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MURAKAMI, Isamu

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ROLE OF MACROPHAGES AND THYMUS IN ANTI-DENGUE ANTIBODY PRODUCTION IN MICE

Isamu MURAKAMI

Department of Microbiology and
International Center for Medical Research
Kobe University School of Medicine

INDEXING WORDS

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SYNOPSIS

The role of macrophages (Mø) and thymus in anti-dengue (DV) antibody production was studied in mice inoculated with DV via the subcutaneous route. The production of both 2-mercaptoethanol (2-ME) sensitive (regarded as IgM) and 2-ME resistant (regarded as IgG) antibodies was dependent upon Mø and thymus. The Mø depressing agents (anti-Mø agents), carrageenan and trypan blue, depressed mainly production of IgM and also of IgG. Nude (nu/nu) mice reconstituted with thymocytes (2.5×10^7 cells) from their heterozygotes (nu/+) failed to produce hemagglutination-inhibition (HI) and neutralization (NT) antibodies, although the NT kinetic curves of their sera intermediated between those of the sera of nu/nu and nu/+ mice. The comparative study on the neutralization kinetic curves of sera from nu/nu, nu/+, and 'thymocyte-reconstituted' nu/nu mice implied that the sera of DV-infected nu/nu mice possessed the plaque-member-increasing (PNI) activities upon the infective DV introduced into neutralization tests. In the DV-infected nu/nu mice, the PNI activities of sera on 7 to 21 days postinfection (dpi) were significantly higher than those on 32 and 41 dpi. Unexpectedly, the PNI activity was also recognized in highly diluted sera from the DV-infected or control non-infected nu/+ mice. The phenomena observed are discussed in relation to the anti-DV antibody activity and the disfunction of Mø by anti-Mø agents.

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INTRODUCTION

Knowledge has been accumulated that macrophages play an important role in natural host defense against viral infections.[21] Regarding the host defense mechanism in togavirus infections of mice, however, data so far obtained have not been consistent. It was reported that cell-mediated immunity (CMI) was essential for recovery of mice from tick-borne encephalitis virus infection,[16] but that the recovery of mice from dengue virus (DV) infection was influenced to higher extents by humoral antibodies than by CMI and macrophages.[3, 4] T cells and macrophages are known to have an essential effect for triggering and differentiation of B cells.[17] The present studies were carried out to investigate the role of macrophages and thymus in the anti-DV humoral antibody production in BALB/c mice.

MATERIALS AND METHODS

Media

A medium (5% CS-MEM, pH 7.2-7.4) consisting of Eagle's minimal essential medium (MEM, Nissui Seiyaku, Japan), Kanamycin (60 μg/ml), sodium bicarbonate and 5% heat-inactivated calf serum (CS, Wako, Japan) was used for subculture of BHK-21 cells. For preparation of virus antigen and diluent, another medium (0.2% BA-MEM) was used, which consisted of MEM, kanamycin, 0.2% bovine albumin (BA, Fraction IV, Armour, USA) and sodium bicarbonate to adjust pH at 7.2 before membrane-filtration for sterilization.

Seed virus

Mouse-passaged dengue virus (DV, type 1, Mochizuki strain)[13] was employed.

A 10% homogenate of infected mouse brains in 0.2% BA-MEM was centrifuged at 9,500 x g for 15 min at 2 C, and the supernatant was stored at -80 C; this was used as seed DV throughout the present experiments.

Virus antigens

BHK-21 cells subcultured with 5% CS-MEM were infected with DV at a moi of 0.5. After an incubation at 37 C for 2 h for cell-virus adsorption, unadsorbed virus was discarded. The infected cells were then refed

with 0.2% BA-MEM and maintained at 37 C. The infected culture fluid harvested on 4 or 5 days postinfection (dpi) was centrifuged at low speeds (2,400-6,000 x g for 10-15 min) and the supernatant was subjected to the following procedures.

1. Crude DV antigen (Cru-DV-Ag)

The supernatant was saturated at 70% with solid ammonium sulfate and stirred magnetically for 30 min in an ice bath. The mixture was spun down at 8,700 x g for 20 min and the pellet was resuspended in borate-buffered saline, pH 9 (BS 9.0).[5] After centrifuging twice at 8,000 x g for 10 min each, the final supernatant was used as 'crude-DV-antigen.' 'Mock-infected antigen' (Mock-Ag) was prepared from non-infected BHK-21 cultures by the same manner as above.

