MA2 (antisense):
TGTGCCCTGCATGACGTCCTT←(for 2G9);
MS2 (sense):
TCCCGACTCCATCACTTTCTCC←(for 2G9);
MS4 (sense): CGCAAGTCCAAGCTCATCTGCC;
MA92 (antisense):
CAGATGAGCTTGGACTTGCGGG←(for 9F11);
MS91 (sense):
AGCTGAACTCTGTGACTCCC←(for 9F11);
KA1 (antisense):
ACTTTGGCCTCTCTGGGATAG; and
KS1 (sense): TGTTGTGTGCCTGCTGAA.

Anti-HIV infectivity assay using KC57-FITC anti-p24 mAb. Naive MOLT4 cells (2×10^5) were mixed with 4×10^3 HIV-IIIB-infected MOLT4 cells (50:1) in a 24-well plate with 1 ml of medium containing one or more of the following reagents: 20% FHS, 9F11 (5 μg/ml) or 2G9 (100 μg/ml) or a combination. The mixtures were incubated at 37 C in a CO_2 incubator. After 3 days, 0.9 ml of each culture was collected and the percentage of infected cells was assessed using KC57-FITC anti-p24 mAb (Coulter, Healeah, Fla., U.S.A.). The remaining cells were suspended in RPMI1640 medium containing 20% FHS with or without the relevant mAb for a further 2 days of cultivation to determine the percentage of infected cells on day 5.

Apoptosis of HIV-infected cells. Apoptosis of MOLT4/IIIB cells was assessed by the TUNEL method using an In Situ Cell Death Detection Kit (Roche Diagnostics Corp., Indianapolis, Ind., U.S.A.) (4). MOLT-4/IIIB cells were suspended in culture medium at 1×10^5/ml and incubated in the presence of either 2G9 or 9F11 (50 μg/ml). After 2 days incubation, fluorescence intensity was determined on a FACSCalibur. Apoptosis was also determined using FITC-conjugated Annexin V, known as an early apoptosis marker. OM10.1 cells were suspended in culture medium at 1×10^5/ml and incubated in the presence of either 2G9 (12.5 μg/ml) alone, 20% FHS alone or their combination. Apoptosis was determined after a 2-day incubation at 37 C in a CO_2 incubator. The cells that reacted with Annexin V were fixed in 1% paraformaldehyde and the intensity of fluorescence was measured by flow cytometry.

Elimination by 2G9 of HIV-1 latently infected OM10.1 cells. OM10.1 cells (untreated or treated with 1 ng/ml TNF-α) were suspended in culture medium at 1×10^5/ml and incubated in the presence of either 2G9 (12.5 μg/ml) alone, 20% FHS alone or their combination. After a 2-day incubation at 37 C, the extent of apoptosis was determined by staining with FITC-Annexin V followed by flow cytometric analysis.

Effect of 2G9 on HIV-1 replication in latently infected OM10.1. Cells of the latently infected cell line OM10.1 produce little HIV-1 under basal conditions but do produce a significant level of virus after stimulation with TNF-α. OM10.1 cells (1×10^5/ml) were stimulated with TNF-α (1 ng/ml) in the presence of either 2G9 (12.5 μg/ml or 50 μg/ml) alone, 20% FHS alone, or their combination. After 48 hr or 72 hr incubation at 37 C, the percentage of HIV-infected cells was assessed by flow cytometry using a KC-57-FITC anti-p24 Ab (Coulter).

Human material and vertebrate animals. The use of human material was approved by the Ethical Committee of Nagoya City University Graduate School of Medical Sciences. Animal experiments with mice were carried out in the Center for Experimental Animal Science of Nagoya City University Graduate School of Medical Sciences. All protocols for animal experiments were approved by the Experimental Animal Committee of Nagoya City University Graduate School of Medical Sciences.

Results

Production and Purification of Human IgM mAbs

To select hybridomas secreting human IgM, culture supernatants of hybridoma cells generated by fusing spleen cells from a TC mouse immunized with U937-IIIB and mouse myeloma cells (P3U1) were screened with a sandwich ELISA using anti-human IgM polyclonal Ab and anti-human κ-chain mAb labeled with HRP. The culture supernatants containing human IgM Abs were then tested for their reactivity to MOLT4/IIIB cells by indirect immunofluorescence staining followed by flow cytometric analysis. From the results of screening, 19 hybridomas were chosen for further cloning. Cloning was performed by limiting dilution in RPMI1640 medium containing 10% FCS (Hyclone, Logan, Ut., U.S.A.), 5% Bri-clone (Bioresearch Ireland, Glasnevin, Ireland) and 3 μg/ml puromycin (Sigma Chemical Co., St. Louis, Mo., U.S.A.). After performing three series of limiting dilutions, two hybridoma clones termed 9F11 and 2G9 were established, and these were adapted to a serum-free medium, SFM101 (Nissui Seiyaku Co., Ltd., Ibaraki, Japan). The mAbs secreted by the respective clones were also designated 9F11 and 2G9. Both mAbs were purified from the serum-free culture supernatants by passing through a MonoQ column followed by gel filtration chromatography. Briefly, cell culture supernatants (5–10-fold concentrations) were dialyzed against 50 mM sodium phosphate, pH 7.2, and were then passed through a MonoQ HR16/10 column (Pharmacia Biotech, Uppsala, Sweden). The column was then eluted by applying a linear gradient of 50–300 mM sodium phosphate and the

activity of each fraction against HIV-infected cells was assessed by indirect immunofluorescence staining followed by flow cytometric analysis; positive fractions were pooled and dialyzed against PBS and were then separated on a Hiload-Superdex 200gp 26/60 column (Pharmacia Biotech) using PBS as an elution buffer.

Reactivity of 9F11 and 2G9 with Cells

On flow cytometric analysis, 9F11 stained MOLT4/IIIB but not uninfected MOLT4, U937 or CEM cells. U937 cells also became reactive with 9F11 following infection with HIV-MN or a primary isolate of HIV-1 (HIV-momo) as shown in Fig. 1. 2G9 also stained HIV-infected cells while they did not stain uninfected cells as far as tested. MOLT4/IIIB cells were cultivated in the presence or absence of tunicamycin for 2 days before the flow cytometric analysis. Reactivities of 9F11 and 2G9 to the tunicamycin-treated cells were reduced as shown in Fig. 2, indicating that both of the antigenic epitopes for these Abs involved a sugar moiety.

Complement-Mediated Cytolysis

MOLT4 and MOLT4/IIIB cells were labeled with ^{51}Cr and incubated with varying concentrations of 9F11 in the presence of 20% FHS as a source of complement. The degree of lysis of MOLT4/IIIB cells was dependent on the dose of 9F11 and 0.4 μg/ml of 9F11 caused cytolysis of more than 50% of these cells. MOLT4 cells were not lysed, even with 20 μg/ml of 9F11 and FHS. Furthermore, although C9-deficient human serum (C9DHS) could not induce cytolysis of MOLT4/IIIB cells in the presence of 20 μg/ml 9F11, C9DHS supplemented with purified C9 could (Fig. 3).

