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# Monoclonal Anti-Cardiolipin Antibodies from New Zealand Black $\times$ New Zealand White F<sub>1</sub> Mice React to Thrombomodulin<sup>1</sup>

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The reactivity with and affinity for thrombomodulin (TM) of monoclonal anti-cardiolipin Abs (MoaCL), derived from a New Zealand Black  $\times$  New Zealand White F<sub>1</sub> (NZB/W F<sub>1</sub>) mouse, were studied to investigate the pathogenicity of anti-cardiolipin Abs (aCL). Four of eighteen MoaCL were found to react with rabbit TM when examined using ELISA. These four MoaCL also reacted with synthetic peptide that included the epidermal growth factor-like domain of human TM, a binding site for thrombin. The reaction with TM of these four MoaCL was inhibited by bovine thrombin. When the affinity for TM of the MoaCL was determined, the dissociation constants ( $K_d$ ) ranged from  $4.8 \times 10^{-9}$  to  $4.7 \times 10^{-8}$  M. By contrast, examination of the affinity for cardiolipin (CL) gave values from  $8.3 \times 10^{-6}$  to  $7.4 \times 10^{-5}$  M. Thus, these MoaCL reacted to TM with a higher affinity than to CL. Moreover, these MoaCL also bound to TM on HUVEC and down-regulated the expression level of TM on the surface of HUVEC due to internalization of TM. The binding of thrombin to TM is known to initiate rapid protein C activation, and complexes of activated protein C and protein S show anticoagulatory activity. Thus, the present studies suggest that certain pathogenic aCL cross-react with TM and induce down-regulation of TM on endothelial cells, followed by induction of thrombosis. *The Journal of Immunology*, 1998, 160: 253–258.

It has been reported that anti-phospholipid Abs (aPL)<sup>3</sup> are closely associated with thrombosis, thrombocytopenia, and intrauterine fetal death in patients with systemic lupus erythematosus (SLE) as well as antiphospholipid syndrome (APS) (1). However, anti-cardiolipin Abs (aCL) have also been detected in sera from subjects who seldom have thrombosis, such as those with juvenile rheumatoid arthritis, AIDS, or syphilis, and even in sera from healthy individuals (2–4). A relatively new method for determination of aCL revealed that aCL in the presence of  $\beta_2$ -glycoprotein I ( $\beta_2$ GPI) has additional pathogenic implications (5, 6). Moreover, Matsuura et al. reported that aCL recognized  $\beta_2$ GPI, but not cardiolipin (CL) (7). However, the mechanism of the pathogenicity of aCL is not well understood.

It was postulated that aCL affects the thrombin-thrombomodulin (TM) complex and inhibits the activation of protein C (8–10). TM is an integral glycoprotein present on the surface of endothelial cells and serves as a potent receptor of thrombin. When thrombin

binds to TM, the former loses procoagulatory and platelet-stimulating actions, then the thrombin-TM complex activates the zymogen, protein C (11, 12). Activated protein C together with protein S degrade the active blood coagulant cofactors, factor V and factor VIII, and stimulate anticoagulatory activity (13). Thus, TM plays an important role in preventing coagulation on blood vessel walls. Since some monoclonal aCL (MoaCL) exhibit polyreactivity (14, 15), it seems important to determine whether TM is one of the cross-reactive Ags responsible for the development of thrombosis in APS. In the present studies, we established MoaCL from a 6-month-old NZB/W F<sub>1</sub> mouse to determine whether aCL could react with TM.

## Materials and Methods

### Reagents

mAbs against human TM (3H1 and 9H12) were gifts from Fuji Chemical Industries Ltd. (Toyama, Japan). Goat polyclonal Abs against rabbit TM and against human TM were purchased from American Diagnostica, Inc. (Greenwich, CT), the TM preparation from rabbit lung was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), a synthetic EGF-5 peptide that includes the thrombin binding site of human TM (residues Glu<sup>408</sup> to Glu<sup>426</sup> of human TM corresponding to the fifth epidermal growth factor-like domain) (16) was purchased from Peptide Institute, Inc. (Osaka, Japan), CL was obtained from Avanti-Polar Lipids Co. Ltd. (Alabaster, AL), bovine thrombin was purchased from Sankyo Co. Ltd. (Tokyo, Japan), human IgG-Fc was obtained from Cappel (Durham, NC), and  $\beta_2$ GPI was purchased from Yamasa Corp. (Chiba, Japan).

