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Genital Ulcers Facilitate Rapid Viral Entry and Dissemination following Intravaginal Inoculation with Cell-Associated Simian Immunodeficiency Virus SIVmac239▽

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Here we report the results of studies in the simian immunodeficiency virus (SIV)-rhesus macaque model of intravaginal transmission of human immunodeficiency virus type 1 in the setting of genital ulcerative diseases. We document preferential association of vRNA with induced ulcers during the first days of infection and show that allogeneic cells of the inoculum traffic from the vaginal lumen to lymphatic tissues. This surprisingly rapid systemic dissemination in this cell-associated SIV challenge model thus reveals the challenges of preventing transmission in the setting of genital ulcerative diseases and illustrates the utility of this animal model in tests of strategies aimed at reducing transmission under these conditions.

Young women are the fastest-growing segment of the population afflicted by the AIDS pandemic (19, 24). Their risk of human immunodeficiency virus (HIV) acquisition is significantly increased by concurrent genital ulcerative diseases (GUD) (3, 5, 17, 21) and other nonulcerative but inflammatory infections or medical conditions (10, 14, 20). Urogenital inflammation in HIV type 1 (HIV-1)-infected men may also play a role in facilitating transmission, as their semen can contain as many as several thousand infected cells per milliliter of ejaculate capable of delivering cell-associated virus to target cells in the female reproductive tract (1, 4, 18, 25). Defining the events of virus transmission in the setting of genital tract ulceration and inflammation is thus important for the evaluation of vaccines or microbicidal strategies aimed at preventing HIV transmission (6–8, 11, 15, 23).

To that end, we have developed an animal model to investigate vaginal transmission by HIV-1-infected cells in the setting of GUD. In this model, we apply 3% benzalkonium gel to the cervical and vaginal mucosa of macaques to induce epithelial disruption and acute inflammation. The irritation is visible by the second day of treatment (9, 16) and lasts 7 to 9 days before resolving spontaneously. We have recently shown that intravaginal inoculation of surprisingly few SIV-infected cells established systemic infection with a high level of viremia (9).

A major question about cell-associated viral transmission is whether infected donor cells traverse the reproductive tract mucosa or whether they remain in the vaginal lumen and release infectious virions that only later cross the epithelial barrier (22). To successfully locate those few donor cells that may migrate into and beneath the recipient's epithelium, we must pinpoint the anatomical site of entry and possibly the initial rounds of viral proliferation. Following our success in establishing the GUD-infection model, we hypothesized that genital ulceration could be used to define likely portals of entry for SIV, enabling us to dissect the earliest events in vaginal transmission. In the present study, we induced lower genital tract ulcers in female macaques and exposed the animals to cell-associated SIVmac239 as described previously (9). Subsequently, we investigated the cervix and vaginal mucosa for the presence of viral RNA (vRNA) and vRNA$^+$ cells within the first 5 days of infection. We also collected the draining and distal lymph nodes (LNs) to gather information about the initial kinetics of viral dissemination.

In these experiments, we used sexually mature, cycling female cynomolgus macaques. Animals were housed at the Wisconsin National Primate Research Center (WNPRC) according to the NIH Guide to the Care and Use of Laboratory Animals. All procedures were performed according to an experimental protocol reviewed and approved by the Research Animal Resource Committee of the University of Wisconsin—Madison. Induction of transient lower female genital tract ulcers and intravaginal viral challenge were performed as described previously (9).

Association of vRNA and vRNA$^+$ cells with visible disruption of the mucosal barrier. We initially hypothesized that increased transmission in the setting of GUD is the result of immediate access to target cells in the submucosa that ulcers provide for infected cells. We tested the major prediction of this hypothesis, that ulcers are sites where infected cells from the inoculum and secondarily infected cells concentrate, by examining the anatomical location of SIV RNA$^+$ cells following exposure to infected cells.