Defect in the C-Activating Capacity of 2G9

MOLT4/IIIB cells and U937/IIIB cells were incubated with varying concentrations of 9F11 and 2G9 in the presence of 20% FHS. Although 0.07 μg/ml 9F11 induced C-mediated cytolysis, 2G9 had no such capacity, even at a concentration as high as 10 μg/ml (Fig. 4, A and B). C3 deposition on MOLT4/IIIB cells following incubation with 9F11 or 2G9 in the presence of 20% FHS was evaluated by flow cytometric analysis using FITC-labeled anti-C3. At 2 μg/ml, 9F11 stained MOLT4/IIIB cells with more than 30 times higher intensity than the background level. On the other hand, C3 staining by 2G9 of the HIV-1-infected cells was limited, even at 100 μg/ml. Increasing the amount of 2G9 to 300 μg/ml did not substantially increase the level of staining (Fig. 4C).

Amino Acid Sequences Coding for 2G9 and 9F11

Base sequences of the cDNA coding for the μ- and κ-chains of 2G9 and 9F11 were determined, and Fig. 5 shows the deduced amino acid sequences of these chains. There was no difference in the constant regions of μ- and κ-chains between 2G9 and 9F11 (Fig. 5, A and B), and we could not find any distinguishing feature that would account for the inability of 2G9 to activate C.

Effect against HIV-IIIB Propagation

MOLT4/IIIB cells and naive MOLT4 cells were mixed at a ratio of 1:50, and the mixtures cultivated with 20% FHS in the presence or absence of 9F11 or 2G9. The percentage of infected cells on days 0, 3 and 5 was determined by staining with anti-p24 Ab (KC57) following fixation with paraformaldehyde. The presence of 5 μg/ml 9F11 suppressed the increase in HIV-1-infected cells as shown in Fig. 6A. Although 2G9 had little capacity to induce C activation (Fig. 4), the presence of 100 μg/ml 2G9 suppressed the increase in HIV-1-infected cells to some extent (Fig. 6B).

Induction of Apoptosis by 2G9

Incubation of MOLT4/IIIB cells in the presence of 50 μg/ml 2G9 for 48 hr induced apoptosis as detected by the TUNEL method (Fig. 7). On the other hand, 9F11 did not induce cytolysis in the absence of FHS. 2G9-antigen (Ag) might be closely related to a receptor that induces a signaling cascade for apoptosis.

Reactivity to OM10.1 Cells of a Latently Infected Cell Line

OM10.1 is a cell line established from HL60 infected with HIV-IIIB, and can be maintained in a latently infected state in the presence of 1 μg/ml AZT. Although OM10.1 cells were not stained with 0.5β (anti-gp120), UCHT-1 (anti-CD3), 4G6 (anti-gp41) or 9F11, they were stained with 2G9 on flow cytometric analysis (Fig. 8A). Cultivation of OM10.1 cells in the absence of AZT and in the presence of 1 ng/ml TNF-α activated the replication of HIV-1 and cultivation for 10 days made the cells reactive with 0.5β and 4G6. On HIV-1-activated OM10.1 cells, expression of 2G9-Ag increased only slightly (Fig. 8B).

Apoptosis of OM10.1 Cells by 2G9

OM10.1 cells and TNF-α-activated OM10.1 cells were cultivated in the presence or absence of 12.5 μg/ml 2G9 for 2 days. Both types of cells were stained with Annexin V after incubation with 2G9 regardless of the presence or absence of FHS (Fig. 9). OM10.1 cells stimulated with 1 ng/ml TNF-α were cultivated for 2 and 3 days in the presence of 12.5 or 50 μg/ml 2G9. Following culture, cells were subjected to staining with KC57 to detect p24-positive cells. Among control

