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Role of the Humoral Immune Response in Resistance To Theiler's Virus Infection

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Theiler's virus, a murine picornavirus, persists in the central nervous system of susceptible strains of mice, causing chronic inflammation and demyelination in the white matter of the spinal cord. Resistant strains, however, clear the virus and do not develop late disease. In this study, we compared the characteristics of T and B lymphocytes in C57BL/6 (resistant) and SJL/J (susceptible) mice 1 week after intracerebral infection. We detected a marked increase of the number of immunoglobulin M (IgM)-secreting cells in the spleens of C57BL/6 mice (but not in those of SJL/J mice), which correlated with higher levels of serum IgM antiviral antibodies. The role of the humoral response in virus clearance and resistance was demonstrated by a marked decrease in the number of infected spinal cord cells in SJL/J mice after passive transfer of serum from infected C57BL/6 donors. The B-cell response was found to be partly T cell independent. These results suggest an important role of the early humoral immune response in resistance to Theiler's virus-induced disease.

Theiler's virus is a picornavirus that causes a natural enteric infection in mice and, more rarely, paralysis (27). After intracerebral (i.c.) inoculation, some strains of mice develop a biphasic disease of the central nervous system (CNS). The first phase, or early disease, is an acute encephalitis which occurs during the first few days of infection. After this encephalitis subsides, a different pathological entity, or late disease, develops. It consists of disseminated lesions of inflammation in the white matter, accompanied by primary demyelination. The virus is present in the lesions during chronic disease (7). Not all mice develop late disease: while some strains, such as SJL/J, are susceptible, others, such as C57BL/6, clear the virus and do not progress to chronic infection (8).

The susceptibility to late disease is controlled in part by two unlinked loci, one of which is associated with the H-2 complex (11, 18, 21, 24), suggesting a T-cell participation in the pathogenic mechanisms. The importance of Theiler's virus-specific delayed-type hypersensitivity in the mechanism of demyelination has been demonstrated (9), and treatments with monoclonal antibodies to CD8 (25), to CD4 (28), and to major histocompatibility complex class II antigens (14, 23) decrease inflammation and demyelination. Most of these studies, however, have focused on the susceptibility of mouse strains to late disease, and little is known about the immune response during the first few days of infection or about the mechanisms of viral clearance in resistant mouse strains.

Our aim was to characterize and compare lymphocyte populations implicated in the early response to infection in the resistant and susceptible strains and their effects on viral persistence. Our results strongly suggest an important role of B lymphocytes in the control of early disease.

Comparison of lymphocyte populations. SJL/J and C57BL/6 mice were inoculated i.c. with 10^5 PFU of Theiler's virus strain DA or with phosphate-buffered saline as a control. We quantified lymphocytes isolated from the thymuses, spleens and mesenteric lymph nodes 1 and 4 weeks postinfection (p.i.). Surface expression of B- and T-cell markers was quantitated in a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.). The data were obtained by flow fluorimetric analysis of 10^6 cells for each reaction with the respective monoclonal antibody. Purified antibodies were labeled either directly with fluorescein isothiocyanate, or with biotin (15) and detected with R-PE streptavidin (Becton Dickinson). Data were processed with a Consort 30 computer program. A total of 5,000 to 10,000 events was scored for each experiment. The lymphocyte populations isolated from thymuses and mesenteric lymph nodes did not change after infection of mice of the two strains. In contrast, as shown in Table 1, we detected an increase of splenic μ^+ and immunoglobulin-positive (Ig^+) cells in infected C57BL/6 mice at 1 week p.i. No increase was detected in SJL/J mice. Interestingly, in the spleens of infected mice, we did not find any significant difference regarding T-cell populations or cell size distribution of B and T lymphocytes, as assessed by forward light scattering.

We quantified the total number of Ig-secreting cells, as a general measure of B-lymphocyte activation in vivo, by using the plaque-forming cell (PFC) assay previously described (2). One week p.i., we found increased numbers of Ig-secreting cells of all isotypes except IgA in the spleens of C57BL/6 mice (Fig. 1). They were predominantly IgM-secreting cells and showed a four- to fivefold increase. No increase was detected in the susceptible SJL/J strain. Four weeks p.i., the levels of activated B cells had returned to normal (data not shown). These results show that shortly after viral infection the resistant strain develops a marked B-cell response, which is essentially of the IgM isotype.

