

## Virus Levels in Untreated African Infants Infected with Human Immunodeficiency Virus Type 1

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**In developed areas, human immunodeficiency virus (HIV)–infected infants have high virus levels and rapidly progress to death. HIV levels were assessed in 1994–1997 in untreated infants in Malawi by analysis of dried blood spots tested by nucleic acid silica-bound amplification. Of 24 umbilical cord blood (CB)–positive samples, 83% had >10,000 copies/mL. The median virus level was 78,000 copies/mL. First positive sample median levels were 355,000 copies/mL among 52 perinatally infected infants and 130,000 copies/mL among 43 infants infected by breast-feeding. Virus levels were stable, and initial levels predicted levels 1 year after infection ( $P = .005$ ), at which time levels did not significantly differ among in utero, perinatally, or postnatally infected infants. Thus, neither age at infection nor route of infection significantly influenced HIV levels measured 1 year after infection. Most (87%) CB-positive infants were infected before labor onset, since virus levels greatly exceeded those expected in their mothers.**

In some cities in Africa, about one-third of women giving birth are infected with human immunodeficiency virus (HIV) type 1 [1]. In the absence of antiretroviral treatments, a third of their babies will become infected in utero, during the peripartum period, or postnatally through breast-feeding [2–4]. These infants will develop immunosuppression and progress to AIDS more quickly than adults [5, 6]. In the United States and Europe, HIV-1 levels are much higher in infants than in adults, and in both infants and adults, CD4 cell declines are associated with higher initial virus loads [7–9]. Since treatment with antiretroviral drugs is now standard [4, 10], infants known to be infected cannot be followed without treatment. Thus, in industrialized countries, only cohorts assembled before therapies were available can be used to determine the natural virologic events surrounding HIV infection.

The virologic patterns of HIV infections among African infants have not been reported. To understand the natural history of these infections, we developed methods to determine HIV levels using dried blood spots. We then applied these methods to samples collected from infants born to HIV-infected women in Blantyre, Malawi, during their first 2 years of life. At the time of this study, 1994–1997, antiretroviral therapies were not available for adults or infants in Malawi. We examined differ-

ences in virus levels according to the route of infection (in utero, peripartum, and breast-feeding) and the timing of infection among infants infected while breast-feeding.

### Methods

**Subjects.** Between June and November 1994, all women delivering at Queen Elizabeth Central Hospital, Blantyre, were offered enrollment in an intervention trial in which the birth canal was washed with chlorhexidine [3]. Women who agreed were tested for antibodies to HIV-1 (HIV-1 EIA; Genetic Systems, Seattle) and were confirmed by immunoblotting of sera from the umbilical cord blood (CB) of their infants. Infected mothers and some uninfected controls were asked to bring their infants to a follow-up clinic at regular intervals, when data were obtained on mothers and infants by questionnaire and physical examination. At each visit, a heel stick blood sample was obtained from the infant and spotted on filter paper cards. These cards were allowed to air-dry for several hours, then stored at  $-20^{\circ}\text{C}$  until testing. As described previously [11], HIV-infected infants were identified by qualitative DNA polymerase chain reaction (PCR; Roche HIV-1 Amplicor kit, Branchburg, NJ) testing of dried blood spot specimens.

Of 7959 women seen initially, 88% agreed to participate. Tests showed that 2094 women were HIV infected. These women delivered 2157 babies, including multiple births, and 1406 (65%) had  $\geq 1$  follow-up visit. Among infants, 405 (29%) were HIV positive by DNA PCR at age  $\leq 1$  year or by antibody tests after age 1 year. Many had only a single positive sample, sometimes obtained late ( $>4$  months) in follow-up. These infants were excluded as being uninformative. For this study, we selected infected infants who died in the first year even if there was only 1 positive postnatal sample, provided it was obtained by age 4 months, and infants surviving to age 1 year with  $\geq 2$  positive samples (possibly including a CB

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sample). We also selected all infants infected by breast-feeding whenever they were infected, even if we had only 1 positive sample. In both categories, samples for some selected subjects had already been exhausted. In all, we studied samples from 135 infants, 24 of whom were CB positive, 68 of whom were infected at or near delivery, and 43 of whom were infected by breast-feeding. The estimated time of infection within each group is shown in figure 1.

