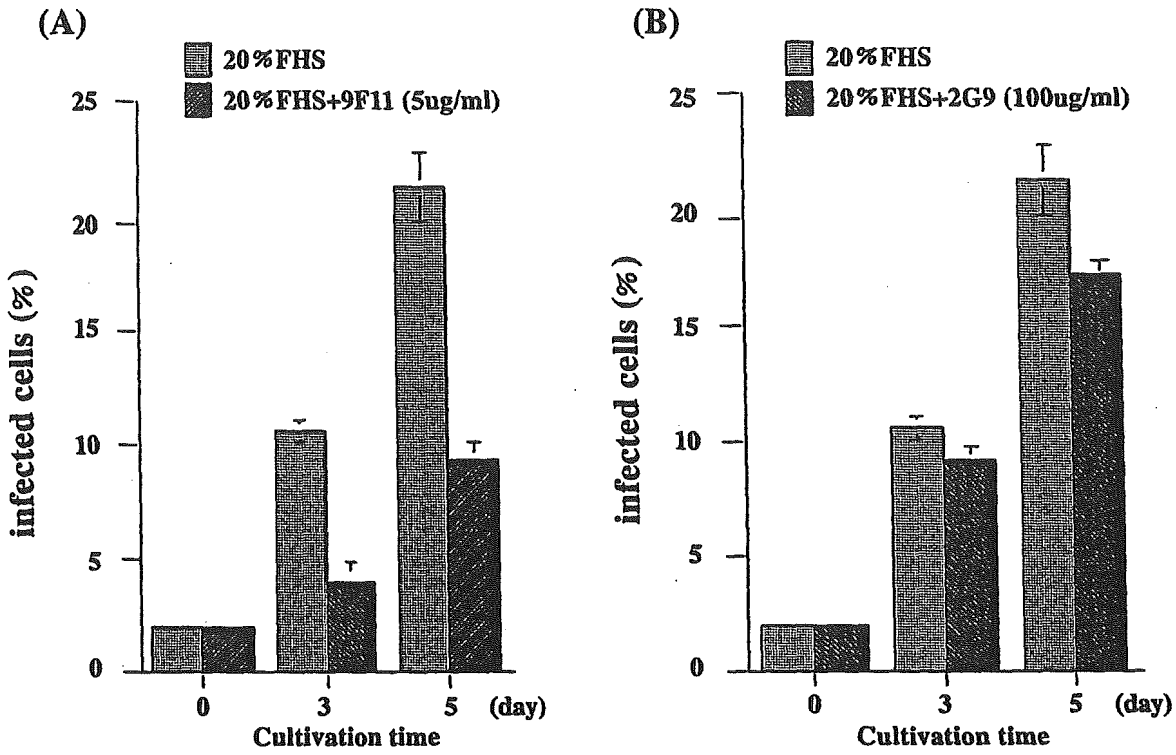

Fig. 6. Effect of (A) 5 μg/ml 9F11 and (B) 100 μg/ml 2G9 on expansion of infection in a mixed culture of MOLT4/IIIB and naive MOLT4 cells at a ratio of 1:50 in the presence of 20% FHS. The percentage (%) of infected cells was determined on days 0, 3 and 5 of cultivation.

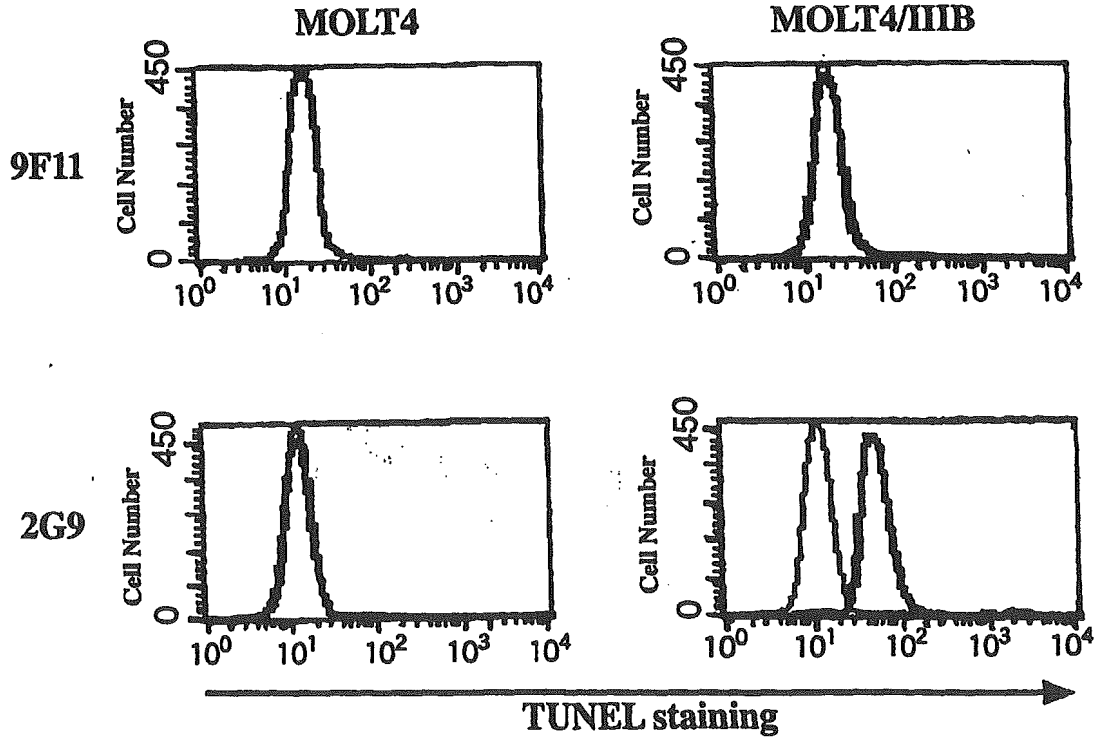

Fig. 7. Detection of 2G9-induced apoptosis with the TUNEL method. MOLT4 cells and MOLT4/IIIB cells were cultivated for 3 days in the presence of 50 μg/ml 9F11 or 2G9.

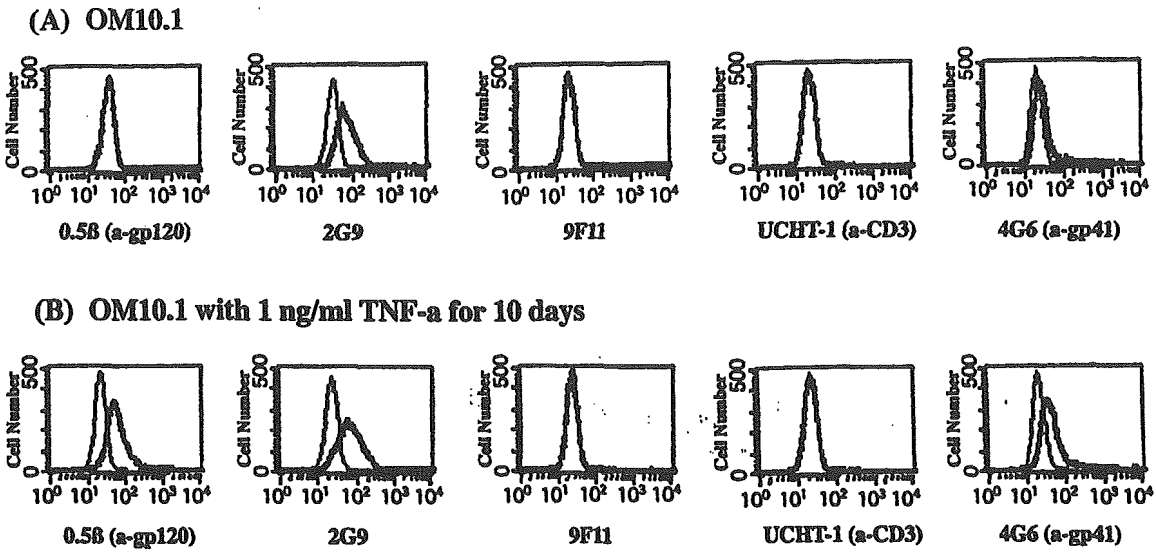

Fig. 8. Reactivity of (A) OM10.1 cells and (B) TNF-α-stimulated OM10.1 cells with various Abs as determined by flow cytometric analysis. 0.5β, UCHT-1 and 4G6 are mAbs to gp120, CD3 and gp41, respectively.

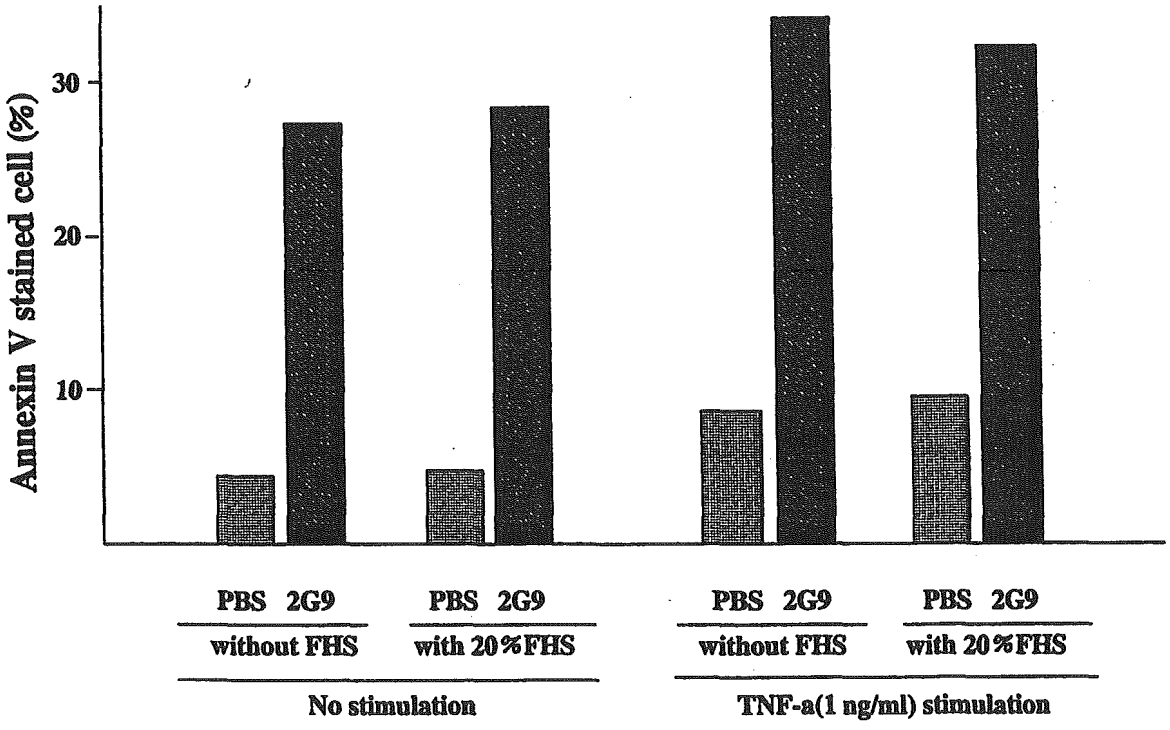

Fig. 9. Effect of 2G9 on OM10.1 cells and TNF-α-stimulated OM10.1 cells. Annexin V staining following incubation with PBS or 2G9 in the presence or absence of 20% FHS.