Specificity of antibody responses. To analyze the specificity of this B-cell activation, we tested supernatants of lipopolysaccharide-activated B cells isolated from the spleens of infected and uninfected C57BL/6 and SJL/J mice for the presence of antibodies specific for Theiler's virus. To culture B cells, splenocytes were obtained 1 week p.i., depleted of T cells with anti-Thy-1 antibodies and complement, and maintained in culture for 6 days. Depletion was complete as

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TABLE 1. Lymphocyte populations isolated from spleens of susceptible and resistant mice[a]

Mouse strain and infection status	No. of cells (10^6) expressing surface markers[b]				
	μ	Ig	CD4	CD8	CD3
SJL/J					
Uninfected	18	21	15	5	20
Infected	18	23	16	5	22
C57BL/6					
Uninfected	22	24	12	4	17
Infected	30[c]	35[d]	14	5	19

[a] Cells were collected 1 week after i.c. infection and stained at 4°C for 20 min as described previously (13).

[b] Values represent arithmetic means of 2 to 3 experiments, with a pool of 3 mice per group in each experiment.

[c] The increase in the absolute number of μ^+ cells in infected C57BL/6 mice was statistically significant, $P < 0.05$.

[d] The increase in the absolute number of Ig^+ and μ^+ cells in infected C57BL/6 mice was statistically significant ($P < 0.01$).

assayed by fluorescence-activated cell sorter (FACS) analysis. Virus-neutralizing titers of B lymphocyte culture supernatants were measured as described elsewhere (6), by using a 50% endpoint dilution assay. Neutralizing antibodies were considered present if there was a 99% reduction of the viral titer. Neutralizing titers were 1/10 dilutions for infected SJL/J mice and 1/60 dilutions for infected C57BL/6 mice. They were calculated by linear regression analysis and expressed as the antibody dilution which neutralized 100 50% tissue culture infective doses. Supernatants from splenocytes of uninfected mice had no neutralizing activity even at a 1/2 dilution. These results demonstrate that viral infection enriches B-cell repertoires in virus-specific clones and that this effect is larger in C57BL/6 mice than in SJL/J mice.

Titers of virus-specific antibodies and total Ig concentration in sera were analyzed by enzyme-linked immunosorbent assay (ELISA) 2 weeks p.i. Plates were coated with DA virus purified as previously described (5). We detected the presence of antiviral antibodies of the IgM and IgG2b isotypes. As shown in Fig. 2, the level of virus-specific IgM was sevenfold higher in infected C57BL/6 than in infected SJL/J mice, while the level of virus-specific IgG2b was slightly lower in infected C57BL/6 than in infected SJL/J mice. The concentration of total Ig in serum was only slightly higher in C57BL/6 than in SJL/J mice (data not shown), and none of the sera contained antibodies to an irrelevant antigen, such as polio virus (Lansing strain).

Serum transfers. To confirm the role of the humoral response in the clearance of Theiler's virus, we studied the effect of sera from C57BL/6 mice passively transferred into SJL/J mice. For this purpose, a group of i.c. infected SJL/J mice received four serial intraperitoneal injections of 0.25 ml of a serum pool obtained from C57BL/6 mice on day 14 after i.c. infection. The injections were performed at days 7, 10, 13, and 20 p.i. Control SJL/J mice were also infected and treated in parallel with the same doses of sera from uninfected C57BL/6 or infected SJL/J mice. The titers of antiviral antibodies in the serum pools are presented in Fig. 2. The three groups were tested 1 month p.i. for the presence of virus and inflammation in the spinal cord. The detection of viral RNA by in situ hybridization was performed by using a cRNA probe specific for Theiler's virus as described previously (4, 12). SJL/J mice that had received sera from infected C57BL/6 mice showed a striking decrease in the number of infected cells and in the extent of inflammation compared with the two control groups (Fig. 3 and Table 2).

