

# Neutralization of HIV-1 by secretory IgA induced by oral immunization with a new macromolecular multicomponent peptide vaccine candidate

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**Control of pandemic infection of human immunodeficiency virus type 1 (HIV-1) requires some means of developing mucosal immunity against HIV-1 because sexual transmission of the virus occurs mainly through the mucosal tissues. However, there is no evidence as yet that the secretory immunoglobulin A (IgA) antibody induced by immunization with antigens in experimental animals can neutralize HIV-1. We demonstrate here that oral immunization with a new macromolecular peptide antigen and cholera toxin (CT) induces a high titre (1:2<sup>11</sup>) of gut-associated and secretory IgA antibody to HIV-1. Using three different neutralizing assays, we clearly demonstrate that this secretory IgA antibody is able to neutralize HIV-1<sub>IIB</sub>, HIV-1<sub>SF2</sub> and HIV-1<sub>MN</sub>. Our new approach may prove to be important in the development of a mucosal vaccine that will provide protection of mucosal surfaces against HIV-1.**

Heterosexual and homosexual transmission of human immunodeficiency virus type 1 (HIV-1) is one of the principal routes of the disease. Since sexual transmission of HIV-1 occurs mainly through the mucosal tissues, the induction of effective mucosal immunity against HIV-1 is of primary importance in protecting against this infection. It has been shown that mucosal immunization of rhesus macaque with p27:Ty-VLP (hybrid virus-like particle) elicited simian immunodeficiency virus (SIV)-specific secretory immunoglobulin A (IgA) antibodies, which were detectable in vaginal fluids and rectal washings<sup>1-3</sup>. Serum IgA antibodies isolated from HIV-1-infected individuals were also reported to be capable of neutralizing HIV-1<sub>MN</sub> *in vitro*<sup>4</sup>. However, neutralization of HIV-1 by mucosal IgA antibodies induced by immunization has not yet been described. The objective of the work presented here was to examine whether mucosal immunization with a newly developed macromolecular multicomponent peptide antigen induces antigen-specific IgA antibodies with HIV-1-neutralizing activity.

We have been working on the development of a vaccine against HIV-1 infection<sup>5,6</sup> and have recently constructed a new macromolecular multicomponent peptide antigen, designated VC1, which is composed of HIV-1 peptides from the third hypervariable region (V3), a CD4 binding site, and a Gag region. In this study, we tested the neutralizing activity of mouse fecal IgA antibodies against HIV-1 produced using VC1 with cholera toxin (CT), a potent adjuvant when given orally with various antigens<sup>7-9</sup>. Our results clearly demonstrate that oral immunization

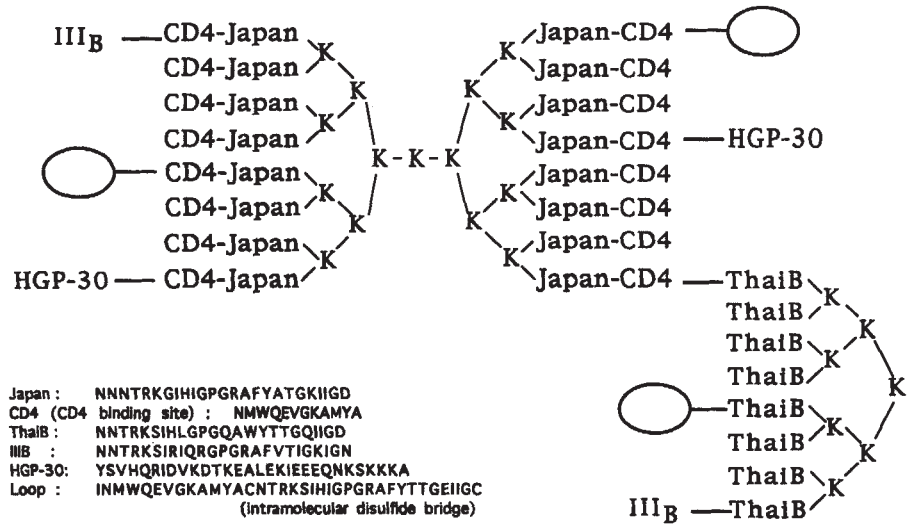
with VC1 induces both serum IgG and fecal IgA antibodies against HIV-1 and that this type of IgA antibody is capable of neutralizing HIV-1<sub>IIB</sub>, HIV-1<sub>SF2</sub> and HIV-1<sub>MN</sub> *in vitro*.

