American Journal of Pathology, Vol. 151, No. 5, November 1997

Expression of the Complement Regulatory Proteins Decay Accelerating Factor (DAF, CD55), Membrane Cofactor Protein (MCP, CD46) and CD59 in the Normal Human Uterine Cervix and in Premalignant and Malignant Cervical Disease

Karen L. Simpson, Anita Jones, Susan Norman, and Christopher H. Holmes

From the Department of Clinical Medicine, Division of Obstetrics and Gynaecology, University of Bristol, St. Michael's Hospital, Bristol, United Kingdom

The membrane-bound complement regulators decay-accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46), and CD59 are broadly expressed proteins that act together to protect host tissues from autologous complement. Comparison of expression profiles of these proteins between normal and pathological tissues could reveal a mechanism by which tumor cells evade complement-mediated killing. Expression of the regulators was therefore examined in the normal human uterine cervix, in cervical intraepithelial neoplasia (CIN; n = 23), and in cervical squamous carcinomas (n = 6). DAF and MCP were reciprocally expressed in normal ectocervical epithelium. MCP was confined predominantly to the basal and parabasal layers with more extensive expression in metaplastic squamous epithelium. An apparent expansion in MCP expression was observed in more severe premalignant lesions whereas cervical carcinomas were uniformly MCP positive. By contrast, DAF expression appeared unaltered in premalignant lesions and variable in carcinomas. However, increased DAF was observed in stromal cells directly adjacent to infiltrating tumor cells. A low molecular weight DAF product was detected in tumors, and preliminary evidence suggests this may be derived from stromal cells. Overall, changes in expression of C3 convertase regulators in both the stromal and epithelial compartments may be important for evasion of immune surveillance in cervical cancer. *(Am J Pathol 1997, 151:1455–1467)*

Complement is a powerful immune effector that can directly eliminate pathogens and cells by cytolysis and can also mediate inflammatory responses through the action of potent complement derivatives termed anaphylatoxins.[1] Activation of complement through either the antibody-mediated classical pathway or the antibody-independent alternative pathway leads to formation of C3 convertase enzymes that cleave the central complement component C3 leading to the deposition of activated C3b on cell surfaces. Subsequent amplification of the complement cascade initiates assembly of the terminal complement components into the cytolytic membrane attack complex (MAC, C5b-9).

To allow immune surveillance, complement undergoes a continuous low-level activation. Tight regulation of activated complement components is therefore essential to prevent nonspecific damage to host tissues. This is achieved by a number of complement control proteins.[2] In particular, three membrane-bound regulatory molecules act specifically to protect cell surfaces from complement-mediated damage. Two of these, decay-accelerating factor (DAF, CD55) and membrane cofactor protein (MCP, CD46), act at the level of the C3 convertases. DAF acts reversibly, preventing the formation of C3 convertases and accelerating their decay[3] whereas MCP acts irreversibly as a cofactor for factor-I-mediated cleavage of C3b and C4b.[4] A third protein, CD59, interacts with the terminal complement components C8 and C9, preventing the formation of MAC.[5,6] The three complement regulatory proteins are broadly expressed on normal human cells and tissues and are generally thought to act together to provide effective protection from complement-mediated damage.[7–9]

It is now widely envisaged that modulated or inappropriate expression of complement regulatory proteins may play a role in disease processes and that intervention in complement regulation may offer therapeutic opportunities. Several studies have examined the status of primary tumors for complement regulators both at the level of protein[10–13] and mRNA,[14,15] and these suggest that expression of complement regulatory proteins in tumors

Supported by the Medical Research Council (grant G9413534).

Accepted for publication August 20, 1997.

Address reprint requests to Dr. C. H. Holmes, Division of Obstetrics and Gynaecology, St. Michael's Hospital, Southwell Street, Bristol BS2 8EG, UK.

may be altered by comparison with normal tissues. Functional studies also suggest a role for complement regulatory proteins in tumor cell survival.[13,16,17]

Invasive squamous carcinomas of the human uterine cervix are usually preceded by a spectrum of lesions known as cervical intraepithelial neoplasias (CINs). These are characterized by a disturbed cell organization and maturation in which the severity of disease is related to the degree of nuclear dysplasia and the depth of the involved epithelium. Studies by Medof et al[18] have shown that expression of DAF in normal stratified epithelia, including skin and cervix, is related to cellular maturation. This has been confirmed more recently by Oglesby et al[19] who also found that maturation influences expression of C3 convertase regulators in the normal ectocervical epithelium. In the present study we provide a detailed analysis of complement regulatory protein expression on normal ectocervical squamous epithelium, metaplastic squamous epithelium, and endocervical columnar epithelium. We have also examined premalignant cervical lesions and primary cervical squamous carcinomas to determine whether changes in the expression of these proteins are associated with the development of cervical disease.

Materials and Methods

Tissues

Normal cervical tissue was obtained from women undergoing hysterectomy for unrelated pathology and in whom the last cervical smear was reported as normal. At surgery, uteri were collected dry, and a sample of tissue was dissected for the study. Normality was subsequently confirmed independently by routine histological examination. Cervical large loop excision of the transformation zone (LLETZ) excisions containing CIN lesions were obtained from patients attending for colposcopy after an abnormal smear. A fragment of the biopsy was snap frozen in liquid nitrogen for immunohistochemical examination of complement regulatory protein expression. The lesions were graded independently on conventionally processed material by standard histopathological criteria. The study included 3 specimens containing CIN1, 10 containing CIN2, and 10 containing CIN3 lesions. Multiple tissue blocks were obtained from six cervical squamous epithelial carcinomas and snap frozen at the time of resection. Normal and tumor tissues were dissected by an independent clinical pathologist.

Term placentae were obtained from apparently normal full-term deliveries. Human semen was obtained from normal fertile donors and liquefied at 37°C for 30 minutes, and motile sperm were recovered by a swim-up procedure. Local ethical approval was obtained before commencing this study and, as appropriate, tissue was collected with informed consent.

Monoclonal Antibodies (MAbs)

The MAbs BRIC 110, BRIC 216, BRIC 220, and BRIC 230 against DAF[20] and BRIC 229 against CD59[21] were obtained as tissue culture supernatants from the International Blood Group Reference Laboratories, Bristol, UK. These reagents can also be obtained commercially from Blood Products Laboratory, Elstree, UK. The anti-MCP MAb E4.3[22] in the form of ascites fluid and J4.48[23] in the form of affinity-purified reagent were both obtained from Serotec, Oxford, UK. The MAb NDOG2 (Serotec) against placental alkaline phosphatase was used as a tissue culture supernatant and has been described previously.[24]

Dilutions of the two MCP MAbs ranging from 1:100 to 1:500 were included in each test and gave similar results. The four anti-DAF MAbs were used undiluted and showed similar reactivities. NDOG2 was also used undiluted whereas BRIC 229 was used at a dilution of 1:10.

The MAb to complement regulatory proteins described above have all been used extensively in immunohistochemical and immunoblotting studies both in our own laboratory and in those of other investigators. They all lack nonspecific cross-reactivity when used in these techniques.

Immunohistochemistry

Tissue blocks were placed in OCT embedding compound (Lab-Tek Products, Naperville, IL), frozen in liquid-nitrogen-cooled isopentane (2-methylbutane) on small cork boards, and stored in liquid nitrogen. Immunostaining was carried out as previously described on 6-μm cryostat tissue sections that were mounted on gelatin-coated slides and then air dried and fixed for 10 minutes in ice-cold acetone.[20] Briefly, after incubation with primary antibody, sections were washed using Tris-buffered saline (TBS), pH 7.6, and incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts, Copenhagen, Denmark) diluted 1:40 in TBS containing 10% normal human serum. Slides were developed using Sigma Fast diaminobenzidine tetrahydrochloride/H_2O_2 (Sigma, Poole, UK), counterstained with hematoxylin, progressively dehydrated in graded alcohol solutions, cleared (Histoclear, National Diagnostics, Atlanta, GA), and mounted. Sections incubated with MAb NDOG2 or in which primary MAb was omitted were included in all tests as negative controls.

Tissue Preparation, Enzyme Treatment, and Immunoblotting

Normal cervical squamous epithelium was disaggregated from underlying stroma using dispase. Briefly, fresh cervical tissue was washed extensively in Ca^{2+}/Mg^{2+}-free Hanks' buffered saline solution (HBSS; Gibco BRL, Paisley, UK), and excess stroma was removed. The tissue was floated on a solution of dispase II (Boehringer, Sussex, UK) diluted to a final concentration of 1.2 U/ml in TBS with the epithelial face oriented toward the medium and incubated overnight at 4°C. Epithelial sheets were then teased from the stromal tissue and washed extensively in HBSS. Detergent extracts were prepared from the isolated epithelial sheets as described previously.[25] Briefly, the washed epithelial sheets were homogenized

in 8 mmol/L CHAPS (Sigma) in TBS supplemented with 1 mmol/L phenylmethylsulfonyl fluoride (PMSF; Sigma) and 1 μg/ml aprotinin (Sigma) and solubilized for 30 minutes at 20°C. Debris was removed by centrifugation at 10,000 × *g* for 10 minutes. Extracts were either used immediately or stored at −70°C.