2. Partially purified DV antigen (SDC-DV-Ag)

To one volume of the supernatant described above was added 2.3 volumes of saturated ammonium sulfate, pH 7.4 (according to T. Matsumura, Kobe University). After being stirred for 30 min in an ice bath, the mixture was spun down at 12,000 x g for 30 min. The pellet was resuspended in BS 9.0 and recentrifuged at 100,000 x g for 90 min. The second pellet was resuspended in 0.1 M Tris-HCl (pH 7.2) and 0.9 ml thereof was put onto the top of a 4 ml sucrose gradient (10-40%, w/v) in 0.1 M Tris-HCl (pH 7.2). After centrifuging at 100,000 x g for 90 min, 34 fractions were collected through a hole punctured at the bottom of tube. Fractions Nos. 7, 8, and 9 which showed the highest hemagglutination (HA) activity and infectivity (PFU) were pooled and used as partially purified DV antigen (SDC-DV-Ag). The Cru-DV-Ag, Mock-Ag, and SDC-DV-Ag were stored at -80 C until ready for use.

Infectivity assay

Infectivity of virus was determined by a plaque assay on BHK-21 monolayer cultures under 1% methyl cellulose overlay medium.[7, 14, 24]

HA and hemagglutination-inhibition (HI) test

The test sera were pretreated with acetone and goose red blood cells (RBC) and determined for HA- and HI- titers by the Clarke-Casals methods[5] modified as adapted to a microtiter technique.[7, 25] The tests were done in duplicate and the mean values were calculated.

2-Mercaptoethanol (2-ME) treatment

The sera which showed the positive HI titers (more than 1 : 10) were treated with 2-ME by a modification of the procedures proposed by Griffin et al.[8] as follows: To one volume of test sera (1 : 10 diluted) pretreated with acetone and RBC was added four volumes of 0.1 M 2-ME in PBS[6] or PBS alone (as a control). The mixtures, after incubated at 37 C for 1 h, were dialysed against PBS overnight at 4 C. The dialysed sera were treated once with acetone, vacuumed for 1 h for concentrating and then dissolved in BS 9.0 at a final dilution of 1 : 10.

Neutralization test (NT)

NT-titers were determined by a plaque reduction method on BHK-21 monolayers by a modification of the procedures previously described.[7, 14] The fifty percent plaque reduction neutralizing titers ($PRNT_{50}$) were calculated.[23] In addition, ratio [$\overline{PFU(S+V)}/\overline{PFU(Dil+V)}$] of the mean number of PFU in the serum-virus (S+V) mixture to that in the diluent-virus (Dil+V) mixture were calculated for each dilution of test sera.

Heat-stability of DV at 37 C

DV adjusted as to contain 1,000 PFU/ml was dispensed, 0.5 ml each, in test tubes which were exposed to 37 C for t min. At intervals, 0.1 ml of the virus was taken and similar samples from 3 tubes were pooled. The infective titers thereof were determined in triplicate by the plaque method on BHK-21 monolayers. Ratios of PFU at t min to that o min PFU (t/o) were calculated.

Mice

Thymus-bearing (nu/+ or +/+) and thymus-lacking (nu/nu) BALB/c mice at the age of 7 to 9 weeks (procured from CLEA JAPAN Inc., Japan) was used.

'Thymocyte-reconstitution' in nu/nu mice

A modification of the procedure proposed by Miller et al.[19] was adopted: Thymus lobes aseptically removed from 8-week-old male nu/+ mice were teased with glass rods through a stainless mesh in cold Hanks' balanced salt solution (HBSS, Nissui, Japan). The suspension of single cell, pooled from three mice similarly treated, was washed twice with cold HBSS and dispensed in cold HBSS. Numbers of viable cells were counted by the trypan blue dye exclusion test using a hemocytometer. The cell

viability was around 95% in the most cases. Five-tenth ml of the cell suspension so prepared (including 2.5 x 10^7 cells) was inoculated slowly (spending over 2 min) into the lateral caudal vein of 7-week-old male nu/nu mice.

Carrageenan and trypan blue

Carrageenan (type IV, Sigma, USA) and trypan blue (Wako, Japan) suspensions (10 mg/ml in 0.15 M NaCl) were injected into mice by the procedures described by the previous investigators. [2, 10]

Statistics

P values were calculated by the Student's t-test.

