Antibody-dependent enhancement representing *in vitro* infective progeny virus titer correlates with the viremia level in dengue patients

Running title: Relationship between ADE and viremia in DENV infection

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ABSTRACT (222 words)

Dengue virus (DENV) distributes throughout tropical and subtropical countries and causes dengue fever (DF) and dengue hemorrhagic fever in humans. Some DF patients suddenly develop severe symptoms after the defervescent period. Although the pathogenic mechanism of the severe symptoms has not been fully elucidated, the viremia level in the early phase has been shown to correlate with the disease severity. One of the hypotheses is that a phenomenon called antibody-dependent enhancement (ADE) of infection leads to a high level of viremia. To examine the plausibility of this hypothesis, we examined the relationship between *in vitro* ADE activity and *in vivo* viral load quantity in six patients with dengue diseases. An autologous DENV strain was isolated from each of the six patients. Blood samples were then collected at multiple time points between the acute and defervescent phases, and the balance between neutralizing and enhancing activities against the autologous and prototype viruses was examined. As the antibody levels against DENV were rapidly increased, ADE activity was decreased over time or partially maintained against some viruses at low serum dilution. In addition, positive correlations were observed between ADE activity representing *in vitro* progeny virus production and viremia levels in patient plasma samples. Therefore, the measurement of ADE activity in dengue-seropositive samples

48 may help to predict the impact of viral load in the subsequent DENV infection.

49

50 **IMPORTANCE** (149 words)

51 It has not been fully elucidated how the phenomenon of antibody-dependent

52 enhancement (ADE) affects the pathogenesis of severe dengue diseases, although high

53 viremia levels have been epidemiologically demonstrated to be associated with the

54 disease severity. Here, we show that ADE in the acute-phase patient sera exhibited

55 significantly different activities against autologous and lab strains than ADE in the

56 defervescent-phase sera. Further, the enhancement of progeny virus production activity,

57 which is one of the factors to evaluate ADE *in vitro*, was significantly correlated with

58 the levels of viral load in the patient blood circulation. This suggests that measurement

59 of the *in vitro* enhancing progeny virus titers might be used to predict the impact of *in*

60 *vivo* DENV viremia level. Our present findings could contribute to a method to forecast

61 disease severity for seropositive populations who would be at risk of developing severe

62 disease in the event of heterotypic DENV infection.

63

64 **Keywords**

65 Dengue, Antibody dependent enhancement, Viremia, Pathogenesis, Deterioration

66

INTRODUCTION

Dengue virus (DENV), belonging to the family *Flaviviridae*, genus *Flavivirus*, is distributed throughout tropical and subtropical areas of the world (1). DENV is transmitted by *Aedes* mosquito species and causes dengue fever (DF) and severe dengue in humans. Approximately 3.9 billion people are under the risk of infection (2). An estimated 390 million people are infected with DENV annually, and 100 million of these individuals show clinical symptoms (3). Therefore, dengue is one of the most important mosquito-borne viral diseases worldwide, and it should be controlled to the greatest extent possible.

The four serotypes of DENV (DENV-1, DENV-2, DENV-3 and DENV-4) are genetically distinct, and there is complicated immunological cross-reactivity among them (4). Secondary heterotypic infection has been epidemiologically demonstrated to give rise to the severe forms—namely, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (5). Patients showing dengue with warning signs have a risk of developing disease severity, with the emergence of severity usually occurring around the defervescence phase, beginning at days 3-7 of illness (6, 7). The mortality rate of cases with DSS is much higher than that of cases without DSS (8). Although a mechanism associated with the severity and a surrogate marker predicting the

85 deterioration have not been fully identified yet, high levels of viremia have been shown
86 to be related to disease severity (6, 9, 10). Antibody-dependent enhancement of
87 infection (ADE) has been proposed as one of the pathogenic mechanisms that increases
88 the viremia level; in the case of ADE the increase occurs by viral internalization via Fc
89 gamma receptors (11). Recently, a potential relationship between ADE and human
90 disease severity in DENV infection has been reported (12). However, it is still unclear
91 whether *in vitro* ADE can be used for the prediction of subsequent clinical outcomes.
92 Enhancing antibodies (EAbs), which exclusively play a role of the ADE
93 phenomenon (13), may be associated with an increase in the viremia level in DENV
94 infection (14, 15). In contrast, neutralizing antibodies (NAbs) have a biological function
95 to decrease the viremia level to protect the host from DENV infection (16), while most
96 NAbs show ADE activity at subneutralizing doses (17). A DENV-immune serum
97 (polyclonal form) could be represented with a cocktail of these intricate functional
98 antibodies (monoclonal EAbs and NAbs) (18), and could be introduced by one of three
99 routes: (i) DENV infection, (ii) maternal antibody from a DENV-seropositive mother
100 and (iii) other flavivirus infection. Therefore, the balance activity between EAbs and
101 NAbs might be critical to control the outcome (protection or pathogenesis). We
102 previously developed a simple method to detect the balance between the enhancing and

neutralizing activities (19), and demonstrated that mouse monoclonal EAbs and NAbs competed over the neutralizing activities *in vitro* (18, 20). Specifically, the neutralizing activity of an NAb was reduced in the presence of a sufficient level of an EAb, suggesting that the relative capacity for neutralization might be easily affected by the balance between NAbs and EAbs.

In the present study, we evaluated the balance between neutralizing and enhancing activities in sera collected from dengue patients at multiple time points between the acute and defervescent phases. The six autologous viruses isolated from the respective patients were used as assay antigens, allowing us to examine the balance antibody assay with autologous combinations between patient sera and virus antigens. We also measured the number of viral RNA copies in plasma samples collected at multiple time points, and revealed a correlation between the *in vitro* ADE activity and *in vivo* viral load quantity.

