Genital Herpes Simplex Virus Type 2 Infection in Humanized HIV-Transgenic Mice Triggers HIV Shedding and Is Associated With Greater Neurological Disease

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Background. Epidemiological studies consistently demonstrate synergy between herpes simplex virus type 2 (HSV-2) and human immunodeficiency virus type 1 (HIV-1). Higher HIV-1 loads are observed in coinfected individuals, and conversely, HIV-1 is associated with more-severe herpetic disease. A small animal model of coinfection would facilitate identification of the biological mechanisms underlying this synergy and provide the opportunity to evaluate interventions.

Methods. Mice transgenic for HIV-1 provirus and human cyclin T1 under the control of a CD4 promoter (JR-CSF/hu-cycT1) were intravaginally infected with HSV-2 and evaluated for disease progression, HIV shedding, and mucosal immune responses.

Results. HSV-2 infection resulted in higher vaginal HIV loads and genital tissue expression of HIV RNA, compared with HSV-uninfected JR-CSF/hu-cycT1 mice. There was an increase in genital tract inflammatory cells, cytokines, chemokines, and interferons in response to HSV-2, although the kinetics of the response were delayed in HIV-transgenic, compared with control mice. Moreover, the JR-CSF/hu-cycT1 mice exhibited earlier and more-severe neurological disease. The latter was associated with downregulation of secretory leukocyte protease inhibitor expression in neuronal tissue, a molecule with antiinflammatory, antiviral, and neuroprotective properties.

Conclusions. JR-CSF/hu-cycT1 mice provide a valuable model to study HIV/HSV-2 coinfection and identify potential mechanisms by which HSV-2 facilitates HIV-1 transmission and HIV modulates HSV-2–mediated disease.

Keywords. herpes simplex virus-2; human immunodeficiency virus-1; coinfection; sexually transmitted infections; mouse model; female genital tract.

Genital herpes is one of the most prevalent sexually transmitted infections worldwide, and its synergistic relationship with human immunodeficiency virus (HIV) amplifies its public health implications. Herpes simplex virus type 2 (HSV-2) seroprevalence rates approach 90%–95% among HIV-infected individuals in developing countries, where HSV-2 remains the dominant cause of genital ulcerative disease [1]. Clinical or subclinical (presence of viral DNA in the absence of clinical signs) HSV-2 reactivation increases the risk of acquiring and transmitting HIV [2, 3]. Epidemiological studies consistently demonstrate higher plasma viral loads and increased genital tract HIV during episodes of HSV-2 reactivation, which may increase the risk for sexual and mother-to-child transmission and accelerate HIV disease progression [3–6]. Conversely, HIV-infected individuals are at greater risk for more frequent and severe HSV-2 recurrences, compared with HIV-uninfected persons, and the risk is only partly reduced by highly active antiretroviral therapy [7, 8]. These clinical observations have led to recommendations to consider valacyclovir/acyclovir.
prophylaxis in HIV-positive pregnant women who may be at increased risk of transmitting HSV to their newborns [9]. Although it is presumed that the increase in HSV infection recurrences in HIV-infected individuals reflects impaired T-cell responses, alterations in the genital tract mucosal immune environment may also contribute. Together, these epidemiological observations highlight the need to define the molecular mechanisms underlying the biological synergy between HSV and HIV.