## Construction of a new macromolecular peptide antigen

VC1, the vaccine candidate used in this study, is a new macromolecular multicomponent antigen composed of peptides from four subtypes of the V3 region (cyclized form of common consensus PND, PND common in Japan<sup>10</sup>, IIB, and Thai-B (refs 11, 12)), one CD4 binding site, and one Gag region (HGP-30) (Fig. 1). This cyclized form of PND peptide induced an antigen-specific serum IgG antibody response 10-fold as great as that obtained using the linear peptide when subcutaneously immunized into rabbits<sup>13</sup>. We previously determined the sequence of the PND common in Japan used here by amino acid analysis of V3 regions from 34 Japanese patients (K. Okuda *et al.*, unpublished data) and 26 reported cases<sup>10</sup>. It has been shown that a monoclonal antibody to the CD4 binding site neutralizes HIV-1 synergistically when a monoclonal antibody to the V3 region is added simultaneously<sup>14</sup>. Therefore, VC1 was designed to elicit antibodies directed to both the CD4 binding site and the V3 region in order to induce a synergistic effect in the neutralization of HIV-1. A Gag region peptide was also included in order to compensate for the heterogeneity of HIV-1 (refs 15, 16). Since short synthetic peptides themselves were poorly immunogenic, they were partially synthesized with multiple antigenic peptides (MAP)<sup>17,18</sup> and then were coupled at the  $\alpha$ -amino terminus of each peptide using glutaraldehyde

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Fig. 1 Schematic representation of VC1. After synthesis, the CD4 binding site—common consensus PND peptide was reduced and then re-oxidized for construction of a loop configuration. Both the peptide consisting of a CD4 binding site coupled to the PND common in Japan and the Thai-B PND peptide were synthesized using the MAP method<sup>17,18</sup>. They were then conjugated at the  $\alpha$ -amino terminus with GA (refs 13, 19, 20) at pH 7.5, below the pK of the amino group, since the NH<sub>2</sub> group ( $\alpha$ -amino terminus) and not the NH<sub>3</sub> group ( $\epsilon$ -amino terminus) is the target for GA.



(GA)<sup>13,19,20</sup> (Fig. 1) (see Methods). Conjugation at the  $\alpha$ -amino terminus of each peptide using GA maintained and increased antigenicities to HIV-1. The molecular mass of VC1 used in this study was presumed to be more than 10<sup>5</sup> daltons.

**Induction of HIV-1-specific antibodies by oral immunization**

In order to investigate the feasibility of inducing a mucosal immune response using VC1, BALB/c mice were immunized by means of oral intubation with VC1 and CT, or VC1 alone. CT and not CT-B (Sigma) was chosen as the mucosal adjuvant<sup>7-9</sup> for the induction of a maximum antigen-specific mucosal immune response to orally administered VC1, since it has been reported that CT suppresses oral tolerance<sup>7,21</sup> and the adjuvant activity of CT is associated with the holotoxin<sup>21,22</sup>. VC1 alone (150  $\mu$ g) induced production of antigen-specific IgA when HIV-specific antibody responses were examined in orally immunized mice (Table 1). As one might expect, coadministration of CT enhanced the HIV-specific IgA response and helped maintain a high titre of antigen-specific IgA antibody. The highest and most prolonged production of HIV-1-specific fecal IgA was observed when 150  $\mu$ g of VC1 and 10  $\mu$ g of CT were administered orally (Table 1). Even