Nef. Recently, it has been reported that in preparation of the natural IgM fraction, antibodies reactive with Fas were shown to have the capacity to induce apoptosis of lymphoblastoid cells (19). On the other hand, 2G9 reacted with HIV-1-infected cells, in which Fas expression is downregulated indicating that it does not react with Fas. Although caspase inhibitor restricted the induction of apoptosis of HIV-1-infected MOLT4 cells by 2G9 (data not shown), the intracellular reaction cascade for apoptosis remains to be clarified.

This result indicates that 2G9 may be able to eliminate provirus-positive cells which cannot be eliminated by combined chemotherapy with RT inhibitors and proteinase inhibitors, which is the basis of the so-called

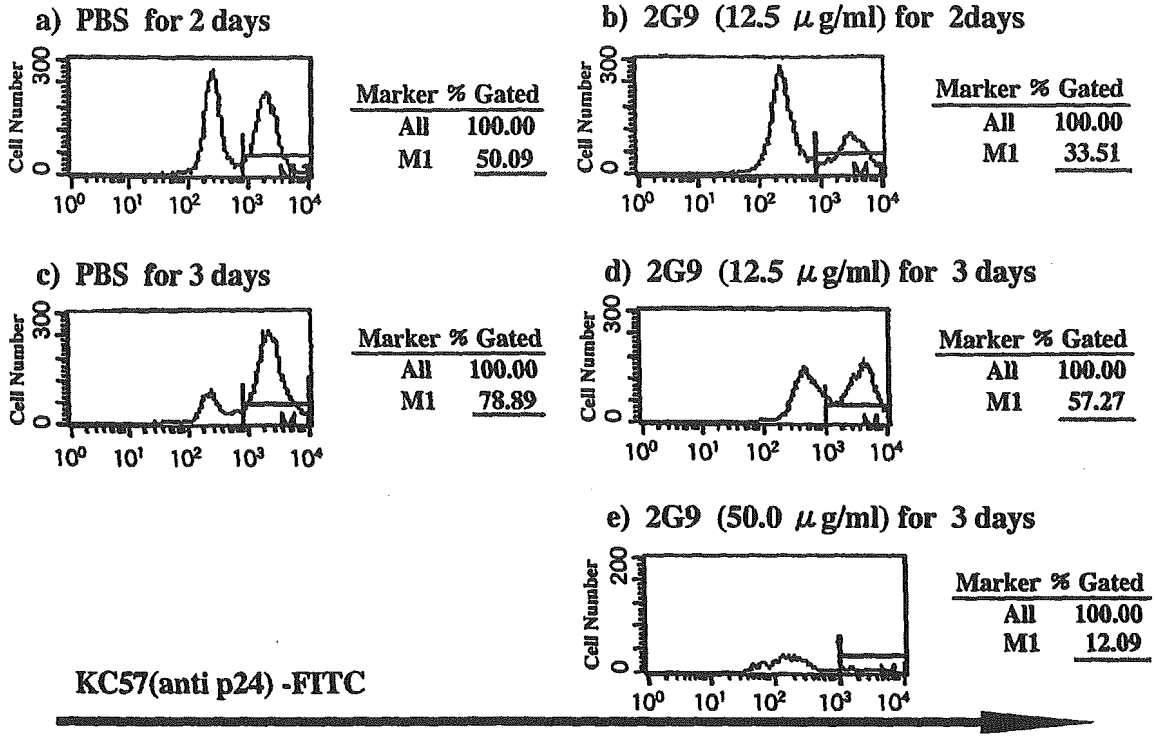

Fig. 10. Effect of 2G9 on the increase in HIV-p24-positive cells among OM10.1 cells stimulated with TNF-α. Without 2G9, p24-positive cells rose to 50.1% in 2 days (a) and 78.9% in 3 days (c). In the presence of 12.5 μg/ml 2G9, they were suppressed to 33.5% (b) and 57.2% (d), respectively. In the presence of 50 μg/ml 2G9, p24-positive cells were suppressed to 12.1% 3 days after the TNF-α stimulation. KC57 was used to detect p24 following fixation of cells with paraformaldehyde.

highly active antiretroviral therapy (HAART) (16).

Although the HIV-1 level in a patient's plasma becomes undetectable by RT-PCR following HAART, provirus DNA-positive cells remain in the individual's lymphocyte population (2, 3). Therefore, removal of latently infected dormant cells is a serious goal. We used OM10.1 as a model of cells latently infected with HIV-1. Since 2G9-Ag was expressed on OM10.1 cells cultivated in the presence of AZT which restricts expression of HIV-1-related Ags such as gp120, gp41 and Nef, 2G9-Ag may be a good target Ag for detection and/or immunological treatment of cells latently or dormantly infected with HIV-1. Therefore, identification of the Ag reactive with 2G9 is an urgent priority, and cloning of its cDNA is currently being carried out in our laboratory.

It is interesting that the latently infected OM10.1 cells were sensitive to cytolysis by IL-2-activated lymphoblasts armed with bifunctional (bispecific) Abs (BFA) against CD3 and gp41 (24). This would suggest that a combination of BFA-armed lymphoblasts and 2G9 may facilitate the elimination of latently HIV-1-infected cells *in vivo* and increase the efficacy of HAART in treating HIV-positive patients.

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Editor-Communicated Paper

Hepatitis Induced by an IgM Monoclonal Antibody against Procarboxypeptidase R

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Abstract: **Procarboxypeptidase R (proCPR), also known as thrombin-activatable fibrinolysis inhibitor (TAFI), is present in plasma and can be activated to carboxypeptidase R (CPR) by trypsin-like enzymes such as thrombin and plasmin. CPR has the carboxypeptidase B-like activity that can inactivate the inflammatory peptides such as C5a by removing the C-terminal arginine and can interfere with fibrinolysis by removing C-terminal lysine residue of fibrin. In the present study, we conducted to produce monoclonal antibodies (mAbs) by using spleen cells from proCPR-deficient mice immunized by partially purified mouse proCPR. The mAbs obtained were IgM isotype and reacted with proCPR and interfered with activation of proCPR to CPR by thrombin-thrombomodulin complex. Some BALB/c mice implanted with the hybridoma died in 7 days, and intravenous injection of the mAb to BALB/c mice induced transient elevation of GOT and GPT in plasma although injection to the deficient mice did not. Furthermore, the histological features showed the focally lesions in liver tissue of BALB/c mice injected with the mAb. Since liver is the major site of proCPR synthesis, IgM mAb to proCPR should have induced local inflammation at the side resulting in induction of hepatitis.**

Key words: **Hepatitis, Procarboxypeptidase R, TAFI, Monoclonal antibody**

Carboxypeptidase R (CPR) (6), also known as plasma carboxypeptidase B (CPB) (10), carboxypeptidase U (CPU) (19, 32) or activated thrombin-activatable fibrinolysis inhibitor (TAFIa) (3) is generated from its precursor, proCPR, by trypsin-like enzymes such as thrombin and plasmin (8, 29). The activation of proCPR by thrombin is enhanced by more than 1,000-fold in the presence of thrombomodulin (4). Furthermore, proCPR is also activated by neutrophil elastase (21). High plasma levels of proCPR may generate excess CPR and interfere with fibrinolysis resulting in thrombotic disorders (2). There are several methods to detect the function of proCPR/TAFI or proCPU (15, 23). We also generated enzyme linked immunosorbent assay (ELISA) to quantitate human proCPR (16, 24, 31). However to establish ELISA for detection of mouse proCPR, antibody to mouse proCPR is desirable.

We have generated proCPR-deficient mice by knocking out exons 4 and 5 of the proCPR gene (1), which are regarded as essential for CPR function. In the present study, we generated mouse monoclonal antibodies (mAbs) specifically reactive with proCPR by using this mutant mouse. The mAbs generated were IgM isotype and inoculation of the hybridoma cells to BALB/c mice induced serious effect resulting in death in 7 days of some portion of the recipient mice. Therefore we analyzed the effect of intravenous injection of the mAb to BALB/c mice as well as proCPR deficient mice.

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Abbreviations: CP, carboxypeptidase; CPB, carboxypeptidase B; CPN, carboxypeptidase N; CPR, carboxypeptidase R; CPU, carboxypeptidase U; DAB, 3,3'-diaminobenzidine-4-HCl; Hip-Arg, hippuryl-L-arginine; HRP, horseradish peroxidase; mAb, monoclonal antibody; proCPR, procarboxypeptidase R; TAFI, thrombin-activatable fibrinolysis inhibitor; TAFIa, activated TAFI; t-PA, tissue-type plasminogen activator; T-TM, thrombin-thrombomodulin complex.

Materials and Methods

Materials. Diethylaminoethyl (DEAE) cellulose was purchased from Whatman (Maidstone, Kent, U.K.). Polyacrylamide gel electrophoresis reagents and equipment were from Bio-Rad (Richmond, Calif., U.S.A.) and were used according to the manufacturer's recommended protocols. Protein concentration was determined by absorbance at 280 nm using a Double-Beam Spectrophotometer (Hitachi Co., Tokyo). Other mentioned agents were purchased from Sigma (St. Louis, Mo., U.S.A.).

Collection of blood samples. For purification of mouse proCPR, blood samples from BALB/c mice were collected into tubes containing sodium citrate dehydrate and kept on ice before treatment. After centrifuging at 3,000 rpm for 15 min at 4 C, the plasma supernatant was transferred to a clear tube and stored at −80 C until use. Rat and human citrate plasma were also prepared for proCPR analysis. For biochemical analysis of mouse plasma, heparinized blood samples were prepared. All animal experiments described in the manuscript were performed under the permissions of Animal Studies Committee of Nagoya City University Graduate School of Medical Sciences.

Measurement of CPR activity. CPR activity was determined with colorimetric assay using hippuryl-L-arginine (Hip-Arg) (Peptide Institute, Inc., Osaka, Japan) as a synthetic substrate as described previously (22). Briefly, 8 μl of diluted samples in 50 mM Tris-HCl (pH 8.0) were mixed with 1 μl of 20 nM thrombin (T) in 5 mM $CaCl_2$ and 1 μl of 5 nM thrombomodulin (TM). After incubated at room temperature for 20 min, 5 μl of 50 mM Hip-Arg in 50 mM HEPES (pH 8.2) as the substrate solution were added and then incubated at room temperature for 1 hr. After incubation, 100 μl of 250 mM phosphate buffer (pH 8.3) was added to the mixtures and placed on ice. Then, 75 μl of 3% cyanuric chloride (Tokyo Kasei Kogyo Co., Tokyo) in 1,4-dioxane (Wako, Tokyo) were added and mixed well. Denatured protein and excess cyanuric chloride were removed by centrifugation. Supernatant was transferred to a 96-well microtiter plate. Absorbance of supernatant was measured at 405 nm, and the values obtained for T-TM activated samples over the values for samples without T-TM were regarded as T-TM generated CPR activity.

