

## 研究成果の刊行に関する一覧表

雑誌

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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kimbara N., Dohi, N., Miyamoto, M., Asai, S., <u>Okada, H.</u> and <i>Okada, N.</i>	Diagnostic surface expression of SWAP-70 on HIV-1 infected cells.	Microbiol. Immunol.	50(3)	235-242	2006
<u>Okada, N.</u> , Yin, S., Asai, S., Kimbara, N., Dohi, N., Hosokawa, M., <u>Wu, X.</u> , <u>Okada, H.</u>	Human IgM monoclonal antibodies reactive with HIV-1 infected cells generated using a trans-chromosome mouse.	Microbiol. Immunol.	49(5)	447-459	2005
Joh, T., Sasaki, M., Kataoka, H., Tanida, S., Itoh, K., Kondo, Y., Ogasawara, N., Oshima, T., <u>Okada, N.</u> , Ohara, H., Nomura, T., and Itoh, M	Helicobacter pylori eradication decreases the expression of GPI-anchored complement regulators, DAF and HRF20 in human gastric epithelium.	J.Gastro enterol Hepatol	20	1344-1351	2005
He, L., Asai, S., Kawamura, T., Kimbara, N., Tada, T., <u>Okada, H.</u> and <u>Okada, N.</u>	Hepatitis induced by an IgM monoclonal antibody against procarboxypeptidase R.	Microbiol. Immunol.	49(4)	373-380	2005
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<u>岡田則子</u>	HIV 感染症におけるヒト IgM 抗体療法の可能性- 抗体医療の最前線 -	第257回 CBI学会研究講演要旨集	257	2	2005
<u>岡田則子</u>	HIV 感染症におけるヒト IgM 抗体の研究	第16回 日本生体防御学会抄録集	16	47	2005
<u>岡田則子</u>	HIV 感染症治療に置ける IgM 抗体の療法の可能性	Medical Tribune	38	67	2005

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<u>Okada, N.</u> , Yin, S., Asai, S., Kimbara, N., Dohi, N., Hpsokawa, M., <u>Wu. X.</u> , <u>Okada, H.</u>	Human IgM monoclonal antibodies reactive with HIV-1 infected cells generated using a trans-chromosome mouse	Molecular Immunology.	41	288-289	2004
Hosokawa, M., Imai, M., <u>Okada, H.</u> , <u>Okada, N.</u>	Inhibition of HIV-1 infection in cells expressing an artificial complementary peptide.	BBRC	324	236-240	2004

## 研究成果の刊行物別刷

## Editor-Communicated Paper

## Diagnostic Surface Expression of SWAP-70 on HIV-1 Infected T Cells

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**Abstract:** Following immunization with HIV-1 infected cells, a hybridoma cell line termed 9F11 was established from the P3U-1 myeloma line fused with lymphocytes from a trans-chromosome (TC) mouse, that harbors human chromosomes containing immunoglobulin genes. The 9F11 human IgM monoclonal antibody (9F11 Ab) reacts with HIV-1 infected MOLT4 cells but not with uninfected MOLT4 cells, and causes immune cytotoxicity with homologous human complement at a concentration as low as 0.4 µg/ml. This Ab was used to perform immunoscreening of a cDNA expression library derived from HIV-1 infected cells. All positive cDNA clones contained SWAP-70 cDNA. SWAP-70 RNA and protein expression are much stronger in HIV-1 infected cells. SWAP-70 was also detected on the surface of HIV-1 infected cells by flow cytometric analysis. The monocyte cell line U937 cells expresses SWAP-70 on its cell surface regardless of whether it was infected with HIV-1. Furthermore, among PBMCs surface expression of SWAP-70 was detected on CD21+, CD56+ and CD14+ cells. Although CD3+ cells scarcely express SWAP-70 on their surface, once activated, they become positive. SWAP-70 may therefore serve as a marker for T cell differentiation as well as for HIV-1 infection.

**Key words:** IgM monoclonal antibody, HIV, SWAP-70

Cells are normally protected from homologous complement (C) by species-specific membrane inhibitors (14, 18) such as decay accelerating factor (DAF, CD55) (17), membrane cofactor protein (MCP, CD46) (26) and 20 kDa homologous restriction factor (HRF20, CD59) (7, 11, 19, 28). Therefore, HIV-1 infected cells cannot be cytolysed by human C even when they react in the presence of an enormous amount of IgG Abs in plasma or serum of HIV-1 infected patients (21, 31). However, human IgM Abs, including a mAb against GM2 ganglioside (termed L55) (12), can induce cytotoxicity of HIV-infected cells by homologous human C (20, 32). IgM antibody may escape regulation by membrane inhibitors of C due to its large size. We expected that an IgM mAb to HIV-1 infected cells might induce C-mediated cytotoxicity. In order to generate human IgM mAb

specifically reactive with HIV-1 infected cells, cells infected with HIV-1<sub>IIIIB</sub> were used to immunize trans-chromosome mice (TC mice) (13, 29). We established hybridoma clones that produce human IgM mAbs reactive to HIV-1 infected MOLT4 cells but not to uninfected MOLT4 cells. One of the mAbs, 9F11 Ab was highly efficient in causing C-mediated cytotoxicity of HIV-1 infected cells at a concentration as low as 0.4 µg/ml (22) and may cause a transient elimination of these cells.

To identify the antigen reactive with 9F11 Ab, we performed immunoscreening using 9F11 Ab to probe a cDNA expression library. All of the 5 clones detected contain cDNA of human SWAP-70 (4, 16). Therefore, we assayed for the surface expression of SWAP-70 on HIV-1 infected cells as well as on PBMCs.

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**Abbreviations:** Ab, antibody; C, complement; HTLV-1, human T-lymphotropic virus type 1; MOLT4<sub>IIIIB</sub> cells, HIV<sub>IIIIB</sub>-infected MOLT4 cells; rSWAP-70, recombinant SWAP-70; TC mouse, trans-chromosome mouse.

## Materials and Methods

**Antibodies (Abs).** The hybridoma producing 9F11 was grown in Hybridoma-SFM (Gibco BRL, Gaithersburg, Md., U.S.A.) supplemented with 3  $\mu\text{g/ml}$  puromycin. Culture supernatants were collected and filtered through 0.22  $\mu\text{m}$  syringe filters. Partial purification of the monoclonal IgM utilized hydroxyapatite and ion-exchange chromatography. A final purification was carried out by size exclusion chromatography utilizing Superdex 200gp 26/60 (Amersham Pharmacia Biotech, Uppsala, Sweden).

Rabbit Ab specific for SWAP-70 was kindly provided by Professor Rolf Jessberger. The Ab was raised immunizing rabbit with the denatured fusion protein of His-SWAP-70. We also raised rabbit specific Ab generating the recombinant SWAP-70 (rSWAP-70) as follows. *E. coli* BL21 was transformed with pET-15b (Novagen, Wis., U.S.A.) containing the open reading frame of human SWAP-70 between the *Bam*HI-sites. Transformed *E. coli* BL21 was cultured in LB medium and the rSWAP-70 was induced by adding IPTG at a final concentration of 0.1 mM. The cells were harvested and sonicated in PBS containing 1% Triton X-100. The rSWAP-70 was purified using a His Trap Kit (Amersham Pharmacia Biotech). Rabbit immune sera were purified by ammonium sulfate precipitation, protein A chromatography (Amersham Pharmacia Biotech) and chromatography on an affinity column conjugated with rSWAP-70.

**Cell lines and viruses.** Human T cell lines (MOLT4 and Jurkat), a human monocyte cell line (U937), a human B cell line (Raji), a human T-cell lymphotropic virus type 1 (HTLV-1) infected cell line (MT4) and a chronically HIV-1 infected T cell line (ACH-2) were cultured in RPMI1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin and 100  $\mu\text{g/ml}$  streptomycin. HIV-1<sub>III</sub> and HIV-1<sub>MN</sub> were prepared from a culture supernatant of persistently infected MOLT4 cells or U937 cells.

