

## Levels of Complement Regulatory Molecules in Lung Cancer: Disappearance of the D17 Epitope of CD55 in Small-cell Carcinoma

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The levels of complement-regulatory molecules (complement receptor type one [CR1], decay-accelerating factor [DAF], membrane cofactor protein [MCP], and an inhibitor of membrane attack complex [CD59]) in lung cancer cells were analyzed to investigate the relation between their expression and histological subtypes, and the possibility of homologous complement deposition on cancer cells. In 25 cell lines (10 adenocarcinoma, 3 large-cell carcinoma, 7 small-cell lung cancer [SCLC], and 5 squamous cell carcinoma), flow cytometric analysis revealed that MCP was expressed in all cell lines, whereas none of the cell lines was CR1-positive. CD59 was detected in all cells. The DAF epitope defined by IA10 was expressed in all cells except one large cell carcinoma cell line. However, another epitope for anti-DAF monoclonal antibody, D17, was not detected in 5 (71.4%) SCLC and in 4 (22.2%) non-small-cell lung cancer. This disparity was seen in most cell lines, irrespective of histological subtypes. The loss of D17 reactivity seemed to be pertinent to malignant phenotype, because most of the normal pulmonary cells possessed the D17 epitope. Furthermore, a cell line lacking DAF (IA10<sup>-</sup>/D17<sup>-</sup>) allowed alternative pathway-mediated homologous complement (C3) deposition after pretreatment with anti-MCP antibody. This raises a new possibility for immunotargeting of cancer. These cell lines should be useful in studying the biology of lung cancer.

Key words: Decay-accelerating factor (DAF, CD55) — Flow cytometry — Lung cancer — Monoclonal antibody

Lung cancer is the leading cause of cancer death in the United States and in some Western countries.<sup>1,2</sup> Lung cancer is clinically divided into SCLC<sup>7</sup> and NSCLC. SCLC is a highly malignant tumor that grows rapidly and disseminates widely, and is treated with chemotherapy and/or radiation.<sup>3</sup> NSCLC, which is usually not chemo- or radiosensitive, is best treated with surgical resection.<sup>4</sup> Besides these differences in clinical behavior, SCLC shows neuroendocrine properties, such as the presence of neurosecretory dense core granules,<sup>5</sup> secretion of multiple hormones/peptides, and the expression of neural-type isozymes.<sup>6</sup> Some of them are available as tumor markers of SCLC.<sup>7</sup> Recently, DAF, one of the complement-regulators, has been reported to be absent from SCLC.<sup>8</sup> This fact indicates that complement-regulatory molecules may be novel tumor markers of SCLC.

Not only foreign organisms but also host cells are continuously exposed to autologous complement system. Uncontrolled activation of the complement could result in formation of MAC on self tissues and activation of C3 receptor-positive host effector cells, in addition to generation of inflammatory mediators. However, host cells can avoid complement attack because they are endowed with complement-regulatory molecules: CR1 facilitates dissociation of classical and alternative pathway C3 convertases, and acts as a cofactor for Factor I-mediated cleavage of C3b and C4b<sup>9</sup>; DAF accelerates dissociation of classical and alternative pathway C3 convertases, C4b2a and C3bBb complex<sup>10</sup>; MCP serves as a cofactor for Factor I-mediated cleavage of C3b and C4b<sup>11</sup>; and CD59 blocks C9 binding to C5b-8 to prevent formation of MAC.<sup>12,13</sup> DAF, MCP and CD59 thus serve as safeguards against autologous complement attack. Cancer cells, during the processes of invasion and metastasis, are also exposed to complement in blood. If the complement-regulators are insufficiently expressed or functionally impaired in cancer cells, the cells can be selectively damaged by complement.

With mAbs raised against these complement-regulators, we evaluated the expression of these molecules in lung cancer cell lines, both SCLC and NSCLC, and found that DAF was present in almost all cell lines.

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<sup>7</sup> Abbreviations: SCLC, small-cell lung cancer; CR1, complement receptor type one (C3b/C4b receptor, CD35); DAF, decay-accelerating factor (CD55); mAb, monoclonal antibody; MAC, membrane attack complex that is formed by C5b-9; MCP, membrane cofactor protein (CD46); NSCLC, non-small-cell lung cancer (adeno-, squamous, and large cell carcinomas); CD59, an inhibitor of MAC formation.