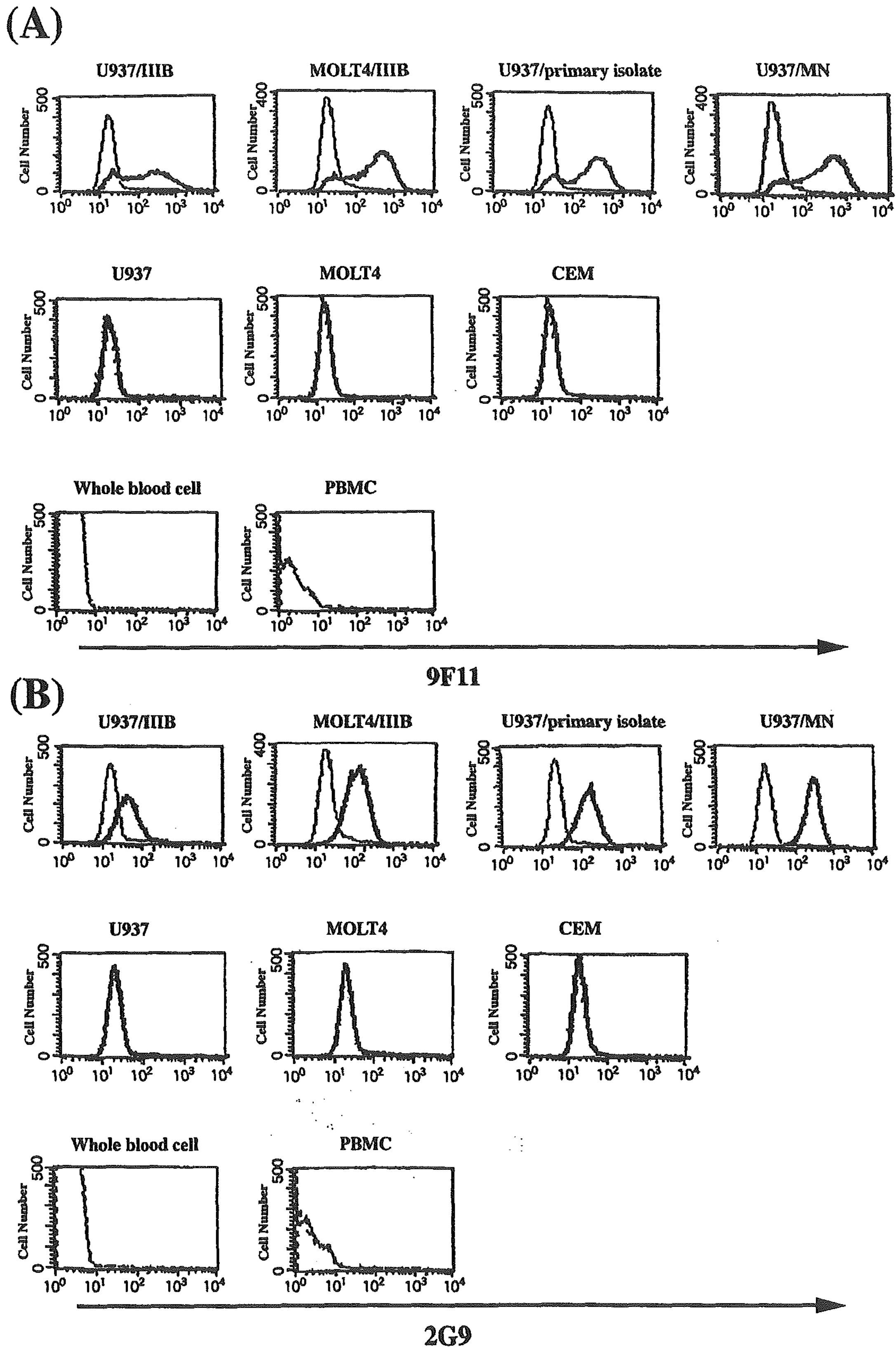

Fig. 1. Reactivity of 9F11 (A) and 2G9 (B) with HIV-1-infected cell lines, uninfected cell lines, whole blood cells and PBMC analyzed by flow cytometry using FITC-labeled anti-human IgM as the second Ab.

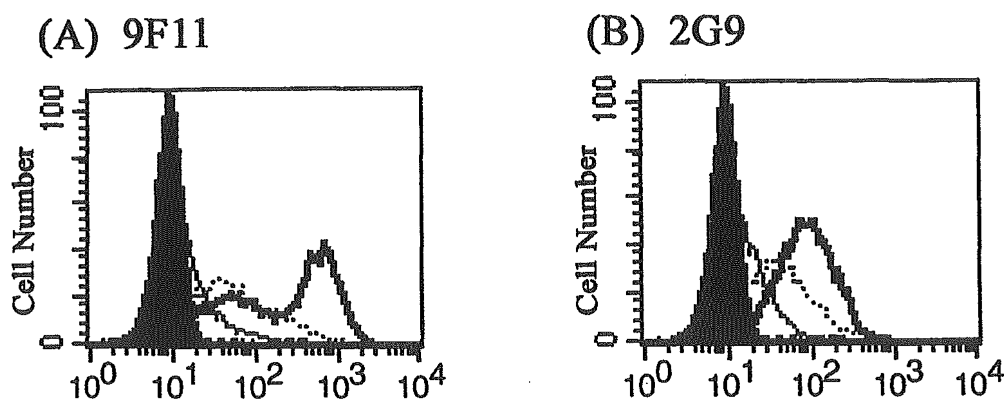

Fig. 2. Reactivity of 9F11 (A) and 2G9 (B) with MOLT4/IIIB cells cultivated in the absence (bold line), or presence of 5 μg/ml tunicamycin for 2 days (dotted line) and for 4 days (broken line).

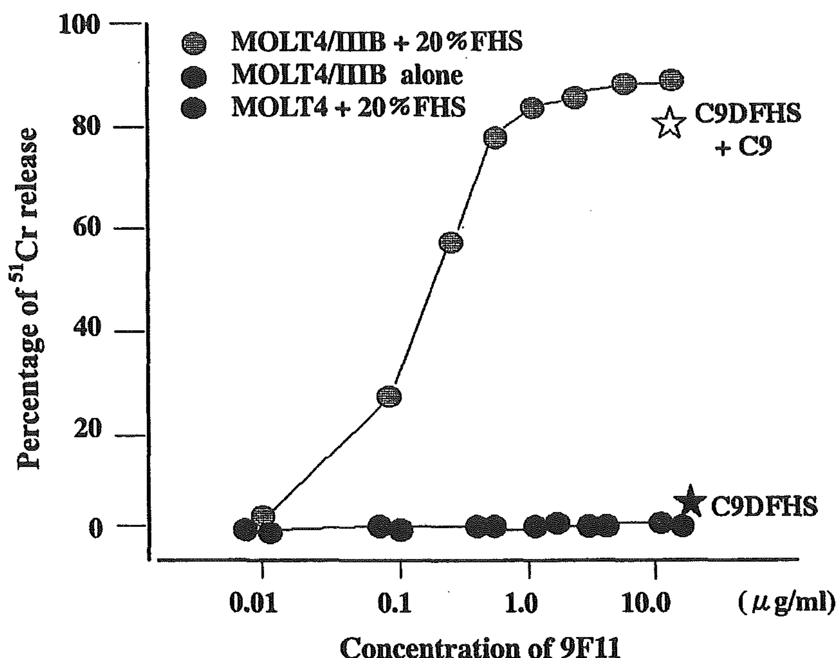

Fig. 3. Complement-mediated cytolysis of ^{51}Cr-labeled cells in the presence of varying amounts of 9F11. Cytolysis of MOLT4/IIIB cells and MOLT4 cells. The solid star indicates 20% C9-deficient fresh human serum (C9DFHS) was used instead of fresh human serum (FHS). The open star indicates C9DFHS supplemented with purified C9.

cells cultivated without 2G9, KC57-positive cells comprised 50.0% and 78.9% of the total cell population after 2 days and 3 days of culture, respectively. In the presence of 12.5 μg/ml 2G9, these values were 33.5% and 57.3% after 2 and 3 days, respectively. Cultivation in the presence of 50 μg/ml 2G9 for 3 days reduced the percentage of K57-positive cells to 12.1% (Fig. 10).

Sequence Data

The GenBank accession numbers for heavy and light chain mRNA of 9F11 are AY510105 and AY510107, and each of 2G9 are AY510104 and AY510106, respectively. Those protein IDs are AAS01770, AAS01772 and AAS01769, AAS01771, respectively.

Discussion

The two hybridoma cell lines obtained produced human IgM mAbs harboring human μ- and κ-chains, and were designated 9F11 and 2G9. 9F11 was highly efficient in causing C-mediated cytolysis of HIV-1-infected MOLT4 cells at a concentration as low as 0.4 μg/ml or less. The high efficiency of 9F11 also indicates that this human IgM mAb is stable because its

