Inoculation of Dengue Virus into Nude Mice

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SUMMARY

When athymic nude (nu/nu) and heterozygous littermate (nu/+ ) mice were injected intraperitoneally (i.p.) with a mouse-adapted strain of dengue virus (DV), the following differences were noted in the course of infection. (i) The average survival time of nu/nu mice was longer than that of nu/+ mice, although the mortality ratios were not significantly different. (ii) DV persisted in some of the nu/nu mice for long periods of time without exhibiting any symptoms but they died after prolonged incubation periods. These aspects were not observed in the nu/+ mice. (iii) Infected nu/nu mice produced IgM antibody only transiently in the early stage of infection but they did not subsequently show regular IgG antibody production which normally occurred in nu/+ mice. (iv) Piamatral and perivascular mononuclear cell infiltration in the infected brain was more intense in nu/+ than in nu/nu mice. It is suggested from these data that the course of DV infection in mice is affected by the availability of thymus-derived lymphocytes (T-cells).

Infectious virus was detected in various organs and tissues of infected mice. The hearts of nu/nu mice tended to show higher virus titres than those of nu/+ mice, whereas the virus concentrations in the brain, skeletal muscle and lymph node were the same in both groups of mice. Specific DV antigen was revealed by the fluorescent antibody (FA) technique in cells located in the infected tissues.

INTRODUCTION

Mouse-adapted strains of dengue virus (DV) cause an acute lethal infection in adult mice when injected intracerebrally (i.c.). However, they are almost harmless for the same animals when injected by the intraperitoneal (i.p.) route (Thind & Price, 1969; Tandon & Chaturvedi, 1977). Little is known about the behaviour of DV i.p.-inoculated into mice.

Athymic nude mice or mice lacking in thymus-derived lymphocytes (T-cells) have been used as an experimental model for microbial infections and the course of infection compared with normal mice is altered. Studies dealing with togavirus infections in the T-cell-depleted mice have been reported by several investigators, but the results obtained were not necessarily compatible with each other, as will be discussed later. In the present paper we describe the responses of athymic nude (nu/nu) mice to the i.p. inoculation of DV, comparing the data with those of heterozygous littermate (nu/+) mice which possess the thymus.

METHODS

Virus. Dengue type 1 virus, Mochizuki strain (Hotta, 1952), was used throughout. A 10% homogenate of infected suckling mouse brains was centrifuged at 10000 rev/min for 15 min at 2 °C and the supernatant was stored at −80 °C until ready for use. As a rule, aliquots from a particular lot were used in a series of similar experiments.
**Mice.** Four- to 6-week-old male Balb/c nu/nu and Balb/c nu/+ mice, bred under specific pathogen-free conditions, were obtained from Japan Clea Co., Osaka, Japan. Throughout the experiments these mice were housed under ordinary conditions. The virus (10⁷.⁸ p.f.u. in 0.5 ml) was inoculated i.p. into each of 5- or 6-week-old mice; a fivefold dilution thereof was similarly inoculated into each of 4-week-old mice. The infected mice were observed for the appearance of signs of infection, especially paralysis. Some mice showing no apparent signs were killed for control tests.

**Excision of organs.** At fixed times after the virus inoculation, each mouse was bled and the organs (axillary lymph node, thymus of nu/+, heart, lung, liver, spleen, kidney, skeletal muscle of the thigh and brain, in order) were removed. The excised organs were divided into two portions: one was immediately frozen at −80 °C and used for titration of virus while the other was subjected to histological and cytological observations.

**Titration of virus.** When ready for testing, portions of the organs were ground up in a mortar with Eagle’s minimum essential medium (MEM) supplemented with heat-inactivated calf serum (2%), sodium bicarbonate (1.2 g/l) and kanamycin (60 μg/ml). The organ suspensions were centrifuged at 10000 rev/min for 15 min at 2 °C and the supernatants assayed for virus by the plaque method using BHK-21 monolayer cultures under methyl cellulose overlay medium (Schulz & Schlesinger, 1963; Hotta et al., 1966).