We inoculated animals cy0089 and cy0096 with a total of 10^5 and animals cy0070 and cy0147 with 2.3×10^6 and 3.0×10^6 in

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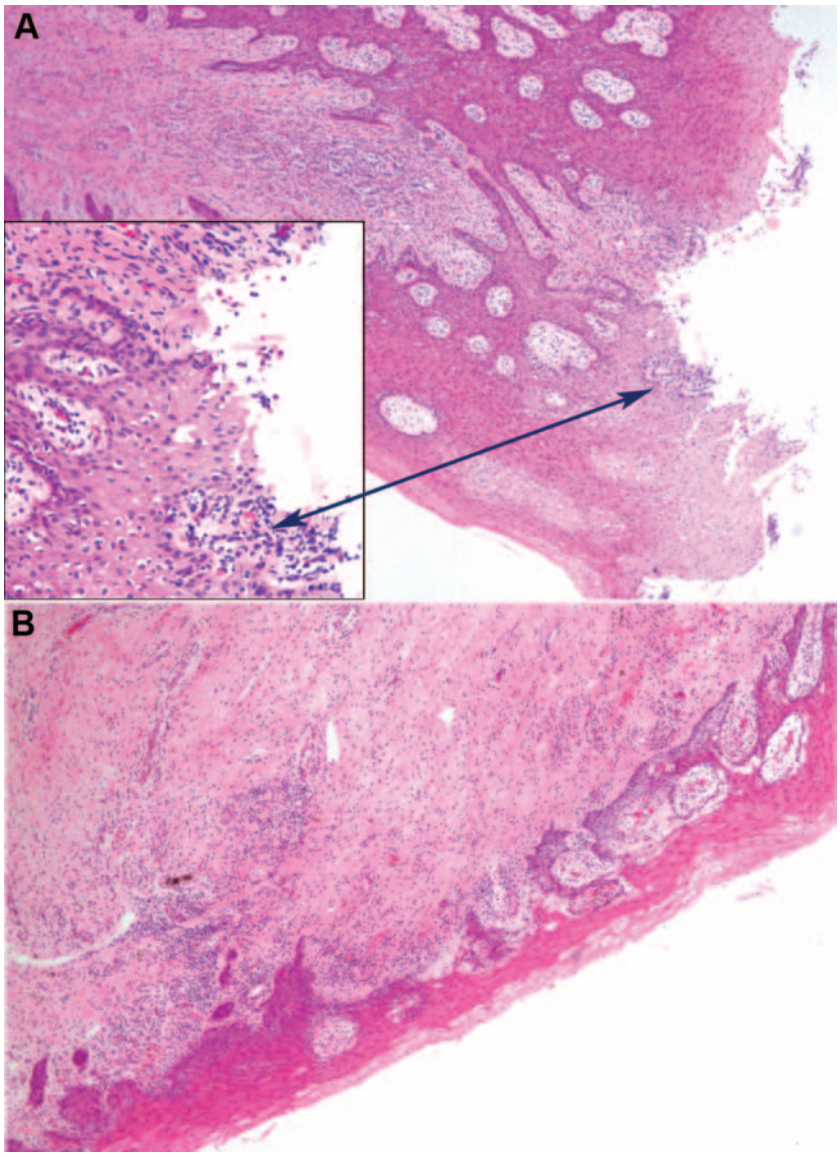

FIG. 1. Benzalkonium-induced inflammation and ulceration. (A) Vaginal ulcer with dark-staining inflammatory infiltrate and associated denuded epithelium at an original magnification of ×10. The double-headed arrow points to the inset showing the ulcer and inflammation at a higher magnification (×20). (B) Intact vaginal epithelium distal to the ulcer shown in panel A at an original magnification of ×4. There is inflammation in the submucosa, but the epithelium is not disrupted.

vitro-infected allogeneic peripheral blood monocytes, respectively. On days 2 (cy0089, cy0096, cy0147) and 3 (cy0070) after inoculation, we inspected the lower female reproductive tract for signs of inflammation by using a rigid fiber-optic scope and then euthanized the animals and processed the cervix and three evenly separated sections encompassing the entire length of the vaginal mucosa. Additionally, we collected genital, inguinal, axillary, and mesenteric LN samples. We fixed a portion of each sample with 4% paraformaldehyde and processed them for in situ hybridization to examine them for SIVmac239 RNA^+ cells as previously described (26). We tested the remaining portion of every sample for SIVmac239 vRNA burden by using a quantitative reverse transcription-PCR as detailed previously (2, 13).