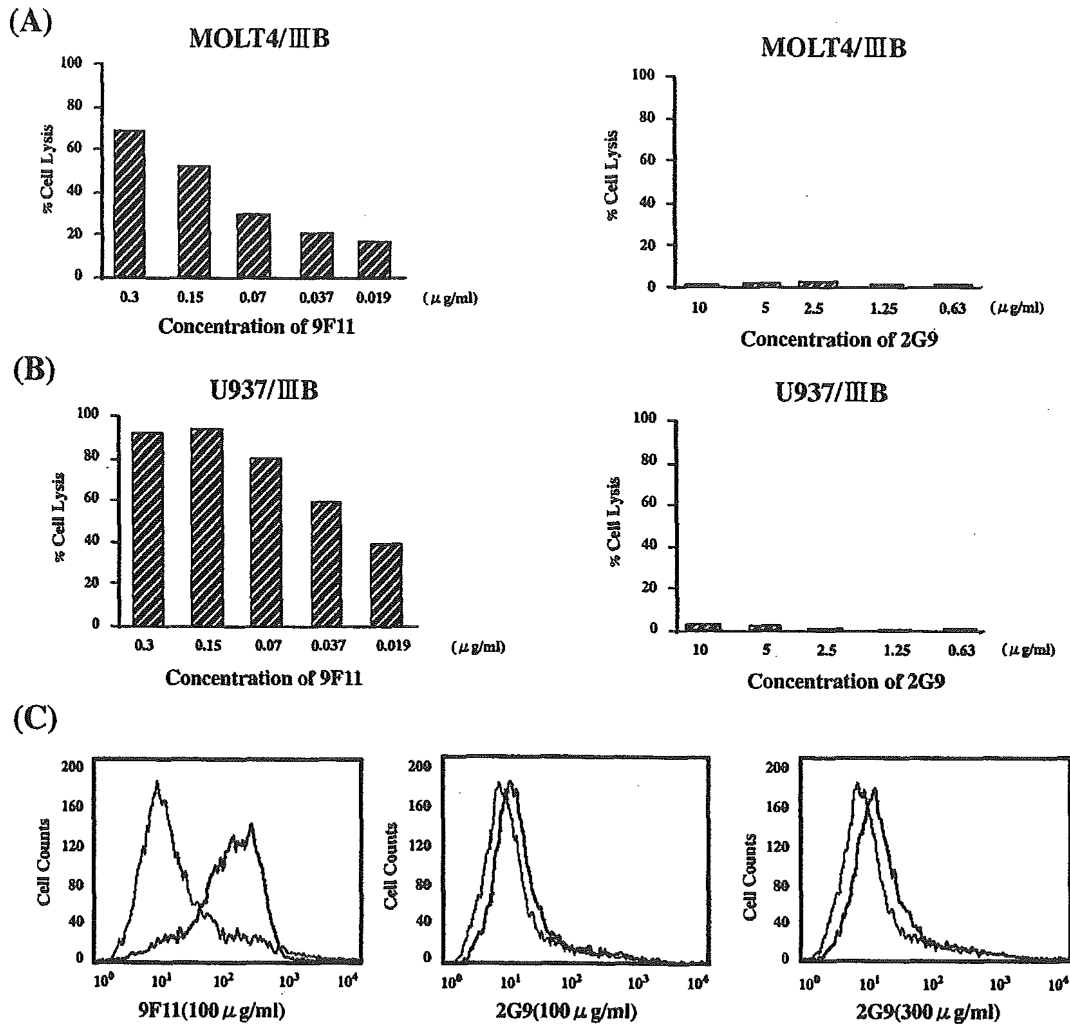

Fig. 4. Comparison of 9F11 and 2G9. Cytolytic effect in the presence of 20% FHS on (A) MOLT4/IIIB and (B) U937/IIIB, and (C) C3 deposition, as determined by flow cytometry of MOLT4/IIIB following incubation with 20% FHS in the presence of 9F11 or 2G9.

activity remained intact after several steps of purification. On the other hand, mAb 2G9 had no ability to induce C-mediated cytolysis of HIV-1-infected MOLT4 cells. This difference in C activation by 9F11 and 2G9 is an interesting finding and deserves investigation. We determined the cDNA sequences coding for these mAbs to compare their primary structures. However, we found no difference in amino acid sequences between the two with respect to the constant regions of their μ- and κ-chains (Fig. 5). Amino acid sequences might not be responsible for the difference in efficiency at activating C. C activation might depend on the concentration of the target Ag and/or the mobility of the IgM-reactive antigenic molecules on the cell membranes. The number of antigenic epitopes, which can bind to an IgM Ab might also influence its C-activating efficiency. A possible approach to understanding this phenomenon would be the identification of the reactive antigens. As far as analyzed, neither of the antigens could be regarded as direct gene products of HIV-1 such as gp120, gp41 and Nef that can be detected on the cell surface of HIV-1-infected cells. Since tunicamycin treatment abolished the reactivity of HIV-1-infected cells with 9F11 and 2G9, the antigenic epitopes would likely involve a sugar moiety (Fig. 2). It is possible that these antigens could be undetectable under normal conditions and be upregulated or modified following HIV-1-infection. In a preliminary trial with PM1 cells infected with JRFL or SF162, which are both R5 type HIV-1, 9F11 stained the infected cells to some extent, although 2G9 did not (personal communication from Dr. Shuzo Matsushita, Kumamoto University, Japan). This evidence indicates that the antigenic molecules might be upregulated as a consequence of a disturbance in the cellular metabolism due to HIV-1-infection. Our efforts for identifying the antigenic molecules continue.

(A) IgH μ chain

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2G9u-aa    1  PWIPRPIHLVISTEHRGFTMELGLRWVFLVAI-LE-GVQCEVQLVESGGGLVKPGGSLRL   58
9F11u-aa   1  -------LNSGPGQSPELQTMSVSFLIFLPVLGLPWGVLSQVQLQQSGPGLVKPAQTLSL   53

2G9u-aa   59  SCAASGFTFSTYSM--NWVRQAPGKGLEWVSSIS-SSSSYIYYADSVKGRFTISRDNAKN  115
9F11u-aa  54  TCAISGDSVSSNSATWNWIRQSPLRGLEWLGRTYYRSKWYNDYAVSVKSRITINPDTSKN  113

2G9u-aa  116  SLYLQMNSLRAEDTAVYYCARDLLIAVA-----GHWGQGTLVTVSSGSASAPTLFPLVSC  170
9F11u-aa 114  QFSLQLNSVTPEDTAVYYCARENYYGSGRYNWFDPWGQGTLVTVSSGSASAPTLFPLVSC  173

2G9u-aa  171  ENSPSDTSSVAVGCLAQDFLPDSITFSWKYKNNSDISSTRGFPSVLRGGKYAATSQVLLP  230
9F11u-aa 174  ENSPSDTSSVAVGCLAQDFLPDSITFSWKYKNNSDISSTRGFPSVLRGGKYAATSQVLLP  233

2G9u-aa  231  SKDVMQGTDEHVVCKVQHPNGNKEKNVPLPVIAELPPKVSVFVPPRDGFFGNPRKSKLIC  290
9F11u-aa 234  SKDVMQGTDEHVVCKVQHPNGNKEKNVPLPVIAELPPKVSVFVPPRDGFFGNPRKSKLIC  293

2G9u-aa  291  QATGFSPRQIQVSWLREGKQVGSGVTTDQVQAEAKESGPTTYKVTSTLTIKESDWLSQSM  350
9F11u-aa 294  QATGFSPRQIQVSWLREGKQVGSGVTTDQVQAEAKESGPTTYKVTSTLTIKESDWLSQSM  353

