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 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 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 analysed, 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.

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 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 intra cellular 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 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 (ref. 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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Foot Notes:

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

**Figure legends:**

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.

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.

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.

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.

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.

# 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$0.4\mu g/mL$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

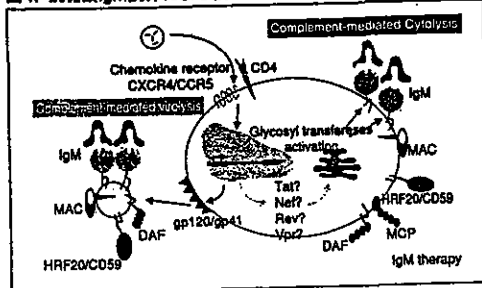
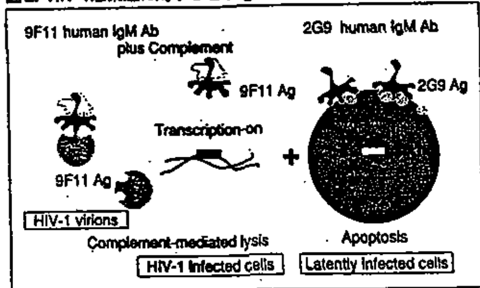



図2. HIV-1感染症に対するヒトIgMモノクローナル抗体の効果



## Human IgM mAbs reactive with HIV-1-infected cells generated using a trans-chromosome mouse

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The trans-chromosome (TC) mouse that we used, provided by Kirin Brewery Co. Ltd., 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. 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.

Further analysis revealed that 9F11 reacts not only on HIV-1 infected MOLT4 cells but also on MT2 and MT4 cells which are HTLV-I infected cells. Those HTLV-I infected cells were also efficiently cytolysis by human complement in the presence of 9F11. Therefore, 9F11 should react on a molecule induced to express on cell membranes following HIV-1 and HTLV-I infection.

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# Inhibition of HIV-1 infection in cells expressing an artificial complementary peptide☆

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**Abstract**

TLMA2993 peptide (N′-TLMALELKGKLLLAGLAPSAFLPLSFPEGL-C′) which was designed by a computer program (MIMETIC) inhibited the activity of HIV-1 reverse transcriptase in a cell-free system. Therefore, we constructed a TLMA2993 expression vector containing an artificial cDNA for TLMA2993 to generate the peptide in cells. The cell lysate of transfected U937 cells contained a detectable level of TLMA2993 peptide using competitive ELISA. The transfectants were resistant to HIV-1 infection due to expression of TLMA2993 peptide in the cells. The use of MIMETIC to design an inhibitory peptide to any intracellular target molecule, followed by transfection of the artificial cDNA for the peptide, could afford a new approach for treatment and/or prevention of viral infection.
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*Keywords:* Complementary peptide; Transfection; HIV-1; Reverse transcriptase; Connection domain; Shot-gun ligation method

MIMETIC is a novel computer program for designing complementary peptides that may interact with a target amino acid sequence of a protein [1]. Complementary peptides targeted to regions regarded to be essential for function of HIV-1 reverse transcriptase (RT) inhibited reverse transcription in vitro [1]. Three complementary peptides (TLMA2993, PSTW1594, and ESLA2340) out of 10 peptides synthesized inhibited RT function in a cell free system. TLMA2993 (N′-TLMALELKGKLLLAGLA PSAFLPLSFPEGL-C′; the name of peptide consists of the one letter code for the first four amino terminal amino acids and its molecular weight) was the strongest of the peptides tested, and 32 μM TLMA2993 could inhibit reverse transcription [1]. TLMA2993 targets the connection domain of RT and we assumed that it could restrict RT function in cells if we transfected cells with the corresponding cDNA. In this work, we generated an artificial cDNA coding for TLMA2993 and demonstrated that transfection of the cDNA induced resistance to HIV-1 infection.

## Materials and methods

*Preparation and phosphorylation of oligonucleotides.* The following oligonucleotides were synthesized: 5′-AATTCCCCACCATGACTTT AATGGCTCTCGAGCTCAA-3′ (TLMA-(i)); 5′-AGGTAAGCTTT

☆ *Abbreviations:* AG promoter, modified chicken β-actin promoter; Amp$^r$, ampicillin resistance; bp, base pair; CMV-IE, cytomegalovirus immediate early; DNA, deoxyribonucleic acid; EDT, 1,2-ethanedithiol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate isomer; HIV, human immunodeficiency virus; KLH, Hemocyanin, Keyhole Limpet; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; Neo$^r$, neomycin resistance; NP-40, nonidet P-40; ori, origin of DNA replication; PE, phycoerythrin; PBS, phosphate-buffered saline; RNA, ribonucleic acid; SDS, sodium dodecyl sulfate; SV40, simian virus 40; TE, Tris–HCl/EDTA buffer; TFA, trifluoroacetic acid; RT, reverse transcriptase.

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TATTAGCTGGGCTAGCGCCTAGCG-3′ (TLMA-(iii)); 5′-CTT TCTTACCCTTAAGTTTTCCGGAAGGACTTTAGG-3′ (TLMA-(v)); 5′-TTACCTTTGAGCTCGAGAGCCATTAAAGTCATGG TG GGG-3′ (TLMA-(ii)); 5′-AGAAAGCGCTAGGCGCTAGCCC AGC TAATAAAAGC-3′ (TLMA-(iv)); and 5′-AATTCCTAAAGTCCT TCCG GAAAACTTAAGGGTA-3′ (TLMA-(vi)). For gel purification of oligonucleotides, 20 μl of MG dye (80% formamide solution containing 1% xylene cyanol and bromophenol blue) 10 mM NaOH, and 1 mM EDTA were added to the oligonucleotide pellets (approximately 100 μg). After polyacrylamide gel electrophoresis on a sequencing-type gel [14% polyacrylamide (acrylamide:bis-acrylamide = 19:1), 8 M urea, 2 mm thickness], the oligonucleotides, which were detected by ethidium bromide staining, were cut out from the gel and eluted in 1 ml G buffer (0.5 M $NH_4OAc$, 10 mM $Mg(OAc)_2$, 1 mM EDTA, and 0.1% SDS) at 37 °C for overnight. The eluted oligonucleotides were purified by the column that was made of Whatmann DE-52 resin (diethylaminoethyl cellulose resin, Whatmann BioSystem, Kent, UK) and recovered by ethanol precipitation, and then resuspended in 25 μl water. Oligonucleotides were phosphorylated at a concentration of 100 pmol in a final volume of 20 μl containing polynucleotide kinase buffer (50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, and 5 mM DTT), 10 mM γ-ATP, and 20 U T4 polynucleotide kinase (TaKaRa Biomedicals, Shiga, Japan), incubated at 37 °C for 1 h. The kinase was subsequently inactivated by incubation at 70 °C for 10 min.

*Shot-gun ligation method.* The pCR2.1 vector (Invitrogen, Carlsbad, California, USA) was cut with *Eco*RI and was dephosphorylated with alkaline phosphatase (TaKaRa Biomedicals). Then shot-gun ligation was performed as described previously (Fig. 1) [2,3]. For shot-gun ligation, 0.5 pmol of the six phosphorylated oligonucleotides was mixed in 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 100 mM NaCl, and hybridized for 1 h at 37 °C. One microliter of hybridized DNA and 50 ng of vector fragment were ligated using DNA Ligation Kit Ver. 1 (TaKaRa Biomedicals). The ligation reaction was performed overnight at 16 °C. The nucleotide sequence was determined by the chain termination method using an ABI PRISM 310 genetic analyzer (PE Biosystems, Tokyo, Japan) with M13 forward or reverse primers. The appropriate sequence was excised from TLMA2993/pCR2.1 vector using *Eco*RI, and ligated into pCXN2 vector which was a pCAGGS derivative [4], was cut with *Eco*RI, and dephosphorylated. For linearization, this vector was cut with *Pvu*I in the ampicillin resistant gene.