Preparation of membranes from cervical tumors was based on the method described by Simpson and Holmes.[26] For this, snap-frozen tissue or 30-μm tissue sections cut from mounted tissue blocks were homogenized in TBS/PMSF/aprotinin. The homogenate was centrifuged at 10,000 × *g* for 10 minutes to remove debris, and the supernatant was centrifuged at 100,000 × *g* at 4°C to pellet membranes. The resulting pellet was resuspended in PBS and used immediately. Homogenates of spermatozoa (approximately 2×10^7/ml) were prepared by brief sonication in PBS. Syncytiotrophoblast membranes were prepared from fresh term placental chorionic villi by the saline extraction procedure of Smith et al.[27]

For neuraminidase treatment, the enzyme (Boehringer) was added to placental trophoblast, cervical tumor membrane preparations, or spermatozoal homogenates at a concentration of 0.2 U/mg protein and incubated at 37°C for 2 hours. Control samples with neuraminidase omitted were incubated in parallel.

Immunoblotting was carried out using a modification of the method described by Towbin et al.[28] Briefly, membrane preparations and tissue extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis under nonreducing conditions on gels containing 10% acrylamide according to the method of Laemmli,[29] and separated proteins were transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, Watford, UK). The membrane was blocked with 5% (w/v) dried milk powder in PBS containing 0.2% (v/v) Tween-20, incubated with MAb overnight at 4°C, and developed using an enhanced chemiluminescence kit (Amersham International, Little Chalfont, UK). Membrane strips incubated with secondary antibody alone were developed in parallel as negative controls.

Results

Distribution of Complement Regulatory Proteins in Normal Human Cervix

Immunohistochemical staining using a panel of MAbs to complement regulatory proteins consistently revealed marked differences between the patterns of expression of DAF, MCP, and CD59 in the normal uterine cervix. Ten normal cervical specimens obtained after hysterectomy were examined in detail. Reactivities on ectocervical squamous epithelium are illustrated in Figure 1, on endocervical columnar epithelium in Figure 2, and on epithelium at the squamo-columnar junction in Figure 3.

CD59 was broadly distributed throughout the squamous epithelium of the ectocervix with all cells staining intensely (Figure 1c). In contrast, DAF (Figure 1a) and MCP (Figure 1b) both showed a much more restricted distribution. The patterns exhibited by the two C3 convertase regulators were also found to be quite distinct from each other. In the case of DAF, basal and parabasal cells showed little or no staining whereas more superficial cells were positive (Figure 1a). The reverse pattern occurred for MCP; reactivity was most intense in the basal and parabasal layers whereas staining progressively decreased toward the superficial aspect of the epithelium (Figure 1b). Although antibodies to all three regulators apparently stained the cell membranes (see arrows in Figures 1, a–c), anti-MCP MAb displayed an additional diffuse cytoplasmic staining in cells at the basal aspect of the epithelium. DAF and MCP therefore exhibit reciprocal patterns of expression that appear related to maturation of the ectocervical squamous epithelium.

In the underlying stromal compartment, both DAF (Figure 1a) and CD59 (Figure 1c) showed an intense expression throughout mesenchymal cells and vessels. Additionally, DAF exhibited a pronounced fibrillar reactivity within the stroma (Figure 1a). By contrast, MCP staining was limited to isolated mesenchymal cells and to vessel endothelial cells (Figure 1b); diffuse staining was noted within some mesenchymal cells, which may be consistent with that noted recently by Oglesby et al.[19]

Columnar epithelial cells of the endocervix displayed strong reactivity with MAb to DAF (Figure 2a), MCP (Figure 2b), and CD59 (Figure 2e). However, there were striking differences in the cellular localization of the proteins on these cells, as illustrated in detail for DAF and MCP in Figure 2, c and d, respectively. The glycosylphosphatidylinositol-anchored regulators, and especially DAF (Figure 2c), were intensely expressed at the apical aspect of columnar epithelial cells, but little or no staining was observed on basolateral membranes. In contrast, MCP exhibited a distinct basolateral localization with little or no staining on the microvillous brush border (Figure 2d).

Five of the normal cervical samples examined were found to contain an intact squamo-columnar junction, which allowed expression of the complement regulators to be examined on metaplastic squamous epithelium (Figure 3). The broad anti-CD59 reactivity characteristic of the ectocervix was also associated with metaplastic squamous epithelium (Figure 3c). Again in common with the ectocervix, anti-DAF reactivity was associated with intermediate and superficial cells of the metaplastic squamous epithelium (Figure 3a); interestingly, at the junction itself DAF-negative squamous epithelial cells were clearly overlaid by DAF-positive columnar epithelial cells (arrow in Figure 3a). In contrast to the restricted distribution observed in the ectocervix, anti-MCP reactivity consistently occurred throughout the metaplastic squamous epithelium (compare Figure 3b with Figure 1b).

Demonstration of Complement Regulatory Proteins in Cervical Squamous Epithelium by Immunoblotting

Immunoblotting was carried out to confirm expression of DAF, MCP, and CD59 in the cervix (Figure 4). For this, detergent extracts were made from squamous epithelial

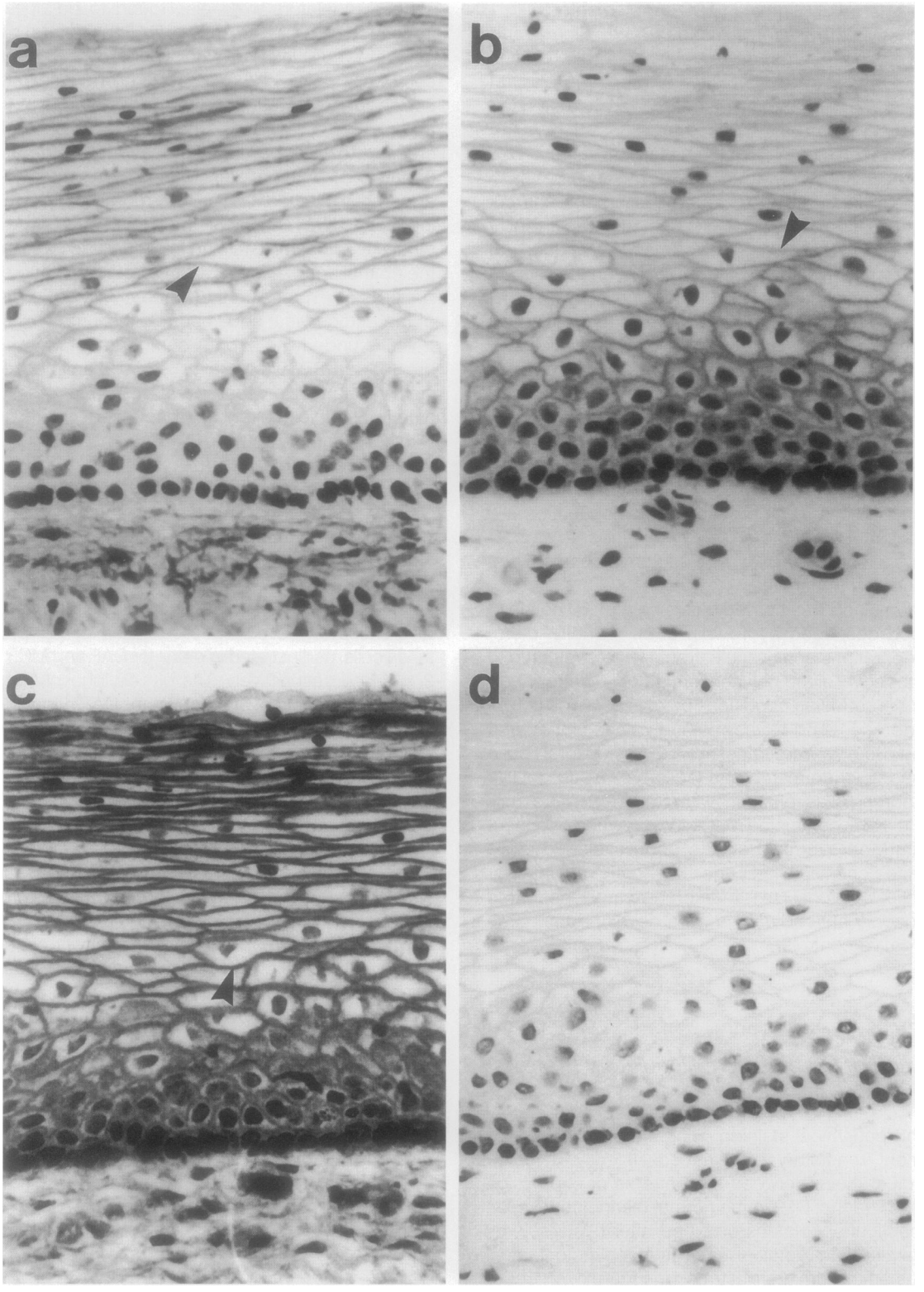

Figure 1. Immunoperoxidase staining for complement regulatory proteins on serial frozen sections of normal ectocervical squamous epithelium. **a:** Anti-DAF (MAb BRIC 230) staining is localized to superficial and intermediate squamous epithelial cells, but there is little or no staining of basal or parabasal cells; strong reactivity is present in the underlying stroma, which also displays an intense fibrillar staining. **b:** Anti-MCP (J4.48) reactivity is localized predominantly on basal and parabasal cells, but staining progressively diminishes toward the superficial aspect of the epithelium; there is limited staining in the stromal compartment. **c:** Anti-CD59 (MAb BRIC 229) reactivity is broadly distributed throughout the squamous epithelium and stroma. **d:** The negative control (primary antibody omitted) shows no staining. **Arrows** in **a** to **c** indicate membrane-type localization of reactivity. Magnification, ×125.

