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Selective Amplification of Simian Immunodeficiency Virus Genotypes after Intrarectal Inoculation of Rhesus Monkeys†

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Animal models for sexual transmission of human immunodeficiency virus can define the influences of virus type, dose, and route of inoculation on infection and clinical outcome. We used an uncloned simian immunodeficiency virus stock (SIVmac) to inoculate cells in vitro and to inoculate rhesus monkeys by intravenous and intrarectal routes. The distribution of virus genotypes present in each of these infection examples was characterized by DNA sequence analysis of viral long terminal repeats (LTRs). Our analysis of LTR sequences from in vitro and in vivo infections revealed three main genotypes: one genotype was observed only for in vitro infection, and two other genotypes were recovered only from infected animals. By comparing animals inoculated with high intrarectal doses of SIVmac and those inoculated with low doses, we demonstrated that unique subsets of the stock were selected after intrarectal infection. Our findings indicate that minor genotypes present in the stock cross the rectal mucosa and are amplified selectively to become prominent in peripheral blood mononuclear cells from acutely infected animals. Studies with a molecular recombinant of SIV and human immunodeficiency virus type 1 sequences, SHIV, showed that viral LTR sequences do not undergo especially rapid sequence variation or rearrangement after intrarectal inoculation. The mucosal barrier exerts a significant influence on infection and disease progression by reducing the efficiency of SIVmac infection and by permitting distinct, pathogenic genotypes to become established in the host.

Sexual transmission is the dominant mode for epidemic spread of human immunodeficiency virus type 1 (HIV-1). Virus or virus-infected cells present in genital fluids are deposited on mucosal surfaces to initiate a new infection. The portal of infection may be epithelial cells that are infected directly (11), specialized M cells that transport virions to the underlying cellular layers (1), inoculation of blood mononuclear cells through tears in the mucosa (2), or infection of lymphoid and myeloid cells present transiently in the mucus layer or interdigitated in the mucosal epithelium (6). Although the cellular targets are not well defined, several studies have described the unusual consequences of intramucosal retrovirus infections. Studies of HIV-1 sexual transmission showed that recently infected recipients manifested only a subset of viruses that were present in the donor (14, 16, 17). In these cases, virus populations were characterized by limited sequence analysis of viral DNA recovered from peripheral blood mononuclear cells (PBMCs) and it was not possible to discern specific sequence motifs within envelope genes that were highly associated with transmission (17). Model studies with simian immunodeficiency virus of macaques (SIVmac) infection in rhesus monkeys provided preliminary indications that different virus populations were enriched after intravenous and intrarectal inoculation and that these differences were related to the marked differences in disease progression rates (8).

Two models were advanced to account for the differences between peripheral blood virus populations in donors and those in recently infected recipients (9, 17). The titration model argues that low doses of virus or inefficient transmission across the mucosal barrier results in a small number of virus strains becoming established in the recipient. The selection model argues that specific properties of individual virus strains facilitate their crossing the mucosal barrier. These two models differ fundamentally in their potential impact on efforts to develop effective vaccines. We initiated studies of intrarectal SIVmac inoculation in rhesus monkeys to provide a model for sexual transmission that was controlled for virus dose, route of inoculation, date of administration, and virus stock. These studies begin the process of discriminating titration and selection models for retrovirus transmission across mucosal surfaces.

In a manner similar to studies of sexual transmission in humans, we characterized the distribution of virus sequences present in rhesus PBMCs soon after intramucosal or intravenous inoculation. The SIVmac stock employed in these studies was an uncloned, biological isolate containing multiple virus strains. We decided to focus attention on the virus long terminal repeat (LTR) sequences that provided a convenient marker for different virus types (8). The SIVmac LTR is a 799-bp element duplicated directly at both ends of the complete viral DNA. The LTR constitutes a promoter for expression of the integrated provirus, it contains recognition sequences for transcription factors, and it includes the TATA box for transcription initiation (3, 4, 15). Among the transcription factor recognition sites are sequences designated Sp1 binding sites, although whether the nuclear factor Sp1 actually recognizes this sequence is controversial (12). Within the SIVmac virus group, the molecular clone SIVmac251 (mc251) contains two copies of the Sp1 binding sites and the molecular clone SIVmac239 (mc239) contains three copies of the Sp1

