibody and enzyme-linked streptavidin: non-specific reactions caused by a substance(s) with biotin-like activity in stools. Biotin, a water-soluble vitamin which is important in gluconeogenesis and fatty acid synthesis,[13]

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is present in various foods, including liver, soy, beans, vegetables, and egg yolks, and is usually bound to protein in foods. In our initial assay, protein(s) coupled with biotin probably non-specifically bound to the Fc portion of IgG on the ELISA plate wells. Characterization of the biotin-binding proteins will require further study.

The non-specific interactions we observed could be overcome by the use of 0.4 M NaCl in the buffer. Use of inorganic salts has also been shown to minimize serum interference in a sandwich ELISA for human growth hormone.[14] However, because the non-specific interaction was observed often in stool samples in the initial study, we did not rely on the use of 0.4 M NaCl alone, but also abandoned the use of the biotin-labeled antibody. Instead, we used HRP-labeled 1C6 anti-DAF antibody as a second antibody, as well as the buffer containing 0.4 M NaCl in the new ELISA system, and we achieved consistent results which correlated well with those in the previous ELISA.

The two monoclonal antibodies used in the new ELISA, i.e., 4F11 antibody and the 1C6 antibody, recognize SCR 4 and SCR 3 domains of DAF molecule, respectively.[8] For full DAF function as a complement regulator, three SCR domains, SCR2, 3, and 4 are necessary.[8] Whether DAF in stools is active in terms of regulation of the complement activation and, if so, the pathophysiological function of stool DAF, await further clarification.

The improvements we have made in the DAF assay have brought it close to being a clinically applicable test. The assay is sufficiently sensitive to detect low concentration (0.4 ng/mL) of DAF in stool. Concentrations greater than 12 ng/mL exceed the concentration range of the assay, requiring dilutions of some stool samples for accurate measurement. We are currently working to develop an immunoassay with a wide assay range to overcome this inconvenience.

Reliable noninvasive methods for the detection of colonic neoplasms is a highly desirable goal, since the currently used radiologic and endoscopic methods are expensive and cumbersome, and fecal occult blood testing is insufficiently sensitive and specific. Other workers are measuring altered DNA in stool samples as a noninvasive test of colonic neoplasms.[15] Measurement of DAF now also seems worthy of further consideration for this important purpose.

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