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Elucidation of the time course of adenosine deaminase *APOBEC3G* and viral infectivity factor *vif* in HIV-2₂₈₇-infected infant macaques

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Abstract

Background—Although the interactions of cellular cytidine deaminase A3G and viral infection factor (*vif*) of human immunodeficiency virus (HIV) were reported, regulation of A3G after *in vivo* HIV infection and disease progression is not known.

Methods—Time courses of plasma virus, CD4⁺ T lymphocyte Macaca levels, and concentrations of *A3G* and *vif* transcripts were determined in infant macaques infected with HIV-2₂₈₇. These *in vivo* results were compared with those collected *in vitro* in HIV-2-infected T cells.

Results—Human immunodeficiency virus-infected macaques exhibited plasma viremia (10^8 copies/ml) followed by a precipitous CD4⁺ T-cell (from 40–70 to 5%) decline. An initial increase in *A3G* transcripts coincides with early increases in virus and *vif* RNA. As virus load continues to increase, *A3G* RNA decreases but recovers at a later phase as virus level stabilizes. Pearson correlation analysis revealed strong interactions of A3G–CD4, *vif*–CD4, and A3G–*vif*.

Conclusions—There is a time-dependent *A3G* and *vif* RNA interaction throughout the course of HIV infection.

Keywords

A3G; disease progression; human immunodeficiency virus

Introduction

The clinical course and outcome of human immunodeficiency virus (HIV) infection are highly variable among individuals. Typically, individuals (including infants) infected with HIV-1 develop acquired immunodeficiency syndrome (AIDS) within 6–8 years [13]. While a small percentage progress to AIDS within the first 2–3 years [1], approximately 1–5% remain relatively healthy for more than 10 years [2, 14, 21]. Although advances in highly active antiretroviral therapies (HAART) have delayed the time from HIV-1 infection to AIDS from about 6–9 years or more years [23], the cure for AIDS remains elusive.

Cumulative data indicate that the virus rebounds immediately after cessation of HAART or upon development of drug resistance. As 27 of 30 currently marketed FDA-approved anti-HIV drugs are inhibitors targeting two viral enzymes (reverse transcriptase and protease), the high rate of viral replication and mutation of these enzymes coupled with incomplete elimination of virus from the body readily renders chemotherapy ineffective. Recent cohort

studies show drug failure at 6 years in 11–21% of individuals infected with HIV-1 [12]. Therefore, additional therapeutic options must be sought. In addition to drug targets that are viral factors (including reverse transcriptase, integrase, protease, nef, tat, and rev), several host factors or receptors (CD4, CCRs, CXCRs, A3G, Trim5 α , and DDX3) have been proposed as drug candidates and are being evaluated clinically for anti-HIV therapy [18].

The cellular cytidine deaminase enzyme, apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (A3G), has been proposed as a novel target [4, 8, 19, 25]. A3G inhibits viral replication through conversion of cytidine to uracil in HIV nucleotide sequences, thus hypermutating the virus and rendering it replication incompetent [4, 8, 19, 25], and may further act to induce adaptive immunity [3]. However, this is counteracted by viral infectivity factor (vif), which binds to A3G protein and prevents its inclusion into virus particles by multiple mechanisms that include reducing synthesis of A3G enzymes [6, 25] or promoting degradation by the proteasome through protein ubiquitination [9, 20, 22].

Although A3G is inhibited by vif, scattered evidence suggests enhancement of A3G expression after immunization or virus challenge [16]. This possible cellular response has yet to be studied in depth, both at points early after infection and as the disease progresses. It is well known that significant changes to immune cell populations and virus levels occur soon after infection. Therefore, we sought to better define the role of A3G as it relates to HIV disease progression, so that potential use of A3G as a drug target for inhibiting HIV replication can be addressed. We have evaluated the time course profiles of *A3G* RNA, *vif* RNA, and HIV-2 RNA in HIV-2₂₈₇-infected *Macaca nemestrina*, and A3G RNA, protein, and *vif* RNA in HIV-2₂₈₇-infected CEMx174 cells. Our data indicate that changes in cellular HIV-2₂₈₇ relate to changes in *A3G* and *vif* RNA and to other markers of HIV disease progression.

