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Antibody responsiveness during immunization and challenge of genetically modified antibody responder mice with murine hepatitis virus 3

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Abstract

The aim of this study was to evaluate some immunological patterns involved in natural and acquired resistance against MHV3 using the original model of genetically modified lines of mice selected for high (H_{III}) and low (L_{III}) antibody responsiveness. As previously shown, a lower pre-existing anti-MHV antibody level was found in susceptible H_{III} mice as compared to resistant L_{III} mice. Mortality rates of the F_1 ($H \times L$) hybrids and F_2 and backcross segregants reflected co-dominance of both characters and the survivors had higher pre-existing anti-MHV antibody titers. The present data show that both lines had the potential to synthesize antibodies and that the resistance acquired by the susceptible H_{III} mice paralleled the antibody synthesis. Nevertheless, higher antibody titers were necessary to confer resistance in H_{III} mice than in L_{III} ones. When compared to uvMHV3, a single immunization with a related infectious MHV strain induced a higher antibody synthesis and led the H_{III} mice to resist the MHV3 challenge. A direct correlation between the antibody level and resistance to infection was always observed in H_{III} mice. Although mounting a Th2 response as indicated by IgG1 responses, they were also able to readily synthesize large amounts of IgG2a antibodies after immunization or during infection, reflecting a Th1 response. The transfer of anti-MHV antibodies to susceptible H_{III} mice was capable of conferring resistance to MHV3, providing the antibodies were present before virus infection and in large amounts. The resistance and the survival time of these animals increased with the level of antibody administered. If these direct and clear data suggest that H_{III} mice can acquire resistance through antibodies, the basis of the resistance of the resistant L_{III} mice may rely on mechanisms less dependent on antibodies.

Introduction

Murine Hepatitis Virus (MHV) species of the genus *Coronaviridae* are widespread and found endemically in mouse colonies worldwide (Rowe et al., 1963; van der Riet & Kahn, 1973; Hierholzer et al., 1979; McIntosh, 1985). In these contaminated colonies most of the animals show no clinical signs

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but have serum antibodies against different types of MHV, such as MHV3, which was isolated by Dick et al. (1956) and has been used as a model of viral infection.

Mouse resistance to an experimental MHV3 infection varies according to the genetic background of the mouse strain and the environmental conditions of the colony in the animal facility (Lamontagne et al., 1989; Lucchiari & Pereira, 1989; Lucchiari et al., 1991; Pope et al., 1995). Several studies using inbred lines of mice reported that A/J mice show an innate resistance and BALB/c mice an innate susceptibility to MHV3 experimental infection. It was also shown that in coronavirus-free colonies, the A/J mice could rapidly acquire resistance through natural or experimental infection with other strains of MHV and BALB/c mice do not (Lucchiari et al., 1991).

Genetically heterogeneous lines of high- and low-responder mice, which better represent a natural population, are useful for the determination of genetic and immunological parameters of general and specific immune responsiveness. Homozygosity for the high or low production of antibodies was attained by bi-directional selective breeding after several generations and in addition to differences in their responses to the selection antigen, the animals also show high or low responsiveness to non related immunogens (Siqueira et al., 1976; Nilsson et al., 1979; Ibanez et al., 1988; de Franco et al., 1996). As previously stated and recently expressed in an editorial (Boyartchuk & Dietrich, 2002), the advantage of this experimental model in relation to inbred lines of mice is the availability, in the H and L lines, of genetically homogeneous relevant loci controlling the general character, that allow the study of correlations between distinct immunobiological parameters. We have shown that the selected multi-specific immune response genes may act on a virus-specific immunity, as in a study of rabies infection where the selected high and low lines exhibited, respectively, a high or low degree of resistance to rabies challenge following specific vaccination, correlating with the high or low specific antibody responsiveness (Nilsson et al., 1979).