Purification of proCPR. Mouse proCPR was purified with the method previously described (16). Mouse plasma was subjected to precipitate at between 45% and 65% saturation of ammonium sulfate and the precipitate was collected by centrifugation. The pellet was dissolved in 10 mM Tris-HCl (pH 7.2, Tris buffer) containing 30 mM NaCl and dialyzed against the same buffer. The equilibrated sample was applied to a DEAE cellulose column (5×80 cm) previously equilibrated with Tris buffer containing 30 mM NaCl. Proteins were eluted with a linear gradient of NaCl from 30 to 300 mM in Tris buffer at 48 ml/hr flow rate. Fractions containing proCPR which eluted at 80–120 mM NaCl were pooled and concentrated. These fractions were applied to a HiLoad 26/60 Superdex 200 pg column (2.6×60 cm) previously equilibrated with phosphate buffered saline (PBS) and eluted at 1 ml/min flow rate. Partially purified proCPR fractions (proCPR preparation) eluted at 60 kDa were collected. The proCPR concentration was derived using $A_{280\,nm}=1.49\ (mg/ml)^{-1}$.

Immunization of proCPR deficient mice with proCPR. The $proCPR^{-/-}$ female mice were immunized by intraperitoneal injection of 50 μg partially purified BALB/c mice proCPR (mouse proCPR preparation) in complete Freund's adjuvant, followed by intraperitoneal injection of 50 μg partially purified proCPR in incomplete Freund's adjuvant once per week for 3 weeks.

Detection of antibodies to proCPR. Antibodies to proCPR were detected by enzyme linked immunosorbent assay (ELISA) and western blotting. For ELISA, 10 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in PBS was added to 96-well ELISA plate (Sumitomo Bakelite Co., Tokyo) and incubated at 37 C for 2 hr. After washing with PBS, 100 μl of 50 μg/ml mouse proCPR preparation was added to each well and incubated at 4 C for over night. The plate was washed again and blocked with 200 μl 25% Block Ace (Dainihon Pharm. Co., Osaka, Japan) at 4 C for over night. Wells were washed and 100 μl diluted immunized or unimmunized mice serum from caudal blood was added to the well and incubated at 4 C for 1 hr. After washing with 0.1% (v/v) Tween-PBS, the wells were reacted with horseradish peroxidase (HRP)-linked goat anti-mouse immunoglobulin (Ig) polyclonal antibody (Sigma Antibody, St. Louis, Mo., U.S.A.) for 1 hr. After washing, *O*-phenylenediamine (OPD) solution was added to the plate and incubated at room temperature for 10 min. The absorbance at 492 nm of each well was measured. For western blotting, the partially purified mouse proCPR was run on the 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The immunized and unimmunized mice sera from caudal blood were reacted with the membrane as the primary antibody, and HRP-linked goat anti-mouse Ig polyclonal antibody was used as the second antibody. The band of each sample was developed by using 3,3′-diaminobenzidine-4-HCl (DAB) and $NiCl_2$.

Generation of hybridoma cell lines. The spleen cells

of the antibody positive proCPR$^{-/-}$ mice were fused with P3U1 myeloma cells at a ratio of 2.5:1 in the presence of 50% polyethylene glycol (PEG-1500, Roche Diagnostics GmbH, Mannheim, Germany) according to the standard method (14). The hybridoma cells were cultured in a hypoxanthine, aminopterin, thymidine (HAT; Sigma Chemical Co., St. Louis, Mo., U.S.A.) selection medium and the cells producing proCPR antibody were screened to establish mAb-producing cell lines. Culture supernatants of hybridoma cells were screened by ELISA and western blotting as described above to establish mAb producing cell lines. Immunoglobulin class and subclass of mAbs were determined with a mouse mAb isotyping kit (Amersham, Buckinghamshire, U.K.). From the culture supernatant, mAbs were precipitated between 30% and 60% saturation of ammonium sulfate and fractionated with Mono Q 10/100 G1 column (Amersham Biosciences, Amersham, U.K.). The mAbs were combinated with *N*-hydroxysuccinimidobiotin (Pierce Biotechnology, Rockford, Ill., U.S.A.) according to the manufacturer's recommended protocols and kept in sodium bicarbonate buffer at 4 C until used.

Western blotting analysis. The proCPR preparation (1 mg/ml) was activated by T-TM (20 nM T and 5 nM TM) solution as mentioned above. At the different reaction time (0, 2, 4, 8, 16 min), 10 μg/ml D-Phe-Pro-Arg chloromethylketone (PPACK), the thrombin inhibitor, was added to stop the reaction. The activated proCPR solution was denatured by boiling for 3 min in the sample buffer and cooled on ice before subjecting to the 10% SDS-PAGE and transferring to a nitrocellulose membrane. After reaction with biotinized mAb (2D1), the membrane was incubated with HRP-streptavidin and the band was developed by DAB. Since biotinization of the other mAb (4H5) was not successful due to causing precipitation, HRP-anti mouse polyclonal antibody was used for western blotting analysis with the mAb (4H5).

Inhibition of CP activity by mAbs. Different concentrations (0, 7.5, 15, 30, 60, 120, 240 μg/ml) of mAbs were preincubated with diluted mouse plasma for 5 min. As a control, 60 μg/ml mouse IgM (Sigma-Aldrich, St. Louis, Mo., U.S.A.) was incubated with diluted mouse plasma. ProCPR in the mixture was activated by T-TM. After reacted for 20 min at room temperature, 10 μg/ml PPACK was added to stop the reaction. The total CP activity (include CPN and CPR) was determined with the methods mentioned above. The mixture without T-TM was examined with the same method to determine the CPN activity. The diluted rat and human plasma was incubated with or without the mAbs for 5 min and the activity of total CP and CPN activity was determined.

Histopathology and blood examination. BALB/c and proCPR$^{-/-}$ mice, 8 weeks of age, received intravenous injection of 100 μg of purified proCPR mAb (4H5). The mice injected with sodium chloride were used for control studies. The days 1, 3 and 7 following the injection, blood samples were drawn and the mice were killed under anesthesia with pentobarbital. The livers and kidneys of mice were immediately fixed in 4% phosphate-buffered formalin, then dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Sections were cut at 3 μm thickness and subjected to hematoxylin and eosin (H&E) staining. Livers and kidneys of BALB/c and proCPR$^{-/-}$ mice who were intraperitoneal injected with 10^7 of 4H5 hybridoma cells 7 days before sacrifice were also fixed in the paraformaldehyde solution, and the sections were stained by H&E. GOT and GPT of plasma samples were determined with standard methods.

Statistical analysis. Each experiment was performed in at least triplicate and results are presented as means±SEM for a representative experiment. Differences among experimental groups were assessed by analysis of variance (Scheffe's test). Significance was defined as P value <0.05.

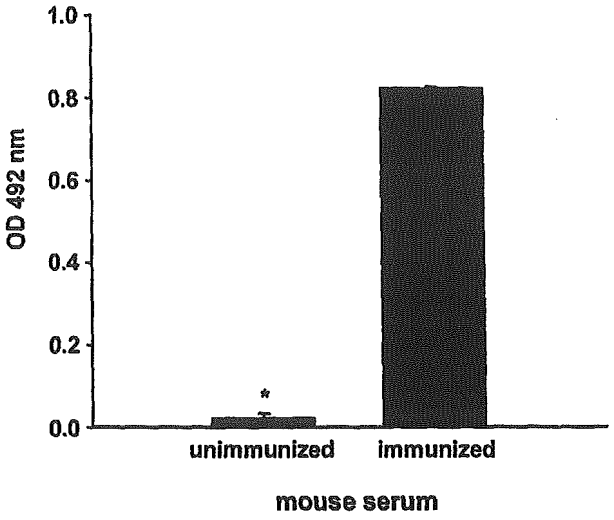
Results

ELISA and Western Blotting of Immunized Mouse Serum

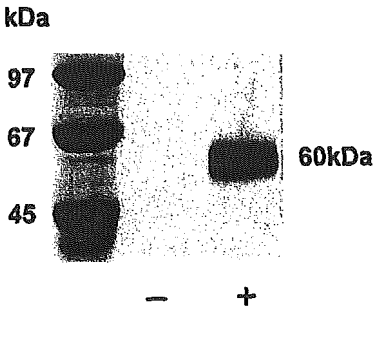
ELISA and western blotting was used to determine the reactivity of immunized mouse serum to proCPR. We coated the wells of 96-well ELISA plate with partially purified mouse proCPR as the antigen, and used this plate to detect anti-proCPR antibodies in immunized proCPR$^{-/-}$ mouse serum. We found that the serum of immunized mouse showed a significantly high absorbance at 492 nm, while that from unimmunized mouse did not react with proCPR (0.823±0.009 vs. 0.024±0.002) (Fig. 1A). In western blotting, the serum from immunized mouse produced a clear band at 60 kDa, while unimmunized mouse serum did not. (Fig. 1B).

Cloning of Hybridoma Producing Anti-proCPR mAbs

Hybridoma cells that produce mAbs to proCPR were cloned monitoring with proCPR coated ELISA plate. The culture supernatants of 6 hybridoma cells had positive reaction with proCPR in ELISA. However, only two mAbs, 2D1 and 4H5, developed a clear band in western blotting (Fig. 2, Table 1). The immunoglobulin classes of these two antibodies were IgM isotype as determined by the isotyping kit.



A



B

Fig. 1. Reactivity of proCPR immunized mouse serum with partially purified proCPR. A: Serum from proCPR immunized proCPR-deficient mouse significantly reacted with partially purified mouse proCPR, while the serum from unimmunized proCPR-deficient mouse did not. *$P<0.05$ vs. immunized. B: Western blotting of immunized (+) or unimmunized (−) mouse serum reacted with partially purified proCPR. Immunized serum showed a clear band at 60 kDa and unimmunized serum did not.

Reactivity of mAbs to proCPR

To determine whether the mAbs specifically react with proCPR, and/or CPR, western blotting was performed on proCPR and T-TM treated proCPR that becomes CPR. As shown in Fig. 2, T-TM treatment of proCPR reduced the signal of the band. After incubation of proCPR with T-TM for 16 min, the band diminished. No signal was observed at any lower molecular size position. The results indicate that the mAbs we generated specifically reacted with proCPR, but not

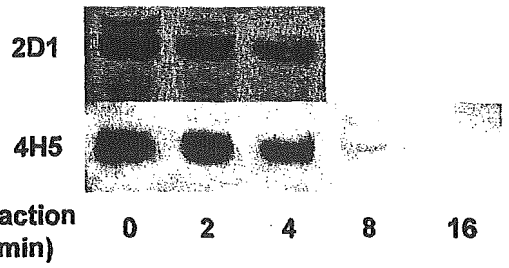


Fig. 2. The reactivity of purified mAbs to proCPR. mAbs 2D1 and 4H5 were subjected to react with partially purified proCPR incubated with T-TM for indicated periods. Both experiments were executed for at least three times to confirm repeatability.

