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Analysis of Age-Dependent Resistance to Murine Coronavirus JHM Infection in Mice

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Resistance to intraperitoneal murine coronavirus JHM infection in mice develops with age. C3H mice were found to be fully susceptible up to the age of 20 days and resistant after 23 days of age. Protection of susceptible animals from death due to infection could be achieved by maternal antibodies or by transfer of spleen cells from immunized, but not from nonimmunized, donor mice. Lack of protection by transfer of unprimed adult spleen cells was not related to immunosuppression by the host. Moreover, resistance of adult mice could not be abrogated by application of lymphocytes from suckling mice, although immune suppression by other means did affect the resistance of adult animals. On the other hand, spleen cells from nonimmunized mice could be primed with inactivated JHM virus in suckling mice and protected these mice from death due to a subsequent virus infection. Thus, the outcome of infection with JHM virus in suckling and adult mice can be influenced by immunological events, but is not exclusively due to the different stages of immune competence.

It has been observed that mouse hepatitis, herpes, sindbis, and other experimental neurotropic virus infections display an age-dependent course. Suckling animals appear to be more susceptible to these agents than adult animals (5, 7, 8, 10). This age-related development of resistance can be best seen after peripheral inoculation of the virus and is less frequent when routes such as intracerebral injection are used. The genetic background of the animals, metabolic and hormonal changes, inhibitory substances including interferon, and anatomical and immunological developments have all been claimed to be implicated in the development of resistance (6, 21). In many systems macrophages are assumed to play a crucial role, because they show increased restriction of virus replication in vitro with age, or could confer resistance when adoptively transferred from adult to young mice or both (24, and reviewed in reference 13). Moreover, the outcome of viral infections has also been shown to be dependent on the functional effectiveness of the immune system. Immune suppression or deficiency rendered resistant animals susceptible to infection (1, 3, 20, 27), and resistance could be bestowed upon susceptible animals by transfer of components of the immune system of resistant animals (11, 26).

In the present investigation the mechanism of the age-dependent development of resistance to murine coronavirus strain JHM in mice was studied. JHM virus is a neurotropic variant of mouse hepatitis virus. This virus strain has been associated with acute, subacute, and chronic central nervous system disease processes in rodents (15, 16). It has been observed that the development of these different central nervous system diseases depends not only on the biological properties of the virus strain, but also on the age of the animals (H. Wege, M. Koga, H. Wege, and V. ter Meulen, *JHM Infection in Rats as a Model for Acute and Subacute Demyelinating Disease*, in press). Virus infection shortly after birth always leads to acute disease, whereas the subacute or chronic form is seen when the animal is infected later in life. An understanding of the mechanisms responsible for age-dependent resistance might help to elucidate pathogenesis of these diseases.

We report here that after JHM virus infection in suckling mice the acquirement of resistance to intraperitoneal infection parallels in time the acquirement of immunological competence.

MATERIALS AND METHODS

Virus. JHM virus, a neurotropic strain of mouse hepatitis virus originally derived from suckling mouse brain (15), was propagated in the C3H mouse fibroblast cell line L 929. Cells were infected with JHM virus at a multiplicity of infection of 0.01. Nonadsorbed virus was removed after 1 h at 37°C, and fresh minimal essential medium containing 5% fetal calf serum was added. The supernatant containing 1×10^5 to 5×10^5 plaque-forming units (PFU) per ml was harvested after an incubation period of 15 to 20 h at 37°C and

stored at −70°C. The titer was evaluated by plaque assay on L 929 cells. After ultraviolet (UV) irradiation of such virus preparations containing 2×10^5 PFU/ml with 30.000 erg/mm^2, no residual infectivity could be detected by plaque assay. The virus was injected intraperitoneally (i.p.) in all experiments.

Mice. C57Bl/6 (B/6) and (B/6 × DBA/2) F_1 hybrid (BDF_1) were bred in our mouse colony from breeding couples obtained from Jackson Laboratories (Bar Harbor, Maine). C3H mice were purchased from Bomholtgart (Ry, Denmark).

GvHR in mice. The graft-versus-host reaction (GvHR) was induced by injecting 10^8 B/6 spleen cells in the tail vein of BDF_1 mice ($BDF_1 \leftarrow B/6$).

The expression "baby mice" is used for mice younger than 25 days; "adult mice" is used for mice older than 2 months.