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by Ficoll-Hypaque (Amersham Pharmacia Biotech) density-gradient centrifugation. Activated PBMCs were stimulated with a mAb against CD3 (UCHT1) (0.5  $\mu\text{g/ml}$ ) in RPMI1640 medium. On day 3, the cells were washed and suspended in RPMI1640 medium containing IL-2 (50 U/ml). On day 7, the cells were collected and analyzed.

**Isolation of RNA and construction of a cDNA library.** Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, Calif., U.S.A.) according to a protocol supplied by the manufacturer. Poly(A)<sup>+</sup> RNA

was prepared from MOLT4<sub>III</sub> cells using a mRNA Purification Kit (Amersham Pharmacia Biotech). The cDNA library was constructed using a ZAP-cDNA Synthesis/Gigapack Gold Packaging Kits (Stratagene, La Jolla, Calif., U.S.A.).

**Immunoscreening of a phage library.** *E. coli* XL-1-Blue was infected with recombinant phages on NZY agar broth supplemented with 0.5 M IPTG and 250 mg/ml X-gal. The formed plaques were transferred onto a Hybond N+ membrane (Amersham Pharmacia Biotech) and blocked overnight at 4 C with 1% skim milk. The membranes were incubated with 1  $\mu\text{g/ml}$  of 9F11 Ab at room temperature for 1 hr. After incubation with anti-human IgM- $\mu$  chain HRP-conjugated goat Ab (Cappel, Cochranville, Pa., U.S.A.) at room temperature for 1 hr, positive plaques were visualized by ECL (Amersham Pharmacia Biotech). Immunoreactive plaques were purified, and phagemids were rescued and used to infect *E. coli* XL0LR to obtain plasmid subclones, as described previously.

**Flow cytometry analysis.** Cells were incubated in 1 mg/ml goat IgG for 10 min at room temperature to block Fc receptors. After blocking, cells were incubated in 9F11 culture supernatant or anti-SWAP-70 polyclonal Ab for 30 min on ice. Cells were stained for 30 min on ice with anti-human IgM- $\mu$  chain FITC-conjugated Abs or anti-rabbit Ig FITC-conjugated Abs. After staining and washing, cells were fixed in 1% paraformaldehyde, and at least 10<sup>5</sup> events were acquired on a FACScalibur (BD Biosciences, Calif., U.S.A.) for subsequent analysis using CellQuest software (BD Biosciences).

In the case of two-color flow cytometric analysis, we used the lineage markers CD3-PE, CD4-PE, CD8-PE, CD14-PE, CD21-PE and CD56-PE (BD Biosciences).

**Northern blot analysis.** For Northern hybridization, total RNA (20  $\mu\text{g}$ ) was fractionated in a formaldehyde-1.25% (w/v) agarose gel and transferred onto a Hybond N+ membrane (Amersham Pharmacia Biotech). Hybridization with digoxigenin-labeled probes and chemiluminescent detection were carried out according to a protocol supplied by Roche Diagnostics (Mannheim, Germany).

**Protein immunoblot analysis.** Cells were pelleted, washed with PBS and lysed in a buffer containing 20 mM Tris, pH 7.4, containing 1% CHAPS. The lysates were subjected to SDS-PAGE, and the separated proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, Mass., U.S.A.). The membranes were saturated with a blocking buffer containing 1% skim milk overnight, then were incubated with 9F11 Ab or rabbit anti-SWAP-70 polyclonal Ab. Reactive Abs were detected with an anti-human IgM- $\mu$  chain or anti-rabbit Ig HRP-conjugated-goat Ab (Cap-

pel) and subsequent visualization using ECL (Amersham Pharmacia Biotech).

**Expansion of HIV-1 infection and detection.** Naïve MOLT4 cells and MOLT4<sub>IIIb</sub> cells were cocultured at a ratio of 50:1 in RPMI1640 medium. Every 2 days, cells were collected for flow cytometric analysis and the medium was changed. The ratio of HIV-1 infected cells to uninfected cells was monitored through measurement of the intracellular HIV p24 by flow cytometric analysis using a KC-57-FITC anti-p24 Ab (Coulter, Healeah, Fla., U.S.A.).

## Results

### Library Screening for 9F11 Ag

Colonies reactive with the 9F11 Ab were searched for among the colonies transfected with an expression vector containing a library of cDNAs derived from the HIV-1 infected cells (MOLT4<sub>IIIb</sub>). Screening of approximately  $5.0 \times 10^5$  independent clones obtained from a ZAP cDNA library with 9F11 Ab revealed 12 positive clones. The cDNA insert sizes of these clones were analyzed by PCR with primer of T3 and T7. Out of the 12 clones, 5 clones were clearly positive in the PCR analysis and these clones were converted to pBK-CMV by auto-excision and sequenced. All of the 5 clones of about 4.5 kbp length showed 100% homology to the human SWAP-70 (16) (accession number AF134894) using the BLAST program at the NCBI.

### Reactivity of HIV-1 Infected Cells to 9F11 Ab and Anti-SWAP-70 Ab

To assess the expression of SWAP-70 protein on HIV-1 infected cells, we used polyclonal Ab specific

for SWAP-70. The purified Ab showed a single band at the expected molecular mass of 70 kDa in Western blotting of the recombinant protein kindly provided by Professor Rolf Jessberger (Fig. 1A). Similarly, 9F11 recognized rSWAP-70 protein. Anti-SWAP-70 polyclonal Ab stained native HIV-1 infected cells as determined by flow cytometry, but not the uninfected MOLT4 cells, as was the case with 9F11 Ab (Fig. 1B). On SDS-PAGE of a crude cell lysate of HIV-1 infected MOLT4 cells and uninfected MOLT4 cells, anti-SWAP-70 Ab recognized a protein of 70 kDa from the infected MOLT4 cells that was not present in the uninfected MOLT4 cells (Fig. 1C). On the other hand, 9F11 Ab showed a band at 30 kDa of a protein from infected MOLT4 cells that was not present in the uninfected MOLT4 cells. The discrepancy in the molecular size of SWAP-70 and 9F11 Ag in the lysate of the HIV-1 infected cells remains to be elucidated.

### SWAP-70 mRNA and Protein Expression

Using SWAP-70 cDNA as a hybridization probe, Northern blot analysis was performed on total RNA prepared from MOLT4 cells, MOLT4<sub>IIIb</sub> cells, MT4 cells and Raji cells. RNA was loaded in approximately equal amounts (10 µg each), as confirmed by ethidium bromide staining of the agarose gels using 28S and 18S species as markers. The SWAP-70 cDNA probe reacted strongly with the RNA from the MOLT4<sub>IIIb</sub> cells but weakly with that from uninfected cells (Fig. 2A).

Each cell lysate was subjected to SDS-PAGE followed by Western blot analysis. The SWAP-70 protein signal was strong in MOLT4<sub>IIIb</sub> cells and Raji cells but no signal was detected on uninfected MOLT4 cells or MT4 cells (Fig. 2B).