**Histological and cytological examinations.** The portions to be used for these tests were further divided into three parts. The first, fixed in formalin, was sectioned and stained with haematoxylin–eosin (HE) and/or periodic acid–Schiff (PAS). The second was frozen-sectioned with a cryostat and subjected to the indirect fluorescent antibody (FA) method using anti-DV rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG. The third was used for electron microscopic observations through procedures consisting of double-fixing in 2.5% glutaraldehyde and 1% osmium tetroxide, ultrathin sectioning and double-staining with uranyl acetate and lead.

**Titration of anti-DV antibodies.** The serum taken from each mouse at the time of organ excision was titrated for anti-DV neutralizing antibodies. Fifty percent plaque reduction neutralizing titres (PRNT₅₀) were determined by the method of Russell et al. (1967).

**RESULTS**

**Mortality ratios of infected mice**

Typical examples of the results obtained are shown in Fig. 1. Of the nu/+ mice, the final mortality ratios were about 40% and all the deaths occurred between 5 and 11 days after infection. In the case of nu/nu mice, the mortality ratios within 2 weeks after infection were not significantly different from those of the nu/+ mice; however, smaller numbers of the infected nu/nu mice were paralysed after 3 weeks or later, so that the overall mortality ratios at 4 weeks reached about 60%. Average survival periods of nu/nu mice dying within 2 weeks after infection were longer than those of nu/+ mice.

**Neutralizing antibody titres**

Fig. 2 shows titres of anti-DV neutralizing antibodies in the serum of the infected mice. Up to 7 or 8 days after infection, the antibodies possessed by nu/nu mice were lower in titre than those by nu/+ mice and were only transiently detected. The antibodies during those periods were mainly IgM, which was identified by inactivation with 2-mercaptoethanol (2-ME). Ten days and longer after infection, the nu/nu mice, either paralysed or asymptomatic, had no detectable antibodies. In contrast, the nu/+ mice showed antibodies at 3 weeks after infection, which were resistant to 2-ME treatment and therefore regarded as IgG.
Distribution of virus in infected mice

Fig. 3 indicates examples of the data on virus concentrations in various organs examined. Both in nu/nu and nu/+ mice, the brain showed the highest virus titre, usually about $10^8$ p.f.u./g. The skeletal muscle and heart tissues of nu/nu mice were the second highest, having virus titres of $10^4$ p.f.u./g or more. Replicating virus was detected also in the lymph node, lung, liver, spleen and kidney, although their titres varied from one sample to another. In a few cases, the virus appeared in the thymus of nu/+ mice, showing titres of about $10^3$ p.f.u./g. Viraemia was not detected in any animal. Generally, the hearts of infected nu/nu mice contained virus at significantly higher concentrations than that of nu/+ mice, while the virus titres in the brain, skeletal muscle and lymph node were practically of the same level in the nu/nu and nu/+ mice.

Of the nu/nu mice, no definite difference was noted in the virus distributions between individuals dying earlier and those dying after 3 weeks or later.

Histological and cytological findings

In the brains of paralysed mice, mononuclear cell infiltration foci were observed in pia mater and/or around small vessels; the changes were more intense in nu/+ than in nu/nu mice (Fig. 4). Specific DV antigen was detected in the cytoplasm of neurons by the FA technique, and DV particles by electron microscopic observations.

The skeletal muscle fibres exhibited the specific FA reaction in the sarcoplasmic reticulum, with little fluorescence in the myofibrils. Cells in the connective tissues of the skeletal and cardiac muscle also revealed the specific fluorescence in the cytoplasm. Kupffer cells of the liver exhibited acidophilic hyaline degeneration and reacted to the specific anti-DV fluorescent antibody.

In both nu/nu and nu/+ mice, the spleens of paralysed mice atrophied; the average weight in paralysed mice (76 ± 23 mg) was less than that of uninfected controls receiving i.p. injection of the diluent (177 ± 52 mg).

No distinct haemorrhagic changes were found in either nu/nu or nu/+ mice injected with DV.
DISCUSSION

Although suckling or weanling mice can be infected by i.p. injection of highly mouse-adapted strains of DV, the response of adult mice is variable. We examined the responses of nu/nu mice to an i.p. inoculation of DV, comparing the data with those of nu/+ mice. While the mortality ratios of nu/nu and nu/+ mice were not significantly different from each other, the patterns of infection differed as follows: (i) nu/+ mice died earlier than the
nu/nu and (ii) some of the nu/nu mice retained DV in their bodies for a longer time without exhibiting any symptoms and died after prolonged incubation periods. The latter were positive for infectious virus and virus antigens.

