INTRODUCTION

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

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

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

METHODS

Patients

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

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

Tissue samples

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

Antibodies against complement membrane inhibitors and complement

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

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

Evaluation of mucosal inflammation

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

Immunohistochemistry of complement membrane inhibitors and complement

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

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

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

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

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

Diagnosis of *H. pylori* infection

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

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

Statistical analysis

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

RESULTS

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

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

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

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

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

DISCUSSION

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

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

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

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

*$P < 0.05$ vs before.

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

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

*$P < 0.05$ vs before.

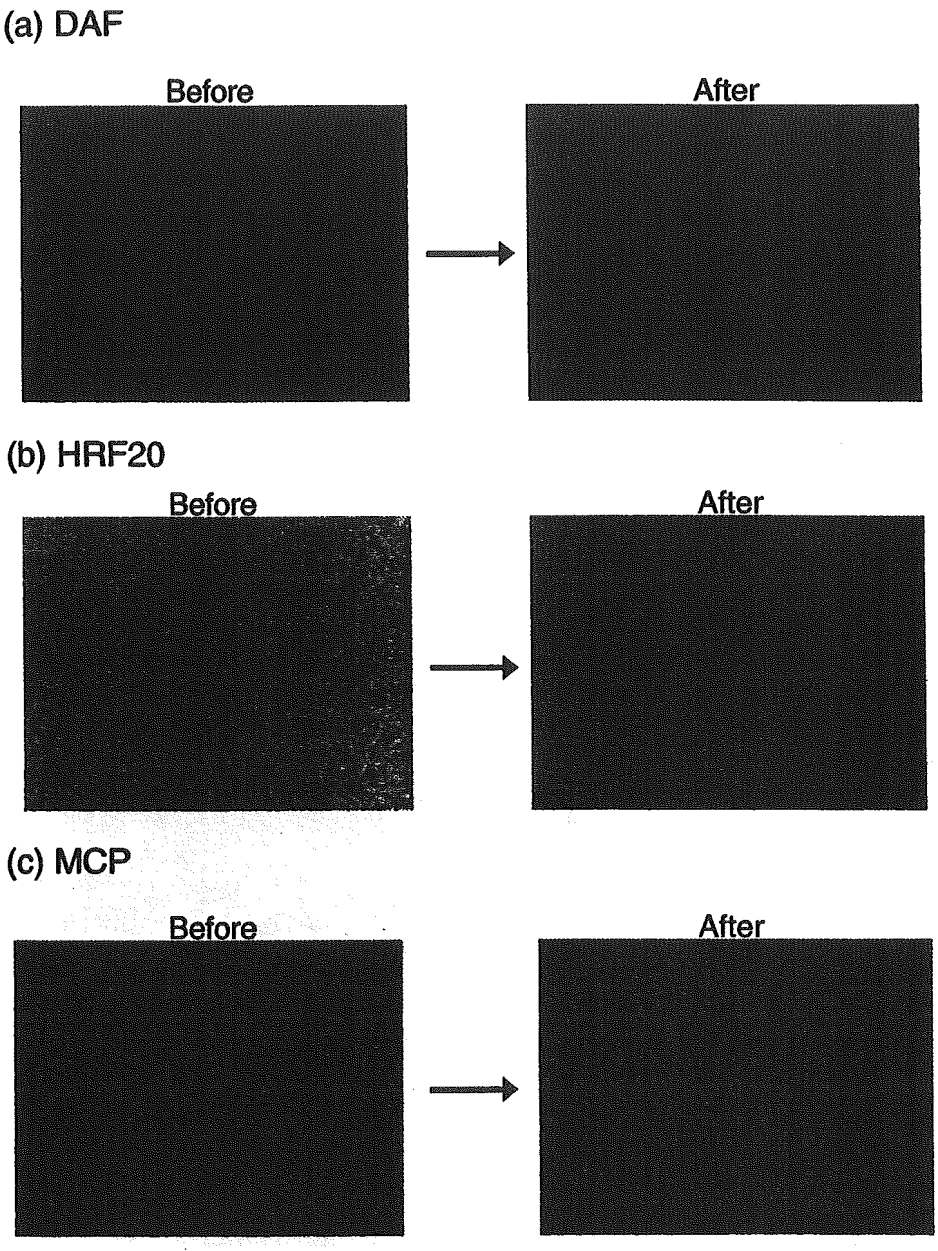

Figure 1 Expression of complement membrane inhibitors before and after eradication of *Helicobacter pylori*. (a) Decay-accelerating factor (DAF) was expressed on the apical side of the gastric epithelium, and the expression decreased after eradication of *H. pylori*. (b) Homologous restriction factor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

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

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

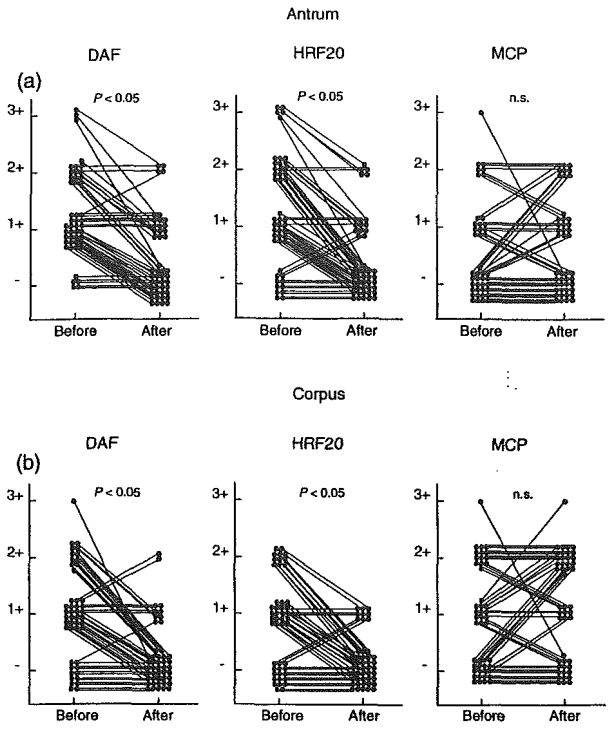

Figure 2 The change in the expression of complement membrane inhibitors in the eradication group. In both antrum and corpus specimens, the expression of decay-accelerating factor (DAF) and homologous restriction factor 20 (HRF20) on gastric epithelium was significantly decreased after eradication of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

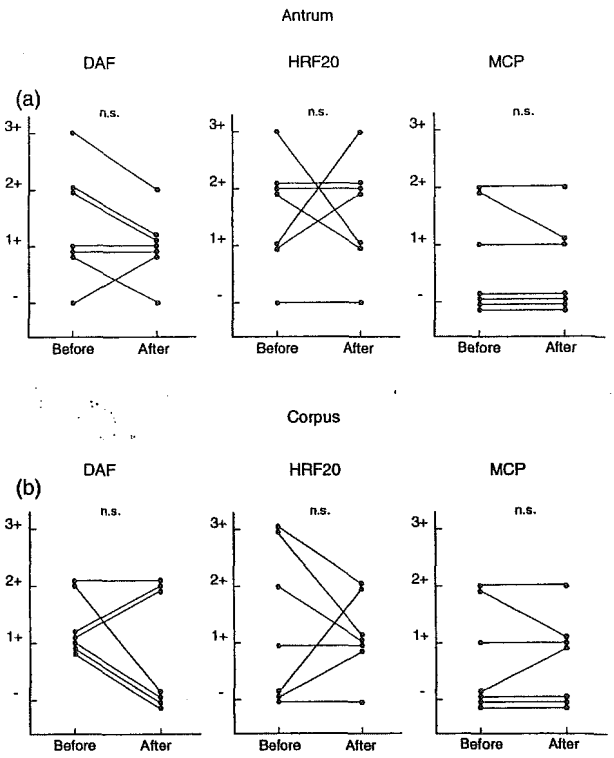

Figure 3 Change in the expression of complement membrane inhibitors in the non-eradication group. The expression of the three inhibitors on *Helicobacter pylori*-infected gastric mucosa did not change after eradication (a–c).