Methods

Virus and macaque inoculation

Pathogenic HIV-2₂₈₇ was isolated originally from the lymph nodes of a macaque with the clinical manifestations of AIDS. The HIV-2₂₈₇ inoculum was prepared by diluting the stock (10^8 tissue culture infectious dose-50, TCID₅₀) in complete tissue culture medium, and three infant macaques (*M. nemestrina*) were administered 10^3 TCID₅₀ HIV-2₂₈₇ as described previously [5].

Quantification of HIV, *vif*, and A3G RNA concentrations

Total RNA was isolated from plasma and PBMC using a Purescript RNA Isolation kit (Gentra Systems, Minneapolis, MN, USA). HIV-2, *A3G*, and *vif* RNA were measured using a real-time quantitative PCR. Briefly, cellular and plasma RNA samples were analyzed in triplicate with 1 reverse transcriptase-free reaction as a DNA control; 250–500 ng per well of *A3G*, *vif*, and HIV-2 RNA was used from the PBMC preparations. RNA was first reverse transcribed at 25°C for 15 minutes, 42°C for 40 minutes, 85°C for 10 minutes, 5°C hold (ABI 7900; AME Bioscience, Foster City, CA, USA). TaqMan PCR (ABI 7900; AME Bioscience) was then performed at 95°C for 10 minutes, 45 cycles of 95°C for 15 s, and then 60°C (*A3G* and *vif*) or 55°C (HIV-2) for 1 minutes. The designs for specific primers and fluorescent probes for HIV-2 gag RNA and *vif* RNA were based on HIV-2_{EHO} sequences: HIV-2_{EHO} (GenBank no. U27200) primers F'1493-TTATTCCCACCTGCCGCTAA-1512, R'1569-CTGCCCCGAACCTTCTTCTCTT-1549; probe P'1515-CCCCGAACCTTAAATGCCTGGG-1537. HIV-2 *vif*, Primers F'5534-AGGATGGGCTTGGTGGACTT-5553, R'5662-

TTAGTCTTACAGCATAGGAAGTCAAGAATC-5632 Probe P'5581-AAGGAGCACATCTAGAAGT-5599.

The design of specific primers and fluorescent probes for *A3G* was based on human (GenBank no. NM_021822) and Rhesus monkey *A3G* sequences. These primers were confirmed by BLAST sequence alignment to be specific for *A3G* with no homology to APOBEC3F. Primers F'920-ACACCTGGGTCCTGCTGAAC-939 R'1035-AAAGGGAATCACGTCCAGGAA-1115. Probe P'999-CCATGCAGAGCTGTG-1113.

HIV-2 and *A3G* RNA copy numbers in the plasma and cell preparations were determined by generating standard curves using serial dilutions of RNA transcribed from plasmids containing the desired gene.

Lymphocyte subset analysis

Fluorescent-labeled monoclonal antibodies to lymphocyte surface markers were used to quantify populations of helper T cells (CD4⁺), suppressor T cells (CD8⁺), and B cells (CD20⁺) in blood using procedures previously described [5].

Statistical analysis

Linear correlations were assessed by Pearson correlations and significance assessed by Student's *t*-test at $P < 0.05$.

In vitro cell infection

The human T cell line, CEMx174, was grown in RPMI (Invitrogen, Carlsbad, CA, USA) with 10% FBS (Invitrogen) at 37°C. Cells were infected with 0.1 or 1 multiplicity of infection (MOI) of HIV-2₂₈₇ in 2 ml and incubated for 30 minutes at 37°C. Cells were then pelleted, resuspended in virus-free media, and plated in duplicate into six well plates, 3 million cells per well corresponding to each day of sampling that would be performed post-infection. RNA samples were isolated and analyzed as described earlier.