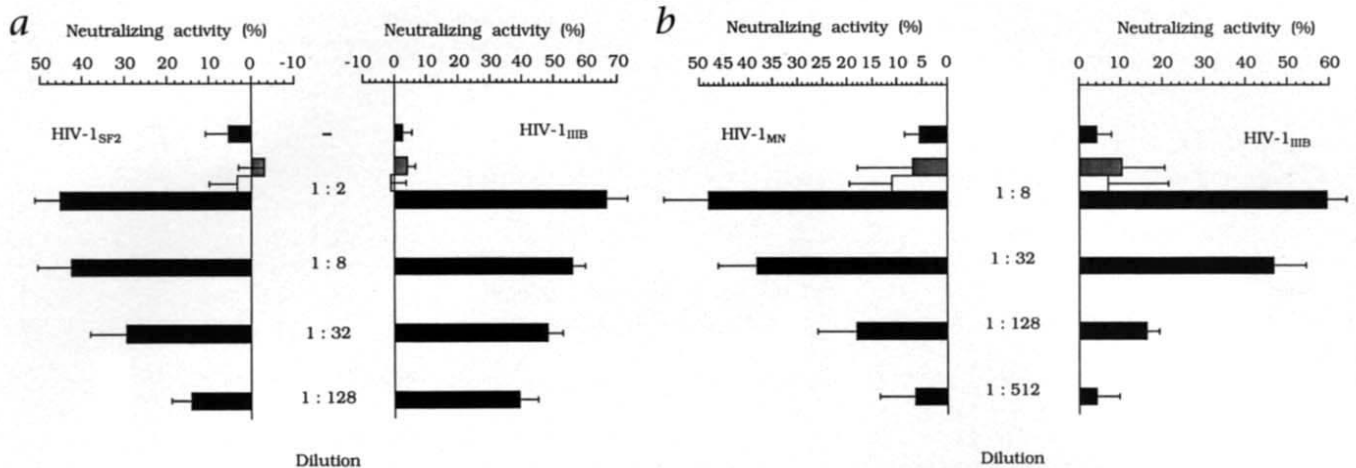
when mice were immunized with 20  $\mu$ g of CT, they exhibited no abnormal symptoms such as diarrhoea or body weight loss. Antigen-specific serum IgA and serum IgG antibodies were also induced by oral immunization with VC1 and CT. Oral administration of 150  $\mu$ g VC1 and 10  $\mu$ g CT elicited the strongest and most prolonged production of serum IgA and serum IgG antibodies as well as production of fecal IgA antibody. In general, higher titres of serum IgG antibody were generated than of serum IgA antibody. However, antigen-specific fecal IgG titres were low and negligible.

In order to determine whether HIV-1-specific fecal IgA antibody raised against VC1 was secretory IgA, the secretory component of the IgA antibody was examined using an enzyme-linked immunosorbent assay (ELISA). The secretory component titre of HIV-1-specific fecal IgA antibody was 1:2<sup>11</sup> when the fecal IgA antibody titre was 1:2<sup>11</sup>. In contrast, the secretory component titre of HIV-1-specific serum IgA was not detected when the serum IgA antibody titre was 1:2<sup>5</sup>. The secretory component of pre-immune fecal antibody directed to HIV-1 was also not detected. These results demonstrate clearly that HIV-1-specific fecal IgA antibody raised against VC1 was mainly the secretory form.

**Table 1 Induction of HIV-1-specific antibodies by oral administration of VC1 with CT**

Mucosal vaccine ( $\mu$ g/dose)	Reciprocal log <sub>2</sub> antibody titres																			
	Serum IgA antibody					Serum IgG antibody					Fecal IgA antibody					Fecal IgG antibody				
	pre	1st	2nd	3rd	4th	pre	1st	2nd	3rd	4th	pre	1st	2nd	3rd	4th	pre	1st	2nd	3rd	4th
VC1 CT	pre	1st	2nd	3rd	4th	pre	1st	2nd	3rd	4th	pre	1st	2nd	3rd	4th	pre	1st	2nd	3rd	4th
15 10	ND	2.2±1.0	2.5±0.7	2.8±1.1	3.2±1.0	ND	5.0±0.7	5.3±0.8	5.7±1.1	4.6±0.5	0.2±0.3	5.4±1.9	10.4±0.3	8.7±0.8	6.7±1.1	ND	1.7±1.1	0.3±1.0	0.3±0.4	ND
150 0	0.3±0.4	1.8±0.7	2.8±0.5	1.2±0.6	0.3±1.0	ND	5.3±0.4	5.0±0.0	6.0±0.7	5.7±0.8	ND	7.2±0.5	8.8±1.4	8.4±0.9	6.0±1.0	ND	0.7±0.8	ND	ND	0.2±0.3
150 10	ND	3.0±0.7	3.3±0.5	5.1±0.8	5.4±0.5	0.2±0.3	5.3±1.1	6.0±1.2	8.0±0.9	7.3±2.0	ND	9.4±0.6	11.2±0.5	10.0±0.5	9.0±0.3	ND	0.7±0.4	0.3±1.0	ND	ND
150 20	ND	3.2±0.5	3.8±0.7	3.8±0.7	4.5±0.9	0.3±0.4	5.0±0.4	5.7±1.1	7.7±0.8	5.7±1.5	ND	9.6±1.1	9.9±1.4	9.3±1.1	7.0±1.2	ND	1.3±1.1	0.3±1.0	ND	0.2±0.3
300 10	ND	3.2±0.7	3.6±0.5	4.6±0.7	5.0±0.5	ND	5.7±0.8	4.7±0.4	6.7±1.1	6.5±1.2	0.3±1.0	9.4±1.3	9.8±1.1	9.2±0.3	7.3±1.1	ND	1.0±0.7	0.7±0.4	ND	ND
0 10	ND	0.3±0.4	ND	ND	ND	ND	0.2±0.3	ND	0.3±0.7	ND	ND	0.4±0.3	0.2±0.3	ND	ND	ND	0.3±1.0	ND	ND	ND