### Generation of MoaCL

MoaCL were obtained by hybridizing the myeloma cell line P3-X63-Ag8.653 with spleen cells from a 6-month-old female NZB/W F<sub>1</sub> mouse, as described previously (17). Binding activities of culture supernatants to CL were screened by ELISA. Namely, 50  $\mu$ l of CL solubilized in ethanol (10  $\mu$ g/ml) were immobilized in each well of a microtiter plate (Immulon 2, Dynatech Laboratories, Inc., Chantilly, VA) by evaporation of the ethanol. After blocking the nonspecific binding sites by incubation with 1% BSA,

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<sup>3</sup> Abbreviations used in this paper: aPL, antiphospholipid Abs, SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome; aCL, anti-cardiolipin antibodies;  $\beta_2$ GPI,  $\beta_2$ -glycoprotein I; CL, cardiolipin; TM, thrombomodulin; MoaCL, monoclonal anti-cardiolipin antibodies; NZB/W F<sub>1</sub>, New Zealand Black  $\times$  New Zealand White F<sub>1</sub>; EGF, epidermal growth factor.

the plate was washed with 0.05% Tween-20 in PBS (PBS/Tween-20) and incubated with culture supernatant. The plate was washed with PBS/Tween-20 followed by incubation with 50  $\mu$ l of appropriately diluted biotinylated Ab against mouse IgG. The preparation was then washed with PBS/Tween-20 followed by incubation with 50  $\mu$ l of avidin-conjugated horseradish peroxidase. Each incubation was performed for 1 h at 37°C. After washing with PBS/Tween-20, 200  $\mu$ l of 0.4 mg/ml substrate (*o*-phenylenediamine dihydrochloride) diluted in 0.1 M citrate/0.2 M phosphate buffer, pH 4.2, with 0.012% H<sub>2</sub>O<sub>2</sub> was added, and the reaction was stopped by adding 50  $\mu$ l of 2.5 N H<sub>2</sub>SO<sub>4</sub> to each well after 5 min. Reactivity (OD) was determined using a MicroPlate Reader (model 450, Bio-Rad, Hercules, CA) at 490 nm. Positive clones were subcloned at least twice by limiting dilution, and 18 MoaCL clones were obtained.

#### Determination of isotypes of MoaCL

The isotypes of MoaCL were determined using Mouse Mono Ab-ID EIA Kits (Zymed Laboratories, San Francisco, CA).

#### Reactivities of MoaCL to TM, EGF-5 peptide, and IgG-Fc

Reactivities of MoaCL with TM, EGF-5 peptide, and IgG-Fc were determined using the ELISA procedures described above, except for immobilization of Ags, which was performed by incubation of each Ag (50  $\mu$ l/well) in wells of a microtiter plates for 1 h at 37°C.

#### Inhibition of reactivity with TM by thrombin

To determine whether MoaCL react with the thrombin binding site on TM, an inhibition assay was performed. After the TM-coated plate had been incubated for 1 h with 1% BSA, culture supernatants of MoaCL or polyclonal Abs against rabbit TM (1  $\mu$ g/ml) were added in the presence of various concentrations of bovine thrombin. As a negative control, BSA was added instead of thrombin. After washing the wells with PBS/Tween-20, MoaCL or polyclonal Abs against rabbit TM (1  $\mu$ g/ml) were added. The percentage of TM binding activity was determined using the following equation: % activity = (OD with thrombin or BSA/OD without thrombin or BSA)  $\times$  100.

#### Effects of $\beta_2$ GPI on binding of MoaCL to CL

In experiments to examine the effects of  $\beta_2$ GPI on binding of MoaCL to CL, the reactivity of protein G-Sepharose column-purified MoaCL (500 ng/ml) to CL was determined in the presence or the absence of  $\beta_2$ GPI in PBS, using the ELISA procedure described above.

#### Competitive inhibition of TM binding activity by TM, EGF-5 peptide, CL, and $\beta_2$ GPI

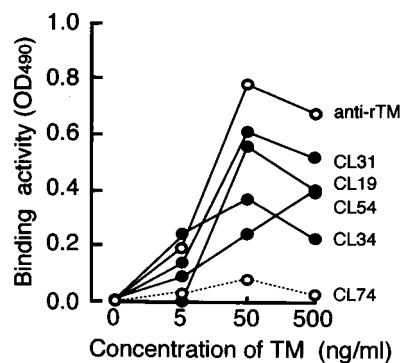
TM binding activities of MoaCL were determined by ELISA in the presence or the absence of several concentrations of TM, EGF-5 peptide, CL,  $\beta_2$ GPI, or BSA as competitive inhibitors. In this experiment, CL was solubilized in PBS by sonication. The percentage of TM binding activity was calculated as follows: % activity = (OD with inhibitor/OD without inhibitor)  $\times$  100.