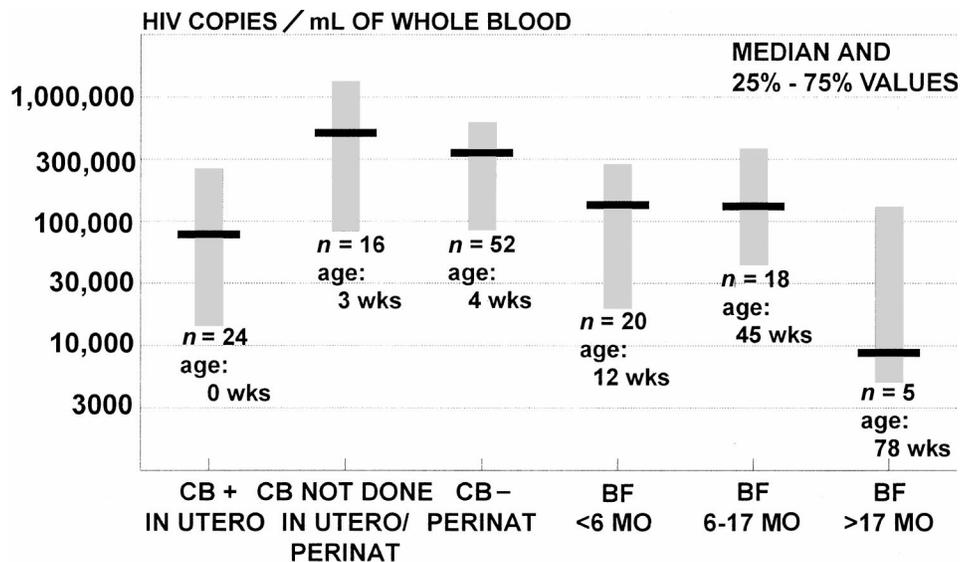
**Laboratory methods.** Guthrie (filter paper) cards were tested for HIV RNA by a second-generation isothermal nucleic acid silica-bound amplification (NASBA) assay (NucliSens HIV-1 RNA Q-T kit; Organon Teknika, Durham, NC [12]) adapted for use with dried blood spots [13]. All tests were performed over 8 months in a single laboratory that also participated in the AIDS Clinical Trials Group HIV-1 Virology Quality Assurance Program.

At the time of analysis, half of a dried blood spot containing an estimated 25  $\mu$ L of whole blood was excised using sterile, acid-depurated scissors, cut into four equal strips, and placed in a 10-mL nuclease-free polypropylene tube. An aliquot of NucliSens lysis buffer (9.0 mL) containing guanidine thiocyanate, Triton X-100, and internal kit RNA calibration standards of 4500 (3.653  $\log_{10}$ ), 35,000 (4.544  $\log_{10}$ ), and 510,000 (5.707  $\log_{10}$ ) HIV-1 RNA copies/mL was added to each tube. After 1 h of incubation at room temperature with intermittent vortexing, tubes were centrifuged at 11,000 g for 10 s, and the supernatant containing the released nucleic acid and calibrators was transferred to a fresh tube. Silicon dioxide particles, provided as part of the kit, were added as a solid support system to isolate and purify the nucleic acids. After several washes, the bound nucleic acids and calibrators were eluted and subjected to NucliSens amplification. The amplified RNA transcript was then detected by electrochemiluminescence. The results were calculated by comparing each sample with the internal calibrators and expressed as copies of HIV-1 RNA per milliliter equiv-