Immunoblot analysis for A3G protein expression

Cells were lysed in Nonidet P-40, and total protein was quantified (Bio-Rad DC Protein Assay; Hercules, CA, USA). Twenty micrograms of total protein was loaded into a Biodot apparatus (Bio-Rad) containing a nitrocellulose membrane that had been pre-incubated with methanol for 5 minutes, deionized water for 5 minutes, and Tris-buffered saline (TBS) buffer for 5 minutes at room temperature. A partial vacuum was applied to transfer protein to the membrane. Membrane blocking was in 5% milk diluted in TBS with 1% Tween overnight at room temperature. A3G primary polyclonal antibody (ab54257; Abcam, Cambridge, MA, USA) was diluted to 1:1000 in 5 ml of blocking buffer and incubated for 2 hours rocking at room temperature. Membrane was washed three times in TBST for 5 minutes each. Goat anti-Rabbit secondary antibody (Affinipure peroxidase-conjugated Goat anti-Rabbit IgG; Jackson ImmunoResearch, West Grove, PA, USA) was diluted to 1:2000 in 5 ml of blocking buffer and incubated for 1 hour rocking at room temperature. Membrane was washed three times in TBST for 5 minutes each. Membrane was developed with ECL-Plus Reagent (BD Bioscience, San Diego, CA, USA) as per manufacturer instructions. Densitometry analysis was performed with AxioVision software (Zeiss, Thornwood, NY, USA).

Results

Infection and disease progression in macaques

To determine the time course of *A3G* in peripheral blood mononuclear cells (PBMC) of HIV-infected infant macaques, we inoculated three infants with pathogenic HIV-2₂₈₇ (10^3 TCID₅₀, IV). Under these experimental conditions, macaque infants developed a characteristic high plasma virus, which peaked at $10^{7.5}$ – 10^8 viral copies/ml at day 7 and declined to a set point of 10^5 – 10^6 viral copies/ml by day 60 (Fig. 1A). This viremia event is followed by a precipitous CD4⁺ decline observed with all three animals, with minor exceptions for infant M03001 (Fig. 1B). This infant exhibited an inconsistent, uncharacteristic early decline and fluctuation in plasma viral RNA that was verified. These animals also showed abnormality in behavioral development similar to those previously reported [5].

Time course of *A3G* and *vif* transcripts in HIV-2-infected macaques

The time courses of *A3G* and *vif* transcripts in these animals were evaluated to examine time-dependent changes following infection. As shown in Fig. 2, a dynamic relationship between *A3G* and *vif* in mononuclear cells of HIV-infected macaques was apparent. After HIV infection, all infants exhibited a rapid and prominent increase in *A3G* RNA transcripts to 1.4 – 2.4×10^5 copies/ μ g RNA, followed by a rapid decline around day 38–42 to $\sim 2.5 \times 10^4$ copies/ μ g RNA, a value lower than that at time zero. Subsequently, an equally rapid rebound to starting levels was seen, which remained constant for ~ 60 days. Finally, *A3G* began to decline just prior to termination of the experiment (day 154; Fig. 2A–C). Analysis of the mean profile (Fig. 2D) revealed a time-dependent inverse trend of *vif* to *A3G* levels in HIV infection. Because of infant sampling limitations (including number of blood draws and collectable volumes), *A3G* levels at time zero were not available. However, cellular *A3G* levels in 3 age-matched controls ranged from 3.6 to 13.1×10^4 copies/ μ g RNA.

Pearson correlation analysis was performed to determine whether the apparent relationship between *A3G* and *vif* could be explained by accounting for relative amounts of cells in the sample population (Table 1, Panels A–C). For *A3G-vif* as well as *A3G-CD4* interactions, we analyzed correlation both early (day 0–49) and overall (day 0–156) phases (Table 1, Panel A) to capture any correlations during the acute phase of infection. These analyses of the data revealed strong and significant correlations between intracellular *vif* and both plasma and intracellular HIV-2 RNA copy numbers (Table 1, Panel B). There were moderately strong and significant correlations between intracellular *vif* and CD4⁺-T-cell percentage (Table 1, Panel C), intracellular *A3G* and CD4⁺-T-cell percentage up to day 42 (Table 1, Panel A). There were no significant linear correlations observed between intracellular *A3G* and intracellular *vif* (Table 1, Panel A), CD4⁺-T-cell percentage (Table 1, Panel A), CD8⁺-T-cell percentage (Table 1, Panel C), or CD20⁺-B-cell percentage (Table 1, Panel C) for any period during the time course after HIV infection.