RESULTS

Effect of carrageenan or trypan blue on anti-DV antibody production in mice

The results obtained are summarized in Fig. 1 and 2. The thymus-bearing mice showed the typical HI and NT antibody production which was depressed by the carrageenan or trypan blue treatment. This depression seemed to be concerned mainly with 2-ME sensitive antibodies (regarded as IgM) and also with the 2-ME resistant antibodies (regarded as IgG). On the contrary, the thymus-lacking nude mice showed no definite HI and NT antibody production regardless of the carrageenan or trypan blue treatment.

Antibody responses in 'thymocyte-reconstituted' nude mice

In nu/nu mice reconstituted by thymocytes (2.5 x 10^7 viable cells) from nu/+ mice, the IgM HI antibodies were not produced; and the IgG HI antibodies were not detectable or detected faintly after comparatively long period of time, e.g., on 41 dpi, as shown in Fig. 2.

Neutralization kinetics of sera at different stages of infection

Sera (1 : 10 diluted) from DV-infected nu/nu mice on 7 to 24 dpi showed the increase in the ratio of $\overline{PFU(S+V)}/\overline{PFU(Dil+V)}$ in NT, compared with sera of the same dilution as above from mice on 32 and 41 dpi or from control non-infected mice or ($p<0.005$ or $p<0.10$, respectively, in Fig. 3). The sera (1 : 320 diluted) on 7 to 24 dpi from nu/nu mice showed the

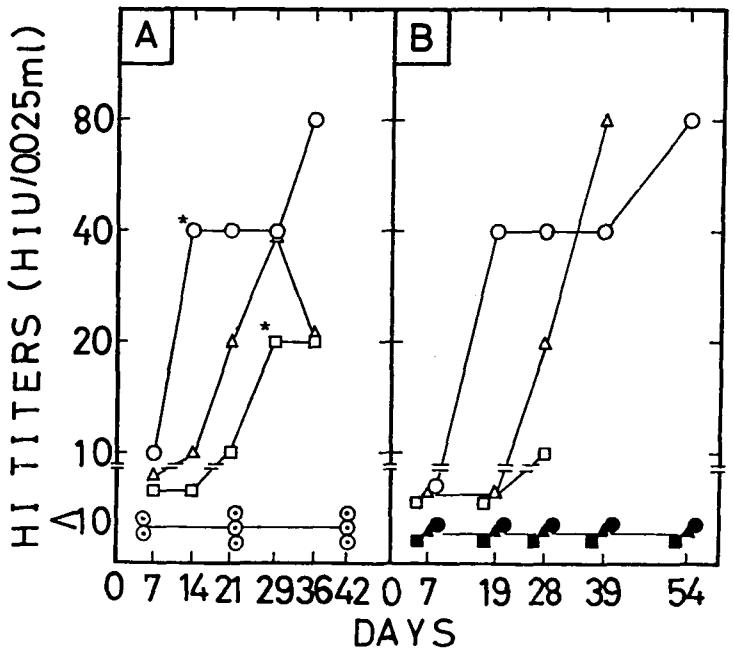

Fig. 1 Effect of carrageenan or trypan blue on anti-DV HI antibody production in BALB/c mice. Each point represents the antibody titer in one mouse; and an asterisk means significant decrease of titer after 2-ME treatment. (A) Female 8-week-old thymus-bearing (+/+) mice were given carrageenan (CAR), trypan blue (TRY) or saline (SAL) and inoculated subcutaneously (sc) with 64 and 32 hemagglutination units (HAU) of crude DV antigen (Cru-DV-Ag) on 0 and 7 days, respectively. The control mice received sc mock-infected antigen (Mock-Ag) on 0 and 5 days. Symbols : △, □, ○, Cru-DV-Ag-infected mice; △, CAR, 5 mg intravenously (iv) prior to the first virus inoculation; □, TRY, 5 mg intraperitoneally (ip) prior to the first virus inoculation, and 1 mg sc, thereafter, twice per week; ○, SAL, iv, similarly as CAR; ⊙, Mock-Ag-infected mice. (B) Female 9-week-old nude (nu/nu) mice and their heterozygotes (nu/+) were given CAR (triangle), TRY (square), or SAL (circle), as indicated in (A), and then were inoculated sc with 32 and 64 HAU of Cru-DV-Ag on 0 and 7 days, respectively. Symbols : ▲, ■, ●, nu/nu mice; △, □, ○, nu/+ mice.

