Cancer Immunol Immunother (1996) 42: 185–192 

ORIGINAL ARTICLE

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# Characterisation of the complement-regulatory proteins decay-accelerating factor (DAF, CD55) and membrane cofactor protein (MCP, CD46) on a human colonic adenocarcinoma cell line

Received: 18 July 1995 / Accepted: 4 January 1996

**Abstract** To avoid destruction by complement, normal and malignant cells express membrane glycoproteins that restrict complement activity. These include decay-accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46) and protectin (CD59), which are all expressed on colonic adenocarcinoma cells in situ. In this study we have characterised the C3/C5 convertase regulators DAF and MCP on the human colonic adenocarcinoma cell line HT29. DAF was found to be a glycosyl-phosphatidylinositol-anchored 70-kDa glycoprotein. Blocking experiments with $F(ab')_2$ fragments of the anti-DAF monoclonal antibody BRIC 216 showed that DAF modulates the degree of C3 deposition and mediates resistance to complement-mediated killing of the cells. The expression and function of DAF were enhanced by tumour necrosis factor α (TNFα) and interleukin-1β (IL-1β). Cells incubated with interferon γ (IFNγ) did not alter their DAF expression. Two MCP forms were expressed, with molecular masses of approximately 58 kDa and 68 kDa, the lower form predominating. MCP expression was up-regulated by IL-1β, but not by TNFα or IFNγ. Expression of DAF and MCP promotes resistance of colonic adenocarcinoma cells to complement-mediated damage, and represents a possible mechanism of tumour escape.

**Key words** Complement · CD55 (DAF) · CD46 (MCP) · Cancer biology

## Introduction

Both clinical and experimental studies suggest complement activation to be involved in immunological surveillance against tumour cells [2, 8, 24, 38]. Complement (C) may be activated on tumour cells by immune complexes [8], spontaneous activation of C3 during "tick-over" of the alternative pathway [25], as a consequence of tissue ischaemia and necrosis [9], and by tissue destruction during the invasion process. In addition tumour cells are also directly exposed to complement components in blood during the processes of invasion and metastasis [38]. Despite deposits of immune complexes and complement activation products [3, 7, 24, 38], tumour cells have restricted susceptibility to complement-mediated damage [9, 25]. This resistance is, to a great extent, mediated by membrane-bound complement-regulatory proteins [2, 6, 9, 11, 14, 26, 34, 37, 38] and cell membrane repair mechanisms [20, 31].

The formation of the C3/C5 convertase enzymes is a critical event in the complement-activating sequences [30], supporting the main functions of complement; opsonization via generation of C3b and C4b, intensifying inflammation by generation of anaphylatoxins (C3a, C4a and C5a) and chemoattractants (C3a and C5a) and initiation of MAC assembly via the formation of C5b. The formation and activity of the C3/C5 convertase enzymes are regulated intrinsically on cells by the membrane-bound proteins decay-accelerating factor (DAF, CD55) and membrane cofactor protein (MCP, CD46) [17, 23]. DAF is a glycosyl-phosphatidylinositol-anchored 70-kDa glycoprotein that prevents the assembly and promotes the decay of the C3 and C5 convertases of both the classical and alternative pathways. However, DAF does not inactivate C3b/C4b irreversibly, and leaves C3b/C4b with the potential to generate new C3/C5 convertases [23]. MCP is a widely distributed C3b/C4b-binding dimeric protein with molecular masses of 50–58 kDa (lower form) and 59–68-kDa (upper form) and serves as cofactor for the plasma serine protease factor I, which irreversibly inactivates C3b and C4b [16]. Thus, DAF and MCP act in complementary fashion in protecting host cells from complement-mediated damage.

On normal colorectal epithelial cells DAF is sporadically expressed apically [12, 18]. The expression pattern is changed, and the expression is up-regulated on the malig-

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nant cells of a considerable percentage of colon carcinomas [12, 32]. In addition, the presence of MCP has been described throughout the epithelial compartment of colorectal mucosa and carcinomas [10, 13, 32, 33], and the MCP antigen density also appeared to be higher in the neoplastic than in the adjacent non-neoplastic epithelium [13, 32]. These observations drew our interest to the possible mechanisms of up-regulation, and subsequently the role of these proteins in colorectal malignancies.

## Materials and methods

### Reagents and antibodies

Trypsin and phosphatidylinositol-specific phospholipase C (PI-PLC) (Boehringer Mannheim, Mannheim, Germany), RPMI-1640 medium and glutamine (Bio Whittaker, Md., USA), fetal calf serum (Sera Lab, Sussex, UK), penicillin (AL, Oslo, Norway), streptomycin (Glaxo, Middlesex, UK), fungizone (Flow Laboratories, Irvine, Scotland), rabbit anti-(human carcinoembryonic antigen) (CEA, purified immunoglobulin fraction), fluorescein-isothiocyanate(FITC)-conjugated $F(ab')_2$ rabbit anti-(mouse Ig), biotinylated rabbit anti-(mouse Ig), avidin/biotin/horseradish-peroxidase complexes (ABComplexes/HRP; Dakopatts, Glostrup, Denmark), human recombinant interleukin-1β (IL-1β) (sp.act $2.5 \times 10^8$ IU/mg), human recombinant tumour necrosis factor α (TNFα) (sp.act $2.0 \times 10^7$ IU/mg) (British Bio-Technology Products, Oxon, UK), and human recombinant interferon γ (IFNγ) (sp.act $2.5 \times 10^8$ IU/mg) (Genzyme Corporation, Mass., USA) were purchased as indicated.

The mAb BRIC 220 (IgG1) and BRIC 230 (IgG1) directed against DAF were supplied by the International Blood Group Reference Laboratory (IBGRF, Bristol, UK). The mAb BRIC 216 (IgG1) directed against DAF, J4-48 (IgG1) directed against MCP, NAMB-1 (IgG1) directed against β2-microglobulin and FITC-conjugated goat anti-(human C3c) were purchased from Bioproducts Laboratories (Herts, UK), Serotec (Oxford, UK), Boehringer Mannheim (Mannheim, Germany) and Nordic Immunological Laboratories (Tilburg, The Netherlands) respectively. $F(ab')_2$ fragments of the mAb BRIC 216 and NAMB-1 were prepared according to the method described by Parham [27].

