NOTES

Persistent Infection of Rabbits with Bovine Immunodeficiency-Like Virus

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Chronic infection of rabbits was induced by a single intraperitoneal injection of bovine immunodeficiency-like virus (BIV)-infected cells. Ten BIV-infected animals were monitored serologically for up to 2 years. Results of serologic and virus rescue assays indicated that all animals became infected and demonstrated a rapid and sustained BIV-specific humoral response. BIV was rescued by cocultivation from spleen, lymph nodes, and peripheral blood leukocytes of infected animals. Viral DNA in immune tissues was confirmed by polymerase chain reaction amplification of BIV sequences. These data and specific immunohistochemical staining of mononuclear cells of the spleen for BIV antigen suggest that the infection is targeted to immune system cells.

The bovine immunodeficiency-like virus (BIV) is an infectious retrovirus and a unique member of the lentivirus subgroup of retroviruses, which also includes human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2, respectively) (12, 16, 17). BIV, like the primate lentiviruses, possesses a complex genome with at least five putative accessory genes (6, 7, 12, 16, 20, 22, 30, 31). The accessory genes of lentiviruses play a significant role in regulating virus gene expression and may also be involved directly or indirectly in their in vivo pathogenesis (6, 7, 20-22).

BIV was originally isolated from a cow with persistent lymphocytosis, lymphadenopathy, central nervous system lesions, weakness, and emaciation; experimental inoculation of specific-pathogen-free calves with tissue culture-propagated BIV induces a mild, persistent lymphocytosis and lymphadenopathy (5, 39). Seroprevalence studies suggest that BIV is sporadically present in cattle populations in the United States (15). BIV is most efficiently transmitted via infected blood and cell-free and cell-associated tissue culture-derived virus; calves experimentally inoculated with BIV appear to become chronically infected and mount a virus-specific humoral response (5, 32, 39, 41). Little else is known about the biology of experimental and natural BIV infections, including BIV’s role in bovine disease and the host’s immune response to BIV infection.

A unifying feature of lentiviruses is the ability to replicate in, and cause chronic infection of, immune system cells; the usual targets are monocytes/macrophages and lymphocytes (11, 13, 14, 19, 27). In many cases, the virus may remain latent in individual cells for long periods of time, although virus replication in the organism is probably continuous. The fact that lentiviruses are retroviruses, and thus can integrate into the host genome, largely accounts for their persistence. In this regard, cellular as well as viral factors may be responsible for regulating virus gene expression and replication (6, 7, 20).

We are interested in developing a BIV–small-animal model that may be useful in studying mechanisms of lentivirus persistence and methods of intervention relevant to HIV-1 infections. The host range of lentiviruses is normally restricted to the natural host and closely related species (19, 29). In the process of characterizing the in vitro and in vivo host range of BIV, we found that the virus can replicate efficiently in cells of rabbit as well as bovine origin. In light of this observation, we attempted to infect rabbits with BIV in vivo. We inoculated specific-pathogen-free juvenile New Zealand White rabbits (Oryctolagus cuniculus) with either a natural isolate or progeny of a proviral molecular clone of BIV; as shown below, all became chronically infected. Virus replication and the host’s humoral immune response to BIV were studied in these animals for up to 2 years.

For in vitro and in vivo studies of BIV infection, two different BIV stocks, BIVαp3, produced from cells infected with the original isolate of BIV (39), and BIV127, produced from cells transfected with a functional molecular clone of BIV (3), were used to inoculate the rabbits. Virus stocks were propagated in either bovine leukocyte adherent (BLAC-20) or embryonic rabbit epidermal (EREp) cells. EREp cells were obtained from the American Type Culture Catalog (catalog no. CRL 6498). BLAC-20 cells were derived from the long-term culture of Ficoll-Hypaque-separated bovine peripheral blood leukocytes (PBLs) obtained from a BIV- and bovine leukemia virus-negative calf. The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics. Phytohemagglutinin at 1 μg/ml was included in the culture medium during the first 48 h, and human interleukin-2 (Collaborative Research) at 500 U/ml was included for the following 14 days. These cultures, which contained adherent cells with the morphology of monocytes/macrophages, were trypsinized when confluent and replated in the original flask in Dulbecco’s modified Eagle’s high-glucose medium (DMEM) containing 10% fetal

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bovine serum, 2 mM l-glutamine, and antibiotics. The infection of both BLAC-20 and EREP cells with BIV results in virus production as measured by reverse transcriptase activity in the culture supernatants and cytopathic effects on these cells, as shown previously for bovine embryonic spleen (BESp) cells (17). BIV infection can also be passed to uninfected EREP or BLAC-20 cells by using cell-free supernatants from the infected EREP cells, indicating that the virus retains its infectiousness after passage through rabbit cells (data not shown).