Table 1. Subclasses analysis with ELISA and western blotting analysis of mAbs

mAbs to proCPR	Second Ab to	OD_{492}[a)]	Western blotting
2D1	IgM	1.62±0.12	60 kDa
2D6	IgM	1.12±0.06	No band
2D8	IgM	0.79±0.07	No band
2F7	IgM	1.15±0.03	No band
4H5	IgM	1.92±0.14	60 kDa
5B5	IgM	0.95±0.11	No band

[a)] Essentially no reactivity was observed with second anti bodies to IgG, IgA or IgE.

CPR.

Inhibition of mAbs on CP Activities

CP activity in plasma was determined by the method mentioned above. Although a portion of proCPR is activated to CPR during coagulation, the addition of T-TM and Ca^{2+} can activate all the remaining proCPR. As shown in Fig. 3, both the mAbs 2D1 (A) and 4H5 (B) significantly inhibited T-TM induced proCPR activation in mouse plasma. 2D1 at 7.5 μg/ml significantly inhibited T-TM induced proCPR activation to 62.23±0.11% from 76.87±0.47% at PBS control ($P<0.001$). 4H5 also inhibited T-TM induced proCPR activation at 15 μg/ml to 48.32±0.26% from 74.58±0.27% at PBS control ($P<0.001$). Both 2D1 and 4H5 had no effect on CPN activity in plasma as shown with dark area in each column in Fig. 3, A and B. Furthermore, mAbs reacted with rat and human plasma and inhibited the T-TM induced proCPR activation (Fig. 3, C and D). In this case, CPN activity (activity in untreated plasma) was subtracted from CP activity in T-TM treated plasma. Therefore, the resulting CP activity of PBS control was regarded as 100% activity of T-TM activated proCPR. In rat plasma, mAb 2D1 inhibited T-TM induced proCPR activation at 15 μg/ml to 61.08±0.65% ($P<0.001$), while 4H5 inhibited T-TM induced proCPR activation at 7.5 μg/ml to

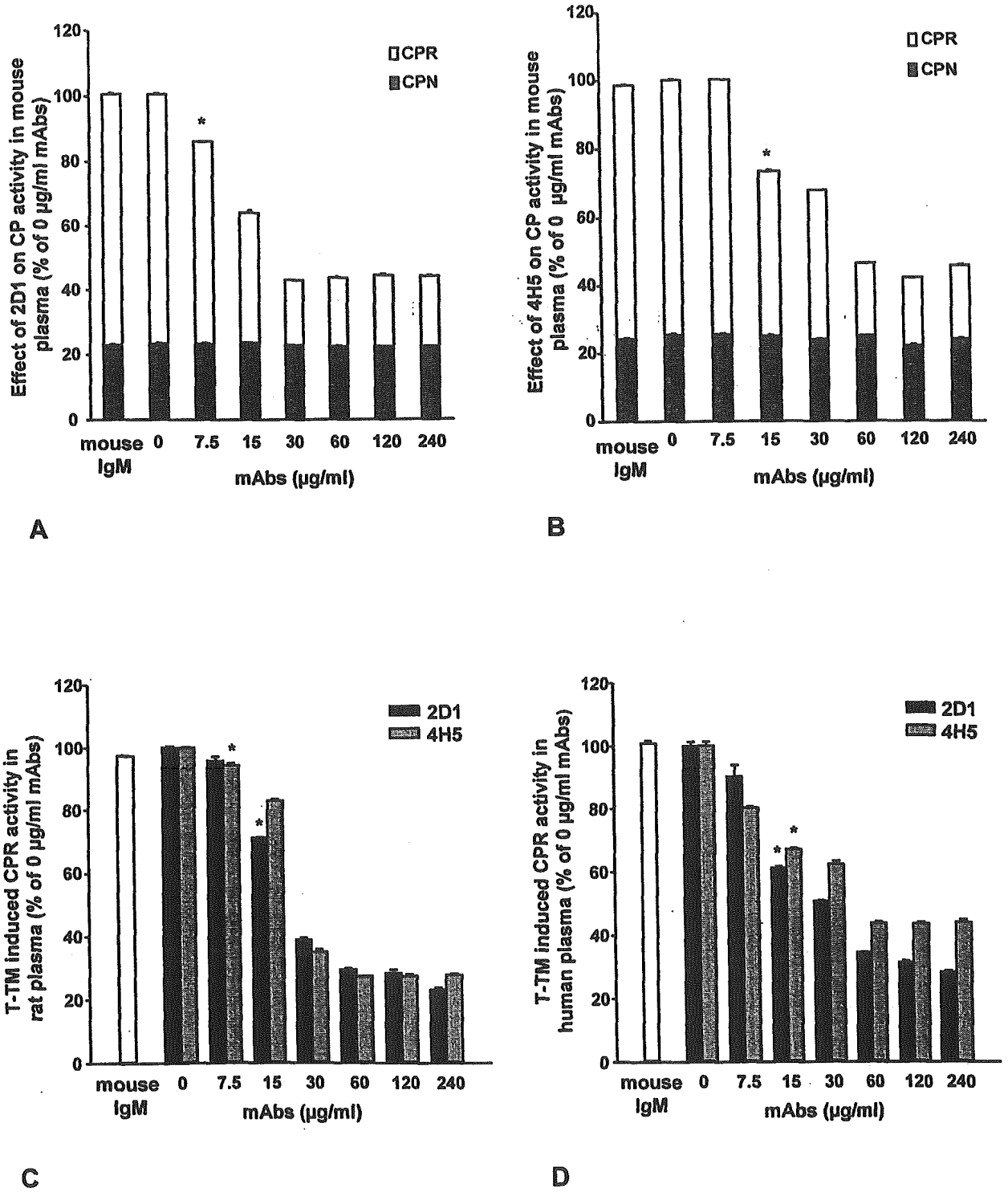


Fig. 3. Effect of mAbs on CP activities. CPN activity was determined as CP activity in plasma before addition of T-TM and $CaCl_2$. The CP activity after addition of T-TM and $CaCl_2$ is total activity of CPN and CPR generated by T-TM. Effects of 2D1 (A) and 4H5 (B) on CP activity in mouse plasma are shown. Before and after incubation with T-TM and $CaCl_2$, both 2D1 and 4H5 showed no effect on CPN activity (dark portion of each column). Effects of 2D1 and 4H5 on T-TM induced proCPR activation in rat plasma (C) and in human plasma (D) are shown. CPN activity was not shown in both rat and human CP activities. Data are shown as the percentage of the value obtained without mAb (PBS control). *$P<0.001$ in comparison with controls without mAbs.

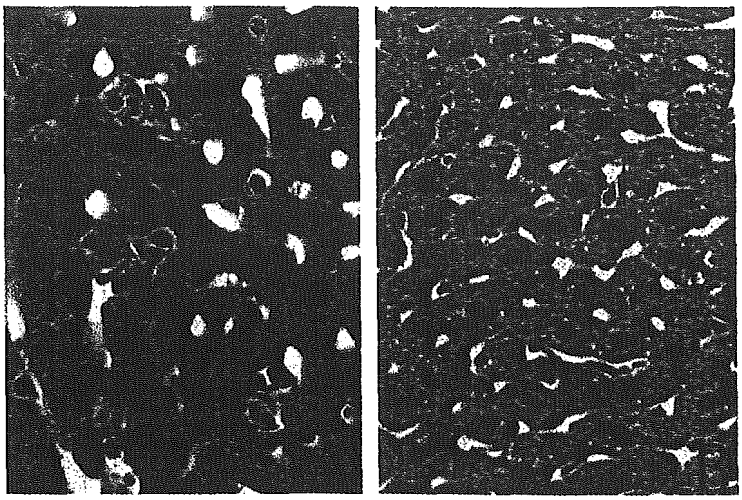


Fig. 4. The histological features of the liver. A: The liver of BALB/c mice sacrificed 7 days following intravenous injection of anti-mouse proCPR mAb (100 μg of 4H5) showed focally minute lesions composed of dense collection of mononuclear and polymorphonuclear inflammatory cells. Such lesions were not observed in the level of proCPR$^{-/-}$ mice injected with 100 μg 4H5 or transplanted with 4H5 hybridoma (data not shown). B: The same lesions were rarely seen in the liver of the control BALB/c mouse with no injection of the anti-mouse proPCR antibody. No collection of inflammatory cells is seen here.

Table 2. Liver function test of BALB/c and proCPR$^{-/-}$ mice injected with or without 100 μg 4H5 mAb (n=5)

(IU/liter)	BALB/c				proCPR$^{-/-}$	
	Control	1 day	3 days	7 days	Control	1 day
ALP	124.25±8.34	71.40±3.70	82.80±5.43	79.00±3.51	220.60±8.78	216.40±11.69
GOT	36.75±3.28	66.50±7.01	50.40±4.07	38.75±3.59	43.80±3.25	43.00±4.06
GPT	12.00±2.00	40.75±5.09	15.50±2.02	16.33±1.45	12.20±1.36	12.20±2.20

80.19±0.43% (P<0.001). In human plasma, both 2D1 and 4H5 significantly inhibited T-TM induced proCPR activation at 15 μg/ml to 71.11±0.28% (P<0.001) and 82.87±0.57% (P<0.001), respectively. Although the mAbs significantly inhibited the proCPR activation of mouse, rat and human, it did not reach the complete inhibition even with increased amount of mAbs indicating that the affinity of those mAbs are not high.

Histopathological Study and Blood Examination

Out of 5 BALB/c mice inoculated with 4H5 hybridoma, 2 mice died in 7 days inducing a large amount of lipid in their abdominal cavity. Therefore, we investigated the effect of intravenous injection of 4H5 (100 μg/head) on BALB/c and proCPR$^{-/-}$ mice. The kidneys showed no histological abnormal features in any experimental groups. The livers of BALB/c mice sacrificed 7 days following intravenous injection of anti-mouse proCPR mAb showed focally minute lesions composed of dense collection of mononuclear and polymorphonuclear inflammatory cells (Fig. 4), as if they reacted to and replaced one or two injured hepatocytes. More lesions of this type were also observed in the livers of BALB/c mice injected with hybridoma cells. In contrast to this, the livers of proCPR$^{-/-}$ mice showed rarely these lesions, as well as livers of mice sacrificed 1 and 3 days following the mAb injection. The blood examination (Table 2) showed that the liver function test indicators (GOT and GPT) in BALB/c mice were significantly changed 1 day after the mAb injection, while there was almost no change in proCPR$^{-/-}$ mice. The increased level of GOT and GPT in the BALB/c mice tended to return to the normal level in 7 days.