RESULTS

Balance between the neutralizing and enhancing activities against autologous viruses

Six hospitalized adult patients, who were enrolled in the previous study (21), were recruited into the present study, and an autologous DENV clinical strain was successfully isolated from each of them. The demographic information (infecting serotype, diagnosis [DF or DHF], ID number, days after fever onset and sample collection period [h] after the hospitalization) is shown in Fig. 1A. Serum samples, which were collected at several time points between the acute and defervescent phases, were subjected to an antibody assay to determine the balance between neutralizing and enhancing activities (NAb/EAb-balance assay) using each autologous virus. Fig. 1B shows that the serum dilution displaying the highest number of infected cells clearly shifted from low to high over time after the first sampling in four of the patients (#44, #57, #12 and #49). These results indicate that their antibody levels rapidly increased by 16~256 fold during the early disease stage. Similar dose-dependent patterns were observed in patients #44, #57 and #49, even though these patients had different infecting serotypes and diagnoses. On the other hand, patients #14 and #46 did not show a remarkable shift during the observation period. As patient #14 was classified as

primary infection by IgG/IgM immunochromatography tests in the previous study (21), no functional antibody activity was observed. Although patient #46 showed strong neutralization with a wide range of serum dilutions (1:10~1:2560), this patient was diagnosed with DHF with severe clinical symptoms.

Balance between neutralizing and enhancing activities against four prototype viruses

Dose-dependent neutralizing/enhancing activity curves were obtained against four prototype laboratory strains (DENV-1: Mochizuki; DENV-2: NGC; DENV-3: H87; and DENV-4: H241). As described in the above assays using the autologous viruses (Fig. 1B), increases in the antibody levels against four prototype DENVs were observed in four patients (#44, #57, #12 and #49) (Fig. 2). Although patients #12 and #49 were currently infected with DENV-4, they displayed higher levels of cross-neutralization against heterologous DENV-2 and/or DENV-1. Similarly, patient #57, who was currently infected with DENV-2, showed cross-neutralization against DENV-4. These results suggest that our assay system may be able to reveal the previous infection history in dengue patients by comparing their cross-neutralizing activities. On the other hand, patients #14 and #46 did not show any remarkable antibody shift against the four

prototype viruses. Patient #46 displayed strong neutralization against all serotypes, while patient #14 showed enhancing activities against DENV-1 and DENV-3 at 1:10 serum dilution. Based on these findings, it seems likely that a history of previous exposure to and infection by DENV affected the antibody response patterns in our patients.

Evaluation of progeny virus titers by NAb/EAb balance assay

To evaluate the levels of progeny virus secreted from the infected cells using the NAb/EAb balance assay system, patient #49 was selected as a representative patient with a range of neutralizing and enhancing activities (Figs. 1 and 2). Culture supernatants were harvested from the infected cells 24 h after the set-up of the NAb/EAb-balance assay, and were titrated on Vero cells. The dose-dependent patterns of progeny virus titers (Fig. 3A) were roughly similar to those from the infected-cell counts (Figs. 1 and 2), with minor differences at lower serum dilutions. Specifically, the defervescent sample (65 h) displayed a trend of neutralization against four prototype viruses in approximately 1:10–1:100 serum dilutions. Fold enhancements were calculated from progeny virus titers at 1:10 serum dilutions (Fig. 3A), and were plotted as the ordinate against time (h) as the abscissae (Fig. 3B). The time-course patterns

tended to decline in parallel, except for DENV-2. The autologous virus showed the higher fold enhancement (>1000 fold on 0 h) than the other four prototype assay antigens. In contrast, no infective progeny virus was detected in DENV-2, suggesting that patient #49 had previously been infected with DENV-2, and possessed sufficient neutralizing antibody against DENV-2. Similar declining trends were observed in graphic data based on infected-cell counts as the ordinate (Supplemental Fig. S1).

Relationship between the *in vitro* infective progeny virus and *in vivo* viral load

Using the same method described above, the infection-enhanced progeny virus titers were determined with the autologous combination (using 1:10 serum dilution) in the other five patients (#14, #44, #57, #46 and #12) (blue triangles in Fig. 4A). In addition, the quantity of viral RNA was directly determined from the corresponding clinical plasma samples (red circles in Fig. 4A). Both the ADE progeny virus titer (blue triangle) and viral RNA copy number (red circle) basically declined in parallel over time. Significant correlation was observed between the ADE progeny virus titers and the viral RNA copy numbers ($r=0.69$; $P<0.05$) (Fig. 4B). These results indicate that there might be positive correlations between (i) the *in vitro* progeny virus titers obtained from the NAb/EAb-balance assay and (ii) the *in vivo* viral quantity in the plasma. In contrast, no

189 correlations were observed between the laboratory results (the intensity of ADE
190 activity) and the clinical disease outcome (DF or DHF). Patients #57, #46, #12 and #49
191 deteriorated after the beginning of the defervescent phase (*pink shading* in Fig. 4A), but
192 the levels of progeny virus titers and viral load in #46 (DHF patient) were not as high as
193 those in other DHF patients. Likewise, #44 showed DF manifestations, but the levels of
194 progeny virus titers and viral load were as high as those of DHF patients.
195

DISCUSSION

In the present study, to elucidate the relationship between antibody status and disease severity in dengue patients, we analyzed the balance between neutralizing and enhancing activities in serum samples which were collected from dengue patients at multiple time points between the acute and defervescent phases (Figs. 1 and 2). Patient #46 showed DHF manifestations in spite of the strong neutralization against both autologous and prototype viruses, and the dose-dependent curve was not meaningfully shifted over 0–19 h in this patient. This suggests that the strong neutralizing antibodies that had been induced by four days after the fever onset might not have protected the patient from the subsequent severe disease, although the antibody status in the pre-infection phase was not known. In contrast, DF patient #44 did not show severe manifestations, even though the ADE activity patterns of this patient were as high as those of the DHF patients (#57 and #49). Therefore, when samples from post-hospitalized patients were used, it was still difficult to predict disease outcomes by measuring the balance between neutralizing and enhancing activities. Although other factors might be involved in determining the immune correlation (such as the treatment conditions in the hospital, current infecting serotype and previous infection history, etc.), the most valuable factor (and also the most

213 difficult) is the collection of samples in the pre-infection or pre-hospitalization period
214 through prospective cohort studies (22-25).