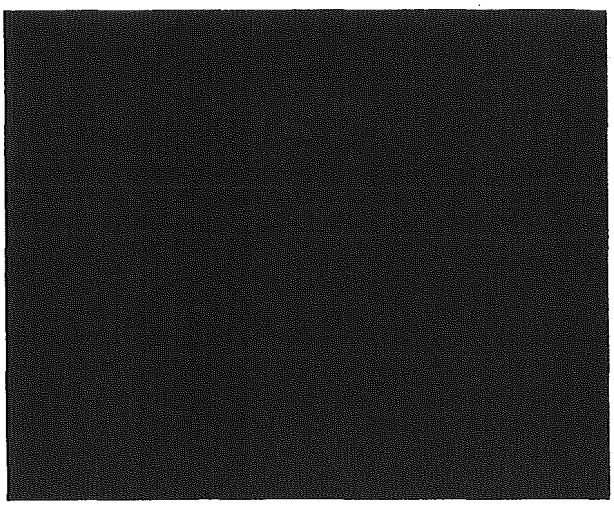

Figure 4 Localization of complement fragment C3c. The main complement fragment C3c staining was recognized on the apical side of gastric epithelial cells and in the mucus layer.

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

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

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

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

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

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

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Special Issue:
XXth International Complement Workshop
13–18th June 2004, Honolulu, Hawaii

Guest Editor: Carl-Wilhelm Vogel

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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.

This work was supported by a Research Grant from the Organization for Pharmaceutical Safety and Research, and a Grant from the University Start-Ups Creation Support System.

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Biochemical and Biophysical Research Communications 324 (2004) 236–240
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Inhibition of HIV-1 infection in cells expressing an artificial complementary peptide☆

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Received 25 May 2004
Available online 25 September 2004

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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doi:10.1016/j.bbrc.2004.09.038

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 5×10^6 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