Discussion

CPR was found in fresh serum (6) in addition to CPN (11, 26), which had been thought to be the only CPB present in plasma and serum. CPR was also reported independently by other groups who termed it CPU (18), plasma CPB (10), or TAFIa (29). CPR is generated from its zymogen (proCPR) by proteolytic enzymes such as trypsin, thrombin and plasmin (8, 10, 17). Although CPR was shown to be a possible inactivator of bioactive peptides such as C3a, C5a and

bradykinin (7, 9, 29), proCPR turned out to be the same molecules as thrombin-activatable fibrinolysis inhibitor (TAFI) (3), with CPR corresponding to TAFIa, which removes C-terminal lysine from fibrin. Since the C-terminal lysine is the binding site for plasminogen to be activated to plasmin by t-PA (4, 27), TAFIa (CPR) interfere with fibrinolysis. Therefore, proCPR plays an important role not only in restriction of inflammation, but also in the regulation of fibrinolysis.

Thrombomodulin (TM) is a component of the blood vessel wall which binds thrombin and changes its specificity from fibrinogen to protein C, yielding anticoagulant rather procoagulant activity (12). The thrombin-thrombomodulin complex (T-TM) catalyzes cleavage of protein C to activated protein C, which then downregulates the coagulation cascade by proteolytically inactivating the essential cofactors (13). These events are essential in the regulation of the coagulation cascade (12). The study of Bajzar's group (5) reported that activated protein C is anticoagulant because it prevents the thrombin-catalyzed activation of TAFI (proCPR) to TAFIa (CPR). They also suggested that T-TM increases proCPR activation to 1,250-fold than thrombin alone (4).

Current interest in TAFI (proCPR) is mainly focused on its role as a fibrinolysis inhibitor. While this notion is amply supported by *in vitro* studies, the *in vivo* role of proCPR/TAFI remains uncertain. ProCPR-deficient mice had no gross phenotypic abnormalities and no differences in the rate of endogenous clot lysis could be demonstrated using a variety of acute or subacute clot lysis models (25). On the other hand, enhanced pulmonary clot lysis was found in proCPR/TAFI deficiency superimposed on a partial plasminogen deficiency setting (30). It is interesting that in these compound deficient mice, increased number of leukocytes was demonstrated in thioglycollate-induced peritoneal inflammation. While the increased leukocyte influx could be due to the enhanced cell migration secondary to unimpeded cell surface fibrinolysis, it is also possible that the deficiency of proCPR increased inflammation due to prolonged survival of inflammatory peptides. These two possibilities are not mutually exclusive. Thus, the function of proCPR may not be restricted to inhibition of fibrinolysis, but also be related with anti-inflammatory effects. Actually, proCPR deficient mice died from extensive complement activation by cobra venom factor following LPS sensitization while wild and heterologous mice survived this treatment (1).

In the present study, the focal lesions histologically found in the livers of the mAb injected or hybridoma cells injected BALB/c mice are considered to be inflammatory in nature because the lesions are composed of mononuclear and polymorphonuclear inflammatory cells. Morphologically, its pathogenesis might be suggested to be a reactive phenomenon against hepatocytes injured immunologically by the IgM antibody against proCPR that is produced in the hepatocytes to secrete into the blood. On the other hand, proCPR$^{-/-}$ mice inoculated with the hybridoma cells did not show such lesions. Biochemical analysis of mice plasma indicated a transient increase of GOT and GPT following intravenous infection of anti-proCPR IgM mAb in BALB/c mice (Table 2). We have previously shown that proCPR production is increased under inflammatory condition (20, 28). Therefore, immune complexes of proCPR and anti-proCPR IgM mAb might have increased proCPR production at the liver and should have extended generation of immune complexes. Furthermore, inhibition of proCPR activation by the mAb should have contributed to the enhanced inflammation. It is also possible that immune complexes of IgM antibodies activated complement efficiently *in vivo* and circumvented the regulation of complement activation by species specific membrane inhibitors of complement (33).

Although the precise mechanism of this phenomenon is remaining to be analyzed, administration of anti-proCPR IgM mAb could be an experimental model of an autoimmune hepatitis in mice.

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HIV感染症に対するIgM抗体療法

エイズ完治に向けた新たな治療法の可能性

HIV1型(HIV-1)感染症であるエイズ病態の解明に伴い，治療法も逆転写酵素阻害薬やプロテアーゼ阻害薬の開発から，多剤併用療法(HAART)へと進展しており，間欠投与療法(SIT)によるウイルス増殖制御に期待が寄せられたが，否定的な現状にある。そこで，慢性感染細胞を排除することにより，エイズ完治へ向けた新たな免疫賦活法や抗体療法に期待が寄せられている。名古屋市立大学大学院医学系研究科生体防御学の岡田則子助教授らは，HIV感染症におけるIgM抗体の感染防御への関与と治療応用の可能性を検討している。同助教授に同療法の開発状況について聞いた。

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感染細胞でのGM2発現を確認

IgM抗体は，自然抗体の1つとして古くから知られている。岡田助教授によると，抗体活性の検索にはin vitroで希釈してその活性を検討解析することから，多くの場合，自然抗体は反応のバックグラウンドとして無視されてきた。これは，補体系における抗体依存性の活性化経路である古典的経路が先に重要視されていたのと同様の経過であろう，と同助教授は見ている。

しかし，補体系において副経路がより重要な生体防御機構であることがのちに判明したように，ノックアウトマウスの解析などと相まって，自然抗体IgMの感染防御における重要性がその分子機構とともに解明され始めているという。

このような現状を踏まえ，同助教授らの研究グループは，健常者血清中の約3％にHIV-1感染細胞を補体反応依存的に破壊する活性があることを見出した。また，HIV感染者のなかでも，頻回の輸血により感染した血友病患者のなかで経過が良好であった10年以上の長期生存者の血清を検討した結果，8割以上の血清でHIV感染細胞に対する強い細胞傷害活性が検出された。この傷害活性はHIV感染細胞に反応するIgM抗体量との間に強い相関を示した。さらに，これらのIgM抗体の多くは抗原としてガングリオシドGM2を認識することが確認された。ガングリオシドとは，スフィンゴ糖脂質のうちシアル酸を持つものの総称で，脳神経系の細胞に高発現し，神経突起の伸長などに関与する免疫系の細胞ではGM3発現が認められるという。

そこで，HIV-IIIB株を用いた感染細胞でGM2発現を解析したところ，感染細胞特異的なGM2抗原の膜への発現誘導が認められた。感染細胞の糖脂質解析を行った結果でも，中性糖脂質における糖脂質プロファイルは，非感染細胞との間に大きな差異は認められなかったが，酸性糖脂質解析において，感染細胞における明瞭なGM2の発現誘導が確認された。以上から，HIV-IIIB株感染により誘導される糖脂質変化はGM2発現がメジャーな変化であるとの結論を得た。

感染細胞は種特異的補体防御膜因子群を発現し，補体反応から自身を保護しているが，HIV-1感染により特に膜傷害複合体形成阻止因子のHRF20/CD59の顕著な発現低下が起こり，IgM抗体による強力な補体活性化能が優位となり，膜傷害複合体生成による細胞破裂が誘起されると考えられたという。さらに，HIV感染者血清中の抗GM2-IgM抗体量は，CD4数と正の相関を，またHIV-RNAロードと負の相関を示し，「感染者体内における感染細胞反応性IgM抗体の重要性が示唆された」と同助教授は言う。

抗GM2-IgM抗体の効果を検証

このような研究結果を踏まえ，岡田助教授らはヒトメラノーマ患者末梢血リンパ球よりEpstein-Barrウイルス(EBV)を用いて作製したヒト抗GM2-IgMモノクローナル抗体L55を用いて抗HIV活性を検討したところ，L55はヒト血清中自然抗体と同等の補体依存性細胞死を誘導できることが確認された。

これらの抗GM2-IgM抗体は，感染細胞のみならず，ウイルス粒子に対しても強い溶解活性を示すことが判明した(図1)。感染母体と感染粒子の両方を同時に破壊することにより感染阻止効果が誘導されることが期待されており，実験室株での感染拡大に対し強力な阻止活性が得られたという。

同助教授らは，ex vivo解析で5例のHIV感染者末梢血リンパ球からCD8⁺細胞を除去した後にL55を添加して，抗CD3抗体とインターロイキン(IL)-2存在下にリンパ球培養を行い，培養上清中のp24-HIVコア蛋白質量を測定したところ，3例はリンパ球でウイルス検出が可能となり，全例でL55の感染拡大阻止効果が認められた。さらに，アズトレオナム(AZT)の併用によりウイルスは検出限界以下に抑制され，強力な相乗効果が認められた。

こうした膜傷害活性を発現するモノクローナル抗体と中和抗体を組み合わせることにより，「抗体治療効果，あるいは化学療法との併用効果なども期待される」と同助教授は話す。

2つのIgM抗体を作製

さらに，岡田助教授らはHIV感染細胞に特異的に反応するヒトIgMモノクローナル抗体を得るために，ヒト免疫グロブリン遺伝子を含むヒト染色体導入マウスを感染細胞で免疫して，抗体産生ハイブリドーマ9F11と2G9のクローンを得た(図2)。

ヒトIgM抗体9F11はHIV感染MOLT4細胞に0.4μg/mLの低濃度で補体による細胞溶解を起こすことができ，HIV感染者末梢血リンパ球を用いたex vivo解析で高い抗HIV活性が検出された。9F11はMOLT4や正常末梢血細胞には反応性を示さないが，PHA刺激Tリンパ球では反応性が誘導されたため，9F11抗原はHIV感染でも発現誘導される分化抗原であることが考えられた。サル免疫不全ウイルス(SIV)感染サルのリンパ球でも9F11抗原が発現していることから，現在はSIV感染サルで前臨床試験を試みている。

一方，別のヒトIgM抗体2G9は感染細胞に特異的に反応するが，不思議なことに補体依存性の細胞傷害活性は全く誘導できない。しかし，補体存在下あるいは非存在下に関係なく，2日間培養により感染細胞が抗体反応依存的に死滅することが認められた。この細胞死はアポトーシス死であることが確認された。2G9遺伝子の解析結果では，補体活性化能を有する他のIgM遺伝子との間に，定常領域に差は認められておらず，この抗体の生物活性の違いは抗原分子のオリエンテーションが影響を及ぼすことが推測されている。

2G9は潜伏感染細胞OM10.1にも反応性を示し，アポトーシスを誘導できるので，HIVがプロウイルス化して潜んでいる細胞をも認識して，生体から排除できる可能性を示唆しているものと期待できるため，現在2G9抗原分子の解析を進めている。

新しい視点からのエイズ治療へ

これまでエイズ治療を目指しての免疫学的治療法としては，感染者血清中の中和抗体活性の詳細な検討の結果により，正常な抗原エピトープの同定に基づいたモノクローナル抗体の開発研究が進められてきた。しかし，岡田助教授は「中和抗体の例で知られるように，HIV-1の高度変異性によって長期的な有効治療が困難となる問題が挙げられている」と話す。これは，化学療法においても同様であり，治療の長期化や不完全性はさらなる耐性ウイルスの産生を加速することにつながる。したがって，ウイルス感染によって二次的に誘導される異常を標的にしたり，あるいはケモカイン受容体などの生体側の分子を標的にした抗体の適用も試行されている。

