Table 1

患者CD4陽性Tリンパ球　　9F11陽細胞

	9F11(+) positive (cells)	9F11(+) count (cells)	(%)	9F11(-) positive (cells)	9F11(-) count (cells)	(%)	CD4 (cells/μl)	VL (copies/ml)	log(VL)
1	282	905	29	8	384	2	86	4.2*10^2	2.6
2	71	328	22	7	312	2	172	2.3*10	1.4
3	126	609	21	0	300	0	173	50	1.7
4	103	610	17	5	327	2	393	1.3*10	1.1
5	106	644	16	8	300	3	139	1.4*10^5	5.1
6	148	999	15	2	302	1	137	50	1.7
7	85	623	14	3	300	1	344	7.7*10^3	3.9
8	90	627	13	2	328	1	313	3.9*10^4	4.6
9	80	632	13	2	307	1	339	50	1.7
10	89	706	13	1	303	0	1077	50	1.7
11	165	1514	11	36	933	4	235	50	1.7
12	70	659	10	3	313	1	665	1.1*10	1.0
13	68	655	10	1	300	0	598	50	1.7
14	67	677	10	3	302	1	379	50	1.7
15	68	720	9	3	340	1	357	2.6*10^3	3.4
16	85	944	9	4	300	1	440	7.7	1.7
17	53	575	9	1	308	0	755	50	1.7
18	47	652	7	3	331	1	114	9.1*10^2	3.0
19	33	612	5	0	300	0	230	3.4*10	1.5
20	30	600	5	3	300	1	312	50	1.7
21	30	669	4	0	300	0	609	50	1.7
22	56	1285	4	5	816	1	248	50	1.7
	Average		12			1			
	Max		29			4			
	Min		4			0			

Table 2

健常の　CD4陽性Tリンパ球
9F11抗陽細胞

	9F11抗体(+)			9F11抗体(-)　Neg. control		
	Positive (cells)	Count (cells)	陽性 (%)	Positive (cells)	Count (cells)	陽性 (%)
Normal 1	26	58	5	0	30	0
Normal 2	24	52	5	0	30	0

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分担研究報告書

HIV感染患者からのLAK-Tリンパ球に関する研究

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研究要旨 HIV感染患者のCD8陽性Tリンパ球には、HIV感染細胞を攻撃するエフェクターTリンパ球が存在し、生体防御機構の役割をある程度果たしている。癌に対するエフェクターT リンパ球を増殖させて患者に戻す自家養子免疫療法であるLymphokine Avtivated Killer T cells(LAK-T)療法がHIV感染細胞に対しても有効であると考えられる。しかし、Tリンパ球をCD3刺激とIL-2によって増殖させるとHIV感染CD4陽性Tリンパ球も一緒に増殖させてしまうリスクを伴う。そこで、患者末梢血からLAK-T細胞を培養増殖させる際に、HIV感染細胞を攻撃破壊する9F11抗体と補体で処理することにより感染細胞を排除する方法を開発するための基礎的研究を行った。また、生体内でIgM抗体が大量に反応すると過度の補体活性化により深刻な炎症病態を惹起する危険性も想定される。そこで、補体反応系での起炎反応の主要因子であるC5aアナフィラトキシンを制御するペプチド剤についての検討も行った。

A. 研究目的

HIV感染細胞に反応するヒトIgM抗体(9F11等)はHIVが感染した株化培養細胞に補体と共に働いて細胞障害を起こすことができる。そこで、これらのヒト抗体がHIV感染患者の血液中に含まれる感染細胞や潜伏感染細胞を障害除去できる可能性がある。そこで、患者リンパ球から潜伏感染細胞を除去して、抗CD3抗体とIL-2で刺激増殖させたLAK-Tリンパ球を作製して治療に応用する為の基礎的知見を集積する。また、感染細胞に9F11が反応して補体が大量に活性化されるとC5aアナフィラトキシンなどが生成されるので、その制御法についての検討もおこなう。