Normal human serum was obtained from healthy blood donors, divided into aliquots of 5 ml and stored at −70 °C. The same serum batch was used throughout the study.

### Cell line

The human colon adenocarcinoma cell line HT29, which is undifferentiated under the standard culture conditions used [28] was kindly provided by Kvale D (LIIPAT, The National Hospital, Oslo, Norway). The HT29 cells were cultured at 37 °C in a humidified atmosphere of 5% CO2 in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin, fungizone and 1% glutamine. Cells were grown on cell-culture flasks, on 24-well and 48-well tissue-culture plates (Costar, Mass., USA) and on tissue-culture chambers (Lab-Tek Chamber Slides, Nunc, Ill., USA). Suspensions of cells were prepared by incubating the cultures with 0.01% trypsin for 30 min at 37 °C. The cells were washed twice, and resuspended in phosphate-buffered saline (PBS) to a concentration of $1 \times 10^6$ cells/ml. Cell viability, determined by the trypan blue exclusion technique, was consistently above 95%.

### Detection of DAF and MCP

Viable unfixed cultures of HT29 cells grown on tissue-culture chambers were incubated with 1 μg/ml mAb BRIC 216, BRIC 220, BRIC 230 or J4-48 diluted in PBS for 60 min. Subsequently the cultures were incubated with biotinylated rabbit anti-(mouse Ig) (2.2 μg/ml in PBS) for 60 min, before incubation with avidin/biotin/peroxidase according to the manufacturer's instruction. The cultures were finally treated with a buffer containing 3-amino-9-ethylcarbazole for the development of a coloured reaction product. The HT29 cells were mounted in Immu-Mount (Shandon, Pa., USA), and examined by a Nikon light microscope (Tokyo, Japan).

HT29 cells ($0.25 \times 10^6$) in suspension were incubated for 60 min at 4 °C with 1 μg BRIC 216 or J4-48 diluted in 1 ml PBS. Specific binding was revealed using 4 μg FITC-conjugated $F(ab')_2$ rabbit anti-(mouse Ig) diluted in 100 μl PBS. After being washed twice in PBS the cell suspensions were analysed on a Coulter Epics V flow cytometer (Coulter Electronic, Luton, UK). The data are expressed as flow cytometry curves, or as mean fluorescence intensity units corrected for background values. Control preparations were incubated with the corresponding amount of an irrelevant mouse mAb of the IgG1 class (MOPC 21) (Sigma Chemical Corporation, Mo., USA), or normal mouse serum. All cell cultures and suspensions were incubated with PBS containing 1 mg/ml heat-aggregated human IgG for 20 min (Kabi Pharmacia AB, Uppsala, Sweden), prior to the immunostaining procedures, to eliminate Fcγ-receptor interactions. All incubation steps were followed by washing in PBS.

### Treatment with PI-PLC

Cells in suspension ($0.25 \times 10^6$) were treated with PI-PLC (100 μl. 0.1 U/ml) diluted in PBS at 37 °C for 60 min. Cells were washed twice with PBS and stained with the mAb BRIC 216 and J4-48 as described above for the flow cytometry. Control cells were incubated in PBS without PI-PLC.

### Deposition of C3 on HT29 cells

Samples containg $0.25 \times 10^6$ cells in 100 μl PBS were incubated for 1 h on ice with 5 mg rabbit anti-(human CEA) antibodies with or without 2 μg $F(ab')_2$ fragments of BRIC 216. After two washes in barbital-buffered saline, pH 7.2 (barbital/NaCl), barbital/NaCl containing 10 mM EDTA or barbital/NaCl containing 2 mM $MgCl_2$ and 10 mM EGTA the cells were incubated for 30 min at 37 °C with 100 ml 20% normal human serum in one of the three or barbital buffer. After two washes in PBS the cells were immunostained with FITC-conjugated goat anti-(human C3c) diluted 1:40 in PBS and processed for flow cytometry as described above. Controls included unsensitised cells and cells incubated with F(ab')2 fragments of BRIC 216 only.

### Assay for antibody-dependent complement cytotoxicity

HT29 cells ($0.25 \times 10^6$) were incubated with 5 μg rabbit anti-(human CEA) antibodies diluted in 100 μl PBS for 60 min at 4 °C. After being washed twice in barbital/NaCl pH 7.2, the cells were exposed to 100 μl of various dilutions of normal human serum in barbital/NaCl (30 min at 37 °C). The number of cells permeable to 0.2% (w/v) trypan blue divided by the total number of cells gave the percentage of lysis. The degree of lysis was then compared with lysis obtained when cells were incubated with anti-(human CEA) antibodies and various concentrations of $F(ab')_2$ fragments of BRIC 216 or NAMB-1. Controls included BRIC 216-sensitised, NAMB-1-sensitised, and unsensitised cells incubated with NHS. In regulation experiments, both the cytokine-stimulated and non-stimulated HT29 cells were examined, using a fixed concentration of both mAb; BRIC 216 (20 μg/ml) or NAMB-1 (20 μg/ml) and NHS (20%).

### Immunoblotting analysis

Lysates of HT29 cells, human erythrocytes and peripheral blood cells, were prepared by incubating 100 mg cells in lysis buffer (10 mM TRIS HCl, pH 8.2, containing 140 mM NaCl, 2 mM EDTA (Sigma Chemical Co, Mo., USA), 1 mM phenylmethylsulphonyl fluoride (PMSF, Sigma)