First we confirmed in hematoxylin-and-eosin-stained sections that epithelial disruption was associated with those foci of ulceration that we had previously identified by visual inspection (Fig. 1A). We investigated the association of initial infection with these sites of epithelial disruption by focusing on the 16 female genital mucosa samples obtained from animals euthanized 2 and 3 days after a cell-associated viral challenge, excluding the one animal (cy0155) euthanized 1 day after a cell-associated viral challenge, where detection of vRNA in samples from the female genital mucosa was likely due to residual inoculum (Table 1). We detected vRNA in 7 out of the 16 female genital mucosa samples obtained from these four macaques. Five of the 7 positive samples were associated with visible ulcer/inflammation versus 2 positive out of the 10 nonulcerated mucosal samples (83% versus 20%, $P = 0.035$ by Fisher's exact test) (Table 1). As additional evidence of the

TABLE 1. Cell-associated virus inocula and subsequent vRNA burdens of tissue samples from cynomolgus macaques challenged in this experiment

Animal	DPI[e]	Cell-associated virus inocula			vRNA burden of tissue sample[d]							
		CD4[a] (%)	p27[b] (%)	No. of infectious cells[c]	Vaginal section 1	Vaginal section 2	Vaginal section 3	Cervix	Genital LNs	Inguinal LNs	Mesenteric LNs	Axillary LNs
cy0121	5	35.49	2.19	1.1E + 03	**9.4E + 04**	1.9E + 04	2.9E + 03	6.5E + 03	1.9E + 04	4.6E + 03	ND[f]	5.6E + 03
cy0070	3	72.55	7.72	3.7E + 04	**+**[g]	−	5.3E + 01	−	1.1E + 02	1.8E + 03	1.1E + 02	+
cy0147	2	70	11.3	9.8E + 04	−	+	**−**	−	−	+	−	−
cy0089	2	19.28	0.48	5.4E + 02	**+**	−	**+**	−	−	−	ND	+
cy0096	2	19.28	0.48	5.4E + 02	**9.9E + 01**	−	**5.1E + 01**	−	+	−	4.1E + 01	−
cy0155	1	26.7	0.6	ND	**1.1E + 02**	3.9E + 02	3.5E + 02	**1.8E + 02**	2.2E + 01	+	−	−

[a] Frequency of $CD4^+$ cells in the inoculum as determined by fluorescence-activated cell sorter analysis.
[b] Frequency of *gag* $p27^+$ cells in the inoculum as determined by intracellular staining and fluorescence-activated cell sorter analysis (9).
[c] Number of infectious cells in the inoculum as determined by coculture with CEMx174 cells (9).
[d] Number of vRNA copies per microgram of tissue. Bold entries indicate association with visible mucosal ulcer/inflammation.
[e] DPI, days postinfection.
[f] ND, not done.
[g] Positive sample; viral burden is below the quantification limit (<15 copies/μg).

association of initial infection with epithelial disruption, we found that $vRNA^+$ cells were concentrated in an ulcer (Fig. 2) in one animal (cy0121) euthanized 5 days after a cell-associated viral challenge, and the ulcer site also had the highest number of copies of vRNA (Table 1).