The absence of animal models of coinfection has constrained investigation of the interactions between these viruses and has limited our ability to identify strategies to disrupt the synergy. Nonhuman primates provide insights into HIV pathogenesis but rely primarily on simian immunodeficiency virus or SHIV and are limited by their variable susceptibility to HSV infection and the high costs of experiments [10]. Mouse models provide several potential advantages, including increased feasibility of conducting studies with larger numbers of animals because of lower costs and availability of reagents to characterize immune responses [11]. Wild-type mice, however, have 2 major species-specific restrictions [1–3]. Entry is blocked because the HIV envelope glycoprotein, gp120, does not engage mouse CD4 and CCR5 [4], and HIV Tat does not bind to mouse cyclin T1 and consequently cannot activate HIV-1 transcription [5]. Several humanized mouse models have been developed to circumvent these problems with variable success. One study, which focused on susceptibility to HIV infection, found that mice transgenic for human CD4, CCR5, and cyclin T1 were more susceptible to HIV infection if they were first intravaginally infected with HSV-2, although the mechanisms for the increased susceptibility were not fully delineated, and the synergy could not be further studied because the mice do not support efficient HIV replication [12, 13]. We adopted a different strategy by developing the JR-CSF/hu-cycT1 mouse, which is transgenic for an integrated full-length infectious HIV-1 provirus derived from the primary R5-tropic clinical isolate JR-CSF regulated by the endogenous HIV-1 long terminal repeat (LTR) and for the human cyclin T1 gene under the control of a mouse CD4 promoter [14]. Selective expression of human cyclin T1 by mouse CD4-expressing immune cells specifically amplifies HIV production by the cells that are the usual target for HIV infection. The current studies were undertaken to explore whether JR-CSF/hu-cycT1 mice could provide a model of HIV/HSV coinfection and to test the hypotheses that HSV-2 infection increases HIV shedding and, conversely, that HIV modulates HSV-2 disease.

METHODS

Murine Model
JR-CSF/hu-cycT1 (HIV-TG) and control nontransgenic littermates (6–8 weeks old) were generated and maintained as described elsewhere [14]. Mice were pretreated subcutaneously with 2.5 mg of medroxyprogesterone acetate (Sicor Pharmaceuticals, Irvine, CA) 5 days before intravaginal inoculation with 10⁴, 10⁵, or 10⁶ plaque-forming units (PFU)/mouse of clinical isolate HSV-2(4674) delivered in 30 µL of phosphate-buffered saline (PBS) or PBS alone (mock infection) [15]. Mice were evaluated daily and scored for epithelial disease (erythema, edema, genital ulcers, and hair loss around the perineum) and neurological disease (urinary and fecal retention and hind-limb paresis/paralysis) on a scale that ranged from 0 (no disease) to 4 (severe ulceration, hair loss, or hind-limb paralysis) [15, 16]. Mice that reached a clinical score of 4 were euthanized. Vaginal washes were collected in 150 µL of normal saline; mice were euthanized at specified times, and genital tract tissue, lumbar and sacral lymph nodes, and dorsal root ganglia and lower spinal cord (neuronal tissue) were harvested for analyses described below.

Cytokine and Chemokine Measurements
Vaginal washes were clarified by centrifugation (210 x g) for 10 minutes at 4°C in the presence of protease inhibitors (Complete Protease Inhibitor Cocktail; Roche Applied Science, Indianapolis, IN), and the supernatants were stored at −80°C. The washes were pooled (2–5 mice per pool), and from each group 6–14 pools were evaluated for interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6), macrophage inflammatory protein 1β (MIP-1β), RANTES, monocyte chemotactic protein 1 (MCP-1), CXCL-1, and MIP-2, using a multiplex proteome array with beads from Millipore (Billerica, MA), measured with a Luminex 100 system (Luminex, Austin, TX), and analyzed using StarStation (Applied Cytometry Systems, Sacramento, CA).

Measurement of HIV and HSV-2
HIV RNA was quantified in vaginal washes after pooling 50 µL from 3 mice per group and diluting the pool 1:10 in PBS (m2000 system; Abbott Molecular). Data are presented as log₁₀ HIV copies per 150 µL of vaginal wash. To quantify HIV in tissue, RNA was extracted, reverse transcribed to complementary DNA (cDNA), and measured by real-time quantitative PCR (qPCR), using HIV-1 LTR–specific primers (Applied Biosystems, Carlsbad, CA) run in duplicate (ABI Prism 7000 detection system; Applied Biosystems). Serial dilutions of U1 cell lysate DNA were included as a positive control. U1 cell DNA has 2 copies of HIV [17]; the number of U1 cells per dilution and the resulting LTR threshold cycle value for that dilution were used to calculate the HIV copy number for each tissue sample.

To quantify HSV-2, genital tract and neuronal tissues were weighed, homogenized in serum-free Dulbecco’s modified Eagle’s medium, using RNase-free pestles, sonicated for 30 seconds, and centrifuged at 10,000 x g for 5 minutes. Supernatants were assayed for HSV-2 in a standard Vero cell plaque assay. Data are presented as log₁₀ PFU per gram of tissue. HSV-2 loads in neuronal tissue were also measured by real-time
qPCR, using primers for the immediate early gene, ICP4, and β-actin (Applied Biosystems) on DNA that was extracted from the tissue using a DNeasy blood and tissue kit (Qiagen, Valencia, CA).