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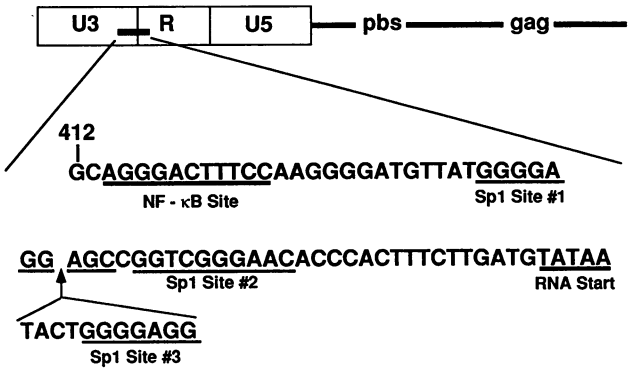

FIG. 1. Diagrammatic representation of LTR sequences of SIVmac. The region spanning the U3-R boundary is represented; it begins at nucleotide 412 near the NF-κB site. The Sp1 binding sites present in mc251 are designated site 1 and site 2. The duplication that produces a third partial Sp1 binding site (site 3) is indicated below the line, and the site of duplication is shown by a vertical arrow. The RNA start site is shown for reference. The primer binding site (pbs) and *gag* structural gene regions are also shown. The viral genome is the integrated provirus form.

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Mc239       TTCCACAAGGGGATGTTACG GGGAGGTACTGGGGAGGAGC CGGTCGGGAACGCCCACTTT
Mc251       ..................--- -------.-.......... ..........A.........
3 Sp1/T478  ..................... ..................... .....................(8/10)
3 Sp1/T478* ..................... .............A....... .....................(1/10)
3 Sp1/T478* ..................... ..................... .................C..(1/10)
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FIG. 2. LTR sequences from in vitro infection of CEMx174 cells with SIVmac. Nucleotide sequences begin at base 421 of the viral LTR and end at position 479; the reference sequences are mc239 and mc251 (7). Dots indicate sequence identity, and dashes indicate deletions. The sequence of 10 deletions, 1 identity, and another deletion in the mc251 reference sequence shows the difference between LTRs with three Sp1 sites (mc239) and those with two Sp1 sites (mc251).

binding sites (7). The third Sp1 site (Fig. 1) in the mc239 sequence is a partial duplication and is inserted into the second Sp1 binding site that is present in the mc251 sequence. The LTR sequence differences may be associated with the increased virulence of mc239 (7), and studies with HIV-1 have related Sp1 binding sites to virulence (12). We examined the distribution of viral LTRs with three or two Sp1 binding sites and closely associated single-nucleotide changes to assess the complexity of viruses present in our stock and in animals infected by intravenous or intrarectal inoculation.

For animal and in vitro infection studies, we used the SIVmac251 uncloned biological stock (denoted here as SIVmac) obtained from Ronald C. Desrosiers at the New England Regional Primate Research Center. The virus was propagated in our laboratories by several passages on mitogen-stimulated rhesus PBMCs with a final passage on CEMx174 cells to increase the titer (8). The titer of this virus stock was determined previously by intravenous inoculation in rhesus monkeys to define the animal intravenous infectious dose (ID). The minimal dose was defined as the amount of virus required to render animals positive for virus isolation from PBMCs.