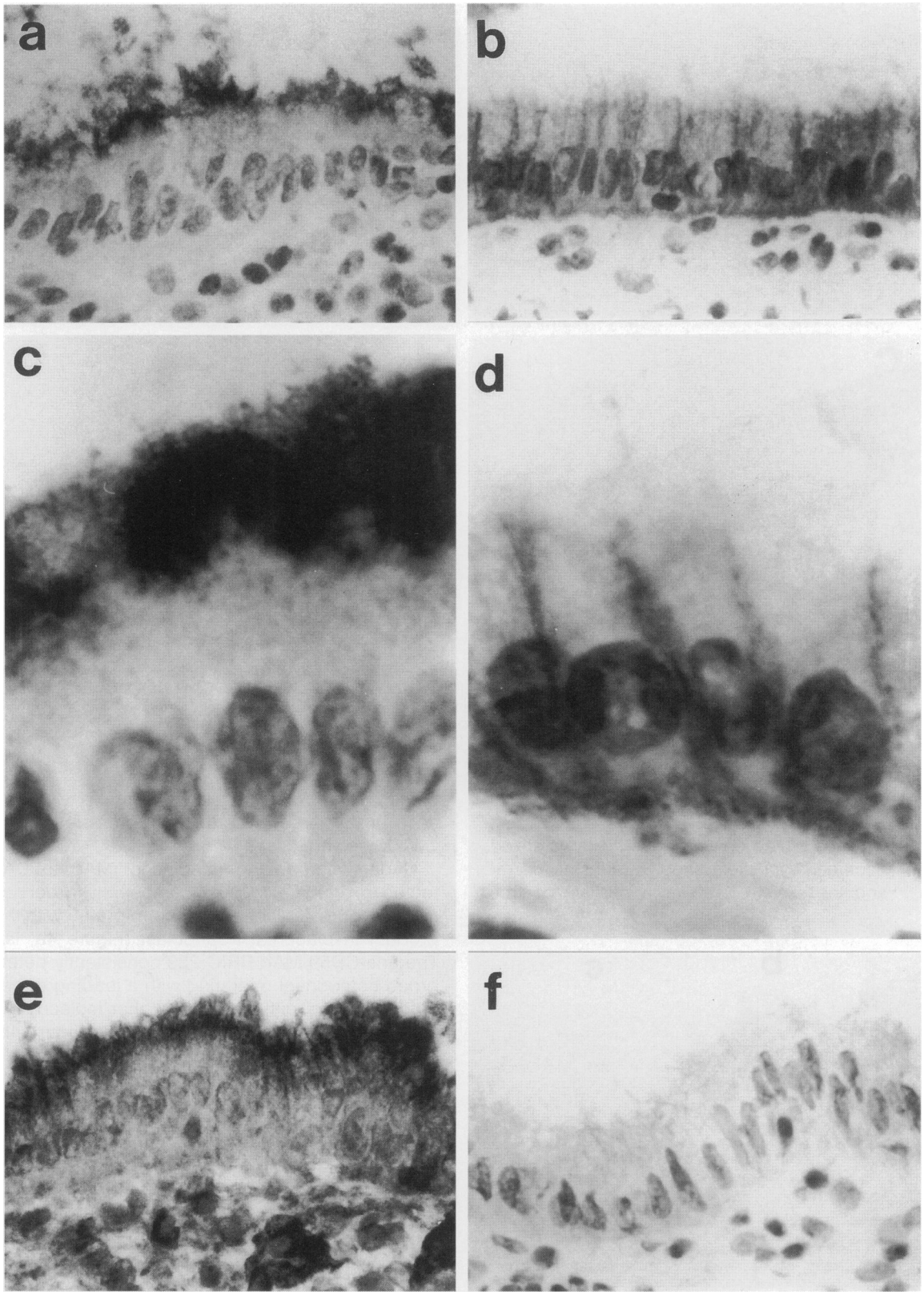

Figure 2. Immunoperoxidase staining for complement regulatory proteins on serial frozen sections of normal endocervical columnar epithelium. **a:** Antibody BRIC 220, against DAF, predominantly stains the apical aspect of columnar epithelial cells. Magnification, ×160. **b:** Anti-MCP staining (MAb E4.3) is more broadly distributed in columnar cells. Magnification, ×160. **c:** The intense localization of anti-DAF reactivity at the apical cell membrane is shown in detail. Magnification, ×630. **d:** The pronounced basolateral reactivity of anti-MCP MAb E4.3 is shown in detail. Magnification, ×630. **e:** Antibody BRIC 229 against CD59 stains throughout columnar cells, but there is prominent reactivity at the apical membrane. Magnification, ×160. **f:** There is no staining in the negative control (primary MAb NDOG2). Magnification, ×160.

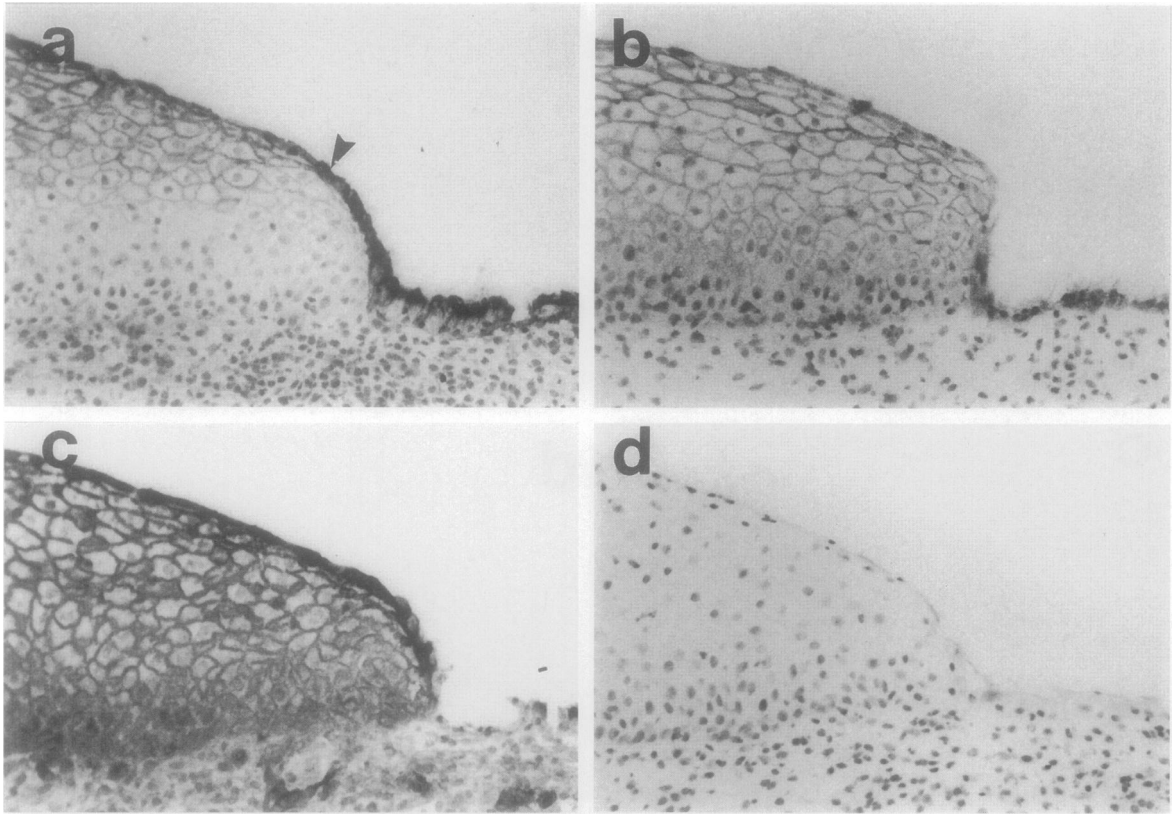

Figure 3. Immunoperoxidase staining for complement regulatory proteins on serial frozen sections of the normal cervical squamo-columnar junction. **a:** Anti-DAF (BRIC 110) staining is localized to the superficial aspect of the squamous epithelium; note that DAF-positive columnar epithelial cells (**arrow**) overlie squamous epithelium lacking DAF. **b:** Anti-MCP (MAb E4.3) reactivity occurs throughout metaplastic squamous epithelium. **c:** Anti-CD59 (BRIC 229) reactivity is broadly distributed at the squamo-columnar junction. **d:** There is no staining of the negative control MAb NDOG2. Magnification, ×50.

sheets that had been separated from underlying stromal tissue by incubation in dispase. The three complement regulatory proteins have been previously characterized on term placental syncytiotrophoblast plasma membranes,[30] and these were used as a positive control.