Time course of *A3G* and *vif* in HIV-2-infected CEMx174 cells

To assess the magnitude of the interaction between *A3G* and *vif* in a system free from potentially confounding cellular and environmental factors *in vivo*, we performed three sets of time course analyses of *A3G* and *vif* in both HIV-2₂₈₇-infected and uninfected CEMx174 cells at 0.1 and 1 MOI. These cells were readily infected by HIV-2₂₈₇ and showed high viral *vif* copy numbers similar to levels seen *in vivo* for both experiments peaking at day 6 at 5.3 – 6.4×10^7 RNA copies/ml (Fig. 3A). Ratios of intracellular *A3G* RNA were taken to normalize for cell growth and health differences between infected and uninfected cells. Immediately after infection, *A3G* levels in both levels of infected cells increased 1.6 times above the uninfected cells (Fig. 3B). At day 3, differential expression depending on the

infection level of the cells was seen. Interestingly, cells infected by 0.1 MOI HIV-2 had higher *A3G* expression at day 3, of three times that of uninfected cells; however, cells infected at 1 MOI, although 2.3 times higher than uninfected cells, were lower than the cells infected at 0.1 MOI. After day 3 and continuing to the end of the experiment at day 7, *A3G* RNA declined from the peak, and 0.1 MOI infected cells maintained an *A3G* expression level higher than the uninfected cells. Conversely, the 1 MOI infected cells declined rapidly after day 3, and by day 7, *A3G* expression was less than half of uninfected cell expression.

Time course of A3G Protein in HIV-2-infected cells

To determine whether the *A3G* RNA changes reflected expressions of A3G protein concentrations, we determined changes in A3G protein levels with Western immunoblot analysis. The A3G antibody used for this assay was specific in that only a single band at 46 kDa was detectable for cellular A3G within total cell lysates (Fig. 4). Quantitative densitometry analysis shows no significant differences in A3G level in uninfected host cells (Table 2). After infection at 0.1 MOI HIV-2₂₈₇, A3G protein levels are not significantly different from the uninfected control cells until day 7, where A3G protein levels are significantly lower. In contrast, after infection at 1 MOI HIV-2₂₈₇, the A3G protein level decreases far more rapidly than at the cells interacting with lower HIV-2₂₈₇ infection level. The A3G protein in HIV-infected cells was significantly lower than that in uninfected control at day 1, 5, 6, and 7. No protein was detectable on either day 6 or day 7. These data indicate that the rate of A3G protein decreases and rebound is reflective of HIV-2 infection level, which is also the case in the RNA level. Collectively, these data demonstrate that after viral infection, intracellular *A3G* RNA in both *in vitro* and *in vivo* systems changes dynamically over time in a manner inverse to plasma HIV, intracellular HIV, and *vif* RNAs that are unexplained simply by changes in lymphocyte subset populations.

Discussion

Because of *A3G* deamination of cytidine to uracil on viral sequences that inactivate HIV and *vif* counteraction of this process, the *vif*-*A3G* interaction has received significant interest as a potential HIV drug target. We have demonstrated for the first time that dynamic changes occur over time after infection in primates, especially during the first month, in the RNA transcripts of both *A3G* and *vif* in mononuclear cells. This is characterized by the initial decline in cellular *A3G* and the subsequent rebound to initial levels with an apparent inverse relationship to *vif* time course. After infection with HIV *in vivo*, *A3G* expression is induced from day 7. However, the rapid build-up of high levels of virus and *vif* combined with a rapid depletion of CD4⁺ T cells may cause suppression and an overall reduction of *A3G* transcripts (day 7–40). As virus and *vif* concentrations decline soon after the peak infection, A3G inhibition may also decrease, allowing normal transcription to resume, thus achieving a dynamic steady state in the later stage (~day 140; Fig. 2).

The correlation analysis indicates that the cycling of *A3G* over the time course of infection is not merely owing to time-dependent fluctuations in cell populations (Table 1). Had the overall fluctuations in *A3G* been caused by changes in the CD4⁺ and CD8⁺ T cells, and CD20⁺ B cells distribution, correlations between these and *A3G* would have been apparent (Table 1). However, we observed a significant correlation between *A3G* and CD4⁺ T cell percent from day 0–42 (Table 1, Panel A). This is most likely a function of the precipitous decline in CD4⁺ T-cell numbers early in the infection. It is interesting to note, however, that the resulting rebound and cycling in *A3G* levels is not correlated to the proliferation of CD20⁺ B cells nor CD8⁺ T cells (Table 1, Panel C). The significant correlation between *vif* and CD4⁺ positive T-cell percent is reasonable, as HIV preferentially infects these cells. Whether intracellular *A3G* levels in CD20, CD8, or other lymphocyte subsets are different from that of CD4 and whether HIV *vif* generated in CD4 cells may influence *A3G* in other

cells is not known. Nevertheless, our results suggest a more complex interaction is likely occurring between A3G and vif than that which can be explained by simple linear correlations and protein/protein interactions only.