B. 研究方法

HIV感染患者の末梢血リンパ球(必要に応じCD8陽性細胞は除去しておく)の初代培養に9F11等と新鮮ヒト血清補体を添加することによる感染リンパ球の排除を検討する。HIV感染患者の末梢血リンパ球をCD3に対する抗体で刺激したあとIL-2で増殖させてLymphokine Avtivated Killer T cells(LAK-T)を作成するとき、活性化リンパ球にも9F11抗原が出現することが分かったので、9F11と新鮮血清補体の作用を前処理として行い、その後でLAK-Tを増殖させる方法について検討をおこなう。なお、HIV感染患者リンパ球の増殖は、名古屋市立大学分子医学研究所のPC実験施設を用いる。また、過剰な補体反応による副作用の可能性に対処するため、C5a阻害ペプチド等の

活用法も検討する。

(倫理面への配慮)

HIV 感染患者の末梢血を用いての解析に際しては、国立名古屋病院倫理委員会の承認のもとに、実験目的などを明確に説明して、書面による同意を得た上で患者末梢血の採取を行って実験を実施した。また、癌患者や非癌患者などの末梢血リンパ球をモデル解析として使用する際にも、福祉村病院倫理委員会で承認を受け、書面による同意書を得たうえで実験解析を行った。その際、得られた個人情報が守られるよう慎重に配慮し、情報の管理担当は、秘守義務が課せられる看護士、薬剤師および医師に限定している。

C. 研究結果

LAK-T を培養増殖させるための、倍地条件についての基礎的比較検討を行い、T リンパ球サブセットの特徴の差異についての解析も行った。基礎的比較検討に用いる末梢血としては、LAK－T 療法を希望した癌患者および非癌患者のものを用いた。Regulatory T リンパ球である CD25 陽性 CD4 リンパ球の比率は、個人差が大きいことが分かった。ので、その比率を下げるためのサイトカインの添加を試みたが、一定の成果を得られるには至っていない。また、がん患者では CD8a,b 発現細胞の比率が上昇する事が確認された。

LAK-T 細胞に 9F11 とヒト補体血清を作用させると大半のリンパ球の細胞溶解が認められた。そこで、HIV 感染患者の末梢血リンパ球を 9F11 と血清補体で処理するのは抗 CD3 抗体で T リンパ球を刺激する前に行う必要があると考えられた。HIV 感染患者の末梢血に 9F11 と補体として新鮮ヒト血清を添加して翌朝まで培養し、その後で抗 CD3 で刺激すると共に、IL2 を添加して 10 日間培養を行った。9F11 を作用させない場合のコントロールでは、殆どの症例で HIV コア蛋白の p24 抗原が検出されたが、9F11 処理した群では、p24 抗原が抑制されていた。

C5a を阻害する相補性ペプチド PepA を創生できたので、PepA の改良を行い N 末のアラニンをアセチル化した AcPepA は安定性が増し、ラットやマウスで炎症抑制作用を示すことがわかった。LPS 投与で血圧低下を起こさせたサルに AcPepA を投与するとショックを軽減させる効果があった。

D. 考察

LAK-T 細胞を効率よく培養増殖させる培養条件を確立できたが、Regulatory T リンパ球の増殖を抑える方法の開発が必要である。がん患者からのこの LAK-T 細胞では、CD8 細胞の比率が上昇するので、CTL 誘導が期待される。9F11 抗原は、HIV 感染により発現誘導されて細胞膜上に現れる分子であるが、正常 T リンパ球が抗 CD3 抗体と IL-2 で活性化されても発現する膜抗原であるので、HIV 感染患者末梢血リンパ球を 9F11 と血清補体で処理する場合には、抗 CD3 抗体での活性化を行う前に行う必要がある。9F11 とヒト血清補体の処理で、10 日後の培養上清中の p24 抗原を激減できることがわかった。一方、9F11 は細胞溶解を

起こすほどの補体反応を誘起するので、アナフィラトキシンによるショック病態への対応も必要であると想定される。AcPepA は有効な対策剤となる可能性があるので、SHIV 感染サルなどを用いて、AcPepA の有用性の解析も前臨床実験段階では必要であろう。

E. 結論

9F11 ヒト IgM 抗体で HIV 感染患者末梢血を処理してから抗 CD3 抗体と IL-2 で刺激して培養増殖させるとHIV感染細胞を有意に抑制と考えられる。また、強い補体活性化反応で懸念される過剰炎症病態の制御に AcPepA の有用性が示唆された。