However, another anti-DAF mAb we newly obtained gave different results, especially in SCLC. The results may imply the expression of structurally altered DAF molecules in the tumor cells.

Homologous C3 deposition on cancer cells due to blocking of MCP was anticipated if DAF were deficient, since all the cells lacked CR1. We found this was the case. These results raise a new possibility for immunological targeting of cancer cells.

MATERIALS AND METHODS

**Cell lines** Seventeen cell lines were established from patients with histologically and cytologically proven primary lung cancer (Table I). Pleural effusions were obtained aseptically and heparinized, and cancer cells were collected by gradient centrifugation or centrifugation after hemolysis. Solid tumors were finely minced with a scalpel and plated into culture vessels.

Lu24 was grown in serum-free medium (SFM 101, Nissui Pharmaceutical Co., Tokyo) at 37°C, under a 5% CO<sub>2</sub> humidified atmosphere. Adherent cells were cultured in Eagle's minimum essential medium (Nissui Pharmaceutical Co.) containing kanamycin and 10% heat-inactivated fetal calf serum (FCS). For other cells, RPMI-1640 (GIBCO Lab., Grand Island, NY) medium

supplemented with 10% FCS, streptomycin and penicillin G was used. Lu24 and Lu134A cells<sup>14)</sup> were kindly supplied by Dr. T. Terasaki (National Cancer Center, Tokyo). Four cell lines of squamous cell carcinoma of the lung (EBC-1, LC-1 sq, LK-2, and VMRC-LCP), and two cell lines of SCLC (SBC5 and RERF) were obtained from the Japanese Cancer Research Resources Bank (Tokyo). Tumorigenicity was determined by inoculating cancer cells into nude mice subcutaneously.

**Monoclonal antibodies** Preparation of the mAbs against MCP (M177),<sup>15, 16)</sup> CR1 (31R)<sup>16)</sup> and DAF (D17)<sup>17)</sup> has been described elsewhere. Another anti-DAF (IA10)<sup>18)</sup> and anti-CD59 (5H8)<sup>19)</sup> mAbs were generous gifts from Dr. T. Kinoshita (Institute for Microbial Diseases, Osaka University) and Dr. M. Tomita (Showa University, Tokyo), respectively.

**Analysis of complement-regulatory molecules in cancer cells** Carcinoma cells were harvested by centrifugation (suspension culture) or trypsinization (0.25% trypsin for 10 min; monolayer). Spheroids were disaggregated with trypsin. Trypsin treatment does not affect DAF,<sup>20)</sup> MCP,<sup>21)</sup> or CD59<sup>19)</sup> molecules. Although CR1 is labile to proteolysis,<sup>22)</sup> our trypsinization conditions did not abrogate CR1 of granulocytes (data not shown). Cancer cells (10<sup>6</sup> cells suspended in 100 μl of Dulbecco's modified phosphate-buffered saline containing bovine serum albumin [0.5% w/v]) were incubated with 10 μg of mAbs for 45 min on ice with 50 μl of human plasma containing 10 mM EDTA. Murine non-immune IgG (Sigma Chemicals, St. Louis, MO) was used as a negative control. The cells were washed twice and incubated with 5 μg of fluorescein isothiocyanate (FITC)-conjugated caprine anti-mouse IgG (Cappel, Organon Teknika Corp., West Chester, PA) for 45 min on ice. The cells were rinsed twice, then fixed with 0.5% (w/v) paraformaldehyde.<sup>16)</sup> The samples were analyzed with an EPICS Profile II (Coulter Electronics, Hialeah, FL) within a week.

Mean fluorescent shift (MFS) was calculated by subtracting the values of mean peak channel of controls from those of the corresponding samples. MFS greater than 1.0, which is the minimal significant shift on Profile II, was defined as positive expression. Each sample was assayed at least twice independently, and the mean MFS value was adopted.