However, $vRNA^+$ samples were not invariably associated with sites of visible ulceration, as we detected SIVmac239 vRNA in two samples with an intact epithelium (Fig. 1B). This is consistent with our earlier observation that cell-associated virus, with less frequency, can establish a persistent infection in the apparent absence of visible ulceration (9). It is quite possible that transmission still occurs at sites of disruption of the mucosal barrier that were not detected visually or in the sections examined, as even in the section shown with an intact epithelium (Fig. 1B) the epithelium is thinned and there is inflammation in the underlying epithelium.

Rapid systemic dissemination of donor cells throughout lymphatic tissues. In this study, we detected vRNA (Table 1) and $vRNA^+$ cells (not shown) throughout the lymphatic tissues

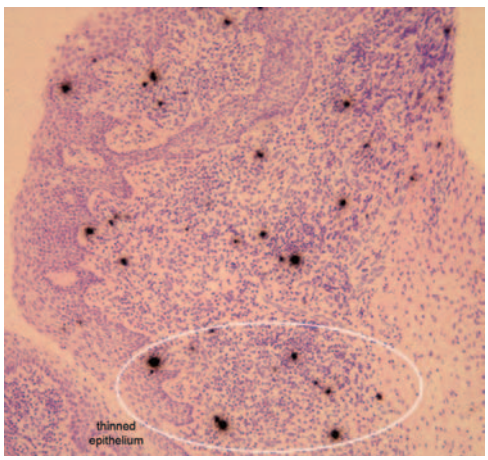

FIG. 2. SIV RNA^+ cells concentrated at sites of ulceration and inflammation at day 5. SIV RNA^+ cells were detected by in situ hybridization (black cells). The circled area is a portion of the extensive inflammatory infiltrate and associated thinned epithelium with several $vRNA^+$ cells. Original magnification, ×100.

5 days after a viral challenge. While this is already faster systemic spread of infection than has been reported following intravaginal inoculation of cell-free virus (13), we were surprised to detect vRNA in the draining LNs already by day 1 postinoculation and at sites as distal as the axillary and mesenteric LNs at days 2 and 3 postinoculation. It seemed unlikely in this time frame that vRNA at these distal sites reflected secondary infections but rather likely that infected allogeneic donor cells quickly entered the submucosa via the ulcer and then traveled to distal sites.

To test this issue, we inoculated animals cy0070 and cy0147 with cells that were not only infected but also labeled with bromodeoxyuridine (BrdU) in vitro (53% and 55.7% $BrdU^+$ cells in the inoculum). Using anti-BrdU specific histochemistry on 6-μm paraffin-embedded tissue sections, we tracked the allogeneic donor cells. We did not detect $BrdU^+$ cells in the cervical and vaginal tissues. We detected $BrdU^+$ cells in peripheral LNs, most strikingly in the mesenteric LNs of both animals. Most of the $BrdU^+$ cells appeared to be macrophages that had ingested apoptotic allogeneic cells (Fig. 3B). However, we also found intact $BrdU^+$ donor cells (Fig. 3A). Despite observing a small number of intact donor cells up to 3 days postchallenge, we were unable to detect SIVmac239 $vRNA^+$ $BrdU^+$ cells by in situ hybridization. This we attribute to the low frequency of infected intact donor cells at these locations. Collectively, these findings are consistent with the concept that ulceration/inflammation supports the rapid entry and widespread dissemination of allogeneic donor cells, which the host immune system then eliminates.

In summary, our in vivo results suggest that mucosal disruption and inflammation provide a portal and environment that support rapid dissemination of cell-associated virus. While the mechanism(s) and anatomic routes of dissemination have not been defined, our model offers further insight into the increased chance of acquisition of HIV-1 in the setting of GUD and highlights the challenges for a vaccine to provide sterilizing immunity. Indeed, this fast dissemination is more akin to intravenous challenges, where the major effect of vaccination may be limited to controlling rather than preventing infection because too many cells are infected initially to be eradicated by