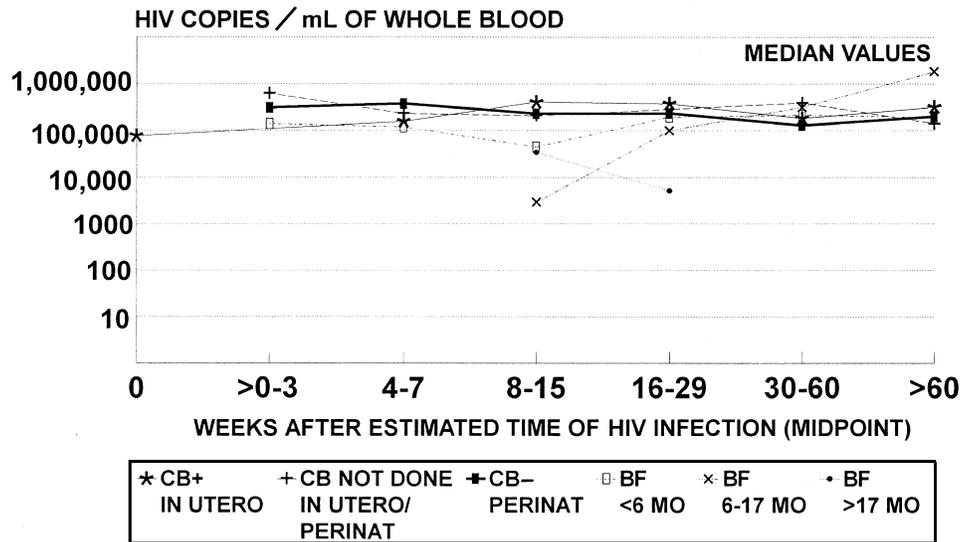
alent of liquid blood, uncorrected for hematocrit. This assay can detect a 4- $\log_{10}$  variation in viral RNA copies and has a threshold sensitivity of 80 (1.903  $\log_{10}$ ) HIV-1 RNA copies per input volume. For the 25- $\mu$ L volume of blood used in this study, the lower limit of detection was 3200 (3.505  $\log_{10}$ ) HIV-1 RNA copies/mL; for a few spots, only a limited amount of sample remained, and the sample size was 15  $\mu$ L (lower limit of detection: 5333 copies/mL [3.727  $\log_{10}$ ]). The observed mean geometric titer and SD of the three internal calibrators used in each assay were 3.653  $\pm$  0.02  $\log_{10}$ , 4.544  $\pm$  0.21  $\log_{10}$ , and 5.708  $\pm$  0.37  $\log_{10}$ . The specificity of the DNA method has been assessed in both adult and pediatric populations with HIV clade C, the subtype found in Malawi [11], and shown to be >98.9% in this and other studies [14–16].

**Analysis.** For comparative analysis of test sensitivity on the same sample, HIV-1 DNA PCR qualitative positive and negative results were compared with RNA results and tested quantitatively but analyzed qualitatively as negative when the result was less than the limit of detection.  $\log_{10}$  transformations of the RNA copy number was used because the virus levels were not normally distributed. Data are presented as geometric mean titers. Statistical comparisons between groups were done by a general linear model on the  $\log_{10}$  transformed values. Rank order significance values were evaluated by paired *t* tests. Virus level associations with other variables were examined by Spearman's correlations because some variables were not well log transformed into a normal distribution.

Results were analyzed by pooling samples of infants infected by the same exposure route according to their time of first PCR positivity. These groups are shown in the figures, with the median age at infection (in weeks) given in figure 1 and range of weeks considered in each group given in figure 2. Table 1 also provides the median age at and after infection for each group. Infants who were umbilical CB positive were assumed to have been infected in utero,



**Figure 1.** Human immunodeficiency virus (HIV) type 1 levels of initially positive samples in infants, sorted by route of exposure and time of infection. Age at infection is set as birth for cord blood (CB)-positive (+) infants. Age at first positive sample in weeks (median, in weeks) was established by using midpoint between last negative (-) and first positive infants. Infants without CB results available were considered CB negative. BF, breast-feeding; mo, months; perinat, perinatally; wks, weeks.



**Figure 2.** Median human immunodeficiency virus (HIV) type 1 levels over time by subgroup of infants sorted by route of exposure (CB, cord blood; perinat, perinatally; BF, breast-feeding) and months (mo) after infection.

those who were CB negative but positive on the first postnatal sample were assumed to have perinatal infections, and infants with a documented postnatal negative sample who became infected later were assumed to have been infected by breast-feeding. To address the influence of age, we further divided infants infected by breast-feeding into age groups on the basis of the time at which infection occurred as estimated by the midpoint between last negative and first positive results: <6, 6–17, and >17 months.

We also grouped data by time from the estimated infection date to evaluate the profile of virus level over time. Time of infection was set at birth for CB-positive infants, although we acknowledge that the actual time of infection was in utero and therefore unknown for most CB-positive infants. For CB-negative infants and infants for whom no CB result was available, the midpoint between the last negative and first positive sample was used. Since virus levels at conversion could have been influenced by the primary viremia, we also assessed whether the route or time of infection affected virus levels 1 year after the estimated date of infection, a time by which virus levels should have stabilized.