*Cell cultures.* U937 cells were maintained in suspension culture in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 nM of 2-mercaptoethanol. U937 cells transfected with the TLMA2993/pCXN2/*Pvu*I vector were maintained in the presence of 400 μg/ml G418 (Gibco Life Technologies, Rockville, Maryland, USA). Cultures were kept humid field at 37 °C in 5% $CO_2$ and passaged every 3–4 days. U937 cells chronically infected with the HIV-1 IIIB strain were maintained under the same conditions.

*Stable expression of TLMA2993 peptide.* TLMA2993/pCXN2 was transfected into U937 cells by electroporation using a Gene Pulser (Bio-Rad, Hemel Hempstead, UK). Twenty micrograms of plasmid DNA (TLMA2993/pCXN2/*Pvu*I digested) in TE was added to $5 \times 10^6$ cells in 400 μl RPMI 1640 medium (without antibiotics) in a 0.4 cm cuvette (Bio-Rad), and given a single pulse at 960 μF, 250 V [5]. The cells were added to 25 ml medium, left to recover overnight in normal medium, and then grown in selective medium (400 μg/ml G418). The cells were dispensed into a 24-well plate. Individual clones from a mixed population of stably transfected cells were isolated by using micropipette.

*Screening by genomic PCR and RT-PCR.* The oligonucleotides synthesized were as follows: 5′-TCCTACAGCTCCTGGGCAAC-3′ (sense strand), 5′-GAGCCAGGGCATTGGCCACA-3′ (antisense strand). These oligonucleotides were able to amplify the region that

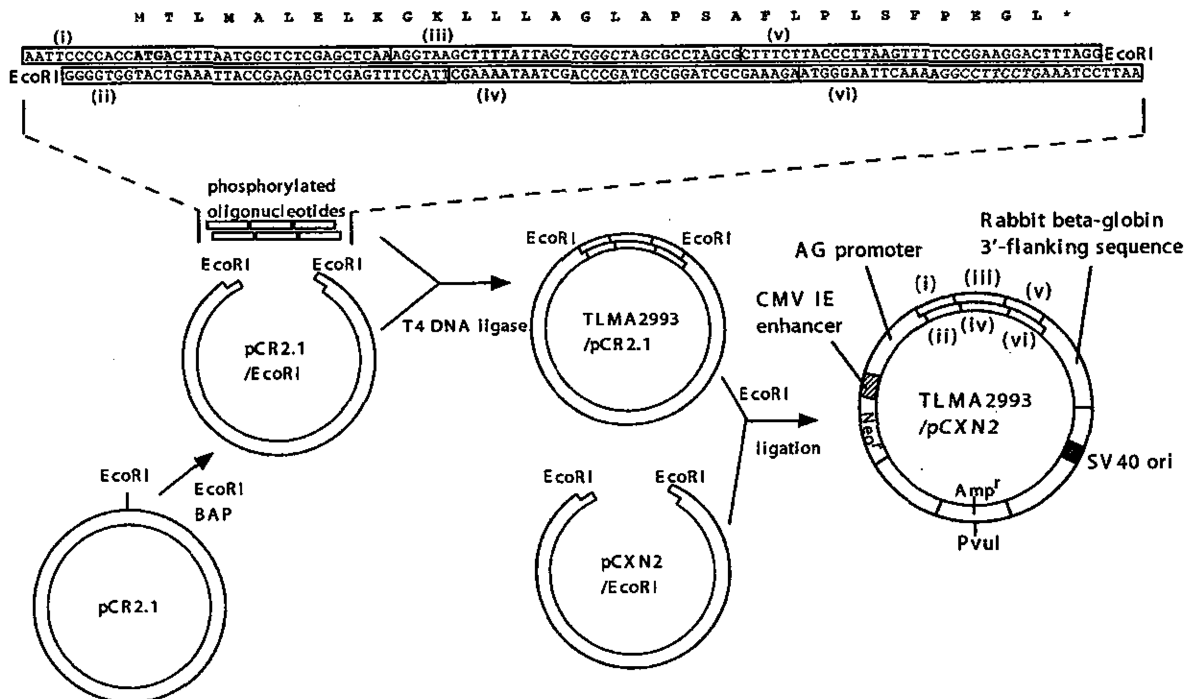


Fig. 1. Outline of the shot-gun ligation method. The vectors were constructed as described in Materials and methods. pCR2.1/*Eco*RI was employed for cloning of six overlapping synthetic oligonucleotides ((i)–(vi), in the upper part of the figure) that constructed the TLMA2993 peptide to which was added an additional N-terminal methionine.

was inserted into the *Eco*RI site of the pCXN2 vector. On genomic DNA isolated using standard methods, PCR amplification was performed at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and ending with 72 °C for 7 min using a Gene Amp PCR System 9700 (PE Biosystems).

To detect TLMA2993 mRNA, RT-PCR was performed on total RNA from TLMA2993 transfected cells. Following reverse transcription, PCR amplification was performed at 94 °C for 1 min, followed by 30 cycles of 94 °C for 5 s, 50 °C for 15 s, and 72 °C for 1 min, and ending with 72 °C for 2 min TLMA-(i) and TLMA-(vi) oligonucleotides were used in this RT-PCR.

*Flow cytometric analysis of surface protein.* Cells were harvested and washed in PBS, and resuspended at $2 \times 10^6$ cells/ml. Aliquots (100 μl) were plated in a V-bottomed 96-well plate ($2 \times 10^5$ cells/well). Plate was centrifuged at 1000 rpm at 4 °C for 5 min, supernatant was removed, and the cells were gently resuspended in 50 μl of anti-CD4-PE, anti-CXCR4-PE (Pharmingen, San Diego, California, USA), or anti-CCR5-FITC (R&D Systems, Minneapolis, Minnesota, USA), and the plates were placed on ice for 30 min. The cells were washed twice in PBS, resuspended in FACSFlow Sheath Fluid (Becton–Dickinson, San Jose, California, USA), and then analyzed by FACS Calibur (Becton–Dickinson) [6].

*Co-cultivation experiment.* $2 \times 10^4$ U937/TLMA-15, U937/TLMA-18 or U937/N2 cells were mixed with $1 \times 10^2$ (200:1) or 40 (500:1) HIV-1 infected U937 cells (U937/IIIB), and the mixtures were cultured in 1.0 ml RPMI1640 containing 10% FCS in a 24-well plate, as described previously [7,8]. Every fourth day, 0.5 ml of the cultures was collected and the percentage of infected cells was determined using a Coulter Colon KC-57-FITC anti-p24 monoclonal antibody (Coulter, Hialeah, Florida, USA) following the manufacturer's protocol for staining p24 and the percentage of HIV infected cells was determined using FACS Calibur. Eighty microliters of residual cell was transferred to 920 μl of culture medium [7,8].

*Peptide synthesis.* Peptides were synthesized by the solid phase method with F-moc chemistry using an AMS peptide synthesizer (ABIMED, Langenfeld, Germany). These were then cleaved from the resin, with the concomitant removal of side-chain protecting groups by treatment with trifluoroacetic acid (TFA), 80%; thioanisol; 12%; 1,2-ethanedithiol (EDT), 6%; and *m*-cresol, 2%. Peptides were then purified by high performance liquid chromatography on a reversed C18 column with 0.1% TFA/water–acetonitrile. All peptides were confirmed using time of flight mass spectrometry on a KOMPACT MALDI II (Kratos-Shimadzu, Kanagawa, Japan) [9].

*Production of antiserum against TLMA2993.* The carrier protein, Hemocyanin, Keyhole Limpet (KLH; Calbiochem, San Diego, California, USA) was linked to *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS; Pirece, Rockford, Illinois, USA) forming an MB/KLH conjugate using standard methods. After column purification, the MB/KLH conjugate was cross-linked to the cysteine-containing synthetic peptide corresponding to the N-terminal 10 amino acids of TLMA2993 peptide (N′-TLMALELKGKC-C′, termed TLMA(Nt) peptide), suspended in approximately 80 μg of peptide/carrier conjugate in PBS, and mixed with complete Freund's adjuvant. Three rabbits were immunized subcutaneously with this mixture and 2 weeks later, each rabbit was boosted with 50 μg of the conjugate in PBS in incomplete Freund's adjuvant. Additional boosts were administered at the 5th and 21st weeks. Rabbits were bled 1 week after the last immunization to obtain antipeptide serum.