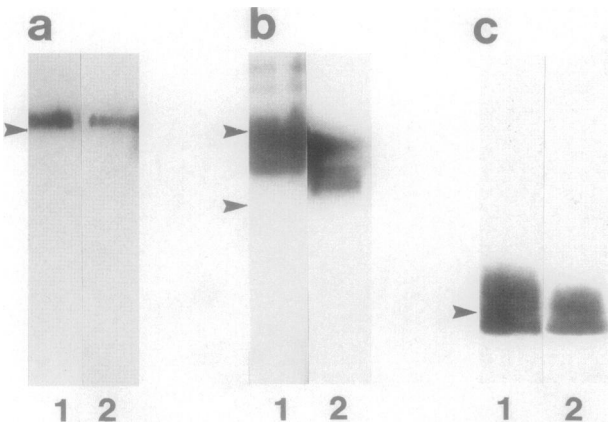

Figure 4. Immunoblotting analysis of complement regulatory proteins in normal cervical squamous epithelium by comparison with control placental membranes. Components detected by MAb BRIC 230 to DAF (**a**), E4.3 to MCP (**b**), and BRIC 229 to CD59 (**c**) are shown in placental syncytiotrophoblast plasma membranes (**lane 1**) and in detergent extracts of cervical squamous epithelium (**lane 2**). **Arrows** indicate the position of molecular weight markers at 66 kd (top) and 45 kd (bottom) in **a** and **b** and at 21 kd in **c**.

Extracts were immunoblotted in parallel with MAbs to each of the three regulators. No cross-reactive bands were observed.

Under nonreducing conditions, MAb to DAF detected a prominent 70-kd component in cervical extracts (Figure 4a, lane 2) that co-migrated with the DAF product in placental trophoblast (Figure 4a, lane 1). The anti-CD59 MAb BRIC 229 consistently detected a broad component migrating with a leading edge of 18 to 20 kd and a diffuse trail of higher molecular weight material in term placental membranes (Figure 4c, lane 1). This pattern is consistent with the detection of CD59 by immunoblotting as reported by Fletcher et al.[21] A CD59 product migrating with a similar leading edge was also observed in cervical extracts (Figure 4c, lane 2). Two broad MCP components were typically detected in cervical extracts (Figure 4b, lane 2), and these migrated approximately 4 to 5 kd ahead of the 50- to 54-kd and 58-kd syncytiotrophoblast MCP products (Figure 4b, lane 1). Such tissue-specific variations in the molecular weight of MCP proteins are well established in other systems.[31] In addition, the relative proportion of the components detected in cervical extracts varied between individuals (not shown). This is consistent with a known inherited variability in the quantities of the two MCP products, which is related to a restriction fragment length polymorphism.[32]

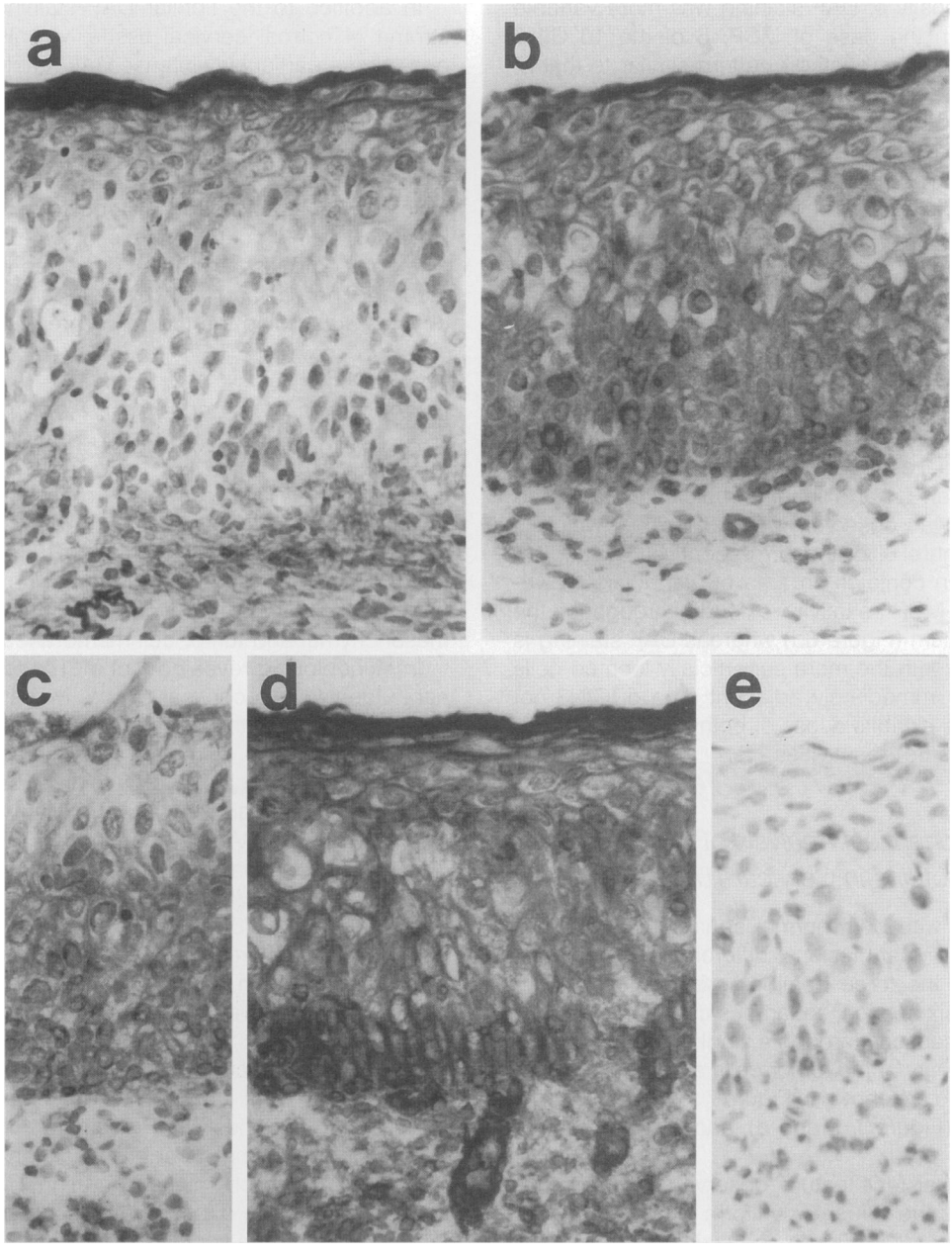

Figure 5. Immunoperoxidase staining for complement regulatory proteins in moderate (CIN2) cervical disease. The distribution of the three regulators is illustrated in a single CIN2 lesion. **a:** Anti-DAF (BRIC 230) reactivity is localized to the superficial aspect of the epithelium. **b:** In some areas of the lesion, MCP expression (MAb J4.48) occurs throughout the epithelium. **c:** In an area adjacent to that shown in **b**, anti-MCP staining is more restricted with superficial cells showing little or no staining. **d:** Anti-CD59 (BRIC 229) reactivity occurs throughout the epithelium. **e:** There is no staining in the negative control (primary antibody omitted). Magnification, ×100.

Immunohistochemical Analysis of Complement Regulatory Proteins in Premalignant Cervical Lesions

Complement regulatory protein expression was examined in 23 cervical biopsies exhibiting CIN (3 CIN1, 10 CIN2, and 10 CIN3). The distribution of all three regulators in the mesenchymal/stromal compartment of cervical biopsies containing CIN was similar to that observed in normal cervical tissue. Also in common with the normal cervix, CD59 was broadly distributed throughout the squamous epithelium of all CIN lesions examined (Figure 5d shows anti-CD59 reactivity in a CIN2 specimen). However, changes in the expression of the C3 convertase regulators DAF and MCP were observed in some CIN lesions. The profile of DAF and MCP reactivity typical of the normal ectocervical squamous epithelium occurred in all three CIN1 lesions examined (not shown). More complex patterns were apparent in biopsies containing CIN2 and CIN3. In all 10 cases of CIN2, as in the normal cervical squamous epithelium, DAF staining was restricted to the superficial aspect of the epithelium (Figure

5a), but the intensity of DAF staining was more variable than in CIN1. In the case of MCP, 5 of the 10 CIN2 specimens showed a reactivity pattern similar to that of the normal ectocervix in which staining was restricted predominantly to the lower layers of the epithelium. The remaining five specimens also displayed this pattern (Figure 5c), but in addition, these lesions contained areas displaying a marked expansion of MCP reactivity in which staining extended to the superficial aspect of the epithelium (Figure 5b).