#### Measurement of affinity

The affinities of MoaCL for CL, TM, EGF-5 peptide, and IgG-Fc were determined according to the method of Friguet et al. (18). A constant amount (1  $\mu$ g/ml) of each MoaCL suspended in PBS/Tween-20 in 5-ml tubes was incubated with serial dilutions of CL, TM, EGF-5 peptide, or IgG-Fc for 2 h at 37°C. CL was sonicated for preincubation. These samples were then transferred to ELISA wells that had been precoated with each corresponding Ag, CL at 10  $\mu$ g/ml, TM at 50 ng/ml, EGF-5 peptide at 5  $\mu$ g/ml, or IgG-Fc at 50 ng/ml. The dissociation constant ( $K_d$ ) of each MoaCL was calculated from the formula reported by Friguet et al. (18):  $A_0/(A_0 - A) = 1 + K_d/a_0$ , where  $A_0$  is the OD measured in the absence of Ag,  $A$  is the OD measured in the presence of Ag, and  $a_0$  is the concentration of Ag. When  $A_0/(A_0 - A)$  and  $1/a_0$  are plotted against one another, the slope yields  $K_d$  for binding of the MoaCL to the Ag (Klotz plots). The affinity is expressed as  $1/K_d$ .

#### Flow cytometry

The reactivity of MoaCL to HUVEC was determined using flow cytometry, as previously described (19). HUVEC (Clonetics Co., San Diego, CA) was used between the third to ninth passages. Cells in the flask were removed using a cell scraper and washed with medium 199 containing 5% FCS (washing solution), then incubated with MoaCL for 30 min at 4°C. After washing with washing solution, cells were stained with phycoerythrin-con-



**FIGURE 1.** Binding of MoaCL to TM. Of 18 MoaCL, CL 19, 31, 34, and 54 reacted with rabbit TM. Binding to TM was not observed with CL 74. anti-rTM, polyclonal goat Abs against rabbit TM (1  $\mu$ g/ml).

jugated goat anti-mouse IgG for 30 min at 4°C and analyzed by flow cytometry using Coulter EPICS Elite (Hiאה, FL). Mouse IgG2a (myeloma protein UPC10, 1  $\mu$ g/ml) was used as the background Ab control for these studies. To determine the reactivity of MoaCL to TM on the HUVEC, MoaCL was incubated with HUVEC preincubated with excess amounts of polyclonal Abs against human TM (100  $\mu$ g/ml) for 30 min at 4°C, and the reactivity was examined by flow cytometry, as described above.

#### Cell-ELISA for detection of TM on cell surface and in cytoplasm of HUVEC

To determine whether binding of MoaCL to TM modulates the level of expression of TM on HUVEC, the amount of TM on the cell surface was measured by cell-ELISA, as previously described (19). Briefly, a confluent culture of HUVEC in the gelatin-coated 96-well plate (Iwaki Glass, Tokyo, Japan) was washed with PBS and incubated with MoaCL for 30 min at 37°C. After washing with PBS, HUVEC were fixed by adding 0.2% glutaraldehyde in PBS at room temperature for 20 min, followed by incubation with 1% BSA for 1 h at 37°C. HUVEC was then incubated with polyclonal Abs against human TM (1  $\mu$ g/ml) for 1 h at 37°C, washed, and incubated with peroxidase-conjugated anti-goat IgG. To determine the total amounts of TM (on cell surface and in cytoplasm), the same procedure was performed, except for treatment of HUVEC with 0.1% Triton X-100 in PBS for 2 min after fixation.

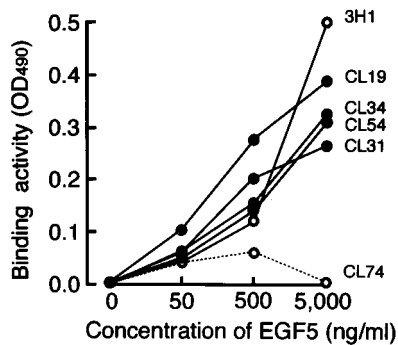
## Results

#### Cross-reactivities of MoaCL with TM, EGF-5 peptide, and IgG-Fc

Four of 18 MoaCL (CL 19, 31, 34, and 54) reacted with TM and also the EGF-5 peptide, a synthetic peptide that includes the thrombin binding site of TM. The maximum reactions of these MoaCL (with the exception of CL 54) and polyclonal Abs against TM were observed when TM at 50 ng/ml was used to coat the microtiter plates. No changes in OD were observed with the other 14 MoaCL, including CL 74, with changes in the concentration of TM (Fig. 1). The reaction of each MoaCL with the EGF-5 peptide depended on concentration of the peptide (Fig. 2). Two of these four MoaCL (CL 31 and 34) reacted to IgG-Fc. The reactivities of these MoaCL with CL, TM, EGF-5 peptide, and IgG-Fc are summarized in Table I. The isotype of all MoaCL reacted with TM was IgG2a.