2G9u-aa  351  FTCRVDHRGLTFQQNASSMCVPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTTYDS  410
9F11u-aa 354  FTCRVDHRGLTFQQNASSMCVPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTTYDS  413

2G9u-aa  411  VTISWTRQNGEAVKTHTNISESHPNATFSAVGEASICEDDWNSGERFTCTVTHTDLPSPL  470
9F11u-aa 414  VTISWTRQNGEAVKTHTNISESHPNATFSAVGEASICEDDWNSGERFTCTVTHTDLPSPL  473
                                                                    * * *
2G9u-aa  471  KQTISRPKGVALHRPDVYLLPPAREQLNLRESATITCLVTGFSPADVFVQWMQRGQPLSP  530
9F11u-aa 474  KQTISRPKGVALHRPDVYLLPPAREQLNLRESATITCLVTGFSPADVFVQWMQRGQPLSP  533

2G9u-aa  531  EKYVTSAPMPEPQAPGRYFAHSILTVSEEEWNTGETYTCVVAHEALPNRVTERTVDKSTG  590
9F11u-aa 534  EKYVTSAPMPEPQAPGRYFAHSILTVSEEEWNTGETYTCVVAHEALPNRVTERTVDKSTG  593

2G9u-aa  591  KPTLYNVSLVMSDTAGTCYPCWPAHRLGAAGRSVCAFS                        628
9F11u-aa 594  KPTLYNVSLVMSDTAGTCYPCWPAHRLGAAGRSVCAFS                        631
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(B) IgL κ chain

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2G9k-aa    1  QSGHSMDMRVPAQLLGLLLLWLPDTRCDIQMTQSPSSLSASVGDRVTITCRASQGISNYL   60
9F11k-aa   1  -SGHSMDMRVPAQLLGLLLLWFPGSRCDIQMTQSPSSVSASVGDRVTITCRASQGISSWL   59

2G9k-aa   61  AWYQQKPGKVPKLLIYAASTLQSGVPSRFSGSGSGTDFTLTISSLQPEDVATYYCQKYNS  120
9F11k-aa  60  AWYQQKPGKAPKLLIYDASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQANS  119

2G9k-aa  121  APYTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNA  180
9F11k-aa 120  FPLTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNA  179

2G9k-aa  181  LQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKLYACEVTHQGLSSPVTKSFNRGE  240
9F11k-aa 180  LQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKLYACEVTHQGLSSPVTKSFNRGE  239

2G9k-aa  241  CREKCPHLLLSSSLTPSHPLASDPFSTGDLPLLR                            274
9F11k-aa 240  CREKCPHLLLSSSLTPSHPLASDPFSTGDLPLLR                            273
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Fig. 5. Amino acid sequences deduced from base sequences of cDNA coding for (A) μ-chain and (B) κ-chain of 2G9 and 9F11. Stars indicate amino acids considered to be responsible for C1q binding in μ-chains. Arrows indicate constant regions of μ- and κ-chains, respectively.

Although 2G9 could not induce C-mediated cytolysis of HIV-1-infected cells, it could induce apoptosis of the cells following a prolonged incubation. Furthermore, it is interesting that 2G9 reacted with OM10.1 cultivated in the presence of AZT acting as a means of maintaining these cells in a latently infected state. Although cultivation of OM10.1 in the absence of AZT-induced cell surface expression of Nef before gp120 expression (7), 2G9-Ag was detected before the expression of Nef, indicating that 2G9-Ag would not be directly related to

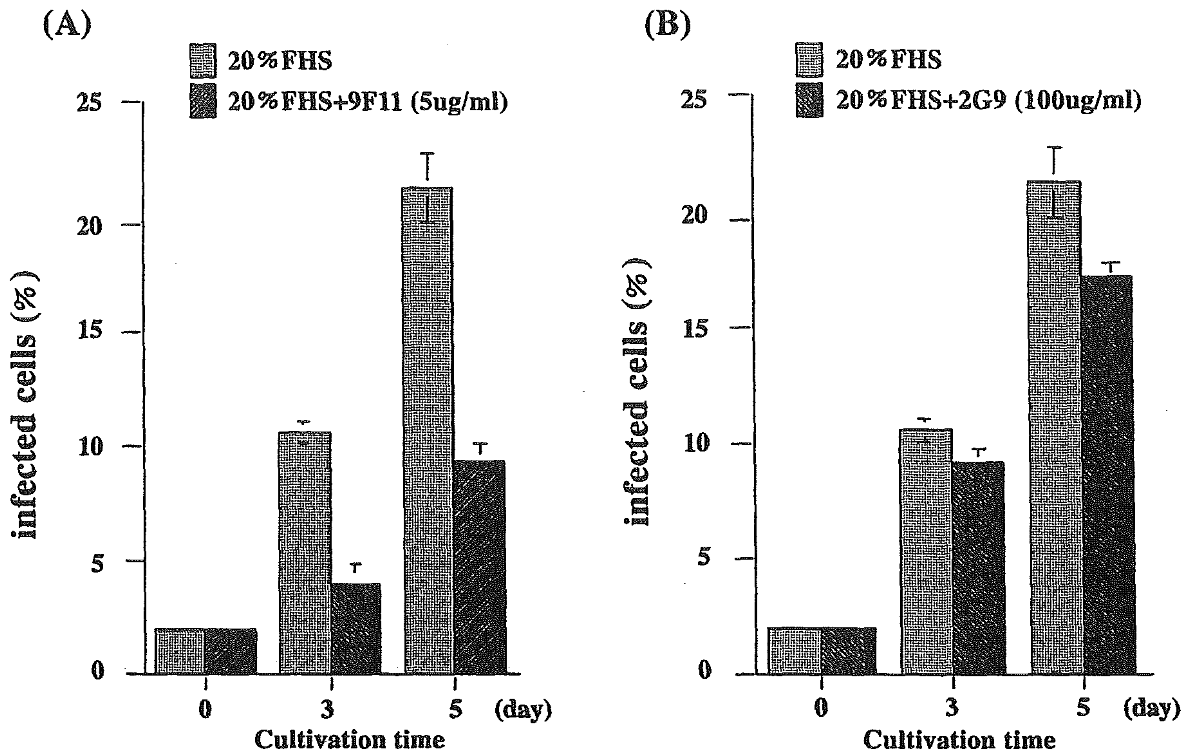

Fig. 6. Effect of (A) 5 μg/ml 9F11 and (B) 100 μg/ml 2G9 on expansion of infection in a mixed culture of MOLT4/IIIB and naive MOLT4 cells at a ratio of 1:50 in the presence of 20% FHS. The percentage (%) of infected cells was determined on days 0, 3 and 5 of cultivation.

