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LYMPHOKINE RELEASE AS MEASUREMENT OF ANTI-MOUSE HEPATITIS VIRUS TYPE 3 (MHV3) CELLULAR REACTIONS IN VARIOUS MOUSE LINES EXHIBITING DIFFERENTIAL SUSCEPTIBILITIES TO MHV3-INDUCED PARALYSIS

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Summary. — We found that susceptibility to Murine Hepatitis Virus, type 3 (MHV3)-induced paralysis is controlled by genes of the H-2 complex. In this article, we compared MHV3 antigen specific cellular reactions, in congenic mice harbouring different H-2 genes (or gene). In a first set of experiments, paralysis susceptible (B10 . A × A/J)F_1, partly susceptible (B10 . AQR × A/J)F_1 and resistant (B10 . Q × A/J)F_1 hybrids were infected with live MHV3. Three weeks or more post-infection (p. i.), the spleens and peritoneal exudate (PE) cells from the mice were put into culture. Killed MHV3 was added to cultures, and antigen specific lymphokine production and utilization were measured: IL-1 production by PE cells after 24 hr in culture, IL-2 production by splenocytes after 24 hr in culture, IL-2 utilization (as appraised by splenocyte proliferation) after 96 hr in culture. No clearcut difference, resulting from genetic disparity, could be observed in the antigen-specific responses. In a second set of experiments, mice were primed with ultra-violet radiation killed MHV3. In that case, increases of IL-1 production by PE cells, of IL-2 production by splenocytes and splenocyte proliferation were always observed, compared to PE cells and splenocytes from non-primed (control) donor mice. However, in latter case, addition of MHV3 antigen to cultures did not result in augmentation of antigen specific IL-2 production and utilization. Here again, no genetic effect was observed. We conclude from these results that MHV3 infection elicited strong lymphokine responses, but that antigen-specific IL-1 and IL-2 production did not correlate with the susceptibility to MHV3-induced paralysis.

Key words: *mouse hepatitis virus type 3*; *interleukin-1*; *interleukin-2*; *susceptibility to paralysis*; *genetic analysis*

Introduction

Certain virus infections in experimental animals result in brain lesions and paralysis; some of these phenomenons are considered for possible models in the study of human demyelinative diseases. To obtain insights on some pathological processes, several murine models have been used in immunologic and immunogenetic studies (McFarlin and Waksman, 1982). Infection of mice with Theiler virus, for instance, produces demyelination and paralysis which have been found to be associated with cellular immune reactions, and partly controlled by a class I gene of the H-2 complex (Clatch *et al.*, 1986). On the other hand, different members of a coronavirus family, such as the Mouse Hepatitis Viruses (MHVs), are able to induce paralyses in intracerebrally (Wege *et al.*, 1981) or intraperitoneally (Levy-Leblond *et al.*, 1979) infected mice. Infection with the MHV type 3 (MHV3) results in a complex pathology pattern, beginning with an "acute disease" which kills variable proportions of the infected mice 5—15 days p.i., followed by a "chronic disease" which produces paralyses in animals which survived the "acute disease", several weeks to several months later (Levy-Leblond *et al.*, 1979). The genetic control of the two forms of MHV3-induced disease is complex and involves several genes. In particular, strong resistance to paralysis has been shown to be dominantly associated with the H-2^f haplotype (Levy-Leblond *et al.*, 1979), which also appeared to influence the susceptibility to the "acute disease", but on a non-dominant fashion and with incomplete penetrance (Sabolovic *et al.*, 1982).

Later on we found that the H-2^q haplotype is also strongly associated with resistance to MHV3-induced paralysis. In that framework, a partial but significant protective role of the class I H-$2K^q$ allele was observed (Oth *et al.*, submitted). In the present article, some parameters of the cellular immune reaction against MHV3 antigens were studied in mice of different genomes, presenting different susceptibilities to MHV3-induced paralysis. Because the congenic mice which harbour the B10 genetic background completely succumb to "acute disease" (Levy-Leblond *et al.*, 1979), we used F_1 hybrids that were partially resistant to lethal effect. The F_1 hybrids were prepared by crossing parents belonging to 3 different congenic-resistant strains with the A/J strain in one set of experiments, and with the C3H strain in the other.

Materials and Methods

Virus. Supernatants of MHV3-infected L2 cells (a C3H fibroblast line) were clarified, determined for virus infectivity, aliquoted and stored at −80°C.