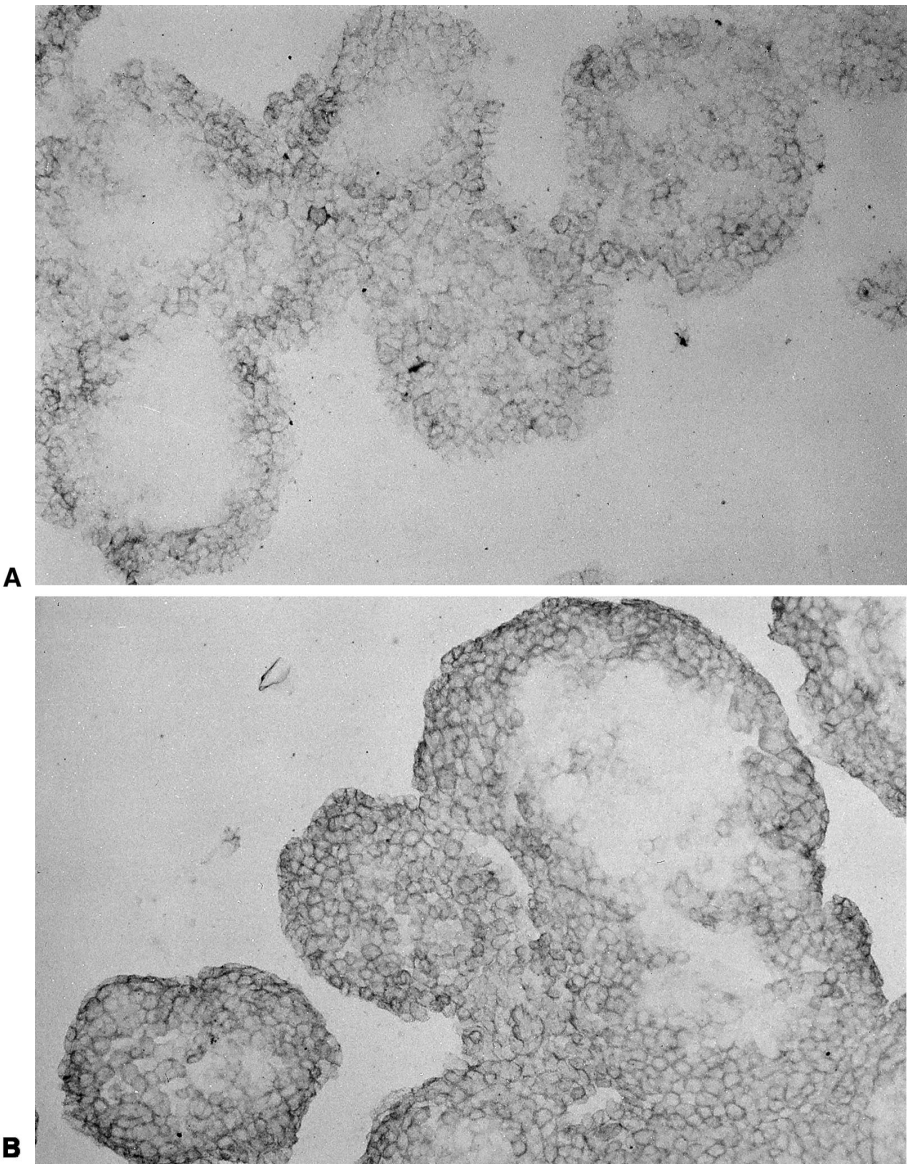


**Fig. 1** Immunohistochemical study of HT29 cells grown on tissue-culture chambers. Cells were stained with (**A**) anti-(decay-accelerating factor, DAF) mAb (BRIC 230, 1 μg/ml) and (**B**) anti-(membrane cofactor protein, MCP) mAb (J4-48, 1 μg/ml). Magnification: × 225

and 2% Nonidet P-40 (NP-40, Fluka AG, Buchs, Germany) for 60 min at 4 °C in a total volume of 1 ml. The extracts were centrifuged for 30 min at 14 000 *g* to remove undissolved cells and debris, and the supernatant was dialysed against PBS with 0.05% azide for 12 h, divided into aliquots and stored at −70 °C until use. Cell culture supernatants were harvested from confluent cells grown in 5 ml RPMI medium in 75 $cm^2$ tissue-culture flasks for 3 days. Cells and debris were removed by centrifugation (14 000 *g*, 30 min). The supernatants were concentrated 20 times on an ultrafiltration unit (Microsep, Filtron, Mass., US), divided into aliquots and stored at −70 °C until use.

In some experiments HT29 cells and human neutrophils ($10 \times 10^6$) were lysed in 500 μl HEPES/Triton X-100 buffer [25 mM HEPES (Bio Wittaker), pH 7.5, containing 1% Triton X-100 (Sigma), 0.5 mg/ml aprotinin (Sigma), 4 mM EDTA, 200 mM PMSF, 100 nM sodium fluoride (Merck, Darmstadt, Germany) and 20 mM pyrophosphate (Merck)] at either 4 °C or 37 °C for 20 min with brief intermittent vortexing, as earlier described [5]. Following the solubilisation the 37 °C lysates were chilled on ice for 5 min before all samples were centrifuged (14 000 *g*, 30 min). The resultant soluble (supernatant) and insoluble (pellet) fractions were separated and processed for immunoblotting.

The immunblotting method was used for detection of DAF and MCP in NP-40 extracts and HT29 cell culture medium and to determine the relative portion of DAF and MCP in pellets and supernatants from Triton-X-100-extracted cells at 4 °C and 37 °C. Briefly, protein fractions of equal concentrations were subjected to electrophoresis under non-reducing conditions in 12% polyacrylamide gels containing sodium dodecyl sulphate (SDS-PAGE) and subsequent Western blotting onto nitrocellulose sheets. The sheets were washed in PBS containing 0.05% (v/v) Tween 20 (PBS/Tween 20), and blocked with 5% dried bovine milk powder in PBS/Tween 20. The blot was incubated with BRIC 216 (1 μg/ml) or J4-48 (1 μg/ml) in PBS/Tween 20 for 12 h at 4 °C, biotinylated rabbit anti-(mouse Ig) 2.2 μg/ml in PBS for 60 min at room temperature, and avidin/biotin/peroxidase for 20 min at room temperature. Washing for 2 × 10 min in PBS was done after each incubation step. The blots were developed using 4-chloro-1-naphthol (Sigma) and hydrogen peroxide. Prestained SDS-PAGE molecular mass standards (Bio-Rad Laboratories, Calif., USA) were used as molecular mass markers.