## Results

We measured virus levels in 583 samples from 135 infants (mean, 4.3 samples per subject). Quantitatively, PCR and NASBA HIV results agreed in 294 positive and 117 negative samples and disagreed in 11. Thus, the qualitative agreement between assays was 97.2%. Among discordant results, 5 were positive only by NASBA (4 at very low levels); for these we used the virus level determined by NASBA. Six were positive only by PCR. We assigned virus levels for these samples as half the minimum threshold of NASBA.

HIV-1 levels grouped by exposure route and age at infection are shown in table 1. Figure 1 displays the median and inter-

quartile ranges in initial positive samples. The median level in 24 CB-positive samples was 78,000 copies/mL. The CB-positive virus levels were significantly lower than levels in the first positive samples of CB-negative perinatally infected infants (median, 355,000;  $P < .0001$ ) obtained on average 8 weeks after birth. However, levels in first postnatal samples of CB-positive infants, also obtained on average at age 8 weeks, were similar to those of perinatally infected infants because levels in 3 CB-positive infants who had barely detectable levels in CB samples rose to high levels by their first postnatal visit, including 1 infant whose CB sample was positive only by PCR. One CB-positive infant had low but persistently positive virus levels at all visits.

First positive levels in infants infected by breast-feeding at ages 0–5 months were lower (median, 135,000 copies/mL) than in perinatally infected infants ( $P = .047$ ). Among 43 infants infected by breast-feeding, virus levels in first positive samples did not vary significantly ( $r = -.14$ ,  $P = .384$ ) by the age the child became infected (median, 29 weeks; range, 4–88). Levels in the visit after the initial positive findings were similar to levels in other groups.

Once infection was established, HIV levels remained fairly constant in all groups (figure 2). At least 1 sample was available from 87 infants 1 year after infection. By comparing geometric mean titers in the first positive result, including CB-positive results, to the first sample available 1 year after infection (193,000 and 212,000 copies/mL, respectively), levels in paired samples remained stable (paired  $t$  test,  $P = .66$ ). Within subgroups, virus levels in the first sample available 1 year after infection were fairly similar (figure 3). In these data, obtained from samples spaced weeks to months apart, we saw no general pattern of a peaking viremia following infection.

**Table 1.** Geometric mean titer and median human immunodeficiency virus type 1 (HIV-1) levels in first positive samples and after 1 year of infection in infants in Malawi.

Infection source, sample	<i>n</i>	Estimated age at infection <sup>a</sup>	Age at sampling	Infection duration	Geometric mean titer (copies/mL)	Median virus level (copies/mL)
CB+, in utero						
1st positive	24	In utero	0	?	54,000	78,000
1 year <sup>b</sup>	21		66	?	331,000	320,000
In utero/perinatal <sup>c</sup>						
1st positive	16	3	6	3	385,000	514,000
1 year	12		65	63	240,000	205,000
CB-, perinatal						
1st positive	52	4	8	4	244,000	355,000
1 year	48		65	61	163,000	214,000
Breast-feeding, 0-5 months						
1st positive	20	12	15	3	93,000	135,000
1 year	6		65	57	158,000	227,000
Breast-feeding, 6-17 months						
1st positive	18	45	66	21	101,000	133,000
1 year	1		95	62	1,800,000	
Breast-feeding, ≥18 months						
1st positive	5	78	91	13	25,000	9000
1 year	0					

NOTE. Age estimates are median values (week). CB, cord blood; +, positive; -, negative.

<sup>a</sup> Estimated by age at midpoint between last negative and 1st positive HIV result.

<sup>b</sup> One year after the estimated date of infection.

<sup>c</sup> CB not analyzed.

Initial virus levels predicted levels 1 year after infection among all 87 subjects ( $P < .005$ ) and in the 3 largest subgroups considered separately: CB-positive ( $n = 21$ ;  $r = .43$ ,  $P = .049$ ); CB not done but first sample positive ( $n = 11$ ;  $r = .56$ ,  $P = .071$ ); CB-negative perinatally infected ( $n = 48$ ;  $r = .38$ ,  $P = .008$ ). Only 7 infants infected by breast-feeding had samples available 1 year after the estimated infection date.