There are two possible explanations for the prolongation of the average survival time in nu/nu mice: (i) the injury of the brain due to DV infection in normal mice is reinforced by mechanism(s) that can be regarded as cell-mediated immunity (CMI). The fact that the piamatral and perivascular mononuclear cell infiltration in DV-infected brain was more intense in nu/+ than in nu/nu mice can be related to this concept; (ii) the prolonged survival of infected nu/nu mice may be due to the activities of macrophages which are thought more effective for defence against infection during the initial stage of infection (Rodda & White, 1976). Moreover, it has been reported that macrophages of nu/nu mice are more effective in combating infection than those of nu/+ mice (Cheers & Waller, 1975; Rao et al., 1977; Emmerling et al., 1977; Mogensen & Andersen, 1978).

Woodman et al. (1975) reported that treatment of mice with anti-thymocyte serum (ATS) prolonged the average survival time of mice receiving a lethal infection of Venezuelan equine encephalitis virus (VEEV), an alphavirus. However, data have been presented that virulent strains of VEEV killed nu/nu mice faster than nu/+ mice (LeBlanc et al., 1978), and that brain lesions produced by infection with Semliki Forest virus (SFV), another alphavirus, developed earlier in nu/nu than in nu/+ mice (Bradish et al., 1979). According to Chaturvedi et al. (1978), the ATS treatment did not reinforce DV infection in i.c.-inoculated mice. These results conflict with our own and further investigations are needed to clarify the mechanism of prolonged survival.

In our experiments, DV persisted in nu/nu mice for long periods of time. Similar observations were reported by other investigators who showed that Japanese encephalitis virus (JEV) persisted in the lymph nodes of infected nu/nu mice in the absence of symptoms and in the presence of anti-JEV humoral antibodies (Sugano et al., 1978). These data suggest that CMI may play some role in exclusion of infecting virus.

In our histopathological examinations, there were apparent differences in response to DV infections between nu/nu and nu/+ mice. The piamatral and perivascular mononuclear cell infiltration in the infected brain was more distinct in nu/+ than in nu/nu mice. This may well suggest the participation of T-cells in normal inflammatory responses occurring in the murine DV infection.

The anti-DV antibodies produced in the nu/nu mice were of IgM type and lower in concentration than those of nu/+ mice, appearing only transiently in the early stage of infection. These aspects are compatible with those reported for VEEV (LeBlanc et al., 1978) or SFV (Bradish et al., 1979) in nu/nu mice. In contrast, data have been presented that neither nu/nu nor nu/+ mice showed detectable neutralizing antibodies at 7 or 8 days after primary i.p. infection of yellow fever virus (YFV), and that the nu/nu mice exhibited significant, though depressed, antibody activities at 2 to 5 weeks after infection (Bradish et al., 1980). These authors concluded that the course of antibody stimulation by alphaviruses and by flaviviruses was different and this was a result of differences in immunogenicity between virus components. However, the same authors noted that A2G mice infected with YFV produced neutralizing antibodies at 7 days after infection. Apparently, the course of infection differs with the mouse strain used. As far as our experiments are concerned, it is very likely that the production and maintenance of IgG antibody in DV-infected Balb/c mice are dependent on T-cells, whereas the transient production of IgM antibody is not.

The heart tissues of nu/nu mice showed significantly higher virus concentrations than those of nu/+ mice, while the virus titres in the brain, skeletal muscle and lymph node were of the same level both in the nu/nu and nu/+ mice. Pathophysiological conditions controlling particular virus—tissue affinity deserve further investigations.
It has been reported that infective DV was detected not only in the brain but also in the heart, liver, spleen (Chaturvedi et al., 1978) and skeletal muscle (Agrawal et al., 1978) of infected mice. However, the target cells in these organs were not completely investigated. In our own FA experiments, the DV-specific fluorescence was found in the skeletal muscle fibres, connective tissue cells of the skeletal and cardiac muscle, and Kupffer cells of the liver. From these observations, together with the results of virus titration, it is logical to assume that the virus replicated in these cells.

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REFERENCES


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