**Immune Mediator Gene Expression**
Genital tract and neuronal tissue were homogenized, and total RNA was extracted using the Absolutely RNA Miniprep Kit (Agilent Stratagene, Santa Clara, CA). Reverse transcription was performed using 400 ng of RNA and the StrataScript cDNA Synthesis Kit (Applied Biosystems), and real-time qPCR was performed with 50 ng of cDNA [18]. Primers for β-actin, interleukin 1α (IL-1α), IL-1β, IL-6, interleukin 12α, interleukin 17α (IL-17α), CXCL-1, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, IFN-α4, IFN-β, IFN-γ, IRF-7, Ifit1, Mx1, Oas2, PKR, and secretory leukocyte protease inhibitor (SLPI) were obtained from Applied Biosystems.

**Hematoxylin-Eosin Staining**
Genital tract tissue was harvested on days 2 and 8 after infection, fixed in 4% paraformaldehyde overnight, transferred into 70% ethanol, and stored at 4°C. Tissues were embedded in paraffin and transversely sectioned using a microtome, and serial sections were stained with hematoxylin-eosin. Stained sections (2–3 per group at each time point) were evaluated in a blinded fashion for inflammatory cell counts in a 625 µm² area (original magnification, 40×).

**Flow Cytometric Analysis of Lymph Nodes**
Lymph nodes were excised on necropsy and stored on ice in PBS. Intact tissue was disassociated through a 70-µm filter, and intact cells were isolated by filtration through a 40-µm filter. A total of 5 × 10⁵ cells per sample were stained with combinations of anti-CD3-APC or anti-CD3-FITC, anti-CD4-PerCP, and/or anti-CD19-FITC (e-Bioscience, San Diego, CA) and analyzed on a Becton Dickinson FACSCanto II analyzer, using FlowJo v9.3.1 software (Tree Star, Ashland, OR). Ten thousand live events were acquired following gating, using an IR-conjugated Live/Dead Marker (Invitrogen, Carlsbad, CA).

**Statistical Analysis**
Immune responses to HSV-2 were compared between HIV-TG and control mice by unpaired t tests, and survival and disease scores were compared by the log-rank test (GraphPad Prism, version 6; GraphPad Software).

**RESULTS**

**HIV-Positive Transgenic Mice Are More Susceptible to HSV-2 Infection and Demonstrate More-Severe Neurological Disease Than Controls**
HIV-TG and control mice were challenged intravaginally with approximately 10⁴, 10⁵, and 10⁶ PFU/mouse of HSV-2(4674) (Figure 1A–C). Significantly greater mortality was observed in HIV-TG mice, compared with control mice, following infection with the lower dose of HSV-2 (10⁴ PFU), with both groups showing >90% mortality following exposure to 10⁶ PFU. To ensure that differential rates of HSV-2 infection did not impact outcomes in subsequent studies, the higher dose of HSV-2 was used to further study coinfection. Mice (23–25 per group) were intravaginally inoculated with HSV-2 (10⁶ PFU/mouse), and a log-rank test was used to compare clinical response over time by dichotomizing mice into those with a score of ≥3 and those with a score of <3. There was a statistically nonsignificant trend toward greater epithelial disease (P = .07; Figure 1D) and a significant increase in neurological disease in HIV-TG over time, compared with control mice (P < .05; Figure 1E).

**Coinfection Is Associated With Increased HIV Replication**
Consistent with clinical experiences in coinfected individuals, the quantity of HIV RNA detected in vaginal washes was significantly greater in HSV-2–infected HIV-TG mice, compared with mock-infected HIV-TG mice, on days 1, 2, and 3 after infection and compared with baseline levels (Figure 2A). There was also a statistically significant increase in HIV LTR expression in genital tract tissue in HSV-2–infected HIV-TG mice, compared with mock-infected HIV-TG mice, on days 1 and 8 after infection (Figure 2B). To investigate whether the differences in neurological disease observed in the coinfected mice reflected differences in local viral replication, HIV and HSV-2 expression were examined in neuronal tissue harvested on day 8. There was a statistically nonsignificant trend toward increased HIV LTR RNA levels in neuronal tissue (Figure 2C) but no difference in HSV-2 ICP4 expression (Figure 2F). There were also no differences in the quantity of infectious HSV-2 recovered from neuronal or genital tract tissue in HIV-TG mice, compared with control mice (Figure 2D and 2E), suggesting that differences in neurological disease are not attributable to greater HSV-2 replication but may reflect immune responses.