F. 健康危険情報

9F11 抗原は正常の活性化 T リンパ球などにも発現するので、ヒトに投与する場合には、一時的に免疫抑制作用が現れる可能性がありうる。補体活性化の副作用についての考慮も必要である。

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活性化自己リンパ球投与による癌の再発予防

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Fig. 1 The intensity of CD8αβ double staining of lymhocytes from cancer bearing patients

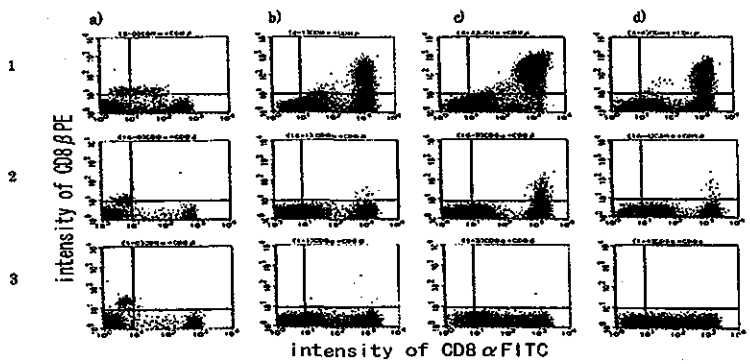


Fig. 2 The intensity of CD8αβ double staining (normal subjects)