Genetic analysis. Since the early B-cell response to viral infection was much stronger in C57BL/6 than in SJL/J mice, we determined whether this response is a dominant genetic trait. For this purpose, F_1 (SJL/J × C57BL/6) mice were inoculated i.c., in parallel with progenitor mice. Their lymphocyte populations were analyzed 1 week p.i. The only detectable difference in lymphocyte populations from infected and uninfected F_1 mice was the increase of Ig-secreting cells in the spleens (Fig. 4). However, this B-cell response was weaker in F_1 animals than in infected C57BL/6 mice. Such intermediate B-cell responses to viral infection in F_1 mice correlate with an intermediate susceptibility to demyelination (19) and a low level of virus in the CNS, as compared with SJL animals (5a). Taken together, these observations suggest that resistance and B-cell response to viral infection are quantitatively correlated.

Partial T-cell independence. To establish the role of T lymphocytes in activating the B-cell response in the resistant strain, we studied T-cell-deprived mice. We infected nude and euthymic C57BL/6 mice orally and analyzed their B-cell populations in spleens and mesenteric lymph nodes 1 week

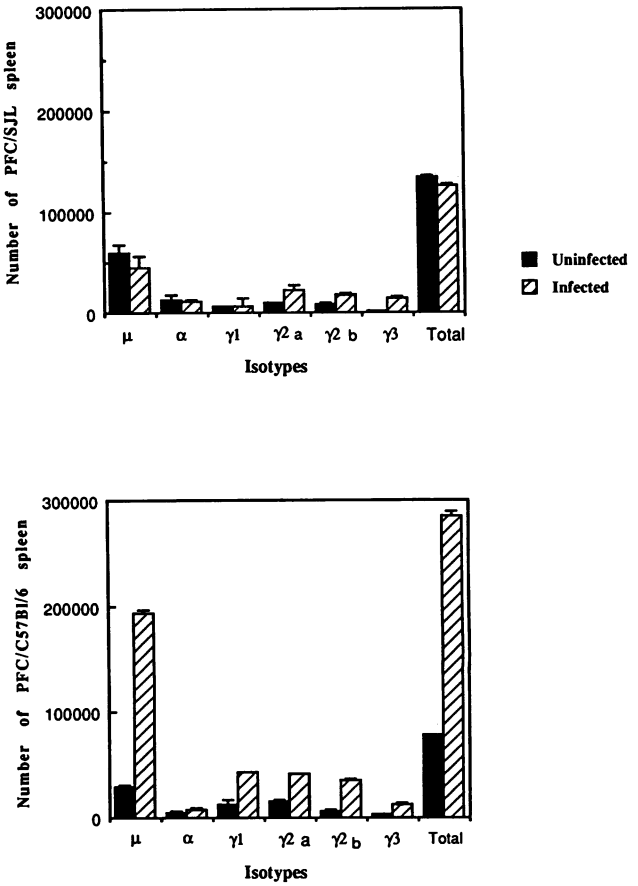

FIG. 1. Ig-secreting PFC in the spleens of SJL/J and C57BL/6 mice 1 week after i.c. infection with Theiler's virus. PFC were detected with a protein A plaque assay with Ig class- and subclass-specific antisera. Standard deviation bars are calculated from three independent experiments, each using a pool of three mice. The increase of IgM-, Ig2a-, Ig2b-, and total Ig-secreting cells is statistically significant ($P < 0.01$) in infected C57BL/6 mice.