#### Inhibition of reactivity of MoaCL with TM by thrombin

The reactivity of one MoaCL (CL 19) with TM decreased when increasing concentrations of thrombin were used for inhibitor (Fig. 3), even though the reaction with TM of polyclonal Abs against rabbit TM, which reacted with various epitopes of TM, was not inhibited by thrombin. The inhibition of reactivity with TM by thrombin was also observed with CL 31, 34, and 54. However, the BSA pretreatment did not affect the reactivity of MoaCL with TM



**FIGURE 2.** Binding of MoaCL with EGF-5 peptide. CL 19, 31, 34, and 54 reacted with EGF-5 peptide. No reaction was observed with CL 74. 3H1, mAb against human TM, which reacts with the fifth EGF-like domain of human TM, (1  $\mu$ g/ml).

Table I. Binding activity of MoaCL to CL, TM, EGF-5 peptide, and IgG-Fc

MoaCL	CL	TM	EGF-5 Peptide	IgG-Fc
CL 19	1.129	0.550	0.381	0.022
CL 31	1.471	0.615	0.257	0.472
CL 34	1.192	0.361	0.332	0.496
CL 54	1.144	0.244	0.302	0.000
CL 5	1.189	0.019	0.037	0.060
CL 7	0.450	0.037	0.036	0.065
CL 16	0.417	0.061	0.075	0.025
CL 24	0.955	0.087	0.047	0.055
CL 25	1.163	0.062	0.007	0.025
CL 27	1.144	0.073	0.000	0.028
CL 49	1.187	0.054	0.000	0.013
CL 59	1.144	0.000	0.000	0.003
CL 74	0.801	0.081	0.061	0.000
CL 90	1.175	0.067	0.069	0.000
CL 100	1.169	0.074	0.023	0.058
CL 102	0.782	0.032	0.048	0.052
CL 104	1.074	0.083	0.023	0.038
CL 154	1.109	0.021	0.047	0.023
Anti-rTM	0.000	0.771	0.000	0.000
3H1	0.000	0.000	0.487	0.000

The reactivity was determined by ELISA and expressed in OD<sub>490</sub>. ELISA plate was coated with CL (10  $\mu$ g/ml), TM (50 ng/ml), EGF 5 peptide (5  $\mu$ g/ml), and IgG-Fc (50 ng/ml). Anti-rTM, polyclonal goat antibodies against rabbit TM (1  $\mu$ g/ml); 3H1: mAb against human TM that reacts with the fifth EGF-like domain of human TM.

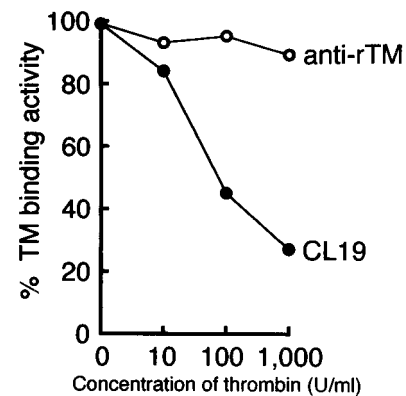
(Table II). These findings suggest that these four MoaCL reacted with thrombin binding sites of TM.

#### Effects of $\beta_2$ GPI on binding of MoaCL to CL

The reactivities of purified MoaCL (CL 19, 31, 34, and 54) with CL decreased in the presence of a high dose (30  $\mu$ g/ml) of  $\beta_2$ GPI compared with reactivities in the absence of  $\beta_2$ GPI. The reactivity of CL 74, which showed no binding activity to TM (Table I), also decreased; however, that of CL 104 increased in the presence of  $\beta_2$ GPI. The serum binding activity with CL in 8-mo-old NZB/W F<sub>1</sub> mice decreased, while that in 4-mo-old MRL/Mp-*lpr/lpr* (MRL/l) mice increased in the presence of  $\beta_2$ GPI (Fig. 4).

#### Competitive inhibition of reactivity of MoaCL with TM

The reactivity of CL 19 with TM decreased when increasing concentrations of TM and EGF-5 peptide, but not of  $\beta_2$ GPI or BSA, were added as competitors. The apparent TM binding activity of CL 19 increased in the presence of high concentrations of CL (Fig. 5A). This may be due to the binding of CL to TM in the ELISA

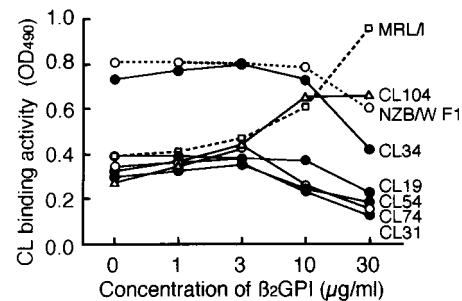


**FIGURE 3.** Inhibition of the binding of CL 19 to TM by thrombin. Binding of CL 19 or anti-rTM (polyclonal goat Abs against rabbit TM; 1  $\mu$ g/ml) to a TM-coated plate in the presence of the indicated concentration of bovine thrombin was measured, and the percent binding activity was determined.