BIV-infected cells, to be used as rabbit inocula, were monitored visually for maximum syncytium production (>50% of the cell sheet being involved in the cytopathic effects of the virus); this coincided with the peak reverse transcriptase values. Infected and uninfected cells were harvested for inoculation into animals by scraping the cells into sterile 1× Dulbecco’s phosphate-buffered saline without Ca²⁺ and Mg²⁺. Inocula were diluted to 10⁷ cells per 5 ml in 1× phosphate-buffered saline and stored on ice for use within 2 h. Outbred, specific-pathogen-free, barrier-raised female New Zealand White rabbits (Hare Marland) were obtained through slaughter after weaning and maintained in a barrier facility throughout the experiment. Rabbits were inoculated intraperitoneally according to protocols previously described (9). The animals were bled by ear venipuncture once prior to inoculation and, in most cases, biweekly for the first 5 months and monthly thereafter until euthanized, to evaluate the serologic response of the BIV-infected animals and to determine differential and total leukocyte counts. The health status of the rabbits was monitored regularly throughout the study. Animal care was provided in accordance with federal guidelines (29a).

The criteria used to assess the infection of rabbits with BIV were (i) a sustained BIV-specific humoral response in inoculated rabbits, (ii) the rescue of virus with a lentivirus morphology in cocultures of tissues from inoculated rabbits with replication- permissive cells, (iii) the detection of amplified BIV sequences in the DNA of tissues by using polymerase chain reaction (PCR) and BIV-specific primers and probes, and (iv) the immunohistochemical staining of BIV antigens in fixed tissues of inoculated rabbits. The humoral response to BIV was monitored by immunoblot, enzyme-linked immunosorbent assay (ELISA), and radioimmunoprecipitation, performed essentially as described previously (2, 34, 35). Metabolic labeling of cells and viruses with [³⁵S]methionine and [³⁵S]cysteine and preparation of lysates for immunoprecipitations were performed as described elsewhere (34, 35). Animals were euthanized at various intervals from 1 to 22 months postinoculation, and selected organs and tissues (liver, lung, brain, spleen, lymph nodes, kidney, omentum, thymus, intestines, bone marrow, PBLs, appendix, heart, and peritoneal macrophages) were obtained for virus isolation, PCR, and histological examination. For virus isolation, sections of organs were removed under sterile conditions and cocultured with subconfluent monolayers of BESp cells in DMEM supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and antibiotics. The BESp cells were negative for both BIV and bovine leukemia virus DNA sequences by PCR (data not shown). Buffy coat PBLs were isolated from sodium citrate-anticoagulated whole blood by Ficoll-Hypaque separation after lysis of erythrocytes with ACK lysis buffer (Quality Biologicals, Inc.). The PBLs were plated onto subconfluent BESp cells in DMEM supplemented as described above for solid tissues but with phytohemagglutinin added at a concentration of 1 µg/ml. After 24 h, the culture medium was removed and replaced with fresh medium containing interleukin-2 (500 U/ml). The cultures were visually monitored daily for syncytium induction and were split 1:3 when cells reached confluency. Cultures were carried for four passages, at which time they