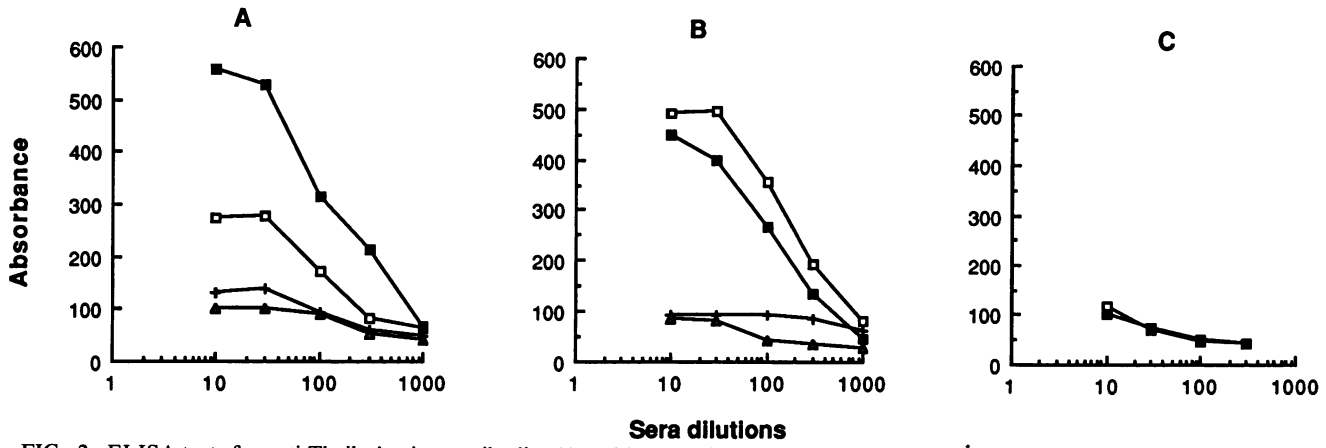

FIG. 2. ELISA tests for anti-Theiler's virus antibodies (A and B) or anti-polio virus antibodies (C) in sera from infected (□) or uninfected (△) SJL/J and infected (■) or uninfected (+) C57BL/6 mice. IgM antibodies are represented in panels A and C, while IgG2b antibodies are represented in panel B. The results represent titration of serum pools obtained from three mice in each group, 2 weeks after infection, tested in duplicate assays. The data shown are a representative example of three independent experiments, including the pools utilized for transfers.

p.i. FACS analysis showed an important increase in splenic μ^+ and Ig^+ cells in both groups (data not shown). As shown in Table 3, the total number of IgM-secreting cells in spleen, as detected with the PFC assay, was lower in nude than in euthymic mice. Surprisingly, in the mesenteric lymph nodes of nude C57BL/6 mice, we detected an increase of Ig-secreting cells which was not observed in euthymic C57BL/6 mice. Circulating antiviral IgM antibodies were measured,