Mice. C3H mice were purchased from Charles River Inc., St Constant, Quebec, whereas A/J, as well as C3H/He/J and B10.A (H-2^a) mice, were obtained from the Jackson Laboratory, Bar Harbor, ME. The B10.Q (H-2^q) and B10.AQR (H-2^{y1} and H-$2K^q$) B10 congenic mice were supplied by Dr. Chella David, Mayo Clinic, Rochester, MN, U.S.A. Young adults of both sexes (equally reparted) were used. Experiments were conducted under conventional conditions, in air-conditioned facilities.

A first set of F_1 hybrids was made as follows: (B10.A × A/J) F_1, (B10.AQR × A/J)F_1 and (B10.Q × A/J)F_1 (Groups 1, 2 and 3), which exhibit "susceptibility", "partial resistance" and "full resistance", respectively, to MHV3-induced paralysis (Oth *et al.*, submitted). Due to the presence of the A/J genetic background these hybrids were expected to present good resistance to

the lethal effect of the "acute disease" resulting from infection with the live MHV3 (Levy-Leblond *et al.*, 1979).

Another set of F_1 hybrids was prepared, i. e., (C3H × B10.A) F_1, (C3H × B10.AQR) F_1 and (C3H × B10.Q) F_1 (Groups 4, 5 and 6), which also permitted comparison of H-2 or H-2K influences. In addition, the presence of the C3H genome in all these hybrids ensured absence of any immune reaction against C3H tissue which could have been accidentally contaminated the MHV3 preparations from L2 cells. These hybrids have always been immunized with killed MHV3, to which liposomes were added, in order to increase lymphokine production (Mansour *et al.*, 1988).

Primings. Mice were primed with either live or killed MHV3. In the first case they received an i. p. injection of 10^3 LD_{50}, and the mice which survived the lethal "acute disease" were found to be immune. In the second case they received 2 i. p. injections of the equivalent of 10^3 LD_{50} inactivated MHV3 (2 hour ultraviolet exposure at 1.6 μwatt/cm², in phosphate buffered saline - PBS). In addition 0.005 μmol of antigenically inert liposomes, prepared as usual (Mansour *et al.*, 1988), were injected together with the killed virus preparation, as priming material. Control mice received 0.1 ml PBS (i. p.). Optimal conditions of immunization were first determined in a preliminary experimental series.

Lymphokine production and determination. Immune or control donor mice were killed, and their spleens as well as their peritoneal cells were harvested. For each experimental group, cells from two donors were pooled. The cells were washed with RPMI-1640 medium (Flow Lab., inc., Mississauga, Ontario) and put into culture for lymphokine production.

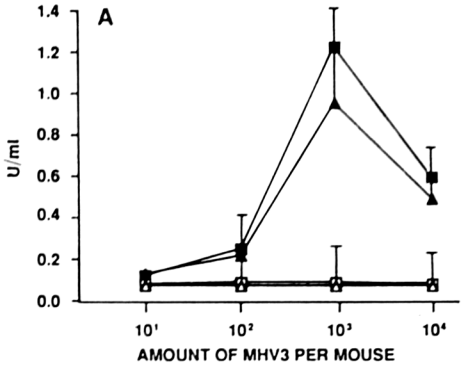
For IL-1 production, 1×10^6 peritoneal cells were seeded, 1 ml per well, in flat bottommed, 24-well culture plates (Corning, Corning, NY, cat. nr. 25820). They were incubated, for adherence, in RPMI-1640 supplemented with 10% foetal bovine serum ("complete medium"), at 37°C for 2 hr. The non-adherent cells were then carefully removed (3 washings with culture medium). In some wells, 100 μl of either U.V.-killed MHV3 (amount equivalent to 10^3 LD_{50} and hereafter referred to as "MHV3 antigen") or polio vaccine (5 μg/ml) was added, whereas 100 μl of medium alone was added to control wells. After a 24 hr incubation at 37°C in humid atmosphere, the supernatants were harvested and stored at −20°C until IL-1 activity determination was performed after thawing the supernatants, as described by Mizel *et al.* (1978). Briefly, dilutions of supernatants were added, together with PHA, to thymocytes from 4—8 week old C3H/HeJ mice, and incubated for 3 days. Thymocytes proliferation was tested for ^{3}H-thymidine (^{3}H-TdR) incorporation (New England Nuclear, Boston, MA; 15 Ci/mmol) after being harvested on a filter paper as described (Oth *et al.*, 1987). The results were expressed as arbitrary units/ml, using a supernatant from LPS stimulated C3H peritoneal cells as a reference. The supernatants were tested on IL-2 sensitive CTLL cells and always found negative. For IL-2 production and determination, splenocytes were used after red cells depletion with brief osmotic shock. Three million cells per well, in 1 ml aliquots, were cultured in 24 well culture plates in "complete medium", antibiotics and 5×10^{-5} mol/l 2-mercaptoethanol, as described (Oth *et al.*, 1987). In some wells, MHV3 antigen (100 μl of a suspension of killed virus corresponding to 10^3 LD_{50}/ml) was added ("restimulated wells") whereas 100 μl of medium alone was added in control ("non-restimulated") wells. Non-specific Con A stimulations ("quality controls") were always performed as described (Oth *et al.*, 1987). The culture supernatants were collected after a 24-hr incubation at 37°C (humid atmosphere, 5% CO_2) and stored at −20°C until IL-2 determinations with CTLL cells, and the results were expressed as units/ml as described (Oth *et al.*, 1987).