FIG. 1. Serologic responses of rabbits to infection with BIV. (A) Whole-virus ELISA (34). Sera from rabbits inoculated intraperitoneally with 10⁷ BIV127-infected EREP ( ▼, ▲), BIV127-infected BLAC-20 ( ■, ▲), BIVm²-infected BLAC-20 ( △, ◆), uninfected BLAC-20 ( △, ◆), or uninfected EREP ( ◆) cells were tested. The control animals ( △, ◆) used are those maintained in the study for a period of up to 22 months. Data for animals euthanized prior to 5 months postinoculation are not shown. (B) Immunoblots of recombinant BIV Pr5³⁵⁹ (34). The serologic responses of rabbits to BIV were analyzed by using denatured recombinant BIV Pr5³⁵⁹, separated on 13% polyacrylamide gels, and transferred to Immobilon. BIV antigen strips were immunostained with sequential bleeds from rabbits inoculated with either BIV-infected or uninfected cells; bound antibody was visualized by using the immunoperoxidase enzyme method and a precipitable substrate (34, 35). The Pr5³⁵⁹ region of each set of immunostained strips representing a single animal were cut out and arranged chronologically according to the bleed dates. Sera from animals inoculated with BIV127-infected BLAC-20 cells (rows A, B, C, E, G, and J), BIV127-infected EREP cells (rows D and J), BIVm²- infected BLAC-20 cells (rows F and H), and uninfected BLAC-20 cells (row K) were tested. Rows represent sera from animals euthanized at the following months postinoculation: A, 1; B and C, 2; D, 5; E, 7; F, 9; G and H, 17; and 1 to K, 22. Numbers at the bottom represent months postinoculation. Prebleeds are shown at time point 0. X, time point for which sera were not available.
were considered negative if syncytia had not appeared. Cells from the virus rescue cocultures were analyzed by thin-
section electron microscopy as previously described (18).
Tissue samples (2 mm³) for PCR were taken at necropsy and
processed for genomic DNA. The DNA was resuspended in
sterile H₂O and sonicated for 15 min. Synthetic oligomers
(GTCTCTGTGTGGTACCCTTTA and CATATGGGACAA
CACAATGTGGGACAG) selected from the central region
of the BIV genome (nucleotides 5091 to 5110 and 5364 to
5338, respectively) (12) were used for the PCR amplification
of BIV sequences in rabbit tissue DNA, using components of
a Gene-Amp kit (Perkin-Elmer Cetus). PCR products were
fractionated on 1% agarose gels and transferred to Gene-
Screen Plus (Du Pont/NEN) for hybridization using a ³²P-
labeled, gel-purified, PCR-generated probe representing nu-
nucleotides 5091 to 5364 of BIV127 (12), using conventional
molecular biology methods (36). BIV-specific PCR products
were visualized by autoradiography. Tissue samples from
most major organs were taken at necropsy, fixed in formalin,
and embedded in paraffin; sections were stained with hema-
toxylin and eosin for histopathologic examination. For
immunohistochemical staining, embedded tissue sections
from both infected and uninfected animals were cut and
deparaffinized. These tissue sections were immunostained
with a polyclonal rat antiserum to recombinant BIV Pr53⁷⁹⁰⁸⁹
(34) or a normal rat antiserum and the avidin-biotin peroxi-
dase-complex immunohistochemical technique (40), using a
Vectastain anti-rat immunoglobulin G (IgG) kit according to
the recommendations of the manufacturer (Vector Labora-
tories). Immunostained slides were counterstained with he-
matoxylin.

Since we established that BIV could grow in rabbit cells,
we wanted to determine whether BIV would replicate in
rabbits. Rabbits were inoculated with a single dose of 10⁶⁰
BIV, or BIV127-infected BLAC-20 or EREP cells. The
animals were monitored for a humoral response to the
infection by ELISA (Fig. 1A). Of the 10 animals that were
given a BIV-infected inoculum, the majority convincingly
seroconverted to BIV by 2 weeks postinoculation, while the
others took up to 6 weeks; none of the animals that were
given uninfected EREP or BLAC-20 cells seroconverted to
BIV. There appeared to be no significant difference in the
antibody response obtained with either the BIV or BIV127
inoculum or whether the viruses were propagated in EREP
or BLAC-20 cells. The BIV-specific antibody response in-
creased over time (Fig. 1A), with maximum ELISA titer of
1:25,000 (data not shown). This strong immunologic
response persisted until the animals were euthanized; for two
animals, this was a period of 22 months. IgM and IgG
antibody responses were also measured. By 2 weeks post-
inoculation, it appeared that the IgM response had already
subsided, in that no BIV-specific IgM was detectable by
ELISA (data not shown); sera were not taken at earlier time
points. A similar early decline in IgG antibody titers has been
seen in animals infected with other viruses (33). We used a
sensitive Western immunoblot assay using recombinant BIV
Gag precursor (Pr53⁷⁹⁰⁸⁹) made in the baculovirus-insect cell
expression system (34) to detect early seroconversion to
BIV and to confirm the ELISA results. All animals inocu-
lated with BIV seroconverted specifically to BIV Pr53⁷⁹⁰⁸⁹
between 2 and 4 weeks postinoculation; the relative strength
of the antibody response rose rapidly before appearing to
level off as in the ELISA, although absolute antibody titers
to Pr53⁷⁹⁰⁸⁹ were not determined (Fig. 1B). The antibody
response to Pr53⁷⁹⁰⁸⁹ in rabbits E and H appeared to be lower
than in the other rabbits at the time they were euthanized.