same increase, compared with the sera (of the same dilution) on 32 and 41 dpi from the nu/nu mice ($p<0.05$, Fig. 3). On the contrary, the 'thymocyte-reconstituted' nu/nu mice infected with DV did not show this plaque-number-increasing (PNI) activity in 1 : 10 to 1 : 320 dilutions of sera (Fig. 3). The plaque reductions in NT were observed in 1 : 10 to 1 : 160 dilutions of sera from the DV-infected nu/+ mice. The PNI activ-

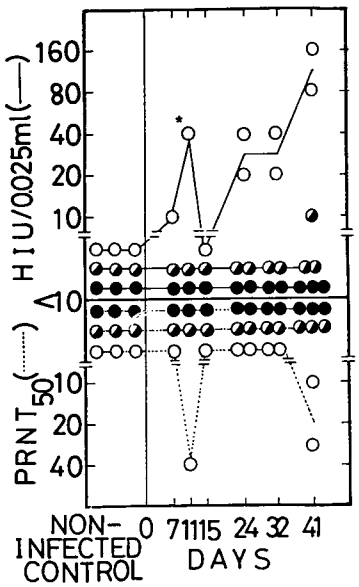

Fig. 2
Antibody responses in 'thymocyte-reconstituted' nude mice. Each point represents the antibody titer in one mouse and the asterisk indicates the same meaning as described in Fig. 1. Male 7-week-old BALB/c nude (nu/nu) mice, their heterozygotes (nu/+) and nude mice which were reconstituted iv by thymocytes (2.5 x 10^7 cells) from nu/+ mice 18 h prior to the first virus inoculation (nu/thy) were inoculated sc with 8 HAU (3.4 x 10^3 PFU) and 50 HAU (3.3 x 10^4 PFU) of SDC-DV-Ag on 0 and 7 days, respectively. Symbols : ●, nu/nu, ○, nu/+, ◕, nu/thy.

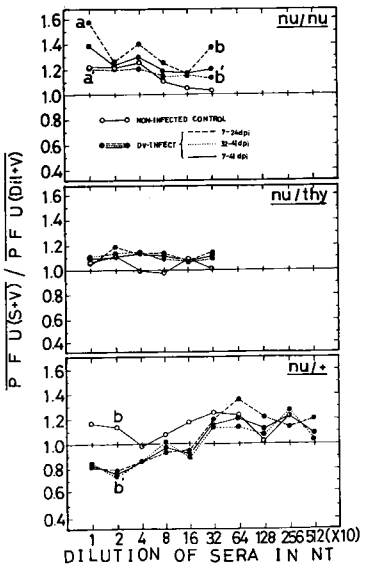

Fig. 3
Neutralization test (NT) of sera from DV-infected mice. All the test sera in this figure are the same as those in Fig. 2. Each point represents the mean of the ratios of $\overline{PFU(S+V)}$ to $\overline{PFU(Dil+V)}$ in NT for test sera at different stages of infection in each group of mice. The meaning of this ratio is described in MATERIALS AND METHODS. P values are compared with these mean ratios. Symbols : ●——●, whole course of infection, i.e., on 7 to 41 dpi; ●---- ●, early stage of infection, i.e., on 7 to 24 dpi; ●···· ●, late stage of infection, i.e., on 32 and 41 dpi; ○——○, control non-infected mice.
P values : a vs. a', $p<0.005$; b vs. b', $p<0.05$.

ities were also recognized in the high dilutions (more than 1 : 80 or 1 : 320) of sera from the control non-infected nu/+ mice or from the DV-infected nu/+ mice, respectively.

Neutralization kinetics of sera from mice treated with carrageenan or trypan blue

The carrageenan or trypan blue treatment depressed the neutralizing activities of sera from DV-infected nu/+ mice and enhanced the PNI activities of those from DV-infected nu/nu mice (Fig. 4).