**HSV-2 Induces Mucosal Inflammation, but Responses Are Delayed in HIV-TG Mice**
There was a significant increase in concentrations of IFN-γ, IL-6, CXCL-1, MCP-1, MIP-1β, and RANTES secreted into the vaginal washes on days 1 and 3 following HSV-2 infection in both HIV-TG and control mice, compared with their respective mock-infected controls (Figure 3A and 3B). This response likely contributed to the increased local HIV replication observed in response to HSV-2. There were no statistically significant differences in the concentrations of cytokines or chemokines in vaginal washes from mock-infected HIV-TG mice, compared with control mice.

To evaluate whether the increased levels of mediators in vaginal washes were associated with increased gene expression (and not only release of intracellular stores), RNA levels were
measured in excised tissue. The only significant difference in mock-infected mice was decreased expression of RANTES in HIV-TG mice, compared with control mice, on day 2 (Figure 4A and 4B). Compared with respective mock-infected mice, significant increases in IL-1α, IL-1β, MCP-1, MIP-1β, RANTES, and TNF-α RNA levels were detected on day 2 after infection in control mice but not HIV-TG mice (Figure 4A). However, significantly increased gene expression of these
Herpes simplex virus (HSV) triggers an increase in human immunodeficiency virus (HIV) shedding and production in the genital tract of HIV-transgenic (HIV-TG) mice. The numbers of HIV RNA copies were measured in pooled vaginal washes (3 mice per pool) at baseline (before HSV exposure) and 1, 2, and 3 days after infection in HIV-TG mice (A). Data are mean values + standard errors of the mean (SEM) from 3 separate pools. RNA was extracted from tissue harvested 1, 2, and 8 days after infection from 3–15 mice per group. RNA was reverse transcribed to complementary DNA, and the gene expression of HIV LTR was determined by real-time quantitative polymerase chain reaction (qPCR) in genital tract (B) and neuronal (C) tissue, using a standard curve of U1 cell lysate DNA to determine the relationship between threshold cycle values and the number of HIV copies. Data are graphed as mean + SEM. *P<.05, by the unpaired t test, vs mock-infected HIV-TG mice. Tissue was also harvested on days 1, 2, 3, and 8 after infection, weighed, homogenized, and plated on Vero cells. Forty-eight hours later, plaques were counted, and the number of plaque-forming units (PFU) per gram of tissue were determined for genital tract (D) and neuronal (E) tissue (3 mice per group). As a second means of quantifying HSV loads in neuronal tissue, DNA was extracted from neuronal tissue on day 8 after infection and was assayed for HSV genomes (using primers for ICP4) by real-time qPCR (F). Data are expressed relative to findings for an uninfected mouse, with 5 mice per group. All data are graphed as mean values + SEM.
mediators was observed in both groups of HSV-infected mice on day 8 after infection; IL-17α gene expression was only increased in HIV-TG mice (Figure 4B). Significantly increased expression of inflammatory mediators was also observed in both groups on day 8 after infection in neuronal tissue (Figure 4C), a time coinciding with a trend toward increased levels of HIV RNA in coinfected mice (Figure 2C). IL-17α was not detected in neuronal tissue.

HSV-2 Infection Induces IFN Responses
Type 1 IFN responses play important roles in host defense but may also contribute to pathology. While HSV-2 induced an increase in the expression of several IFN-related genes, including those encoding Ifit1, Mx1, IRF-7, Oas2, and PKR, in genital tract tissue on day 2 after infection, only the control mice exhibited significant increases in IFN-β and IFN-γ expression (Figure 5A). However, as with the cytokine/chemokine responses, both groups of HSV-2–infected mice displayed a significant increase in IFN-γ expression on day 8 after infection (Figure 5B). Notably, expression of IRF-7, Oas2, and PKR genes was modestly downregulated in mock-infected HIV-TG mice, compared with control mice, although these differences were only seen in tissue isolated on day 2. We also observed a significant increase in expression of IFN genes in response to HSV-2 in neuronal tissue on day 8 (Figure 5C), with little or no difference between HIV-TG and control mice in their responses.