FIG. 2. Reactivity of sera from experimentally infected rabbits and naturally infected bovines to BIV proteins. (A) Whole-virus
immunoblots. For immunoblots, BIV virions obtained from BIV-infected C/GTh cells were denatured, separated on an 8 to 16% poly-
acrylamide gel, and electrophoretically transferred to Immobilon membranes. BIV antigen strips were reacted with sera from a BIV-infected
bovine (lane 1), a BIV-negative bovine (lane 2), a postinoculation bleed from an experimentally infected rabbit (lane 3), and a prebleed from an
experimentally infected rabbit (lane 4). The bound antibody was visualized with peroxidase-labeled goat anti-bovine IgG (lanes 1 and 2) and goat
anti-rabbit IgG (lanes 3 and 4) and visualized by using the enhanced chemiluminescence system (Amersham Corp.). The mobilities of
BIV-specific protein bands (gPr145, gp100 SU, Pr53, Pr49, p32, p26 CA, p19, p16 MA, and p13 CA) are indicated on the left. (B) Radioimmunoprecipitation of native and recombinant BIV antigens. Immunoprecipitations were carried out by using native and recombinant
BIV Gag and Env proteins metabolically labeled with [³⁵S]methionine and [³⁵S]cysteine and polyclonal sera from naturally and experimentally
infected animals. Lanes: 1 to 8, proteins immunoprecipitated with serum from a bovine naturally infected with BIV; 9 to 16, proteins
immunoprecipitated with serum from a rabbit experimentally infected with BIV. The BIV lysed antigens used in these immunoprecipitation
reactions were uninfected BLAC-20 cells (lanes 1 and 9), BIV-infected BLAC-20 cells (lanes 2 and 10), uninfected EREP cells (lanes 3 and 11),
BIV-infected EREP cells (lanes 4 and 12), BIV virions (lanes 5 and 13), recombinant baculovirus-infected insect cells overproducing BIV Env
proteins (lanes 6 and 14), recombinant baculovirus-infected insect cells overproducing BIV Gag precursor protein (lanes 7 and 15), and wild-type
baculovirus-infected insect cells (lanes 8 and 16). Proteins were resolved by sodium dodeyl sulfate-polyacrylamide gel electrophoresis on an 8
to 16% gradient gel. The mobilities of native BIV proteins (gp100 SU, Pr53, p26 CA, and p16 MA) and recombinant BIV Env (gp100 SU) and
Gag (Pr53) are designated on the left, and positions of molecular weight standards (in thousands) are indicated on the right.
(Fig. 1B). Nevertheless, virus was rescued from the spleens of both animals. BIV DNA sequences were also demonstrated by PCR in the small intestine, peritoneal wash, and omentum of rabbit E (no spleen was available for testing) and the spleen, bone marrow, peritoneal wash, and lymph nodes of rabbit H. The serologic results in Fig. 1 demonstrate that antibodies directed to epitopes on the BIV Gag precursor are an early and positive indicator of seroconversion, and the retention of a strong antibody response to Gag over time is indicative of active virus replication; the contribution of Env antibodies to the humoral response over time has not been studied in this investigation but may differ from that of Gag.