By comparison with less severe disease, CIN3 lesions exhibited a more pronounced variability in both DAF and MCP reactivities. All 10 CIN3 specimens examined contained areas in which MCP staining extended throughout the lesion to the superficial aspect of the epithelium (Figure 6b). In general, DAF reactivity was associated only with the superficial aspect of the affected epithelium (Figure 6a). However, an additional complexity in DAF and MCP reactivity was observed in CIN3 lesions, which is illustrated in Figure 6, c and d, respectively. The CIN3 specimen shown contains an area of glandular involvement in which MCP reactivity extends throughout the involved epithelium (Figure 6d) whereas DAF staining is associated only with the more superficially located cells (Figure 6c). An immediately adjacent area of CIN3 not involving glands exhibits a loss of both MCP (Figure 6d) and DAF (Figure 6c) reactivities.

Immunohistochemical Analysis of Complement Regulatory Proteins in Cervical Tumors

By comparison with both normal squamous epithelium and early premalignant lesions, differences in the staining patterns of the three complement regulators were apparent in all six cervical squamous carcinomas examined. Adjacent sections through a single tumor nest illustrating the reactivity of the tumor cells and the surrounding mesenchyme are shown in Figure 7 for DAF (Figure 7, a and b), MCP (Figure 7, c and d), and CD59 (Figure 7, f and g).

The most consistent feature observed in squamous carcinomas was the apparent uniform staining of tumor epithelial cells for MCP (Figure 7c); staining intensity did not appear to depend on the position of a tumor cell in relation to adjacent stroma or basement membrane, nor did we observe the heterogeneity characteristic of MCP expression in premalignant lesions. By contrast, DAF expression in tumors was more variable; tumor cells generally appeared to lack DAF (Figure 7a), but restricted areas of DAF expression were also observed in all six carcinomas. In areas of squamous differentiation, DAF-positive cells were generally localized toward the center of tumor nests distal to the surrounding stroma (not shown) whereas in areas with no such organization clusters of DAF-positive cells were apparently randomly distributed and surrounded by DAF-negative cells (Figure 7e). Inspection of multiple tissue blocks revealed that variations of tumor cell phenotype and intensity of reactivity with respect to DAF occurred within an individual tumor.

In addition to the fibrillar DAF staining noted in the stroma of normal cervical tissue, we also consistently observed a particularly intense DAF reactivity on mesenchymal cells located directly adjacent to the tumor cells (Figure 7b). In contrast, stromal tissue adjacent to the tumor epithelium was MCP negative (Figure 7d). In common with both normal cervical tissue and premalignant lesions, CD59 was broadly distributed in cervical tumors. However, staining of tumor cells in some specimens was weak by comparison with that observed in normal squamous epithelium and also by comparison with mesenchyme surrounding the tumor (Figure 7, f and g).

Examination of Complement Regulatory Proteins in Cervical Tumors by Immunoblotting

In place of detergent extracts of dispase-separated epithelial sheets as described for studies on the normal cervix (Figure 4), immunoblotting of tumors was carried out using membranes prepared from whole tissue blocks (Figure 8). These preparations therefore contained both mesenchymal and tumor cell membranes.

Immunoblotting revealed that MCP and CD59 components identified in cervical tumors migrated with similar characteristics to those in normal cervical tissue (not shown), and these were not investigated further. However, in addition to a 70-kd DAF product typical of that observed in normal cervical epithelium another, lower molecular weight DAF component migrating with a leading edge of approximately 54 to 56 kd was clearly evident in cervical tumors (Figure 8a, lanes 2 and 3). In some experiments, the 70-kd and 54- to 56-kd DAF components were of approximately equal intensity (Figure 8a, lane 3), whereas in others, the lower product was predominant and migrated as a broad component (Figure 8a, lane 2).

The lower molecular weight DAF product could be derived either from DAF-positive tumor cells or, alternatively, from the intensely DAF-positive stroma at the tumor margin. In an attempt to address this an approach was developed in which mini-preparations of tumor membranes were made directly from tissue sections. By immunostaining tissue sections cut immediately before and subsequent to those used to prepare membranes, the phenotype of tumor cells with respect to DAF could be established for immunoblotting studies.

Membranes prepared from tissue blocks in which the tumor cells appeared to lack DAF but where the surrounding stroma was intensely DAF positive contained a prominent 54- to 56-kd DAF product (Figure 8a, lane 2; membranes used in this experiment were prepared from tissue sections adjacent to that illustrated in Figure 7, a and b). By contrast, immunoblots of membranes made from tissue sections in which DAF-positive tumor cells were present contained both 70-kd and 54- to 56-kd DAF components (Figure 8a, lane 3; membranes used in this experiment were prepared from tissue sections adjacent to that illustrated in Figure 7e).

DAF is a highly glycosylated protein, and a low molecular weight DAF variant has been described in human

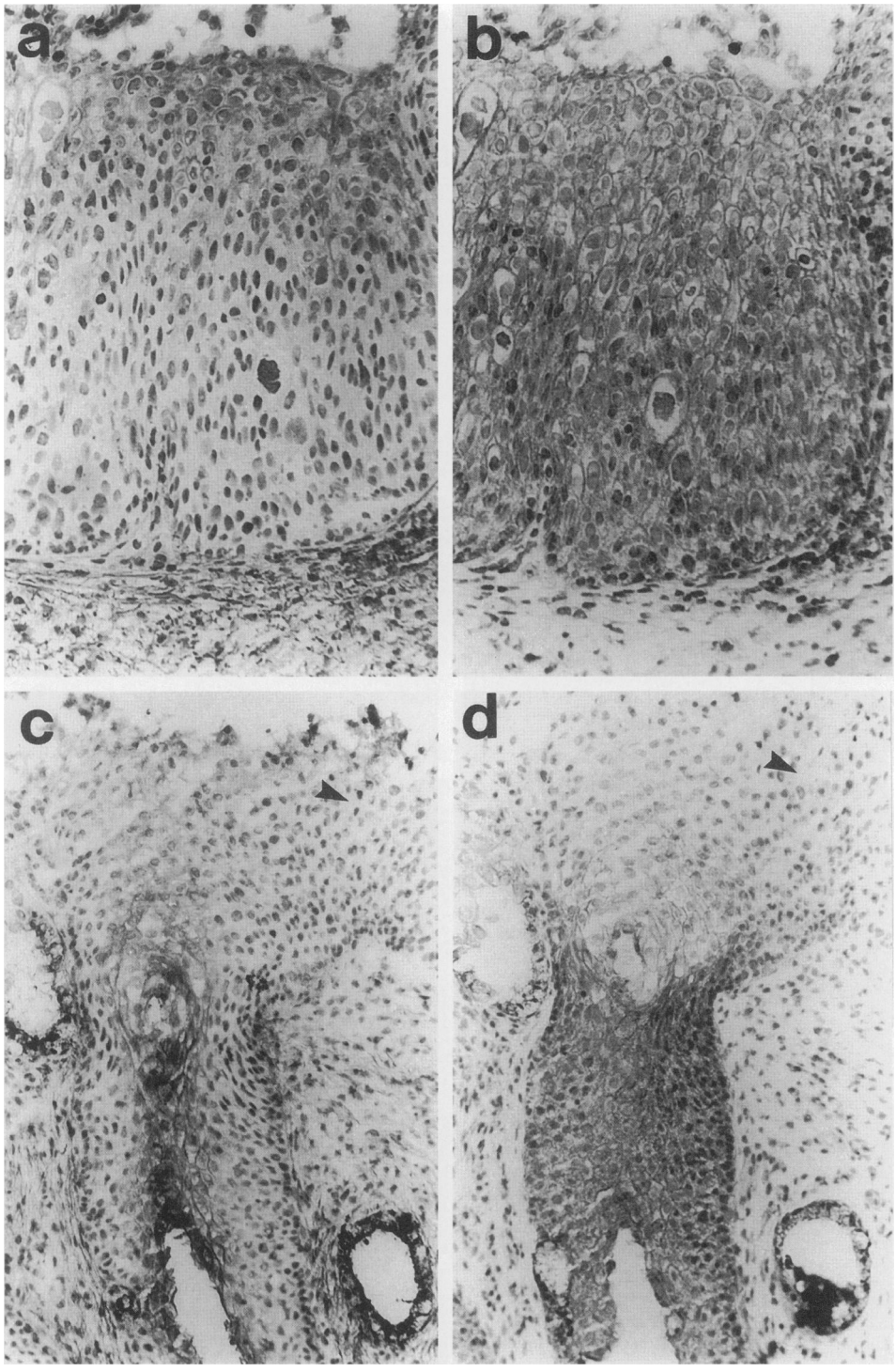

Figure 6. Localization of DAF and MCP reactivity in severe cervical lesions (CIN3) by immunoperoxidase staining. **a:** Anti-DAF (BRIC 230) staining occurs on cells located toward the superficial aspect of the lesion. Magnification, ×63. **b:** In an adjacent section, anti-MCP (MAb J4.48) reactivity occurs throughout the lesion. Magnification, ×63. **c:** In a specimen showing glandular involvement (lower field), anti-DAF (BRIC 216) reactivity is predominantly associated with superficial cells, but overlying epithelium shows little or no staining. Magnification, ×50. **d:** Anti-MCP reactivity (MAb J4.48) occurs throughout the area of glandular involvement, but the overlying epithelium shows much less staining or is negative. Magnification, ×50. **Arrows** in **c** and **d** indicate areas of disease with an apparent loss of both DAF and MCP.