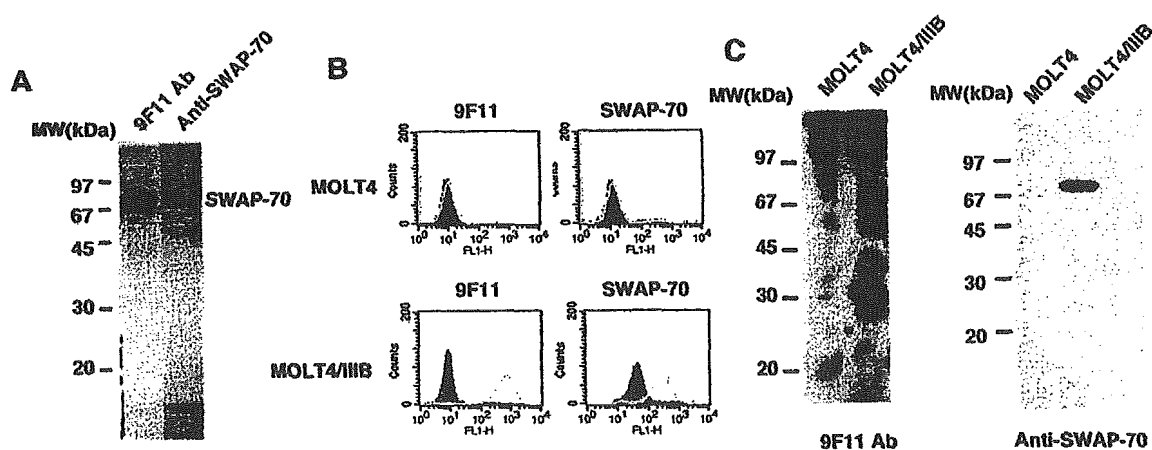


Fig. 1. 9F11 Ab and anti-SWAP-70 Ab reactivity. A, Western blotting of recombinant SWAP-70 protein with 9F11 Ab and anti-SWAP-70 Ab. B, Flow cytometric analysis using 9F11 Ab and anti-SWAP-70 Ab. Control cells (without first Ab) are shown using a filled histogram and Ab-treated cells are shown with an empty histogram. C, Western blotting of cell lysates prepared from MOLT4 cells and MOLT4<sub>IIIb</sub> cells.

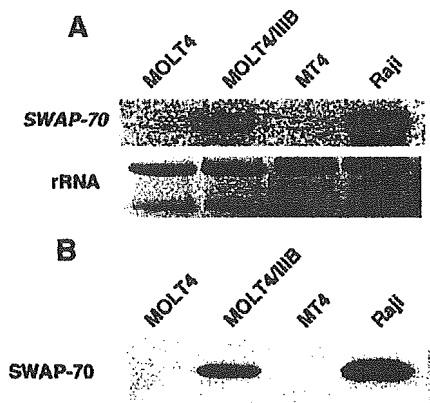


Fig. 2. SWAP-70 mRNA and protein expression in different cell types. A, Northern blot of 10  $\mu$ g each of total RNA from MOLT4 cells, MOLT4<sub>IIIB</sub> cells, MT4 cells and Raji cells. The bottom panel shows an ethidium-bromide-stained-agarose gel with 28S and 18S RNA as loading controls. B, Total cell lysates of  $1 \times 10^7$  MOLT4 cells, MOLT4<sub>IIIB</sub> cells, MT4 cells and Raji cells were subjected to SDS-PAGE and Western blotting with anti-SWAP-70 Ab.

#### Detection of SWAP-70 on the Surface of HIV-1 Infected Cells

On flow cytometric analysis (Fig. 3), HIV-1 infected T cell lines (MOLT4<sub>IIIB</sub> and MOLT4<sub>MN</sub>) were stained by the anti-SWAP-70 polyclonal Ab but the Ab failed to stain HIV-1 uninfected cells (MOLT4 and Jurkat) including HTLV-1 infected cells (MT4) and a latently infected HIV-1 T cell line (ACH-2). However, U937 cells (the monocyte cell line) were stained by anti-SWAP-70 Ab whether infected or not. Raji cells (the B cell line), which contain a high level of SWAP-70, scarcely expressed SWAP-70 on their cell surface. Although only a few positive cells were detected in nonactivated PBMCs, after anti-CD3 stimulation and growth in the presence of IL-2, activated PBMCs showed appreciable increase in surface reactivity with anti-SWAP-70 Ab.

#### Detection of SWAP-70 in Specific Subpopulations of PBMCs

Mononuclear cell subpopulations were analyzed for surface SWAP-70 immunoreactivity. We performed two-color FACS analysis of PBMCs using polyclonal Ab to SWAP-70 and the lineage markers CD3, CD4, CD8, CD14, CD21 and CD56. Among nonactivated PBMCs, T cells (CD3-positive cells) were not stained with anti-SWAP-70 Ab, but monocytes, B cells and NK cells were stained to an appreciable extent (Fig. 4). On the other hand, after activation with mAb against CD3 and IL-2, both CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells became reactive to SWAP-70 Ab (Fig. 5).

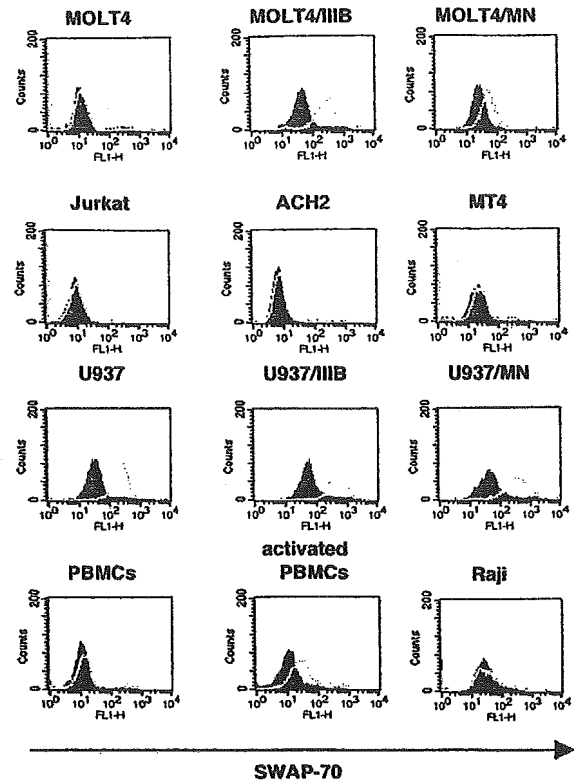


Fig. 3. Reactivity of anti-SWAP-70 Ab with cells of HIV-1 infected lines, uninfected cell lines, and PBMCs on flow cytometric analysis using FITC-labeled anti-rabbit Ig as the second Ab. Control cells (without first Ab) are shown using a filled histogram and Ab-treated cells are shown with an empty histogram.

#### Correlation between Surface Expression of SWAP-70 and HIV-1 Infection

As described above, SWAP-70 is expressed on the cell surface of HIV-1 infected cells. Surface expression of SWAP-70 during HIV-1 expansion was next investigated on newly infected cells. Naïve MOLT4 cells were mixed with MOLT4<sub>IIIB</sub> cells at a ratio of 1 to 50 and incubated at 37 C in a CO<sub>2</sub> incubator. Every 2 days, cells were collected and the percentage of infected cells and surface expression of SWAP-70 was assessed. The rate of SWAP-70 expression increased with a rise in the HIV-1 infected cell number determined by intracellular HIV p24 expression (Fig. 6) was increased to reach steady state within 12 days. However, SWAP-70 expression on these cells required 20 days to acquire steady state.

#### Discussion

9F11 Ab is a human IgM mAb reactive to HIV-1 infected cells, and causes efficient cytolysis of these cells which is mediated by homologous human C (22).