また，感染の初期段階に発現するNef蛋白質やHIV-1感染による細胞膜変化を標的とした有効な感染阻止効果やアポトーシスを誘導するIgM抗体の作製も試みられている。同助教授は「今後はIgG抗体のレベルまでIgM抗体の応用研究を進めることにより，近い将来はIgM抗体療法を実現したい」と言う。既にエイズは，HAARTやSITの登場によって"死の病"から"制御可能な疾患"へと変貌した。

「少しでも化学療法から離れることができるよう，新しい視点から治療法が求められており，その1つとしてIgM抗体療法は有効な治療法になりうるのではないか」と同助教授は期待する。

図1. 抗糖鎖IgM抗体による抗HIV活性

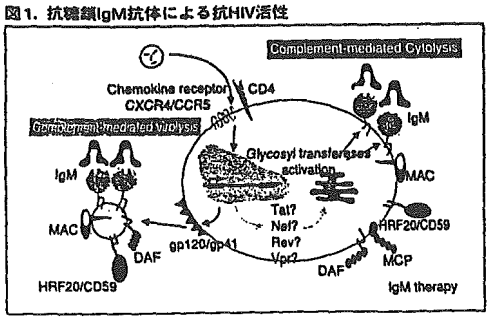
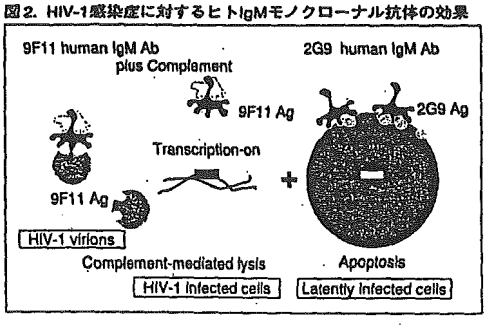


図2. HIV-1感染症に対するヒトIgMモノクローナル抗体の効果



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GASTROENTEROLOGY

Helicobacter pylori eradication decreases the expression of glycosylphosphatidylinositol-anchored complement regulators, decay-accelerating factor and homologous restriction factor 20, in human gastric epithelium

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Abstract

Background: It has previously been reported that there is a strong correlation between the expression of glycosylphosphatidylinositol (GPI)-anchored complement membrane inhibitor in gastric epithelium and the severity of inflammation of gastric mucosa. To investigate the regulation of complement activity in gastric epithelium during *Helicobacter pylori* (*H. pylori*)-associated gastritis, the expression of GPI-anchored complement membrane inhibitors, decay-accelerating factor (DAF) and 20-kDa homologous restriction factor 20 (HRF20), and membrane cofactor protein (MCP), which is a transmembrane protein, were evaluated after removal of the *H. pylori* stimulus. Furthermore, the expression of the complement fragment, C3c, was also investigated.

Methods: Forty-six patients with epigastric symptoms and endoscopically confirmed peptic ulcer or gastritis who had *H. pylori* infection of the gastric mucosa were enrolled in the present study. Biopsy specimens were obtained from the gastric antrum and corpus 1 month before and after eradication. *Helicobacter pylori* infection was determined by the rapid urease test, histology, and culture before eradication, and by histology, culture, and urea breath test after eradication. Gastric biopsy specimens obtained before and after eradication were evaluated for infiltration by neutrophils and mononuclear cells. The expression of complement membrane inhibitors, DAF, HRF20, and MCP and that of the main complement fragment, C3c, was immunohistochemically evaluated.

Results: One month after the eradication of *H. pylori*, the infiltration by neutrophils and mononuclear cells in the gastric mucosa decreased significantly ($P < 0.0001$) as compared with that before eradication. The expression of DAF, HRF20, and C3c on gastric mucosal epithelium also significantly decreased in both the antrum and the corpus ($P < 0.05$) 1 month after eradication. However, no change was observed in the expression of MCP.

Conclusions: The decrease in the expression of GPI-anchored complement regulator and the complement after removal of a chronic microbial stimulus suggests that the gastric epithelium appears to undergo an aggressive stress of complement during *H. pylori* infection. Conclusively, DAF and HRF20 may play an important protective role against complement-mediated damage induced by chronic microbial stimuli in such a pathological condition.

Key words: C3c, complement activation, DAF, eradication, *Helicobacter pylori*, HRF20.

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INTRODUCTION

Helicobacter pylori is a Gram-negative bacterium that frequently infects the human stomach and leads to gastric inflammation that is histologically characterized by surface epithelial degeneration and infiltration of gastric mucosa by acute and chronic inflammatory cells.

In general, the human complement system plays a major role in resistance against microbial infection because it participates in both specific and non-specific immunity.[1] Activation of the alternative pathway is induced directly and independent of antibody by the surface structures on the bacteria. In addition to antibodies, the classical pathway can be directly activated by bacterial lipopolysaccharides (LPS). The lectin pathway can recognize foreign targets by a carbohydrate binding protein, mannose-binding lectin. The final phase of cytolytic membrane lesions involves the membrane attack complex (MAC), which can penetrate the bacterial cell membranes and cause cell death via the collapse of membrane potential.[2] However, fragments of complement proteins may be deposited on non-target cells[3] and thereby be of immunopathological importance in acute and chronic inflammatory diseases of the gastrointestinal tract.[4] In order to prevent complement lysis, the complement system is regulated at multiple levels, and several proteins on cell membranes are known to inhibit the activation of the complement cascade.[5,6] Most proximally, the enzymatic subcomponents of C1 can be bound by C1 inhibitor and removed from C1q. Fragments of the structurally related C4 and C3 molecules are controlled by the following group of six regulators of complement activation encoded in the RCA gene cluster: membrane cofactor protein (MCP; CD46), CR1 (CD35) and CR2 (CD21), decay-accelerating factor (DAF; CD55), C4 binding protein (C4bp), and factor H. The first four regulators are expressed as membrane-bound proteins, and the last two circulate in the plasma. Membrane cofactor protein and DAF are present on a wide variety of cells including leukocytes and vascular endothelial cells whereas CR1 and CR2 are expressed on selected leukocytes and antigen-presenting cells. Decay-accelerating factor dissociates the classical and alternative C3 convertase.[7,8] The other regulators (MCP, CR1, C4bp, and factor H) function as cofactors for factor I, allowing it to cleave C4b and C3b into biologically active fragments.[9] Lysis of autologous or allogeneic cells is further inhibited by regulatory proteins such as the 20-kDa homologous restriction factor 20 (HRF20; CD59), also referred to as protectin, which inhibits the formation of MAC by preventing the polymerization of C9 on C5b-8.[10,11] These proteins, which are widely distributed in normal tissues, are believed to protect host tissues from autologous complement-mediated damage.[12–14]

Expression of these three membrane-bound proteins, DAF, MCP, and HRF20, has been reported in *H. pylori*-associated gastritis,[15–17] sepsis, autoimmune disease, and gastric cancer, as well as in normal humans.[18–21] Moreover, complement activation in the gastric mucosa has been determined to be a part of the pathogenesis of *H. pylori*-associated gastritis.[4,15,17] In patients with *H. pylori*-associated gastritis, the expression of DAF was markedly enhanced and was proportional to the severity of mucosal inflammation.[16] Several publications have reported that *H. pylori* induces complement activity[17] and that various proinflammatory activators upregulate glycosylphosphatidylinositol (GPI)-anchored complement regulators, such as DAF and HRF20.[22] However, a change in the expression of these complement membrane inhibitors as well as complement is not known to occur in the case where gastric inflammation is decreased following the eradication of *H. pylori*. Therefore, we conducted an *H. pylori* eradication study to specifically evaluate the role of DAF, HRF20, and MCP, as well as the main complement fragment C3c, in gastric mucosal inflammation.

METHODS

Patients

From April 1998 to March 2000, 56 patients (23 women, 33 men; mean age: 52 years) with epigastric symptoms and endoscopically confirmed peptic ulcer or gastritis who had visited Nagoya City University Hospital and were found to have *H. pylori* infection of the gastric mucosa were enrolled in the present study after informed consent was obtained. Exclusion criteria were as follows: *H. pylori*-negative diagnosis, intake of non-steroidal anti-inflammatory drugs (NSAIDs), severe concomitant diseases, and gastric cancer.

Patients having open peptic ulcer received histamine-receptor antagonist (HRA) treatment, and ulcer healing was endoscopically confirmed after 6 or 8 weeks. Patients received 1 week of eradication therapy of omeprazole, 20 mg twice daily; clarithromycin, 200 mg twice daily; and amoxycillin, 500 mg three times daily. One month after eradication, endoscopy was performed and gastric biopsies were taken.

Tissue samples

Three biopsy specimens were obtained individually from each antrum and corpus before eradication and two were obtained individually 1 month after eradication. One individual sample from antrum and corpus before and after eradication were promptly fixed in pure acetone at 4°C for 3 days, cleared in a series of xylene rinses, and embedded in paraffin. Other samples were used for the diagnosis of *H. pylori*.

Antibodies against complement membrane inhibitors and complement

We utilized mouse monoclonal antibodies against complement inhibitors: 1C6 (IgG1 isotype, Wako Pure Chemical Industries, Osaka, Japan), antibody to DAF (CD55); 1F5 (IgG1 isotype),[10,16] antibody to HRF20 (CD59); and J4-48 (IgG1 isotype, Immunotech, Marseilles, France), and antibody to MCP (CD46). We also used rabbit polyclonal antibody (Dako, Glostrup,

Denmark) against human C3c complement as primary antibody. Original preparation of monoclonal antibodies against DAF, HRF20, MCP, and C3c, was diluted in phosphate-buffered saline (PBS) in the ratios 1:500 (2 μg/mL), 1:100 (1 μg/mL), 1:1000 (1 μg/mL), and 1:100 (100 μg/mL), respectively.

Evaluation of mucosal inflammation

Routine hematoxylin and eosin (HE)-stained sections were made from all tissue blocks. For comparative study and establishment of the precise distributions of neutrophils and mononuclear cell infiltration, serial sections were used for HE and immunohistochemical staining. Inflammation in the gastric mucosa was graded from 0 to 3 using the Updated Sydney System.[23] Pathological analysis was performed three times independently without knowledge of the patient treatment status. If the results of these three analyses differed, the score chosen by the majority was used.

Immunohistochemistry of complement membrane inhibitors and complement

Paraffin sections of 3-μm thickness were serially cut, deparaffinized with xylene, and dehydrated with acetone. After washing with PBS, the sections were first treated with 3% (v/v) H_2O_2 for 15 min at room temperature to inactivate the endogenous peroxidase, and subsequently, with a 1:10 dilution of non-immune rabbit serum for 10 min to block non-specific immunoglobulin binding sites. After blotting the excess serum, the sections were incubated with each of the primary antibodies for 1 h at room temperature; control sections were incubated with PBS or non-immune mouse IgG1 (Dako), the concentration of which was matched with that of the respective primary antibody. The sections were rinsed, incubated with biotin-labeled rabbit anti-mouse IgG antibody for 10 min, washed with PBS, and incubated with peroxidase-labeled streptavidin–biotin for 5 min (Histfine SAB-PO [M] kit; Nichirei, Tokyo, Japan). After washing with PBS, the sections were stained with 0.02% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (Sigma, Tokyo, Japan) containing 0.003% (v/v) H_2O_2 and 10 mmol/L sodium azide. The sections were counterstained with hematoxylin, washed with PBS, dehydrated in graded concentrations of ethanol, and mounted.