Logarithmically growing cultures of CEMx174 cells or mitogen-stimulated PBMCs from uninfected rhesus monkeys were infected with SIVmac (multiplicity of infection equal to 1.0 tissue culture infective dose per cell). At 18 h after infection, cells were collected and processed for DNA purification. For each sample, 10^6 PBMCs were lysed in buffer containing sodium dodecyl sulfate and incubated for 4 h at 56°C with 200 μg of protease K per ml (Boehringer Mannheim). DNA was precipitated upon addition of freshly made dimethyl formamide solution (70% acetone, 5% dimethyl formamide in water), and the precipitates were rinsed twice with 70% ethanol (10). The DNA samples were resuspended in 10 mM Tris-HCl–1 mM EDTA (pH 7.5), and total cellular DNA concentrations were determined by optical density at 260 nm. The titers of the samples were determined by limiting dilution analysis to estimate the relative content of viral DNA sequences. An amount of infected-cell DNA equivalent to between 25 and 100 copies of SIVmac DNA was added to each PCR mixture, and the total template DNA did not exceed 250 ng for a single reaction. Two round (nested) PCRs were performed routinely. For each sample included in the study, five parallel primary amplification reactions were set up for a single PBMC DNA preparation. The five parallel secondary reactions used 1 microliter of each primary reaction mixture as template. The primary amplification primers were SU3.58 (GGAAGAAGGCATCATACCAGATTGGC) and SU5.774 (GCAGAAAGGGTCCTAACAGACCAGGG). The second set of primers were SU3.82 (CAUCAUCAUCAUGGTCTC CCTCTCTTGGAGGC) and SU5.721 (CUACUACUACUA CCAGGCGGCGACTAGGAGAG). The sequences underlined in secondary primers were included for convenient cloning into the precut pAMP vector (Gibco BRL, Gaithersburg, Md.) after uracil deglycosylase treatment to reveal single-stranded ends complementary to the vector. The secondary amplification primers generate products for cloning that are 639 bases in length. The analysis of LTR sequences present in the population of viruses was performed by direct sequencing of the bulk amplification product (consensus) and by sequencing of a representative number of individual plasmid clones (13).

The consensus sequence analysis for LTRs amplified from the in vitro infection of CEMx174 cells or rhesus PBMCs showed that the LTRs with three Sp1 sites were most abundant (not shown). Molecular clones were generated from these DNA amplifications; 10 random clones were picked and sequenced from the CEMx174 infection experiment. The sequences were compared with those of molecular clones mc239 and mc251 as references for LTRs containing three and two Sp1 sites, respectively. All 10 clones were similar to mc239, and they contained three Sp1 sites in the U3 region of the LTR (Fig. 2). We observed the same spectrum of LTR sequences in several independent amplification, cloning, and sequencing experiments for in vitro SIVmac infections of CEMx174 or rhesus PBMC cultures.

The LTR sequences present in PBMCs at 8 weeks after intravenous SIVmac inoculation of rhesus monkeys with 10 ID of SIVmac were examined for distribution of individual genotypes. Two rounds of PCR amplification and molecular cloning generated LTR clones from animals 90090 and 90100; we sequenced 10 clones selected at random from each of these sample sets. In both cases, 10 of 10 clones contained three Sp1 sites in the U3 region. However, the LTR sequences from intravenous animal infection samples were not identical to sequences obtained after in vitro infection of CEMx174 cells. The LTRs from intravenously infected animals all contained a C at position 478 (C-478) that was not present among the in vitro infection samples (Fig. 3). This sequence was not reported previously for either mc239 or mc251 and was not observed after in vitro infection. In addition to the C-478, we observed other single-base changes in the LTRs from intravenously infected animals. On the basis of repetitive amplification cloning and sequencing of control templates, we estimate our combined error rate to be less than 1 error in 5,000 bases

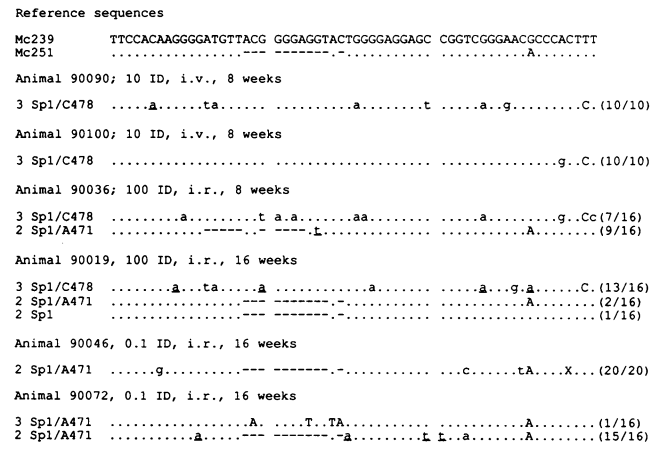

FIG. 3. LTR sequences (nucleotides 421 to 479) from rhesus monkeys inoculated intravenously (i.v.) or intrarectally (i.r.) with SIVmac251. The reference sequences mc239 and mc251 are listed at the top. Experimental sequences are listed with the animal identification number, the dose (ID) and route of inoculation, and the weeks after inoculation for sampling. For each animal, we show the predominant sequence types, with an indication in parentheses as to the frequency of that sequence in the population. Nucleotides in lowercase designate minor sequence variations among the set of LTR sequences. Individual variants observed twice or more are underlined. The X indicates positions at which more than one nucleotide substitution was noted.