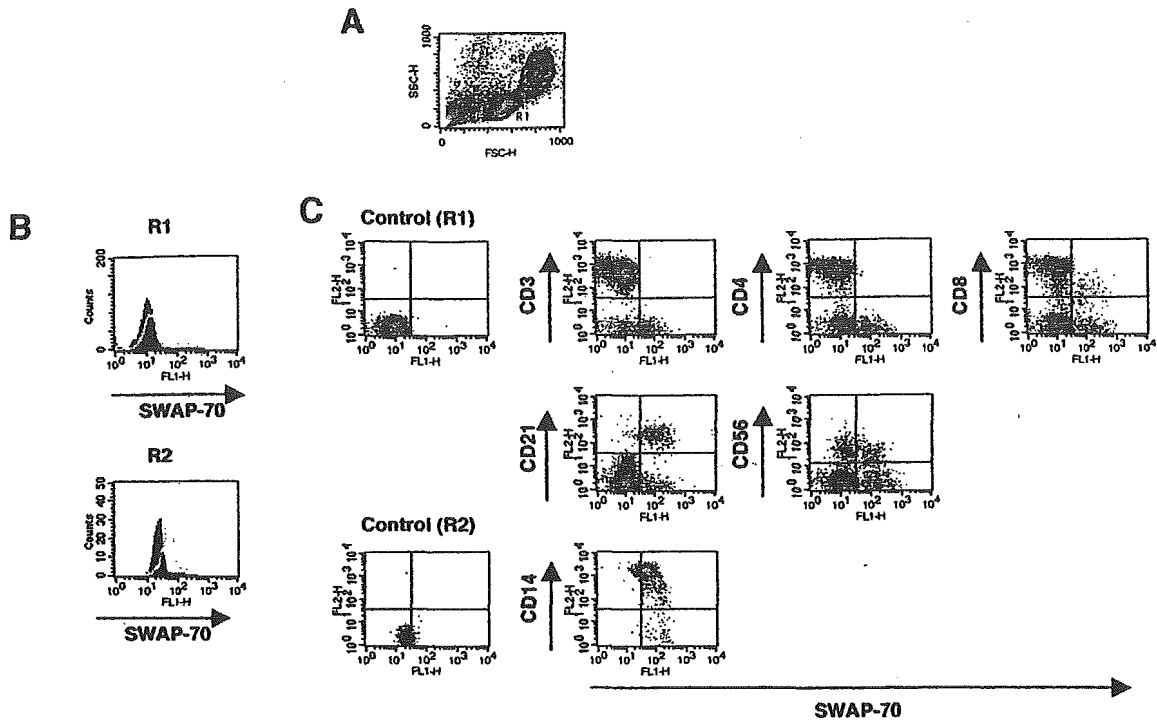


Fig. 4. Detection of SWAP-70 in the subpopulation of nonactivated PBMCs. PBMCs were isolated from healthy donors. A, Side scatter vs. forward scatter of the cells. Region 1 (R1), which includes lymphocytes, and R2, which includes monocytes, are indicated in the dot blot. B, These panels show anti-SWAP-70 Ab reactivity to total lymphocytes (R1) and monocytes (R2). C, Cells were stained with anti-SWAP-70 Ab and anti-CD3, anti-CD4, anti-CD8, anti-CD21, anti-CD56 for flow cytometric analysis. Cells were analyzed by gating R1 except for the anti-CD14 panel. Cells stained with anti-CD14 Ab were analyzed by gating R2. Control cells were not reacted with the first Ab and stained with control mouse IgG-conjugated PE.

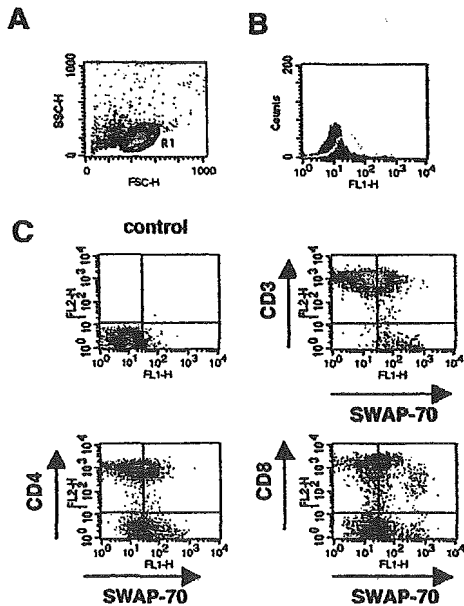


Fig. 5. Detection of SWAP-70 in the subpopulation of activated PBMCs. PBMCs were cultured in RPMI medium supplemented with anti-CD3 (UCHT1) for 3 days. The cells were then washed and the cultures supplemented with IL-2 for 4 days. A, Side scatter vs. forward scatter of the cells is indicated in the dot blot. B, This panel shows the reactivity of anti-SWAP-70 Ab to activated lymphocytes. C, Cells were stained with anti-SWAP-70 Ab and anti-CD3, anti-CD4 or anti-CD8 for flow cytometric analysis.

Therefore, 9F11 Ab may be useful for transient elimination of HIV-1 infected cells which harbor proviral DNA in their chromosomes. To determine the target molecule (9F11 Ag) for 9F11 Ab, we performed immuno-screen-

ing of a cDNA expression library prepared from mRNA of HIV-1 infected cells. All clones isolated bear cDNA of human SWAP-70 (16). SWAP-70 is presumed to play a role in various signaling pathways and was origi-



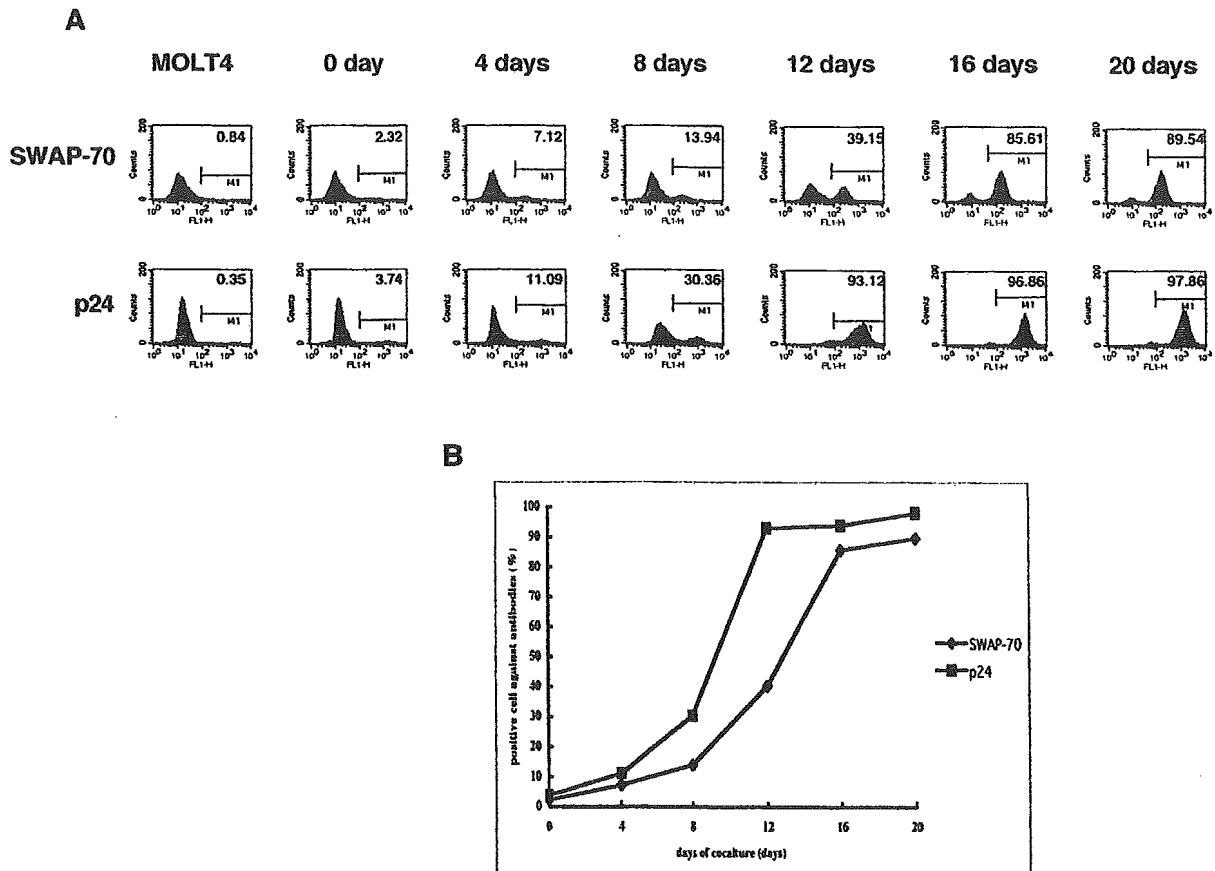


Fig. 6. Surface expression of SWAP-70 increased with expansion of the HIV-1 infection. Naïve MOLT4 cells were mixed with HIV<sub>III</sub> infected MOLT4 at a ratio of 1 to 50. A, Upper panel, surface expression of SWAP-70 in the mixture was determined by flow cytometry. Lower panel, the population of intracellular HIV-1-p24-positive cells was determined by flow cytometry using KC57. B, The rates of SWAP-70-positive cells (◆) and p24 positive-cells (■) are shown.

nally identified as a protein involved in B-cell activation and heavy-chain immunoglobulin class switching (4).