In previous studies with heterogeneous genetically selected mouse lines (Vassão et al., 1993, 1995) we demonstrated that the H_{III} mice are fully susceptible and the L_{III} mice are resistant to an experimental infection with MHV3. Although the resistance/susceptibility is not influenced by the route of virus inoculation, animals infected subcutaneously (sc) show a mean survival time (mst) twice as high in comparison to animals infected intravenously (iv) or intraperitoneally (ip) (Vassão et al., 1994a). These findings indicated that resistance against MHV3 depends on both genetic and environmental factors. These studies showed a direct inter- and intra-populational correlation between the pre-existing antibody titers against MHV3 and the mortality rate (Vassão et al., 1994b).

Pope et al. (1996), working with genetically homogeneous mouse lines, have shown that after MHV3 immunization, the resistant A/J mice mount preferentially a Th1 cellular immune response and the susceptible BALB/c mice preferentially a Th2 one, as evaluated by the ability of primary cell lines to produce a cytokine profile consistent with Th1 or Th2 phenotype (IL-2 and IFNγ or IL-4 respectively). Their experiments, designed to alter resistance/susceptibility by neutralizing *in vivo* the Th1 cytokine IFNγ in A/J mice or the Th2 cytokine IL-4 in BALB/c mice, led to loss of resistance among A/J mice but failed to improve survival among BALB/c mice.

Altogether, the MHV3 resistance studies performed with genetically homogeneous (A/J and BALB/c) or with genetically heterogeneous (H_{III} and L_{III}) mice suggest that the virus replication in the target cells, the antiviral interferon action, and the expression of a monokine with pro-coagulant activity, as well as the antibody response, are implicated in the resistance/susceptibility of individuals and populations to the MHV3 infection (Taguchi et al., 1980; Pereira et al., 1984; Nakanaga et al., 1986; Lecomte et al., 1987; Barthold & Smith, 1989; Lamontagne et al., 1989; Lucchiari & Pereira, 1989; Lamontagne & Jolicoeur, 1991; Lucchiari et al. 1991, 1992; Vassão et al., 1993, 1994a, b, 1995; Lamarre & Talbot, 1995; Gustafsson et al., 1996; Pope et al., 1996). One of the main pieces of evidence concerning resistance mechanisms is that, although submitted to the same environment, only some mouse strains (A/J and L_{III}) easily acquired resistance (Lucchiari et al., 1991; Vassão et al., 1994b, 1995). Efforts towards alterations of the mouse resistance/susceptibility pattern often fail to protect susceptible animals but easily induce a loss of resistance among resistant ones (Lucchiari et al., 1992; Pope et al., 1996). These findings suggest that the development of resistance is based on different mechanisms, which account for defining resistant and susceptible strains.

The present paper shows a comparative study of development of resistance against MHV3, focusing on the humoral immune response, in natural and experimental conditions. The participation of antibodies in the acquisition of resistance by mice genetically modified for high or low humoral

responsiveness was evaluated after active or passive immunization.

Materials and methods

Viruses and mice

MHV3 cultivated and titled by plaque assay on L929 cells at 37 °C were cloned by limiting dilution. One plaque was selected and amplified on L929 cells to serve as the inoculum for future stock (Martin et al., 1988), in order to limit spontaneous mutations. Aliquots containing 2×10^5 PFU/ml were stored at -80 °C and used in the experiments. MHV4 and MHVA59 strains were also cultivated and titled by plaque assay on L929 cells and stored in aliquots with 1 to 5×10^5 PFU/ml at -80 °C. Short-wave ultraviolet (UV) irradiation was used to inactivate the previously titled MHV3 stocks (uvMHV3), and was accomplished by exposing a thin layer of virus in a glass Petri dish within an ice bath to a GTE 30W germicidal lamp, at a distance of 15 cm for 10 minutes (Hanson, 1983). Each virus preparation submitted to inactivation was assayed by intraperitoneal (ip) inoculation in susceptible BALB/c mice, which were observed daily for mortality.