Various doses of VC1 with CT were administered orally on days 0, 7, 14 and 21. pre indicates a pre-immune sample. 1st, 2nd, 3rd and 4th represent samples collected every 7 days after each immunization. Antigen-specific antibody titres are expressed as the reciprocal log<sub>2</sub> of the final detectable dilution, which gave an optical density of 0.1 A above the pre-immune control at 490 nm. Data are means  $\pm$  s.e.m. of 3–5 experiments. ND, not detected.



**Fig. 2** Neutralization of HIV-1 by fecal IgA raised against VC1. *a*, A previously described<sup>23,24</sup> anti-fusion assay was used for measuring the neutralizing activity of the fecal extract solution. (-), 1:2 diluted solution of fecal extract from a mouse injected with CT alone. Shaded bar, 1:2 diluted immune sample adsorbed on an anti-IgA column<sup>29</sup>. White bar, 1:2 diluted immune sample adsorbed on a VC1 antigen column<sup>29</sup>. Data are means  $\pm$  s.e.m. of 3–5 experiments. *b*, The neutralizing activity of the fecal extract solution was measured using an MTT assay, as described previously<sup>25,26</sup> (-), 1:8 diluted solution of fecal extract from a mouse injected with CT alone. Shaded bar, 1:8 diluted immune sample adsorbed on an anti-IgA column<sup>29</sup>. White bar, 1:8 diluted immune sample adsorbed on a VC1 antigen column<sup>29</sup>. Data are means  $\pm$  s.e.m. of 3–5 experiments.

### Neutralization of HIV-1 by antigen-specific fecal IgA

We next tested whether the fecal IgA antibodies induced by oral immunization were able to neutralize three types of HIV-1 strains. Neutralizing activity against HIV-1 was measured using three different methods; an anti-fusion assay<sup>23,24</sup>, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay<sup>25,26</sup>, and a p24 protein assay<sup>27,28</sup> (Fig. 2, Table 2). Strong inhibition of HIV-specific syncytium formation (cell-to-cell infection) was noted not only with HIV-1<sub>IIIIB</sub> but with HIV-1<sub>SF2</sub> as well when fecal extract solutions from mice that had been immunized orally with VC1 were examined by the anti-fusion assay (Fig. 2*a*). In contrast, fecal extract antibodies that were adsorbed on anti-IgA antibody or VC1 antigen columns<sup>29</sup> failed to inhibit syncytium formation, indicating that HIV-1 neutralizing activity was due to the presence of HIV-specific fecal IgA antibodies (Fig. 2*a*). It was also shown that fecal IgA antibodies prevented infection (virus-to-cell infection) not only with HIV-1<sub>IIIIB</sub> but also with HIV-1<sub>MN</sub>, as determined by the MTT assay (Fig. 2*b*). However, the fecal IgA antibodies failed to neutralize HIV-1 strains after adsorption on the columns<sup>29</sup>. Further HIV-specific p24 protein production was also prevented when HIV-1 was cocultured with fecal IgA antibodies obtained from orally immunized mice (Table 2). Results of these three different assays indicated that antigen-specific fecal IgA antibody produced by oral administration of VC1 with CT was capable of neutralizing HIV-1<sub>IIIIB</sub>, HIV-1<sub>SF2</sub> and HIV-1<sub>MN</sub>. Serum IgG antibodies (1:2<sup>8</sup>) induced by oral immunization with VC1 and CT also inhibited approximately 28% of syncytium formation of HIV-1<sub>IIIIB</sub> at a 1:8 dilution, but failed to inhibit it at a 1:32 dilution.

