BF23, which used *btuB* as a cell surface receptor to gain entry into *E. coli*. Because *birA* is an essential gene, recombinants at *birA* were screened by electron microscopy.
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# Induction of Mucosal and Systemic Immunity to a Recombinant Simian Immunodeficiency Viral Protein

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**Heterosexual transmission through the cervico-vaginal mucosa is the principal route of human immunodeficiency virus (HIV) infection in Africa and is increasing in the United States and Europe. Vaginal immunization with simian immunodeficiency virus (SIV) had not yet been studied in nonhuman primates. Immune responses in macaques were investigated by stimulation of the genital and gut-associated lymphoid tissue with a recombinant, particulate SIV antigen. Vaginal, followed by oral, administration of the vaccine elicited three types of immunity: (i) gag protein p27–specific, secretory immunoglobulin A (IgA) and immunoglobulin G (IgG) in the vaginal fluid, (ii) specific $CD4^+$ T cell proliferation and helper function in B cell p27-specific IgA synthesis in the genital lymph nodes, and (iii) specific serum IgA and IgG, with $CD4^+$ T cell proliferative and helper functions in the circulating blood.**

Vaginal transmission of SIV has been achieved experimentally in macaques, resulting in the development of an acquired immunodeficiency syndrome (AIDS)–like syndrome (*1*). This route of infection resembles heterosexual transmission of HIV in humans. Although systemic immunization strategies have protected against intravenous challenges with infectious SIV (*2*), they have not prevented vaginal transmission (*1*). In view of these observations and the prevalence of heterosexual transmission of HIV in humans, the development of a simian model of vaccination that can prevent genital transmission of SIV has been emphasized (*3*).

We have shown that protein p1, encoded by the yeast retrotransposon Ty, can be used as a carrier for recombinant antigens and that p1 fusion proteins self-assemble into hybrid virus-like particles (Ty-VLP) (*4*). Systemic immunization studies demonstrated that hybrid particles that carry the SIV gag protein p27 (SIV p27:Ty-VLP) induce both circulating antibody and T cell responses in macaques (*4*). Administration of $SIVmac_{251}$ whole, inactivated vaccine or synthetic peptides by the mucosal route did not induce an effective immune response (*5*). We therefore attempted to use SIV p27:Ty-VLP to stimulate the mucosal-associated lymphoid tissue (*6*). The SIV p27:Ty-VLP was conjugated to the GM1 ganglioside receptor–binding subunit of cholera toxin (CTB), which has potent mucosal adjuvant properties (*7*). We used two separate immunization regimes to assess the p27:Ty-VLP/CTB vaccine that was administered by nontraumatic vaginal and oral instillation. Three macaques received two oral, followed by three vaginal, administrations (O-V), and four macaques received two vaginal, followed by three oral, administrations (V-O) of the vaccine. Four macaques were used as controls by administration of p27:Ty-VLP without CTB, p27 conjugated to CTB (p27/CTB), or p27 alone, all by the O-V route to one macaque each, or by administration of Ty-VLPs conjugated to CTB (Ty-VLP/CTB) by the V-O route to one macaque.

Sequential examination of vaginal, rectal, salivary, and serum antibodies before and after each of the oral and vaginal immunizations with p27:Ty-VLP/CTB showed that IgA (Fig. 1 and Table 1) and IgG (Fig. 2 and Table 1) were specifically raised to p27 (anti-p27). IgA and IgG anti-p27 in vaginal fluid were found after the first or second oral or vaginal immunization in one macaque in each group. In the other macaques immunized by the O-V route, antibodies increased after the fifth mucosal immunization. The macaques in the second group immunized by the V-O route showed an increase in antibodies after the fourth or fifth mucosal immunization. Similar results were found with serum IgA and IgG, except that these were detected earlier (Figs. 1 and 2). Salivary IgA, but not IgG, appeared early, after the first or second vaginal immunization in the V-O group and to a lesser extent in the O-V group (Table 1). Rectal washings showed anti-p27 IgA in only three out of seven macaques and IgG in one out of seven macaques after the fifth mucosal immunization. Of the four control macaques, those immunized with p27:Ty-VLP, p27 alone, or Ty-VLP/CTB did not show IgA or IgG in vaginal and rectal fluids, whereas p27/CTB elicited some antibodies in vaginal fluid (Figs. 1 and 2). However, all but Ty-VLP elicited serum IgA and IgG anti-p27. Immunization with p27/CTB or p27 also induced salivary IgA and IgG (Table 1). One-way analysis of variance (ANOVA) of vaginal anti-p27 IgA in macaques immunized with p27:Ty-VLP/CTB by the V-O route, as compared with the control group, showed a significant difference ($P = 0.017$), and anti-p27 IgG was also significant ($P = 0.022$). Although vaginal IgA elicited by the O-V sequence of immunization was significant ($P = 0.025$), IgG ($P = 0.44$) did not reach the 5% level of significance. By either sequence of immunization, serum anti-p27 IgA was not significant if the immunized group was compared with the control group ($P = 0.057$ and 0.143) because among the control macaques all but Ty-VLP/CTB elicited serum IgA (Figs. 1 and 2). However, serum

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IgG by the O-V route reached a significant difference ($P = 0.006$) but not by the V-O sequence ($P = 0.056$).