Regulation of DAF and MCP expression

HT29 cells were seeded on 24-well (2 $cm^2$) Costar tissue-culture plates at a density of $5 \times 10^4$ cells/$cm^2$, in a final volume of 1 ml growth medium. After 72 h the cells had reached 60%–70% confluence, and each well received 1 ml freshly prepared growth medium supplied with either IL-1β, TNFα or INFγ (final cytokine concentrations are given in the respective figures). Control wells contained medium without supplements. The plates were incubated for 18 h at 37 °C. The cells were harvested and washed in PBS, and processed for flow cytometric analysis, and a complement-mediated cytotoxicity test (see above). Each experiment was performed in duplicate and repeated three times. Results from the six experiments are expressed as means ± 1 SD. In some experiments stimulated and non-stimulated HT29 cells were extracted and analysed by SDS-PAGE and Western blotting.

Proliferative responses

As preliminary results showed that the expression of DAF and MCP was confluence-dependent, the influence of INFγ, TNFα and IL-1β on the proliferation of the HT29 cells was examined. The number of cells per well was calculated after the cell suspensions had been counted on a Coulter counter (Coulter Electronic, Luton, UK).

## Results

Expression of DAF and MCP on HT29 cells

Almost all HT29 cells grown on tissue-culture chambers showed a membranous staining by anti-DAF mAb. On cells growing in clusters, the intensity was greatest on the most peripherally located cells (Fig. 1A). Flow cytometry was used to analyse the surface expression further, and confirmed that almost all HT29 cells expressed DAF. The unimodal fluorescence profile was broad (Fig. 2A). Anti-DAF staining of HT29 cells pre-incubated with PI-PLC was reduced by approximately 90% (Fig. 2B) when compared to untreated controls. The cultured HT29 cells showed a strong well-defined homogeneous membranous staining for MCP (Fig. 1B). MCP expression on cells in clusters was greatest on the most peripherally located cells, while flow cytometric analysis showed a homogeneous pattern of expression (Fig. 2D). HT29 cells showed a bimodal fluorescence profile after pre-incubation with PI-PLC for 1 h at 37 °C in PBS (Fig. 2F). Control cells resuspended for 1 h at 37 °C in PBS showed the same bimodal fluorescence profile (Fig. 2E). Control flow cytometry studies of HT29 cells incubated with the corresponding amount of an irrelevant mouse mAb of the IgG1 isotype or normal mouse serum were negative.

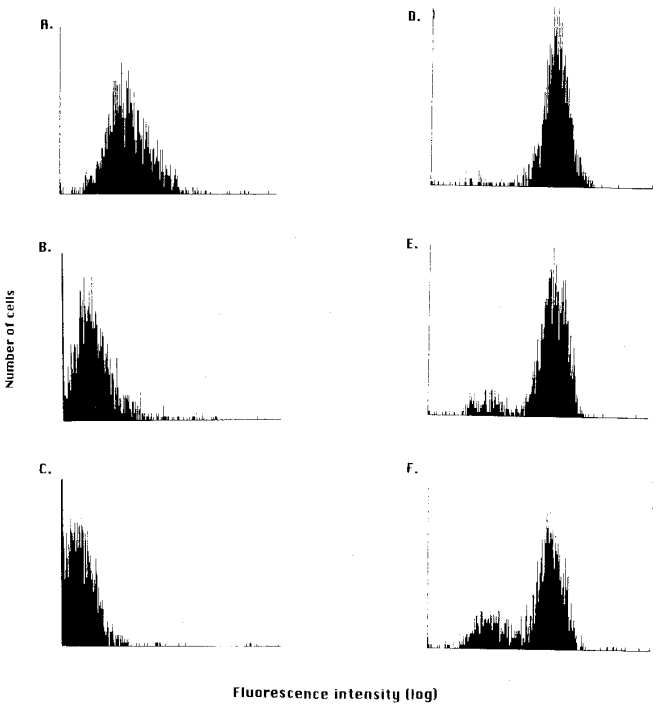


**Fig. 2A–F** Flow-cytometric analysis of DAF (CD55) and MCP (CD46) expression on HT29 cells. HT29 cells were stained with (**A**) anti-DAF mAb (BRIC 216), (**B**) anti-DAF mAb (BRIC 216) after treatment with phosphatidylinositol-specific phospholipase C (PI-PLC), (**C**) non-specific mAb (MOPC 21), (**D**) anti-MCP mAb (J4-48), (**E**) anti-MCP mAb (J4-48) after incubation in PBS for 60 min, (**F**) anti-CD46 mAb (J4-48) after PI-PLC treatment. The gate was set in the same position in each case

Complement-regulatory role of DAF

HT29 cells showed positive staining for C3 when complement was activated by the classical pathway (barbital/NaCl). No staining could be detected when complement was activated by the alternative pathway (Mg/EGTA/barbital/NaCl) or if 10 mM EDTA was added to the reaction mixture (EDTA/barbital/NaCl) (data not shown).

CEA-sensitised cells treated with barbital/NaCl/normal human serum was used for examining the DAF function. When the function of DAF was blocked by $F(ab')_2$ fragments of BRIC 216 the amount of C3 detected on the HT29 cells was increased by 79% compared with cells not treated with BRIC 216 (mean flourescence intensity increased from 28 to 50 units). In contrast, C3 depositions on unsensitised cells were similar whether the cells were preincubated with BRIC 216 or not.

The functional significance of DAF in the protection against complement-mediated cytolysis was also assessed. CEA-sensitised HT29 cells were highly resistant to antibody-dependent complement-mediated cytotoxicity, as earlier described [3]. Adding $F(ab')_2$ fragments of mAb BRIC 216 to the CEA-sensitised HT29 cells enhanced the susceptibility of the cells to complement-mediated lysis. When the concentrations of anti-CEA and normal human serum were kept constant, mAb BRIC 216 had a dose-dependent effect on the susceptibility of HT29 cells to complement-mediated killing. Using 20% NHS and BRIC 216 at concentrations of 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.25 μg/ml and 0.0 μg/ml led to 19%, 17%, 14%, 14%, 6% and 5% lysis respectively (Fig. 3). The results are means of