**Immunohistochemical analysis of complement regulators in normal lung** Normal pulmonary tissues were obtained from an autopsy case of non-malignant disease. The excised lungs were fixed with acetone. Endogenous peroxidase activity was blocked with 2% H<sub>2</sub>O<sub>2</sub> for 10 min. The specimens were reacted with primary antibodies diluted 50–200 fold for 1 h at room temperature. Color was developed with HRP-labeled anti-mouse IgG (Cappel) and 3,3'-diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka; 1 mg/ml).<sup>23)</sup>

Table I. Profiles of Cell Lines Established

Cell line	Histology	Origin	Growth behavior	Tumorigenicity in nude mice
CADO LC1	LG <sup>a)</sup>	BR <sup>b)</sup>	Mono <sup>c)</sup>	+
CADO LC3	LG	LN	Mono	+
CADO LC5	AD	PF	Mono	-
CADO LC6	SM	PF	Susp	+
CADO LC7	AD	PF	Mono	-
CADO LC8	SM	HE	Susp	+
CADO LC9	AD	PF	Susp	+
CADO LC10	AD	PF	Mono	+
CADO LC11	AD	PF	Mono	+
CADO LC15	SQ	BR	Mono	ND <sup>d)</sup>
CADO LC17	SM	LN	Susp	+
CADO LC20	AD	PF	Sphe	+
CADO LC21	AD	PF	Susp	+
CADO LC22	SM	PR	Susp	+
CADO LC24	LG	PF	Mono	ND
CADO LC26	AD	PF	Mono	+
CADO LC41	AD	PF	Mono	ND

a) AD, adenocarcinoma; LG, large cell carcinoma; SM, small-cell carcinoma; SQ, squamous cell carcinoma.

b) BR, brain metastasis; HE, hepatic metastasis; LN, lymph node metastasis; PF, pleural effusion; PR, primary tumor.

c) Mono, monolayer; Sphe, spheroid; Susp, suspension.

d) ND, not determined.

**Analysis of C3 deposition on cancer cells** To test if the lack of D17 epitope was associated with homologous C3 deposition, we treated three types of cells, IA10<sup>+</sup>/D17<sup>+</sup>, IA10<sup>+</sup>/D17<sup>-</sup>, and IA10<sup>-</sup>/D17<sup>-</sup>, with anti-MCP mAb and then Mg-ethyleneglycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)-serum (capable of activating the alternative pathway).<sup>24)</sup> The cancer cells (1–2  $\times 10^6$ ) were pretreated with M177 for 45 min at room temperature, then incubated with 50  $\mu$ l of human serum containing 10 mM EGTA and 2 mM MgCl<sub>2</sub>, and 150  $\mu$ l of EGTA-GVB (gelatin veronal buffer; barbital 25 mM, NaCl 0.15 M, EGTA 10 mM, MgCl<sub>2</sub> 2 mM, gelatin 0.5% [w/v]) for 1 h at 37°C. For a control, human serum supplemented with 10 mM EDTA and 150  $\mu$ l of EDTA-GVB (barbital 25 mM, NaCl 0.15 M, EDTA 10 mM, gelatin 0.5% [w/v]) was employed. The cells were stained in the same way as in the assay of complement-regulatory molecules except that leporine anti-human-C3c (Behring, Marburg, Germany) and FITC-conjugated caprine anti-rabbit IgG (Cappel) were used.

## RESULTS

**Cell lines** The characteristics of the cell lines established are listed in Table I. SCLC cell lines (CADO LC6, LC8, and LC22) grew as tightly packed floating aggregates, as did LU24 and LU134A. Three adenocarcinoma lines (CADO LC9, LC17 and LC21) expanded in loosely aggregated cell clusters when cultured in plain dishes. One adenocarcinoma (CADO LC20) proliferated as spheroids that had a central cavity. Other adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma cells multiplied as monolayers.

