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Infections of Congenitally Athymic (Nude) and Normal Mice with Avirulent and Virulent Strains of Venezuelan Encephalitis Virus

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Two strains of Venezuelan encephalitis virus that are avirulent for normal BALB/c mice inoculated subcutaneously were also avirulent for infected congenitally athymic (nude) mice of the same strain. Viremias were of similar magnitudes and durations in normal and nude mice. Brain concentrations were higher in nude mice with the one avirulent strain tested, although the periods of detectable virus in brains were similar. No lesions were found in brains, spleens, or lymph nodes by ordinary histopathological examination. Viral neutralizing antibody titers in plasmas at 1 to 3 weeks after infection were lower and more transient in nude than in normal mice, and implantations of thymic tissues into nude mice partially restored their neutralizing antibody responses. Concentrations of spleen cells producing antibodies that lysed sheep erythrocytes 4 days after inoculation of erythrocytes and avirulent virus into nude mice were above the levels of uninfected nude mice. These concentrations were similar in infected and uninfected normal mice. In contrast, two mouse-virulent strains of Venezuelan encephalitis virus killed nude mice faster than normal mice after subcutaneous inoculation. Yet concentrations and durations of virus in bloods and brains were not consistently different between nude and normal mice. There were perivascular monocytes in brains and slight architectural alterations of spleens and lymph nodes. Concentrations of spleen cells producing antibodies hemolytic for sheep erythrocytes 4 days after inoculation with erythrocytes were depressed in nude and normal mice by infection with virulent strains.

Virulence as a measure of pathogenicity of an infectious parasite is a complex phenomenon that involves both parasite and host. Virus diseases often result from destruction of cells in which viruses replicate, but sometimes reactions of host cells participate in pathogenesis through the cellular inflammatory response, formation of antigen-antibody complexes, or stimulation of lymphocyte interactions with virus-infected cells (1, 2, 5, 6, 14, 23).

One approach to studying virus virulence has been to compare strains of virus that differ in virulence levels. A virus which provides a spectrum of virulent and benign strains and can be studied in small laboratory animal models is the alphavirus Venezuelan encephalitis (VE) virus of the Togaviridae family.

To evaluate the possible role of thymus-derived (T) lymphocytes in determining virulence of VE virus, avirulent and virulent strains were tested in congenitally athymic (nude) mice and compared with immunologically competent control mice of the same genetic background.

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MATERIALS AND METHODS

Viruses and virus assays. The two mouse-avirulent strains of VE virus used were BeAr35645 (Pixuna) in suckling mouse (SM) passage (p) 5, chicken embryonic cell culture (CEC) p1, and TC83, used as human vaccine, lot TC83/3-2L3. The two mouse-virulent strains used were 69Z1 in SM-p2, CEC-p1, SM-p1, CEC-p1, and 68U201 in SM-p1, CEC-p2 (10, 17, 19, 20). CECs were prepared as described elsewhere (16). Virus suspensions were fluids from CEC harvested 24 h after inoculation and incubation at 36°C. After centrifugation at 10,000 × *g* for 1 h at 4°C, supernatant fluids were diluted in 1% bovine serum albumin in Hanks solution (BA/H) with 100 U of penicillin per ml and 100 μg of streptomycin per ml and stored in an electric freezer at −60°C.

Virus suspensions and bloods and tissues from mice were titrated for infectious virus by counting plaque-forming units (PFU) in CEC; cells were in 8-cm^2 wells of plastic plates and were maintained under agar medium for 48 to 72 h at 37°C (18). Bloods and tissue suspensions were decimally diluted in BA/H with 2 U of heparin per ml.