The expression of the complement regulatory factors in gastric epithelial cells was rated on a four-point scale: –, faint or no staining; 1+, specific staining in 10–50% of the cells; 2+, specific staining of 50–90% of the cells; and 3+, specific staining of 90–100% of the cells.

To confirm the activation of complements, the main fragment was detected by immunohistochemistry. In order to block non-specific binding sites the sections were treated for 1 h with 5% dry milk in PBS and incubated with the primary antibodies for 1 h at room temperature. After washing, the sections were incubated with Cy3-conjugated AffiniPure goat antirabbit IgH antibody (H+L; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:100, 1 μg/mL) for 10 min. After washing with PBS, the sections were mounted in Dako fluorescent mounting medium.

The staining of the apical side of gastric epithelial cells was semiquantitatively scored from – to 3+: –, no staining; 1+, faint specific staining in 20% of the cells; 2+, specific staining of 20–50% of the cells; and 3+, specific staining of 50–100% of the cells.

Immunohistochemical analysis was performed three times independently without knowledge of patient treatment status. If the results of these three analyses differed, the score chosen by the majority was used.

Diagnosis of *H. pylori* infection

Before eradication we took three biopsy samples individually from the gastric antrum and corpus of each patient to test *H. pylori* infection by rapid urease test, culture, and histological examination. A minimum of two positive results from a total of four tests (rapid urease test, histology, culture, and serological examination) were considered to be confirmatory for *H. pylori* infection. After eradication of the infection, two biopsy samples were taken individually from the antrum and corpus for culture and histological examination. Eradication was considered successful when all the three investigations (culture, histology, and urea breath test) were negative.

A rapid urease test was done by Pyloritek (Serim Research Corporation, Elkhart, IN, USA), and serological examination was carried out using the Determiner Kit (Enteric Products, Westbury, NY, USA). Histological examination was performed by an immunohistochemical study using anti-*H. pylori* polyclonal antibody (Dako) diluted in the ratio 1:100 in PBS, following the same procedure described earlier. In the urea breath test, *H. pylori* infection was considered positive when an increase of 5% or more in the δ-values was observed at 20 min after carbon-13-labeled urea was obtained.

Statistical analysis

Data are reported as mean ± SE, and $P < 0.05$ was accepted as statistically significant. A change in the infiltration by neutrophils or mononuclear cells and the expression of each complement membrane inhibitor before and after eradication was analyzed using the Wilcoxon single-rank test.

RESULTS

Twenty-three patients had gastric ulcer, 18 had duodenal ulcer, seven had both gastric and duodenal ulcer, and eight had chronic gastritis. Fifty-four patients completed the study but two patients were excluded after failed follow-up endoscopy. *Helicobacter pylori* infection was successfully eradicated in 47 patients (87%). In the

eradication group ($n = 47$), the infiltration of the gastric mucosa of the antrum and corpus by neutrophils was dramatically decreased 1 month after eradication as compared with before eradication (0.06 ± 0.04 vs 2.15 ± 0.10; 0.04 ± 0.03 vs 1.72 ± 0.08; $P < 0.05$, Table 1). Mononuclear cell infiltration of the antrum and corpus also decreased compared with before eradication, but this decrease was to a lesser extent than that of neutrophils (1.81 ± 0.07 vs 2.70 ± 0.07; 1.32 ± 0.08 vs 2.47 ± 0.07; $P < 0.05$; Table 1). In contrast, no significant change in the neutrophils and mononuclear cell infiltration of the antrum and corpus was observed in the non-eradication group ($n = 7$; Table 1).

Complement membrane inhibitors (DAF, which binds to C4b or C3b; HRF20, which binds to C8 or C9; and MCP, which binds to C3b) were recognized as membrane-binding proteins both before and after *H. pylori* eradication. The expression of GPI-anchored proteins, DAF and HRF20, was observed mainly on the luminal surface of the gastric epithelium, and the transmembrane protein, MCP, on the basolateral surface. The expression of DAF and HRF20 on gastric mucosal epithelial cells in the antrum and corpus was remarkably decreased after *H. pylori* eradication in both the antrum and corpus as compared with that before eradication ($P < 0.05$, Figs 1,2). However, no change was observed in the expression of MCP (Figs 1,2). In the non-eradication group, no change was observed in DAF, MCP, and HRF20 expression before and after the eradication therapy (Fig. 3).

The main fragment, C3c, was presented in the lamina propria and often recognized on the apical side of gastric epithelial cells as well as in the mucus layer (Fig. 4). A remarkable change was not observed in the staining of lamina propria before and after eradication. The expression of C3c on the apical side of gastric epithelial cells and in the mucus layer reduced significantly after eradication ($P < 0.05$; Table 2).

Any *H. pylori* bacteria were not found to be positive for DAF, HRF20, MCP, or C3c.

DISCUSSION

It has been reported that *H. pylori* infection increases serum C3 levels[24] and leads to the over-expression of complements and complement inhibitors.[15–17,25] Furthermore, it has been suggested that the complement system may play an important role in *H. pylori*-induced gastritis. We had also previously demonstrated that DAF was upregulated in *H. pylori*-associated gastritis and that its expression in gastric epithelial cells was correlated with leukocyte infiltration in the gastric mucosa. We suggested that the *H. pylori* infection in gastric mucosa may mediate the local expression of complement membrane inhibitors on gastric epithelial cells.[16] To cross-examine our conclusions, we conducted a comparative immunohistochemical study before and after eradication to specifically examine whether *H. pylori* infection changes the expression levels of complement inhibitors and complement activation in the human gastric epithelium by eradicating *H. pylori* from the gastric mucosa.

We demonstrated that DAF (CD55), HRF20 (CD59), MCP (CD45), and complement fragment C3c were expressed in gastric mucosa infected by *H. pylori*. The staining pattern of DAF and HRF20,

Table 1 Leukocyte infiltration into the gastric mucosa before and after *Helicobacter pylori* eradication

	Neutrophil		Monocyte	
	Before	After	Before	After
Eradication group ($n = 47$)				
Antrum	2.15 ± 0.10	0.06 ± 0.04*	2.70 ± 0.07	1.81 ± 0.07*
Corpus	1.72 ± 0.08	0.04 ± 0.03*	2.47 ± 0.07	1.32 ± 0.08*
Non-eradication group ($n = 7$)				
Antrum	1.86 ± 0.26	1.57 ± 0.20	2.71 ± 0.18	2.43 ± 0.20
Corpus	1.57 ± 0.20	1.71 ± 0.18	2.29 ± 0.29	2.14 ± 0.34

*$P < 0.05$ vs before.

Table 2 Change in staining of complement fragment C3c before and after eradication of *Helicobacter pylori*

	Antrum ($n = 32$)		Corpus ($n = 34$)	
	Before	After*	Before	After*
C3c staining	% (n)	% (n)	% (n)	% (n)
–	12.5 (4/32)	53.1 (17/32)	17.6 (6/34)	55.9 (19/34)
1+	46.9 (15/32)	28.1 (9/32)	35.3 (12/34)	20.9 (7/34)
2+	12.5 (4/32)	12.5 (4/32)	23.6 (8/34)	14.7 (5/34)
3+	28.1 (9/32)	6.3 (2/32)	23.6 (8/34)	8.8 (3/34)

*$P < 0.05$ vs before.

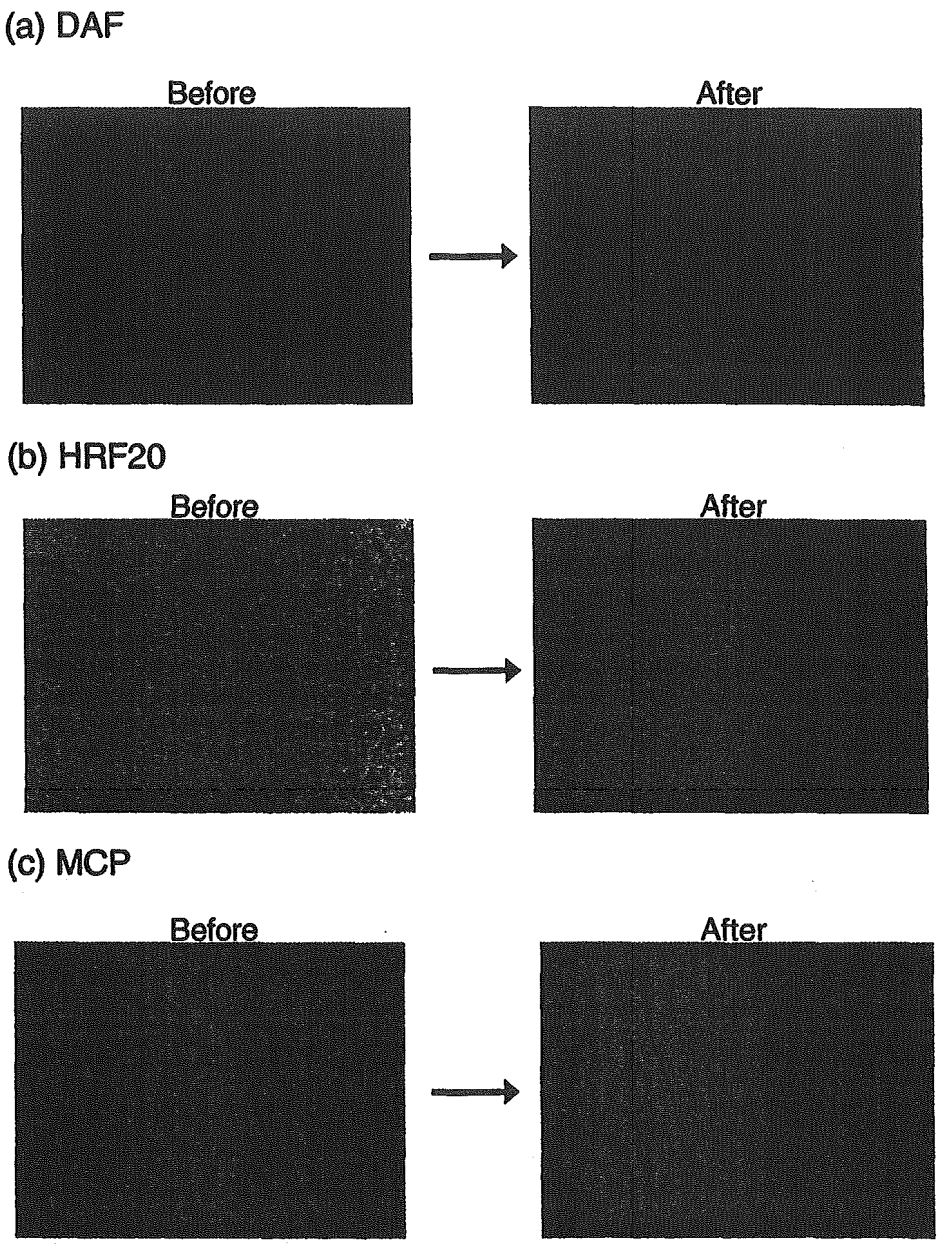

Figure 1 Expression of complement membrane inhibitors before and after eradication of *Helicobacter pylori*. (a) Decay-accelerating factor (DAF) was expressed on the apical side of the gastric epithelium, and the expression decreased after eradication of *H. pylori*. (b) Homologous restriction factor 20 (HRF20) was also present on the apical side, and eradication of *H. pylori* attenuated the expression of HRF20. (c) The staining for membrane cofactor protein (MCP) was positive on the basolateral surface, and there was no change after eradication.

which are GPI-anchored membrane proteins,[10,23,26] was identical to the pattern that was previously observed on the apical side of the epithelium in *H. pylori*-associated gastritis.[15,16,25,27] In contrast, the staining pattern of MCP, a transmembrane protein,[9] was also identical to that previously observed on the basolateral surface of these cells.[16,25] However, we have demonstrated by immunohistochemical studies that C3c localization is similar to that observed previously.[15] Additionally, after eradication, the expression of DAF and HRF20 as well as C3c was significantly decreased. Furthermore, in the non-eradication group, the expression levels of DAF and HRF20 did not change. To our knowledge, such a comparative study on the expression of both complement and complement membrane inhibitors in gastric mucosa, before and after eradication, has not been previously reported.