High and low antibody responder mice of both sexes from Selection III (H_{III} and L_{III}), obtained by selective breeding based on responsiveness to flagellar antigen of *Salmonellae* (Siqueira et al., 1976), were used at ages ranging from 8 to 12 weeks. Reciprocal interline crosses of ($H_{III} \times L_{III}$) F_1 hybrids, F_2 segregants and backcrosses (BcH_{III} and BcL_{III}) were bred in the animal unit of the Laboratório de Imunogenética, Instituto Butantan. Animals were periodically sacrificed and peritoneal exudate, serum and liver tissue samples obtained. No animal was found to have MHV in the liver or in the peritoneal exudate. However, all the animals had antibodies against MHV. The antibodies against MHV were shown to express neutralizing activity as measured by the *in vitro* MHV3 neutralization assay (Vassão et al., 1994b). The laboratories involved in the work have ethical approval for animal experimentation.

Anti-MHV antibody assays

Mice were bled from the retro-orbital venous plexus. Individual anti-MHV titers determined by serum neutralization assays in microtitre plates and reported as $\times \log_2$ ($\pm$ standard deviation) (sd), were expressed as the reciprocal of the highest serum dilution giving a 100% inhibition of the cytopathic effect induced by MHV3 on L929 cells. The individual IgG1 and IgG2a isotypes were measured by ELISA test. 96 well microplates (Nunc) were covered with MHV3 in PBS buffer overnight at 4 °C. The remaining uncoated sites were blocked by incubation for 3 hours at room temperature with blocking buffer (PBS/3% gelatin). Several dilutions of serum samples in ELISA buffer (PBS/0.5% gelatin) were added to the plates. Controls included diluents, serum from an unvaccinated animal and serum containing antibodies against MHV3. After 18 hours at 4 °C the wells were then washed. Biotin-conjugated anti-mouse IgG1 monoclonal antibody (A85–1) and IgG2a monoclonal antibody (R19–15, PharMingen) (8 μg/ml) were added and the plates were incubated for 1 hour at room temperature. After washing, avidin-alkaline phosphatase (Sigma) in ELISA buffer was added and the plates were incubated for 1 hour at room temperature. After washing, the reaction was developed for 30 minutes at room temperature with p-Nitrophenyl Phosphate tablets (Sigma) in diethanolamine buffer pH 9.8. Absorbance was measured at 405 nm and antibody titer was defined as the reciprocal of the highest serum dilution at which the absorbance was equal to three times the background value. Data were expressed as $\times \log_2$ with the respective variance coefficient among individual samples (Sant'Anna et al., 1991).

Immunization and challenge protocols

For the correlation between resistance/susceptibility to MHV3 and pre-existing anti-MHV antibody levels, mice were first identified and bled from the retro-orbital venous plexus for the evaluation of antibody level in their sera, and 1 hour later infected subcutaneously (sc) with 10^3 PFU of MHV3. The mortality rates after 30 days were recorded and the number of survivors or dead mice expressed and analyzed as a function of the anti-MHV antibody titer present in their sera before the experimental infection.

The correlation between the phenotypes *resistance/susceptibility* to MHV3 and *antibody responsiveness* to uvMHV3 was determined. For infectious related MHV strains, mice were ip immunized with 10^3 PFU of uvMHV3 (corresponding to the titer determined before UV-inactivation) or with related MHV (MHV4 or MHVA59) as indicated in the Tables and Fig. 1. Animals were observed daily and the mortality recorded after 30 days. Mice were bled at the indicated days and the level and isotype of antibodies against MHV3 evaluated in their serum. The mortality was expressed and analyzed as a function of the kinetics of anti-MHV antibody

synthesis and of the difference between background and final antibody titers.