We used anti-SWAP-70 Ab purified by an immunosorbent coupled with rSWAP-70 to confirm the expression of SWAP-70 on the surface of the infected cells. Since anti-SWAP-70 developed a single band at 70 kDa on Western blotting of an HIV-1 infected cell lysate as well as of a Raji cell lysate, the Ab was deemed highly specific for SWAP-70. Although anti-SWAP-70 Ab did not react with MOLT4 cells, it did react with HIV-1 infected MOLT4 cells to an appreciable extent at flow cytometric analysis. SWAP-70 is abundantly expressed in activated B lymphocytes and in immature mast cells and its intracellular localization has been reported to depend on B cell activation. Stimulation of the B cell receptor triggers a translocation of SWAP-70 from the cytosol to the plasma membrane (3). In mast cells, the protein is present in the cytoplasm or is localized to the cytoplasmic membrane (10).

SWAP-70 harbors the pleckstrin homology (PH) domain which allows it to bind phosphatidylinositol-3,4,5-triphosphate (PIP3), the second messenger product generated by phosphatidylinositol 3-kinase (PI3K). Binding of PIP3 is necessary for SWAP-70 to localize to membrane actin structure termed membrane ruffles (27). Furthermore, interaction of HIV-1 envelope glycoprotein gp120 with the chemokine receptor CXCR4 caused rapid actin cytoskeleton rearrangements and membrane ruffling (1). We suggest that SWAP-70 associates with the plasma membrane upon HIV-1 infection and appears on the surface of infected cells because of membrane rearrangements such as membrane ruffling. Similar mechanisms were described for annexin V (2, 6, 9, 15).

Furthermore, Western blot analysis with anti-SWAP-70 Ab showed a strong signal at 70 kDa following SDS-PAGE of an HIV-1 infected MOLT4 cell lysate but not following an SDS-PAGE of uninfected MOLT4 cells. It was reported SWAP-70 expression is increased

in spleen of mice affected with murine acquired immunodeficiency syndrome (MAIDS) (25). However, Western blotting with 9F11 Ab showed a specific signal at 30 kDa with HIV-1 infected cells and not with uninfected cells. Although 9F11 Ab reacted with rSWAP-70 at the expected 70 kDa position, there was no signal at this position with the HIV-1 infected cell lysate. Furthermore, SWAP-70 Ab did not show any signal at 30 kDa with HIV-1 infected cells. Therefore, the relationship between SWAP-70 and the 30 kDa 9F11 Ag, and the nature of high molecular weight polypeptides reactive with 9F11 remain to be determined.

HIV-1 can activate multiple signaling pathways within a target cell to facilitate viral entry and replication. Combination of HIV-1 envelope and CD4 causes phosphorylation of receptor tyrosine kinases such as p56<sup>Lck</sup>, which activate the Raf/MEK/ERK and PI3K pathways and indirectly activate calcium channels (5, 8, 23, 24, 30). SWAP-70 binds the PI3K product PIP3 and operates as a signaling protein. It was reported that release of TNF- $\alpha$ , mediator which promotes virus production, is lower in SWAP-70 knock-out mice than wild-type bone marrow mast cells (10). We propose that upregulation of SWAP-70 expression by HIV-1 infection is important for signal transmission within the PI3K pathway and in HIV-1 replication.

Therefore we investigated possible physiological effects of anti-SWAP-70 Ab on U1 cells, a chronically HIV-1 infected monocyte cell line. However, this antibody did not upregulate C5a receptor expression, did not induce apoptosis, did not increase C3 deposition at incubation with human serum and did not induce HIV-1 production. To date we could not detect any physiological effect of cross linking of SWAP-70 on cell surfaces.

Flow cytometric analysis with anti-SWAP-70 Ab indicated that, among fresh PBMCs, SWAP-70 molecules are expressed on CD21-positive cells, CD56-positive cells and CD14-positive cells, although hardly any CD3-positive cells express SWAP-70 on their cell surface. However, among CD3-positive cells, both CD4-positive and CD8-positive cells, once activated, were able to express SWAP-70 on their cell surface. Therefore, SWAP-70 expression on the surface of HIV-1 infected T lymphocytes may be induced by stimulation of the T cell activation cascade following HIV-1 infection. SWAP-70 expression on CD3-positive cells could be a marker of HIV-1 infection.

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Editor-Communicated Paper

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

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**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  $\mu$ - and  $\kappa$ -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  $\mu$ g/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  $\mu$ - and  $\kappa$ -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.

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

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

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**Abbreviations:** Ab, antibody; C, complement; C9DHS, C9-deficient human serum; FCS, fetal calf serum; FHS, fresh human serum; HRP, horseradish peroxidase; mAb, monoclonal Ab; MOLT4/IIIB, HIV-IIIB-infected MOLT4 cells; PBMC, peripheral blood mononuclear cells; PBS-T, PBS with 0.05% tween; PI, propidium iodide; RT, reverse transcriptase; TC mouse, trans-chromosome mouse; U937/IIIB, HIV-IIIB-infected U937 cells.

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<sup>+</sup> 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  $\mu$ M 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-III<sub>B</sub>)-infected cells to immunize trans-chromosome mice (TC mice) harboring human chromosomes 2, 14 and/or 22 (6, 18), whose endogenous murine genes for  $\mu$ - and  $\kappa$ -chains had been knocked out in the TC mice to induce human Abs against the Ags used in immunization (6).

## Materials and Methods

**Cells and viruses.** U937 (a human monocyte cell line), U937/III<sub>B</sub>, U937/MN, U937/momo, MOLT4 (a human T cell line) (8), MOLT4/III<sub>B</sub>, OM10.1 (1) and CEM (a human leukemia cell line) were used. U937/III<sub>B</sub>, U937/MN and U937/momo are U937 cells persistently infected with the HIV-III<sub>B</sub> strain, HIV-MN strain and a primary isolated HIV-momo strain, respec-

tively. MOLT4/III<sub>B</sub> cells are MOLT4 cells persistently infected with the HIV-III<sub>B</sub> 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  $\mu$ M glutamine, 100 IU/ml penicillin and 100  $\mu$ g/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-III<sub>B</sub> cells were cultivated in the presence or absence of 5  $\mu$ g/ml tunicamycin (Sigma, St. Louis, Mo., U.S.A.) 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) and one of these was immunized with U937/III<sub>B</sub> 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 P3U1 cells 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  $\mu$ g/well rabbit anti-human IgM Ab (CAPPEL, Cochranville, Penn., U.S.A.) 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  $\mu$ l/well) at room temperature for 1 hr. Plates were washed three times with PBS-T, and then 50  $\mu$ l of anti-human  $\kappa$  chain-horseradish peroxidase (HRP) conjugate were added and plates were incubated at room temperature for 1 hr. 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, Calif., U.S.A.). 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., Calif., U.S.A.) to block any Fc receptors, were suspended in 50  $\mu$ l 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 hr 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 naive 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  $\mu$ g/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  $^{51}\text{Cr}$  release assay as previously described (15). As target cells, U937, U937/IIIB, MOLT4 and MOLT4/IIIB were labeled with  $^{51}\text{Cr}$ . Various amounts of mAb (9F11 or 2G9) were used in culture with  $2 \times 10^4$  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  $\mu$ l. After 4 hr incubation at 37 C, the amount of  $^{51}\text{Cr}$  released in the supernatants was determined. The percentage of specific  $^{51}\text{Cr}$  release was calculated according to the following formula:

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

The amount of  $^{51}\text{Cr}$  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  $\mu$ g/ml) in the presence of normal FHS, C9-deficient human serum (C9DHS), or C9DHS plus C9 (0.01, 0.1, 1 and 10  $\mu$ g/ml).