215 The ADE activities shown by the low serum dilution (1:10) were gradually decreased
216 over time (Fig 3B and Supplemental Fig. S1). These results suggest that neutralizing
217 antibodies were immediately produced after the infection, and then the original
218 enhancing activity might have been competitively suppressed by the appearance of
219 neutralizing antibodies. However, some cases did not show a decreasing trend in the
220 heterologous combinations (Supplemental Fig. S1). For instance, patient #44 displayed
221 the decreasing trend against DENN-2 (autologous and NGC strains) and DENV-4, but
222 exhibited an increasing or flat trend against DENV-1 and DENV-3. This suggests that
223 patient #44 will be protected from subsequent infection with DENV-2 and DENV-4, but
224 may not be protected from infection with DENV-1 or DENV-3. Such patients should be
225 advised to take care to avoid future infections. Therefore, monitoring the balance
226 between NAb and EAb activities against all serotypes is important for the prediction of
227 disease severity upon secondary heterotypic infection in seropositive individuals. On the
228 other hand, the opposite trend was observed in the high-serum dilution range
229 (1:2560)—that is, the ADE activities were basically increased over time (Supplemental
230 Fig. S2). This suggests that the induced neutralizing antibodies still possess the potential

231 ADE activity at sub-neutralizing doses. Therefore, if the antibody level wanes over time,
232 it might become a risk factor for increased disease severity.

233 The first approved dengue vaccine, Sanofi's Dengvaxia, has been introduced into
234 approximately 20 endemic countries since 2015 (26). However, WHO SAGE revised
235 their recommendation to say that only seropositive individuals, who have previously been
236 infected with DENV, can be inoculated with Dengvaxia (27). Seronegative populations
237 should not be vaccinated (28, 29), because a risk of vaccine-induced infection
238 enhancement cannot be excluded (30-32). Although there are several assay kits to detect
239 dengue antibodies in a quantitative or qualitative manner (e.g., ELISA,
240 immunochromatography assays, etc.), few rapid test kits are available to detect the
241 functional antibody activity. Only an assay with the capacity to detect ADE activity
242 would be able to provide a risk assessment for vaccine recipients. In the present study,
243 five (#44, #46, #57, #12 and #49) of six patients were confirmed to be seropositive
244 (classified as secondary infection). In contrast, patient #14 (classified as primary
245 infection) showed no functional antibody activity against the autologous DENV-1, NGC
246 (DENV-2) and H241 (DENV-4) strains (Figs. 1 and 2), although this patient did show
247 limited enhancing activities against the Mochizuki (DENV-1) and H87 (DENV-3)

248 strains. Thus, such individuals should be advised to delay vaccination until their antibody
249 levels are elevated sufficiently, if they have a plan to get Dengvaxia in the near future.

250 A previous study showed that ADE contributed to an increase in viremia in an animal
251 model (33), and the high viremia levels in dengue patients have been demonstrated to
252 correlate with the disease severity (6, 9, 10). Since the viremia in humans is caused by
253 secretion of the virus from the infected-host cells, the *in vitro* progeny virus infectivity
254 obtained from the NAb/EAb-balance assay might correlate to the clinical viral load.
255 Interestingly, in the present study, significant positive correlations were observed
256 between *in vitro* ADE progeny virus levels (focus forming units [FFU]/ml) in serum
257 samples and *in vivo* viremia levels (copies/ml) in plasma samples (Fig. 4B). This result
258 suggests that measurement of the infective progeny virus titers (or the infected cell
259 counts) in the NAb/EAb-balance assay may enable us to predict the impact of viral load
260 in the subsequent DENV infection. Since patients #14 and #46 showed no enhancing
261 activity against autologous virus in the lower serum dilutions (Fig. 1B), the viral load
262 quantity in the plasma might have been lower in these patients than the other participants
263 (Fig. 4A).

264 In conclusion, the present NAb/EAb-balance assay might be used to predict the
265 impact of the viremia level when a human is subsequently infected with DENV.

Although we could not find a relationship between the ADE activity (viremia level) in the early phase and disease severity in the defervescent phase, the *in vitro* progeny virus titers obtained from the NAb/EAb-balance assay were shown to significantly correlate with the viral load determined by qPCR assay. The major limitation of this study was the small sample size. Nonetheless, although only six autologous virus strains were successfully isolated in this study, we believe that the analysis of the combination of autologous virus and serum is meaningful to understand the relationship between neutralization/ADE and disease outcome.

MATERIALS AND METHODS

Blood samples

The present study was conducted using the serum and plasma collected from confirmed-dengue patients at the Hospital for Tropical Diseases, Bangkok, Thailand, in a previous study (21). Six dengue patients were enrolled from whom clinical virus isolates were obtained. All subjects gave their informed consent for inclusion into the study before they participated. This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Faulty of Tropical Medicine, Mahidol University, Thailand (FTM ECF-019-04).

Cells

African green monkey kidney Vero cells were cultivated in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS) and 60 µg/mL kanamycin (34). Human erythroleukemia K562 cells were cultivated in RPMI 1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin (35). All cell lines were cultivated in a humidified atmosphere of 5% CO_2:95% air at 37 °C.