For the correlation between *resistance/susceptibility* to MHV3 and the *effect of passive immunization* with antibodies against MHV, groups of 8 to 12-week-old mice were intravenously (iv) injected with 0.2 ml serum pools of MHV3 hyper-immunized L_{III} mice (antibody titers ranging from 10 to 12 $\log_2$). The animals were infected with 10^3 PFU of MHV3 24 or 48 hours before or 4 hours after the serum transfer and observed for 30 days. Mortality and mean survival time rates were recorded, expressed and analyzed as a function of the anti-MHV antibody titer transferred by passive immunization and of the time of challenge.

Statistical analysis

Mean, standard deviation (sd) and variances ($V = sd^2$) were calculated. The significance levels were assessed by the Student's t-test at the confidence level of $p < 0.05$.

Results

Pre-existing anti-MHV antibody and resistance to MHV3 infection

As indicated in Table 1, the fully susceptible H_{III} mice were shown to have a lower pre-existing anti-MHV antibody titer (3.2 $\log_2$) when compared to the resistant L_{III} mice (5.9 $\log_2$). The mortality rates of the hybrids and the segregants showed a co-dominance of the susceptible and resistance characters in all these populations, as shown by the mortality analysis. The survivors had pre-existing anti-MHV antibody titers higher (ranging from 7.2 to 8 $\log_2$) than those found in mice which died (ranging from 4.7 to 5.6 $\log_2$) and also higher than those found in the parental resistant L_{III} mice; thus, for this phenotype, there exists an over dominance of high responsiveness. These data indicate a positive correlation between the antibody titers present in the serum and resistance to the experimental infection with MHV3. Animals showing higher antibody titers (> 5.9 $\log_2$) survived the infection and those showing lower antibody titers (< 5.6 $\log_2$) died. No variation of mean survival time was observed among the groups. Thus, it could be deduced from the data in Table 1, that among the genetically heterogeneous mice, anti-MHV antibody titers greater than 5.9 $\log_2$ would predict resistance and mice expressing titers less than 5.6 $\log_2$ should be susceptible to MHV3.

Resistance to MHV3 and antibody response to uvMHV3 or infectious related MHV strains

The kinetics of antibody responsiveness after MHV3 immunization, followed by virus challenge, showed that both mouse lines were capable of synthesizing antibodies. The acquired resistance of the originally susceptible H_{III} mice paralleled the antibody synthesis. In the H_{III} line some individuals became resistant when their anti-MHV antibody titers crossed the barrier of 6 $\log_2$ (Fig. 1 and Table 2). Moreover, it became clear that higher antibody titers are necessary to confer resistance in H_{III} mice than in L_{III} mice. With equivalent antibody titers of about 6 $\log_2$, the mortality rates were 0% in the L and 80% in the H lines (Fig. 1). This suggests the participation of other resistance mechanisms against this virus infection, mainly for the L_{III} mice, which are presented in the discussion.

The genetically selected trait of high or low immune responsiveness was also observed among

Table 1. Resistance/susceptibility to MHV3 infection and the level of pre-existing antibodies in H_{III} and L_{III} mice, their F_1 hybrids, F_2 and Bc segregants. Mice were sc inoculated with 10^3 PFU of MHV3 after their sera were collected for the determination of antibodies against MHV. $F_1 = H_{III} \times L_{III}$; $F_2 = F_1 \times F_1$; $BcH_{III} = H_{III} \times F_1$; $BcLIII = L_{III} \times F_1$. mst = mean survival time. * significance $p < 0.001$.

Mice	Mortality			Mean anti-MHV antibody titers (log2)
	%	mst (sd) days	n Survivors(s)/Total Dead (d)/Total	
H_{III}	100	–	13d/13	3.2 (2.0)*
L_{III}	0	6.8 (0.9)	0d/18	5.9 (2.0)*
F_1	55	7.1 (1.0)	6d/11	5.2 (0.4)
			5s/11	8.0 (1.4)*
F_2	49	6.6 (0.7)	24d/49	4.8 (0.7)
			25s/49	7.9 (1.4)*
BcH_{III}	54	6.9 (1.1)	25d/46	4.7 (0.9)
			21s/46	7.2 (0.8)*
BcL_{III}	17	7.0 (0.6)	5d/30	5.6 (0.5)
			25s/30	7.2 (0.7)*