**C3 deposition on Ab-sensitized HIV-infected cells.** MOLT4/IIIB cells were incubated with 9F11 (2  $\mu$ g/ml)

in the presence of 20% FHS in GVB<sup>2+</sup> (gelatin veronal-buffered saline containing 0.15 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$ ) 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  $\mu$ g/ml propidium iodide (PI) for 2 min, and resuspended in 1% *p*-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, Calif., U.S.A.). The dsDNAs were then generated from the mRNAs. Since the 5' ends were variable regions for immunoglobulin  $\mu$ - and  $\kappa$ -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, Calif., U.S.A.). 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): AGAGTCTGGGCCACGACCT;

O-7 (antisense):

GCTCTAGAATGCACACACAGAGC;

O-14 (antisense): GATCTAGAGGACCGCAATAG;

2O-14 (antisense): GACCGCAATAGGGGTAGGTC;

4A (antisense):

GGGTTTTACAGCTTCGCCATTCT; and

5A (antisense): ACGGCGCTGAAAGTGGCATTG.

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

O-10 (sense): TATAGCGGCCGCGCTCTCCTCA;

O-9 (antisense): AGGTCGTGGGCCAGACTCT;

O-70 (sense): ACTGTGGCGGCCATCTGTC;

1S (sense): GAAAACCCACACCAACATCTCCGA;

1A (antisense):

ATTGGGGCGCTGGTACATACTTCTC;

2S (sense): ACCCAATGCCACTTTCAGCGCCGT;

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 ( $2 \times 10^5$ ) were mixed with  $4 \times 10^3$  HIV-III<sub>B</sub>-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  $\mu$ g/ml) or 2G9 (100  $\mu$ g/ml) or a combination. The mixtures were incubated at 37 C in a CO<sub>2</sub> 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/III<sub>B</sub> cells was assessed by the TUNEL method using an In Situ Cell Death Detection Kit (Roche Diagnostics Corp., Indianapolis, Ind., U.S.A.) (4). MOLT4/III<sub>B</sub> cells were suspended in culture medium at  $1 \times 10^5$ /ml and incubated in the presence of either 2G9 or 9F11 (50  $\mu$ g/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  $1 \times 10^5$ /ml and incubated in the presence of either 2G9 (12.5  $\mu$ g/ml) alone, 20% FHS alone or their combination. Apoptosis was determined after a 2-day incubation at 37 C in a CO<sub>2</sub> 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- $\alpha$ ) were suspended in culture medium at  $1 \times 10^5$ /ml and incubated in the presence of either 2G9 (12.5  $\mu$ g/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- $\alpha$ . OM10.1 cells ( $1 \times 10^5$ /ml) were stimulated with TNF- $\alpha$  (1 ng/ml) in the presence of either 2G9 (12.5  $\mu$ g/ml or 50  $\mu$ g/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-III<sub>B</sub> and mouse myeloma cells (P3U1) were screened with a sandwich ELISA using anti-human IgM polyclonal Ab and anti-human  $\kappa$ -chain mAb labeled with HRP. The culture supernatants containing human IgM Abs were then tested for their reactivity to MOLT4/III<sub>B</sub> 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  $\mu$ g/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  $^{51}\text{Cr}$  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  $\mu\text{g/ml}$  of 9F11 caused cytolysis of more than 50% of these cells. MOLT4 cells were not lysed, even with 20  $\mu\text{g/ml}$  of 9F11 and FHS. Furthermore, although C9-deficient human serum (C9DHS) could not induce cytolysis of MOLT4/IIIB cells in the presence of 20  $\mu\text{g/ml}$  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  $\mu\text{g/ml}$  9F11 induced C-mediated cytolysis, 2G9 had no such capacity, even at a concentration as high as 10  $\mu\text{g/ml}$  (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  $\mu\text{g/ml}$ , 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  $\mu\text{g/ml}$ . Increasing the amount of 2G9 to 300  $\mu\text{g/ml}$  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  $\mu$ - and  $\kappa$ -

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  $\mu$ - and  $\kappa$ -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-III B 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  $\mu\text{g/ml}$  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  $\mu\text{g/ml}$  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  $\mu\text{g/ml}$  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-III B, and can be maintained in a latently infected state in the presence of 1  $\mu\text{g/ml}$  AZT. Although OM10.1 cells were not stained with 0.5 $\beta$  (anti-gp120), UCHL-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- $\alpha$  activated the replication of HIV-1 and cultivation for 10 days made the cells reactive with 0.5 $\beta$  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- $\alpha$ -activated OM10.1 cells were cultivated in the presence or absence of 12.5  $\mu\text{g/ml}$  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- $\alpha$  were cultivated for 2 and 3 days in the presence of 12.5 or 50  $\mu\text{g/ml}$  2G9. Following culture, cells were subjected to staining with KC57 to detect p24-positive cells. Among control