spermatozoa that arises as a result of differential glycosylation.[33] To determine whether the low molecular weight DAF product observed in cervical tumors arose as a result of altered glycosylation, the effect of neuraminidase was examined. In control experiments, the molecular weight of the placental syncytiotrophoblast DAF product was reduced from 70 kd to approximately 52 kd after exposure to neuraminidase (Figure 8b, lanes 1 and

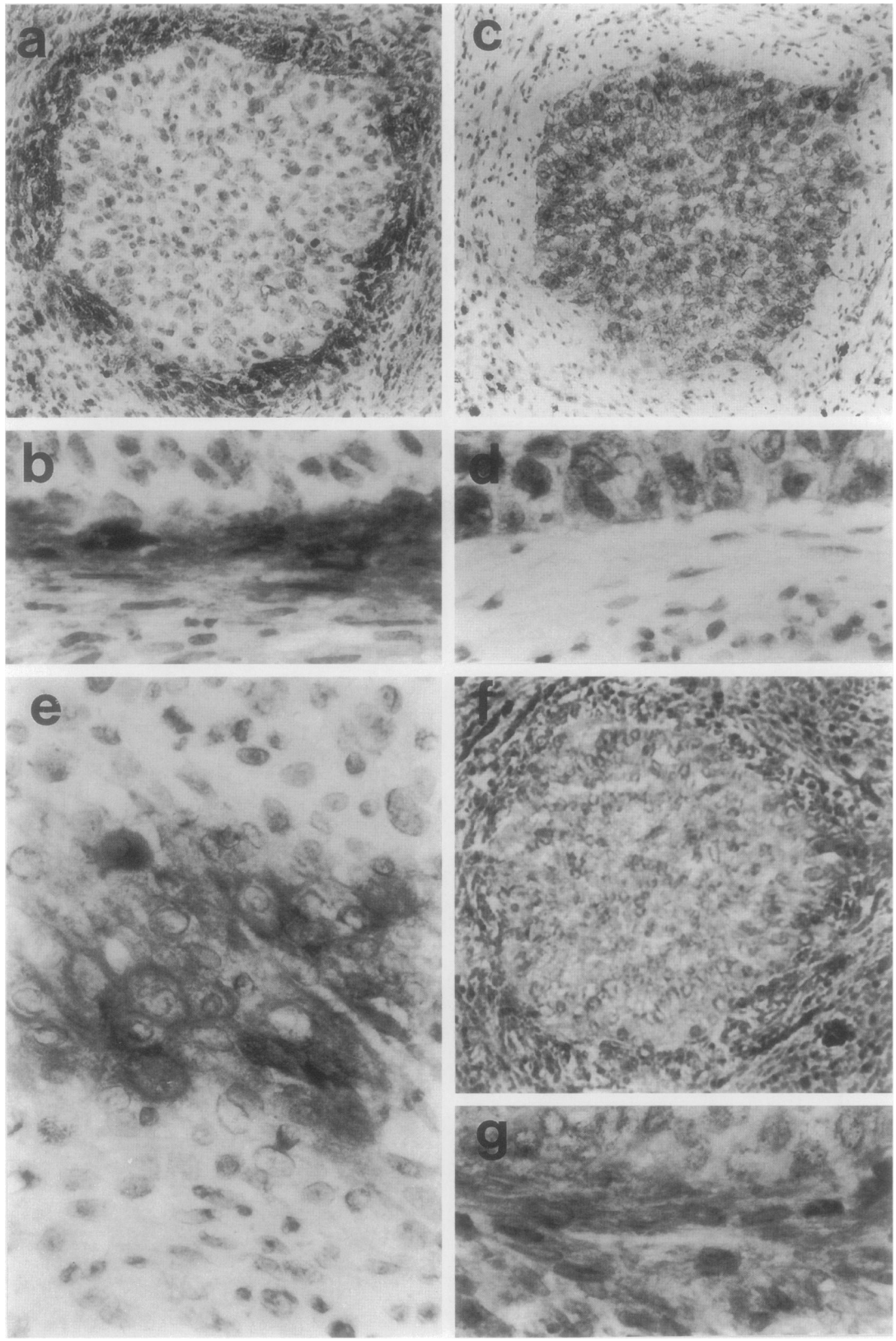

Figure 7. Immunoperoxidase staining for complement regulatory proteins in primary cervical squamous carcinomas. Serial sections through a nest of cervical tumor cells are illustrated in **a**, **c**, and **f** (all magnifications, ×63) and in more detail in **b**, **d**, and **g** (all magnifications, ×160). **a:** Cervical tumor cells appear unstained with MAb to DAF (BRIC 230), but the surrounding stroma is DAF positive. **b:** There is intense anti-DAF reactivity on stromal cells directly adjacent to the tumor. **c:** Tumor cells display uniform reactivity with MAb to MCP (E4.3). **d:** The stromal compartment adjacent to MCP-positive tumor cells is unstained. **e:** The variability in anti-DAF (BRIC 230) reactivity on tumors is illustrated. Magnification, ×160. **f** and **g:** Anti-CD59 MAb (BRIC 229) reacts broadly with cervical carcinomas although staining of tumor cells appears less intense than staining of adjacent stroma.

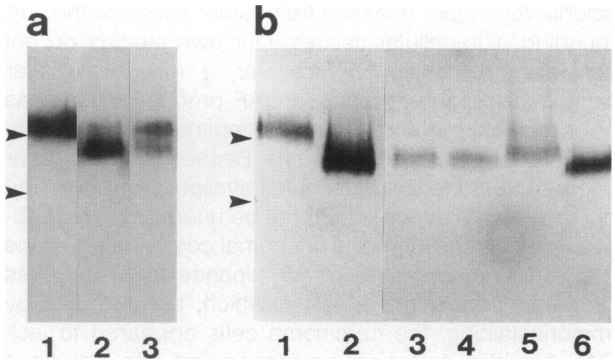

Figure 8. Analysis of DAF components in cervical tumors and assessment of their sensitivity to neuraminidase treatment. **a:** Components identified by anti-DAF MAb BRIC 230 in placental syncytiotrophoblast membranes (**lane 1**), in membranes prepared from frozen sections (see Figure 7a) in which cervical tumor cells appear to lack DAF (**lane 2**) and in membranes from sections (see Figure 7e) of a tumor containing DAF-positive carcinoma cells (**lane 3**). **b:** Immunoblots of placental syncytiotrophoblast membranes (**lanes 1** and **2**), sperm lysates (**lanes 3** and **4**) and cervical tumor membranes (**5** and **6**) in the absence (**lanes 1**, **3**, and **5**) and presence (**lanes 2**, **4**, and **6**) of neuraminidase. **Arrows** mark the position of molecular weight markers at 66 kd (top) and 45 kd (bottom).

2, respectively). Consistent with previous studies,[33] the low molecular weight human spermatozoal DAF product was unaffected by exposure to the enzyme (Figure 8b, lanes 3 and 4, respectively). Neuraminidase treatment of membrane preparations of a cervical tumor in which the lower DAF form predominated resulted in a reduction in the apparent molecular weight of the cervical DAF product (Figure 8b, lanes 5 and 6, respectively). Moreover, the neuraminidase-treated cervical and placental DAF components co-migrated (Figure 8b, compare lanes 6 and 2, respectively).