The enhanced expression of DAF and HRF20 on the epithelial surface is an important protective mechanism for preventing host epithelial cell damage due to complement attack. However, various proinflammatory and immunoregulatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), interleukin-1β (IL-1β), and interleukin-4 (IL-4), increased in *H. pylori*-infected stomach.[28] Moreover, TNF-α, IL-1β, and IFN-γ enhanced the constitutive expression of HRF20 on the human colonic adenocarcinama cell line, HT29, in a dose-dependent manner.[29] Additionally, TNF-α, IL-1β, and particularly IL-4 have been shown to enhance the expression of DAF; in contrast, the expression of MCP is not enhanced.[28,30] However, all these observations were recorded by using human colonic adenocarcinoma cell line or endothelial cells, and not epithelial cells.

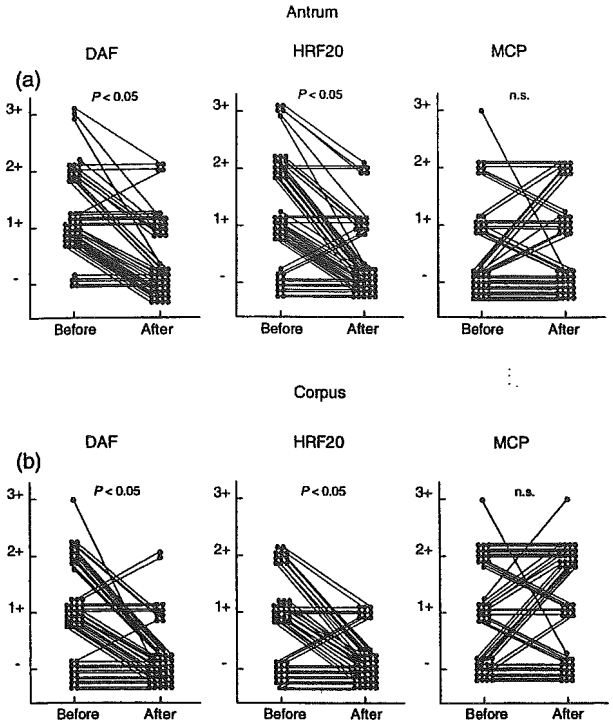

Figure 2 The change in the expression of complement membrane inhibitors in the eradication group. In both antrum and corpus specimens, the expression of decay-accelerating factor (DAF) and homologous restriction factor 20 (HRF20) on gastric epithelium was significantly decreased after eradication of *H. pylori* (b,c). However, no changes were observed in the membrane cofactor protein (MCP) expression (c).

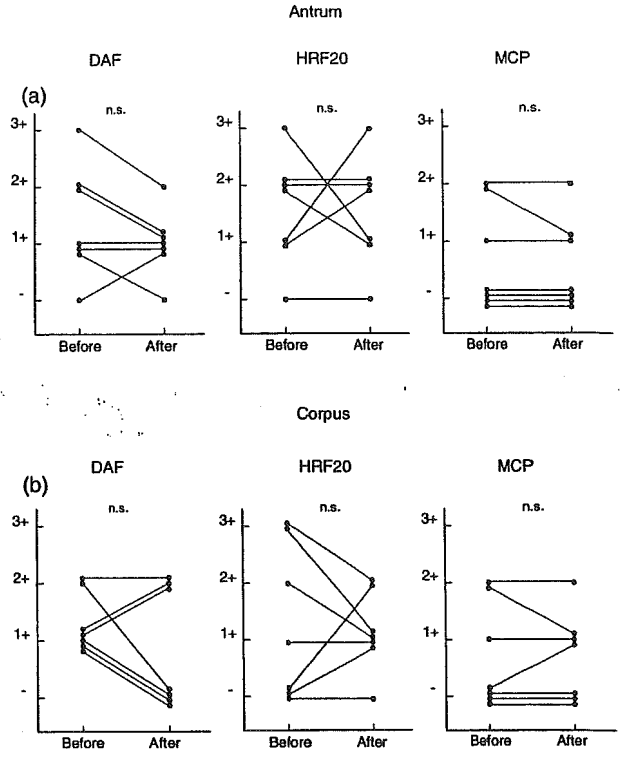

Figure 3 Change in the expression of complement membrane inhibitors in the non-eradication group. The expression of the three inhibitors on *Helicobacter pylori*-infected gastric mucosa did not change after eradication (a–c).

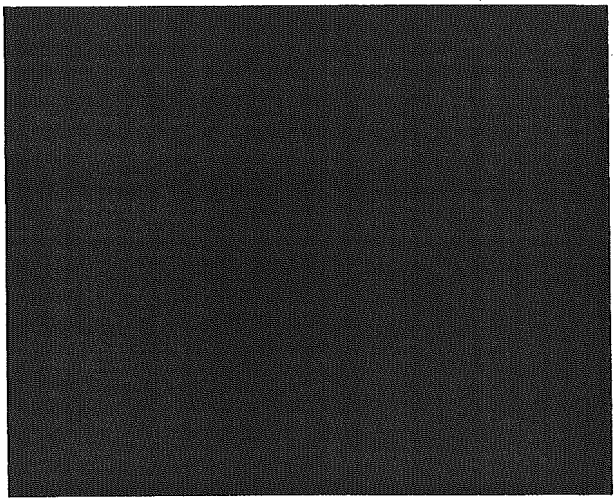

Figure 4 Localization of complement fragment C3c. The main complement fragment C3c staining was recognized on the apical side of gastric epithelial cells and in the mucus layer.

In the present study we have shown that the eradication of *H. pylori* decreased the expression of DAF and HRF20 on the gastric epithelium, suggesting that eradication changed the activation of complement, such as by C3 convertase and MAC. The eradication of *H. pylori* decreases the level of various cytokines, including IL-1β, and thereby possibly resulting in the decrease in DAF and HRF20 after eradication. However, the complement products were localized in only the areas that were associated with *H. pylori*-induced local activation of complement.[15] Moreover, the level of DAF and HRF20, which were expressed on the apical side (the side on which *H. pylori* was present) of the endothelium, decreased after eradication. Based on these considerations, although gastric mucosal cytokines were not analyzed in the present study, the expression of complement membrane inhibitors in gastric epithelium is more dependent on *H. pylori* stimuli than on cytokines. We demonstrated that the main complement fragment, C3c, which is present on the apical side of the epithelium, as well as distribution of DAF and HRF20, decreases after eradication, provided that *H. pylori* actually activated the complement in gastric mucosa. We also stained C9 but no specific staining was observed on epithelial cells. The staining of lamina propria was found to remain unchanged after eradication (data not shown). Therefore the level of MCP, which was expressed on the basolateral side (the side opposite to that of *H. pylori*), did not decrease, suggesting the regulatory role of complement in the expression of complement inhibitors. The observation of unaltered expression of MCP on the basolateral membrane, 1 month after eradication, could be attributed to the

apparently unaltered expression of C3c in the lamina propria after eradication (data not shown).

We followed several patients for 6 months after eradication and observed that the expression level of DAF and HRF20 was less than that at 1 month after eradication. Furthermore, the expression of MCP was greater than that observed 1 month after eradication. We reported that the expression of MCP had a negative relation to mucosal inflammation, and the downregulation of MCP might be caused by consumption of complement regulators to regulate complement activation.[16] Further experiments need to be performed to clarify whether the upregulation of complement membrane inhibitors is lowered to normal levels several months after eradication.

During the activation of complement in *H. pylori*-associated gastritis, the bacteria do not appear to survive in the stomach. In fact, *H. pylori* and its LPS generated a large amount of complements such as C3bc and MAC. These complements were observed to have formed a coating on *H. pylori*, thereby inducing the elimination of *H. pylori* in more than 30% of the serum samples *in vitro*.[17] In contrast, it has been suggested that *H. pylori*-bound MAC can bind vitronectin (S-protein), which is a multifunctional adhesive plasma protein that binds to soluble TCC intermediates and inhibits complement-mediated cell lysis.[31,32] Furthermore, during inflammation and tissue degradation, the endogenous phospholipase A_2 from neutrophils may induce mucosal tissue to lose its phospholipid-anchored regulator. Additionally, *H. pylori* produces a similar phospholipase, PldA.[33] Micell-formed soluble protectin (HRF20, CD59), which is a phospholipid that is a part of the GPI-anchored protein, is capable of translocating from one surface to another.[34,35] Rautemaa *et al.* demonstrated the binding of protectin (CD59) on *H. pylori* and suggested a reason for the complement resistance of *H. pylori* and hence its survival in the stomach.[36] In the present study, however, *H. pylori* was not detected by HRF20 (CD59) staining. In addition, *H. pylori* produces urease enzyme; the ammonia produced by this enzyme can directly inhibit complement activation.[37]

Not only gastric epithelial cells but also parietal cells of gastric body were suggested as being vulnerable to complement attack and as contributing to the development of atrophic gastritis.[25] There were no reports of complement inactivation after eradication, but decline of the expression of DAF, HRF20, and C3c that was demonstrated in the present study may be sufficient to suggest the degradation of complement activity. We did not consider the parietal cells, but after eradication the parietal cells may be free from complement attack, and the development of atrophic gastritis starts declining.

In summary, the results show that the *H. pylori* infection-induced upregulation of GPI-anchored complement regulators in gastric epithelium is rapidly lowered after the eradication of the microbe. Our study indicates that the gastric epithelium appears to undergo an aggressive complement stress during *H. pylori* infection and that DAF and HRF20 may play a pivotal protective role against complement-mediated damage in such a pathological condition.

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