**Complement-regulatory molecules in lung cancer cells** The flow cytometric analysis of complement-regulators is shown in Table II. Whereas MCP was ubiquitously present in all cell lines, CR1 was not detected in any of the cells. CD59 was found in all cells tested. Unexpectedly, almost all cells expressed the IA10 epitope except CADO LC3. We therefore used another anti-DAF mAb (D17) and found that five (71.4%) SCLC and four (22.2%)

Table II. Expression of Complement-regulatory Molecules

Cell line	Mean fluorescence shift				
	MCP	CR1	DAF(D17)	DAF(IA10)	CD59
<b>Small cell carcinoma</b>					
CADO LC6	6.72	0.363	0.845	25.3	35.7
CADO LC8	12.1	~0	~0	1.67	24.2
CADO LC22	13.5	~0	4.38	34.9	1.53
LU24	12.1	~0	~0	25.4	34.3
LU134A	6.62	0.108	0.548	1.67	19.9
SBC5	7.30	ND <sup>a)</sup>	~0	0.773	ND
RERF	30.3	ND	12.6	23.4	ND
<b>Adenocarcinoma</b>					
CADO LC5	35.0	~0	0.517	3.60	35.0
CADO LC7	35.0	~0	14.5	38.6	49.8
CADO LC9	26.2	~0	0.300	27.0	175
CADO LC10	25.7	~0	3.29	8.03	69.1
CADO LC11	47.5	~0	3.63	10.8	23.6
CADO LC17	36.7	~0	5.05	25.8	6.79
CADO LC20	23.2	0.506	10.3	25.9	86.2
CADO LC21	31.2	~0	8.22	23.2	72.7
CADO LC26	53.8	~0	~0	3.24	51.1
CADO LC41	29.0	~0	13.4	30.3	8.42
<b>Squamous cell carcinoma</b>					
EBC-1	41.7	~0	1.96	9.06	127
LC-1 sq	133	~0	8.72	23.2	110
LK-2	109	~0	2.40	11.9	33.7
VMRC-LCP	158	~0	3.58	16.8	153
CADO LC15	22.3	0.178	2.97	8.87	34.3
<b>Large cell carcinoma</b>					
CADO LC1	9.31	~0	3.61	21.3	151
CADO LC3	29.2	~0	~0	~0	6.94
CADO LC24	148	~0	11.5	36.4	35.7

a) Not determined.

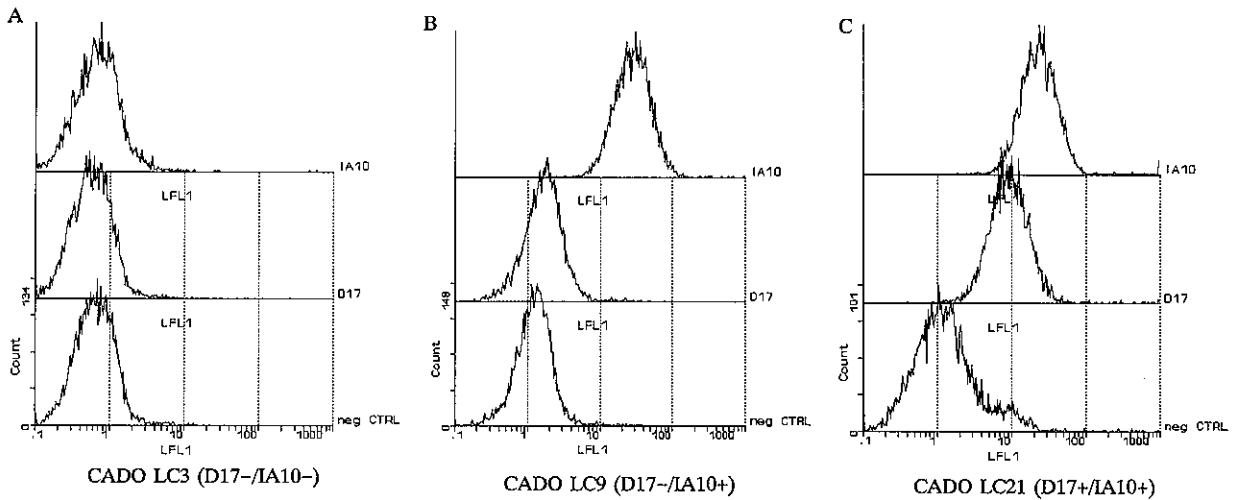


Fig. 1. Three patterns of DAF expression in lung cancer cells. Cells were treated with IA10 or D17, then FITC-labeled secondary Ab, and analyzed by flow cytometry. Non-immune mouse IgG was used as a background control. A, CADO LC3 cells (large cell carcinoma) lack both IA10 and D17 epitopes. B, CADO LC9 cells (adenocarcinoma) express IA10 but not D17. C, CADO LC21 cells (adenocarcinoma) possess both IA10 and D17.