All mice used were specific pathogen free and kept in laminar-flow hoods. Neutralization antibodies against JHM virus were not found in these mice before experimental manipulation.

Cells. Spleen cell suspensions were prepared in minimal essential medium containing 5% fetal calf serum and injected i.p. unless otherwise stated.

Normal spleen cells (NSC) were derived from nonimmune adult C3H mice; immune spleen cells (ISC) were derived from adult C3H mice which were immunized i.p. once with 10^5 PFU of JHM virus. Immune spleen cells were obtained 4 days, 14 to 30 days, or 50 to 100 days after immunization. Serum antibody titers as measured by a neutralization test (50% reduction of 100 PFU) were 1:16 at day 4 and 1:64 to 1:512 at days 14 to 100 after priming. All ISC populations were tested for the presence of infectious virus in vitro by plaque assay and in vivo by injecting the cells into susceptible baby mice. There was a strict correlation between the results obtained by either test.

Populations containing predominantly T cells were prepared by passing spleen cells through nylon wool columns as described previously (9).

Adherent peritoneal exudate cells were prepared by washing the peritoneal cavity with 3 ml of minimal essential medium containing 5% fetal calf serum. The cells were allowed to attach to the surface of plastic petri dishes for 2 h, and then nonadherent cells were removed by several washings. Adherent cells were harvested with a rubber policeman.

Design of experiments. To circumvent maturation differences between different litters experiments were set up in the following way: when differently treated groups were compared in an experiment in which more than one litter was used the animals of the different litters were dispensed in such a way that each experimental group was represented in each litter.

RESULTS

Pathological changes after JHM virus infection. Histopathological investigations revealed inflammatory changes mainly in the liver and central nervous system tissue. They consisted in the liver of nodular necrosis with mononuclear cell infiltrations and giant cell formations. Similar changes were also observed in mouse brain and spinal cord with the exception of giant cell formation.

Age and outcome of infection. C3H mice were infected with 20 PFU of JHM virus per animal on different days postpartum (p.p.). Table 1 shows a representative set of experiments. It demonstrates that infection up to 20 days p.p. was always lethal for the animals, leading to death within 4 to 8 days. Clinical disease, characterized by paralyzed hind legs and impaired balance, was observed 8 to 4 h before death occurred. Mice infected with 20 PFU on day 23 p.p. or later showed no clinical signs of illness and survived. Only when animals were infected at 21 and 22 days p.p. was an essentially intermediate result observed. Mice either shared the fate of the animals of one of the groups described above, or compared with the first group their death was slightly delayed. The data in Table 1 also demonstrate that there was no qualitative change of the results even when the dose of infection was increased 1,000-fold. Comparing different sets of experiments, the occurrence of resistance was shifted by maximally 2 days, probably reflecting the variation in the maturation of different litters. Mice older than 25 days were only occasionally affected by intraperitoneal JHM virus infection.

Effect of GvHR on the course of infection in adult mice. The resistance of adult mice to JHM infection could be affected by immunosuppression caused by a GvHR (18). Adult BDF_1 mice injected intravenously with 10^8 parental B/6 spleen cells developed a GvHR, and their resistance to intraperitoneal JHM virus infection was strongly impaired (Table 2). The rate of survival of infection was influenced by the

TABLE 1. *Age and dose dependence of the outcome of JHM virus infection*

Infection (i.p.)		Survivors/total group	Time of death (days postinfection)
Days p.p.	PFU per mouse		
14	2×10^1	0/10	4 to 8
	2×10^2	0/10	4 to 6
	2×10^3	0/5	4 to 5
16	2×10^1	0/6	5 to 6
18	2×10^1	0/5	6 to 8
20	2×10^1	0/5	5 to 7
21	2×10^1	3/5	8 to 11
22	2×10^1	7/9	9
	2×10^2	3/4	7
	2×10^3	2/3	7
	2×10^4	1/3	7
23	2×10^1	7/7	
24	2×10^1	8/8	
	2×10^2	3/3	
	2×10^3	1/2	7
	2×10^4	4/7	7 to 11
25	2×10^5	49/50	18