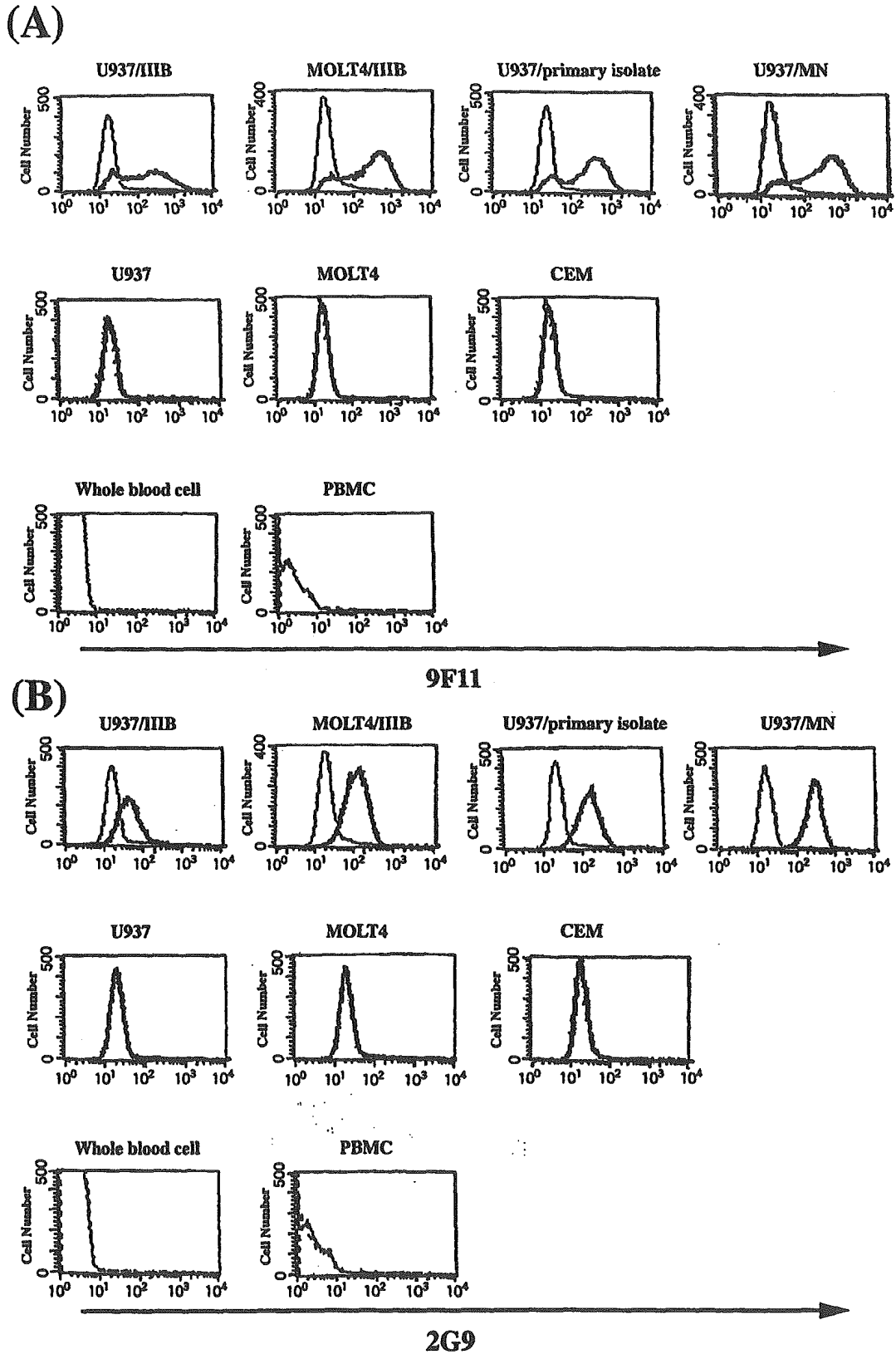


Fig. 1. Reactivity of 9F11 (A) and 2G9 (B) with HIV-1-infected cell lines, uninfected cell lines, whole blood cells and PBMC analyzed by flow cytometry using FITC-labeled anti-human IgM as the second Ab.

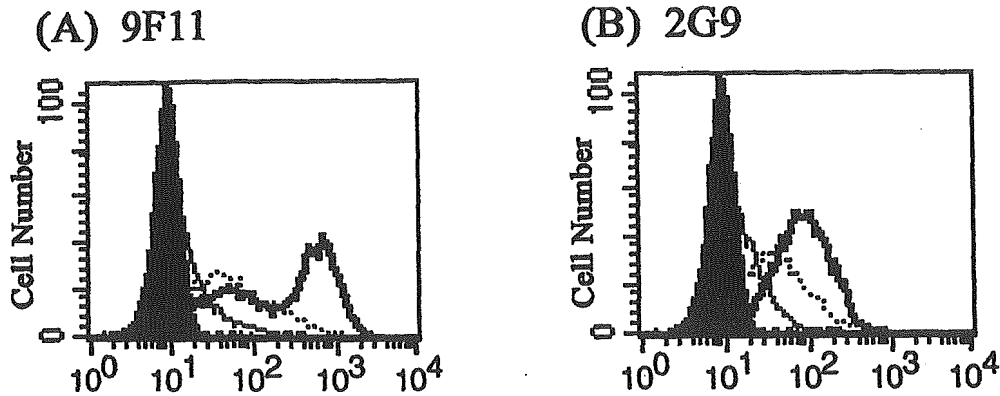


Fig. 2. Reactivity of 9F11 (A) and 2G9 (B) with MOLT4/IIIB cells cultivated in the absence (bold line), or presence of 5  $\mu$ g/ml tunicamycin for 2 days (dotted line) and for 4 days (broken line).

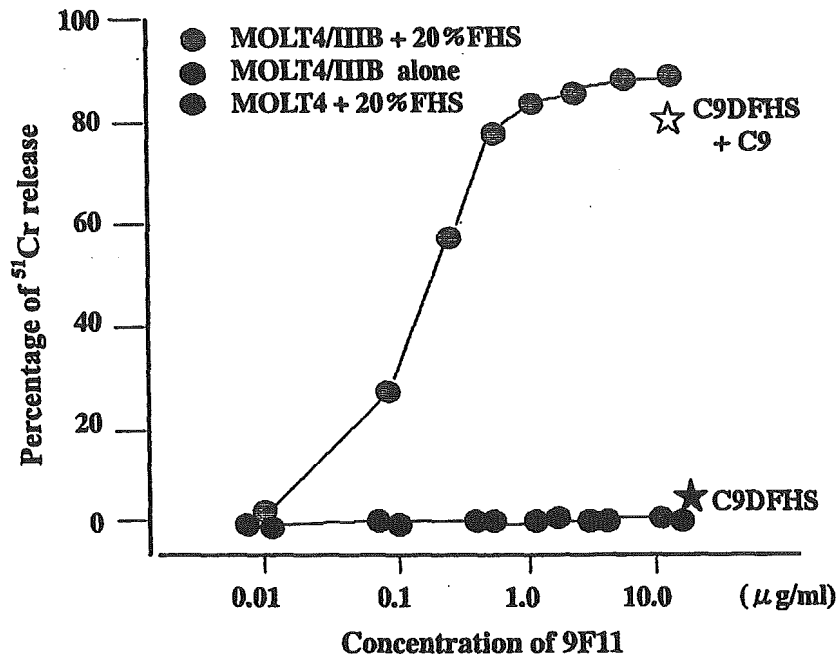


Fig. 3. Complement-mediated cytotoxicity of  $^{51}\text{Cr}$ -labeled cells in the presence of varying amounts of 9F11. Cytotoxicity of MOLT4/IIIB cells and MOLT4 cells. The solid star indicates 20% C9-deficient fresh human serum (C9DFHS) was used instead of fresh human serum (FHS). The open star indicates 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  $\mu$ g/ml 2G9, these values were 33.5% and 57.3% after 2 and 3 days, respectively. Cultivation in the presence of 50  $\mu$ g/ml 2G9 for 3 days reduced the percentage of KC57-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, respec-

tively. Those protein IDs are AAS01770, AAS01772 and AAS01769, AAS01771, respectively.