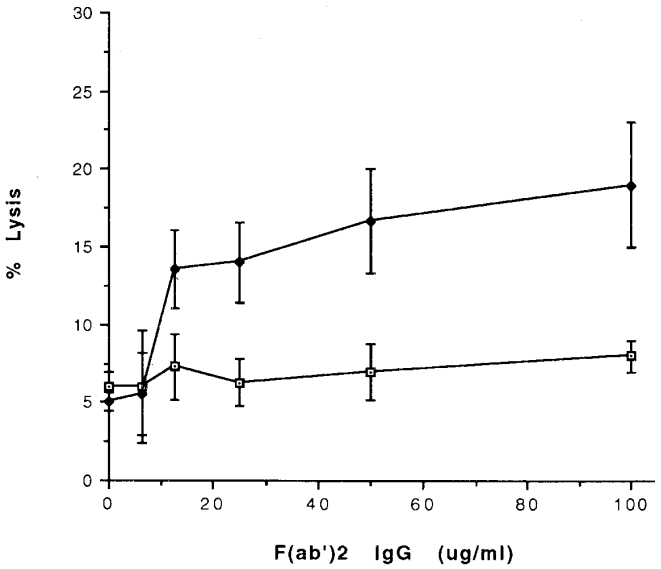


**Fig. 3** Percentage lysis of carcinoembryonic-antigen-sensitised HT29 cells in the presence of 20% normal human serum and various amounts of F(ab′)$_2$ fragments of the anti-DAF mAb BRIC 216 [●] and F(ab′)$^2$ fragments of the anti-β$^2$ microglobulin mAb NAMB-1 [❑]. The results are means of triplicate determinations and error bars represent ± 1 SD

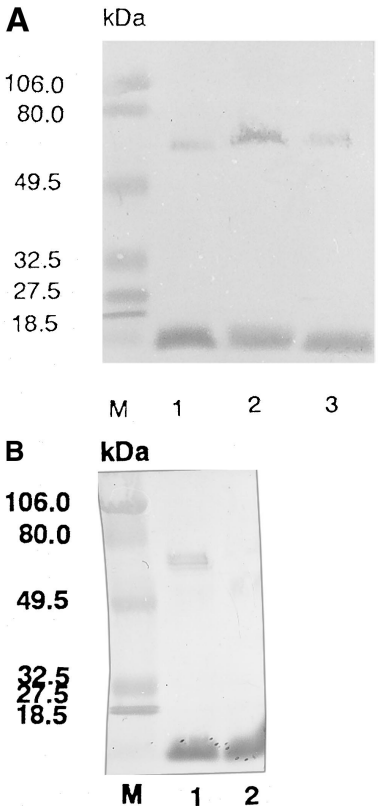


**Fig. 4** **A** Western blot stained with anti-DAF mAb (BRIC 216): *1* membrane extract of human erythrocytes, *2* membrane extract of the HT29 cells, *3* concentrated supernatant from the HT29 cell cultures, *M* prestained molecular mass markers. **B** Western blot stained with anti-MCP mAb (J4-48): *1* membrane extract of human lymphocytes, *2* membrane extract of the HT29 cells, *M* prestained molecular mass markers

triplicate measurements. Increased lysis was not observed when unsensitised cells were incubated with F(ab′)$_2$ fragments of BRIC 216 up to a concentration of 100 μg/ml and normal human serum, or when sensitised cells were incubated with different concentrations of F(ab′)$_2$ fragments of the isotype-matched anti-β$^2$-microglobulin mAb, NAMB-1 and normal human serum.

## Biochemical characterisation of DAF and MCP

DAF and MCP were readily detected in the NP-40 extracts of the HT29 cells, while only DAF was detected in HT29 cell supernatants following SDS-PAGE and Western blotting (Fig. 4). The molecular masses of both the membrane-associated and soluble form of DAF were approximately 70 kDa. The anti-DAF reactive bands comigrated with erythrocyte DAF (Fig. 4A). Two MCP forms were expressed on the HT29 cells, with bands of approximately 58 kDa and 68 kDa, shown by Western blotting, the lower form predominating (Fig. 4B). The anti-MCP-reactive bands comigrated with MCP from lymphocyte extracts, where the upper form predominated.

At 37 °C, Triton X-100 extraction almost completely solubilised DAF from the HT29 cells. However, at 4 °C the efficiency of the extraction was greatly reduced as almost all DAF remained associated with the Triton X-100-insoluble cellular material. In contrast, equal amounts of MCP were extracted at 4 °C and 37 °C (data not shown). Examination of extracts of neutrophils that were used as controls revealed similar results [5].

## Regulation of DAF and MCP expression

The results from flow-cytometric analysis of the DAF and MCP expression on HT29 cells grown in media with cytokine added are shown in Fig. 5.

TNFα enhanced the expression of DAF in a dose-dependent manner; 100 IU/ml, 250 IU/ml or 1000 IU/ml increased the MFI by 43%, 58% and 64% respectively. IL-1β also showed an effect on DAF expression. When the same IL-1β concentrations were used as for TNFα, the expression of DAF was increased by 4%, 15% and 13.5% respectively. Incubation with various concentrations of IFNγ did not appreciably affect the expression of DAF on the HT29 cells (Fig. 5A).

Examining the expression of MCP we found that 100 IU/ml, 250 IU/ml or 1000 IU/ml IL-1β increased the mean fluorescence intensity by 25%, 43% and 45% respectively, and SDS-PAGE and Western blotting showed that the expression of MCP of both molecular masses was enhanced (Fig. 6). Incubation with various concentrations of TNFα and IFNγ did not appreciably affect the expression of MCP on the HT29 cells (Fig. 5B).