After the final mucosal immunization, the macaques were immunized by the intramuscular (IM) route with the vaccine used for mucosal immunization. Increases in vaginal, serum, and salivary IgA and IgG were recorded in three out of four macaques immunized by the V-O route. However, O-V immunization resulted in an increase of vaginal IgA in two out of three animals but not in IgG. No increases were seen in serum or salivary IgA and IgG after O-V immunization; salivary, and to a lesser extent, vaginal and serum antibodies actually decreased in some macaques (Figs. 1 and 2 and Table 1). IM challenge did not increase rectal antibody titers. One-way ANOVA of the V-O sequence followed by IM immunization showed a significant increase in IgA ($P = 0.027$) and IgG ($P = 0.008$) when the titers after the three oral immunizations were compared with that of the IM immunization; significant results were not found with the O-V sequence of immunization ($P > 0.2$).

The sensitivity of the enzyme-linked immunosorbent assay (ELISA) method was determined by means of affinity-purified anti-p27 IgA and IgG (8). These antibodies were used in an ELISA and showed that the sensitivity for anti-p27 IgA reached 59 ng/ml and that for IgG it was 18 ng/ml. A comparison of serum with vaginal antibodies revealed that representative serum anti-p27 IgA was 168.4 μg/ml and that for IgG it was 62.1 μg/ml, whereas in vaginal washings anti-p27 IgA was 490 ng/ml and IgG was 60 ng/ml. In addition, vaginal IgA is underestimated by a factor of about 3 because we used monomeric serum IgA standard to estimate polymeric IgA. Furthermore, both vaginal IgA and IgG antibodies are greatly underestimated because washings were used, which introduces an unknown dilution factor.

The specificity of anti-p27 was demonstrated by the fact that antibodies were not raised in the vaginal washings, sera, or saliva to a random peptide or to Ty-VLP after mucosal immunization. Furthermore, adsorption of vaginal fluid, serum, or saliva by Sepharose beads coated with p27 abrogated IgA and IgG titers, unlike beads coated with control tetanus toxoid (TT) antigen that had little effect on the antibody titers (9). The vaginal IgA was of the polymeric secretory type, as demonstrated by ELISA with a goat antibody to human IgA secretory component and a goat antibody to human IgA J chain. Thus, anti-p27 in vaginal fluid detected by antibodies to IgA secretory component or J chain yielded titers of 1:8 to 1:16. In contrast, serum anti-p27 IgA in the same macaque revealed a titer of 1:1600 with antibody to IgA but only 1:100 with antibody to secretory component. To examine for J chains, the serum IgA had to be separated from IgM (which also has J chains), and the pure IgA showed an anti-p27 titer of 1:128 with the antibody to IgA but only 1:2 with the antibody to J chain.

The detection of p27-specific antibodies in the serum of macaques immunized through the mucosa suggested that IgA- and IgG-producing B cells migrated from the mucosal-associated lymphoid tissues into the circulation (6). These cells could then recirculate and go to other mucosal-associated

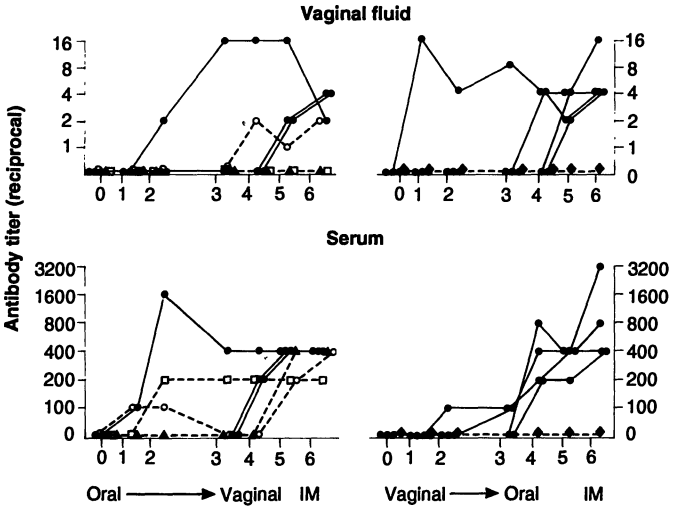


**Fig. 1.** IgA anti-p27 in vaginal fluid or serum were determined by ELISA after O-V or V-O immunization of macaques with SIV p27:Ty-VLP/CTB (●, solid lines), p27:Ty-VLP (□, dotted lines), p27/CTB (○, dotted lines), p27 (▲, dotted lines), or Ty-VLP/CTB (◆, dotted lines) (*16, 17*).