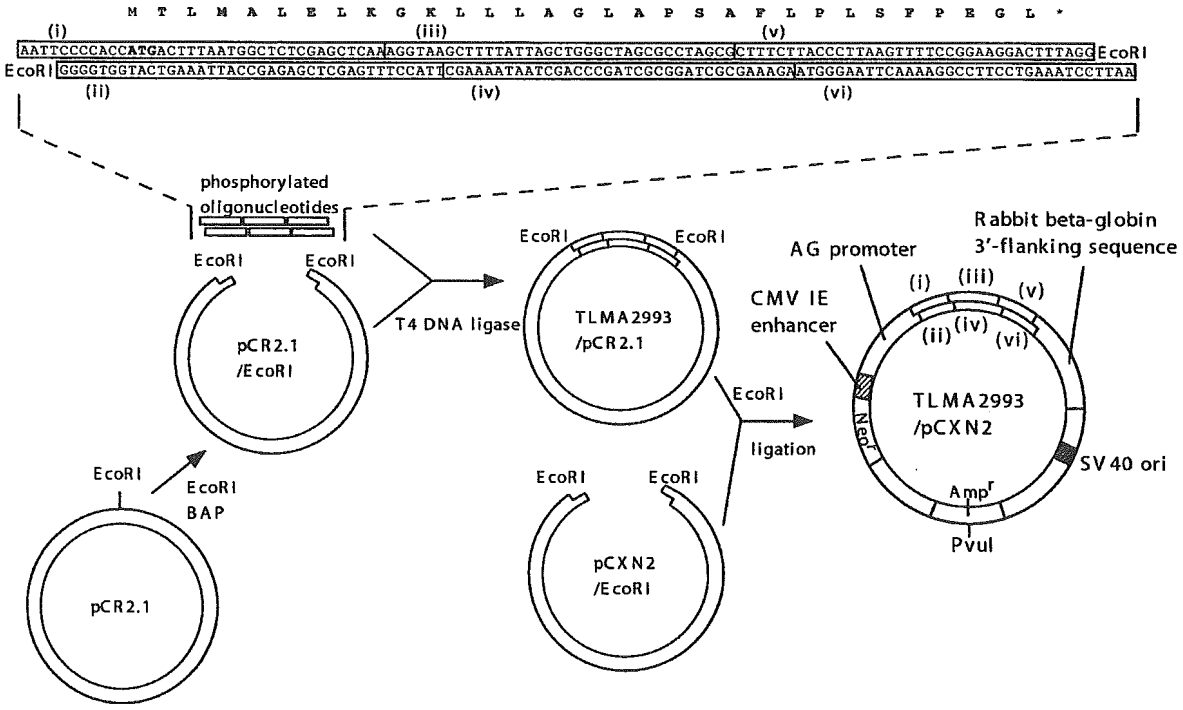

Fig. 1. Outline of the shot-gun ligation method. The vectors were constructed as described in Materials and methods. pCR2.1/*Eco*RI was employed for cloning of six overlapping synthetic oligonucleotides ((i)–(vi), in the upper part of the figure) that constructed the TLMA2993 peptide to which was added 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 2×10^6 cells/ml. Aliquots (100 μl) were plated in a V-bottomed 96-well plate (2×10^5 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. 2×10^4 U937/TLMA-15, U937/TLMA-18 or U937/N2 cells were mixed with 1×10^2 (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 (5×10^6 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 (2.5×10^5 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 (2×10^5 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′-GGTCTCTCTGGTTAGACCAGAT-3′ (RU5-5′ primer), 5′-CTGCTAGAGATTTTCCACACTG-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′-GAAATCGTGCGTGACATTAAG-3′ (β-actin 5′ primer) and 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC-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, 2×10^4 U937/TLMA-15, U937/TLMA-18 or U937/N2 cells were mixed with 1×10^2 (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-

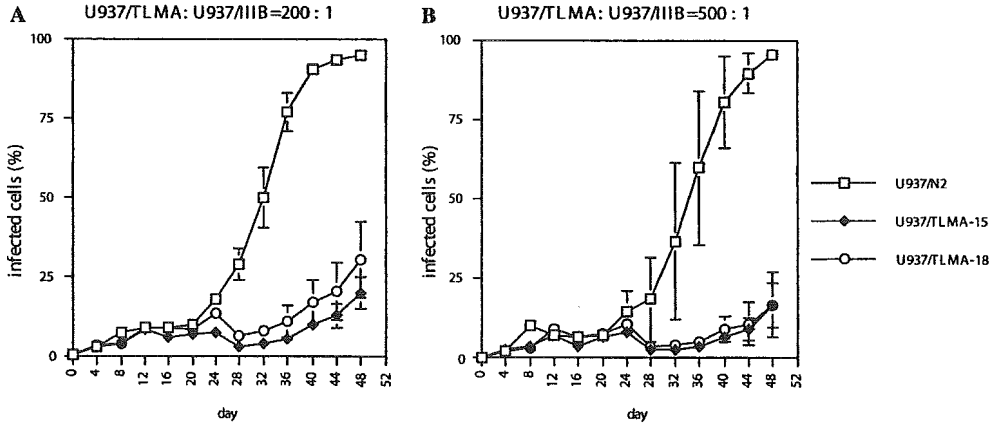

Fig. 2. Inhibition of HIV-1 spread in a mixed culture. TLMA2993 transfected cells (2×10^4) were mixed with 1×10^2 (A) or 40 (B) HIV-1 IIIB-infected U937 cells in 24-well plates with 1 ml medium. U937/TLMA-15 (♦), U937/TLMA-18 (○), and U937/N2 (□) are shown. The number of HIV-infected cells was assessed as described in Materials and methods. The values shown represent means of assays performed in triplicate (SD shown).