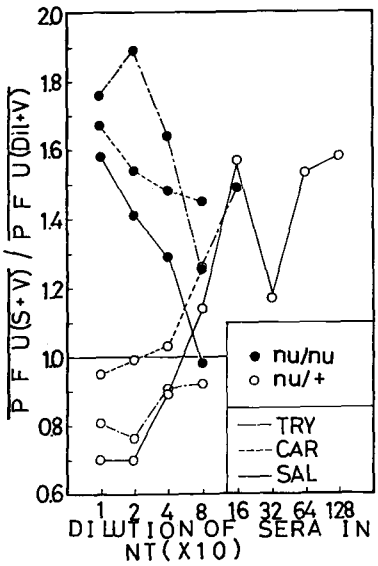

Fig. 4
NT of sera from DV-infected mice administered with macrophage-depressing agents. The test sera in this figure are identical to those in Fig. 1-B. The solid lines (——) refer to NT of test sera from saline-administered mice; the broken lines (----) to those from carrageenan-administered mice; and the semi-broken lines (— - —) to those from trypan blue-administered mice. Symbols : ●, nu/nu mice; ○, nu/+ mice.

Heat-stability of DV at 37 C

The DV diluted in the diluent used in NT was relatively stable when exposed to 37 C for 60 min, compared to the same virus in stabilizer-free diluent (Fig. 5).

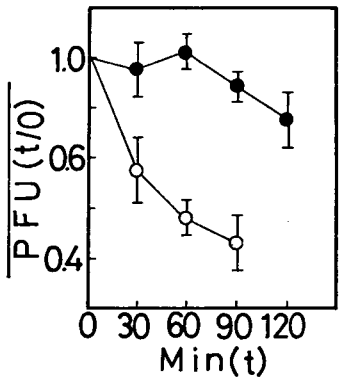

Fig. 5
Heat-stability of DV at 37 C. The bars represent standard deviations of the means; the mean at 0 min is considered as 1.0. Symbols for diluents used : ●, Eagle's MEM containing 0.2 % bovine albumin (BA); O, MEM without BA.

Comparison of anti-DV HI and NT titers

The sera which consisted mainly of IgG-type HI antibodies seemed to be weaker in neutralizing activity than those of IgM-type antibodies (Table 1).

Table 1 Comparison of HI- and NT-titers.

Day of serum collection[a]	Sample No.	Fraction of antibody[b]	Antibody titer[c] HI	Antibody titer[c] NT	HI/NT ratio	
11	1	IgM	40	40	1	
24	1	IgG	40	<10	4<	
	2		20	<10	2<	
32	1	IgG	40	<10	4<	5.2<[d]
	2		20	<10	2<	
41	1	IgG	160	10	16	
	2		80	25	3.2	

a The test sera are identical to those in Fig. 2.

b The immunoglobulin fractions were estimated by HI titers after 2-ME treatments as stated relative to Fig. 2.

c Both HI- and NT-titers of the same test serum were determined as described in MATERIALS AND METHODS and compared with each other.

d Mean HI/NT ratio of sera consisting mainly of IgG-HI antibodies.

DISCUSSION

In the present study, the patterns of immune responses of mice to DV-infection were investigated using carrageenan or trypan blue as the Mø-depressing agents (anti-Mø agents). Crude and partially purified antigens were obtained from infected tissue culture fluids and mice (+/+, nu/+, nu/nu and 'thymocyte-reconstituted' nu/nu) were used as experimental animals.

The data obtained under the present experimental conditions indicated: (i) In the +/+ or nu/+ mice, trypan blue depressed production of HI antibodies more strongly than did carrageenan (Figs. 1-A and -B); (ii) both HI- and NT-antibodies (regarded as IgG) were not detected in the nu/nu mice regardless of the treatment with anti-Mø agents (Fig. 1-B); (iii) both IgM- and IgG-HI antibodies were dependent upon Mø and thymus (Figs. 1 and 2); (iv) neutralizing ability of the IgG-HI antibodies was nil or lower than that of the IgM-HI antibodies (Fig. 2 or Table 1); (v) the reconstitution of nu/nu mice by 2.5×10^7 thymocytes had no or little effect on HI and NT antibody production (Fig. 2) but the neutralization kinetics in their sera were intermediate between those in the sera of nu/+ and nu/nu mice (Fig. 3).

The timing of treatment with anti-Mø agents and the doses of these agents prior to or after DV inoculation influenced the HI antibody titers in the sera and the amount of DV in the brains at the early stage of infection in mice (unpublished data). In brief, the pretreatment of even low doses of anti-Mø agents seemed to act on both the antigen-processing Mø and other scavenger Mø for eliminating the antigen-antibody complexes from the body. The post-treatment of even relatively high doses of anti-Mø agents seemed not to depress the antigen-processing Mø but to depress scavenger Mø. Regarding the event (i) stated above, the depressed antibody production seemed to be concerned mainly with the strong depression of the antigen-processing Mø due to the high dose of anti-Mø agents.