of sequence information. The nucleotide sequence variation among clones confirms that we are detecting individual viral DNA templates and that we are not simply resequencing amplification products from a single viral DNA molecule. The common pattern observed for intravenous infection samples was an LTR with three Sp1 sites and a C-478.

We next analyzed the distribution of LTR sequences in PBMCs from two animals inoculated intrarectally with 100 ID and from two animals inoculated with 0.1 ID of SIVmac. LTR was amplified from PBMC DNA collected from high-dose (100 ID) or low-dose (0.1 ID) intrarectally infected animals. Consensus sequence analysis showed that viral DNA from high-dose intrarectally infected animals was a mixture of LTRs with three and two Sp1 binding sites. Seven of 16 clones sequenced from high-dose animal 90036 8 weeks after infection had three Sp1 sites in the LTR and the T-to-C change at position 478 (Fig. 3). The remaining nine clones from the 90036 sample had two Sp1 sites and a unique A at position 471. Sixteen clones were analyzed for animal 90019 by using PBMCs that were collected 16 weeks after infection (Fig. 3). Three of 16 clones had two Sp1 sites, and 2 of these clones also had an A-471. The remaining 13 clones had three Sp1 sites and the C-478. In animals that received low-dose intrarectal SIVmac inoculation, we observed mostly LTRs with two Sp1 sites. The LTRs with two Sp1 sites were present in 20 of 20 clones sequenced from animal 90046 and 15 of 16 clones from animal 90072 16 weeks after inoculation (Fig. 3). The single unusual clone in animal 90072 contained sufficient DNA to encode three Sp1 sites, although a number of nucleotide changes were present in the third Sp1 site, and the G-to-A change at position 471 was present. All other clones from low-dose intrarectally infected animals also contained A-471 and T-478.

There was a distinct difference between LTR sequences from intravenously infected animals and those from intrarectally infected animals early after inoculation. This pattern of

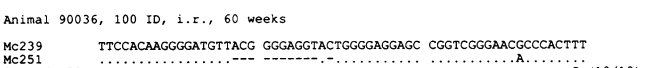

FIG. 4. LTR sequences for animal 90036 (high dose, intrarectal [i.r.]) 60 weeks after inoculation. All clones showed three Sp1 sites and the T-to-C change at position 478. The position of a minor C-to-T variant is indicated by a "t."

LTR sequences was not maintained in later samples. Consensus sequence analysis of LTRs from PBMCs of the high-dose intrarectally infected animals (90036 and 90019) at 60 weeks showed a clear predominance of LTRs with three Sp1 sites for both animals. All 18 clones sequenced from animal 90036 at 60 weeks showed three Sp1 sites and a C-478 (Fig. 4). The LTRs from low-dose intrarectally infected animals at 60 weeks or later did not show this shift in the population of virus sequences, and they remained predominately LTRs with two Sp1 sites and A-471 (not shown). It is important to note that the high-dose intrarectally infected animals were positive for virus isolation, were seropositive throughout the 60-week sampling period (the last sample from animal 90036 was at 60 weeks), and were characterized as having an active and progressive virus infection. The low-dose animals manifested an indolent infection that was characterized by no seroconversion and the inability to isolate SIVmac from PBMCs (7a).