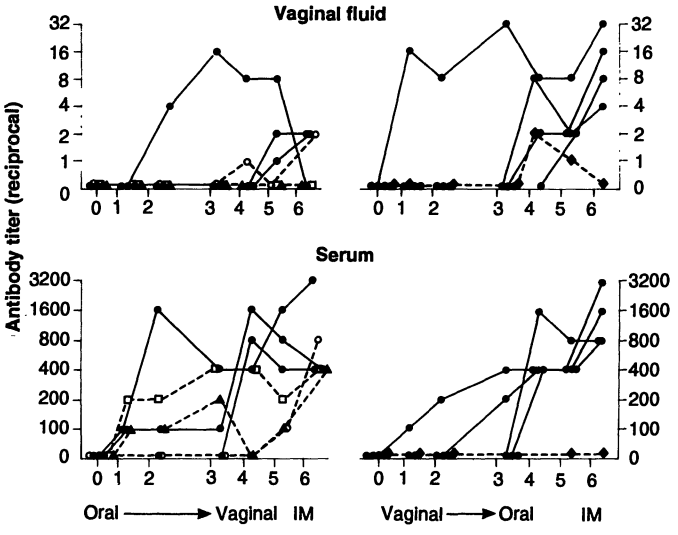


**Fig. 2.** IgG anti-p27 in vaginal fluid or serum after O-V or V-O immunization of macaques with SIV p27:Ty-VLP/CTB (●, solid lines), p27:Ty-VLP (□, dotted lines), p27/CTB (○, dotted lines), p27 (▲, dotted lines), or Ty-VLP/CTB (◆, dotted lines). The methods used were as described (*16, 17*), except that IgG anti-p27 was detected with a rabbit IgG to monkey IgG (2 μg/ml; Nordic Immunological Laboratory).

**Table 1.** Salivary IgA and IgG anti-p27 antibodies were determined by ELISA (*16*). Saliva was collected in petri dishes after pilocarpine stimulation. The results are expressed as reciprocal titers of the lowest dilution that gives an absorbance of 0.15 units above the background sample. PI, before immunization; ND, not determined.

| Vaccine | PI | Immunizations | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| | *IgA anti-p27* | | | | | | |
| | | Oral | | Vaginal | | | IM |
| p27:Ty-VLP/CTB | 0 | 2 | 1 | 0 | 32 | 8 | 8 |
| p27:Ty-VLP/CTB | 0 | 0 | 16 | 0 | 0 | 8 | 0 |
| p27:Ty-VLP/CTB | 0 | 2 | 0 | 0 | 0 | 8 | 8 |
| p27:Ty-VLP | 0 | 0 | 0 | 0 | 0 | 0 | ND |
| p27-CTB | 0 | 32 | 8 | 8 | 16 | 4 | 2 |
| p27 | 0 | 16 | 8 | 0 | 1 | 16 | 16 |
| | | Vaginal | | | Oral | | IM |
| p27:Ty-VLP/CTB | 0 | 8 | 4 | 2 | 16 | 4 | 4 |
| p27:Ty-VLP/CTB | 0 | 0 | 2 | 2 | 16 | 2 | 16 |
| p27:Ty-VLP/CTB | 0 | 8 | 4 | 0 | 2 | 2 | 4 |
| p27:Ty-VLP/CTB | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Ty-VLP/CTB | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *IgG anti-p27* | | | | | | |
| | | Oral | | Vaginal | | | IM |
| p27:Ty-VLP/CTB | 0 | 0 | 0 | 0 | 32 | 2 | 4 |
| p27:Ty-VLP/CTB | 0 | 0 | 0 | 0 | 0 | 8 | 8 |
| p27:Ty-VLP/CTB | 0 | 0 | 0 | 0 | 0 | 4 | 2 |
| p27:Ty-VLP | 0 | 0 | 0 | 0 | 0 | 0 | ND |
| p27-CTB | 0 | 0 | 0 | 2 | 2 | 1 | 0 |
| p27 | 0 | 4 | 0 | 0 | 0 | 8 | 16 |
| | | Vaginal | | | Oral | | IM |
| p27:Ty-VLP/CTB | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| p27:Ty-VLP/CTB | 0 | 0 | 0 | 0 | 4 | 2 | 8 |
| p27:Ty-VLP/CTB | 0 | 0 | 2 | 0 | 2 | 0 | 4 |
| p27:Ty-VLP/CTB | 0 | 0 | 0 | 0 | 0 | 2 | 4 |
| Ty-VLP | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

lymphoid tissues to produce antibodies (for example, in saliva) after vaginal and oral immunization. These observations are consistent with other studies, mostly in rodents, in which oral immunization with a variety of antigens (but not HIV or SIV) induced antibodies in the genital tract (*10*) and cervico-vaginal or uterine immunization (*11*) resulted in the production of local and, in some cases, systemic antibodies. The induction of systemic antibodies after mucosal immunization with p27:Ty-VLP/CTB led us to investigate whether p27-sensitized T cells could be detected in the circulation.