Antigen-driven splenocyte proliferation. Splenocytes from the same donors as those of the IL-2 production experiments were cultured for 96 hr in AIM-V culture medium (Gibco Labs inc., Grand Island, N.Y.) supplemented with 2% foetal bovine serum. One μCi of ^{3}H-TdR was added to each well for the last 18 hr of culture. The cells were harvested and counted in a liquid scintillation counter, using the same procedures as for the thymocytes and the CTLL cells in the IL-1 and IL-2 determinations, respectively.

Results

Determination of optimal conditions for priming with MHV3

Optimal conditions for antigen-induced IL-2 production were first set with C3H mice, for *in vivo* priming with MHV3. When live MHV3 was



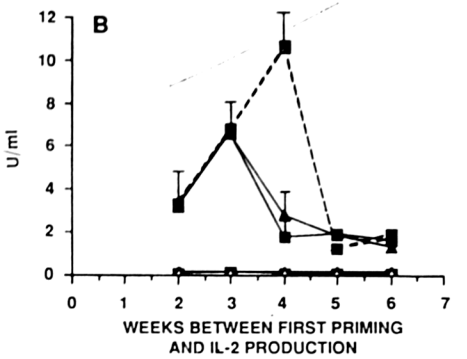


Fig. 1

IL-2 production (units/ml ± standard deviation) by 3×10^6 splenocytes from primed and control C3H mice

A: Mice were killed 7 days post priming with various amounts of killed MHV3 virus. Open symbols: priming with killed MHV3 alone. Black symbols: priming with killed MHV3 + 0.005 μmol/l of liposomes. Triangles: MHV3 antigen was added to cultured cells, for *in vitro* specific restimulation. Squares: no antigenic restimulation *in vitro*.
Abscissa: amounts of the equivalent of LD_{50} MHV3 per mouse.
Ordinate: IL-2 IU per ml.

B: Mice received two primings with an amount of killed MHV3 equivalent to 10^3 LD_{50} + 0.005 μmol/l of liposomes each. They were killed at different times (abscissa) after the first priming, one week after the second one. Same symbols as for A. An additional group was included (dotted lines): two indomethacin treatments were given, together with the MHV3 + liposome primings.
Abscissa: weeks between first priming and IL-2 production.
Ordinate: IL-2 IU per ml.

injected, the mice which survived the "acute disease" always exhibited significant antigen-specific IL-2 production, up to 3 months p. i. (not shown). We choosed to perform the lymphokine tests 3 weeks after injection of live MHV3, as a standard procedure.

When killed virus was used for priming, different amounts were injected in a first step, either alone or in combination with 0.005 μmol/l liposomes per mouse. We previously found that these liposomes alone did not induce IL-2 level different from the baseline (Mansour *et al.*, 1988). As seen in Fig. 1A, the highest IL-2 production was obtained when a priming dose of killed MHV3, corresponding to the same amount as 10^3 LD_{50} per mouse, was injected. This amount was therefore routinely used in the subsequent experiments. It can also be noticed that no significant IL-2 production was observed if no liposome was added to the killed virus, and that the addition, to the cultured cells, of MHV3 antigen did not elicit significant increase of IL-2 production. Therefore, in the subsequent experiments, liposomes were added to priming antigen.