Mice and observations. The original breeding nucleus of congenitally athymic (nude) mice which were heterozygous for the nude gene (+/nu) was kindly donated by S. P. Flanagan from his closed but not inbred colony at the Institute for Animal Genetics, Edinburgh, Scotland. Nude mice for experiments were derived from heterozygous breeders that had been backcrossed a minimum of six times to BALB/c mice, obtained from The Jackson Laboratory, Bar Harbor, Me. BALB/c mice from the same supplier also served as normal (+/+) controls. Animals were housed under ordinary conditions and given a standard diet.

Mice were inoculated subcutaneously (s.c.) with virus at 28 to 35 days of age. Uninfected nude and normal mice were housed separately from virus-infected mice. Mice were bled from the retro-orbital sinus under light ether anesthesia. They were killed by cervical dislocation. Organs for virus titrations were removed under sterile conditions and weighed, and 10% (wt/vol) suspensions were made in BA/H containing 2 U of heparin per ml, using mortars and pestles. Suspensions were centrifuged at 10,000 × *g* for 1 h at 4°C. Bloods and supernatant fluids of tissue suspensions were stored at −60°C until assayed for virus content.

Tissues for histological evaluation were fixed in 10% Formalin in phosphate-buffered saline for at least 1 day. Sections were stained with hematoxylin and eosin and examined under magnification of ×50 to 400.

Neutralizing antibody assays of plasma. For antibody titrations, whole blood was diluted in heparinized BA/H, and plasma was removed after centrifugation and stored at −20°C. Plasma was heated at 60°C for 20 min and diluted serially in BA/H with 2 U of heparin per ml, using separate pipettes for each dilution. The neutralizing antibody titer was the highest dilution of serum that reduced the PFU titer of an equal volume of virus suspension containing 2,000 PFU/ml by 90% or more.

Thymus implantation. Three-week-old nude mice each received an implant of a complete thymus from sex- and age-matched syngenic donors. Tissue was implanted s.c. over the rib cage. Virus was inoculated 2 weeks later. Only mice bearing macroscopically visible implants at the end of an experiment were considered successfully reconstituted.

Splenic anti-SRBC response in virus-infected mice. Mice were injected intraperitoneally with 0.01 ml of a 20% sheep erythrocyte (SRBC)-saline suspension per g of body weight and simultaneously with 1,000 PFU of virus per mouse s.c. After 4 days mice were killed by cervical dislocation, and spleens were assayed for anti-SRBC plaque-forming cells (PFC) by using the hemolysis-in-gel assay of Jerne and Nordin (9), as modified for slides by Mishell and Dutton (12). Plaque-forming activity was expressed as the number of PFC per 10^6 nucleated cells. Total numbers of viable nucleated cells in the spleen of each animal were also determined by counting in a hemocytometer with trypan blue to stain dead cells.

RESULTS

Lethality patterns of avirulent and virulent strains of VE virus in nude and normal BALB/c mice. VE virus strains that were avirulent for normal mice inoculated s.c. remained avirulent for nude mice. No deaths occurred in 40 nude and 35 normal mice within 30 days after s.c. inoculation of 1,000 PFU of strain BeAr-35645 (Pixuna) or in 36 nude and 30 normal mice inoculated similarly with vaccine strain TC83. Mice were infected by these avirulent strains because all of 40 nude and 35 normal survivors with strain BeAr35645 resisted s.c. challenge with 1,000 PFU of virulent strain 68U201, and all of 36 nude and 30 normal survivors with strain TC83 remained alive after challenge.

Two VE virus strains that were virulent for normal mice killed nude mice faster than normals (Table 1). For both strains, differences in mean times until death were significant by Student's *t* test and the two-sample rank test ($P < 0.01$). Normal mice infected with either virulent strain exhibited hind limb paralysis for at least 24 h before death. Nude mice showed paralysis for 6 h or less before death, and some nudes died without evident paralysis.