*Competitive ELISA.* Fifty microliters of TLMA(Nt) peptide (1 μg/ml), corresponding to the N-terminal of TLMA2993 peptide, in PBS was added to a Falcon 3911 96-well U-bottomed plate (Becton–Dickinson Labware, Bedford, Massachusetts, USA) and incubated at 4 °C overnight. After washing with 0.05% Tween 20 in PBS (PBST) five times, wells were blocked using 200 μl PBS containing 2% BSA and incubated at room temperature for 2 h, followed by washing with PBST five times. To generate a standard curve, U937/N2 cells (electroporation with empty vector) lysed by TNE buffer ($5 \times 10^6$ cells/1 ml TNE buffer (10 mM Tris–HCl, pH 7.9, 1% NP-40, 0.15 M NaCl, 1 mM EDTA, and 10 μg/ml aprotinin)) were mixed with serial dilutions of TLMA2993 peptide (final concentrations were 32, 12, 6.0, 3.0, 1.5, 0.75, and 0.38 μM). Rabbit serum (final concentration 1:1000) was mixed with U937/N2 cell lysate ($2.5 \times 10^5$ cells/well). A standard inhibition curve was generated for dose dependent inhibition of the ELISA reaction by the peptide mixed with the control cell lysate. U937/TLMA-15 and 18 cells were lysed in the same manner and mixed with rabbit serum at the same ratio of U937/N2 lysate. Sixty microliters of each mixture was added to the micro plate, incubated at room temperature for 2 h, and then washed with PBST five times. Peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, Alabama, USA) was then added to the plate and incubated at room temperature for 1 h. After washing, peroxidase enzyme activity was detected by addition of a solution containing 0.015% hydrogen peroxide and 0.04% *O*-phenylenediamine followed by incubation for 5–10 min. Finally, 2 N $H_2SO_4$ was added to stop the reaction and the absorbance at 492 nm was determined. The immunoreactive peptide in the samples was determined using a standard curve.

*Detection of HIV-1 DNA using PCR.* TLMA2993 transfected cells ($2 \times 10^5$ cells/0.5 ml) were mixed with 0.5 ml of the culture supernatant of HIV-1 IIIB chronically infected U937 cells ($10^{3.2}$ $TCID_{50}$/100 μl) and incubated for 1 h at 37 °C with shaking. After shaking, cells were incubated in a $CO_2$ incubator and collected at 24 and 48 h. The amount of HIV-1 DNA was detected by PCR at 24 and 48 h after HIV-1 infection. After fixation of HIV-1 infected cells with 1% paraformaldehyde for 1 h, genomic DNAs were prepared using standard methods. The following oligonucleotides were synthesized for PCR amplification of 180 bp of the HIV-1 LTR region [10]: 5′-GGTCTCTCTGGTTA GACCAGAT-3′ (RU5-5′ primer), 5′-CTGCTAGAGATTTTCCAC ACTG-3′ (RU5-3′ primer). PCR amplification was performed using 50 ng DNA template from cells to be tested, at 94 °C for 1 min, followed by 30 cycles of 94 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s, and ending with 72 °C for 5 min. PCR products were analyzed on a 4% polyacrylamide gel. Parallel reactions for quality control of the DNA were shown by amplification of the β-actin gene. The annealing temperature was changed to 65 °C. Synthesized oligonucleotides for amplification of the β-actin gene were 5′-GAAATCGTGCGTGA CATTAAG-3′ (β-actin 5′ primer) and 5′-CTAGAAGCATTTGC GGTGGACGATGGAGGGGCC-3′ (β-actin 3′ primer) [11].

## Results

### *Strategy of shot-gun ligation*

TLMA2993 is an artificially generated peptide and produced the gene which coding TLMA2993 artificially. Then shot-gun ligation method was performed by using synthetic oligonucleotides that overlap complementarily. Synthetic DNA molecules that contained both the appropriate 5′ and 3′ sticky ends would allow circularization of the vector DNA during the ligation reaction and created transformants in *Escherichia coli*. The strategy used is shown in Fig. 1 and Materials and methods. The pCR2.1 vector was cut with *Eco*RI, dephosphorylated, and precipitated with ethanol. Sets of six overlapping oligonucleotides corresponding to TLMA2993 peptide that was added to additional N-terminal methionine and generating *Eco*RI sticky ends were synthesized. The oligonucleotides were phosphorylated by T4 polynucleotide kinase, hybridized, ligated with the

vector DNA, and used to transform *E. coli*. Progeny colonies from shot-gun ligation method were screened by nucleotide sequencing, which was determined by the chain termination method using ABI PRISM 310 genetic analyzer with M13 universal or reverse primers. The clones showed apparently correct sequences. Then appropriate sequence was cut out using *Eco*RI and ligated into pCXN2/*Eco*RI vector. The suitable direction was checked by nucleotide sequencing. To produce stable transfectant, it was necessary to linearize the vector. TLMA2993/pCXN2 vector was cut with *Pvu*I on the ampicillin resistant gene before performing the electroporation.

*Characteristic of transfectants*

Stable clones of U937 cells expressing TLMA2993 peptide were generated by electroporation of the linearized TLMA2993/pCXN2. Four clones (U937/TLMA-8, 15, 18, and 20) out of 20 transfectants were selected and cDNA expression was confirmed by genomic PCR and RT-PCR. For a control, U937/N2, which was transfected with empty vector, was established under the same conditions.

Since infectivity of HIV-1 is influenced by the amount of CD4 and chemokine receptors such as CXCR4 and CCR5 which function as HIV-1 receptor and co-receptors on cell surfaces, we determined the level of these molecules on transfected cells. On cytometric analysis, cell surface expression of CCR5 was significantly lower in U937/TLMA-8 cell than in others, and CXCR4 expression was a little higher in U937/TLMA-8 and U937/TLMA-20 cells, although CD4 expression was almost the same in all clones (data not shown). The expression patterns of CCR5 and CXCR4 on U937/TLMA-15, U937/TLMA-18, and U937/N2 were close to the same level. Therefore, U937/TLMA-15, U937/TLMA-18, and U937/N2 were chosen and their resistance to HIV-1 infection was evaluated. The concentrations of TLMA2993 peptide in the U937/TLMA-15 and U937/TLMA-18 determined by the competitive ELISA were 1.8 and 1.3 μM, respectively.

*Anti-HIV infectivity assay*

To evaluate the ability of TLMA2993 peptide to render cells resistant to HIV-1 infection, $2\times10^4$ U937/TLMA-15, U937/TLMA-18 or U937/N2 cells were mixed with $1\times10^2$ (200:1, Fig. 2A) or 40 (500:1, Fig. 2B) HIV-1 IIIB chronically infected U937 cells. Following co-cultivation, we assessed the percentage of HIV-1 infected cells with a Coulter Colon KC-57-FITC anti-

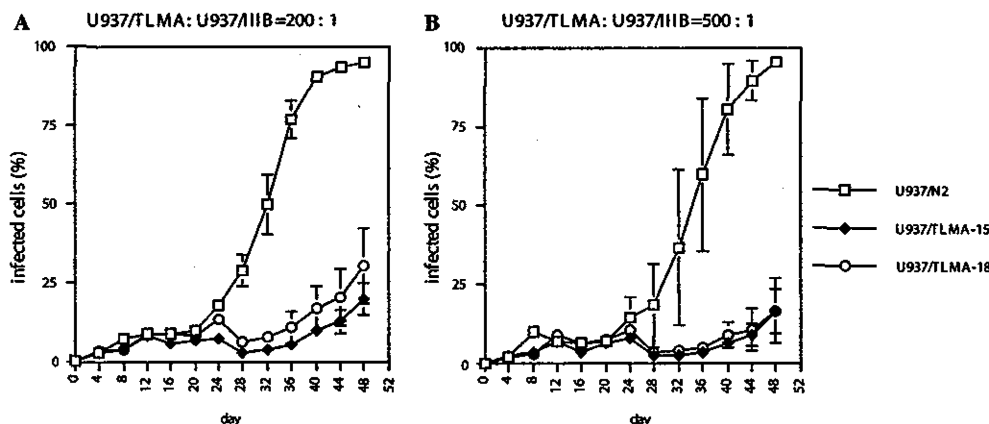


Fig. 2. Inhibition of HIV-1 spread in a mixed culture. TLMA2993 transfected cells ($2\times10^4$) were mixed with $1\times10^2$ (A) or 40 (B) HIV-1 IIIB-infected U937 cells in 24-well plates with 1 ml medium. U937/TLMA-15 (♦), U937/TLMA-18 (○), and U937/N2 (□) are shown. The number of HIV-infected cells was assessed as described in Materials and methods. The values shown represent means of assays performed in triplicate (SD shown).