Peripheral blood lymphocytes from the immunized animals were stimulated in vitro with p27, and the uptake of $^3$H-labeled thymidine was recorded both in absolute counts per minute and as stimulation indices (Fig. 3). Specific proliferation of lymphocytes stimulated with p27 (but not with Ty-VLP/CTB alone or the control peptide) was detected earlier than the corresponding antibodies. In two of the three macaques in the O-V group, the stimulation index was >2 after the first oral immunization; the third macaque showed a specific increase in the lymphoproliferative response only after the first vaginal immunization. In the V-O group, two out of four macaques showed a significant stimulation index after vaginal immunization, with the remaining two macaques converting after subsequent oral immunizations. The lymphoproliferative responses were augmented in all four macaques after oral immunization of the V-O group of animals. Expression of the results of [$^3$H]thymidine uptake also showed an increase in counts per minute in all macaques after vaginal immunization (in the V-O group) or oral immunization (in the O-V group) with gag p27:Ty-VLP/CTB. However, V-O immunization yielded higher stimulation indices after the last mucosal immunization (7.6, 7.0, 5.1, and 2.4) than those after O-V immunization (6.2, 3.0, and 2.6). One-way ANOVA of the stimulation indices of either the V-O or O-V sequence of immunization, as compared with those of the controls, showed a significant difference ($P < 0.0001$).

IM challenge after mucosal immunization elicited a significant increase in T cell p27 stimulation indices in vitro in three out of four macaques immunized by the V-O route (net increases of 7.5, 7.4, and 5.0; $P = 0.01$), as compared with lower and insignificant increases in the O-V immunized group (5.0, 2.8, and 2.8; $P = 0.077$) (Fig. 3). The four control macaques immunized with p27:Ty-VLP, p27/CTB, p27, or Ty-VLP/CTB did not induce significant lymphoproliferative responses to p27 (Fig. 3). To determine which T cell subset mediated the observed proliferative responses, we separated the T lymphocytes from four macaques into CD4- and CD8-enriched populations by panning with a monoclonal antibody to CD4 (*12*). Stimulation of these T cell subsets in vitro with p27 showed that the

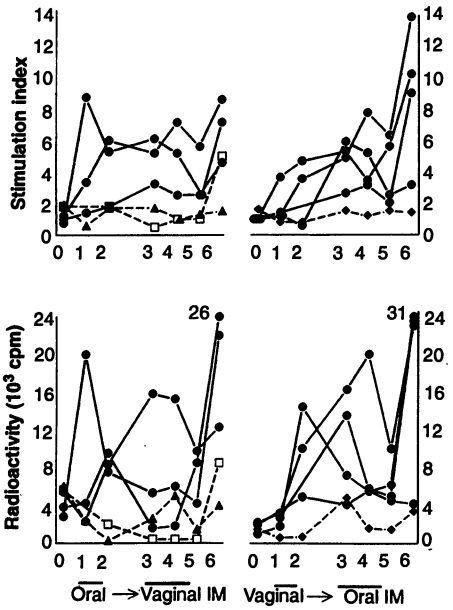


**Fig. 3.** The p27-specific proliferation of peripheral blood lymphocytes after O-V or V-O immunization of macaques with SIV p27:Ty-VLP/CTB (●, solid lines), p27:Ty-VLP (□, dotted lines), p27/CTB (○, dotted lines), p27 (▲, dotted lines), or Ty-VLP/CTB (◆, dotted lines) (*15*).

**Table 2.** We determined in vitro IgA and IgG synthesis by reconstituting B cells, CD4$^+$ cells, macrophages, and antigen (*14*). The results are given as mean (± SEM in parentheses) of absorbance with peripheral blood cells after V-O immunization of three macaques with p27:Ty-VLP/CTB (Exp. 1), the same animals after IM immunization with p27:Ty-VLP in AluGel (Exp. 2), and a macaque immunized only by the IM route with the latter vaccine (Exp. 3). The results are presented for the genital lymph node cells and for splenic cells removed from three macaques immunized by the V-O route and boosted by IM immunization by the vaccines used in Exps. 1 and 2, respectively.

| Exp. | Immunization | p27 | | Ty-VLP | | TT | |
|---|---|---|---|---|---|---|---|
| | | IgA | IgG | IgA | IgG | IgA | IgG |
| | | *Peripheral blood mononuclear cells* | | | | | |
| 1 | V-O | 0.218 (0.025) | 0.102 (0.014) | 0.081 (0.011) | 0.075 (0.014) | 0 | 0 |
| 2 | V-O + IM | 0.044 (0.007) | 0.163 (0.024) | 0.111 (0.028) | 0.100 (0.037) | 0 | 0 |
| 3 | IM | 0.056 | 0.357 | 0.076 | 0.10 | 0.242 | 0.396 |
| | | *Genital lymph node cells* | | | | | |
| 4 | V-O + IM | 0.304 (0.031) | 0.188 (0.003) | 0.162 | 0.180 | 0.161 (0) | 0.172 (0.01) |
| | | *Spleen cells* | | | | | |
| 5 | V-O + IM | 0.201 (0.023) | 0.395 (0.003) | 0.131 | 0.195 | 0.161 (0) | 0.175 (0.013) |