292 **Virus isolation and typing**

293 Vero cells were inoculated with the diluted patient sera (1:10) collected in the acute
294 phase, and then incubated at 37 °C for 7 days. Within five blind passages, viral RNA
295 was extracted from the supernatant of the infected cells, and the serotype was
296 determined by PCR using type-specific primers following a previous report (36).

297

298 **Viruses**

299 Six clinical isolates (1 strain of DENV-1, 2 of DENV-2, 1 of DENV-3, and 2 of
300 DENV-4) and four prototype lab strains (DENV-1: Mochizuki strain; DENV-2: New
301 Guinea C [NGC] strain; DENV-3: H87 strain; and DENV-4: H241 strain) (37) were
302 used in this study. The culture supernatants harvested from the infected Vero cells were
303 used as live virus sources for the antibody assay measuring the balance between
304 neutralizing and enhancing activities (see below).

305

306 **Antibody assay for the balance between neutralizing and enhancing activities**
307 **(NAb/EAb-balance assay)**

308 The NAb/EAb-balance assay was conducted using semi-adherent K562 cells as
309 described previously (19). Briefly, serial dilutions of sera (starting from 1:10 dilution)

were mixed with each DENV strain in a poly-L lysine-coated 96-well microplate and incubated at 37 °C for 2 h. K562 cells (1×10^5 cells per well) were then added to the mixtures and incubated at 37 °C for 2 days. After immunostaining (see below), the infected cells were counted. The cut-off values for neutralizing and enhancing activities were calculated from the means ± three standard deviations (SD) of infected cell counts obtained with eight negative controls adjusted for approximately 100 infected cells. When the number of infected cells was higher than the mean + three SD, it was defined as enhancing activity. In contrast, when the infected cell number was lower than the mean − three SD, it was defined as neutralizing activity.

In addition, enhancing activity was also evaluated by titrating the progeny virus infectivity (FFU/ml) in the culture supernatant of the infected cells. The supernatants were harvested at 24 h after the inoculation, and their titers were determined on Vero cells.

Immunostaining

Immunochemical staining was performed essentially as described previously (34). Briefly, cells were fixed with acetone/methanol (1:1) and incubated serially with a mouse monoclonal antibody D1-4G2 (flavivirus group cross-reactive) purchased from

328 American Type Culture Collection (Manassas, VA), biotinylated anti-mouse IgG, ABC
329 (avidin-biotinylated peroxidase complex) reagent, and VIP substrate (Vector
330 Laboratories, Burlingame, CA).
331
332 **Quantification of the viral RNA copy number in plasma samples**
333 The viral RNA copy number in plasma samples was determined by following a
334 previous study (21). Briefly, viral RNA was extracted from 70 µl plasma using a
335 QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the
336 manufacturer's protocol, then subjected to quantitative real-time RT-PCR using a
337 One-Step SYBR PrimeScript RT-PCR kit II (Takara Bio, Japan). The PCR mixture was
338 mixed with 2 µl of extracted RNA and DENV-specific primer (38) before running on a
339 CFX96TM real-time PCR cycler (BioRad, Hercules, CA, USA) under cycle conditions
340 of 42°C for 5 min, 95°C for 10 sec followed by 45 cycles of 95°C for 5 sec, 55°C for 30
341 sec and 72°C for 30 sec. The viral load quantity was determined by linear regression of
342 the cycle threshold value against the known viral titers quantified by plaque forming
343 unit assay.
344
345 **Statistical analysis**

346 Correlation coefficients were estimated on the basis of the Pearson product-moment

347 correlation coefficient (r). A probability (*p*) less than 0.05 was considered statistically

348 significant.

349

Supplemental Material

Fig. S1: Transition of the NAb/EAb balance activity in a low serum dilution (1:10) with time progression.

Fig. S2: Transition of the NAb/EAb balance activity in a high serum dilution (1:2560) with time progression.

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Conflict of Interest

The authors declare that they have no competing interests.

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515

516 **Figure Legends**

517

518 **FIG 1. Balance between neutralizing and enhancing activities against autologous**

519 **virus.** (A) Demographic data for the six dengue patients enrolled in this study. Blood

520 samples were collected on the day of hospitalization and the following time points at

521 12-24 h intervals until a maximum 65 h after hospitalization. (B) NAb/EAb balance

522 activity against the autologous virus. The NAb/EAb-balance assay was performed using

523 a combination of autologous virus and serum . The *blue*, *green*, *yellow* and *red* triangles,

524 corresponding to Fig. 1A, indicate the hours after hospitalization. The infecting serotype

525 is indicated in parentheses (D1: DENV-1; D2: DENV-2; D3: DENV-3; and D4:

526 DENV-4). The abscissa indicates the serum dilutions and the ordinate shows the

527 numbers of infected cells (both expressed as log10). Each data point represents the

528 average of two separate assays; error bars indicate the SDs. Dotted lines indicate the

529 mean numbers of infected cells plus or minus three times the SD (mean ± 3SD)

530 calculated from eight negative controls.