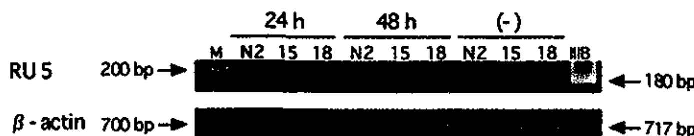


Fig. 3. PCR amplification of HIV-1 DNA in the LTR region. TLMA2993 transfected cells (15 and 18) and control cells (N2) were infected with HIV-IIIB. After cultivation for 24 and 48 h, cells were subjected to PCR amplification of 180 bp of the HIV-1 LTR region. Parallel reactions for quality control of the DNA are shown by amplification of the β-actin gene. Size markers are in the left lane. The right lane is DNA extracted from HIV-IIIB clonically infected U937 cells.

p24 mAb according to the manufacturer's protocol. The percentage of infected cells on day 40 was 90.6% in U937/N2 cells, whereas in U937/TLMA-15 and U937/TLMA-18 cells, these values remained at only 10.2% and 17.0%, respectively (Fig. 2A). By day 48, the percentage of infected U937/N2 cells was 95.4%, whereas U937/TLMA-15 and U937/TLMA-18 cells remained at 16.4% and 16.6%, respectively (Fig. 2B).

### *Detection of HIV-1 DNA using PCR*

TLMA2993 transfected cells were mixed with 0.5 ml of the culture supernatant of HIV-1 IIIB infected U937 cells and incubated for 1 h at 37 °C in a $CO_2$ incubator with shaking. After incubation, cells were collected at 24 and 48 h. The amount of HIV-1 DNA detected by PCR at 24 and 48 h after HIV-1 infection was significantly suppressed in U937/TLMA-15 and U937/TLMA-18 while that of U937/N2 was at an appreciable level (Fig. 3).

## Discussion

Since TLMA2993 significantly inhibited reverse transcription in a cell free system [1], we designed, and synthesized an artificial cDNA to generate TLMA2993 in the transfectants. As expected, the transfectants became resistant to HIV-1 infection. The amount of TLMA2993 peptide detected by competitive ELISA was 1.8 μM for U937/TLMA-15 and 1.3 μM for U937/TLMA-18, and U937/TLMA-15 showed a higher resistance than U937/TLMA-18 (Figs. 2 and 3). It is clear that the data shown in Figs. 2 and 3 reflected the results of competitive ELISA. Since the levels of expression of CD4, CCR5, and CXCR4 were essentially the same among the cells, resistance could be correlated with the amount of TLMA2993 expressed and was dose dependent.

Inhibition of HIV-1 infection in the transfectants was actually due to suppression of RT function, since generation of HIV-1 DNA at an early stage of infection was suppressed (Fig. 3). It is likely that the current dose of nucleoside analogues or non-nucleoside drugs could be lowered by combination with TLMA2993 peptide or other complementary peptides of RT. A stronger effect on HIV-1 infection would be expected if the three kinds of complementary peptides were combined. It will be necessary to also test peptides similar to TLMA2993 such as PSTW1954 and ESLA2340 that are other RT inhibitors [1], and to confirm their effects in the cell. Complementary peptides of RT will have a potential to cure HIV-1 infected patients and this complementary peptide anti-viral therapy provides a novel approach.

The method described here may be applicable to the regulation of any intracellular functional protein. Complementary peptides such as TLMA2993 can be designed using a program such as MIMETIC, and these may be expressed using an artificial cDNA as a means of regulating target molecules in cells.

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# Inactivation of C5a Anaphylatoxin by a Peptide That Is Complementary to a Region of C5a[1]

**Emiko Fujita,* Imre Farkas,*‡ William Campbell,† Lajos Baranyi,† Hidechika Okada,† and Noriko Okada[2]***

**PL37 (RAARISLGPRCIKAFTE) is an antisense homology box peptide composed of aa 37–53 of C5a-anaphylatoxin and is considered to be the region essential for C5a function. Using a computer program, we designed the complementary peptides ASGAPAPGPAGPLRPMF (Pep-A) and ASTAPARAGLPRLPKFF (Pep-B). Pep-A bound to PL37 and to C5a with very slow dissociation as determined by analysis using surface plasmon resonance, whereas Pep-B failed to bind at all. C5a was inactivated by concentrations of 7 nM or more of Pep-A, and this concentration of Pep-A inhibited induction of intracellular $Ca^{2+}$ influx in neutrophils. Patch clamp electrophysiology experiments also showed the effectiveness of Pep-A in C5aR-expressing neuroblastoma cells. Furthermore, Pep-A administration prevented rats from C5a-mediated rapid lethal shock induced by an Ab to a membrane inhibitor of complement after LPS sensitization.** *The Journal of Immunology,* **2004, 172: 6382–6387.**

Complement anaphylatoxin C5a is a 74-aa peptide generated from the fifth component of complement (C5) during complement activation (1, 2). C5a acts efficiently as an anaphylatoxin, stimulating cells such as leukocytes and endothelial cells, and is also a potent chemotactic factor for neutrophils and other inflammatory cells bearing C5aR. Therefore, C5a is considered to be one of the most potent inflammatory mediators (3). Inflammatory cells respond to nanomolar concentrations of C5a with intracellular calcium mobilization, stimulation of chemotaxis, aggregation, degranulation, and production of superoxide anions (4). Some inhibitors such as peptide or nonpeptide C5a receptor antagonists and anti-C5a Ab have already been reported. However, the design of low molecular mass agents that directly *inactivate* C5a has been a challenging problem (4).

Peptide RAARISLGPRCIKAFTE (PL37)[3] is a complement C5a anaphylatoxin fragment (aa 37–53) and is an antisense homology box (AHB) peptide of C5a (5, 6). The sequences within the AHBs were based on the molecular recognition theory, which states that peptides that are encoded on opposite strands of DNA in a given reading frame show affinity in binding each other and that this binding occurs as a result of the hydropathic complementary of the peptides. In addition, such sense-antisense amino acid sequences might represent both intra- and intermolecular interaction sites. Approximately 8- to 15-aa-long regions of this type were found in proteins, which we termed AHBs (5). PL37 is an AHB of C5a; however, it is also antisense to two regions of the C5aR (6). PL37 in multiple antigenic peptide form (PL37-MAP) evoked inward calcium current pulses on human neuroblastoma TGW cells or dibutyryl cAMP-treated U937 cells (6, 7). Therefore, we generated complementary peptides (C-peptides) to PL37, expecting that they could interfere with the function of C5a. To design the C-peptides, we used the software program MIMETIC (8). The algorithm scores several physicochemical parameters of each candidate peptide. C-peptides generated in this manner have already been shown to be inhibitory to HIV-1 reverse transcriptase (8) and thrombomodulin (9).

We synthesized two C-peptides targeting PL-37, and examined their reactivity to PL37-MAP and C5a in various assays such as binding measurements, intracellular calcium mobilization, calcium influx, and in an in vivo C5a-mediated lethal shock rat model (10–12).