Table II. Inhibition of binding activity of MoaCL to TM by thrombin

MoaCL	% Binding Activity	
	Thrombin	BSA
CL 19	54.1	90.3
CL 31	51.8	95.7
CL 34	48.3	97.5
CL 54	57.6	96.6
Anti-rTM	94.1	100.0

Binding activity of MoaCL or anti-rTM to TM-coated plate was measured in the presence or absence of 100  $\mu$ g/ml of bovine thrombin (100 U/ml) or BSA, and % binding activity was determined as described in *Materials and Methods*. Anti-rTM; polyclonal goat antibodies against rabbit TM (1  $\mu$ g/ml).

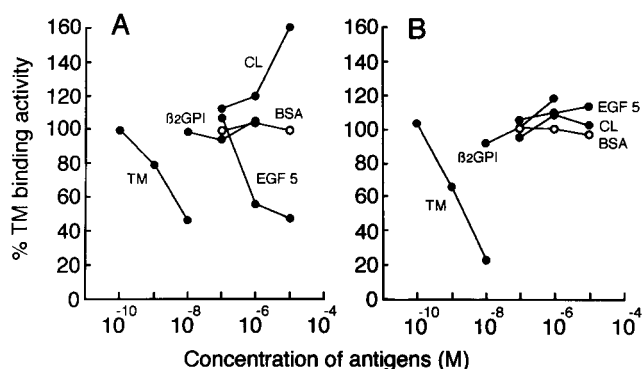


**FIGURE 4.** Effects of  $\beta_2$ GPI on reactivity of MoaCL with CL. Binding to CL was determined in the presence of the indicated concentration of  $\beta_2$ GPI. MoaCL was purified using a protein G-Sepharose column and used at a concentration of 500 ng/ml. Pooled sera from 4-mo-old MRL/l or 8-mo-old NZB/W F<sub>1</sub> mice was used at a 1/3200 dilution.

system because of the avid binding capacity of TM to phospholipids such as CL; thus, CL 19 bound both TM and CL. In contrast, the reactivity of polyclonal Abs against rabbit TM was inhibited only by TM (Fig. 5B). The same results were observed with CL 31, 34, and 54 (Table III).

#### Comparison of affinities of each MoaCL for CL, TM, EGF-5 peptide, and IgG-Fc

The  $K_d$  values of each MoaCL for binding to CL, TM, EGF-5 peptide, and IgG-Fc were determined. As was typical of other MoaCL, the reactivity of CL 31 to each Ag gradually decreased when CL 31 was preincubated with increasing concentrations of

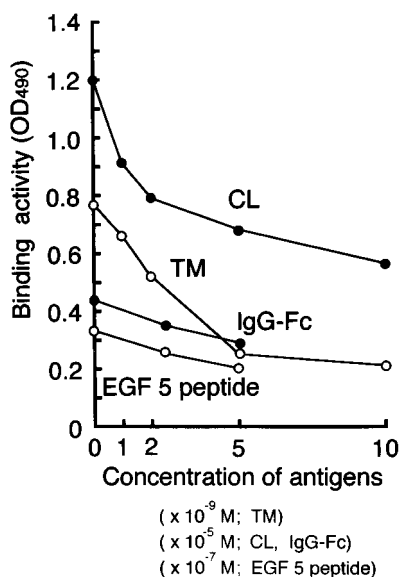


**FIGURE 5.** Competitive inhibition of TM binding with various inhibitors. Binding to TM of CL 19 (A) or anti-rTM (B; polyclonal goat Abs against rabbit TM; 1  $\mu$ g/ml) was determined in the presence of TM, EGF-5 peptide,  $\beta_2$ GPI, CL, or BSA, and the percent binding activity was determined.