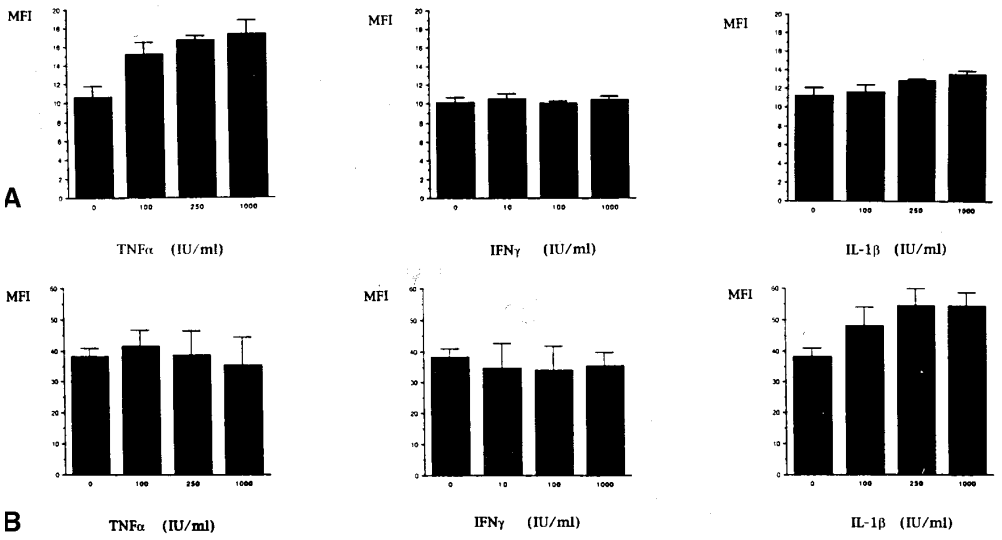


**Fig. 5A,B** Effects of cytokines on (**A**) DAF and (**B**) MCP expression by HT29 cells. Cells were incubated for 18 h with or without various concentrations of different cytokines. Controls included medium only. DAF and MCP expression was analysed with the anti-(human DAF) mAb BRIC 216 and the anti-(human MCP) mAb J4-48 respectively. Mean ± 1 SD values are shown ($n$ = 6)

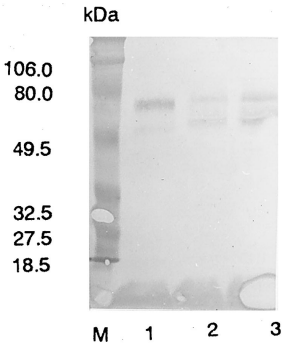


**Fig. 6** Effects of interleukin-1β (IL-1β) on MCP expression by HT29 cells. Cells were incubated for 18 h with or without the presence 1000 IU/ml IL-1β. MCP expression was analysed by SDS-PAGE and Western blotting using the anti-MCP mAb (J4-48). *1* Membrane extract of human lymphocytes, *2* membrane extract of non-stimulated HT29 cells, *3* membrane extract of IL-1β-stimulated HT29 cells, *M* prestained molecular mass markers

**Table 1** Effect of monoclonal anti-DAF (BRIC 216) and control mAb anti-β2-microglobulin (NAMB-1) on HT29 cell killing by complement (20% NHS) after the HT29 cells were cultured in the presence of different cytokines for 18 h.

| Culture conditions | Cells killed (%) after treatment with F(ab′)$_2$ fragments of mAb | |
|---|---|---|
| | Anti-β$_2$-microglobulin, 20 μg/ml | Anti-DAF 20 μg/ml |
| Non-stimulated cells | 7 | 18 |
| Cells stimulated with: | | |
| (TNFα 250 IU/ml) | 8 | 10 |
| (IL-1β 250 IU/ml) | 5 | 12 |
| (INFγ 100 IU/ml) | 9 | 20 |

The effect of an up-regulation of DAF on the resistance of HT29 cells to complement-mediated lysis

To assess possible functional effects of the alterations in DAF expression, both stimulated and non-stimulated HT29 cells were subjected to a complement-dependent cytotoxicity test. We found both stimulated and non-stimulated cells to be highly resistant to lysis (Table 1.). With fixed concentrations of the anti-DAF mAb BRIC 216 (20 μg/ml) and complement (normal human serum, 20%) the TNFα(250 IU/ml)- and IL-1β(250 IU/ml)-treated cells were lysed to a lesser extent than non-stimulated cells (Table 1). Exposure to 100 IU/ml INFγ did not modify the resistance to complement-mediated cytotoxicity.

Proliferative responses

TNFα and IL-1β showed no influence on the proliferation of the HT29 cells, whereas INFγ exerted a moderate growth-inhibitory effect. The inhibition did not exceed 15% for the experiments presented in this study (data not shown).

## Discussion

The complement-regulatory molecules DAF and MCP have previously been shown to be expressed on normal colorectal cells in situ, with an increased expression on malignant colorectal epithelial cells [10, 12, 13, 32, 33]. In the present study we have characterised these complement-regulatory molecules on the colonic adenocarcinoma cell line HT29, and demonstrated their importance in protection against complement-mediated lysis.

DAF was heterogeneously expressed on cultured HT29 cells, which is in line with the report by Koretz et al. [12]. The demonstration of DAF on HT29 cells is interesting also in relation to the observations by Kumar et al. [14], who found that HT29 cells, as well as other tumour cells examined, express DAF mRNA. They suggested that the expression of mRNA coding for DAF may provide some advantage for the cells as a defence against complement-mediated cytotoxicity. The amount of C3 deposits was

increased and the resistance of the HT29 cells to antibody-dependent complement-mediated cytolysis could, in part, be reversed by $F(ab')_2$ fragments of the anti-DAF mAb BRIC 216. Thus DAF expressed on malignant cells may be of significance in the protection against complement-mediated cytolysis, although the contribution of DAF is relatively low. In comparison, in a recent study we demonstrated that blocking of CD59 with an anti-CD59 mAb resulted in a more marked increase in the susceptibility to complement-mediated cytolysis [2]. This suggests that CD59 is a more potent regulator of the complement system on this cell type or, alternatively, that CD59 is expressed in greater quantity on HT29 cells, a possibility suggested by flow cytometry analysis (data not shown).

GPI-anchoring of DAF was suggested by the susceptibility PI-PLC treatment and its resistance to extraction with Triton X-100 at low temperature [4]. Glycosyl-phosphatidylinositol-anchoring may permit fast recruitment of high levels of DAF in defined sites of the cell membrane in which it is functionally required [23].

HT29 cells in culture released DAF into the supernatants. We demonstrated that the membrane-associated and the soluble form had a molecular mass of approximately 70 kDa, which corresponds to that of DAF on non-malignant cells and in some body fluids [15, 18]. It is not clear whether the soluble form of DAF has been secreted, cleaved from the cell membrane by a phospholipase or shed from the surface by some other mechanisms. Soluble DAF has lost the ability to incorporate into cell membranes, and has consequently lost its efficiency as an intrinsic C3/C5 convertase inhibitor [19]. It can, however, inhibit both the classical and alternative pathways of complement extrinsically in a manner similar to the complement regulator factor H [19]. Soluble DAF released from the malignant cells may, in this way, further enhance the resistance of tumour cells to complement-mediated damage.