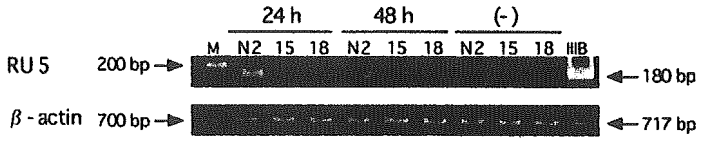

Fig. 3. PCR amplification of HIV-1 DNA in the LTR region. TLMA2993 transfected cells (15 and 18) and control cells (N2) were infected with HIV-IIIB. After cultivation for 24 and 48 h, cells were subjected to PCR amplification of 180 bp of the HIV-1 LTR region. Parallel reactions for quality control of 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.

Acknowledgments

We thank Dr. William Campbell and Dr. Eliada Lazoura for English editing of the manuscript and valuable comments. We also thank Dr. Jun-ichi Miyazaki for providing pCXN2 expression vector. This work was supported by a research grant from the Organization for Pharmaceutical Safety and Research and by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan.

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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、１ug/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。

（本研究は文科省大学発ベンチャー創出事業補助金および厚労省科学研究補助金により一部をサポートされた。）

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Biochemical and Biophysical Research Communications 324 (2004) 236–240
BBRC
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Inhibition of HIV-1 infection in cells expressing an artificial complementary peptide☆

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Received 25 May 2004
Available online 25 September 2004

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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doi:10.1016/j.bbrc.2004.09.038

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 5×10^6 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