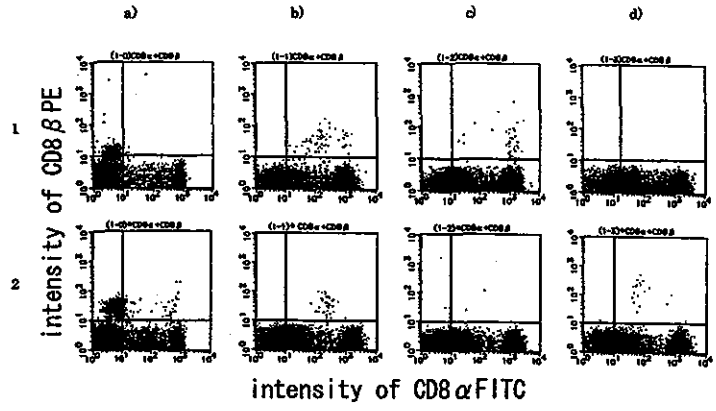


Fig.3 The intensity of staining lymphocytes by different staining method

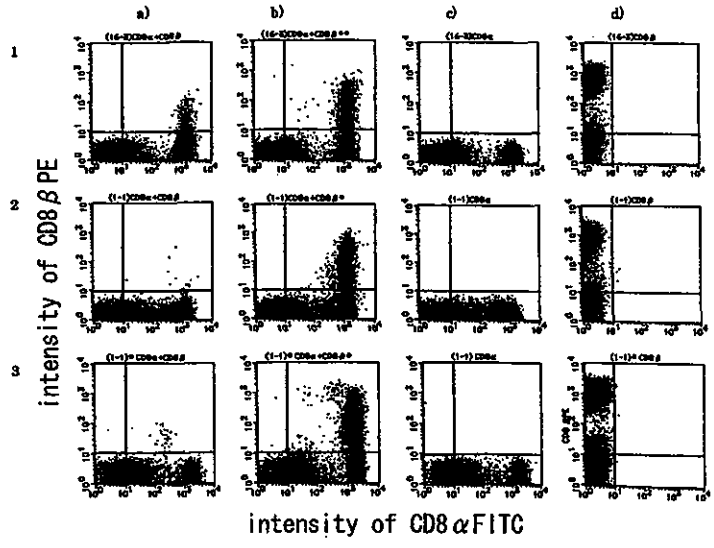


Fig. 4 The percentages of CD8α and CD8β single staining

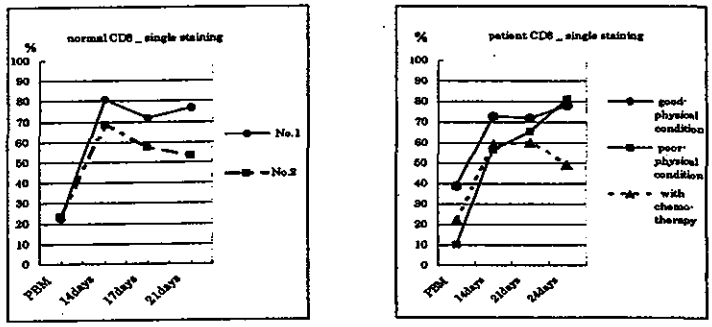


Fig. 5 The percentages of CD8α and CD8β single and double staining

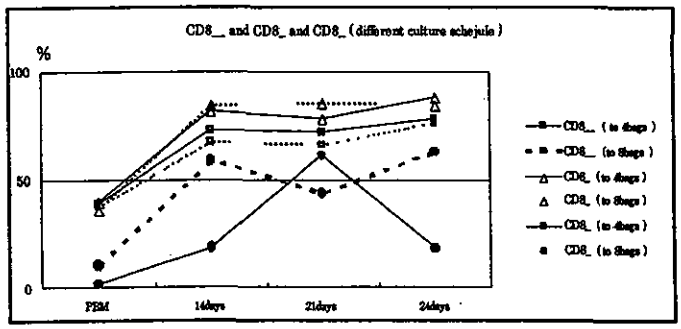
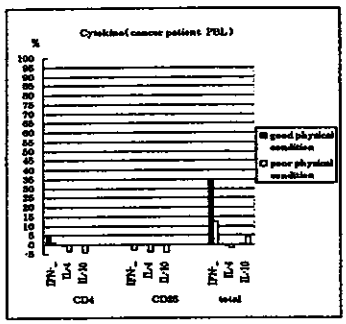
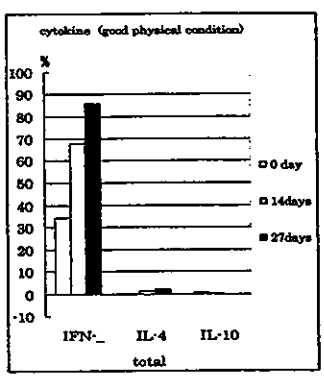


Fig. 6 a The percentage of lymphocytes with the capacity of secreting cytokine

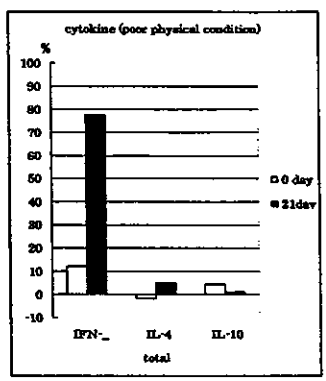
a)



b)



c)



別紙５

研究成果の刊行に関する一覧表

雑誌

岡田 則子（研究代表者）

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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添付資料

(Microbiol. Immunol. 2005 in press)

Human IgM Monoclonal Antibodies Reactive with HIV-1-Infected Cells Generated Using a Trans-Chromosome Mouse

Running title: HUMAN IgM ANTIBODIES to HIV-INFECTED CELLS

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Key words: **Apoptosis, Complement, HIV, Human monoclonal antibody, IgM**

Abstract: **The trans-chromosome (TC) mouse that we used harbors human chromosomes 2, 14 and/or 22, and has undergone knock-out of its endogeneous genes coding for μ- and κ-chains of immunoglobulin. One of these TC mice was immunized with HIV-1-infected U937 cells, and spleen cells from the immunized animal were fused with the mouse myeloma cell line to generate hybridoma cells. We selected hybridomas that produce human IgM antibodies (Abs) reactive with HIV-1-infected MOLT4 cells but not with uninfected MOLT4 cells. Two hybridoma cell lines were established termed 9F11 and 2G9. Although 0.4 μg/ml of 9F11 was able to induce complement-mediated cytolysis of the infected cells in the presence of fresh human serum, 2G9 could not. There was no difference between the two monoclonal Abs in the base sequences of cDNAs coding for the constant regions of μ- and κ-chains. Therefore, we speculate that the ability to activate complement on homologous cell membranes might reflect the structural presentation of antigenic molecules, which could facilitate the binding of an IgM Ab to multiple binding sites resulting in escape from restriction by species-specific inhibitors of complement such as DAF (CD55) and CD59. On the other hand, 2G9 induced apoptosis of HIV-1-infected cells, including latently infected OM10.1 cells, although the Ag for 2G9 remains to be identified. Since both of the Abs had reduced reactivity toward HIV-1-infected MOLT4 cells following cultivation in the presence of tunicamycin, the responsible antigens would involve a sugar moiety.**