Viremia patterns of avirulent and virulent strains of VE virus in nude and normal mice. Mean titers and durations of virus in blood were similar in nude and normal mice with the two avirulent strains of VE virus (Table 2). Differences between nude and normal mice did not exceed 0.6 $\log_{10}$ PFU/ml with strain

TABLE 1. *Lethality patterns of two virulent strains of VE virus in nude and normal BALB/c mice*

Virulent strain	Mice		Cumulative percentage dead on day:[a]								Time of death (days)
	Type	No.	2[b]	3	4	5	6	7	8	9	
69Z1	Nude	20	0	5	35	90	100				4.7 ± 0.16[c]
	Normal	22	0	0	9	45	90	100			5.5 ± 0.17
68U201	Nude	48	0	0	4	16	69	100			5.9 ± 0.10
	Normal	50	0	0	0	8	44	82	90	100	6.6 ± 0.14

[a] A 1,000-PFU quantity of either 69Z1 or 68U201 was inoculated s.c. at zero time.
[b] Number of days after inoculation.
[c] Mean ± standard error of the mean.

BeAr35645. With strain TC83, the titer at 36 h after inoculation was 1.1 $\log_{10}$ PFU/ml greater in nude mice than in normal mice. Virulent strains of VE virus produced higher titers in blood than did avirulent strains in both types of mice, but differences between mean concentrations of virulent strains in nude and normal mice were not consistently in the same direction.

VE virus concentrations in brains of nude and normal mice. Benign strain BeAr-35645 became undetectable in blood of both types of mice by day 3 after inoculation, but virus was found in brain tissues through day 6, presumably a result of viral replication in brain (Table 3). Titers of this virus were higher in brains of nude than of normal mice, but the durations of detectable virus in brains were similar.

Virulent virus concentrations and durations in brains were similar in nude and normal mice (Table 3). Until day 2 after inoculation of virulent strain 69Z1 into either nude or normal mice, concentrations of virus in blood exceeded those in brain (Table 3). Thus, virus in brain during this time period could be attributed to contamination from blood. On days 2 to 6 after inoculation, virulent virus concentrations in brain were equal to or in excess of those in blood and thus reflected viral replication in brain tissues. On day 6, only three of six infected nude mice, but all six normal mice, were alive for titration of virus.

Histopathological studies of brains, spleens, and lymph nodes of nude and normal mice infected with avirulent and virulent strains of VE virus. Two nude and two normal mice were inoculated s.c. with 1,000 PFU of avirulent strains BeAr35645 or TC83 or virulent strain 69Z1, and brains, spleens, and lymph nodes were obtained 5 days later for histopathological examination. Avirulent strains BeAr35645 and TC83 produced no striking abnormalities in brains of either type of mouse. Brains from normal and nude mice infected with

TABLE 2. *Viremias produced in nude and normal BALB/c mice by two avirulent and two virulent strains of VE virus*

Virus strain	Mice		Mean of $\log_{10}$ PFU/ml of blood at:[a]								
	Type	No.[b]	8 h[c]	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
Avirulent BeAr35645 (Pixuna)	Nude	8	1.9	3.0	4.2	4.0	2.9	2.0	<1.4	<1.4	<1.4
	Normal	9	2.0	3.5	4.3	3.5	2.3	1.6	<1.4	<1.4	<1.4
Avirulent TC83	Nude	5		1.8	2.3	3.8	3.0		2.8	2.2	<1.4
	Normal	5		1.8	2.0	2.7	2.2		2.2	1.4	<1.4
Virulent 69Z1	Nude	5	3.3	6.8	6.4	5.5	5.6		5.6	4.1	
	Normal	5	3.5	5.5	6.2	6.9	5.2		6.0	4.1	
Virulent 68U201	Nude	6	1.4	3.1	5.6	6.1	5.3		4.6	3.6	2.5
	Normal	6	1.4	2.5	5.6	6.5	6.2		5.3	2.9	1.5

[a] A 1,000-PFU quantity of virus was inoculated s.c. at zero time.
[b] The same mice were rebled at each time indicated.
[c] Number of hours after inoculation.