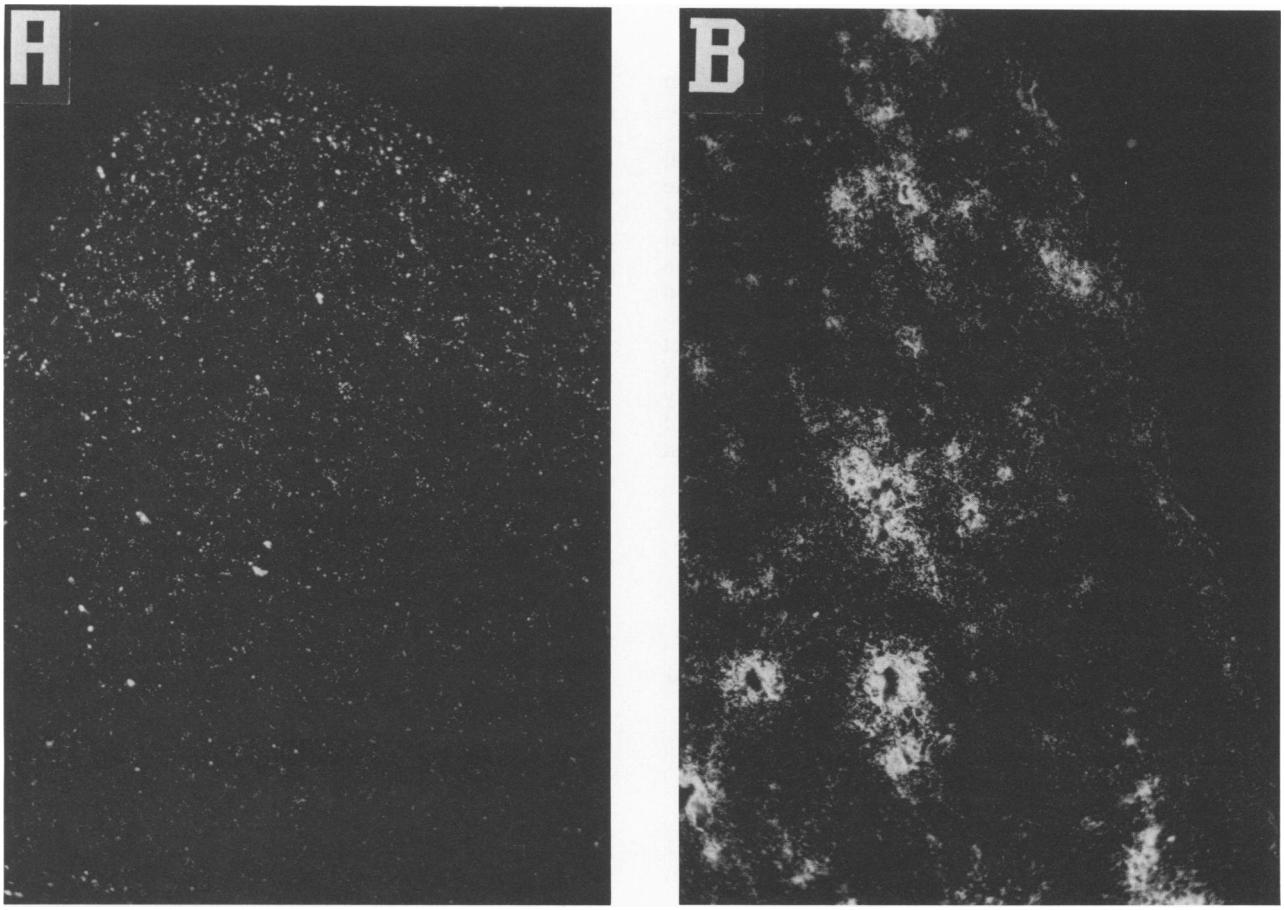

FIG. 3. Detection of Theiler's virus RNA by in situ hybridization. Dark-field examination of longitudinal sections of spinal cord of infected SJL/J mice that were treated with sera obtained from infected (A) or control (B) C57BL/6 mice. The signal observed in panel A is indistinguishable from that observed in normal noninfected mice and represents background hybridization. Original magnification, ×150. Final magnification is the same for panels A and B.

TABLE 2. Viral RNA detected in spinal cord of SJL mice treated with sera from C57BL/6 mice

Serum transfer group[a]	Mice	No. of infected cells[b]
Group I	1	650
	2	700
	3	560
	4	680
Group II	5	160
	6	20
	7	24
	8	6
	9	10
	10	12
Group III	11	750
	12	720
	13	690

[a] Group I was injected with serum from uninfected C57BL/6 mice. Group II was injected with serum from infected C57BL/6 mice. Group III was injected with serum from infected SJL/J mice.

[b] Infected cells were counted in longitudinal sections of the entire spinal cord of each mouse by using a dark-field condenser and a final magnification of ×100. Each grain cluster was considered to correspond to a single cell. Two longitudinal sections of the entire spinal cord were examined for each mouse. The table shows the total number of infected cells in both sections. The *t* test comparing the groups showed statistical significance, $P < 0.01$. Mice were examined 30 days p.i.

TABLE 3. Number of Ig-secreting cells isolated from athymic and euthymic C57BL/6 mice

Mice	Organ and mouse type	PFC[a]/10^6 cells reacting with antibody to:	
		Total Ig	IgM
Uninfected	Spleen		
	Euthymic	1,444	558
	Athymic	1,299	892
	Lymph node		
	Euthymic	940	60
	Athymic	1,598	80
Infected	Spleen		
	Euthymic	4,519	2,499
	Athymic	2,052	1,411
	Lymph node		
	Euthymic	880	40
	Athymic	3,980	325

[a] Cells were collected 1 week after oral infection. PFC were detected with a protein A plaque assay by using anti-total Ig and anti-IgM antisera. The results represent the means of two independent experiments with pools of two or three mice per group.

and the titers were threefold lower in nude mice than in normal mice. These results show that B-lymphocyte activation in infected C57BL/6 mice is partly T cell independent.

The selective B-cell and serum IgM response to viral infection observed in resistant mice suggests a role of the humoral immune response in clearing the virus during the first weeks of viral infection. This hypothesis was supported by the finding that sera transferred from infected C57BL/6 mice to infected SJL/J mice resulted in a virtual elimination of the virus from the CNS. This would suggest that resistance corresponds to the ability of the infected host to react to and clear the virus from tissues, rather than a lack of permissiveness. In fact, resistant mice are infected early after i.c. inoculation, as has been shown by the isolation of virus from the CNS (17). However, the virus does not persist as it does in susceptible mice, in which it can be detected in the CNS for life (8).