MCP was homogeneously expressed on the HT29 cells in suspension, which is in line with the study by Koretz et al. [13]. Surprisingly, the cells showed a bimodal expression profile for MCP after incubation for 1 h at 37 °C in PBS. This seems to be a characteristic of the HT29 cells, as a comparison of the MCP expression between our HT29 cell line and a HT29 cell line newly obtained from American Type Culture Collection (ATCC, Md., USA), showed concordance (data not shown). We did not observe a corresponding change in the expression of the surface antigens CD59, DAF and $\beta_2$-microglobulin (data not shown). As a soluble form of MCP could not be detected in the supernatants, this phenomenon is probably not mediated by vesiculation or secretion, and the possibility of exocytosis has to be considered.

The molecular masses of MCP observed in extracts of the HT29 cells and peripheral lymphocytes were similar, but the phenotypic patterns were different. The upper form was predominantly expressed on the peripheral blood cells, while the colonic adenocarcinoma cell line predominantly expressed the lower form. The lower band phenotype of MCP is predominantly expressed on malignant and immortalised cell lines [17], while it is present in only 6% of the cells of a normal population [1]. The HT29 cells may have been derived from a person expressing this uncommon phenotype, but it is more likely that the phenotypic pattern is modulated by the malignant state.

The relative complement-regulatory function of MCP on the HT29 cells was not examined, as blocking antibodies were not available. Treatment of HT29 cells with PI-PLC reduces the surface expression of DAF and CD59 [2] by 90%, and renders the HT29 cells less resistant to complement-mediated lysis. However, since MCP alone is sufficient to inhibit complement-mediated damage on some malignant cell lines [34, 35], we suggest that at least part of the remaining resistance is mediated by MCP and thus contributes in promoting the resistance of tumour cells to complement-mediated damage when local immune surveillance is effected.

The expression of DAF on HT29 cells was rapidly and dose-dependently increased by IL-1$\beta$ and TNF$\alpha$, while only IL-1$\beta$ increased the expression of MCP. Our data are in agreement with previous reports, which indicate that pro-inflammatory cytokines can enhance the constitutive expression of DAF and MCP molecules on epithelial cells [21, 36]. These differences may indicate that the expression of DAF and MCP is independently regulated. Recent studies have, however, not been able to show any or only moderate alterations in the DAF and MCP expression by endothelial cells after exposure to pro-inflammatory cytokines [7, 22]. Accordingly, different cell populations may show differences in regulation pattern for DAF and MCP expression.

Despite the documented increase in DAF expression, we could not find a clear correlation to the observed increase in resistance to complement-mediated lysis. As the complement cytotoxicity test used examined only the “net” resistance against lysis by complement, and such lysis of nucleated cells is a threshold phenomenon, we presume that the increased resistance is also due to alterations in other cellular resistance mechanisms [20, 31]. Our results indicate a link between pro-inflammatory cytokines, known to be secreted by infiltrating inflammatory mononuclear cells in colonic adenocarcinomas [29], and the regulatory limb of the complement system. The release of pro-inflammatory cytokines may be partly responsible for the enhanced DAF and MCP expression on colonic adenocarcinoma cells in vivo [12, 13, 32], and thus contribute in promoting the resistance of tumour cells to complement-mediated damage when local immune surveillance is effected.

The activation of complement may also have an indirect impact on tumour growth. Local formation of C3a can increase both blood flow and diffusion of proteins into the tumour-containing tissues, while C5a may increase the influx of phagocytes to the tumour site [37]. As a consequence, therefore, even a partial block in DAF, MCP or CD59 regulatory activity alone or in combination might result in a sufficient activation of the cascade to retard tumour growth and may have important clinical implications for targeted immunotherapy using antitumour antibodies.

**Acknowledgements** We thank Dr. D. Kvale (LIIPAT, The National Hospital, Oslo, Norway) for providing of the HT29 cell line and the International Blood Group Reference Laboratory (IBGRF, Bristol, UK) for the gift of anti-DAF monoclonal antibodies. This work was supported by grants from The Norwegian Cancer Society, Norske Hoechst A/S, Bio-Test A/S, Nordic Academy for Advanced Studies Abroad (NorFA) and L. Meltzer's Fund. L. Bjørge is a research fellow of The Norwegian Cancer Society.