There was no correlation between virus level and the infant's sex, birth weight, or age at infection. Higher initial loads were seen in infants of mothers who were younger ( $P = .006$ ), had fewer pregnancies ( $P = .016$ ), and reported being ill or were known to have died within 2 years after delivery ( $P = .06$ ). These effects were generally dominated by their effects in CB-negative perinatally infected infants, the largest group. In CB-positive and breast-feeding-infected infants, we found no significant correlation between virus levels and any transmission variables. However, infants with higher virus levels tended to have more health problems ( $P = .06$ ). These health problems were often poorly defined (diarrhea, poor weight gain, unexplained death) and not clearly attributed to AIDS or AIDS-related conditions.

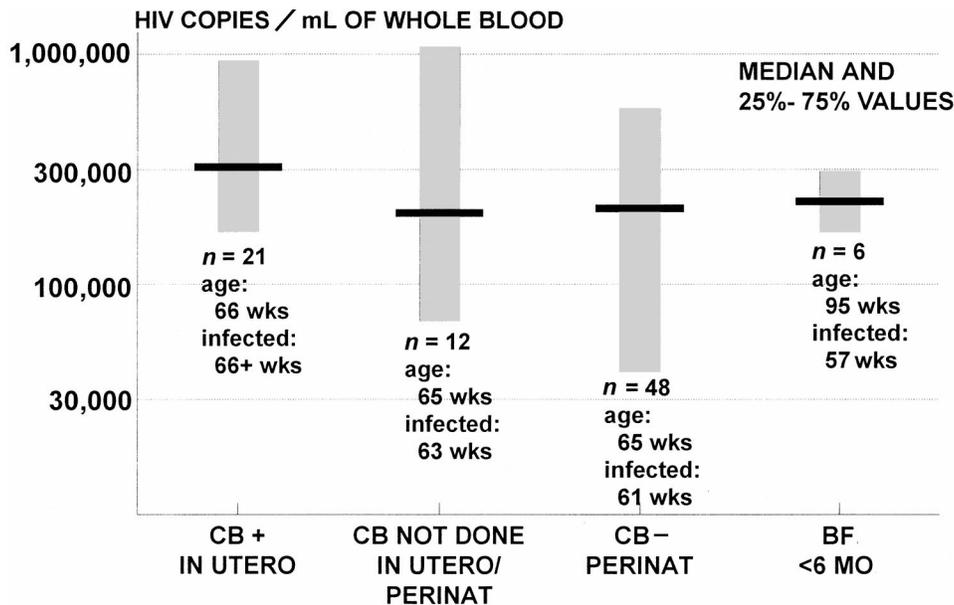
**Discussion**

PCR has become the standard approach for diagnosis of HIV infection in infancy. Typically, non-breast-fed infants, who must have been infected before or after birth, are positive in CB or become positive within 14 days (data summarized by Dunn et al. [17]). Those results are based on qualitative assays. Virus level data are available for only small numbers of infants and none from Africa. This study was possible because we used

newly developed methods to quantify HIV-1 levels from dried blood spots [13]. Another group who recently used a similar technique to detect HIV levels reported that levels in spiked dried blood spots were similar to levels in whole blood samples [18]. Quantitation in dried blood spots is a significant breakthrough in applying technology to field work. This technique will be especially valuable for use in the less developed areas of the world, where both technical support and sample storage capacity are limited. However, this study shows that even in the industrialized world, collection of blood samples on filter papers may be useful.

We compared results from qualitative DNA PCR results in our initial studies with RNA virus levels. We found few discrepant results, and the performance of each test appeared equivalent. In earlier studies, one report [19] suggested that RNA was more sensitive than DNA PCR in this setting, but another [20] concluded that the two approaches were equivalent. Virus levels in infants are very high, and therefore the finding that the two assays gave similar results may not apply to groups other than infants.