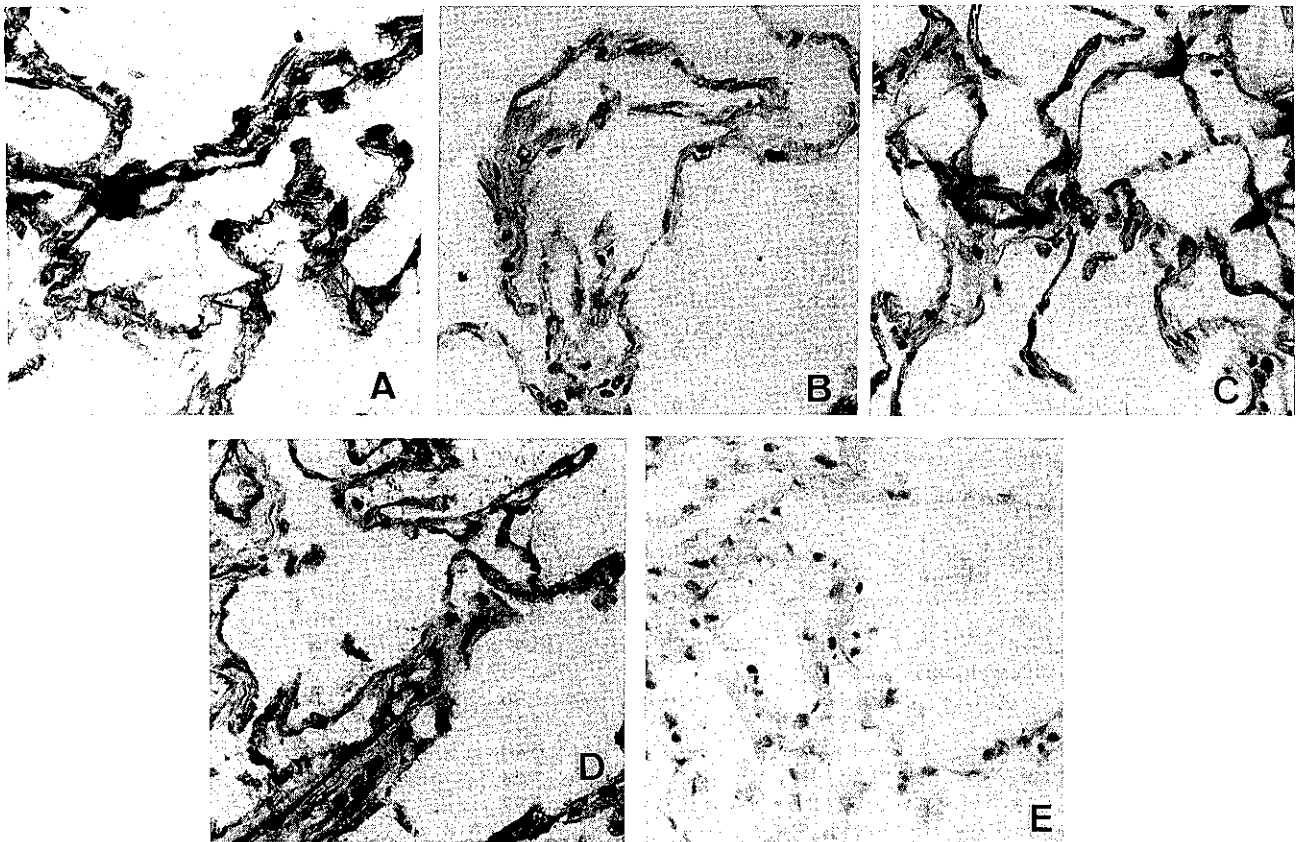


Fig. 2. Immunohistochemistry of complement-regulatory molecules in normal lung. The fixed lung specimens were stained with antibodies against DAF (IA10 and D17), MCP (M177), or CR1 (31R). A, DAF(IA10) and B, DAF(D17) were both expressed in pulmonary tissue, although the level of D17 was less than that of IA10. C, MCP and D, CD59 were detected but E, CR1 was not.