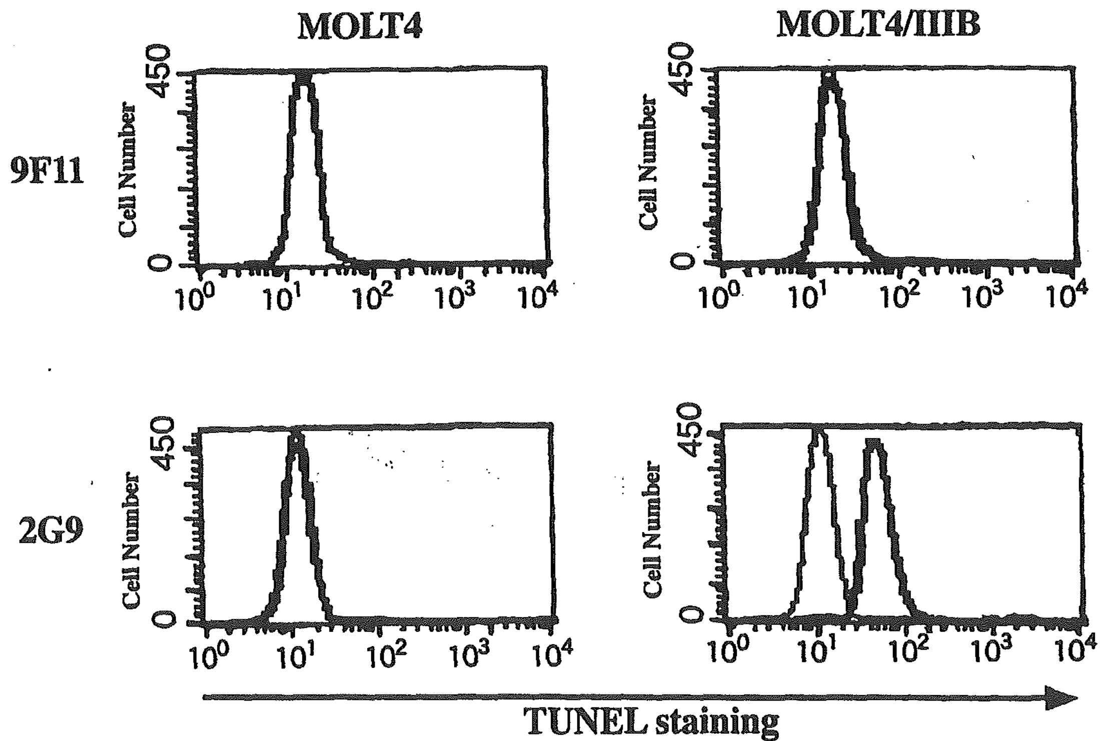

Fig. 7. Detection of 2G9-induced apoptosis with the TUNEL method. MOLT4 cells and MOLT4/IIIB cells were cultivated for 3 days in the presence of 50 μg/ml 9F11 or 2G9.

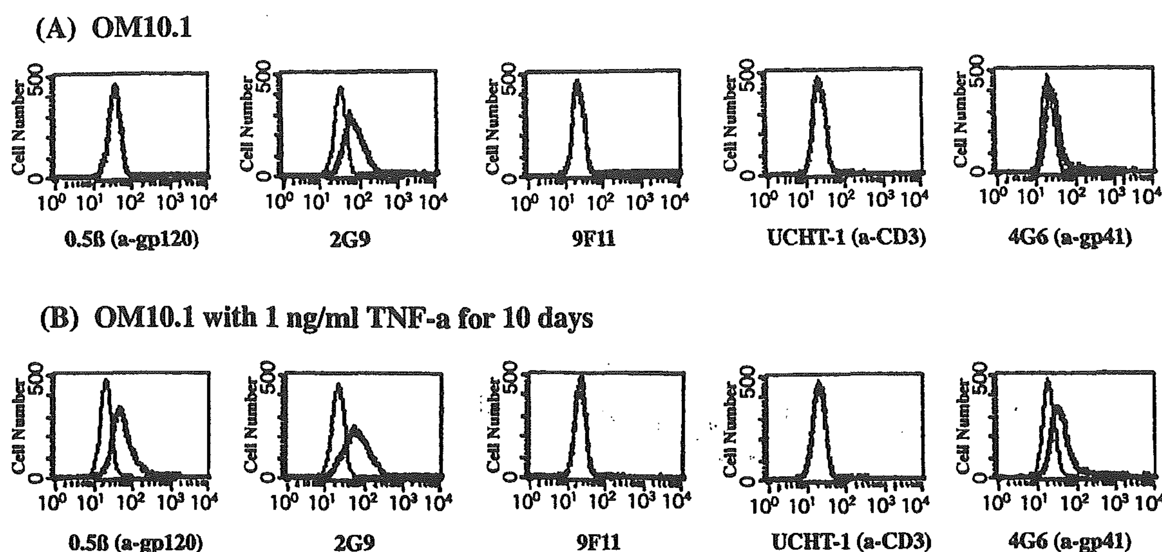

Fig. 8. Reactivity of (A) OM10.1 cells and (B) TNF-α-stimulated OM10.1 cells with various Abs as determined by flow cytometric analysis. 0.5β, UCHT-1 and 4G6 are mAbs to gp120, CD3 and gp41, respectively.