Discussion

The present study has established in detail profiles of complement regulatory protein expression in normal cervical cells and tissues. This has provided an essential framework for assessing the likely role of these proteins in cervical disease. Our observations on the immunolocalization of DAF, MCP, and CD59 in the normal cervix are broadly in line with previous studies and, additionally, provide biochemical evidence demonstrating expression of the proteins in this system. In particular, Oglesby et al[19] recently found that DAF expression appeared increased in superficial layers of the cervical squamous epithelium whereas, conversely, MCP staining was most intense on immature cells in the basal layer. A reciprocal expression of the two C3 convertase regulators on ectocervical squamous epithelium was also observed in our study. Thus, as Oglesby et al[19] have suggested, expression of these proteins in the ectocervical epithelium appears to be influenced by cellular maturity. Such a maturational dependence of DAF expression was also observed in earlier studies by Medof et al[18] in several stratified epithelia, including the cervix. Moreover, Skeie Jensen et al[34] reported that, like endometrial glandular epithelium, cervical mucosa displays an apical distribution for DAF and CD59 whereas MCP showed an additional basal membrane localization. In our hands, the apical localization of the glycosylphosphatidylinositol-anchored proteins DAF and CD59 contrasted sharply with the pronounced basolateral distribution of the transmembrane protein MCP, which showed little or no reactivity on the apical membrane. A novel finding of the present study was the marked apparent expansion of MCP reactivity on metaplastic squamous epithelium of the squamocolumnar junction, a region of particular importance in the development of cervical disease. This change could reflect a difference in maturity relative to the adjacent squamous epithelium. Alternatively, differences in proliferative capacity or cellular origin of the metaplastic epithelium could influence MCP expression at this site.

Our results, in common with those of Oglesby et al,[19] show that the cervical stroma contains abundant DAF and CD59 but relatively little MCP. Several studies have reported an association between DAF and collagen or elastic fibers. Medof et al[18] speculated that DAF on fibers could function to regulate complement after C3b or C4b condensation on hydroxyl or amino groups present at stromal sites. Fibrillar DAF has not been characterized biochemically, and therefore its functional significance is at present unclear. Interestingly, however, Seyama et al[35] recently reported that DAF on elastic fibers was resistant to phosphatidylinositol lipase digestion and suggested that the protein may lack a glycosylphosphatidylinositol anchor. Oglesby et al[19] found that MCP, which contains a putative nuclear localization signal,[36] showed an apparently nuclear reactivity in both cervical and ovarian stromal cells. In our study it was unclear whether the limited and diffuse cytoplasmic staining observed on cervical stromal cells had a specific nuclear involvement.

The factors controlling expression patterns of the complement regulatory proteins remain to be clarified. However, several pro-inflammatory agents are known to influence expression in a cell-specific manner. For example, DAF synthesis in human endothelial cells is increased by phorbol esters, some lectins, and histamine.[37–39] DAF is also up-regulated in response to complement deposition on mesangial cells.[40] MCP expression was found to be increased by histamine, but not by cytokines, on an epidermoid cell line.[41] Tumor necrosis factor-α and interleukin-1β enhanced expression of both DAF and CD59 in a colonic adenocarcinoma cell line, but MCP expression was influenced by interleukin-1β alone.[42,43] In the case of the cervix, it has been established that cervical mucus contains functional complement, which most likely acts to protect against ascending tract infections.[44] Consequently, pro-inflammatory factors generated in the local environment may influence the expression profiles observed both in the normal cervix and in cervical disease.

Complement regulators may have functions in addition to a direct involvement in protection of cells against complement. DAF and CD59 transduce activation signals in T cells, and both proteins can associate into large covalent complexes along with tyrosine kinases, further suggesting a role in signal transduction.[45–47] DAF contains an adhesin-binding site, which may facilitate infection by *Escherichia coli*,[48] and MCP is a receptor for group A streptococci.[49] Both proteins can also act as viral recep-

tors.[50–52] These properties of the regulators may be particularly important in the cervix given its exposure to infectious agents. In addition, the nuclear localization observed in previous studies[19] raises the possibility of an alternative function for MCP in cervical stromal cells. Observed changes in the expression of the complement regulators in disease could therefore have complex consequences in both epithelial and stromal compartments of the cervix.

A significant finding of the present study is that cervical disease is associated with a change in the maturation-dependent MCP expression profile typical of the normal ectocervical epithelium. Although the typical pattern was found in CIN1, a marked expansion in MCP expression was evident in one-half of the CIN2 and all of the CIN3 specimens tested. Moreover, primary cervical carcinomas consistently displayed extensive and uniform MCP expression. This MCP reactivity is reminiscent of immature or metaplastic squamous epithelial cells in the normal cervix. Increased MCP expression has been observed previously in colon, mammary, and ovarian carcinomas.[12,13] Seya et al also found increased levels of MCP on leukemic cells compared with their normal counterparts and concluded that MCP function may be important for tumor cell survival.[17] The observed expansion of MCP expression in the cervix could therefore provide a mechanism for evasion of immune surveillance. It may be significant that this apparent selective advantage can be detected in premalignant disease and is therefore not confined only to the later stages of tumorigenesis.

In contrast to MCP, DAF expression appeared generally unaltered in premalignant lesions and, also, showed a variability in expression both within and between cervical carcinomas. In general, tumor cells appeared to be devoid of DAF, but focal areas of DAF reactivity were revealed in all specimens after examination of several tissue blocks from the same tumor. Such apparent focal DAF staining was observed recently by Bjorge et al[13] in ovarian carcinomas. Variable DAF expression has been reported in basal carcinomas of skin[10] and in cell lines derived from breast carcinomas and melanomas.[16] On the other hand, Niehans et al[12] observed an absence of membrane-associated DAF in breast, colon, kidney, and lung carcinomas. Studies by Cheung et al[16] showed that DAF on tumor cells lines contributed to resistance to complement whereas Bjorge et al[13] found an inverse correlation between DAF and resistance to complement in ovarian carcinomas. The presence of some DAF-positive tumor cells could therefore have functional implications in cervical carcinomas.

An intriguing finding of the present study was the apparent increased DAF expression observed in stromal cells located directly adjacent to infiltrating tumor cell nests. Interestingly, Niehans et al[12] also reported that, by comparison with normal tissue stroma, large quantities of DAF and to a lesser degree CD59 were present in the stroma surrounding tumor cells in a variety of primary carcinomas. Because soluble DAF has been reported in a variety of body fluids and in tissue culture supernatant from the HeLa cell line,[18] these investigators proposed[12] that the stromal localization reflected the presence of soluble regulators released from tumor cells into the surrounding extracellular tissues. Our own studies do not exclude this possibility. However, a lower molecular weight membrane-associated DAF product, which was not detectable in normal cervical epithelium, was demonstrated in cervical carcinomas. Because of the cellular complexity of the tissue samples available for biochemical analysis, this product cannot be unequivocally attributed to either the epithelial or stromal compartment of the tumor. Nevertheless, its predominance in membranes prepared from tumor areas in which, as assessed by immunostaining, the carcinoma cells appeared to lack DAF provides preliminary evidence that this may be a stromal product. Our data also suggest that this protein is incompletely glycosylated. It may be speculated that this could represent an immature glycosylation pattern resulting from rapid turnover or up-regulation of expression. Alternatively, it could represent an *in situ* modification of a mature glycosylated species. Additional characterization of this DAF product will be important because of its potential functional role both in regulating complement deposition and in preventing the recruitment of inflammatory cells at tumor margins. These observations emphasize the importance of considering the stromal environment, in addition to the epithelial compartment, for the development of cervical disease.

References

1. Kinoshita T: Biology of complement: the overture. Immunol Today 1991, 12:291–295
2. Atkinson JP, Farries T: Separation of self from non self in the complement system. Immunol Today 1987, 8:212–215
3. Lublin DM, Atkinson JP: Decay-accelerating factor: biochemistry, molecular biology and function. Annu Rev Immunol 1989, 7:35–58
4. Seya T, Atkinson JP: Functional properties of membrane cofactor protein of complement. Biochem J 1989, 264:581–588
5. Meri S, Morgan BP, Davies A, Daniels RH, Olavesen MG, Waldmann H, Lachmann PJ: Human protectin (CD59), an 18–20000 MW complement lysis restricting factor, inhibits C5b-9 catalysed insertion of C9 into lipid bilayers. Immunology 1990, 71:1–9
6. Rollins SA, Zhoa J, Ninomiya H, Sims PJ: Inhibition of homologous complement by CD59 is mediated by a species selective recognition conferred through binding to C8 within C5b-8 or C9 within C5b-9. J Immunol 1991, 146:2345–2351
7. McNearney T, Ballard L, Seya T, Atkinson JP: Membrane cofactor protein of complement is present on human fibroblast, epithelial and endothelial cells. J Clin Invest 1989, 84:538–545
8. Nose M, Katoh M, Okada N, Kyogoku M, Okada H: Tissue distribution of HRF20, a novel factor preventing the membrane attack of homologous complement, and its predominant expression in endothelial cells in vivo. Immunology 1990, 70:145–149
9. Meri S, Waldmann H, Lachmann PJ: Distribution of protectin (CD59), a complement membrane attack inhibitor, in normal human tissues. Lab Invest 1991, 65:532–537
10. Sayama K, Shiraishi S, Miki Y: Distribution of complement regulators (CD46, CD55 and CD59) in skin appendages and in benign and malignant skin neoplasms. Br J Dermatol 1992, 127:1–4
11. Yamakawa M, Yamada K, Tsuge T, Ohrui H, Ogata T, Dobashi M, Imai Y: Protection of thyroid cancer cells by complement-regulatory factors. Cancer 1994, 73:2808–2817
12. Niehans GA, Cherwitz DL, Staley NA, Knapp DJ, Dalmasso AP: Human carcinomas variably express the complement inhibitory proteins CD46 (membrane cofactor protein), CD55 (decay-accelerating factor), and CD59 (protectin). Am J Pathol 1996, 149:129–142
13. Bjorge L, Hakulinen J, Wahlstrom T, Matre R, Meri S: Complement