By using sera obtained from terminal bleeds, the serologic response of BIV-infected rabbits was determined in Western blots and immunoprecipitations and was compared with the reactivity of serum from a naturally infected bovine (Fig. 2). In the immunoblots using BIV virions, bands corresponding to the envelope precursor and surface (SU) glycoproteins, gp145 and gp100, respectively, and the Gag precursor, capsid (CA), matrix (MA), and nucleocapsid (NC) proteins, Pr53gag, p26, p16, and p13, respectively (2, 26), were observed, as well as several virus-specific bands whose identity has not been established (Fig. 2A). Radioimmunoprecipitation of BIV-infected cell and virion lysates showed that gp100 SU, Pr53gag, p26 CA, and p16 MA proteins were immunoprecipitated by the BIV-infected rabbit sera (Fig. 2B). Recombinant Pr53gag and Env proteins synthesized in the baculovirus-insect cell expression system (34, 35) were also immunoprecipitated by the BIV-infected rabbit sera (Fig. 2B), further substantiating the specificity of the rabbit humoral response to authentic BIV Gag and Env proteins. In Western blots and immunoprecipitations, the recognition of native and recombinant BIV Gag and Env structural proteins by antibodies in BIV-infected rabbits was similar to that of BIV-infected bovines (32, 34, 35). As expected, the sera from rabbits inoculated with uninfected EREp or BLAC-20 cells did not detect any BIV-specific bands in either the Western blots or immunoprecipitations.
Virus rescues were attempted on individual animals that were euthanized at 1, 5, 7, and 9 months postinoculation and two animals each at 2, 17, and 22 months postinoculation. In all cases, BIV was successfully rescued from the spleens, lymph nodes, or PBLs of BIV-infected rabbits by cocultivation with BESp cells; the tissues of one animal were not available for study (Table 1). In most cases, syncytia, indicative of virus infection, were apparent in the BESp cells after the first subculture (day 4) (Fig. 3B). Syncytia were never observed in BESp cells that had been cocultured with tissues from rabbits inoculated with uninfected BLAC-20 or ERp cells or other organs of BIV-infected rabbits (Fig. 3A). In some infected animals, successful virus rescues were made periodically from PBLs in the interval between initiation of infection and euthanasia of the animal. The specific induction of syncytia in BESp cocultures with infected tissues (but not uninfected tissues), the positive reverse transcriptase results with supernatants from cultures containing syncytia, and the identification by electron microscopy of virus particles with a typical lentivirus morphology (Fig. 3C) all indicated that the virus rescued was BIV. In all instances, the presence of BIV was confirmed by specific PCR amplification of BIV sequences in the DNA of cells used in the coculture (data not shown). We wanted to determine whether BIV sequences could be detected in the DNA extracted directly from tissues of BIV-infected rabbits, which may be further indicative of the tropism of the infection. As shown in Table 1, BIV sequences were most frequently detected in the lymph nodes, spleen, peritoneal wash (presumably containing macrophages), and bone marrow. PBL DNAs were not tested. Tissues from animals given uninfected cells were negative for BIV sequences by PCR analysis (data not shown). Our ability to readily rescue virus from spleen, lymph nodes, and PBLs, as well as the positive PCR results, suggested that these tissues may be the sites of active virus replication. Immunohistochemical staining of tissue samples from various organs of several animals was performed by using a rat anti-Pr53Gag or normal rat serum to localize BIV-specific antigens. Antigen-positive cells were detected only in the red pulp of the spleen of infected animals with the rat anti-Pr53Gag serum (Fig. 4A); no BIV-specific staining was observed with the control normal rat serum or in tissues from uninfected rabbits (data not shown). The BIV antigen-containing mononuclear cells in the infected animals appeared to be of the macrophage lineage and were atypical and blastic (Fig. 4B).

All rabbits experimentally infected with BIV appeared clinically normal throughout the experiment, and differential and total leukocyte counts remained within normal ranges for both infected and uninfected animals (data not shown).
BIV-infected and uninfected animals (as controls) were euthanized at various time points and examined for gross pathologic lesions at necropsy. The only differences noted between infected and uninfected animals were the slight enlargement of lymph nodes and spleen in the infected animals terminated 5 and 7 months postinoculation. Histopathologic examinations of major organs of animals terminated prior to 9 months revealed subtle and inconsistent lesions which included multifocal lymphoreticular infiltrates in the lungs, moderate depletion of T-cell regions of the white pulp of the spleen, and multinucleated giant cells in the omentum.