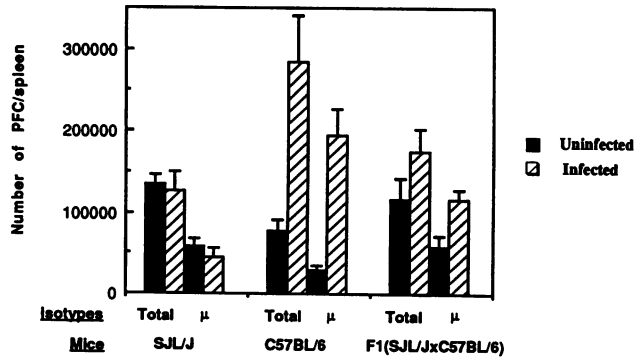

FIG. 4. Ig-secreting PFC in the spleens of SJL/J, C57BL/6, and F_1 (SJL/J × C57BL/6) mice i.c. infected with Theiler's virus. The increase of IgM-secreting cells and total Ig-secreting cells is statistically significant ($P < 0.01$) in infected C57BL/6 and F_1 (C57BL/6 × SJL/J) mice.

Susceptible mice, however, also developed an antiviral immune response by producing detectable levels of circulating IgM and IgG2b antibodies directed at viral particles. This antibody production, particularly for the IgM isotype, was weaker than in C57BL/6 mice and obviously unable to prevent viral persistence. The secretory B-cell responses detected in the spleens of infected SJL/J mice are weak compared with the marked IgM responses found in the spleens of C57BL/6 mice. Since the spleen is the major site of IgM production, while other isotypes are predominantly produced at other anatomic sites, such as the lymph nodes and bone marrow for IgG classes (1), it is possible that the SJL/J defect is limited to the splenic response. In fact, these mice develop serum IgG antibody responses that are equivalent to or higher than those of the resistant strain. This could be due to the alterations in the normal composition of B-cell populations that have been described in SJL/J mice (16) or to the maturational defect of antigen-presenting cells which has also been reported in this strain (3, 20). In any event, these results ascribe a particular advantage to IgM over IgG antibodies in virus clearance.

Clatch et al. (10) detected, 3 to 4 months p.i., a higher titer of antiviral antibody in sera of susceptible strains than in sera of intermediate or resistant mice. This observation does not contradict ours, since at this time the antigenic load is different in each case. Whereas virus has been already cleared in resistant mice, it is still present in susceptible strains, in which it may continuously stimulate B-cell responses. Rodriguez et al. (22) have reported that anti-μ antibody treatments from birth, aimed at suppressing B cells and antibody production, resulted in the aggravation of inflammatory lesions and demyelination in SJL/J mice infected with Theiler's virus. This indicated that antibodies play a role in limiting disease in susceptible strains. The authors also showed that the same treatment had no effect on the limited CNS pathology observed early after infection of C57BL/6 mice. No definite conclusions, however, can be drawn from these experiments regarding the role of antibod-

ies in viral clearance, since anti-μ immunosuppression was incomplete.

Our passive transfer experiments show that serum (possible antibodies) from resistant mice decreases the amount of CNS infection in susceptible mice. A model that includes all observations would suggest that C57BL/6 mice clear the virus primarily on the basis of antibody responses. In this case, no pathology is observed, since the virus does not persist in the CNS. Pathology, however, is likely to require factors other than the mere persistence of the virus in tissues, since demyelination seems to be associated with T-cell responses (24, 25, 28) and is linked with major histocompatibility complex antigens (11, 19, 21). It is possible, therefore, that not all mice which fail to clear the virus with IgM antibodies (e.g., anti-μ-suppressed C57BL/6 mice) will develop pathology, this requiring both viral antigens in the tissues and a particular T-cell repertoire and reactivity.

The fact that nude C57BL/6 mice developed some B-cell response to infection shows that T cells are not strictly necessary for B-cell activation during the acute phase of the disease. Nevertheless, the increase of Ig-secreting cells was quite modest under these conditions, which is in agreement with the reported higher disease susceptibility of nude mice to Theiler's virus-induced disease (26, 29).

Finally, it is interesting to note that the magnitude of the B-cell response was similar for mice inoculated either i.c. or orally. This indicates that i.c. inoculation does not significantly distort the B-cell response observed with the natural route of infection.

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