Table 2. Resistance/susceptibility to MHV3 and the antibody response of H_{III} and L_{III} mice immunized with uvMHV3 or infected with related MHV strains. Mice were ip inoculated with 10^3 PFU of virus (at day 0) and ip challenged (at day 10) with 10^3 PFU of MHV3. Mice were observed daily and mortality recorded after 30 days of infection. p = significance; n.s. = not significant.

Mice	Immunization or Infection	Mortality			Mean anti-MHV antibody titers (log_2)		
		n	%	p	Day 0	Day 10	p
H_{III}	–	8/8	100		4.0 (1.8)	4.2 (1.7)	
H_{III}	uvMHV3	9/10	90	n.s.	3.4 (1.5)	4.3 (1.3)	n.s.
H_{III}	MHV4	0/10	0	< 0.001	5.2 (1.6)	12.2 (1.0)	< 0.001
H_{III}	MHVA59	0/10	0	< 0.001	4.8 (1,4)	12.6 (1.5)	< 0.001
L_{III}	–	0/10	0		5.9 (1.7)	6.1 (0.9)	
L_{III}	uvMHV3	0/10	0		6.2 (0.8)	7.8 (0.8)	$0.001 < p < 0.01$
L_{III}	MHV4	0/10	0		7.5 (1.0)	11.6 (0.5)	< 0.001
L_{III}	MHVA59	0/10	0		7.0 (1.2)	12.0 (0.8)	< 0.001

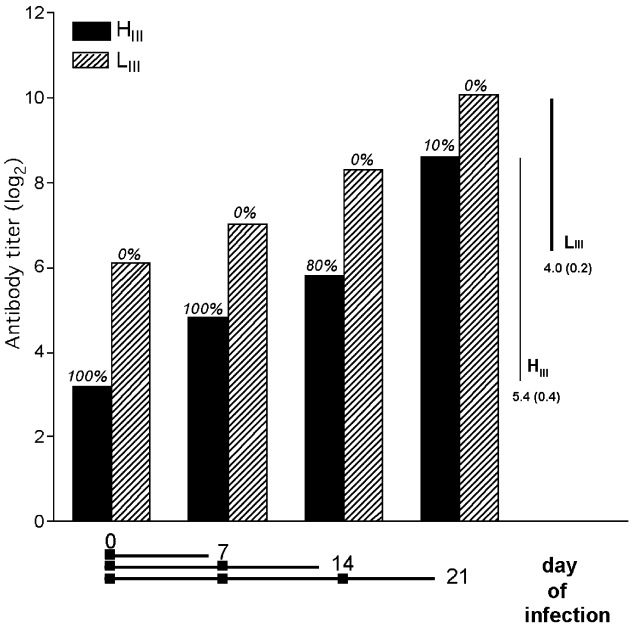

Fig. 1. Resistance/susceptibility to MHV3 infection and kinetics of antibody responsiveness of H_{III} and L_{III} mice after experimental hyper-immunization with uvMHV3. Groups of 10 H_{III} and L_{III} mice were ip immunized with 10^3 PFU of uvMHV3 once (at day 0), twice (at days 0 and 7) or three times (at days 0, 7 and 14). They were then ip infected with 10^3 PFU of MHV3 at, respectively, days 7, 14 and 21. Groups of H_{III} and L_{III} non immunized mice were also infected with 10^3 PFU of MHV3 (day 0). They were observed daily and the mortality recorded after 30 days. The mice were bled at days 0, 7, 14 and 21 and the level of antibodies against MHV evaluated in their sera. The results show the geometric mean antibody titers (log_2), the percent of mortality as indicated by the numbers in italic and the increase in antibody titer in H_{III} and L_{III} mice as indicated by the difference between the final and the background antibody titer.