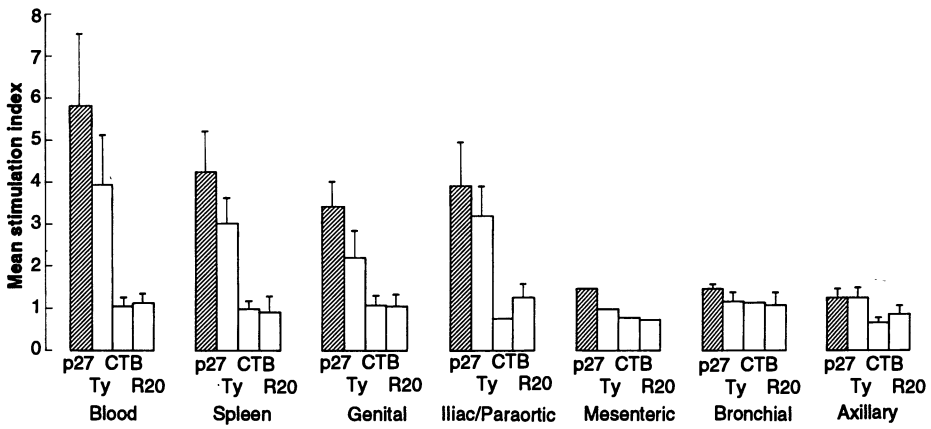


**Fig. 4.** The spleen, genital (obturator), iliac, paraortic, superior mesenteric, bronchial, and axillary lymph nodes were removed at autopsy from four macaques immunized by the V-O ($n = 2$) or O-V ($n = 2$) route and boosted by IM immunization (*12*). The cells were separated and processed after breaking up the tissues (*15*). The cells were cultured without antigen and with p27, Ty-VLP, CTB, or R20 (1, 10, and 20 μg/ml) for 5 days; only the optimal responses are presented. Because the proliferative responses of lymphocytes from the macaques immunized by the V-O route showed little difference from those immunized by the O-V route, the results are expressed as the mean stimulation index (± SEM) of the lymphoid cells of four macaques; for mesenteric lymph node cells only two macaques were examined.

responding cells belonged to the $CD4^+$ subset, which yielded a mean stimulation index of 5.4 ± 1.3 (or 7450 ± 3100 cpm), as compared with the $CD8^+$ subset, which yielded a mean stimulation index of 1.8 ± 0.2 (or 1200 ± 400 cpm).

We killed four macaques (*13*) immunized by the O-V ($n = 2$) or V-O ($n = 2$) route and removed the related and unrelated lymph nodes and spleens. Mononuclear cells isolated from the draining lymph nodes (genital, iliac, and paraortic) showed significant proliferative responses to p27 or Ty-VLP (but not to CTB or the random peptide R20) (Fig. 4). However, superior mesenteric, bronchial, or axillary lymph node cells did not respond to these antigens, although they were readily stimulated with concanavalin A. This suggests that augmented vaginal immunization does not involve the entire mucosal-associated lymphoid tissue because neither the mesenteric nor bronchial lymph nodes yielded specific lymphoproliferative responses. However, spleen cells and circulating blood cells showed significant T cell responses, indicating that vaginal immunization augmented by oral immunization elicits genital and systemic lymphoproliferative responses that are not found with the gut-associated lymphoid tissue (mesenteric) or the unstimulated bronchial-associated lymphoid tissue. We have also shown that the T cell responses involve predominantly $CD4^+$ and not $CD8^+$ cells (*12*).

To establish that mucosal immunization elicits $CD4^+$ cells in the circulation that are capable of helping B cell antibody synthesis, we carried out in vitro reconstitution experiments (*14*). Adding enriched circulating $CD4^+$ cells to B cells and macrophages from the V-O–immunized macaques and stimulation with p27 elicited specific anti-p27 IgA (Table 2, Exp. 1). However, when these macaques were challenged by the IM route, anti-p27 IgG was synthesized (Table 2, Exp. 2). Systemic immunization alone induced IgG but not IgA anti-p27, although the animal was capable of producing IgA antibodies to TT (Table 2, Exp. 3). A series of controls without one of the cells ($CD4^+$ cells, B cells, or macrophages), without antigen, or stimulation with unrelated antigen TT did not elicit anti-p27 IgA or IgG.