(Introduction)

We have previously reported that some normal human sera harboring natural IgM antibody (Ab) against Gg4 ganglioside (Gg4) or GM2 ganglioside (GM2) can induce complement (C)-mediated cytolysis of HIV-1 infected cells (12, 22). Cells are normally protected from homologous C by species-specific membrane inhibitors (11) such as decay accelerating factor (DAF; CD55) (10), membrane cofactor protein (MCP; CD45) (17) and 20 kDa homologous restriction factor (HRF20; CD59) (13). However, on HIV-1-infected cells, C activation by natural IgM Ab may happen to escape restriction by these inhibitors. Although decreased expression of DAF and HRF20/CD59 has been observed to some extent on HIV-infected cells (22) and lymphocytes of AIDS patients (9, 20), HIV-infected cells heavily acted upon by IgG Abs are resistant to C-mediated cytolysis (22). In addition to the high efficiency of IgM Ab in terms of C activation, its large molecular size may allow for C activation at a point somewhat removed from the membrane inhibitors, thus overcoming restriction (22, 23).

To confirm that IgM Ab is really responsible for the C-mediated cytolysis of HIV-1-infected cells, we used a human monoclonal Ab (mAb) against GM2 (L55 Ab) (5), and demonstrated that it has the capacity to cause cytolysis of HIV-infected cells in the presence of fresh human serum (FHS) as a source of homologous human C.

Furthermore, anti-GM2 IgM mAb and C destroyed HIV particles as well as HIV-infected cells (23). In addition, HIV-1 spreading in culture was strongly inhibited by the anti-GM2 human mAb in combination with anti-HIV agents such as AZT or KNI-272 which are a reverse transcriptase (RT) inhibitor and a proteinase inhibitor, respectively (12, 23).

Following depletion of $CD8^+$ cells, primary cultured lymphocytes from HIV-1-infected patients released HIV-1 virions into the culture medium. The addition of L55 in the presence of FHS partially suppressed HIV-1 generation, and in combination with 1 μM AZT, suppression was complete, indicating that HIV-1-infected lymphocytes in these patients would be effectively eliminated by C-mediated cytolysis with IgM Ab in the presence of AZT (12, 14).

We previously reported that seropositive individuals who had survived for over 10 years possessed levels of IgM Abs cytolytic to HIV-1-infected cells which were higher than those of patients who had developed AIDS in a shorter period (15). We also found that most of the cytolytic sera from HIV-infected patients contained IgM Ab against GM2 as determined by ELISA (21). Therefore, the presence of anti-GM2 Ab may be beneficial for prolonged protection against AIDS.

In addition to the human IgM mAb against GM2, we attempted to generate human IgM mAb-producing cells using HIV-1 (HIV-IIIB)-infected cells to immunize trans-chromosome mice (TC mice) harboring human chromosomes 2, 14 and/or 22 (ref. 6, 18), whose endogenous murine genes for μ- and κ--chains had been knocked out in the TC mice to induce human Abs against the Ags used in immunization (6).

Materials and Medthods

Cells and viruses. U937 (a human monocyte cell line), U937/IIIB, U937/MN, U937/momo, MOLT4 (a human T cell line) (8), MOLT4/IIIB, OM10.1 (ref. 1) and CEM (a human leukemia cell line) were used. U937/IIIB, U937/MN and U937/momo are U937 cells persistently infected with the HIV-IIIB strain, HIV-MN strain and a primary isolated HIV-momo strain, respectively. MOLT4/IIIB cells are MOLT4 cells persistently infected with the HIV-IIIB strain. The OM10.1 cell line, which is latently infected with HIV-1, established from the HL60 cell line, was kindly provided by Dr. M. Baba (Kagoshima University, Kagoshima, Japan). All cells were grown in RPMI1640 medium supplemented with 10% FCS, 2 μM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (FCS-RPMI). Whole blood cells

from a healthy adult donor were collected into a sterile heparinized tube and centrifuged at 1,800 rpm for 5 min. The pellets were resuspended in twice the original blood volume of PBS and subjected to Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation to isolate the peripheral blood mononuclear cells (PBMC).