#### Discussion

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

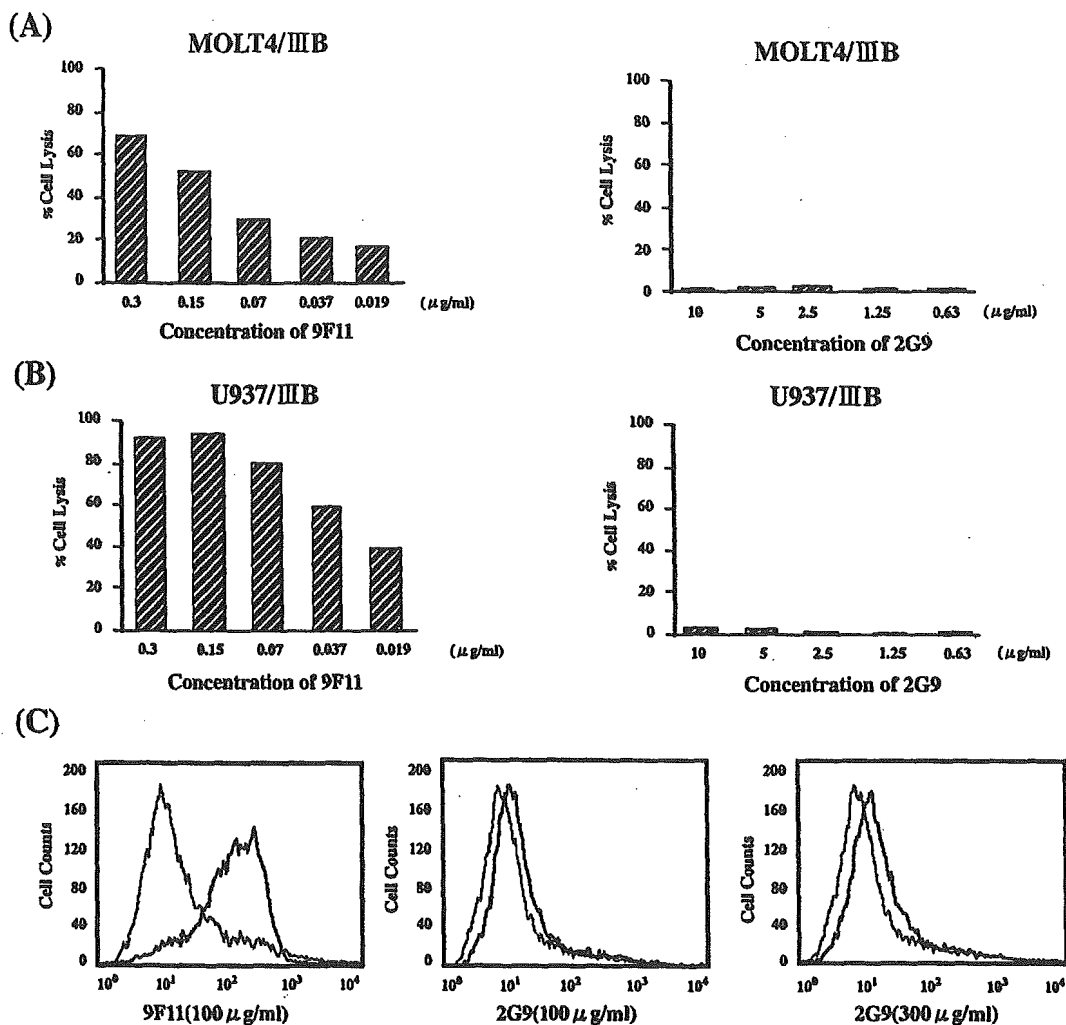


Fig. 4. Comparison of 9F11 and 2G9. Cytolytic effect in the presence of 20% FHS on (A) MOLT4/III B and (B) U937/III B, and (C) C3 deposition, as determined by flow cytometry of MOLT4/III B following incubation with 20% FHS in the presence of 9F11 or 2G9.

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  $\mu$ - and  $\kappa$ -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  $\mu$  chain

2G9u-aa	1	PWIPRPIHLVISTEHRGFTMELGLRWVFLVAI-LE-GVQCEVOLVESGGGLVKPGGSLRL	58
9F11u-aa	1	-----LNSGPGQSPLEQTMSVSFLIFLPVLGLPWGVLQSOVOLQOSGPGLVKPAQTLSL	53
2G9u-aa	59	SCAASGFTFSITYSM--NWRQAPGKGLEWVSSIS-SSSSYIYADSVKGRFTISRDNAKN	115
9F11u-aa	54	TCATSGDSVSNATWNTROSPLRGLEWLRGRYYRSKWNNDYAVSVKSRIITLNPTSKN	113
2G9u-aa	116	SLYLQNSLRAEDTAVYYCARDLLIAVA-----GHNGOGTLVTVSSGSASAPTLFPLVSC	170
9F11u-aa	114	QFSLQNSVTPEDTAVYYCARENYYGSGRYNWFDPNGOGTLVTVSSGSASAPTLFPLVSC	173
2G9u-aa	171	ENSPSDTSSVAVGCLAQDFLPDSITFSWKYKNNSDISSSTRGFPSVLRGGKYAATSQVLLP	230
9F11u-aa	174	ENSPSDTSSVAVGCLAQDFLPDSITFSWKYKNNSDISSSTRGFPSVLRGGKYAATSQVLLP	233
2G9u-aa	231	SKDVMQGTDEHVVCKVQHPNGNKEKNVPLPVIAELPPKVSFVPPRDGFFGNPRKSKLIC	290
9F11u-aa	234	SKDVMQGTDEHVVCKVQHPNGNKEKNVPLPVIAELPPKVSFVPPRDGFFGNPRKSKLIC	293
2G9u-aa	291	QATGFSPRQIQVSWLREGKQVSGSVTTDQVQAEAKESGPTTYKVTSTLTIKESDWLSQSM	350
9F11u-aa	294	QATGFSPRQIQVSWLREGKQVSGSVTTDQVQAEAKESGPTTYKVTSTLTIKESDWLSQSM	353
2G9u-aa	351	FTCRVDHRGLTFQONASSMCPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTYYDS	410
9F11u-aa	354	FTCRVDHRGLTFQONASSMCPDQDTAIRVFAIPPSFASIFLTKSTKLTCLVTDLTYYDS	413
2G9u-aa	411	VTISWTRONGEAVKHTNISESHPNATFSAVGEASICEDDWSNGERFTCTVHTDLPSPL	470
9F11u-aa	414	VTISWTRONGEAVKHTNISESHPNATFSAVGEASICEDDWSNGERFTCTVHTDLPSPL	473
2G9u-aa	471	KQTISRPKGVALHRPDVYLLPPAREQLNLRESATITCLVTGFSPADVFVQWMQRGQPLSP	530
9F11u-aa	474	KQTISRPKGVALHRPDVYLLPPAREQLNLRESATITCLVTGFSPADVFVQWMQRGQPLSP	533
2G9u-aa	531	EKYVTSAPMPEPQAPGRYFAHSILTVSEEWNTGETYTCVAHEALPNRVTERTVDKSTG	590
9F11u-aa	534	EKYVTSAPMPEPQAPGRYFAHSILTVSEEWNTGETYTCVAHEALPNRVTERTVDKSTG	593
2G9u-aa	591	KPTLYNVSLVMSDTAGTCYPCWPAHRLGAAGRSVCAFS	628
9F11u-aa	594	KPTLYNVSLVMSDTAGTCYPCWPAHRLGAAGRSVCAFS	631

(B) IgL  $\kappa$  chain

2G9k-aa	1	QSGHSMDMRVPAQLLGLLLWLFDTRCDIQMTQSPSSLASAVGDRVITICRASQGISNYL	60
9F11k-aa	1	-SGHSMDMRVPAQLLGLLLWFDGSRCDIQMTQSPSSVSASVGDRTVITICRASQGISSWL	59
2G9k-aa	61	AWYQOKPGKVPKLLIYAASLQSGVPSRFSGSGSDFTLTISLQPEDVATYYCQKYS	120
9F11k-aa	60	AWYQOKPGKVPKLLIYDASSLQSGVPSRFSGSGSDFTLTISLQPEDVATYYCQANS	119
2G9k-aa	121	APYITFGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNFPYPREAKVQWKVDNA	180
9F11k-aa	120	FPLITFGGTKMEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNFPYPREAKVQWKVDNA	179
2G9k-aa	181	LQSGNSQESVTEQDSKDYSLSSSTLTLISKADYEKHKLYACEVTHQGLSSPVTKSFNRGE	240
9F11k-aa	180	LQSGNSQESVTEQDSKDYSLSSSTLTLISKADYEKHKLYACEVTHQGLSSPVTKSFNRGE	239
2G9k-aa	241	CREKCPHLLLSSSLTPSHPLASDPFSTGDLPLLR	274
9F11k-aa	240	CREKCPHLLLSSSLTPSHPLASDPFSTGDLPLLR	273

Fig. 5. Amino acid sequences deduced from base sequences of cDNA coding for (A)  $\mu$ -chain and (B)  $\kappa$ -chain of 2G9 and 9F11. Stars indicate amino acids considered to be responsible for C1q binding in  $\mu$ -chains. Arrows indicate constant regions of  $\mu$ - and  $\kappa$ -chains, respectively.

Although 2G9 could not induce C-mediated cytotoxicity 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