It has been reported that nu/nu mice infected via the footpad route with Sindbis virus (SV, a member of alphaviruses) produced anti-SV NT antibodies of IgM-type comparably to nu/+ mice[11] but the nu/nu mice infected ip with a high dose of DV (a member of flaviviruses) produced lower anti-DV NT antibodies of IgM type, compared to the nu/+ mice,[12] and that the nu/nu mice possess the highly-activated Mø.[11, 18, 20, 22] If the activation level of these Mø influenced the anti-DV antibody titers, the treatment of anti-Mø agents might enhance the IgM antibody titers. As

shown in the events (ii) and (iii), the IgM production did not occur. From the discussion mentioned above, the results concerning the post-treatment of anti-Mø agents appear to be interesting. However, the present experiments were done mainly by the pretreatment scheme. It seemed to be influenced more by the inoculum size (PFU) of DV or the route of infection whether or not the IgM-type antibody production occurred in the nu/nu mice.

Regarding the event (iv), the ratios of HI titer to NT titer of IgG antibody seemed to be more than 5 times than those of IgM antibody (Table 1). Supposedly if the identical molecule of antibody shows both HI- and NT-antibody activities, the data seemed to be interesting, since the IgM molecule is a pentamer, having five protruding arms, while the IgG molecule is a monomer.

The antibody-producing ability of nude or thymectomized mice can be resorted by treatments such as transfer of the spleen cells from thymus-bearing mice, injection of extracts from thymus or 'reconstitution of thymocytes', etc.[1, 15, 19]

Regarding the event (v), the anti-DV antibody production was not restored in nu/nu mice given 2.5×10^7 thymocytes 18 h prior to the virus inoculation (Fig. 2). It seems that more numbers of cells must be transferred and longer incubation period from cell transfer to virus inoculation is needed for the maturation of T cells, since 5-week-old nu/nu mice 'reconstituted' by 7×10^7 thymocytes 36 h prior to virus inoculation produced definitely positive HI antibodies on 42 dpi after receiving more amount of HAU (unpublished data). Under the fixed conditions, using the same age of mice, the same inoculum size and same lots of DV, further studies will be necessary to seek for optimal conditions for the restoration of immune responses of nu/nu mice to DV-infections.

The sera of the DV-infected nu/nu mice at relatively early stage of infection; i.e., on 7 to 24 dpi, seemed to possess the PNI activity, compared to the sera at relatively late stage of infection, i.e., on 32 and 42 dpi ($p<0.005$ in 1 : 10 dilution, $p<0.05$ in 1 : 320 dilution); the similar aspects were observed to the intact nu/nu mice ($p<0.10$ in 1 : 10 dilution) (Fig. 3). On the contrary, the 'thymocyte-reconstituted' nu/nu mice infected with DV seemed not to possess the PNI activity in 1 : 10 and 1 : 320 dilutions of sera in NT (Fig. 3) and the anti-Mø agents depressed the neutralizing activity of the sera from the DV-infected nu/+ mice and enhanced the PNI activity of the sera from the DV-infected nu/nu mice (Fig. 4). From these observations, the PNI activity seemed (i) to be induced in DV-infected mice, (ii) to compete with the neutralizing activity of sera

from DV-infected nu/+ mice, and (iii) to correlate with the disfunction of Mø of DV-infected nu/nu mice. The PNI activity seemed not to be due to the heat-decay of infective DV in virus-diluent mixture during 45-min incubation period at 37 C, since the DV was relatively stable at the 60-min exposure to 37 C in the diluent used in NT (Fig. 5). The slight PNI activity noted in the sera of intact mice (Fig. 3) seemed to be partially due to the stabilizing activity of the sera during the adsorption period in plaque-assay.

Unexpectedly, the highly diluted sera of the intact and DV-infected nu/+ mice possessed the PNI activity (Figs. 3 and 4).

A report has been presented on the DV-infection enhancement by addition of highly diluted non-neutralizing antibody in human phagocyte cultures[9] but, to the present author's knowledge, there is no report on PNI activities in sera of DV-infected nu/nu mice and in highly diluted sera of intact nu/+ mice, which were detected in NT tests by the BHK-21 monolayer culture techniques. The biological meanings of these PNI activities are still unknown.

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