Tunicamycin treatment. HIV-IIIB cells were cultivated in the presence or absence of 5 μg/ml tunicamycin (Sigma, St Louis, MO) in FCS-RPMI for 2 or 4 days. After cultivation, cells were washed and subjected to flow cytometric analysis for their reactivity to human IgM monoclonal Abs as described in the section on flow cytometric analysis presented later.

Trans-chromosome mouse (TC mouse) and hybridoma preparation. TC mice were provided by Kirin Brewery Co. Ltd. (Tokyo, Japan) and one of these was immunized with U937/IIIB once a week for a total of three inoculations. Three days after the final immunization, spleen cells prepared from the immunized mouse were fused with murine myeloma P3U1cells at a ratio of 5:1 in the presence of 50 % polyethylene glycol 1500 (Cell Biology Boehringer Mannheim, Mannheim, Germany). The cells were then dispensed into 96-well tissue culture plates containing HAT medium.

ELISA. Hybridoma culture supernatants were subjected to screening for human IgM by ELISA. Briefly, 0.05 μg/well rabbit anti-human IgM Ab (CAPPEL, Cochranville, PA) were added to 96-well flexible polyvinylchloride-activated microtiter plates and incubated at 4 C overnight. After washing three times with PBS-T (0.05% tween 20 in PBS), the plates were blocked with 25% BlockAce (Yukijirushi, Sapporo, Japan). Hybridoma culture supernatants were applied to the plates (50 μl/well) at room temperature for 1 h. Plates were washed three times with PBS-T, and then 50 μl of anti-human κ chain-horseradish peroxidase (HRP) conjugate were added and plates were incubated at room temperature for 1 h. HRP activity was detected by adding a solution containing 0.015% hydrogen peroxide and 0.04% o-phenylenediamine. Absorbance at 492 nm was determined after 20 min.

Flow cytometric analysis. Human IgM Abs reactive with cells were detected by indirect immunofluorescence staining followed by flow cytometric analysis using a FACSCalibur (Becton Dickinson. CA). Briefly, naive U937, HIV/IIIB-infected U937 (U937/IIIB), naive MOLT4 and HIV/IIIB-infected MOLT4 (MOLT4/IIIB) were incubated with goat IgG (10 mg/ml, Chemicon International Inc.,CA) to block any Fc receptors, were suspended in 50 μl of test sample such as hybridoma culture supernatant, and were then incubated at 4 C for 30 min. After washing three times with PBS containing 0.1% BSA (PBS-0.1%BSA), cells were incubated with FITC-conjugated goat anti-human IgM Ab (CAPPEL) at 4 C for 30 min. Following washing, cells were resuspended in 1% paraformaldehyde and incubated for more than 3 h at 4 C for inactivation of HIV-1, and then analyzed using a FACSCalibur. The reactivity of purified human IgM mAbs, 9F11 and 2G9, against HIV-infected cells was determined using U937/IIIB, U937/MN, U937/momo, MOLT4/IIIB and OM10.1. The reactivity of 9F11 and 2G9 against naïve U937, MOLT4, CEM, human blood cells and human PBMC was also tested. After blocking with goat IgG, cells were suspended in PBS-0.1%BSA containing 10 μg/ml purified 9F11 or 2G9, and incubated at 4 C for 30 min. Following staining with FITC-conjugated goat anti-human IgM Ab, FITC staining was analyzed with a FACSCalibur.

Cytotoxicity Assay. Cytotoxicity was measured with a 4-hr ^{51}Cr release assay as previously described (15). As target cells, U937, U937/IIIB, MOLT4 and MOLT4/IIIB were labeled with ^{51}Cr. Various amounts of mAb (9F11 or 2G9) were used in culture with $2x10^4$ labeled target cells in the presence of 20 % FHS as a C source, and each well of the U-bottomed 96-well plate contained a total volume of

100 µl. After 4 h incubation at 37 C, the amount of ^{51}Cr released in the supernatants was determined. The percentage of specific ^{51}Cr release was calculated according to the following formula:

Percentage of specific release

$$= \frac{\text{(release with mAb and FHS - spontaneous release)} \times 100}{\text{(maximum release - spontaneous release)}}$$

The amount of ^{51}Cr released from cells treated with 5 % Triton X-100 was used as the maximum release value. All assays were performed in triplicate.