TABLE 3. *Concentrations of virulent and avirulent strains of VE virus in brains and bloods of nude and normal BALB/c mice*

Virus strain	Mice		Tissue	Mean of $\log_{10}$ PFU/ml of blood or g of brain on day:[a]							
	Type	No.		0.3[b]	1	2	3	4	5	6	7
Avirulent BeAr35645 (Pixuna)	Nude	4–7	Brain		1.7	1.8	3.5	4.1	3.5	2.5	<1.4
		4–7	Blood		4.5	2.5	<1.4	<1.4	<1.4	<1.4	<1.4
	Normal	5–12	Brain		1.5	1.9	2.6	1.6	1.6	2.0	<1.4
		5–7	Blood		3.5	1.8	<1.4	<1.4	<1.4	<1.4	<1.4
Virulent 69Z1	Nude	3–6[c]	Brain	1.6	4.0	6.4	6.7	8.1	8.1	7.9	
		3–6	Blood	4.4	6.2	6.3	6.0	4.8	2.6	2.2	
	Normal	5–6	Brain	1.8	3.6	6.1	6.4	7.8	7.9	7.5	
		5–6	Blood	4.1	6.2	6.5	6.3	4.2	3.0	1.9	

[a] A 1,000-PFU quantity of virus was inoculated s.c. at zero time.
[b] Number of days after inoculation.
[c] Three nude mice died between days 5 and 6.

virulent strain 69Z1 showed perivascular mononuclear cells, perhaps with fewer cells in nude mice.

Infection with avirulent strains BeAr35645 and TC83 produced no striking lesions in spleens or lymph nodes of nude or normal mice. Spleens of nude and normal mice infected with virulent strain 69Z1 showed some disruption of the architecture of the white pulp and macrophages containing cellular debris. There was moderate loss of demarcation between cortical and medullary regions of lymph nodes in both types of mice.

Neutralizing antibody concentrations in plasmas of nude and normal mice infected with avirulent strains of VE virus. Nude mice had lower titers of neutralizing antibody than did normal mice after inoculation with either avirulent strain BeAr35645 or TC83 (Table 4). Moreover, the antibody titers in nude mice began to decrease 1 to 2 weeks after inoculation of virus. Yet both types of mice resisted s.c. challenge with 1,000 PFU of virulent strain 69Z1 at 32 days after inoculation of either avirulent strain.

Effects of thymic implantation into nude mice on blood and brain concentrations of avirulent strain BeAr35645 and on neutralizing antibody titers in plasma. Average viremia levels during days 1 to 3 after s.c. inoculation of 1,000 PFU of strain BeAr35645 into five nude mice with thymus tissue implanted 14 days previously were similar to those of seven control unimplanted nude and eight normal mice (Table 5). The mean titer of virus in brains at 5 days after inoculation of avirulent strain BeAr35645 into nine reconstituted nude mice was $10^{1.8}$ PFU/g. This titer was lower than the mean in unimplanted nudes ($10^{3.5}$ PFU/g), but like that of normal mice ($10^{1.6}$ PFU/g; Table 3).

Mean neutralizing antibody titers in plasmas of thymus-restored nudes were higher than those in nude mice (Table 5). On day 7 after inoculation, antibody titers of thymus-restored nudes were like those of normal mice, but on days 19 and 23 after inoculation, they were lower than those in normal mice.