Table III. Competitive inhibition of binding of MoaCL to TM by various Ags<sup>a</sup>

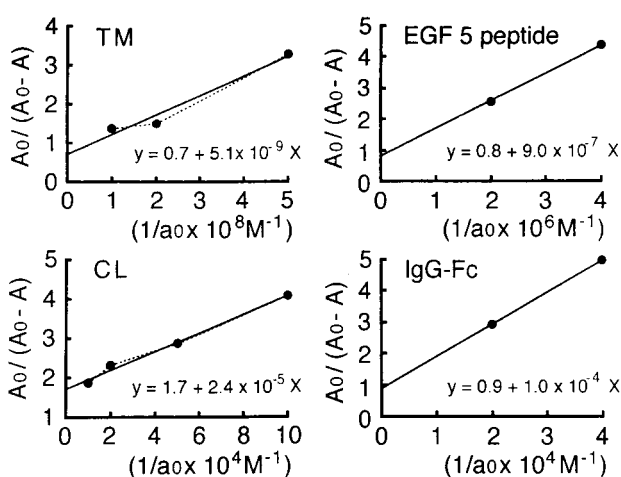
MoaCL	% TM Binding Activity				
	TM	EGF-5 Peptide	CL	$\beta_2$ GPI	BSA
CL 19	42.5	45.9	161.2	106.5	99.6
CL 31	41.9	45.5	208.6	99.7	96.6
CL 34	60.3	17.4	222.0	77.1	105.9
CL 54	34.6	42.2	248.5	105.0	105.3
Anti-rTM	23.9	106.1	100.9	109.2	97.7

<sup>a</sup> Competitive inhibition of binding of MoaCL with TM by TM ( $10^{-8}$  M), EGF-5 peptide ( $10^{-5}$  M), CL ( $10^{-5}$  M),  $\beta_2$ GPI ( $10^{-6}$  M), or BSA ( $10^{-5}$  M). Anti-rTM, polyclonal goat Abs against rabbit TM (1  $\mu$ g/ml).



**FIGURE 6.** Binding activity of CL 31 preincubated with CL, TM, EGF-5 peptide, or IgG-Fc to each CL-, TM-, EGF-5 peptide-, or IgG-Fc-coated plate. Based on these data, the  $K_d$  values were calculated as shown in Figure 7. See details in *Materials and Methods*.

each Ag. We obtained a competitive inhibition curve for CL 31 with each CL, TM, EGF-5 peptide, and IgG-Fc (Fig. 6). Linear plots (Klotz plots) for determination of the  $K_d$  for the binding of CL 31 to each Ag are shown in Figure 7. Table IV shows a sum-



**FIGURE 7.** Klotz plots for the determination of  $K_d$  values for binding of CL 31 to each Ag. See details in *Materials and Methods*.

Table IV. The  $K_d$  values for binding of MoaCL to CL, TM, EGF-5 peptide, and IgG-Fc<sup>a</sup>

MoaCL	$K_d$ Values (M)			
	CL	TM	EGF-5 Peptide	IgG-Fc
CL 19	$7.4 \times 10^{-5}$	$1.7 \times 10^{-8}$	$6.3 \times 10^{-6}$	NR
CL 31	$2.4 \times 10^{-5}$	$5.1 \times 10^{-9}$	$9.0 \times 10^{-7}$	$1.0 \times 10^{-4}$
CL 34	$8.3 \times 10^{-6}$	$4.8 \times 10^{-9}$	$2.5 \times 10^{-6}$	$5.5 \times 10^{-5}$
CL 54	$3.9 \times 10^{-5}$	$4.7 \times 10^{-8}$	$1.6 \times 10^{-6}$	NR
CL 74	$7.4 \times 10^{-5}$	NR	NR	NR
Anti-rTM	NR	$2.2 \times 10^{-10}$	NR	NR
3H1	NR	NR	$3.3 \times 10^{-7}$	NR

<sup>a</sup> NR, no reactivity. Anti-rTM, polyclonal goat Abs against rabbit TM. 3H1, mAb against human TM that reacted with the fifth EGF-like domain of human TM.