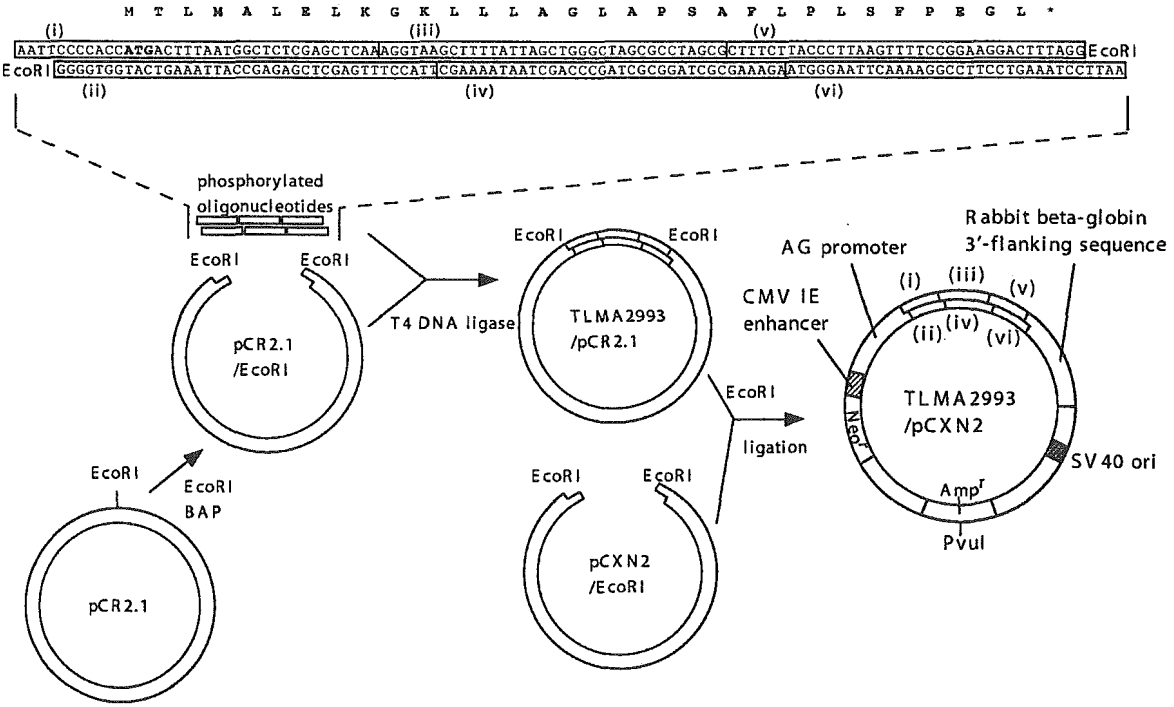

Fig. 1. Outline of the shot-gun ligation method. The vectors were constructed as described in Materials and methods. pCR2.1/*Eco*RI was employed for cloning of six overlapping synthetic oligonucleotides ((i)–(vi), in the upper part of the figure) that constructed the TLMA2993 peptide to which was added 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 2×10^6 cells/ml. Aliquots (100 μl) were plated in a V-bottomed 96-well plate (2×10^5 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. 2×10^4 U937/TLMA-15, U937/TLMA-18 or U937/N2 cells were mixed with 1×10^2 (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 (5×10^6 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 (2.5×10^5 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 (2×10^5 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′-GGTCTCTCTGGTTAGACCAGAT-3′ (RU5-5′ primer), 5′-CTGCTAGAGATTTTCCACACTG-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′-GAAATCGTGCGTGACATTAAG-3′ (β-actin 5′ primer) and 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC-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, 2×10^4 U937/TLMA-15, U937/TLMA-18 or U937/N2 cells were mixed with 1×10^2 (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-

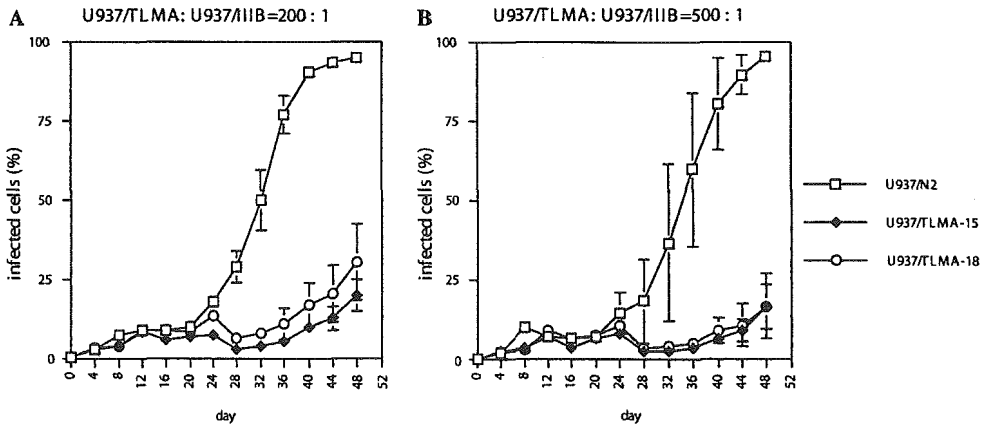

Fig. 2. Inhibition of HIV-1 spread in a mixed culture. TLMA2993 transfected cells (2×10^4) were mixed with 1×10^2 (A) or 40 (B) HIV-1 IIIB-infected U937 cells in 24-well plates with 1 ml medium. U937/TLMA-15 (♦), U937/TLMA-18 (○), and U937/N2 (□) are shown. The number of HIV-infected cells was assessed as described in Materials and methods. The values shown represent means of assays performed in triplicate (SD shown).

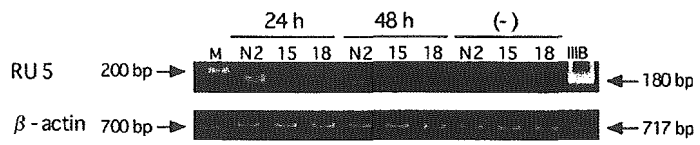

Fig. 3. PCR amplification of HIV-1 DNA in the LTR region. TLMA2993 transfected cells (15 and 18) and control cells (N2) were infected with HIV-IIIB. After cultivation for 24 and 48 h, cells were subjected to PCR amplification of 180 bp of the HIV-1 LTR region. Parallel reactions for quality control of 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.