Effects of virulent and benign strains of VE virus on antibody responses to SRBC in nude and normal mice. Nude and normal mice inoculated only with SRBC had similar numbers of nucleated cells per spleen, but nude mice had less than 0.1% of normal numbers of spleen cells producing anti-erythrocyte antibodies (Table 6). After inoculation of SRBC and avirulent strain BeAr35645 or TC83, numbers of nucleated cells per spleen did not change in either nude or normal mice. However, numbers of cells producing anti-erythrocyte antibodies increased significantly above normal in nude mice, although

TABLE 4. *Neutralizing antibody titers in plasmas of nude and normal BALB/c mice after inoculation with avirulent strains of VE virus*

Avirulent virus strain	Mice		Geometric mean of reciprocals of neutralizing antibody titers of plasmas on day:[a]							
	Type	No.[b]	5[c]	7	9	12	15	18	21	23
BeAr35645 (Pixuna)	Nude	6	16	20	12	5	<5	<5		5
	Normal	6	40	160	100	63	63	160		1,000
TC83	Nude	5	16	80	63	80	12	10	12	
	Normal	6	16	250	800	400	400	500	500	

[a] A 1,000-PFU quantity of virus was inoculated s.c. at zero time.
[b] The same mice were rebled at each time indicated.
[c] Number of days after inoculation.

TABLE 5. *Effect of thymus implantation on viremias and plasma neutralizing antibody titers of nude BALB/c mice inoculated with an avirulent strain of VE virus, BeAr35645 (Pixuna)*

Mice		Mean of log PFU of virus per ml of blood on day:[a]			Geometric mean of reciprocals of antibody titers in plasmas on day:		
Type	No.	1[b]	2	3	7[b]	19	25
Thymus-implanted nude[c]	5	3.9	3.0	<1.4	400	250	500
Nude	7	4.6	3.6	<1.4	160	10	10
Normal	8	3.8	2.7	<1.4	400	2,500	2,500

[a] A 1,000-PFU quantity of virus was inoculated s.c. at zero time.
[b] Number of days after inoculation.
[c] Nude mice were implanted with sex- and age-matched thymus tissue from syngenic donors 14 days before inoculation with virus.

TABLE 6. *Numbers of nucleated cells and anti-SRBC PFC in spleens of nude and normal BALB/c mice 4 days after inoculation with SRBC and virulent or avirulent strains of VE virus*[a]

Substances inoculated into mice[b]	Nucleated cells ($\times 10^6$) per spleen in:		PFC/10^6 nucleated cells in:	
	Nude mice	Normal mice	Nude mice	Normal mice
SRBC	158 ± 25[c]	149 ± 14	8.5 ± 0.2	1,100 ± 106
SRBC + avirulent strain BeAr35645	116 ± 15	163 ± 10	125 ± 14	1,000 ± 89
SRBC + avirulent strain TC83	182 ± 18	155 ± 16	105 ± 33	1,550 ± 245
SRBC + virulent strain 69Z1	35 ± 4	35 ± 4	1.5 ± 0.5	220 ± 45
SRBC + virulent strain 68U201	45 ± 9	50 ± 10	4.1 ± 0.9	30 ± 2.6

[a] Mice were inoculated at zero time with 1,000 PFU of virus s.c. and/or intraperitoneally with 0.01 ml of a 20% SRBC-saline suspension per g of body weight.

[b] Numbers of nude mice were 7 for SRBC or SRBC + strain 69Z1 and 6 for other inoculations; numbers of normal mice were 8 for SRBC, 7 for SRBC plus 69Z1, 6 for SRBC + 68U201 or TC83, and 5 for SRBC + BeAr35645.

[c] Mean ± standard error of the mean.

they remained unchanged in normal mice. After inoculation with SRBC and virulent VE strain 69Z1 or 68U201, numbers of nucleated cells per spleen decreased in both types of mice, as did numbers of spleen cells producing anti-erythrocyte antibodies (Table 6).

DISCUSSION

The results of this study show that the presence of a thymus is not essential to the processes that determine avirulence or virulence of VE virus in mice. Lack of a thymus and T cells only shortened incubation periods slightly for virulent strains, resulted in higher virus titers in brains after infection with an avirulent strain, and depressed viral neutralizing antibody responses to avirulent strains. Histopathological lesions were not greatly affected by the absence of thymus or T lymphocytes. As expected, there were fewer splenic cells producing anti-SRBC antibodies in nude mice than in normal mice, but, unexpectedly, the concentrations of these cells increased in nude mice (but not in normal mice) after infection with avirulent strains. Virulent strains decreased concentrations of these splenic cells in both types of mice.