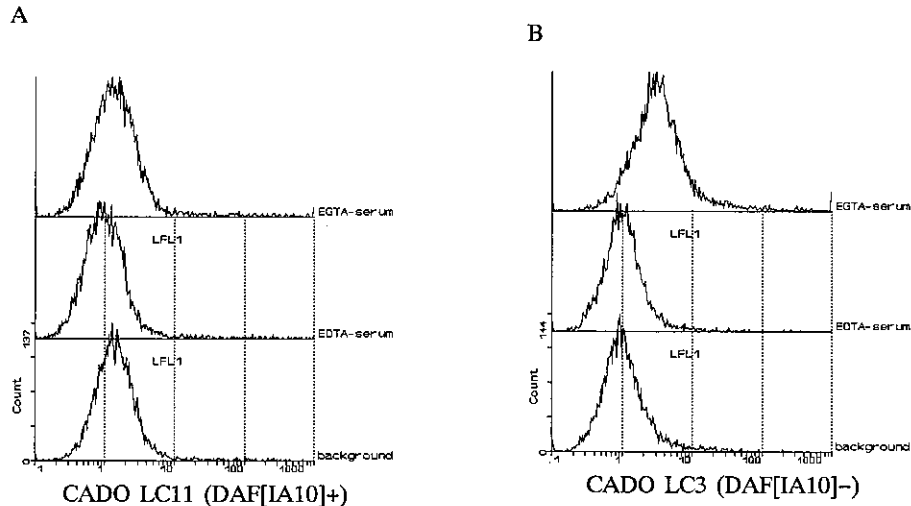


Fig. 3. Induction of homologous complement deposition on cancer cells. Cells were pretreated with M177 (MCP cofactor blocking Ab), then treated with Mg-EGTA normal human serum or EDTA normal human serum, and the deposited C3 was assessed by flow cytometry using anti-C3c Ab. Non-immune mouse IgG was used as a background control. A, cell line expressing DAF showed no C3 deposition while B, cells without DAF allowed complement accumulation.

NSCLC were negative. All D17-positive cells expressed IA10. Only two carcinoma cell lines, CADO LC3 and SBC5, were completely devoid of DAF (IA10<sup>-</sup>/D17<sup>-</sup>). IA10 always yielded a greater fluorescent shift than D17, although the degree of the difference of MFS varied. Typical expression patterns of D17 and IA10 (D17<sup>-</sup>/IA10<sup>-</sup>, D17<sup>-</sup>/IA10<sup>+</sup>, and D17<sup>+</sup>/IA10<sup>+</sup>) are shown in Fig. 1.

**Complement-regulatory molecules in normal pulmonary cells** Normal pulmonary cells including alveolar, endothelial, and interstitial tissues expressed DAF (both IA10 and D17), MCP, and CD59. CR1 was not detected in normal lung cells (Fig. 2). Hence, the D17 epitope seems to be lost during the process of malignant transformation.

**C3 deposition induced by Ab against MCP** Examples of C3 fragment deposition on carcinoma cells are depicted in Fig. 3. A cell line with both D17 and IA10 expression (CADO LC11) refused to accept C3 deposition. A cell line of D17<sup>-</sup>/IA10<sup>+</sup> phenotype (CADO LC9) showed marginal C3 accumulation (data not shown), and one that lacks both epitopes (CADO LC3) displayed C3 accrual on the cell membrane.