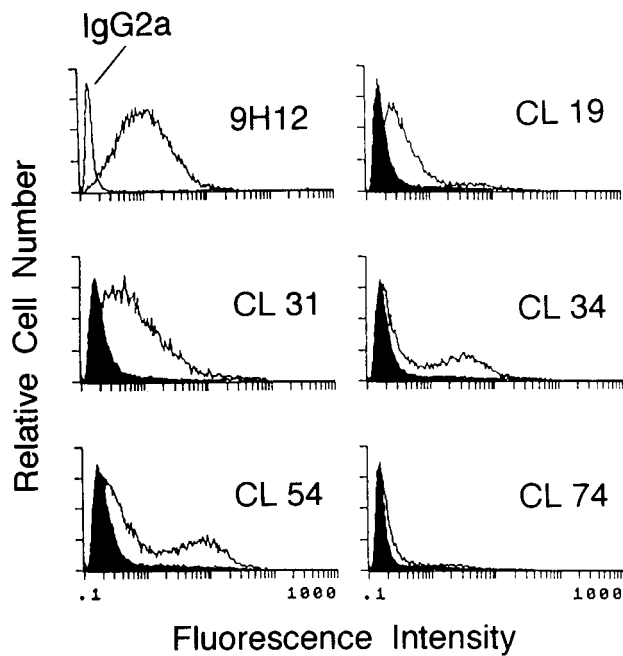
mary of  $K_d$  values for each MoaCL. The  $K_d$  value revealed high affinities to TM ranging from  $4.8 \times 10^{-9}$  M (CL 34) to  $4.7 \times 10^{-8}$  M (CL 54). In case of polyclonal anti-TM, the  $K_d$  value was  $2.2 \times 10^{-10}$  M. Affinities to EGF-5 peptide were lower than those to TM, and the  $K_d$  values ranged from  $9.0 \times 10^{-7}$  M (CL 31) to  $6.3 \times 10^{-6}$  M (CL 19). These MoaCL had low affinities to CL ( $K_d$  values ranging from  $8.3 \times 10^{-6}$  M (CL34) to  $7.4 \times 10^{-5}$  M (CL 19)) and to IgG-Fc ( $5.5 \times 10^{-5}$  M for CL 34 and  $1.0 \times 10^{-4}$  M for CL 31).

#### Modulation of cell surface expression of TM on HUVEC by binding of MoaCL

Figure 8 shows the reactivity of each MoaCL with HUVEC, examined using flow cytometry. CL 19, 31, 34, and 54, but not CL 74, reacted with HUVEC, and these activities were completely inhibited by pretreatment of HUVEC with polyclonal Abs against human TM, thus indicating that these MoaCL bound cell surface TM on HUVEC. When HUVEC were preincubated with these MoaCL, the expression level of TM on the cell surface, detected using polyclonal Abs against human TM, decreased by 14~36% (Fig. 9). In contrast, there was no change in the total contents of TM on the cell surface and in the cytoplasm of HUVEC (data not shown). These results indicate that TM on the cell surface was internalized as a result of MoaCL binding.

## Discussion

We obtained evidence that our murine MoaCL reacted with rabbit TM and that these MoaCL were directed to the thrombin binding

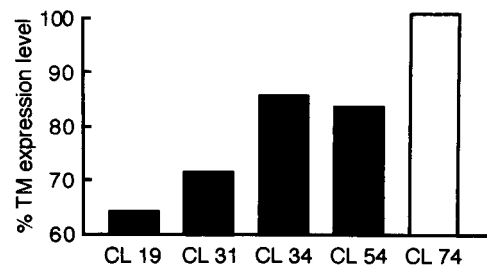


**FIGURE 8.** Flow cytometric analysis of reactivities of MoaCL with HUVEC. CL 19, 31, 34, and 54, but not CL 74, reacted with HUVEC. These reactions were completely inhibited by preincubation of HUVEC with polyclonal Abs against human TM (100  $\mu$ g/ml; solid histograms). 9H12, mAb against human TM which reacts with the third EGF-like domain of human TM; IgG2a, mouse myeloma protein UPC10.

site on TM, since the MoaCL reacted with the synthetic form of the EGF-5 peptide. Furthermore, the reaction with TM was inhibited by bovine thrombin. Reactions of MoaCL with TM and the EGF-5 peptide were true Ag-antibody interactions, since they were competitively inhibited by each respective Ag. Moreover, flow cytometric analysis revealed that these MoaCL reacted to TM expressed on the surface of HUVEC.

There is controversy as to whether autoantibody against TM is present in patients with SLE or APS or in the lupus-prone mouse. Gibson et al. (20) found no autoantibody to TM in patients with SLE. MoaCL from the MRL/l mouse, which has been reported to show polyreactivity, did not react with recombinant human TM (15). However, Ruiz-Arguelles et al. (8) reported that the reactivity of aCL with CL in sera from patients with primary APS was eliminated by preincubation with TM. Furthermore, Oosting et al. (21) reported that autoantibodies against TM were transiently present in the sera from two aPL-positive patients with SLE and in four aPL-negative patients with histories of thrombosis. IgG fractions of these sera inhibited the activation of protein C and bound to a recombinant version of the EGF-like portion of TM. These reports are in agreement with our present findings.