531

532 **FIG 2. Balance between neutralizing and enhancing activities against four**

533 **prototype viruses.** The NAb/EAb-balance assay was performed using a combination of

534 serum and four prototype viruses (DENV-1: Mochizuki strain; DENV-2: NGC strain;
535 DENV-3: H87 strain; and DENV-4: H241 strain). The *blue*, *green*, *yellow* and *red*
536 triangles, corresponding to Fig. 1A, indicate the hours after hospitalization. The
537 infecting serotype is indicated in parentheses (D1: DENV-1; D2: DENV-2; D3:
538 DENV-3; and D4: DENV-4). The abscissa indicates the serum dilutions and the ordinate
539 shows the numbers of infected cells (both expressed as log10). Each data point
540 represents the average of two separate assays; error bars indicate the SDs. Dotted lines
541 indicate the mean numbers of infected cells plus or minus three times the SD (mean ±
542 3SD) calculated from eight negative controls.
543
544 **FIG 3. Progeny virus infectivity in the NAb/EAb balance assay.** (A) Progeny virus
545 titers obtained from the NAb/EAb-balance assay. The NAb/EAb-balance assay was
546 conducted with a combination of 2-fold serial serum dilutions (starting from 1:10) in
547 patient #49, and each DENV strain (the autologous, Mochizuki, NGC, H87 or H241
548 strain). Culture supernatants were harvested at 24 h after the mixture of serum, virus and
549 K562 cells. The infective titers (FFU/ml) were determined on Vero cells. The *blue*,
550 *green*, *yellow* and *red* triangles, corresponding to Fig. 1A, indicate the hours after
551 hospitalization. The abscissa indicates the serum dilutions and the ordinate shows the

552 progeny virus titers (both expressed as log10). *Dotted lines* indicate the mean progeny
553 virus titers calculated from four negative (no serum) controls. (B) Decreasing trend of
554 ADE at low serum dilution with time progression. Fold enhancement was calculated
555 from the infective progeny virus titers obtained in Fig. 3A (specifying data at 1:10
556 serum dilution), and was expressed in log10 as the increase in the progeny virus titer
557 relative to the negative control. The fold enhancement was plotted as the ordinate
558 against time (h) as the abscissa.
559
560 **FIG 4. Relationship between *in vitro* ADE activity and *in vivo* viral load quantity.**
561 (A) The NAb/EAb-balance assay was conducted with a combination of serum diluted at
562 1:10 and the corresponding autologous virus. Culture supernatants were harvested at 24
563 h after the mixture of serum, virus and K562 cells. The infective titers were determined
564 on Vero cells and plotted as the left ordinates (*blue triangles*: expressed as log10
565 FFU/ml). The number of viral RNA copies in plasma samples collected at same time
566 points as well as one extra time point were determined by real time RT-PCR, and plotted
567 as the right ordinates (*red circles*: expressed as log10 copies/ml). The abscissae indicate
568 time (h) after the hospitalization. *Pink shading* indicates the beginning of defervescence
569 on the clinical observation. (B) Correlation between the ADE progeny virus titers and

570 the viral RNA copy numbers. The correlation coefficient (r) was estimated for the

571 individual progeny virus titers and qPCR values obtained in Fig. 4A. The abscissa and

572 ordinate indicate the qPCR values and the progeny virus titers, respectively. Linear

573 regression lines and r values are presented in the panel.

574

Supplemental Figure Legend

Supplemental FIG S1. Transition of the NAb/EAb balance activity in a low serum dilution (1:10) with time progression. The infected cell counts (specifying data at 1:10 serum dilution) obtained from Figs. 1B and 2 were plotted as the ordinate (expressed as log10) against time (h) as the abscissa.

Supplemental FIG S2. Transition of the NAb/EAb balance activity in a high serum dilution (1:2560) with time progression. The infected cell counts (specifying data at 1:2560 serum dilution) obtained from Figs. 1B and 2 were plotted as the ordinate (expressed as log10), and plotted with time (h) as the abscissa.

A

Infecting Type	Diagnosis	ID	Fever day	Blood collection (h) ▲	▲	▲	▲
DENV-1	DF	14	3	0	13	-	-
DENV-2	DF	44	3	0	12	36	60
DENV-2	DHF	57	1	0	15	39	63
DENV-3	DHF	46	4	0	19	-	-
DENV-4	DHF	12	3	0	14	26	-
DENV-4	DHF	49	3	0	17	41	65