The shortening of the incubation period in athymic nude mice infected with virulent strains of VE virus differs from the observation of Woodman et al., who reported that mean times to death of C3H HeJ mice treated with antithymocyte serum and inoculated with Trinidad strain VE virus were extended by 2 days (23). However, it is noteworthy that there are other differences between antithymocyte serum-treated and nude mice. For example, antithymocyte serum injections caused only a prolongation of allograft survival (from 10 days in untreated mice to 30 days in treated mice), whereas nude mice accept allografts and even xenografts indefinitely (11, 15).

The partial thymus dependence of the neutralizing antibody response of mice to avirulent strains of VE virus was consistent with other observations showing that antibody responses of mice to many viruses are T dependent (3, 13). Antibody responses of nude mice to avirulent VE strains were transient, like those shown by Burns et al. to represent immunoglobulin M (3). Inability of nude mice to maintain high levels of antibody can best be understood as failures to switch to an immunoglobulin G response. This pattern of an early immunoglobulin M response that fails to develop into an immuoglobulin G response was also described in nude mice inoculated with a T-dependent antigen, SRBC (4, 22).

The higher virus titers in brains of nude versus normal mice infected with an avirulent strain suggested that T cells participate in preventing maximal viral replication in brain. However, the observation that nude mice survived infection by avirulent strains despite higher than normal virus titers in brains and lower and transient neutralizing antibody responses indicated that virus levels in brain and early neutralizing antibody concentrations in plasma are not critical to resistance of mice to avirulent strains of VE virus. The outcome of a VE virus infection in mice, therefore, appeared to depend more on as yet undetermined virulence properties of the inoculated virus than on presence or absence of an intact T-cell system.

Infection of mice with TC83 was reported to correlate with increased clearance rates for colloidal carbon and aggregated bovine albumin, proliferation of reticuloendothelial cells, and

stimulation of the antibody response to SRBC, if infection preceded SRBC injection by 1 day (7, 8, 21). In view of these nonspecific immunostimulatory effects of attenuated VE virus, we compared numbers of nucleated spleen cells and concentrations of cells forming antibody against an unrelated antigen, SRBC, in nude and normal mice inoculated with virulent or avirulent VE virus. Virulent infection reduced the ability of both nude and intact mice to mount a response to SRBC. This reduction appeared to be primarily due to a loss of nucleated cells from the spleen. Because of the normally weak anti-SRBC response in nudes, depression of the response was much more dramatic in intact animals. On the other hand, infection with avirulent virus enhanced the response of nude mice, although this increase was noteworthy only relative to the low base-line PFC activity in nudes. The enhanced response in nudes was not related to a general increase in nucleated cells. It appears that nude mice are more susceptible to immunopotentiation by avirulent virus than are thymus-bearing mice.

When the activities of virulent and avirulent strains of VE virus in normal mice are compared, the results demonstrate a correlation among lethality of virulent strains and viremia levels of 100- to 1,000-fold higher or more, 1- or 2-day-longer durations of viremia, levels of virus in brain of 10,000-fold higher or more, perivascular monocytes in brain, and decreased numbers of nucleated cells per spleen and of cells producing anti-SRBC plaque-forming antibodies. Virulence of VE virus in normal BALB/c mice, therefore, seemed to be related to the extent of virus replication in tissues, including brain, the resultant destruction of antibody-producing cells, and inflammation of the brain. The rapidity of these processes (within 5 to 7 days) was compatible with direct cytopathic effects of virus, which were related to viral replication in target cells. However, the possibility of participation of viral antigen-antibody complexes and killer lymphocytes in the pathological processes cannot be excluded.

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