The results are consistent with the concept that V-O or O-V immunization activates the mucosal-associated lymphoid tissue to produce IgA, whereas IM immunization favors the IgG class of antibodies. We verified this hypothesis by comparing the isotypes of antibodies synthesized by the genital lymph node B cells, $CD4^+$ cells, and macrophages with those from the corresponding spleens in three macaques immunized by the V-O route and boosted by IM immunization (Table 2). Genital lymph node cells yielded higher anti-p27 IgA levels than for IgG (Table 2, Exp. 4), whereas higher IgG levels than for IgA were found with splenic cells (Table 2, Exp. 5). This was not found with Ty-VLP or the unrelated TT, which stimulated similar readings for both IgA and IgG. The specificity of the in vitro antibody synthesis was established because stimulation with p27 induced only anti-p27, stimulation with Ty-VLP induced only antibodies to Ty-VLP, and stimulation with TT induced only antibodies to TT. Furthermore, these experiments suggest that the $CD4^+$ cells function as helper T cells in antibody synthesis and that mucosal immunization generates both p27-sensitized $CD4^+$ cells and predominantly IgA-producing B cells in the genital lymph nodes, from which they enter the circulation. However, homing of T cells to the genital tract or the cytotoxic potential of these cells has not been explored.

Vaginal transmission of SIV might be prevented by local mucosal IgA and IgG. However, if the mucosal immune barrier were breached, a second line of defense, the genital lymph node T and B cell functions, might prevent infection. A failure of both the mucosal and genital lymph node barriers still leaves the circulating antibodies and T cells against SIV to prevent infection and the development of AIDS. We have shown that the V-O immunization regime with p27:Ty-VLP/CTB generates surface mucosal, genital lymphoid tissue, and systemic immunity, as assessed by IgA and IgG, $CD4^+$ proliferation, and helper cell function in specific antibody synthesis. This mucosal model can now be used to evaluate the ability of candidate vaccines to prevent vaginal transmission of SIV. Augmented V-O immunization strategies might also be applicable to other sexually transmitted diseases.

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8. IgG was separated from serum by ion-exchange chromatography on a DEAE-cellulose column (Whatman, Maidstone, U.K.) in 0.01 M tris-phosphate (pH 7.0). Partially purified IgG was adsorbed with goat antibody to IgA (Nordic Immunological Laboratory, Maidenhead, U.K.), attached to cyanogen bromide (CNBr)–activated Sepharose 4B beads (Kabi-Pharmacia, Milton Keynes, U.K.). The IgG was then affinity-purified on CNBr-activated 4B beads to which SIV p27 had been coupled. Specific monkey anti-SIV p27 IgG was eluted with 6 M guanidine hydrochloride, brought to neutrality with 2 M tris, and dialyzed against phosphate-buffered saline before assay by ELISA. IgA was separated from serum by gel filtration chromatography on a Sephadex G-200 column (Kabi-Pharmacia) in 0.1 M tris-HCl with 1 M NaCl (pH 8.0). The *7S* peak was further chromatographed on a DEAE column as in the case of IgG. After elution of the IgG main peak, IgA was eluted by application of a salt-pH gradient of 0.01 M tris-phosphate (pH 7.0) to 0.3 M tris-phosphate (pH 4.0). We applied IgA to an affinity column of goat antibody to human IgG coupled to Sepharose 4B to remove traces of IgG. Purified IgA was then applied to a Sepharose 4B column with bound p27 and eluted as for the specific anti-p27 IgG. The concentrations of the affinity-purified anti-p27 IgA and IgG were determined by radial immunodiffusion with reference to purified rhesus monkey serum IgA and IgG standards.
9. We tested the specificity of the antibody assay by coupling p27 or TT (at 0.25, 0.5, 1, 2, and 4 μg/ml) to CNBr-activated Sepharose 4B beads. Dilutions of vaginal washings, serum, or saliva samples were incubated with the appropriate volume of beads (1 hour at 37°C and 16 hours at 4°C). The beads were centrifuged, and the adsorbed samples were tested by ELISA. All three test fluids showed complete inhibition of anti-p27 with p27 (at 2 to 4 μg/ml) but negligible inhibition with TT.
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12. Mononuclear cells were separated from defibrinated blood by Lymphoprep (Nycomed, Oslo, Norway) and density-gradient centrifugation, following the manufacturer's instructions. We isolated enriched monocytes by incubating the cells in plastic plates in RPMI 1640 medium (with 10% fetal bovine serum) for 1 hour at 37°C with 5% $CO_2$. Nonadherent cells were removed, and adherent cells were incubated with RPMI-1640 medium overnight at 37°C and recovered by washing the plate. The nonadherent cells were separated by established procedures on the basis of whether they bound 2-aminoethylisothiouronium (AET)–treated sheep red blood cells ($T^+$ cells) or did not ($T^-$; enriched B cells). The T cells were further separated by panning $5 \times 10^6$ cells with an optimal amount of monoclonal anti-T4 culture supernatant (100 μl per $10^6$ cells) in Hanks' solution (with 10% fetal bovine serum) overnight at 4°C. After washing, $15 \times 10^6$ cells were added to petri dishes that had been coated with affinity-purified goat antibody to mouse IgG (at 5 μg/ml in 0.5 M tris-HCl, pH 9.5) for 70 min at 4°C. The nonadherent cells consisted of enriched $CD8^+$ cells, and the adherent cells were enriched $CD4^+$ cells. These enriched T cell subsets were stimulated with p27 and processed as described (*15*).
13. The macaques were killed by injection of IM ketamine hydrochloride (10 mg per kilogram of body mass; Parke-Davis Veterinary, Ponty Poole, U.K., followed by IM Rompun xylazine (20 mg/kg; Bayer Pharmaceuticals, Newbury, U.K.), and finally intravenous pentobarbitone sodium (200 mg/kg; Bayer Pharmaceuticals).
14. R. Fellowes and T. Lehner, *J. Immunol. Methods* **132**, 165 (1990). Mononuclear cells were separated from peripheral blood (*12*), and enriched B cells ($10^5$), $CD4^+$ cells ($4 \times 10^5$), and monocytes ($5 \times 10^4$) were reconstituted and stimulated with