The initially positive virus levels in CB-positive samples (median, 78,000 copies/mL) were lower than those in CB-negative perinatally infected infants, although still quite high. We previously speculated that PCR-positive CB may represent detection of maternal cells transfused into the placenta at birth [21]. However, the median levels in the CB-positive samples were too high to represent contamination by trace amounts of maternal blood on the umbilical cord or even maternal blood transfused at delivery. We did not have maternal blood against which to compare CB virus levels directly; however, in a study of 94 Ugandan women who transmitted HIV to their infants,



**Figure 3.** Human immunodeficiency virus (HIV) type 1 levels in infants 1 year after estimated date of infection, when HIV levels should have plateaued. CB, cord blood; BF, breast-feeding; +, positive; -, negative; mo, months; perinat, perinatally; wks, weeks.

the virus levels measured by Roche Amplicor Monitor were  $3419 + 7489$  copies/mL [22]. Maternal-infant microtransfusions could have been only minute amounts, and any virus transfused with maternal blood would have been diluted further by the much larger volume of infant blood. Thus, virus levels in infants infected at birth and detected in CB would have been very low even if the maternal levels were well above the average. However, the virus levels that we observed in CB-positive samples were far higher than were likely to be present in their mothers [22–24]. Therefore, this study provides compelling evidence that most CB-positive infants are infected in utero. This finding does not exclude the possibility that CB-negative perinatally infected infants may be infected by transfused maternal blood. Virus levels were barely detectable in 4 CB-positive infants. For unknown reasons, 1 infant was persistently positive at a very low level. When we excluded this infant, who perhaps maintained a low virus level because of genetic reasons [25], the 3 remaining infants could have been infected by maternal blood transfused at delivery. If this explanation is correct,  $\geq 20$  (87%) of 23 CB-positive infants in this study represented in utero infections. The fraction of CB-negative perinatally infected infants who were infected at delivery versus by breast-feeding in the first days of life remains unclear.

The high sustained virus levels in grouped subjects were consistent with other reports [7–9]. We did not observe a peak period of viremia, perhaps because we did not collect samples early enough after delivery or frequently enough in infants infected later. If infants with high virus levels died or were selectively lost to follow-up after acute infection, these findings might have been biased, but we are not aware of studies reporting such deaths or follow-up losses following primary HIV

infection during infancy. Virus levels in first positive samples might have been more variable than at later time points because they would be influenced by when, relative to the primary viremia, we obtained the sample. We note that the precision concerning the exact age at infection decreased as the interval between visits increased during the course of the study. However, by 1 year after infection, the virus levels should have stabilized. In the 1-year samples, there were few between-group differences, regardless of the route of or age at infection.

We also found that initial positive virus levels predicted levels in paired samples obtained 1 year later. Thus, in infants, low initial virus levels tended to remain low, suggesting that some host-specific aspect may control virus expression. We examined the infant's sex, birth weight (as a measure of maturity), and age at infection, without finding significant associations between initial positive levels or levels 1 year after infection. We observed a weak correlation between infants of younger mothers and higher virus levels. In this study, younger mothers and more symptomatic women were also more likely to transmit HIV [3, 21]. Virus levels were also higher among infants born to mothers with symptoms or death within 2 years after delivery. Possibly these subsets of mothers had higher average titers because they were recently infected or had more advanced disease and therefore transmitted higher levels of HIV to their infants. However, there are other possible explanations. Perhaps the virus strains in these women were more virulent. Alternatively, newly infected women and women with advanced infections may convey less effective passive immunity to their infants and the infections in the infants therefore may escape initial control.

In summary, most CB-positive infants are infected in utero and have high HIV levels that are similar to plateau levels in

other infants. Infants detected as weakly positive in CB appear to be very recently infected, perhaps by microtransfusion during labor, and rise to plateau levels rapidly. Our data showed no peak of viremia immediately following infection, but we may have missed a viral spike if it occurred during the first month of life. Initial virus levels in these infants were often high, and high early levels predicted high levels of virus 1 year after infection. Neither the route of infection (in utero, perinatal, or breast-feeding in the first year) or age at infection significantly influenced the plateau virus levels 1 year after infection. Thus, control of viral replication may depend on inherent virus-host interactions.

Antiretroviral therapy during the early postnatal period has been reported to reduce transmission risk [26]. In adults, lower levels during primary viremia are associated with lower plateau levels [27, 28]. We found that early lower HIV levels predict later lower levels in infants and, furthermore, that lower levels are associated with fewer health problems. It is possible that postnatal antiretroviral therapy could reduce levels of primary viremia and reset the plateau levels, thereby benefiting infants even if it did not prevent infection.

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