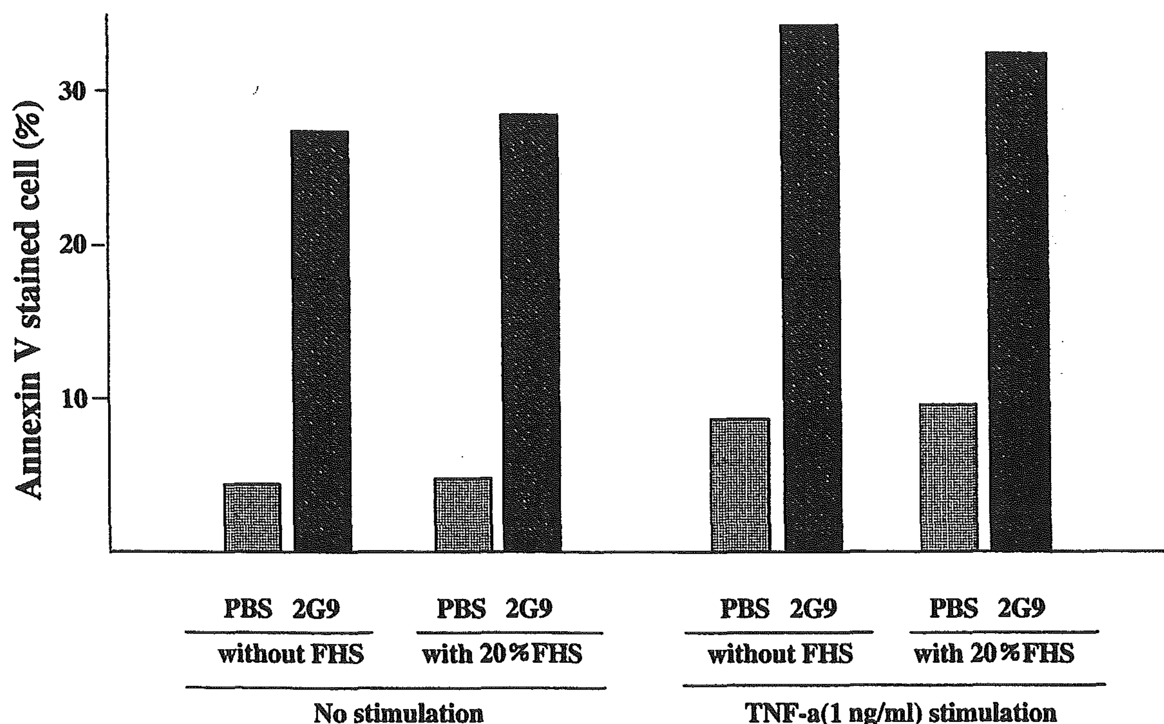

Fig. 9. Effect of 2G9 on OM10.1 cells and TNF-α-stimulated OM10.1 cells. Annexin V staining following incubation with PBS or 2G9 in the presence or absence of 20% FHS.

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

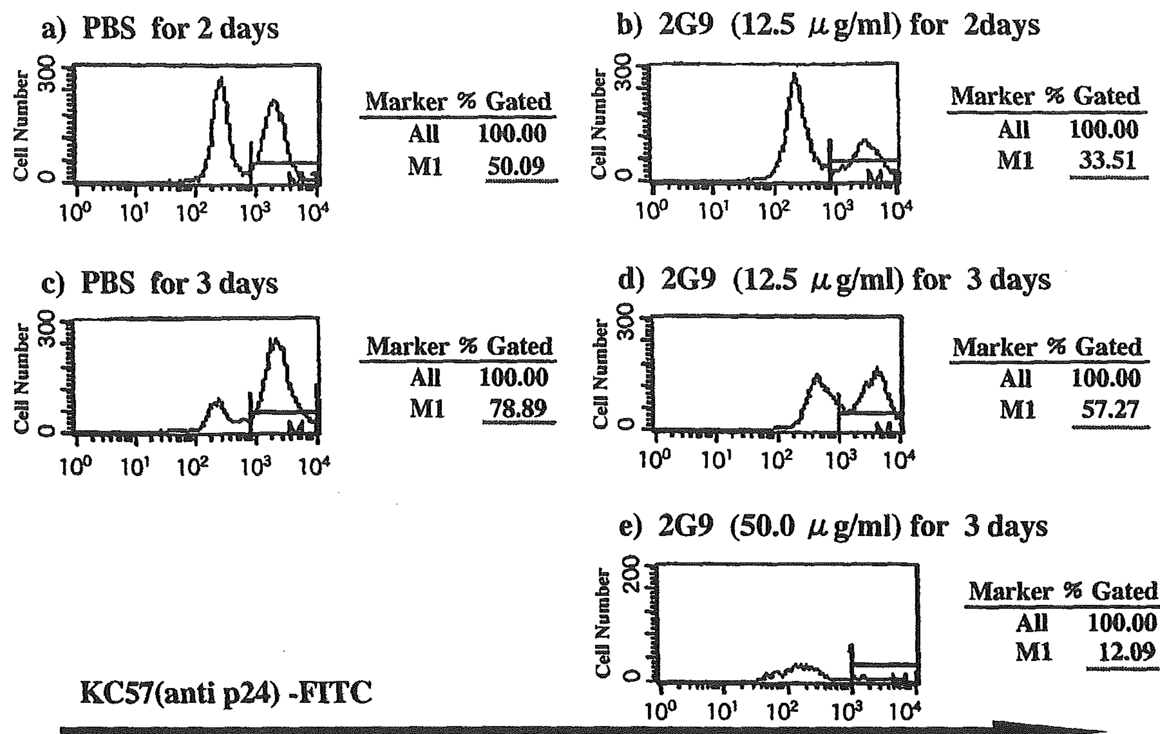

Fig. 10. Effect of 2G9 on the increase in HIV-p24-positive cells among OM10.1 cells stimulated with TNF-α. Without 2G9, p24-positive cells rose to 50.1% in 2 days (a) and 78.9% in 3 days (c). In the presence of 12.5 μg/ml 2G9, they were suppressed to 33.5% (b) and 57.2% (d), respectively. In the presence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

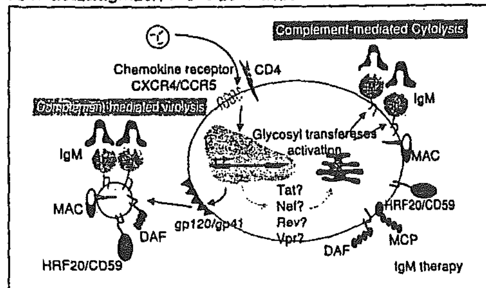
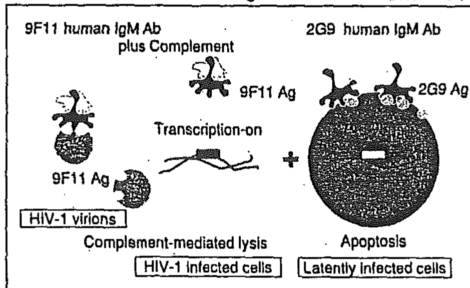


図2. HIV-1感染症に対するヒトIgMモノクローナル抗体の効果



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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.

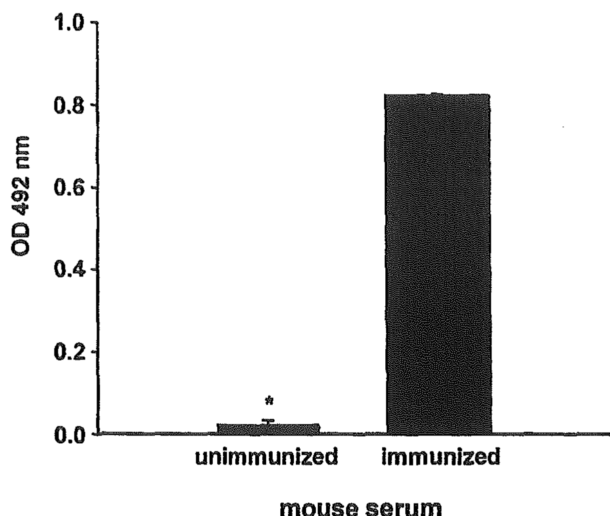
Results

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.



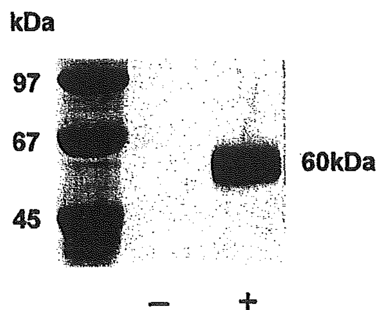


Fig. 1. Reactivity of proCPR immunized mouse serum with partially purified proCPR. A: Serum from proCPR immunized proCPR-deficient mouse significantly reacted with partially purified mouse proCPR, while the serum from unimmunized proCPR-deficient mouse did not. *$P<0.05$ vs. immunized. B: Western blotting 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 CPR.