p27, Ty-VLP, or TT (200 or 20 μg/ml) for 7 days, followed by culture without antigen for 8 days. The culture supernatants were assayed for the corresponding IgA or IgG by a modified ELISA. Microtiter plates were coated with antigen (p27, p27:Ty-VLP, Ty-VLP, or TT; 1 μg/ml). Culture supernatants were diluted 1:1 with RPMI 1640 medium before incubation. Bound antibody was detected with goat antibody to monkey IgA or IgG (Nordic Immunological Laboratory), followed by biotinylated rabbit antibody to goat IgG, horseradish peroxidase, and phenylenediamine dihydrochloride. The reaction was terminated with 2 M $H_2SO_4$. The results are expressed as mean (± SEM) absorbance at a wavelength of 492 nm, with the absorbance of the control culture (of $CD4^+$ cells, B cells, and macrophages but without antigen) subtracted. The results are presented only for stimulation with p27 (200 ng/ml), Ty-VLP (200 ng/ml), or TT (20 ng/ml) and tested against the corresponding antigen. Stimulation with one antigen and tested against another antigen did not show an absorbance greater than 0.15 unit. A control macaque immunized by the V-O route with Ty-VLP/CTB and boosted by IM immunization did not yield anti-p27.

15. Mononuclear cells were separated (*12*) and cultured without antigen and with p27, p27:Ty-VLP, Ty-VLP, CTB, R20, and concanavalin A (1, 10, and 20 μg/ml) in 96-well round-bottomed plates (Costar) containing RPMI 1640 medium (Gibco) supplemented with penicillin (100 units/ml; Sigma), streptomycin (100 μg/ml; Sigma), L-glutamine (2 mmol/liter; Sigma), and 10% autologous serum for 4 days. The cultures were then pulsed with 0.5 μCi of [$^3$H]thymidine for 4 hours. The cells were then harvested on filter paper discs, and the [$^3$H]thymidine uptake was determined by scintillation counting. The results were expressed as stimulation indices (ratio of counts with and without antigen) and as counts per minute for cultures stimulated with p27 (10 μg/ml); those stimulated with p27:Ty-VLP gave similar results. All cultures yielded high stimulation indices and counts with concanavalin A, and no significant counts were found with CTB or R20. The mucosal route of immunization did not elicit a rise in [$^3$H]thymidine uptake when the cells were stimulated with Ty-VLP. However, after IM administration of the immunogens, moderate responses were elicited by stimulation with Ty-VLP.
16. IgA antibodies to p27 and a control random peptide of 20 amino acids (R20) was determined by ELISA. Plates coated with antigen (at 1 μg/ml) were incubated with doubling dilutions of test samples. Bound antibody was detected by incubation with rabbit IgG to monkey IgA at 8 μg/ml or monkey IgG at 2 μg/ml (Nordic Immunological Laboratory), followed by affinity-purified goat antibody to rabbit IgG conjugated to alkaline phosphatase (Sigma Fine Chemicals) and *p*-nitrophenylphosphate disodium (Sigma Diagnostics). The reaction was terminated with 3 M NaOH, and the absorbance measured at a wavelength of 405 nm. Results are expressed as the reciprocal of the lowest dilution that gave an absorbance of 0.15 units above the background sample. The reproducibility of the ELISA after four repeated assays of the same vaginal fluid sample for IgA and IgG was within one dilution. The results with R20 were negative.
17. The construction of hybrid virus-like particles containing the SIV p27 sequence of isolate 32H of $SIVmac_{251}$ fused to the p1 protein of Ty has been described [N. R. Burns, J. E. M. Gilmour, S. M. Kingsman, A. J. Kingsman, S. E. Adams, *J. Mol. Biol.* **216**, 207 (1990); N. R. Burns, in *Methods in Molecular Biology*, M. Collins, Ed. (Humana, Clifton, NJ, 1991), vol. 8, p. 277]. The SIV gag p27 gene was derived from the clone pNIBSCI, and the p27:Ty-VLP and control Ty-VLP were purified from yeast extracts [N. Almond *et al.*, *J. Virol. Methods* **28**, 301 (1990)]. Nonparticulate p27 was prepared by cleavage from the p27:Ty-VLP and further purified by ion exchange chromatography. The absence of any Ty protein in the p27 preparation was confirmed by protein immunoblotting. The recombinant antigens were covalently linked to CTB (Sigma) at a ratio of 1:1 with SPDP (*N*-succinimidyl-3,2-pyridyl dithiopropionate) [C. Czerkinsky *et al.*, *Infect. Immun.* **57**, 1072 (1989)]. Six rhesus macaques received two oral, followed by three vaginal immunizations (O-V) of p27:Ty-VLP/CTB ($n$ = 3), p27:Ty-VLP without CTB ($n$ = 1), p27/CTB without Ty-VLP ($n$ = 1), or p27 ($n$ = 1) at monthly intervals. Three rhesus macaques and one cynomolgus macaque received two vaginal, followed by three oral (V-O) monthly immunizations of p27:Ty-VLP/CTB, and one control rhesus macaque received Ty-VLP/CTB. Topical vaginal administration of 200 μg of p27:Ty-VLP/CTB (or Ty-VLP/CTB) was carried out with soft, lubricated pediatric nasogastric tubes. Oral administration was performed by intragastric intubation of gelatin-coated capsules, containing 500 μg of the vaccine and 400 μl of cholera vibrio (Institut Merieux, Lyon, France) in the presence of sodium bicarbonate. One month after the last mucosal administration, all macaques were challenged by IM immunization with 200 μg of the preparation that we used to immunize that animal and mixed with aluminium hydroxide (AluGel, Uniscience, London, U.K.), except for the p27/CTB-immunized animal, which received 130 μg of p27/CTB in AluGel. Blood was collected from the femoral vessels, and the serum was separated. Vaginal and rectal washings were collected atraumatically with the aid of pediatric naso-gastric tubes that were flexible and lubricated. Samples were collected before and approximately 1 month after each immunization; the results shown are from samples taken approximately a month after the given immunization schedule. All procedures in the macaques were carried out after sedation with I M ketamine hydrochloride (10 mg/kg; Parke-Davis Veterinary).
18. We thank N. Graff and N. Burns (British Biotechnology, Ltd.) for preparing the SIV antigens and P. Kitchin and N. Almond (National Institute of Biological Standards and Control) for supplying plasmid pNIBSCI, containing the p27 gene we used to construct the p27:Ty-VLP. Supported by the Medical Research Council AIDS Directed Programme.