TABLE 2. *Course of infection in adult mice undergoing GvHR*

Mouse strain	Infection (i.p.)		Survivors/total group	Time of death (days postinfection)
	PFU/mouse	Days after induction of GvHR		
BDF_1	10^5	No GvHR	6/6	
B/6	10^5	No GvHR	6/6	
$BDF_1 \leftarrow B/6$[a]	10^5	4	4/7	9 to 13 (11)[b]
	10^5	7	2/14	11 to 18 (12.3)[b]
$BDF_1 \leftarrow B/6$[a]	No infection		10/15	Survived GvHR, 5 of 15 died of GvHR 27 to 32 days after induction[c]

[a] GvHR was induced by intravenous injection of 10^8 NSC of B/6 mice into BDF_1 mice.
[b] Numbers within parentheses indicate averages.
[c] Mice dying of GvHR succumb after several days; death of JHM virus infection occurs within hours.

period between GvHR induction and infection. More animals survived when the virus was administered in the early phase of the GvHR. Death caused by GvHR could be easily distinguished from death due to infection by the clinical course. Animals died of GvHR approximately 30 days after induction and exhibited the symptoms of "wasting disease" for several days before death, whereas clinical signs as a result of virus infection occurred only hours before death.

Effect of anti-JHM antibodies and JHM-immune lymphocytes on the course of infection in baby mice. As resistant adult mice were rendered susceptible by immunosuppression the question was asked whether lack of a competent immune system during ontogeny is responsible for the fatal outcome of JHM virus infection in suckling mice. To investigate this question two experiments were performed. First, female mice were immunized before mating. Their offspring, which carried maternal antibodies and continuously received antibodies by suckling, were consequently found to be well protected against infection with various doses of JHM virus (Table 3). Secondly, a similar protection was observed after i.p. injection of spleen cells from immunized adult mice (ISC) into suckling mice (Table 4). The majority of 14-day-old mice could be protected from death due to infection. Donors of the ISC were adult mice which had been immunized for various periods with 10^5 PFU of JHM virus per animal. Protection by these cells did not depend on the priming period of the immune mice, as resistance could be mediated by ISC from donors primed for 4 days as well as for 100 days, provided their spleens were free of JHM virus. Therefore, experiments with ISC were only evaluated when the spleen cell population did not carry infectious virus. The results of these parallel control experiments are also shown in Table 4. ISC were inoculated in animals which did not receive subsequently infectious virus, and the occurrence of death of JHM infection as the consequence of infected ISC was recorded. All samples of ISC primed for 50 to 100 days, all but one sample of ISC primed for 14 to 30 days, and 5 out of 12 populations of the samples of ISC primed for 4 days were negative for infectious virus. Consequently, in the last group only experiments with those five populations which obviously had already successfully cleared the virus were counted.

TABLE 3. *Influence of maternal antibodies against JHM virus on the course of infection in suckling mice*[a]

Infection		Survivors/total group	Time of death (days postinfection)
Days p.p.	PFU/mouse		
16	2×10^1	3/3	
	2×10^2	5/5	
	2×10^4	5/5	
14	2×10^1	14/15	6
6–8	2×10^1	7/7	

[a] Mothers were given 10^4 PFU of JHM virus i.p. at 6, 5, and 4 weeks before delivery.

ISC, when transferred 2 to 3 days before or 1 day after infection, provided protection as effectively as simultaneous application of virus and cells. They failed, however, to protect when given 2 days after infection.

Immune T cells, or immune T cells to which adherent peritoneal cells from immune mice were admixed, did not confer protection.

Priming of suckling mice with UV-inactivated JHM virus. The possibility was tested whether suckling mice could be protected from lethal infection by active immunization with UV-inactivated JHM virus. Baby mice were injected with UV-inactivated JHM virus and then at different times with 20 PFU of infectious JHM virus. Application of UV-inactivated virus alone did not cause any clinical disease (Table

TABLE 4. *Effect of immune spleen cells on JHM virus infection in baby mice*

Time of infection with 20 PFU/mouse (days p.p.)	ISC transfer (days p.p.)	Priming period (days)	No. of ISC transferred	Survivors/total group	Time of death (days postinfection)
14	14	50 through 100	3×10^7	2/3	7
			1×10^7	6/8	7
16	14		3×10^7	3/5	7 to 8
	16		3×10^7	3/3	
	17		3×10^7	3/3	
	18		3×10^7	0/5	6 to 8
14	14	14 through 30	3×10^7	8/9	11
			1×10^7	2/3	8
			3×10^6	0/4	5 to 6
			3×10^{7a}	0/4	6 to 8
			3×10^{7b} + 1×10^{7b}	0/4	7 to 8
15	12		3×10^7	3/4	7
14	16		3×10^7	0/4	6 to 7
14	14	4	3×10^7	8/8	
No infection	14	50	3×10^7	5/5	
	14	14 through 30	3×10^7	6/7	8
	14	4	3×10^7	5/12	5 to 9
14				0/12	4 to 8

[a] T cells.