As our MoaCL were polyreactive, we examined the affinity of MoaCL for CL, TM, EGF-5 peptide, and IgG-Fc by calculating  $K_d$  values.  $K_d$  values of four MoaCL to TM ranged from  $4.8 \times 10^{-9}$  to  $4.7 \times 10^{-8}$  M, and those to EGF-5 peptide ranged from  $9.0 \times 10^{-7}$  to  $6.3 \times 10^{-6}$  M. In contrast,  $K_d$  values to CL ranged from  $8.3 \times 10^{-6}$  to  $7.4 \times 10^{-5}$  M, and those to IgG-Fc ranged  $1.0 \times 10^{-4}$  for CL 31 and  $5.5 \times 10^{-5}$  M for CL 34. The  $K_d$  values to IgG-Fc were almost of the same magnitude as those of the polyreactive Abs reported in the literature (22, 23). These results suggest that our MoaCL bound more strongly to TM than to CL. As for the affinity of thrombin for TM, the  $K_d$  value was reported to be  $5 \times 10^{-10}$  M (24), thereby suggesting a higher affinity for TM



**FIGURE 9.** Effects of MoaCL on the expression of TM on HUVEC. The TM expression level on the cell surface of HUVEC decreased when HUVEC were preincubated with CL 19, 31, 34, and 54, but not with CL 74. The percentage of TM expression was calculated compared with the level after preincubation with culture medium alone.

than for our MoaCL. This high affinity was shown to be due to the secondary binding site of thrombin, glycosaminoglycan linked to the serine/threonine-rich domain (residues 463–497) on TM (25). Thus, it is not known whether the affinity of thrombin for the thrombin binding site of TM is indeed higher than that of our MoaCL. In fact, thrombin inhibited the binding of MoaCL to TM only when large amounts of thrombin were used (Fig. 3). In our preceding studies, we found that anti-DNA autoantibodies from NZB/W F<sub>1</sub> mice showed an age-associated increase in the number of somatic mutations in IgV region genes, and that these somatic hypermutations correlated well with affinity maturation of anti-DNA Abs (17, 26). Based on these observations, we propose that an age-associated accumulation of somatic hypermutation in the IgV region genes may lead to the formation of highly pathogenic autoantibodies in NZB/W F<sub>1</sub> mice. In this study we obtained MoaCL only from a single 6-mo-old NZB/W F<sub>1</sub> mouse. MoaCL with a higher affinity for TM may be obtained from older mice. Studies are ongoing to clarify relationships between an age-associated somatic hypermutation in IgV region genes and the affinity maturation of MoaCL to TM.

Kumada et al. (27) found that i.v. injection of polyclonal Abs against rat TM accelerated the thrombin-induced thromboembolism in mice. Moreover, it was demonstrated that the levels of expression of TM on endothelial cells were decreased by internalization of the TM-thrombin complex in the presence of anti-TM Abs, resulting in a decrease in the activation of protein C (28). In the present study, addition of MoaCL down-regulated the expression level of TM on the cell surface of HUVEC due to internalization of the TM-MoaCL complex. Thus, it is suggested that our MoaCL in the circulation directly down-regulate the expression of TM on endothelial cells, thereby preserving the procoagulatory action of thrombin.

The mechanism of pathogenicity of aCL is obscure. Vismara et al. (29) demonstrated that affinity-purified aCL from sera of patients with SLE reacted with endothelial cells and suggested that these Abs were responsible for the induction of thrombosis via binding to phospholipids on these cells. Matsuura et al. (6) reported that the reactivity with CL of aCL from patients with APS was enhanced in the presence of  $\beta_2$ GPI. It was also shown that the presence of  $\beta_2$ GPI in the aCL assay was necessary for quantitation of thrombosis-related aCL in sera of patients with SLE or APS (5), but not in the case of thrombosis-unrelated disease, such as syphilis (6). Moreover, aCL from patients with SLE or lupus-prone mice are directed against  $\beta_2$ GPI, but not CL, as demonstrated only when gamma-ray-irradiated microplates are used to coat  $\beta_2$ GPI (7).  $\beta_2$ GPI has been shown to inhibit the intrinsic blood coagulation pathway, the ADP-mediated aggregation of platelets, and the

prothrombinase activity of activated platelets (30). Thus, we speculate that it is the functionally impaired  $\beta_2$ GPI induced by binding of aCL to  $\beta_2$ GPI that induces thrombosis. However, there is a report that these anticoagulatory activities of  $\beta_2$ GPI were enhanced in the presence of aCL (31).

$\beta_2$ GPI-dependent aCL do not always relate to pathologic activities (32, 33). In the present studies, binding activities to CL of our four MoaCL with reactivity to TM did not increase by adding  $\beta_2$ GPI. It is possible that there might be several mechanisms by which aCL induce thrombosis because aCL are, in general, heterogeneous (34). Our present studies suggest that the cross-reactivity to TM of certain aCL is one of the mechanisms of aCL-induced thromboembolism. Further studies on the biologic activity of aCL in relation to reactivity to TM will lead to a better understanding of the pathogenicity of aCL.

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