To explore this interaction in the absence of potentially confounding factors inherent in a complex *in vivo* system, we examined the time courses of *A3G*, *vif*, and HIV-2 RNA and A3G protein CEMx174 cells variably infected with HIV. By variably infecting cells with HIV-2, we were able to determine what role intensity of infection plays in the cellular response. It has been previously shown that vaccination can induce A3G expression [16]. We have shown that after infection with HIV, *A3G* mRNA expression is enhanced in the infected cells up to three times after 0.1 MOI infection and 2.3 times after 1 MOI infection (Fig. 3). As the infection progresses (after day 3), *A3G* transcription declines in comparison with the initial induction. In 0.1 MOI infection, transcription remains high relative to the control cells; however, in the 1 MOI infection, although *A3G* RNA is induced, this occurs at a lower level and declines faster than the lower infected cells. *A3G* RNA is reduced below that of the uninfected cells by day 5 and is less than half of uninfected cell RNA by the end of the experiment at day 7. Despite significant RNA induction, this is significantly attenuated in protein expression. No A3G protein induction is apparent; in contrast, protein content declines relative to the initial infection level (Table 2). The *vif* RNA transcripts initially increase rapidly and maintain high levels for the duration of the experiments, which reflects the pattern seen in the early part of the *in vivo* time course data. It is possible that in addition to the role of Vif protein in enhancing A3G protein degradation, it may also act directly on *A3G* RNA, which has been suggested by others [10]. If this is determined to be the case, it could provide a potential explanation for observed *A3G* RNA fluctuations *in vivo*.

It is now well established that Vif protein promotes the proteosomal degradation of A3G through modulation of ubiquitination [9, 20, 22]. Unfortunately, there are no antibodies or purified macaque A3G or HIV-2 Vif proteins available to probe these interactions in detail in our primate HIV model. Whether the A3G/Vif interaction can be successfully modulated to enhance and maintain *A3G* levels and/or inhibit Vif to disrupt HIV replication has yet to be conclusively determined. Work is currently underway to screen compounds that shift the advantage from Vif back to A3G. Some compounds being evaluated include proteasome inhibitors such as peptide aldehydes, lactacystin, or epoxomicin, which may be able to preserve A3G long enough and to an extent sufficient to influence cellular virus level [7, 17, 20, 22, 24, 26]. The use of deaminase inhibitors such as tetrahydrouridine [11] could occupy the binding site on A3G where Vif interacts. Although this binding may reduce the innate activity of A3G in normal cells, temporary inhibition in the formation of Vif–A3G complex could reduce proteosomal degradation of A3G to a sufficient extent that it could still act to reduce peak plasma virus load and thus may eventually slow the rate of disease progression. Additionally, the development of an inhibitor of Vif may allow A3G to access and act upon the virus [15]. Most likely, a combination of antiviral agents that inhibit viral replication, Vif action, and A3G enhancers will be required to prevent disease progression in HIV infection.

In summary, using an HIV-2 macaque model and supported by CEMx174 experiments, we have demonstrated that there is a time-dependent interaction between *A3G* and *vif* RNA throughout the course of HIV infection. An initial increase in *A3G* RNA coincides with early increases in viral copies and *vif* RNA; however, as virus load continues to increase, *A3G* RNA decreases initially, but recovers at a later phase as virus level stabilizes. These data encourage the further development of drugs that modulate this interaction. Clearly, the roles of A3G and Vif are important within the context of the time course of disease, and the progressive relationship between A3G and Vif invites speculation as to how it may be

shifted to the advantage of A3G to degrade viral RNA and control viral replication for a positive outcome on the HIV pathogenesis.

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Fig. 1.