## References

1. Ballard L, Seya T, Teckman J, Lublin DM, Atkinson JP (1987) A polymorphism of the complement regulatory protein MCP (membrane cofactor protein or gp45-70). J Immunol 138:3850
2. Bjørge L, Vedeler CA, Ulvestad E, Matre R (1994) Expression and function of CD59 on colonic adenocarcinoma cells. Eur J Immunol 24:1597
3. Bora NS, Gobelman CL, Atkinson JP, Pepose JS, Kaplan HJ (1993) Differential expression of the complement regulatory proteins in the human eye. Invest Ophthalmol Vis Sci 34:3579
4. Brown DA, Rose JK (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 68:533
5. Cain TJ, Liu Y, Takizawa T, Robinson JM (1995) Solubilization of glycosyl-phosphatidylinositol-anchored proteins in quiescent and stimulated neutrophils. Biochim Biophys Acta 1235:69
6. Cheung N-KV, Walter EI, Smith-Mensah WH, Ratnoff WD, Tykocinski ML, Medof ME (1988) Decay-accelerating factor protects human tumour cells from complement-mediated cytotoxicity in vitro. J Clin Invest 81:122
7. Favaloro EJ (1993) Differential expression of surface antigens on activated endothelium. Immunol Cell Biol 71:571
8. Guidi L, Baroni R, Bartoloni C, Pellegrino M, Tricerri A, Marciano M, Barone C, Gambassi G (1988) Immune complexes in solid tumours precipitable by 3.5% polyethylene glycol: analysis of some nonspecific components. Diagn Clin Immunol 5:284
9. Hakulinen J, Meri S (1994) Expression and function of the complement membrane attack complex inhibitor protectin (CD59) on human breast cancer cells. Lab Invest 71:820
10. Johnstone RW, Loveland BE, McKenzie IFC (1993) Identification and quantitation of complement regulator CD46 on normal human tissues. Immunology 79:341
11. Junnikkala S, Hakulinen J, Meri S (1994) Targeted neutralization of the complement membrane attack complex inhibitor CD59 on the surface of human melanoma cells. Eur J Immunol 24:611
12. Koretz K, Brüderlein C, Henne S, Möller P (1992) Decay-accelerating factor (DAF, CD55) in normal colorectal mucosa, adenomas and carcinomas. Br J Cancer 66:810
13. Koretz K, Brüderlein C, Henne C, Möller P (1993) Expression of CD59, a complement regulator protein and a second ligand of the CD2 molecule, and CD46 in normal and neoplastic colorectal epithelium. Br J Cancer 68:926
14. Kumar S, Vinci JM, Pytel BA, Baglioni C (1993) Expression of messenger RNAs for complement inhibitors in human tissues and tumors. Cancer Res 53:348
15. Lass JH, Walter EI, Burris TE, Grossniklaus HE, Roat MI, Skelnik DL, Needham L, Singer M, Medof ME (1990) Expression of two molecular forms of the complement decay-accelerating factor in the eye and lacrimal gland. Invest Ophthalmol Vis Sci 31:1136
16. Liszewski MK, Atkinson JP (1992) Membrane cofactor protein. Curr Top Microbiol Immunol 178:45
17. McNearney T, Ballard L, Seya T, Atkinson JP (1989) Membrane cofactor protein of complement is present on human fibroblast, epithelial, and endothelial cells. J Clin Invest 84:538
18. Medof ME, Walter EI, Rutgers JL, Knowles DM, Nussenzweig V (1987) Identification of the complement decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids. J Exp Med 165:848
19. Moran P, Beasley H, Gorrell A, Martin E, Gribling P, Fuchs H, Gillett N, Burton LE, Caras IW (1992) Human recombinant soluble decay accelerating factor inhibits complement activation in vitro and in vivo. J Immunol 149:1736
20. Morgan PB (1989) Complement membrane attack on nucleated cells: resistance, recovery and non-lethal effects. Biochem J 264:1
21. Moutabarrik A, Nakanishi I, Matsumoto M, Zaid D, Seya T (1993) Cytokine-mediated regulation of the secretion of third component of the complement (C3) and surface expression of complement regulatory proteins, CD46 (MCP), CD55 (DAF), and CD59 on human glomerular epithelial cells (GEC). Mol Immunol 30:36
22. Moutabarrik A, Nakanishi I, Namiki M, Hara T, Matsumoto M, Ishibashi M, Okuyama A, Zaid D, Seya T (1993) Cytokine-mediated regulation of the surface expression of complement regulatory proteins, CD46 (MCP), CD55 (DAF), and CD59 on human vascular endothelial cells. Lymphokine Cytokine Res 12:167
23. Nicholson-Weller A (1992) Decay accelerating factor. Curr Top Microbiol Immunol 178:7
24. Niculescu F, Rus HG, Retegan M, Vlaicu R (1992) Persistent complement activation on tumour cells in breast cancer. Am J Pathol 140:1039
25. Panneerselvam M, Welt S, Old LJ, Vogel C-W (1986) A molecular mechanism of complement resistance of human melanoma cells. J Immunol 136:2534
26. Parczyk K, Pilarsky C, Rachel U, Koch-Brandt C (1994) Gp80 (clusterin; TRPM-2) mRNA level is enhanced in human renal clear cell carcinomas. J Cancer Res Clin Oncol 120:186
27. Parham P (1983) On the fragmentation of monoclonal IgG1, IgG2a, and IgG2b from BALB/c mice. J Immunol 131:2895
28. Pinto M, Appay M-D, Simon-Assmann P, Chervalier G, Dracopoli N, Fogh J, Zweibaum A (1982) Enterocytic differentiation of cultured colon cancer cells by replacement of glucose by galactose in the medium. Biol Cell 44:193
29. Ravenswaay Claasen HH van, Kluin PM, Fleuren GJ (1992) Tumour infiltrating cells in human cancer. On the possible role of $CD16^+$ macrophages in antitumor cytotoxicity. Lab Invest 67:166
30. Reid KBM (1986) Activation and control of the complement system. Essays Biochem 22:27
31. Reiter Y, Ciobotariu A, Fishelson Z (1992) Sublytic complement attack protects tumour cells from lytic doses of antibody and complement. Eur J Immunol 22:1207
32. Schmitt C, Schwaeble W, Wittig B, Meyer zum Büschenfelde KH, Dippold W (1994) Expression and regulation of the membrane associated complement-regulators CD46, CD55 and CD59 in gastrointestinal cells. Gastroenterology 106:768
33. Seto N, Takemura S, Ueda M, Nakanishi S, Ichio N, Ashihara K, Nakahara R, Doi T, Kasamatsu Y, Okamoto M, Yanagida K, Fukuda W, Onodera H, Deguchi M, Sugino S, Kondo M (1993) Expression of membrane co-factor protein (MCP) in gastro-intestinal mucosa. Molecular Immunol 30:50
34. Seya T, Hara T, Matsumoto M, Akedo H (1990) Quantitative analysis of membrane cofactor protein (MCP) of complement. High expression of MCP on human leukemia cell lines, which is down-regulated during cell differentiation. J Immunol 145:238
35. Seya T, Hara T, Matsumoto M, Sugita Y, Akedo H (1990) Complement-mediated tumour cell damage induced by antibodies against membrane cofactor protein (MCP, CD46). J Exp Med 172:1673
36. Tandon N, Yan SL, Morgan BP, Weetman AP (1994) Expression and function of multiple regulators of complement activation in autoimmune thyroid disease. Immunology 81:643
37. Terachi T, Stanescu G, Pontes JE, Medof ME, Caulfield MJ (1991) Coexistence of autologous antibodies and decay-accelerating factor, in inhibitor of complement, on human renal tumour cells. Cancer Res 51:2521
38. Yamakawa M, Yamada K, Tsuge T, Ohrui H, Ogata T, Dobashi M, Imai Y (1994) Protection of thyroid cancer cells by complement-regulatory factors. Cancer 73:2808