## Materials and Methods

### *Design of C-peptides*

We used the evolutionary software program MIMETIC (Institute for Protein Science, Nagoya, Japan) to design C-peptide sequences for interaction with PL37 (7). MIMETIC assigns a score using a genetic algorithm based on several physicochemical parameters including hydropathic complementarity optimization, average structural similarity optimization, minimization of bulky side chain interference, and backbone alignment. MIMETIC uses a genetic algorithm that generates a series of peptides by random alteration and by shuffling segments to optimize fitting to the target. MIMETIC then ranks the C-peptides according to their score. We synthesized the two highest score peptides and tested their activity.

### *Measurement of binding interactions by surface plasmon resonance (SPR)*

Binding interactions between PL37 or C5a with C-peptides were evaluated using SPR technology with the Biacore system (Biacore International, Upsala, Sweden). PL37 and C5a were covalently immobilized on the CM5 sensor chip by amine-coupling methods using *N*-ethyl-*N*-(dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) according to the manufacturer's instructions. We activated the surface of the CM5 sensor

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[3] Abbreviations used in this paper: AHB, antisense homology box; P37, peptide RAARISLGPRCIKAFTE; PL37-MAP, PL37 in multiple antigenic peptide form; C-peptide, complementary peptide; SPR, surface plasmon resonance; EDC/NHS, *N*-ethyl-*N*-(dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide; EA, ethanolamine hydrochloride; RU, units of resonance response; Pep-A, Peptide A (ASGAPAPGPAGPLRPMF); Pep-B, Peptide B (ASTAPARAGLPRLPKFF).

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chip with EDC/NHS for 20 min before injection with PL37-MAP (200 μg/ml in 10 mM sodium carbonate buffer, pH 8.5, over flow cell 2) or C5a (100 μg/ml in 10 mM acetate buffer, pH 5, over flow cell 3). Afterward, excess NHS was deactivated for 20 min with 1 M ethanolamine hydrochloride (EA), pH 8.5. The reference flow cell was activated with EDC/NHS and blocked with EA. Coupling was performed at a flow rate of 5 μl/min at 25°C in PBS. Analyte (30 μl of C-peptide A or B) was injected at 10 μl/min at 25°C in PBS. Binding interactions were determined by passing samples simultaneously over both the EA-blocked cell and the flow cell with immobilized PL37 or C5a so as to obtain units of resonance response (RU) by subtraction of the background using Biacore software (BIA evaluation).

### *Patch clamp measurements*

The measurements were conducted on TGW human neuroblastoma cells bearing C5aR. Cells were voltage clamped at room temperature at a holding potential of −70 mV using a whole cell clamp configuration. The instruments used for electrophysiology were an Axopatch 200-A patch clamp amplifier, a Digidata-1200 data acquisition system; and pCLAMP 6.02 software from Axon Instruments (Foster City, CA). The head stage of the amplifier was fitted to an MHW-3 hydraulic manipulator manufactured by Narishige (Tokyo, Japan), and the cells were visualized using an Olympus IMT-2 invert microscope (Olympus, Melville, NY). Data acquisition and analysis were performed using an IBM-compatible personal computer. Patch electrodes (OD = 1.5 mm; thin wall; Garner Glass, Claremont, CA) were pulled with a PP-83 puller and polished with an MF-83 microforge (Narishige). The resistance of patch electrodes was 8–10 MΩ. The solutions used were as follows: an extracellular solution (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, pH 7.34); and an intracellular pipette solution (10 mM HEPES, 110 mM KCl, 15 mM NaCl, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.25). Recordings were conducted on several cells ($n$ = 10 in each experiment) and were initiated simultaneously with the peptide application.

The PL37-MAP peptide or mixture of peptides (PL37-MAP and C-peptides) were incubated in an Eppendorf tube at room temperature for 1 h in the extracellular solution and then applied to the cells via a puff pipette from a distance of 300–500 μm for 2 min.

### *Neutrophil isolation*

Neutrophils were isolated from fresh human blood with 0.2% EDTA as an anticoagulant. Whole blood was collected from a healthy volunteer (a collaborator of this study) via venipuncture. A 2.4-ml aliquot of blood was then layered onto 2 ml of Mono-Poly Resolving Medium (Dainihon Seiyaku, Tokyo, Japan) in a centrifuge tube and centrifuged at 400 × $g$ for 20 min at room temperature. The polymorphonuclear leukocytes (neutrophils) were then harvested, and the cell fraction was washed with HBSS.

### *Intracellular $Ca^{2+}$ mobilization measurement*

The isolated neutrophils were loaded with 2 μM fura-2/AM (Molecular Probes, Eugene, OR) mixed with 0.02% Pluronic F-127 (Molecular Probes) and 0.2% DMSO in HBSS for 40 min at 37°C. The suspension was agitated to prevent sedimentation. After loading, the cells were washed with HBSS twice and suspended in HBSS containing 0.3% BSA (HBSS/BSA). Approximately 1 × $10^6$ cells in 900 μl of HBSS/BSA were added to a poly-L-lysine-coated 35-mm petri dish and allowed to attach to the bottom of the dish for 30 min. Changes in intracellular calcium concentrations in response to C5a or a mixture of peptides (C-peptides and C5a) were determined by monitoring the ratio of fluorescence light emission at 510 nm as a result of excitation at 340 and 380 nm at 37°C using an ARGUS HiSCA calcium imaging system (Hamamatsu Photonics, Hamamatsu, Japan). The mixture of peptides and C5a was incubated in an Eppendorf tube on ice for 30 min in the HBSS solution and then applied to the cells after a 2-min baseline recording.

### *Rat lethal shock induced by anti-Crry Ab after LPS priming*

Administration of anti-rat Crry mAb (5I2) (10) induces lethal shock in rats primed with a trace amount of LPS (11, 12). With this model, all rats sensitized with 0.05 mg/kg LPS died within 30 min of injection of mAb 5I2.

Male Wistar rats weighing ~250 g were purchased from Chubu Kagaku Shizai (Nagoya, Japan) and were allowed free access to food and water. Each rat was injected with 0.05 mg/kg LPS, prepared from a phenol extraction of *Salmonella typhosa* (Sigma-Aldrich, St. Louis, MO) in 250 μl of saline. After 20 h, 0.75 mg/kg 5I2 was administered. Ten minutes before the injection of 5I2, saline or C-peptides in saline were injected. All injections were administered i.v. through the tail vein. Animal experiments were conducted according to the Nagoya City University Guideline for the Care and Use of Experimental Animals and approved by the Nagoya City University Graduate School of Medical Sciences Animal Care Committee.