The observed patterns of viral LTR sequences suggested that the population of viruses in PBMCs was affected by the initial route of infection. However, the main differences observed for LTR sequences involved a duplicated Sp1 site that was present at two or three copies in each LTR. We wanted to rule out the possibility that changes in Sp1 sites were selected after intramucosal transmission by a rearrangement within the LTR. To accomplish this test, we inoculated rhesus monkeys intravenously or intrarectally with molecularly cloned SHIV, a virus composed of SIV and HIV-1 sequences and that includes the mc239 LTR with a three-Sp1 site and an envelope gene from the HIV-1_{HXB} clone (5). One animal was infected intravenously (91021) and three were infected intrarectally (91014, 91023, and 91030) with 2,500 tissue culture infective doses. We prepared DNA from PBMCs collected 4 weeks after inoculation and continued with amplification, cloning, and DNA sequence analysis of LTRs as described above. All sequences from PBMCs of intravenously (not shown) and intrarectally infected monkeys contained only LTRs with three Sp1 sites in the U3 region (Fig. 5). In addition, the nucleotide at position 478 was the T expected for the mc239 sequence in the SHIV construct and was not the C observed for in vivo SIVmac infection. In a situation in which only one sequence was present in the inoculum, we did not observe changes in two individual nucleotide positions or the Sp1 binding sites that were associated previously with intrarectal transmission.

In vitro and in vivo infection studies characterized the LTR genotypes present in our SIVmac virus stock. When we

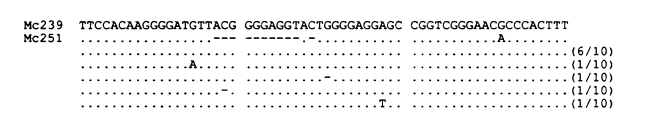

FIG. 5. LTR sequences for animal 91023, which was inoculated intrarectally with 2,500 tissue culture infective doses of SHIV. Template DNA was from PBMCs collected 10 weeks after inoculation. The SHIV LTR sequences from this infected animal closely match the mc239 LTR that was present in the SHIV molecular clone.

focused on a limited region of the LTR extending from nucleotide 440 to nucleotide 479, we recognized three major virus types. One type contains three Sp1 sites in the U3 region and is identical to the published sequence for mc239 (7). A second type contains three Sp1 sites and C-478 in place of the T present in the mc239 sequence. The third type contains two Sp1 sites and a A-471. The A-471 is found in the published sequence for mc251 and replaces G in the mc239 sequence.

The distribution of LTR genotypes differed for the in vitro, intravenous, high-dose intrarectal, and low-dose intrarectal infections. A single-round infection of CEMx174 cells or rhesus PBMCs in vitro produced a population of viral DNA molecules dominated by LTRs with three Sp1 binding sites. Analysis of LTR sequences between nucleotides 360 and 649 from 10 plasmid clones revealed a total of only nine deviations from the published mc239 sequence. Infection of rhesus monkeys uncovered other viral LTR genotypes that were not apparent in the limited sampling from in vitro-infected cells. In two animals infected by intravenous inoculation, the LTR contained three Sp1 sites and the T-to-C change at position 478. Other nucleotide changes were observed among the 20 plasmid clones sequenced from these two animals, although the pattern of three Sp1 sites and C-478 was observed in each of the 20 clones. In two animals inoculated intrarectally with high doses of SIVmac, we observed a mixed population of virus sequences composed of LTRs with three Sp1 sites and C-478 along with LTRs containing two Sp1 sites, T-478 and A-471. In two animals inoculated intrarectally with low doses of SIVmac, we observed 35 of 36 sequences having the LTRs with two Sp1 sites, T-478 and A-471. In addition to these data, we examined either the consensus sequence or a small number of plasmid LTR sequences from all other animals in the study (not shown). The spectrum of LTR sequences present in other infected animals was consistent with the detailed results reported above. High-dose intrarectal infection resulted in a mixed population of viral LTRs, and low-dose inoculation established mostly two-Sp1 LTR sequences. Animals that received 10, 1, or 0.1 ID intrarectally were grouped together as low-dose infections; all animals in this group were also characterized by a predominant appearance of LTRs with two Sp1 binding sites.

The initial pattern of LTR sequences was not stable in high-dose intrarectally infected animals. The example of animal 90036 documents a gradual shift in the virus population from the starting point of mostly LTRs with two Sp1 binding sites, A-471 and T-478, to a situation at 60 weeks that was characterized by a predominance of LTRs with three Sp1 binding sites and C-478. On the basis of physical examinations for lymphadenopathy, erythematous macular rashes, body weight data, and analysis of virus load in PBMCs, animal 90036 showed significant disease progression during this time. We did not observe the full cycle of SIV disease because of this animal's accidental death as a result of fighting with another monkey. Despite this reservation, we generally observe a correlation between the relative proportion of LTRs with three Sp1 binding sites and the severity of clinical disease. Animals inoculated intravenously established infections primarily composed of LTRs with three Sp1 sites, and both of these animals died within the first year after infection from SIV-related outcomes (wasting in one case and lymphoma in the other case). The high-dose intrarectally infected animals (four total) survive to this day (3 years after inoculation), with the exception of 90036 as noted above. The low-dose intrarectally infected animals showed no signs of disease progression and maintained LTRs with two Sp1 binding sites, A-471, and T-478.