[b] T cells (3×10^7) plus 1×10^7 adherent peritoneal exudate cells.

5). Application together with infectious virus to increase the antigenic stimulus did not interfere with the development of the acute disease. When the inactivated virus was given before infection and the animals were infected at the age of 14 to 16 days, only a few survivors were observed. When 18- to 20-day-old mice were infected, pretreatment with UV-inactivated JHM virus slightly enhanced the rate of survival. Thus, sufficient priming of the babies' own lymphocytes to protect the animals did not occur in 2-week-old animals, but cannot be excluded in 3-week-old mice.

Influence of spleen cells from nonimmune adult mice on the course of infection in baby mice. Suckling mice could not be protected from death due to infection by their own immune system, but they could be protected by anti-JHM antibodies or by ISC derived from adult mice. We investigated whether the transfer of nonimmune mature lymphocytes was also sufficient for protection. Spleen cells from adult (>2-month-old) nonimmunized mice (NSC) were used as a source of mature lymphocytes. They were injected i.p. into suckling mice at various times before infection. When these animals were infected at the age of 14 to 16 days (Table 6) the transfer of NSC in general did not prevent the lethal outcome of the infection. It made no difference whether the cells were transferred 2 or 4 days before infection. Transfer together with the virus afforded slightly better protection, probably because the injected spleen cells still remained at the site of virus inoculation and thus encountered the virus at a higher concentration. In contrast, when the mice were infected at the age of 18 to 20 days and supplied with NSC at the same day or 2 to 4 days before infection their chances of survival improved. The lack of protection of 14-day-old mice by

TABLE 5. *Effect of UV-inactivated JHM virus on JHM virus infection in baby mice*

Time of infection with 20 PFU/mouse (days p.p.)	Application of UV-JHM (days before infection)	Survivors/total group	Time of death (days postinfection)
14 to 16	0	0/10	5 to 10
	2	1/8	4 to 8
	4	0/6	4 to 9
19 to 21	0	3/4	11
	1 to 2	6/10	6 to 13
	3 to 4	5/17	4 to 12
	5 to 6	6/8	5 to 13
14 to 16		0/15	5 to 8
19 to 21		6/18	5 to 12
No infection	14 to 16[a]	7/7	
No infection	19 to 21[a]	5/5	

[a] Days p.p.

TABLE 6. *Effect of NSC on JHM virus infection in baby mice*

Time of infection with 20 PFU/mouse (days p.p.)	Cell transfer (days before infection)	No. of NSC transferred	Survivors/total group	Time of death (days postinfection)
14 to 16	0	6×10^7	1/6	5 to 8
		3×10^7	3/8	5 to 10
	2	6×10^7	1/8	5 to 11
		3×10^7	1/10	5 to 8
	4	6×10^7	0/9	6 to 12
		3×10^7	0/5	5 to 7
18	2	3×10^7	2/4	5 to 6
	4	3×10^7	2/4	4 to 5
20	0	3×10^7	5/5	
14 to 16			0/20	4 to 12
18			0/6	5 to 11
20			0/5	6 to 15

NSC could be due to suppression exerted by the host on the transferred NSC. However, injection (intravenous or i.p.) of 1×10^8 to 4×10^8 lymphocytes from 14-day-old mice into adult mice did not abolish the resistance of these animals to JHM virus infection. None of 18 injected mice died (data not shown). The possibility of suppression of adult NSC in the baby host was further tested in the following way. UV-inactivated JHM virus was injected into 12- to 14-day-old babies together with spleen cells from nonimmunized adult mice at different times before challenge with infectious virus (Table 7). When more than 10^7 NSC were transferred over 50% of the baby mice were protected from death due to a subsequent infection. It made no difference whether the mice were challenged with infectious virus 2 or 4 days after cell transfer. Thus, it was possible to prime NSC from adult mice in baby mice.