SLPI Is Downregulated in Neuronal Tissue Harvested From Coinfected Mice
SLPI is an antinflammatory protein expressed by multiple cell types with in vitro activity against HSV-2 [19] and HIV-1 [20], as well as neuroprotective roles [21–23]. We previously found that HSV-2 downregulates SLPI expression in epithelial cells in vitro [24]. To explore whether these in vitro results translated to
Recruitment of Inflammatory Cells Into the Genital Tract in Response to HSV-2 Is Delayed in HIV-TG Mice

Histological examination of genital tract tissue extracted on day 2 after infection in control mice demonstrated acute inflammation with a significant increase in inflammatory cells, compared with uninfected tissue (P < .05), and with neutrophil infiltration into the lamina propria, epithelium, and lumen of the mucosa.
The number of inflammatory cells declined significantly between days 2 and 8 (P < .001), and chronic inflammatory changes were observed on day 8 with lymphocytes, plasma cells, and rare neutrophils (Figure 6G and 6H). In contrast, while there was no increase in inflammatory cells in response to HSV-2 on day 2 after infection in HIV-TG mice, the
The number of inflammatory cells increased over time and was greater on day 8, compared with baseline values and with values for HSV-2–infected control mice; the differences did not reach statistical significance ($P = .10$; Figure 6D–F and 6I–K). These findings are consistent with the delayed cytokine/chemokine response to HSV-2 in the HIV-TG mice.
To explore the effect of HSV-2 infection on immune cell populations in the draining lymph nodes, nodes were harvested from mice 2 and 8 days after infection and stained for the expression of CD3 (T cells) or CD19 (B cells) and then, within the CD3+ population, for expression of CD4 and CD8. There was a decrease in the percentage of CD3+ cells and a concomitant increase in CD19+ cells in both HIV-TG and control mouse lymph nodes in response to HSV-2 infection, suggesting trafficking of T cells from the lymph node to the genital tract (Figure 7A and 7B). However, there were notable differences in the CD4+ and CD8+ T-cell responses to HSV-2 between HIV-TG and control mice (Figure 7C–E). Compared with mock-infected mice, HSV-2 infection of control mice was associated with a significant increase in CD8+ T-cell counts on days 2 and 8 after infection and a decrease in CD4+ T-cell counts on day 8, resulting in a decrease in the ratio of CD4+ T cells to CD8+ T cells, which reached statistical significance on day 8 ($P < .05$). In contrast, there was a significant decrease in CD8+ T-cell counts on day 8 after infection and no change in CD4+ T-cell counts or the ratio of CD4+ T cells to CD8+ T cells in HSV-infected HIV-TG mice, compared with mock-infected HIV-TG mice. The mock-infected HIV-TG mice exhibited a
DISCUSSION

The current study provides the first in vivo model of HSV-2/HIV-1 coinfection in the setting of chronic HIV-1 whose predictive value is indicated by its recapitulation of several key clinical observations, including increased genital tract shedding of HIV following HSV-2 infection and increased susceptibility to HSV-2 in the setting of chronic HIV infection. Use of this model enabled us to identify several molecular mechanisms relevant to the synergistic interactions between HIV and HSV-2 that occur in coinfected individuals. Specifically, we found that, although HIV-TG and control mice responded to HSV-2 infection with local release of inflammatory cytokines and chemokines that was paralleled by upregulated gene expression, the latter responses were delayed in HIV-TG mice. The acute release of cytokines into the genital tract could directly activate the HIV LTR and increase local HIV replication, as evidenced by the significantly higher HIV loads in vaginal washes detected 1 day after infection. In addition, the delayed inflammatory response illustrated by the histological findings of acute inflammation on day 8 after infection in HIV-TG mice could further promote HIV replication through recruitment of immune target cells.