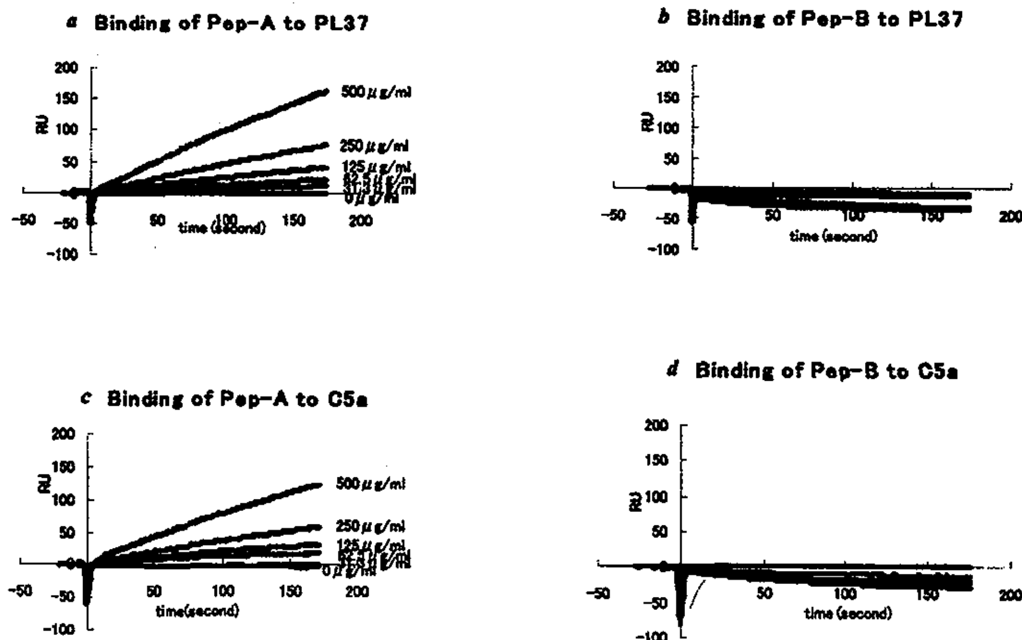


**FIGURE 1.** Binding of C-peptides to immobilized PL37-MAP and C5a using Biacore equipment. An overlay plot of response curves was obtained from the Biacore instrument when various concentrations of Pep-A and Pep-B were injected. All samples were injected at time 0, and the association was monitored as an increase in RU. *a*, Various concentrations of Pep-A were injected over the PL37-MAP-coupled flow cell. *b*, The same concentrations of Pep-B were also injected over the PL37-MAP-coupled flow cell. *c*, Pep-A was injected over the C5a-coupled flow cell at the same concentrations of Pep-A as in *a*. *d*, The same concentrations of Pep-B were also injected over the C5a-coupled flow cell.

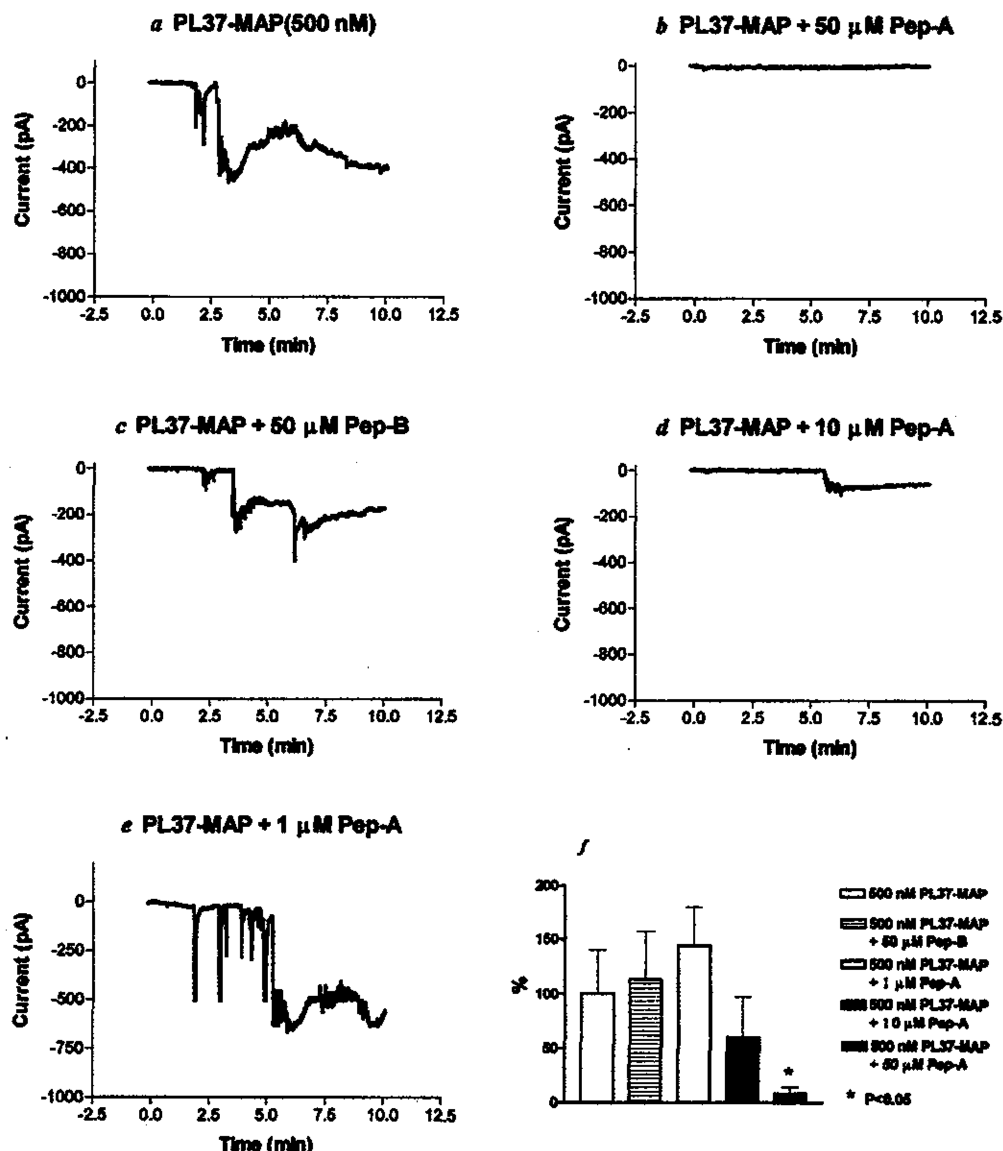


**FIGURE 2.** Inward ion current pulses of human TGW cells treated with PL37-MAP and C-peptides. *a*, 500 nM PL37-MAP induced inward current pulses with an amplitude of 500 pA. *b*, When the PL37-MAP was mixed and incubated with 50 µM Pep-A, a pulse could not be induced. *c*, 50 µM Pep-B did not have any effect on the ion current evoked by PL37-MAP. d, A lower concentration (10 µM) of Pep-A partially inhibited the ion current triggered by PL37-MAP. *e*, When 1 µM Pep-A was mixed and incubated with PL37-MAP, no inhibition was found. *f*, Concentration dependency of the area-under-curve data. These results show that Pep-A had a significant and concentration-dependent effect on the ion current pulses induced by PL37-MAP.

## Results

### *C-peptides generated to a target peptide, PL37*

The best fit peptide sequences to the target region of C5a (sequence of PL37: RAARISLGPRCIKAFTE) designed by MIMETIC were Peptide A (Pep-A: ASGAPAPGPAGPLRPMF) and Peptide-B (Pep-B: ASTAPARAGLPRLPKFF).

### *Binding of C-peptides to PL37-MAP and C5a*

The Biacore instrument that uses SPR technology measures association and dissociation in real time of an unlabeled analyte with an immobilized ligand. PL37-MAP was covalently coupled to a CM5 sensor chip, resulting in a net increase of 8979 RU.

As analytes, Pep-A and Pep-B were injected over the PL37-MAP-coupled flow cell and to the flow cell coupled with EA only. All cycles were performed in PBS at 25°C at a flow rate of 10 µl/min. As shown in Fig. 1*a*, binding data for the PL37-MAP-coupled flow cell were adjusted by subtraction of the data obtained by injection of the same sample over the EA-blocked flow cell (background). Pep-A bound to PL37-MAP, whereas Pep-B did not (Fig. 1, *a* and *b*). Binding of Pep-A was concentration dependent. Pep-A bound to PL37-MAP did not dissociate by increasing the salt concentration or by the addition of DMSO. A 6 M urea treatment was required to dissociate the complex.