### Discussion

It was reported that, in the SIV macaque model, administration of whole inactivated SIVmac251 vaccine or 15-amino acid synthetic peptide by the vaginal or rectal route and boosted by the oral route failed to induce an effective immune response<sup>30</sup>. In contrast, our present results indicate that oral administration of

our recently developed VC1, which contains cyclized peptide with MAP and is polymerized by GA, elicits strongly antigen-specific IgA and IgG antibodies in secretions and serum. This suggests that VC1 possesses much higher immunogenicity than the peptide used in the SIV macaque model. It is characteristic that the fecal IgA antibody response induced was greater than that of the serum IgG antibody following oral immunization with VC1. This may have resulted from the fact that VC1 is an insoluble substance and may exist for long periods without diffusion at locations within the alimentary canal or in inductive tissues such as Peyer's patches by means of M cells. Since mouse serum IgA antibody is reported to be mainly dimeric<sup>31</sup>, the presence of IgA in this form would make it difficult to distinguish secretory IgA from serum IgA. Secretory IgA antibody was therefore determined by the presence of the secretory component of the fecal IgA antibody directed to HIV-1. Mouse serum IgG antibody induced by oral immunization of VC1 showed a low level of neutralizing activity.

Our present observations support the idea that induction of secretory IgA antibody by vaccination might be effective in preventing HIV-1 infection. Results obtained with the three assay systems employed here clearly demonstrate that oral administration of VC1 with CT can induce a high level of secretory IgA antibodies effective in inhibiting HIV-1 replication *in vitro*. Antigen-specific secretory IgA antibodies neutralized not only HIV-1<sub>IIIIB</sub>, whose V3 region peptide was one of components of VC1, but also other HIV-1 strains whose V3 region peptides were not included in the vaccine. Since VC1 was constructed from polyvalent macromolecular peptide antigens, it may be capable of inducing broadly reactive antibodies against HIV-1 strains. It would be also necessary to test neutralization of broader types of HIV-1 strains and primary HIV-1 isolates. This is the first report that the secretory IgA antibody induced by immunization neutralizes HIV-1. This new approach might prove to be very important for the development of a mucosal vaccine capable of providing protection of mucosal surfaces against HIV-1.



## Methods

**Viruses.** HIV-1<sub>IB</sub>, HIV-1<sub>SF2</sub> and HIV-1<sub>MN</sub> strains used in our study were provided by the National Institutes of Health AIDS Research and Reference Reagent Program.

**Experimental animals.** All 8- to 12-week-old BALB/c mice were obtained from the Shizuoka Laboratory Animal Center Co. Ltd. (Japan), and were maintained in horizontal laminar flow cabinets and provided with sterile food and water.

**Peptide synthesis and antigen construction.** A peptide consisting of a 22-amino acid residue of the common consensus PND and a 13-amino acid residue of the CD4 binding site was synthesized using an automated model 430A peptide-synthesizer (Applied Biosystems, California) by a solid-phase procedure. After synthesis, it was reduced and reoxidized for construction of a cyclized form of the V3 loop peptide<sup>13</sup>. Both the PND peptide common in Japan and the Thai-B peptide were synthesized with MAP (refs 17, 18). A Gag-region peptide (HGP-30)<sup>15,16</sup> was similarly synthesized. After all synthesized peptides were purified using reversed-phase high-performance liquid chromatography (HPLC), as previously described<sup>32</sup>, they were conjugated at the  $\alpha$ -amino terminus of each peptide with GA (refs 13, 19, 20) to increase antigenicity. Since the NH<sub>2</sub> group and not the NH<sub>3</sub><sup>+</sup> group is the target for GA, the  $\alpha$ -amino terminus of each peptide was generally an NH<sub>2</sub> group at a pH of 7.5, below the pK of each amino group, enabling it to be conjugated by GA<sup>13</sup>. In this way, the antigenicity of each peptide was maintained and increased.

**Immunization.** Fifteen minutes before immunization, we administered to mice by gavage 250  $\mu$ l of a solution composed of 8 parts Hanks' balanced salt solution and 2 parts 7.5% sodium bicarbonate in order to neutralize stomach acidity<sup>7</sup>. Vaccines containing VC1 (0–300  $\mu$ g) and CT (0–20  $\mu$ g) were then administered orally in 250  $\mu$ l of phosphate-buffered saline (PBS) on days 0, 7, 14 and 21. Both sera and feces were collected every 7 days after each immunization. Fecal extract samples were prepared as described elsewhere<sup>33</sup>. Briefly, after 100 mg of fecal pellets were mixed with 1 ml of PBS, samples were spun in a vortex mixer, left to settle for 15 min, respun until all materials were resuspended, and centrifuged at 12,000 r.p.m. for 10 min. The supernatants were then removed and tested.