H_{III} and L_{III} mice following experimental immunization with MHV3. H_{III} mice had a background serum level of 3.3 log_2 and 21 days after the uvMHV3 hyper-immunization the serum antibody level attained 8.7 log_2 (an increase of 5.4 log_2). The L_{III} mice showed a background and a final serum antibody level of 6.2 and 10.2 log_2 respectively (an increase of 4 log_2) (Fig. 1). This was even more evident when a 10 day single immunization was performed with the related infectious MHV4 (increase of 7 log_2 for H_{III} mice and 4.1 log_2 for L_{III} mice) (Table 2). When compared to uvMHV3, the immunization with related infectious MHV strains induced a significantly higher serum antibody response and led the H_{III} mice to resist the infection with the pathogen (Table 2).

As evaluated by ELISA, in both mouse populations the IgG2a antibody isotype was preferentially produced after infection with MHV3 or MHVA59, and the IgG1 isotype is predominant after the uvMHV3 immunization. When compared to L_{III} mice, the H_{III} mice were capable of producing higher and remarkable levels of both IgG1 and IgG2a antibodies following infection with MHVA59 or hyper immunization with uvMHV3. These data confirm, at the isotype level, the higher ability of the H_{III} line to respond to an antigenic stimulation (Table 3). Moreover, the IgG1 and IgG2a isotype production were more homogeneous for H_{III} than for L_{III} mice, as shown by the variance values.

Passive immunization and resistance to MHV3

As shown in Table 4, the passive transfer of anti-MHV antibody to the susceptible H_{III} mice was capable of conferring resistance against infection. When the animals were infected 24 or 48 hours before receiving a serum transfer of high levels of anti-MHV antibodies (titer = 12 log_2), a high mortality rate following the MHV3 challenge was observed. The longer the period after immunization, the higher was the mortality. On the other hand, when the animals were treated 4 hours before receiving the virus infection, resistance was achieved

Table 3. IgG1 and IgG2a antibody production against MHV3 by H_{III} and L_{III} mice infected with MHVA59 or MHV3 strains. Mice were ip inoculated with 10^3 PFU of MHV3 or MHVA59 or the corresponding 10^3 PFU of uvMHV3 and at day 10 sera were collected and IgG1 and IgG2a against MHV3 were evaluated by ELISA assay. *Animals were immunized at days 0, 7 and 14, the sera collected at day 21. V = variance (sd^2); nd = not determined.

Mice	Infection or Immunization	Mean anti-MHV3 antibody titers of 10 mice			
		IgG1	V	IgG2a	V
H_{III}	uvMHV3	2.7 (1.5)	8.6	< 2.0	–
H_{III}	uvMHV3*	15.2 (1.2)	12.1	10.9 (1.1)	1.1
H_{III}	MHV3	nd		nd	
H_{III}	MHVA59	12.1 (3.4)	11.4	15.3 (0.8)	0.7
L_{III}	uvMHV3	3.4 (2.0)	8.2	< 2.0	–
L_{III}	uvMHV3*	12.9 (0.9)	12.3	6.8 (0.8)	3.1
L_{III}	MHV3	6.0 (5.2)	26.8	10.3 (1.5)	2.3
L_{III}	MHVA59	2.8 (4.2)	17.4	7.8 (2.0)	4.0

Table 4. Resistance/susceptibility to MHV3 and passive immunization with antibodies against MHV in H_{III} mice. H_{III} mice were iv injected with 0.2 ml hyper-immunized L_{III} mouse serum pools with indicated anti-MHV titers. After or before the serum transfer, mice were ip challenged with 10^3 PFU of MHV3. n = number of dead mice/number of infected mice; mst = mean survival time.