Rabbit cells and the laboratory rabbit have been experimentally infected with several types of retroviruses, including bovine leukemia virus, HIV-1, and human T-cell leukemia virus type I (HTLV-I) (1, 4, 8, 9, 23–25, 28). The HTLV-I infection in the rabbit has no associated disease or lesions, but it has been used as a model for HTLV-I transmission and vaccination studies (38). In the present study, the infection of rabbits with BIV derived from bovine or rabbit cells was accomplished with a single intraperitoneal dose of 10^6 infected cells, although we have since shown that a single dose of 10^4 BIV-infected cells given intraperitoneally is sufficient to induce a persistent infection in rabbits (32). HIV-1 is the only other lentivirus that has been shown to replicate in rabbits (8, 9, 23, 25). The BIV infection of rabbits results in a rapid (within 2 weeks of inoculation), high-titered (ELISA titers of up to 1:25,000), and sustained (up to 22 months postinoculation) humoral response and the reliable rescue of virus from immune organs or tissues. The serologic results with BIV are in contrast to those reported for the HIV-1 infection of rabbits, in which antibody titers in ELISA are lower (1:2,560), peak at approximately 3 months postinoculation, and steadily decline (1:320) thereafter (8, 9).

In this study, BIV was rescued from 100% of the rabbits receiving infectious inoculum; virus was also rescued from various tissues of the immune system, including spleen, lymph nodes, and PBLs. By using PCR amplification, BIV DNA sequences were found primarily in the same tissues from which virus was rescued, but they were also found in the bone marrow and brain. Immunohistochemistry with BIV-specific antisera localized BIV antigens in mononuclear cells of the red pulp of the spleen. Taken together, these results suggest that BIV replicates in cells of the immune system, possibly monocytes and macrophages. The fact that BIV can be rescued from infected animals persistently producing BIV-specific antibodies provides direct evidence that the BIV infection is chronic and is not overcome by the host's immune system. The contribution of neutralizing antibodies and cellular immunity to the host defense against BIV infection has not been addressed, but the failure to clear the virus suggests that the virus-specific humoral response, and possibly the cellular response, may be deficient. Additional studies on the virus-specific humoral and cellular immune responses in BIV-infected animals are in progress.

The lack of overt disease in the BIV- and HIV-1-infected rabbits may be analogous to HIV-1 infection of chimpanzees (10). The reasons for the lack of clinical disease in BIV infections of rabbits are not known, but several explanations can be proposed. First, the BIV\textsuperscript{par} isolate, and progeny from proviral molecular clones, may be attenuated; BIV\textsuperscript{par} has been in culture for over 20 years and shows a strong tropism for adherent embryonic bovine cells, which may not be typical of the tropism of new field isolates of BIV (32). Significant lesions and disease development may take longer than 2 years to occur. It is also possible that the rabbit immune response offers protection from disease but not from infection. The possibility also exists that BIV does not directly cause severe lesions in the tissues in which it replicates, but may exert its effects on the immune system in a more subtle way, perhaps through mild immunosuppressive activity. Although BIV has not been demonstrated to cause any immune system dysfunction or blood cell disorder, BIV sequences were found in the bone marrow of infected rabbits by PCR amplification. There was also a subtle depletion of cells in the T-cell regions of the white pulp of spleens in infected animals. HIV-1 has also been demonstrated to infect bone marrow cells in vitro, as well as CD4\textsuperscript{+} cells, and to cause in vitro suppression of bone marrow progenitor cells (37). Adventitious agents may be required to potentiate BIV's effects. The specific-pathogen-free New Zealand White rabbits used in this study were maintained in a barrier facility and therefore were not subjected to normal rabbit pathogens.

Although much information still needs to be gathered, our studies suggest that the chronic infection of rabbits with BIV may be a useful lentivirus-animal model with a number of advantages. Laboratory rabbits are small, inexpensive, and readily available. BIV causes chronic infections in the rabbit immune system. BIV is a lentivirus with a genome complexity that closely correlates with that of the primate and human lentiviruses, but it does not appear to infect humans or cells of human origin (12, 15). This model should offer the opportunity to test novel prophylactic and therapeutic measures of relevance to HIV-1 as well as the opportunity to study mechanisms of lentivirus persistence and replication.

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