Binding of C-peptides to C5a was also examined. C5a was covalently coupled to a CM5 sensor chip resulting in a net increase of 5770 RU. As analytes, Pep-A and Pep-B were injected over both the C5a-coupled and EA-coupled (as the control) flow cells. All cycles were performed in PBS at 25°C at a flow rate of 10 µl/min. In Fig. 1*c*, the binding data for the C5a-coupled flow cell were adjusted by subtraction of the data obtained by injection of

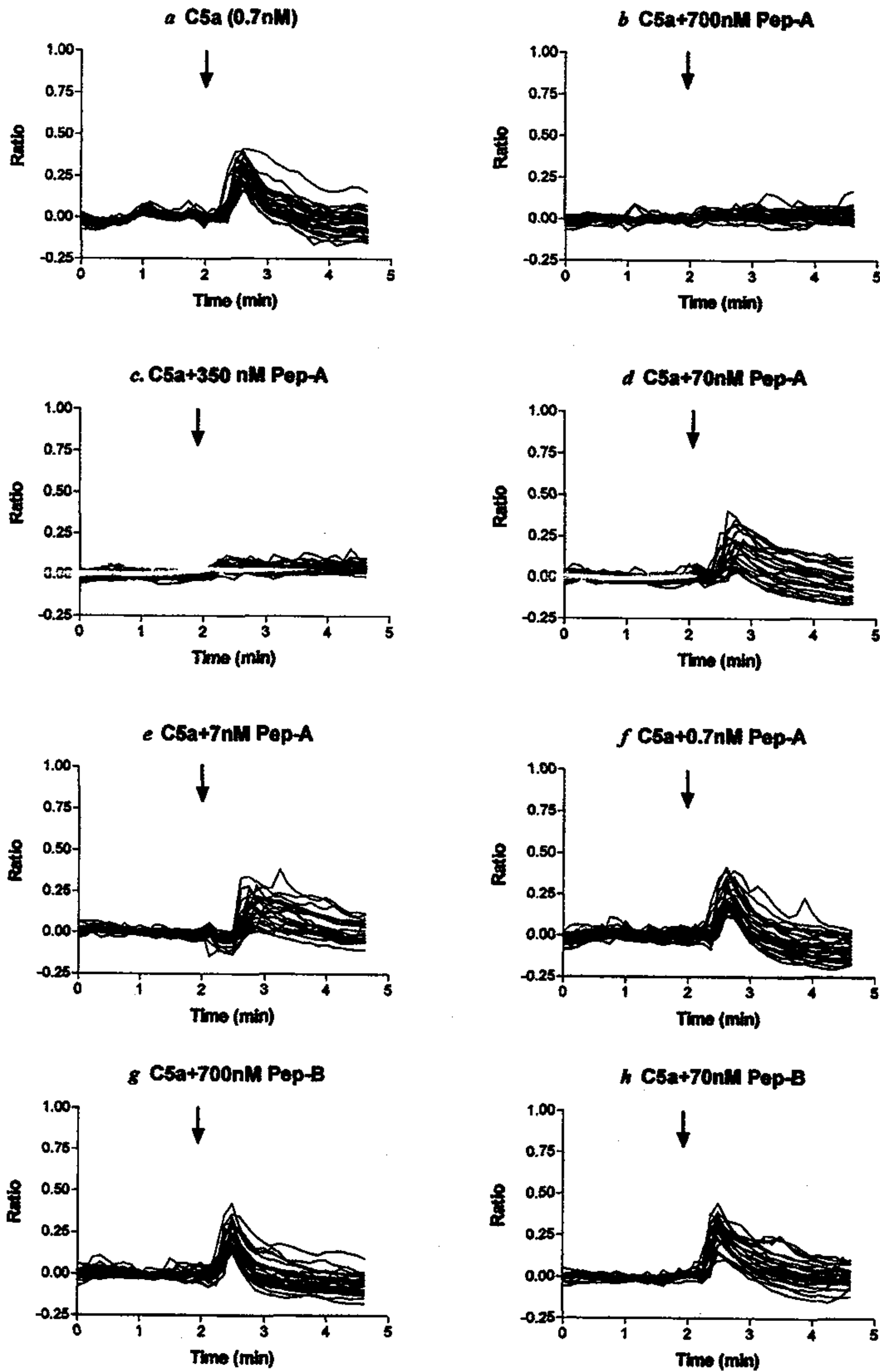


**FIGURE 3.** Measurement of intracellular $Ca^{2+}$ mobilization in neutrophils using a calcium imaging system *a*, 0.7 nM C5a triggered a transient calcium influx in neutrophils. *b*, Incubation with 700 nM Pep-A blocked the effect of C5a completely. *c*, 95% inhibition was found when 350 nM Pep-A was incubated with C5a. *d* and *e*, Lower concentrations of Pep-A (70 or 7 nM) caused partial inhibition. *f*, The inhibitory effect was not detected when 0.7 nM Pep-A was mixed with C5a. *g* and *h*, Pep-B (700 or 70 nM) failed to cause inhibition of the calcium influx triggered by C5a. The area-under-curve data show the concentration dependency of the inhibitory effect of Pep-A.

the same sample over the EA-blocked flow cell (background). Pep-A bound to C5a, whereas Pep-B did not (Fig. 1*d*). Binding of Pep-A was concentration dependent. Furthermore, no dissociation of Pep-A bound to C5a was observed without treatment with 6 M urea.

### *$Ca^{2+}$ influx measurement of TGW cells*

As reported previously, inward calcium current pulses detected by a patch clamp assay, were evoked when TGW cells were exposed to 500 nM PL37-MAP (Fig. 2*a*). However, 50 μM Pep-A inhibited the pulse almost completely (Fig. 2*b*). After washing out, 500 nM PL37-MAP alone was added to the same cells without incubation with C-peptide, and the PL37-MAP peptide triggered an ion current pulse (data not shown). Pep-B did not block the ion current evoked by PL37-MAP (Fig. 2*c*). Inhibition by Pep-A was concentration dependent, because lower concentrations of the C-peptide blocked the effect of PL37-MAP only partially (Fig. 2, *d* and *e*). When the cells were exposed to Pep-A alone, ion current could not be observed even at the highest concentration used (not shown). Normalized data for amplitudes of the ion current responses are shown in Fig. 2*f*.

### *Intracellular $Ca^{2+}$ mobilization measurement of neutrophils*

The effect of C-peptides on C5a function was analyzed by measuring $Ca^{2+}$ mobilization using an ARUGUS HiSCA calcium imaging system (Hamamatsu, Japan). Administration of 0.7 nM recombinant human C5a induced a transient increase in the level of intracellular $Ca^{2+}$ of human neutrophils (Fig. 3*a*). The level of this activation was ~50% of the maximum achieved with higher concentrations of C5a. A mixture of C-peptide and C5a was incubated in an Eppendorf tube on ice for 30 min in the HBSS solution and then applied to the cells. Incubation of C5a with 700 nM Pep-A inhibited the $Ca^{2+}$ mobilization almost completely (Fig. 3*b*). A lower concentration of Pep-A (350 nM) still inhibited ~95% of the effect of C5a (Fig. 3*c*). Inhibition was therefore concentration dependent: lowering the concentration of Pep-A resulted in a higher amplitude of calcium influx triggered by C5a (Fig. 3, *d* and *e*). The inhibitory effect of Pep-A disappeared at 0.7 nM (Fig. 3*f*). However, incubation of C5a with Pep-B showed no inhibition at any concentration used (700 and 70 nM Pep-B; Fig. 3, *g* and *h*). The results were normalized results using the area-under-curve data of the calcium influx and are described in Fig. 4.

### *Effect of C-peptides in a rat lethal shock model*

Administration of 0.75 mg/kg anti-rat Crry mAb (10) induces rapid lethal shock in rats primed with 0.05 mg/kg LPS 20 h earlier (11, 12). The lethal outcome was C5a mediated (12). To investigate the inhibitory effect of the C-peptides in this model, we injected rats with saline (for the control) or C-peptides in 250 μl of saline 10 min before the 5I2 injection. All rats injected with saline died within 30 min (Table I). However, all rats injected with 4 mg/kg Pep-A survived. The inhibition was concentration dependent.

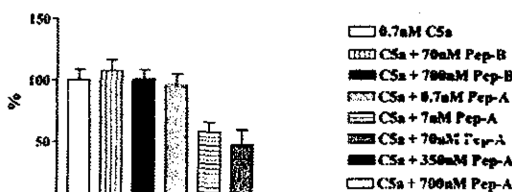


**FIGURE 4.** The area-under-curve graph of the data of Fig. 3 show the concentration dependency of the inhibitory effect of Pep-A.