Cytotoxicity of C9-deficient serum supplemented with C9. The procedure was the same as that described for the cytotoxicity assay, except that the labeled *MOLT4/IIIB or U937/IIIB* cells were incubated with 9F11 (10 µg/ml) in the presence of normal FHS, C9-deficient human serum (C9DHS), or C9DHS plus C9 (0.01, 0.1, 1 and 10 µg/ml).

C3 deposition on Ab-sensitized HIV-infected cells. MOLT4/IIIB cells were incubated with 9F11 (2 µg/ml) in the presence of 20 % FHS in GVB^{++} (gelatin veronal-buffered saline containing 0.15mM $CaCl_2$ and 1 mM $MgCl_2$) at 37 C for 90 min. Cells were washed with PBS-0.1%BSA and then were blocked with 10 mg/ml goat IgG. After washing three times with PBS-0.1%BSA, the cells were incubated with FITC-conjugated goat anti-human C3 at 4 C for 30 min. Cells were suspended in PBS containing 2 µg/ml propidium iodide (PI) for 2 min, and resuspended in 1% para-formaldehyde in PBS for inactivation of HIV-1 before flow cytometric analysis with a FACSCalibur. PI-positive cells were excluded in the analysis.

Sequencing of cDNA coding for mAbs. The mRNAs were isolated from 2G9 and 9F11 hybridoma cells using a Micro-FastTrack Kit (INVITROGEN, Carlsbad, CA). The dsDNAs were then generated from the mRNAs. Since the 5' ends were variable regions for immunoglobulin µ- and κ-chains, they were analyzed by rapid amplification of the cDNA end (5' RACE) on adaptor-ligated double-strand (ds) cDNAs following cloning. First- and second-strand cDNA synthesis was performed to create blunt ends from mRNAs with T4 DNA polymerase. The ds cDNAs were ligated to the Marathon cDNA adaptor using a Marathon cDNA amplification Kit (CLONTECH, Palo Alto, CA). A 5' RACE reaction was performed using an Advantage cDNA polymerase mix (CLONTECH). DNA sequences of the adaptor primers (CLONTECH) and the gene-specific primers designed for the PCR were as follows.

Adaptor Primer 1 AP1): CCATCCTAATACGACTCACTATAGGGC;

Nested Adaptor Primer 2 (AP2): ACTCACTATAGGGCTCGAGCGGC;

O-8(sense): AGAGTCTGGGCCCACGACCT;

O-7(antisense): GCTCTAGAATGCACACACAGAGC;

O-14(antisense): GATCTAGAGGACCGCAATAG;

2O-14(antisense): GACCGCAATAGGGGTAGGTC;

4A(antisense): GGGTTTTCACAGCTTCGCCATTCT; and

5A(antisense): ACGGCGCTGAAAGTGGCATTG.

To confirm the sequences of cDNA, the following primers were also used.

O-10(sense): TATAGCGGCCGCGCTCTCCTCA;

O-9(antisense): AGGTCGTGGGCCCAGACTCT;

O-70(sense): ACTGTGGCGGCGCCATCTGTC;

1S(sense): GAAAACCCACACCAACATCTCCGA;

1A(antisense): ATTGGGGCGCTGGTCACATACTTCTC;

2S(sense): ACCCCAATGCCACTTTCAGCGCCGT;

2A(antisense): TGGTGGCAGCAAGTAGACATCGGGCCT;

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 x 10^5) were mixed with 4 x 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, FL). The remaining cells were suspended in RPMI 1640 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 Corporation, Indianapolis, IN) (4). MOLT-4/IIIB cells were suspended in culture medium at 1x10^5/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 x 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-) were suspended in culture medium at 1 x 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- . OM10.1 cells (1 x 10^5/ml) were stimulated with TNF-

(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 h or 72 h incubatio 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 Centre 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), 5 % Bri-clone (Bioresearch Ireland, Glasnevin, Ireland) and 3 μg/ml puromycin (Sigma Chemical Co., St. Louis, MO) 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., Ibaragi, 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. 4A and 4B). 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. 5A and 5B), 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 h 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