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# Encoding of a Homolog of the IFN-γ Receptor by Myxoma Virus

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**Many poxvirus-encoded virulence factors have been identified as proteins that are secreted from infected cells. The major secreted protein (37 kilodaltons) from cells infected with myxoma virus is encoded by the M-T7 open reading frame. This protein has significant sequence similarity to the human and mouse receptors for interferon-gamma (IFN-γ). Furthermore, the myxoma M-T7 protein specifically binds rabbit IFN-γ and inhibits the biological activity of extracellular IFN-γ, one of the key regulatory cytokines in the host immune response against viral infections.**

The poxviruses comprise a family of large, complex, and relatively autonomous double-stranded DNA viruses that replicate in the cytoplasm of host eukaryotic cells (*1*). Although they have been shown to infect mammals, birds, reptiles, and insects, the severity of the disease produced by different poxviruses varies greatly (*2*, *3*). In general, poxvirus genomes are organized with essential genes clustered in the center, whereas virulence markers that govern pathogenesis tend to map toward the termini of the genome (*4*). A number of these virulence markers are secreted proteins that enhance the ability of the virus to propagate in its natural host but have only a minor effect, if any, in cultured cells. These "virokines" include epidermal growth factor–like growth factors, a complement binding protein, and serine protease inhibitors (*2*, *5*). Shope fibroma virus (SFV) encodes a secreted protein that has significant sequence similarity to the host tumor necrosis factor (TNF) receptor (*6*, *7*); the deletion of the homolog of this gene in the myxoma virus reduces the virulence of this virus considerably (*8*). Wild-type myxoma virus is extremely virulent in adult domestic rabbits and has apparently evolved multiple mechanisms to combat the host immune response to the infection (*9*). It therefore provides a useful model to study the general mechanisms of poxvirus virulence, a greater understanding of which is required for the development of safer poxvirus vaccines for humans and animals (*10*, *11*). The self-defense weapons that are encoded by such highly virulent viral pathogens (*12*) may also be important indicators of the relative value of the various components of the immune system in combating such disease agents in vivo.

Labeling studies have shown that a number of secreted proteins are specific to myxoma infected cells (*13*). Figure 1A shows proteins secreted at early and late (after viral DNA replication begins) times during an infection of BGMK cells by myxoma virus (strain Lausanne) in comparison to mock-infected cells. The most prominent [$^{35}$S]methionine + cysteine protein (arrow in Fig. 1A) was observed at both early (lane E) and late (lane L) times of infection. After concentration, the same protein was readily detected as the predominant band in Coomassie blue–stained polyacrylamide gels and migrated with an apparent size of ap-

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