Table I. *Effect of C-peptides on the rat lethal shock model*

| C-peptides[a] | Surviving/Total No. of Rats | Survival Rate (%) |
|---|---|---|
| Saline alone | 0/8[b] | 0 |
| Pep-B, 4 mg/kg | 0/4 | 0 |
| Pep-A | | |
| 4 mg/kg | 4/4[b] | 100 |
| 2 mg/kg | 2/3 | 67 |
| 1 mg/kg | 1/3 | 33 |
| 0.5 mg/kg | 3/4 | 75 |

[a] C-peptides in 250 μl of saline were injected i.v. 10 min before the i.v. injection of anti-Crry mAb.
[b] Saline control and Pep-A, 4 mg/kg, are statistically significant ($p < 0.003$, Fisher's method).

Lowering the concentration of Pep-A (from 2 mg/kg to 1 mg/kg) resulted in a lower proportion of surviving rats. Some of the surviving rats stopped breathing briefly in the first 1 or 2 min after injection of 5I2, but were soon breathing again, and ~20 ~ 40 min later began moving. When the LPS-sensitized rats were treated with Pep-A without anti-Crry mAb administration, the animals survived with no harmful effect of Pep-A. In contrast, all rats injected with Ab and 4 mg/kg Pep-B died, as did those treated with the saline control.

## Discussion

Various C5aR antagonists have already been reported, and some of these interfered with C5a-mediated functions in vitro and in vivo (3, 13–15). However, the only reported inhibitors of C5a were anti-C5a Abs (16–18). In this study, we designed C-peptides expecting them to interact directly with C5a, resulting in abrogation of C5a function. Two peptides, PepA and PepB, were designed by the computer program MIMETIC to target PL37, an AHB region of human C5a (aa 37–53). Pep-A bound to the target PL37, as determined using SPR technology, and inhibited the inward ion current pulse induced by PL37-MAP in neuroblastoma cells and C5a induction of intracellular $Ca^{2+}$ mobilization in neutrophils. Furthermore, Pep-A bound to the whole C5a molecule so strongly that the complex could be dissociated only with 6 M urea.

Although Pep-B was designed by the same method as used for Pep-A and although both Pep-A and Pep-B showed maximum best fit values using MIMETIC, Pep-B showed no reactivity with PL37-MAP or C5a in any assay conducted. Careful examination of Pep-A and Pep-B might therefore provide useful information for improvement of the algorithm. Furthermore, the information obtained will contribute to a better understanding of peptide characteristics necessary to ensure interaction with a target amino acid sequence.

Two binding sites in C5a to C5aR have been reported. One is at the core of C5a and is centered around Arg 40 (19). The other is contained in the eight amino acids of the C terminus (20). PL37 is located around the first binding site. Pep-A was designed against the PL37 sequence of C5a and inhibited the function of both C5a and PL37. Therefore, our data demonstrate that this AHB-derived PL37 is an important region in C5a.

Treatment with Pep-A was effective in our rat lethal shock model. In this model, rats primed with LPS died within 30 min when injected with anti-Crry mAb. In addition, the lethal outcome was mediated by C5a (12). Hence, our data suggest that Pep-A might bind selectively to C5a in vivo. However, one weak point of using peptide drugs is their short half-life in vivo. Therefore, a time delay between triggering shock and administration of the peptide could be crucial. Administration of 4 mg/kg Pep-A 30 min before the injection of anti-Crry mAb was not effective, indicating

that PeP-A had been degraded in 30 min in vivo. The variable results at lower concentration of Pep-A (Table I) could be due to the short half-life of the peptide in vivo. In contrast, this short half-life could be advantageous. In endotoxic shock, endotoxin induces transient activation of complement and generation of C5a and C3a fragments, which cause lethal shock. In this form of shock, the short duration of the peptide drug and its rapid clearance would be an advantage in avoiding possible long-lasting side effects. Indeed, rats injected with Pep-A survived without any noticeable deleterious side effects.

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# HIV 感染細胞を補体依存性に排除するヒトモノクローナル抗体の研究

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**Study of human monoclonal antibody that eliminates HIV infected cells.**

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## はじめに

我々は約２％の健常人血清に 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いだした。また、１０年以上長期生存 HIV 感染者血清を検討したところ、８０％以上で 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 抗体量との間に高い相関を示した。これらの IgM 抗体の抗原としてガングリオシド GM2 や Gg4 を確認した。さらに、HIV 感染患者血清中の抗 GM2-IgM 抗体量は、CD4 カウントと正の相関を、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。そこで、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(キリンビール社）を感染細胞で免疫して、抗体産生ハイブリドーマ 9F11 クローンを得た。ヒト IgM 抗体 9F11 は、感染細胞特異的に反応し、1 ug/ml 以下の少量で、強力に補体依存性細胞障害活性を示した。そこで、9F11 が反応する細胞についてさらに解析を進めるとともに、HIV 感染者末梢血リンパ球を用いての抗ウィルス活性について検討した。

## 方法

1　ヒトレトロウィルスである HTLV－１が感染して発症したヒト T 細胞白血病細胞株である MT2, MT4, ATN-1, ATL102,HUT102 および T 細胞白血病細胞株 CCRF-CEM, PEER, Jurkat, TALL-1, MOLT3, HPB-ALL などの細胞に対する反応性および細胞障害性を FACS および Cr 放出試験により解析した。

2　サルのエイズウィルスである SIV(Mac 239 株)をサルリンパ球株に感染させた後、その SIV 感染細胞への 9F11 反応性および細胞障害性を解析した。さらに SIV を赤毛サルに感染させて、その１０週間後に生存している感染サルの末梢血よりリンパ球を分離して 9F11 の反応性を FACS 解析した。

3　HIV 陽性ヒト末梢血を Ficol 分離した後、StemSep カラムパスにより、CD4 陽性細胞画分を得た。この感染者 CD4 細胞を 10 ug/ml 9F11 存在下に１０％新鮮 AB 型ヒト血清添加 RPMI1640 にて一晩反応させた後、反応上清を取り除き、1 ug/ml 抗 CD3 抗体添加 10%FCS-RPMI1640 にて培養した。３日間培養後上清を除去して、50U/ml IL2 添加 10%FCS-RPMI1640 にて培養後、１０日目および２０日目の培養上清を回収して、上清中の HIV コア蛋白 p24 量を ELISA にて解析した。

## 結果

1　9F11 は HIV-1 感染細胞に反応する抗体であるが、ATL 由来細胞株にも反応性を示した。また、検討した非 ATL 細胞である白血病細胞などには全く反応性を示さなかった。さらに、MT２や MT４では HIV 感染細胞と同様に細胞障害活性が検出さ

れた。

2　サル SIV サル感染細胞に 9F11 は反応して補体依存性細胞障害活性を示した。また、SIV 感染サルの末梢血リンパ球に 9F11 は反応性を示した。

3　HIV 感染者の末梢血 CD4 陽性細胞を 9F11 と補体で処理した後に、抗 CD3抗体と IL2 培養にてウィルスの叩き出しを行った。末梢血検体３８例中リンパ球培養できたのが２３例であり、このうちの６検体で p24 量が検出された。9F11 添加により、これらのすべての検体で９８％以上の p24 産生の抑制が検出された。そのうちの４例は完全抑制を示した。

**考察**

HIV 感染細胞に反応して補体依存性細胞障害を強力に誘導できる 9F11 は HIV-1 の実験室株を用いて、抗ウィルス効果を示すことが確認されている。今回、レトロウィルス HTLV-1 感染細胞株においても 9F11 は反応し、細胞傷害活性を発揮することが確認された。9F11 は HIV-1 感染のみならず ATL 治療にも応用可能な抗体であることが示唆された。また、サル感染実験における、9F11 の安全性および有効性の検討も可能となった。さらに、HIV 感染者の末梢血リンパ球培養による ex vivo 解析においても 9F11 は優れた抗 HIV 効果を発揮できた。今後、潜伏感染細胞の排除なども対照とした解析を進める予定である。

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