**ELISA.** Antibody responses were determined by ELISA as previously described<sup>7</sup>. Ninety-six-well microplates (Nunc, Denmark) were coated with 5  $\mu$ g per 100  $\mu$ l per well of the peptide mixture of VC1 components overnight at 4 °C. Following blocking with 1% bovine serum albumin, serially diluted samples were incubated in the wells for 2 h. The wells were treated with peroxidase-labeled, affinity purified, anti-mouse IgA or IgG (Organon Teknica, Pennsylvania) or anti-human secretory component (Medical & Biological Laboratories, Japan) for 1.5 h. *o*-Phenylenediamine dihydrochloride (Sigma) in 0.1 M citrate-phosphate buffer (pH 5.0) containing 0.01% H<sub>2</sub>O<sub>2</sub> was added. Antigen-specific antibody titres were expressed as the reciprocal log<sub>2</sub> of the final detectable dilution, which gave an optical density of  $\geq 0.1$  A above the pre-immune control at 490 nm.

**Anti-fusion assay.** A previously described anti-fusion assay<sup>23,24</sup> was used for measuring the neutralizing activity of the fecal solution. CEM cells were infected with HIV-1<sub>IB</sub> or HIV-1<sub>SF2</sub>, and then cultured for approximately 14 days. Stably infected CEM cells were first incubated for

**Table 2 HIV-1 neutralizing activity of fecal IgA antibody measured using a p24 protein assay**

Fecal extract solution	p24 protein production (ng/ml)	
	HIV-1 <sub>IB</sub>	HIV-1 <sub>SF2</sub>
Pre-immune	25.9 $\pm$ 1.4	13.9 $\pm$ 1.8
CT only	26.5 $\pm$ 1.5	11.7 $\pm$ 1.6
Immune	11.1 $\pm$ 1.1*	8.2 $\pm$ 1.5*
Immune (adsorbed on an anti-IgA column*)	24.6 $\pm$ 1.6	13.2 $\pm$ 1.7
Immune (adsorbed on a VC1 antigen column*)	25.7 $\pm$ 1.4	15.1 $\pm$ 1.9

Data are means  $\pm$  s.e.m. of 3–5 assays. \*Mean values are significantly different from each pre-immune control ( $P < 0.01$ ).

\*Purified anti-mouse IgA antibody or VC1 antigen was conjugated to CNBr-activated Sepharose 4B beads according to a previously described method<sup>29</sup>. One milligram of protein or peptide was coupled to 0.5 ml of beads and the product was packed into a 3-ml syringe. Adsorptions were carried out by repeatedly passing 2 ml of sample through the columns.

2 h with several concentrations of fecal extracts or column-adsorbed samples. Uninfected MOLT-4 cells were then added to this culture system (infected cells:uninfected cells = 1:10). After the plates were incubated for 24 h at 37 °C, giant cells were counted. The neutralizing activity was expressed as the percentage of reduction in syncytium formation by the immune fecal solution as compared with the pre-immune fecal solution. An anti-IgA or a VC1 antigen column<sup>29</sup> was used for adsorption of fecal IgA antibodies or HIV-1-specific Abs, respectively, and samples were tested again.

**MTT assay.** One hundred tissue-culture infectious doses (TCID<sub>50</sub>) of HIV-1<sub>IB</sub> or HIV-1<sub>MN</sub> were preincubated with dilutions of fecal extract solution for 1.5 h at 4 °C. Following incubation, 50  $\mu$ l of pretreated virus was plated on a 96-well microplate (Nunc) into wells containing  $2.5 \times 10^4$  MT-4 cells per well in 150  $\mu$ l of complete medium (RPMI-1640 containing 5% heat-inactivated fetal calf serum) and then incubated at 37 °C. As controls, untreated HIV and mock-infected cells were also incubated. Five days after infection, their viabilities were examined spectrophotometrically. The MTT assay<sup>25,26</sup> used is based on the reduction of yellow-coloured MTT (Dojin Co. Ltd., Japan) by mitochondrial dehydrogenases of metabolically active cells. Absorbance measured at 540 nm correlated with cell viability.

**p24 protein assay.** A 2% solution of fecal extract from each immunized or pre-immunized mouse was added to a medium containing HIV-1 and incubated for 1 h. Uninfected CEM was added to the medium containing the virus. After 4 h, the cells were washed and cultured for 5 days. The concentrations of p24 protein in the filtered cell-free supernatants were measured using an HIV-1-specific enzyme immunoassay (Abbott Laboratories, North Chicago, Illinois) according to the manufacturer's instructions<sup>27,28</sup>. The concentration of p24 (nanograms per millilitre) was determined from the standard curve derived from dilutions of the known standard.

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