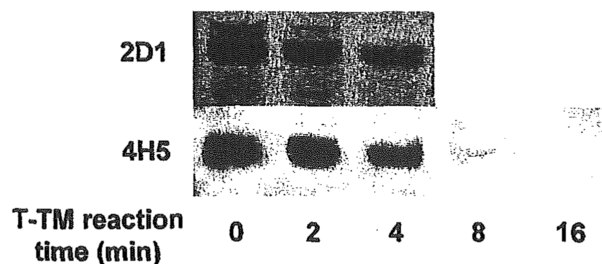


Fig. 2. The reactivity of purified mAbs to proCPR. mAbs 2D1 and 4H5 were subjected to react with partially purified proCPR incubated with T-TM for indicated periods. Both experiments were executed for at least three times to confirm repeatability.

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.

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

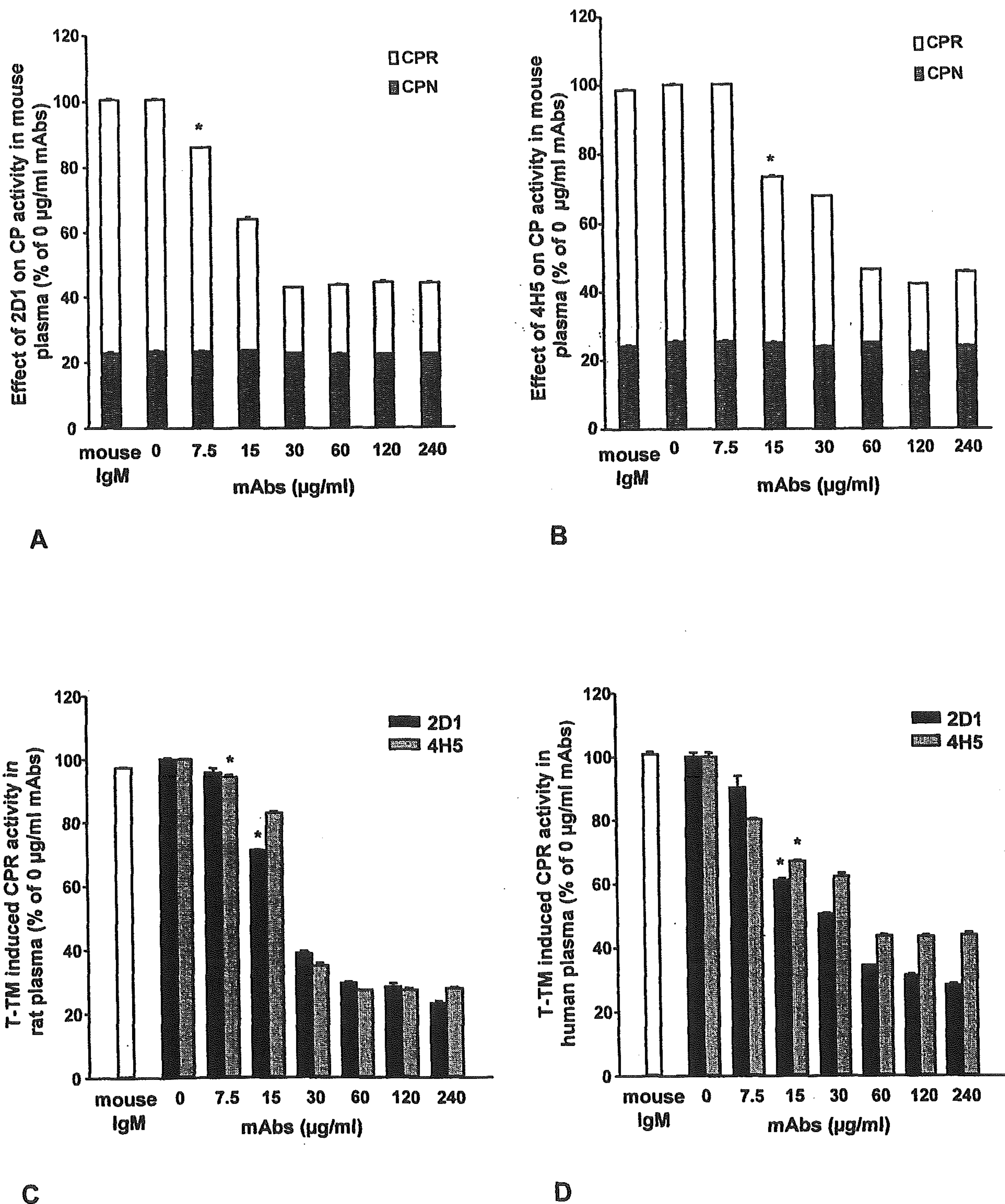


Fig. 3. Effect of mAbs on CP activities. CPN activity was determined as CP activity in plasma before addition of T-TM and $CaCl_2$. The CP activity after addition of T-TM and $CaCl_2$ is total activity of CPN and CPR generated by T-TM. Effects of 2D1 (A) and 4H5 (B) on CP activity in mouse plasma are 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.

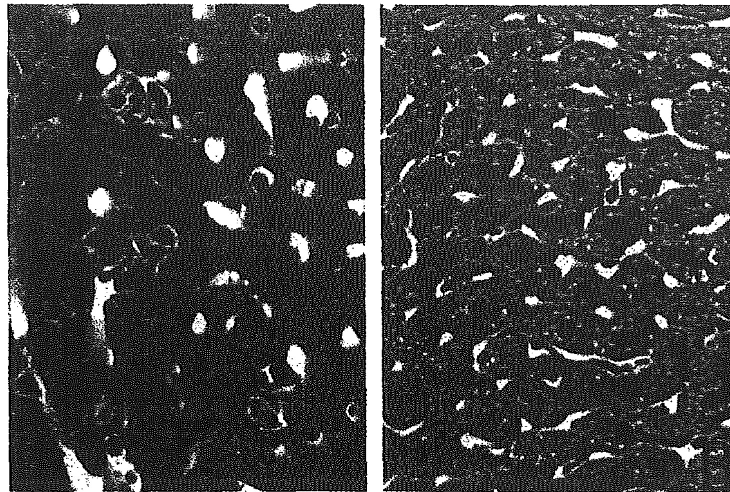


Fig. 4. The histological features of the liver. A: The liver of BALB/c mice sacrificed 7 days following intravenous injection of anti-mouse proCPR mAb (100 µg of 4H5) showed focally minute lesions composed of dense collection of mononuclear and polymorphonuclear inflammatory cells. Such lesions were 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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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.
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Key words: C3c, complement activation, DAF, eradication, *Helicobacter pylori*, HRF20.

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