B

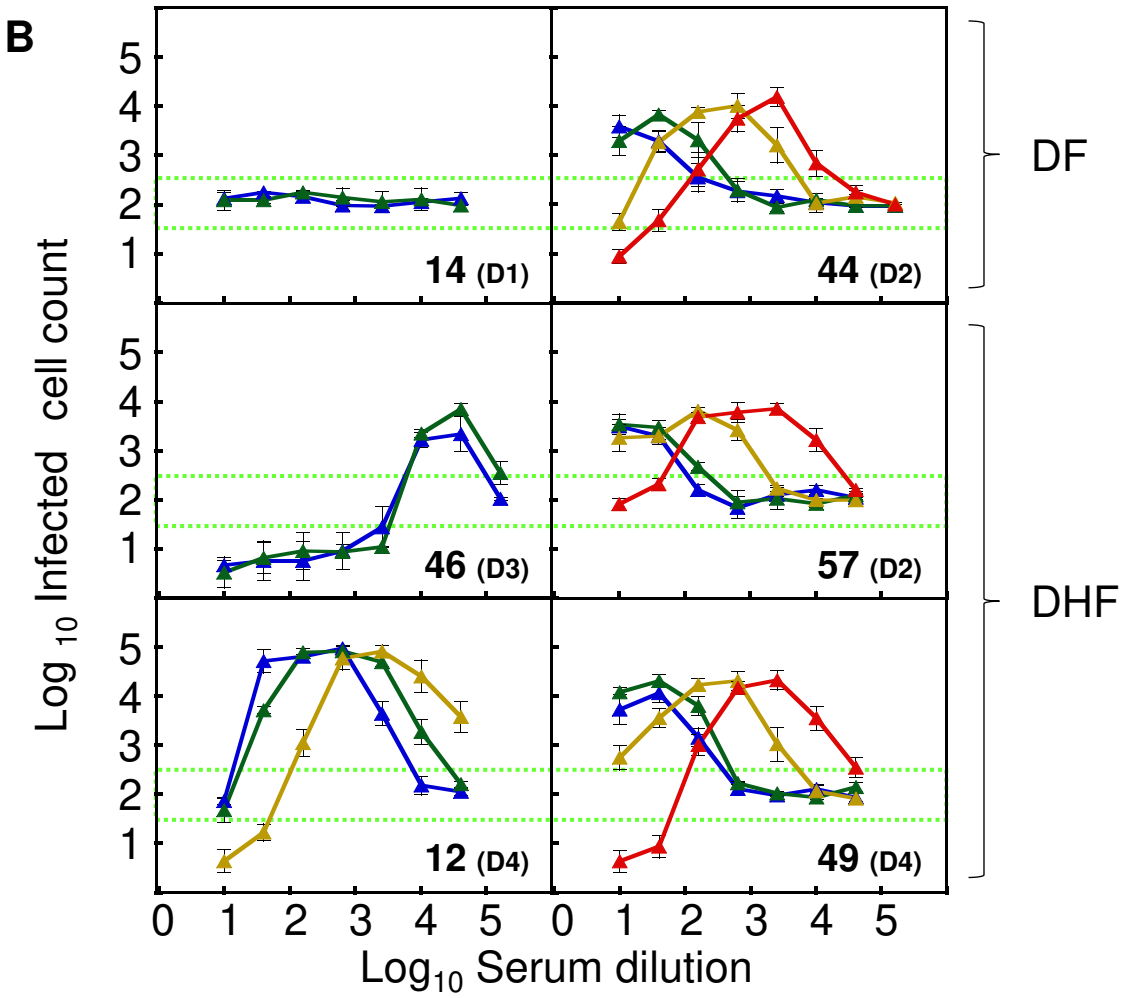
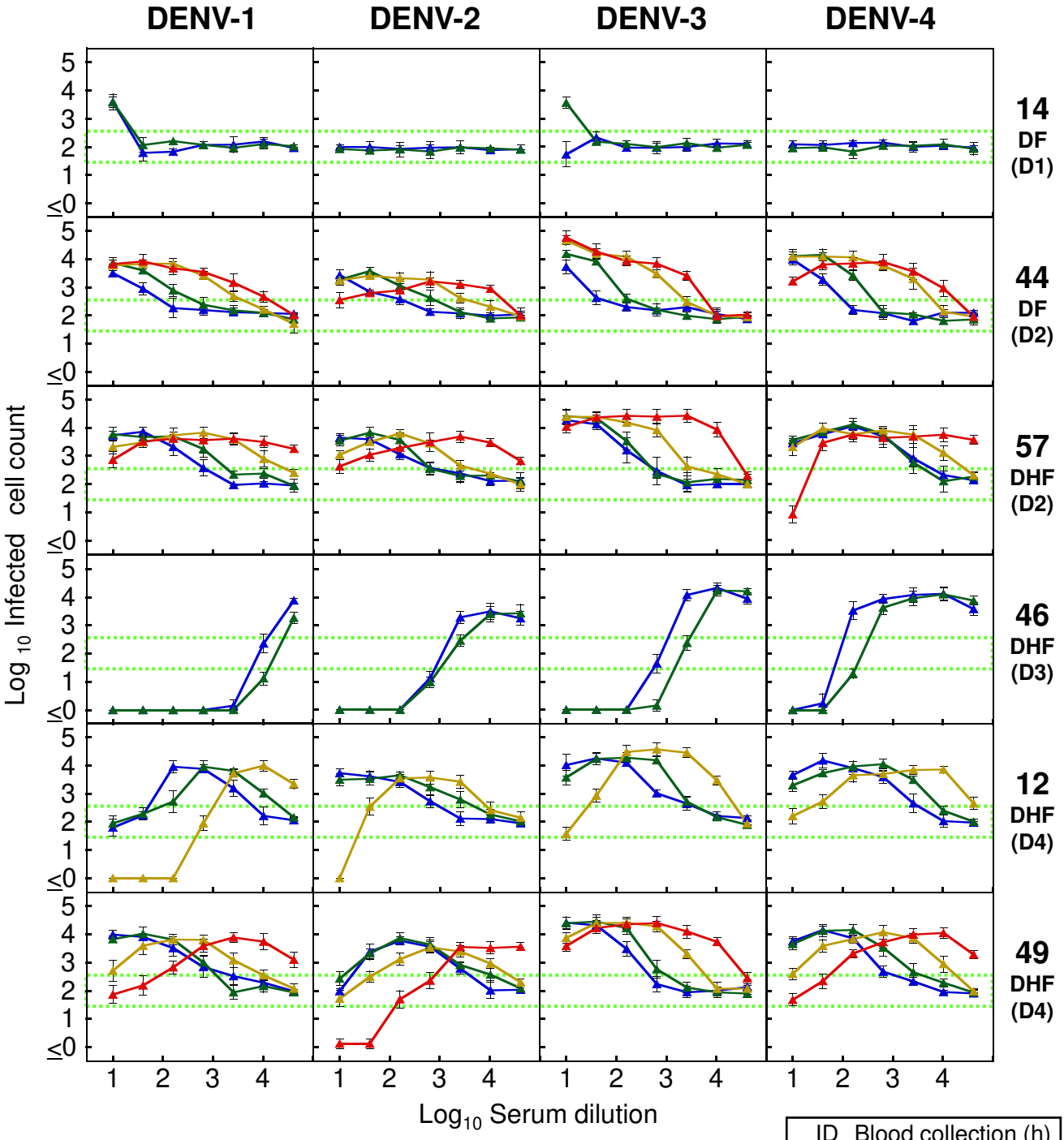


FIG 1. Balance between neutralizing and enhancing activities against autologous virus. (A) Demographic data for the six dengue patients enrolled in this study. Blood samples were collected on the day of hospitalization and the following time points at 12-24 h intervals until a maximum 65 h after hospitalization. (B) NAb/EAb balance activity against the autologous virus. The NAb/EAb-balance assay was performed using a combination of autologous virus and serum . The *blue*, *green*, *yellow* and *red* triangles, corresponding to Fig. 1A, indicate the hours after hospitalization. The infecting serotype is indicated in parentheses (D1: DENV-1; D2: DENV-2; D3: DENV-3; and D4: DENV-4). The abscissa indicates the serum dilutions and the ordinate shows the numbers of infected cells (both expressed as log10). Each data point represents the average of two separate assays; error bars indicate the SDs. Dotted lines indicate the mean numbers of infected cells plus or minus three times the SD (mean $\pm$ 3SD) calculated from eight negative controls.