DISCUSSION

The age-dependent development of natural resistance to JHM virus infection in mice is an all-or-none effect and does not develop gradually over a longer period or by showing clinical symptoms of changing severity. Mice of the C3H strain were chosen because they are genetically not resistant (23), yet under the conditions used—namely, i.p. injection of up to 10^5 PFU of JHM virus—they are not harmed by JHM virus infection once they have reached a certain age. Thus, a system was chosen in which virus replication was possible, independent of the age of the animals at the day of infection. It was assumed that the age-related outcome of the infection—namely, whether the mice survived or died of it—was dependent on the ability of the animals to confine and overcome the infection. Various defense mechanisms are probably involved, one of them being the immune system. As the development of competence of the immune system in mice (22) parallels in time the development of resistance to JHM virus infection it is suggested that this defense mechanism plays a major role in overcoming the infection.

It could be shown that babies from immunized females resisted the infection, and mice also withstood an infection when they were given unseparated ISC, but not when immune T cells alone or in combination with adherent peritoneal cells were transplanted. Further, spleen cells from nonimmunized adult animals were not protective. Thus priming of the transplanted lymphocytes and the presence of B cells was a prerequisite for protection. Yet, it seems to be inconsistent that the application of nonimmune spleen cells from immunocompetent donors did not confer protection, whereas primed lymphocytes were obviously not inhibited to exert their function. This observation suggested that an interference with the process of antigen-induced immune response of the injected unprimed spleen cells took place. One explanation of this phenomenon could be immunosuppression by lymphocytes from neonates as has been shown in several other systems (2, 14, 19). However, simultaneous administration of unprimed spleen cells and inactivated virus protected against death from subsequent infection, indicating that lack of protection by nonimmune spleen cells alone is not the result of immunosuppression by the host. Moreover, the resistance of adult mice to JHM virus infection could nonetheless not be abolished by injection of lymphocytes from suckling mice. On the other hand, adult animals undergoing a GvHR which leads to profound

TABLE 7. *Priming of NSC from adult mice in baby mice*

Time of infection with 20 PFU/mouse (days p.p.)	Cell transfer and application of UV-JHM (days before infection)	No. of cells transferred	Survivors/total group	Time of death (days postinfection)
14 to 16	2	6×10^7	4/7	5 to 8
	2	3×10^7	6/13	5 to 9
	2	1×10^7	3/10	7 to 9
	3	6×10^7	2/3	7
	3	3×10^7	8/12	4 to 10
	4	6×10^7	6/7	5
	4	3×10^7	9/14	7 to 8
	4	1×10^7	5/10	4 to 6
	4	3×10^6	0/5	5 to 6
14 to 16			0/20	4 to 9
No infection	14 to 16[a]	6×10^7	5/5	

[a] Days p.p.

suppression of the humoral immune response (12, 17) have a reduced chance to survive an infection. It has been shown that GvHR proceeds through two phases (18), an early one in which both stimulation and suppression of antibody production occur simultaneously and a later phase in which only suppression is left. Our observation that the impairment of resistance was influenced by the period between GvHR induction and commencement of infection was therefore of interest in that resistance was preferentially abolished during the later phase. These results revealed that adult mice, which normally can cope with a JHM infection, can be rendered susceptible by immunosuppression.

From the results described above it is clear, however, that the different course of infection with JHM virus in suckling and adult mice cannot only be attributed to the different stages of immunocompetence. It is well known that dissemination of virus in the adult animal can also be prevented by non-immunological defense mechanisms which might not yet be fully developed in a baby mouse. Findings reported by Taguchi et al. (25) for the interferon system would be consistent with these interpretations. By comparing suckling and weanling mice, these authors showed that, after infection, the production of interferon was delayed in suckling mice. In addition, target cells for JHM virus might change during development. Baby mice might have relatively more target cells for JHM virus than adult mice, and the change during maturation might reflect a reduction of target cells by loss of virus receptors or by alteration of the cellular competence for virus replication. An age-related conversion from susceptibility to resistance has been shown for infection of mouse fibroblasts with Sindbis virus (8) and for infection of mouse macrophages with mouse hepatitis virus 2 (4). In addition, the relevant cells—namely, the potential target cells—might change with respect to their importance for the organism.

Different findings have been reported for intracerebral JHM virus infection in the genetically resistant mouse strain SJL (24). Resistance could be transferred by unprimed spleen cells from 3-month-old mice to 6-week-old animals. Thus, different mechanisms have to be considered for the age-dependent development of resistance to JHM virus infection, depending on the route of virus application, on the age, and on the genetic constitution of the animals.

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