Antibody (log_2)	Infection with MHV3 (hours)	Mortality		
		n	%	mst +/− sd (days)
12	−48	7/7	100	4.0 +/−0.0
12	−24	6/8	75	4.6 +/−1.2
1	+4	10/10	100	4.2 +/−0.4
7	+4	4/8	50	5.0 +/−0.0
10	+4	1/7	14	7.0
12	+4	0/8	0	–

and was a function of the amount of antibodies transferred. In spite of a drop in anti-MHV antibody titer due to antibody clearance, both the resistance to infection and the mean survival time (mst) increased correlating with the level of antibodies administered. Resistance was never observed in recipients of serum free of anti-MHV antibodies, nor were differences observed using anti-MHV containing sera from different donors, such as H_{III} mice, or donors immunized with different MHV strains (data not shown).

Discussion

MHV-induced disease has been successfully modulated by antibody *in vivo* (Buchmeier et al., 1984). Studies on challenge immunity to some MHV have indicated that the immunity to some strains is strong and highly virus strain-specific (Barthold & Smith, 1989). Antibodies directed to the surface S glycoprotein or to the nucleoprotein and even Fab antiviral antibody fragments were able to protect mice *in vivo* (Lecomte et al., 1987; Barthold & Smith, 1989; Lamarre & Talbot, 1995). Barthold and Smith (1989) have shown that mice immunized with a given live virus strain express high resistance to the same MHV strain, but remain highly susceptible to challenge with another MHV strain. The authors emphasize the hypothesis of host immunity dependence on the variation of the surface glycoprotein found in the virion that could explain the susceptibility to a heterologous challenge. On the other hand, some mouse strains are highly susceptible to MHV and hardly acquire resistance to infection, and others can easily become resistant (Lucchiari et al., 1991). Although different MHV strains differ substantially in their pathogenicity they express similar structure and composition (McIntosh, 1985).

In addition to previous studies dealing with mouse immunity in natural conditions (Vassão et al., 1994b), the main interest of the present work was to investigate acquired immunity to MHV3. We studied host resistance through humoral immune responsiveness to MHV immunization in genetically selected High (H) or Low (L) antibody responder

lines of mice, which were considered as naturally infected by coronaviruses on the basis of anti-MHV antibodies found in their sera.

The relevance of this approach is the use of H and L lines genetically homogeneous at the loci controlling the general character and the possibility to reconstitute, by interline crosses, segregant populations with a genetically heterogeneous background, reproducing natural conditions.

The H_{III} and L_{III} mice, although showing no MHV in their tissues, have possibly been infected with a low pathogenic MHV and developed antibodies cross-reacting with MHV3. As previously demonstrated, the H_{III} line was fully susceptible and the L_{III} line fully resistant to the experimental MHV3 infection and these distinct phenotypes were confirmed here as shown in Tables 1, 2 and Fig. 1. Among hybrids and segregants, that showed codominance for the susceptible and resistant characters, only animals showing an antibody level over 6 $\log_2$ resisted a challenge (Table 1). In kinetic studies of antibody production after immunization, the resistance acquired by H_{III} mice paralleled antibody synthesis and animals crossing a barrier of 6 $\log_2$ titer became resistant (Fig. 1). Hyper-immunization with uvMHV3 or immunization with antigenically related MHV4 or MHVA59 (Table 2) induced the same acquired resistance pattern. These strains were able to provide resistance inasmuch as the antibody titer before challenge was higher than 6 $\log_2$. This observation was confirmed by experiments of passive antibody transfer, which showed that resistance of H_{III} mice to MHV3 infection and also the mean survival time increased as a function of the level of antibodies administered (Table 4). These data demonstrate that memory induction by nonvirulent virus strains provides resistance to the pathogenic variant.

H_{III} mice synthesized higher levels of both IgG1 and IgG2a antibodies against MHV3 (Table 3). This confirms, at the isotype level, the already described multi-specific genetic traits of H_{III} and L_{III} antibody responders, which indicate an involvement of effects that may be attributed to isotype-specific influenced T helper lymphocyte subsets (Buchmeier et al., 1984; Lucchiari et al., 1991).