Effects of human immunodeficiency virus (HIV)-2287 infection in macaques. Three infant macaques were inoculated with 103 TCID₅₀ HIV-2287 and the time course of plasma HIV-2 viral load (Copies/ml) was assessed (Panel A), CD4 and CD8 positive T cells counts, and CD20 positive B cell counts (percent of total) were detected (Panel B–D). Circles, M03001; triangles, M03003; squares, M02395. Data expressed were a mean ± SD.

Fig. 2. Time course of intracellular A3G and human immunodeficiency virus (HIV) viral infection factor (*vif*) RNA in peripheral blood mononuclear cells (PBMC) of HIV infected macaques. Open circles, *APOBEC3G* (copies/ μ g RNA); closed circles, *vif* (copies/ μ g RNA). (A) M03001, (B) M03003, (C) M02395, (D) Average of the three macaque data.

Fig. 3.

Time course of intracellular *A3G* and viral infection factor (*vif*) in an human immunodeficiency virus (HIV) infected CEMx174 cell line. (A) *vif*(copies/μg RNA). (B) Ratio of infected to uninfected CEMx174 *A3G* transcript. Closed circles: 0.1 multiplicity of infection (MOI) HIV-2 infection. Open circles: 1.0 MOI HIV-2 infection.

Fig. 4.

Concentration dependent specific binding of A3G antibody to CEMx174 cell lysates. Cells were pelleted, lysed and total protein concentration was determined. An immunoblot was performed at several total protein concentrations to determine A3G antibody specificity. Only single bands at 46 kDa were observed at all protein concentrations.

Table 1

Correlation analysis of *APOBEC3G* (A3G), human immunodeficiency virus (HIV), and *vif*RNA, and lymphocyte subsets in HIV-infected macaques

Animal ID	<u>A3G–<i>vif</i> (days)</u>		<u>A3G–CD4% (days)</u>	
	0–42	0–156	0–42	0–156
(A)				
M03001	0.19	–0.33	0.69	–0.42
M03003	0.94 ^I	0.19	0.96 ^I	0.15
M02395	0.52	0.46	0.85 ^I	0.59
Average	0.55	0.11	0.83 ^I	0.11

<u><i>Vif</i>–HIV-2</u>		
	Plasma	Cell
(B)		
M03001	0.95 ^{I,2}	0.58 ^I
M03003	0.93 ^I	1.00 ^I
M02395	0.98 ^I	1.00 ^I
Average	0.95 ^I	0.86 ^I

	<u><i>Vif</i>–CD4</u>	<u>A3G–CD8</u>	<u>A3G–CD20</u>
(C)			
M03001	0.69 ^I	–0.1	0.25
M03003	0.97 ^I	0.02	0.75 ^I
M02395	0.61 ^I	–0.63 ^I	–0.19
Average	0.76 ^I	–0.24	0.27

vif, viral infection factor.

Linear correlations were assessed by Pearson correlations and significance assessed by Student's *t*-test for listed pairs and time frames. A3G was divided at 0–42 days and 0–156 days to attempt to capture any potential correlation at the early phase of infection during the rapid CD4 decline.

^ICorrelations significant at $P < 0.05$.

²Correlation was 0.03 when two uncharacteristically low HIV-2 plasma load values were included; however, removal of the points gives the correlation presented.

Time course of intracellular A3G protein in human immunodeficiency virus (HIV)-infected CEMx174 cells. Cells were infected with 0, 0.1, or 1.0 MOI of HIV-2₈₇ and sampled on days 0, 1, 3, 5, 6, and 7. Immunoblots were performed to determine protein content, and signal was quantified by densitometry

Table 2

Multiplicity of infection	Day after infection						
	0	1	3	5	6	7	
0	74.2 ± 6.0	69.4 ± 7.9	55.5 ± 10.4	67.3 ± 7.9	67.4 ± 10.7	67.4 ± 12.3	
0.1	66.5 ± 10.9	70.5 ± 6.6	65.2 ± 13.6	55.9 ± 7.2	70.2 ± 7.8	28.7 ± 4.5 [/]	
1	71.2 ± 8.7	48 ± 8.4 [/]	59.1 ± 11.7	29.2 ± 3.2 [/]	1.0 ± 0.1 [/]	0.9 ± 0.1 [/]	

MOI, multiplicity of infection.

[/] Significantly different from uninfected controlat corresponding day ($P < 0.05$).