The decreased expression of RANTES and several IFN genes in genital tract tissue from mock-infected HIV-TG mice, compared with control mice, on day 2 (but not day 8), coupled with the delayed and/or reduced upregulation of RANTES, MIP-1β, and IFN-γ in response to HSV-2 (Figures 4 and 5), are indicative of immune cell dysfunction and consistent with human studies of chronic HIV infection. For example, one study found that peripheral blood mononuclear cells (PBMCs) isolated from subjects with chronic HIV infection exhibited impaired T-helper 1 cell (Th1) responses to nonspecific stimulation with PHA, including RANTES, MIP-1β, and IFN-γ, and that the responses recovered after introduction of antiretroviral therapy [25]. In that study, PBMC IL-17α responses were also impaired, whereas HSV-2 induced a delayed but significant increase in mucosal IL-17α expression in HIV-TG (but not control) mice. Impaired Th1 responses may be related to the activation and exhaustion status of memory T cells, but this has not been examined in genital tract tissue.

The upregulation of IL-17α, which recruits monocytes and neutrophils into sites of inflammation [26], may have contributed to the inflammation observed histologically on day 8 after infection in the HIV-TG mice, the trend toward greater epithelial disease (P = .07), and the increase in HIV shedding. The role of IL-17α in genital herpes has not been evaluated, although a recent study found that IL-17α–producing CD4+ T cells (T-helper 17 cells [Th17]) contributed to the pathogenesis of HSV stromal keratitis in a murine model [27]. Notably, Th17 may be preferentially infected by HIV, possibly because of their increased activation state and expression of CCR5 coreceptors, and are rapidly depleted in the gut (but not the respiratory tract) following HIV infection [28, 29]. The HIV-TG mice provide a model to study specific T-cell populations in the different anatomic compartments and their response to coinfection.

The observation that HIV-TG mice were more susceptible to HSV-2 following exposure to lower doses of virus (Figure 1) provides a possible mechanistic basis for the epidemiologic findings of an increased risk of acquiring HSV-2 among HIV-infected hosts, compared with HIV-uninfected hosts [30, 31]. The finding of early and more severe neurological disease scores was unanticipated but is consistent with anecdotal case reports suggesting that neurological complications of HSV-2 infection, such as transverse myelitis, lumbosacral radiculoneuropathy, and encephalitis, while rare, are more common in the setting of HIV infection [32–34]. The difference in susceptibility and in disease manifestations may be related to the delayed mucosal innate immune response. Specifically, there was no increase in IFN-β gene expression and a delayed IFN-γ response in HIV-TG mice following HSV-2 infection (Figure 5).

There was no increase in HSV-2 levels and only a trend toward an increase in HIV levels in the extracted neurological tissue (Figure 2), suggesting that the viral loads do not directly contribute to the increase in neurological disease observed in the coinfected mice. The increase in paralysis may reflect the significant downregulation of SLPI in neuronal tissue in response to coinfection. Other studies have shown that SLPI has neuroprotective functions and promoted axonal regeneration [21]; inhibited cleavage of progranulin, a neuronal growth factor [22]; and protected mice from ischemic brain injury [23]. Notably, we previously found that HSV-2 triggers the rapid downregulation of SLPI in vitro in several cell types, although neuronal cells were not studied [24]. However, SLPI was not detected in genital tract tissue harvested from HIV-TG or control mice at any time point or from mice that were not pretreated with medroxyprogesterone (data not shown). Untreated mice were evaluated because human studies suggest that medroxyprogesterone downregulates SLPI expression in endometrial tissue [35]. The inability to detect SLPI in murine genital tract tissue may be a limitation of the model.

In summary, we describe the first small-animal model of mucosal HIV-1/HSV-2 coinfection in the setting of chronic HIV infection, which recapitulated several well-described epidemiological findings, including an increase in HIV shedding following HSV-2 infection and a more rapid course of HSV-2 disease in the setting of chronic HIV infection. Importantly, the model provides several insights into potential molecular mechanisms that could contribute to the clinical associations between these 2 viruses, including the role of genital tract...
IL-17α and neuronal SLPI, and thus identifies potential new targets for intervention. Further experiments with this model may identify additional mechanisms by which HIV and HSV-2 infections synergize to support coinfection and more-severe morbidity. The model could also be used to evaluate the influence of antivirals on coinfection and could be modified to study recurrent HSV-2 infection in the setting of HIV infection by treating the mice with intermittent acyclovir [36].

Notes

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