regulatory proteins in ovarian malignancies. Int J Cancer 1997, 70: 14–25
14. Kumar S, Vinci JM, Pytel BA, Baglioni C: Expression of messenger RNAs for complement inhibitors in human tissues and tumors. Cancer Res 1993, 53:348–353
15. Shinoura N, Heffelfinger SC, Miller M, Shamraj OI, Miura N-H, Larson JL, DeTribolet N, Warnick RE, Tew JJ, Menon AG: RNA expression of complement regulatory proteins in human brain tumors. Cancer Lett. 1994, 86:143–149
16. Cheung N-KV, Walter EI, Smith-Mensah WH, Ratnoff WD, Tykocinski ML, Medof ME: Decay-accelerating factor protects human tumor cells from complement-mediated cytotoxicity in vitro. J Clin Invest 1988, 81:1122–1128
17. Seya T, Hara T, Matsumoto M, Akedo H: 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 1990, 145:238–245
18. Medof ME, Walter EI, Rutgers JL, Knowles DM, Nussenzweig V: Identification of the complement decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids. J Exp Med 1987, 165:848–864
19. Oglesby TJ, Longwith JE, Huettner PC: Human complement regulator expression by the normal female reproductive tract. Anat Rec 1996, 246:78–86
20. Holmes CH, Simpson KL, Wainwright SD, Tate CG, Houlihan JM, Sawyer IH, Rogers IP, Spring FA, Anstee DJ, Tanner MJ: Preferential expression of the complement regulatory protein decay accelerating factor at the feto-maternal interface during human pregnancy. J Immunol 1990, 144:3099–3105
21. Fletcher A, Bryant JA, Gardner B, Judson PA, Spring FA, Parsons SF, Mallinson G, Anstee DJ: New monoclonal antibodies in CD59: use for the analysis of peripheral blood cells from paroxysmal nocturnal haemoglobinuria patients and for the quantitation of CD59 on normal and decay-accelerating factor (DAF)-deficient erythrocytes. Immunology 1992, 75:507–512
22. Sparrow RL, McKenzie IFC: HuLy-m5: a unique antigen physically associated with HLA molecules. Hum Immunol 1983, 7:1–15
23. Pesando JM, Stucki M, Hoffman P: Altered expression of surface antigens with the appearance of class II molecules on a malignant human B cell line. Hum Immunol 1987, 19:235–243
24. Sunderland CA, Davies JO, Stirrat GM: Immunohistology of normal and ovarian cancer tissue with a monoclonal antibody to placental alkaline phosphatase. Cancer Res 1984, 44:4496–4502
25. Simpson KL, Houlihan JM, Holmes CH: Complement regulatory proteins in early fetal life: CD59, membrane cofactor protein (MCP) and decay-accelerating factor (DAF) are differentially expressed in the developing liver. Immunology 1993, 80:183–190
26. Simpson KL, Holmes CH: Differential expression of complement regulatory proteins decay-accelerating factor (CD55), membrane cofactor protein (CD46) and CD59 during human spermatogenesis. Immunology 1994, 81:452–461
27. Smith NC, Brush MG, Luckett S: Preparation of placental villous surface membrane. Nature 1974, 252:302–303
28. Towbin H, Staehlin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979, 76:4350–4354
29. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227:680–681
30. Holmes CH, Simpson KL, Okada H, Okada N, Wainwright SD, Purcell DFJ, Houlihan JM: Complement regulatory proteins at the feto-maternal interface during human placental development: distribution of CD59 by comparison with membrane cofactor protein (CD46) and decay accelerating factor (CD55). Eur J Immunol 1992, 22:1579–1585
31. Russell SM, Sparrow RL, McKenzie IFC, Purcell DFJ: Tissue-specific and allelic expression of the complement regulator CD46 is controlled by alternative splicing. Eur J Immunol 1992, 22:1513–1518
32. Bora NS, Post TW, Atkinson JP: Membrane cofactor protein of the complement system: a *Hin*dIII restriction length polymorphism that correlates with the expression polymorphism. J Immunol 1991, 146: 2821–2825
33. Cervoni F, Oglesby TJ, Fenichel P, Dohr G, Rossi B, Atkinson JP, Hsi B-L: Expression of decay-accelerating factor (CD55) of the complement system on human spermatozoa. J Immunol 1993, 151:939–948
34. Skeie Jensen T, Bjorge L, Wollen A-L, Ulstein M: Identification of the complement regulatory proteins CD46, CD55, and CD59 in human fallopian tube, endometrium, and cervical mucosa and secretion. Am J Reprod Immunol 1995, 34:1–9
35. Sayama K, Shiraishi S, Shirakata Y, Kobayashi Y, Miki Y: Characterisation of decay-accelerating factor (DAF) in human skin. J Invest Dermatol 1991, 96:61–64
36. Liszewski MK, Tedja I, Atkinson JP: Membrane cofactor protein (CD46) of complement: processing differences related to alternatively spliced cytoplasmic domains. J Biol Chem 1994, 269:10776–10779
37. Bryant RW, Granzow CA, Siegel MI, Egan RW, Billah MM: Phorbol esters increase synthesis of decay-accelerating factor, a phosphatidylinositol-anchored surface protein, in human endothelial cells. J Immunol 1991, 147:1856–1862
38. Bryant RW, Granzow CA, Siegel MI, Egan RW, Billah MM: Wheat germ L-agglutinin and other selected lectins increase synthesis of decay-accelerating factor in human endothelial cells. J Immunol 1991, 147:1856–1862
39. Tsuji S, Kaji K, Nagasawa S: Decay-accelerating factor on human umbilical endothelial cells: its histamine-induced expression and spontaneous shedding from the cell surface. J Immunol 1993, 152: 1404–1410
40. Shibata T, Cosio FG, Birmingham DJ: Complement activation induces the expression of decay-accelerating factor on human mesangial cells. J Immunol 1991, 147:3901–3908
41. Cui W, Atkinson JP: The regulation of expression of membrane cofactor protein MCP;CD46. FASEB J 1994, 8:A765
42. Bjorge L, Skeie Jensen T, Ulvestad E, Vedeler CA, Matre R: The influence of tumour necrosis factor-α, interleukin-1β and interferon-γ on the expression and function of the complement regulatory protein CD59 on the human colonic adenocarcinoma cell line HT29. Scand J Immunol 1995, 41:350–356
43. Bjorge L, Skeie Jensen T, Matre R: Characterisation of the complement-regulatory proteins decay-accelerating factor (DAF, CD55) and membrane cofactor protein (MCP, CD46) on a human colonic adenocarcinoma cell line. Cancer Immunol Immunother 1996, 42:185–192
44. Price RJ, Boettcher B: The presence of complement in human cervical mucus and its possible relevance to infertility in women with complement dependent immobilising antibodies. Fertil Steril 1979, 32:61–66
45. Davis LS, Patel SS, Atkinson JP, Lipsky PE: Decay accelerating factor functions as a signal transducing molecule for human T cells. J Immunol 1988, 141:2246–2252
46. Korty PE, Brando C, Shevach EM: CD59 functions as a signal transducing molecule for human T cell activation. J Immunol 1991, 146: 4092–4098
47. Cinek T, Horesji V: The nature of large noncovalent complexes containing glycosyl-phosphatidylinositol-anchored membrane glycoproteins and protein tyrosine kinases. J Immunol 1992, 149:2262–2270
48. Nowicki B, Hart A, Coyne KE, Lublin DM, Nowicki S: Short consensus repeat-3 domain of recombinant decay-accelerating factor is recognised by *Escherichia coli* recombinant Dr adhesin in a model of cell-cell interaction. J Exp Med 1993, 178:2115–2121
49. Okada N, Liszewski MK, Atkinson JP, Caparon M: Membrane cofactor protein (MCP, CD46) is a keratinocyte receptor for the M protein of group A streptococcus. Proc Natl Acad Sci USA 1995, 92:2489–2493
50. Bergelson JM, Chan M, Solomon KR, St John NF, Lin H, Finberg RW: Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. Proc Natl Acad Sci USA 1994, 91:6245–6248
51. Naniche D, Varior-Krishnan G, Cervoni F, Wild TF, Rossi B, Rabourdin-Combe C, Gerlier D: Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. J Virol 1993, 67:6025–6032
52. Dorig RE, Marcil A, Chopra A, Richardson CD: The human CD46 molecule is a receptor for measles virus (Edmonston strain). Cell 1993, 75:295–305