The analysis of LTR sequences in SHIV-infected animals proves that the results described above represent differential virus transmission according to dose and route of inoculation. Irrespective of whether rhesus monkeys were inoculated by the intravenous or intrarectal route, all SHIV LTR clones have three Sp1 sites, with no change at position 478. Consistent with our previous results, we observed a small number of nucleotide changes among individual LTR clones, although the sequence markers of Sp1 sites and positions 471 and 478 were unchanged. These data also show that viruses containing the mc239 LTR are capable of crossing the rectal mucosa and establishing infection in the animal, with the important caveat that we have not yet observed rapidly progressing disease in SHIV-infected animals.

We used a complex virus stock and intramucosal transmission to examine the specific effects of different inoculation routes on SIVmac infection. On the basis of LTR genotypes, we recognize three main virus types in the stock. One type was only observed when SIVmac was used to infect lymphoblastoid cells or PBMCs in vitro. The main LTR sequence observed for in vitro infection was not seen again in any of the infected rhesus monkeys. Animals inoculated intravenously contained a homogeneous population of LTRs; the main LTR sequence observed after intravenous infection was also present within a mixed-virus population after high-dose intrarectal infection. Animals inoculated with low doses intrarectally again contained a homogeneous virus population, and this species was also represented in the mixed infection of high-dose intrarectally inoculated animals. Our conclusions from these data are as follows. (i) Minor species in the virus stock are established after in vivo infection, irrespective of the route of inoculation. Thus, the dominant species observed for in vitro infection does not compete effectively during in vivo infection. (ii) High-dose intrarectal or intravenous inoculation favors infection by the same types of viruses that are present in long-term-infected animals. These viruses have LTRs with three Sp1 sites and C-478. This pattern of LTR accumulation implies that, of all infecting viruses, the three-Sp1-site C-478 viruses were selectively amplified during infection or have an inherent growth advantage once the infection is seeded. (iii) Low-dose intrarectal inoculation was associated with lack of disease progression and was characterized by LTRs with two Sp1 sites, A-471 and T-478. This result suggests that the two-Sp1-site viruses have an inherent advantage in crossing the mucosal barrier or that they were present in the virus stock to a greater extent than the three-Sp1-site viruses. (iv) Selection across the mucosal barrier has a profound influence on the eventual disease outcome. At high intrarectal doses, viruses that were later correlated with disease progression were established during the initial infection event. The same viruses were not seen in a group of low-dose intrarectal infection animals that did not show evidence of disease progression.

Our data support the role of selection mechanisms in determining the population of viruses established after intramucosal SIVmac transmission in rhesus monkeys. Viruses marked by three Sp1 sites and C-478 have a replicative advantage in vivo, as shown by their increasing abundance during the course of progressive infection. The exclusive presence of LTRs with two Sp1 sites in the low-dose intrarectally infected animals implies that this sequence was more abundant in the inoculum than the three-Sp1-site C-478 virus or was much more efficient at crossing the mucosal barrier. Titration operates as expected, and low-dose inoculation can produce a situation in which virulent virus strains are not included in the population of transmitted viruses. The phenomenon of virus transmission across the rectal mucosa in

rhesus monkeys is best explained by the selective amplification model (17). Our data show that viruses with three Sp1 sites and C-478 or two Sp1 sites and A-471 or SHIV molecular clones with three Sp1 sites and T-478 all cross the rectal mucosa and establish infection in the recipient animal. However, the long-term outcome is strongly influenced by the type of virus amplified soon after infection. The high-dose intrarectal infections were sufficient to include the virus with three Sp1 sites and C-478. Once present, this virus established a virulent infection that resulted in a rapidly progressing disease.

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