## DISCUSSION

As yet the expression of complement-regulatory proteins (CR1, DAF, MCP and CD59) in lung cancer has not been extensively investigated. The phenotype of the lung cancer cells was CR1<sup>-</sup>/MCP<sup>+</sup>/CD59<sup>+</sup>, regardless of histological subtypes. The high frequency of the

CR1<sup>-</sup>/MCP<sup>+</sup> profile in lung cancer is consistent with previous reports suggesting that up-regulation of MCP concomitant with loss or down-regulation of CR1 is a characteristic phenotype of tumor cells.<sup>15, 16)</sup>

Of considerable importance was the discrepancy observed between the reactivities of two mAbs against DAF. The D17 epitope was absent in 36.0% of the cell lines, whereas the IA10 epitope was detected in almost all cells. The disparity of reactivity was unique to DAF, since 3 anti-MCP and 3 anti-CR1 mAbs yielded similar fluorescence shifts to M177 and 31R, respectively (data not shown). Normal blood cells expressed both the D17 and the IA10 epitopes,<sup>17)</sup> as did normal pulmonary cells (Fig. 2). The discrepancy of reactivity of the anti-DAF mAbs, therefore, was thought to be relevant to the malignant phenotype of these cells. To our knowledge, this is the first demonstration of a variant form of DAF in cancer cells.

DAF, like CD59, is a glycan phosphatidylinositol (GPI)-anchored protein, and is known to be deficient in paroxysmal nocturnal hemoglobinuria (PNH), in which disorder of GPI-anchoring occurs.<sup>18)</sup> The loss of reactivity to the D17 mAb was not considered to be owing to the aberrant GPI-anchoring, because most of the cells still expressed the IA10 epitope. Furthermore, another GPI-anchored protein, CD59,<sup>13)</sup> was present in all cells including the D17-deficient cells.

We think that a possible mechanism for the discrepancy of the DAF epitopes in lung cancer cells is that the D17 epitope is structurally altered or made inaccessible, for example, being sterically hindered by other moieties

or molecules. The IA10 epitope resides in the first short consensus repeat (SCR) of DAF,<sup>25)</sup> whilst that of D17 is located in the vicinity of a serine/threonine-rich domain that is close to O-linked sugars.<sup>17)</sup> Immunoblot analysis disclosed that O-glycanase treatment increased D17 reactivity for the DAF purified from erythrocytes (data not shown). Moreover, another mAb 1C6, which recognizes the third SCR,<sup>25)</sup> showed similar results to IA10 but not D17 (data not shown). Hence, the altered form of DAF is likely to originate from excessive glycosylation occurring in a malignant state. MCP also has large amounts of O-linked sugars that modulate its function,<sup>21)</sup> although the role of the O-linked sugars in DAF function is unknown. Of course, cDNA analysis of the DAF from these lung cancer cells would be needed before a final conclusion can be reached.

Complement (C3) deposition can be induced on cells with aberrant complement-regulatory proteins,<sup>15, 24, 26)</sup> resulting in cell damage. To ascertain whether the lack of D17 epitope could be associated with C3-targeting, we incubated three types of cells, IA10<sup>+</sup>/D17<sup>+</sup>, IA10<sup>+</sup>/D17<sup>-</sup>, and IA10<sup>-</sup>/D17<sup>-</sup>, with anti-MCP mAb to block MCP, followed by Mg-EGTA serum (Fig. 3). The degree of resultant C3 deposition was in the order of IA10<sup>+</sup>/D17<sup>+</sup> ≈ IA10<sup>+</sup>/D17<sup>-</sup> < IA10<sup>-</sup>/D17<sup>-</sup>. The presence of D17 epitope seemed not to be essential to avoid complement attack.

Cheung *et al.*, using IA10 mAb, have reported the absence of DAF in SCLC cells.<sup>8)</sup> NSCLC cells were not studied, however. In this study, CADO LC3 (large cell carcinoma) and probably SBC5 (small cell carcinoma) totally lacked DAF (IA10<sup>-</sup>/D17<sup>-</sup>). Two SCLC cell lines (CADO LC8 and Lu134A) were not reactive to D17 but were weakly IA10-positive. Our results thus showed that not only SCLC but also NSCLC cells can be DAF (IA10)-deficient. The levels of DAF are known to change during the course of viral infection<sup>27)</sup> or under the influence of other reagents.<sup>28)</sup> Although the origin of the variant DAF and its relation to histological subtypes in lung cancer is unclear at present, the variant form of DAF we found might be significant in relation to the pathogenesis of lung cancer.

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