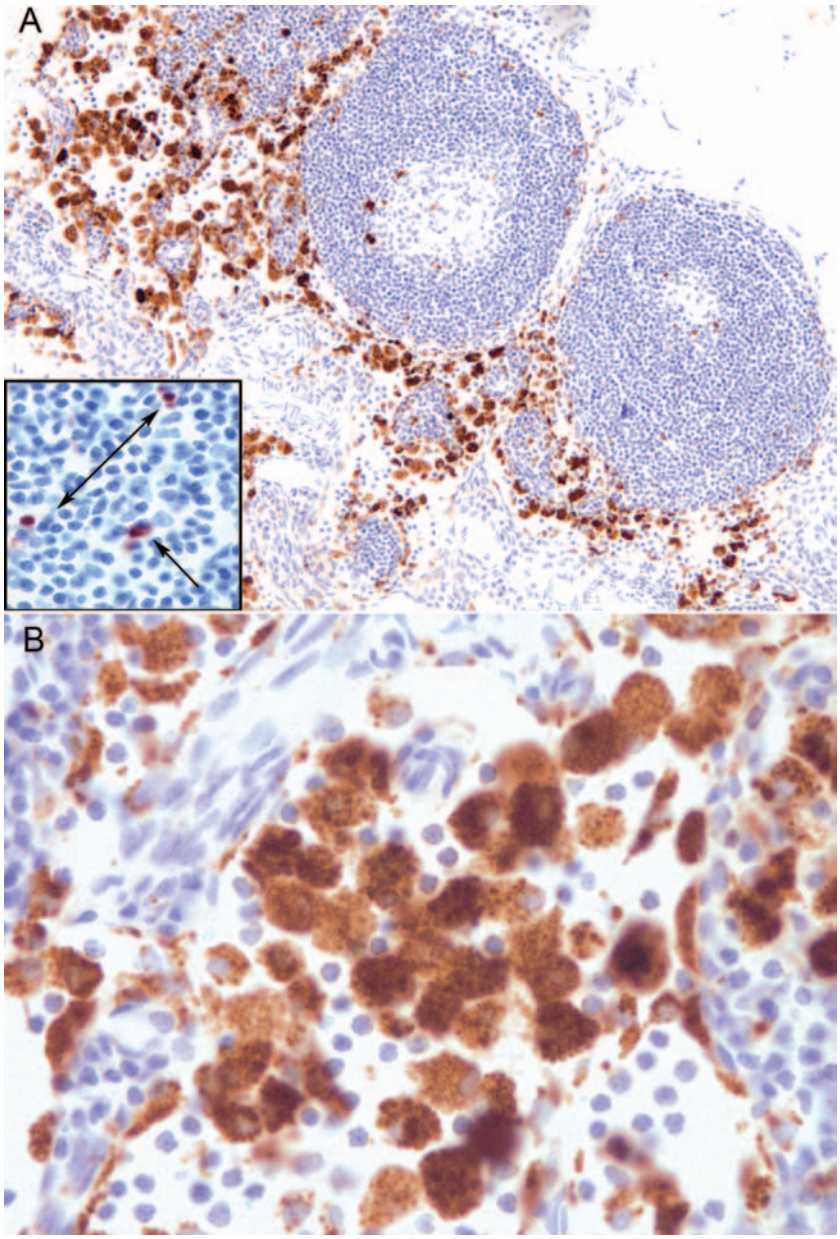

FIG. 3. BrdU$^+$ donor cells in the recipient animal's mesenteric LN 3 days after inoculation. BrdU$^+$ cells were identified by immunohistochemically stained brown with anti-BrdU antibody. (A) BrdU$^+$ cells around follicles and in the T-cell zone (inset arrows point to three intact BrdU$^+$ cells). Original magnifications, ×100 and ×200 (inset). (B) Ingested apoptotic donor cells and nuclear debris from the donor cells are evident in the recipient animal's tingible-body macrophages.

even a robust anamnestic response. Finally, we provide further evidence of the utility of this model to investigate the pathogenesis of transmission and explore new approaches to prevent infection in the setting of GUD.

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