Other relevant data were obtained by comparing the acquisition of resistance against MHV3 infection among H_{III} and L_{III} mice (Fig. 1). With comparable serum anti-MHV antibody titers, the H_{III} mice were shown to be susceptible and the L_{III} mice resistant to MHV3 challenge. It may indicate that lower titers are necessary for the L_{III} mice to express resistance and/or that the resistance is not based only on this trait. The explanation for this observation, although influenced by the inherent high or low response, may be found in the immune response showed by immunized mice: L_{III} mice, in accordance with their genetic trait, showed a modest but preferential Th1 type response after infection, as indicated by the IgG2a isotype synthesis (Table 3). On the other hand, H_{III} mice were shown to mount a Th2 response, reflected by the high levels of the IgG1 isotype, which promotes several inhibitory/suppressive processes. Further, the H_{III} mice, as high responders, following infection or immunization were also capable of mounting a Th1 response, reflected by the high levels of the IgG2a isotype. It may correlate with their resistance acquired after uvMHV3 hyper immunization (Fig. 1) or MHVA59 and MHV4 infection (Table 2).

The pattern of antibody isotype production in both H_{III} and L_{III} mice was different depending on the immunization with live or inactivated virus (Table 3). With live virus the immune response was stronger inducing preferentially the synthesis of IgG2a antibodies and with inactivated virus a higher IgG1 response was detected. This suggests a differential effector mechanism dependent on the mode of antigen presentation.

Our data, produced with genetically heterogeneous mice and evaluating a Th subset response by measuring the antibody isotypes synthesized, confirm the findings published by Pope et al. (1996), showing a correlation between a Th1 response and resistance against MHV3 infection. Our immunization experiments with inactivated MHV3 showed that the acquisition of resistance by susceptible mice correlated with an overall antibody response, including a strong Th1 response. These susceptible animals (H_{III} mice) are genetically high antibody producers and upon immunization synthesize high levels of both IgG1 and IgG2a antibodies (Table 3).

Nevertheless, other mechanisms may play a role in the resistance/susceptibility phenotypes, such as the specific antiviral effect of IFNγ (Lucchiari et al., 1989) on macrophages that has been described only for L_{III} mice (Nakanaga et al., 1986); or the production of the pro-coagulant activity monokine (Levy et al., 1981; Pope et al., 1995) described for H_{III} mice (Vassão et al., 1994a).

It is interesting to note that, in the same environmental conditions, the H_{III} mice expressed a serum antibody level lower than the L_{III} mice (Table 1, Table 2 and Fig. 1). These results contrast with experimental data showing that the selected genetic trait of high or low humoral immune responsiveness was indeed observed among H_{III} and L_{III}, following immunization with MHV (Table 2 and Fig. 1). These findings may be explained by the fact that

these mouse lines were obtained through immunization and the selective process was not based on any parameter of infection or resistance.

The present data show the participation of specific antibodies in the MHV3 resistance process taking place in H_{III} mice. Previous evidence (Vassão et al., 1993, 1994b, 1995), and the fact that L_{III} but not H_{III} mice may easily acquire resistance in natural conditions, lead to the hypothesis that distinct mechanisms can be involved in MHV3 resistance in H_{III} and L_{III} mice. The mechanism involving the antiviral state induced by IFNγ in macrophages may play a central role only in the resistance of L_{III} mice (Vassão et al., 1994a). Susceptible lines, such as H_{III} mice, would not acquire resistance through IFNγ since their macrophages do not respond with an antiviral state.

Besides the evidence that MHV3-neutralizing antibodies, as evaluated by *in vitro* assay, can be generated by related MHV-strains and are capable of conferring *in vivo* protection when present in a certain amount, our studies make clear the importance of the environment and genetic profile in studies of resistance and point out the advantages of approaches using genetically heterogeneous mice, which better represent a population and are closer to what is found in nature.

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