ID	Blood collection (h)			
	▲	▲	▲	▲
14	0	13	-	-
44	0	12	36	60
57	0	15	39	63
46	0	19	-	-
12	0	14	26	-
49	0	17	41	65

FIG 2. Balance between neutralizing and enhancing activities against four prototype viruses. The NAb/EAb-balance assay was performed using a combination of serum and four prototype viruses (DENV-1: Mochizuki strain; DENV-2: NGC strain; DENV-3: H87 strain; and DENV-4: H241 strain). The *blue*, *green*, *yellow* and *red* triangles, corresponding to Fig. 1A, indicate the hours after hospitalization. The infecting serotype is indicated in parentheses (D1: DENV-1; D2: DENV-2; D3: DENV-3; and D4: DENV-4). The abscissa indicates the serum dilutions and the ordinate shows the numbers of infected cells (both expressed as log10). Each data point represents the average of two separate assays; error bars indicate the SDs. Dotted lines indicate the mean numbers of infected cells plus or minus three times the SD (mean $\pm$ 3SD) calculated from eight negative controls.

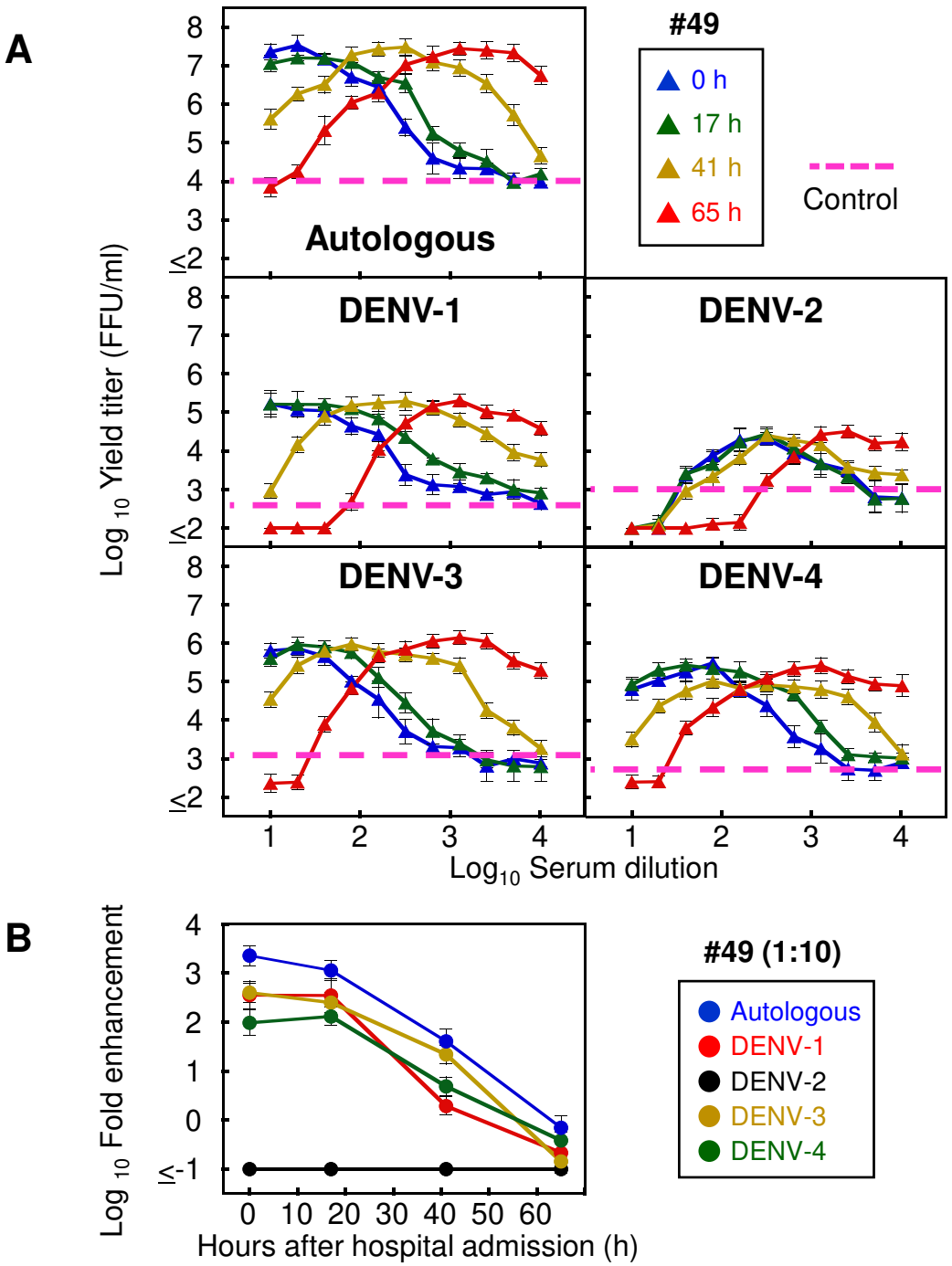


FIG 3. Progeny virus infectivity in the NAb/EAb balance assay. (A) Progeny virus titers obtained from the NAb/EAb-balance assay. The NAb/EAb-balance assay was conducted with a combination of 2-fold serial serum dilutions (starting from 1:10) in patient #49, and each DENV strain (the autologous, Mochizuki, NGC, H87 or H241 strain). Culture supernatants were harvested at 24 h after the mixture of serum, virus and K562 cells. The infective titers (FFU/ml) were determined on Vero cells. The *blue*, *green*, *yellow* and *red* triangles, corresponding to Fig. 1A, indicate the hours after hospitalization. The abscissa indicates the serum dilutions and the ordinate shows the progeny virus titers (both expressed as log10). *Dotted lines* indicate the mean progeny virus titers calculated from four negative (no serum) controls. (B) Decreasing trend of ADE at low serum dilution with time progression. Fold enhancement was calculated from the infective progeny virus titers obtained in Fig. 3A (specifying data at 1:10 serum dilution), and was expressed in log10 as the increase in the progeny virus titer relative to the negative control. The fold enhancement was plotted as the ordinate against time (h) as the abscissa.

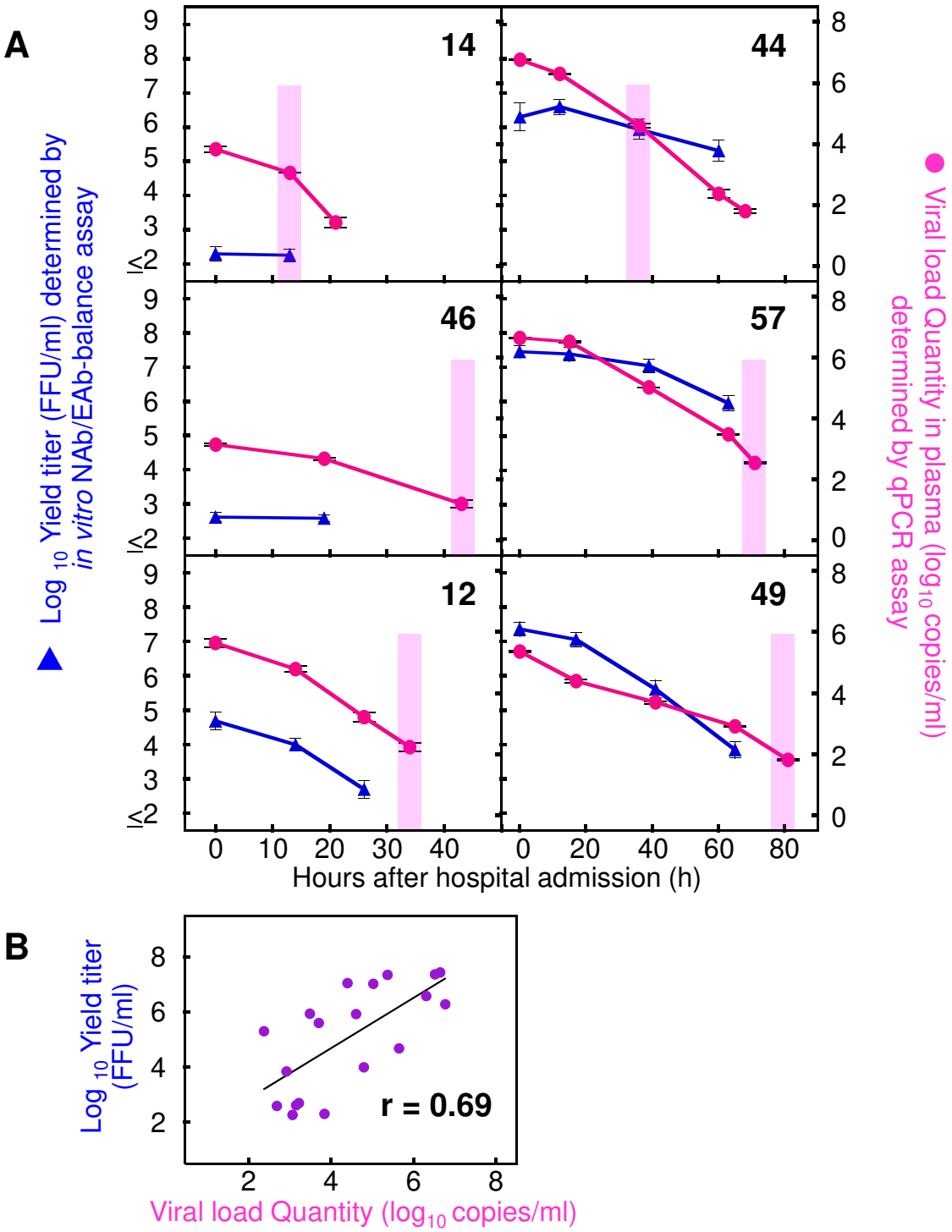


FIG 4. Relationship between *in vitro* ADE activity and *in vivo* viral load quantity. (A) The NAb/EAb-balance assay was conducted with a combination of serum diluted at 1:10 and the corresponding autologous virus. Culture supernatants were harvested at 24 h after the mixture of serum, virus and K562 cells. The infective titers were determined on Vero cells and plotted as the left ordinates (*blue triangles*: expressed as log10 FFU/ml). The number of viral RNA copies in plasma samples collected at same time points as well as one extra time point were determined by real time RT-PCR, and plotted as the right ordinates (*red circles*: expressed as log10 copies/ml). The abscissae indicate time (h) after the hospitalization. *Pink shading* indicates the beginning of defervescence on the clinical observation. (B) Correlation between the ADE progeny virus titers and the viral RNA copy numbers. The correlation coefficient (r) was estimated for the individual progeny virus titers and qPCR values obtained in Fig. 4A. The abscissa and ordinate indicate the qPCR values and the progeny virus titers, respectively. Linear regression lines and r values are presented in the panel.