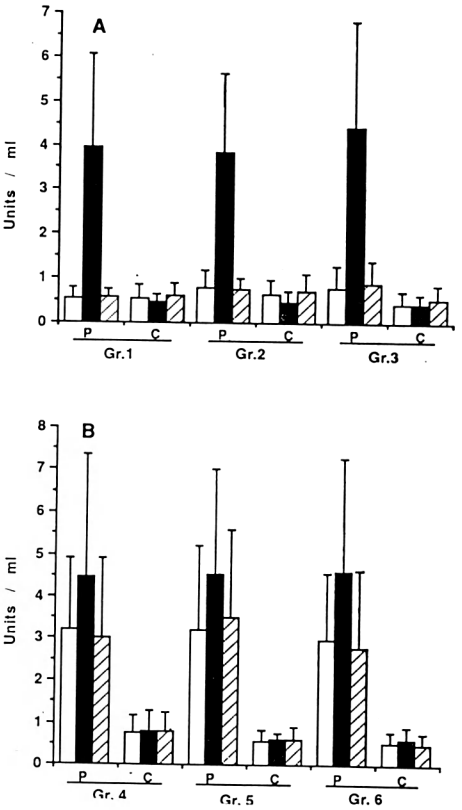


Fig. 2

IL-1 production (units/ml ± standard deviation) of 1×10^6 peritoneal cells of various firoups of primed (P) or control (C) F_1 hybrid mice (see Materials and Methods for details) (Pool of 3 independent experiments)

Black columns: MHV3 antigen added to cultured cells (specific restimulation).
Hatched columns: Poliovaccine added to cultured cells (non-specific restimulation).
Open columns: medium alone added to cultured cells(no antigenic restimulation).
For group designations see Materials and Methods.
A: Priming with live MHV3 infection, 3 weeks before killing.
B: Primings with killed MHV3 + liposomes, 3 weeks (first priming) and 1 week (second priming) before killing.

In a second step, we determined the optimal immunization schedules for the *in vivo* priming with killed MHV3 virus. We found that giving two primings, rather than one, resulted in significantly higher responses. In the subsequent experiment, therefore, we decided to perform two primings. Mice were always killed one week after the second *in vivo* priming, and we found that the optimal schedule for performing the IL-2 production was 3 weeks after the first priming. Addition of liposomes was always mandatory for obtention of good IL-2 production. Again, addition of MHV3 antigen only, to cultured cells, did not result in sizeable increase of the response (Fig. 1B). When indomethacin was injected to mice, together with the primings, IL-2 production was enhanced and the optimal delay between the first priming and the splenocyte cultures shifted from 3 to 4 weeks (Fig. 1B).

Production of IL-1 in MHV3 primed and non-primed mice

Mice primed with either live MHV3 (Fig. 2A) or killed MHV3 + liposomes (Fig. 2B) were used. Their adherent peritoneal cells were tested for IL-1

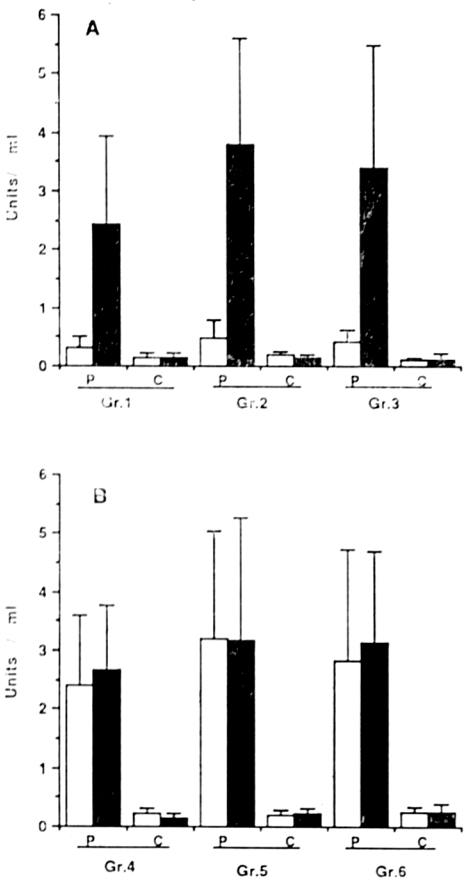


Fig. 3

IL-2 production (units/ml ± standard deviation) of 3×10^6 splenocytes of various groups of primed (P) or control (C) hybrid mice (Pool of 3 independent experiments)
Groups and symbols as in Fig. 2.

production. MHV3 antigen was added to some cultures. Poliovaccine was added to other cultures, and other cultures were left without antigen addition. Fig. 1A shows that addition of MHV3 antigen to the peritoneal cells in culture resulted always in significantly higher IL-1 production than the baseline value, in the case of the mice primed with live MHV3. In the case of mice primed with killed MHV3 + liposomes (Fig. 2B) IL-1 production was always significantly increased if compared with non-primed mice. In addition, in the cultures where MHV3 antigen had been added, this increase was always higher. In both Figs 2A and 2B, no clearcut difference could be observed among mice of different genome type. The results presented were pooled from three experiments.

Production of IL-2 in MHV3 primed and non-primed mice

Splenocytes from the same donor mice as those used for IL-1 determinations were cultured either with or without MHV3 antigen, and tested for IL-2

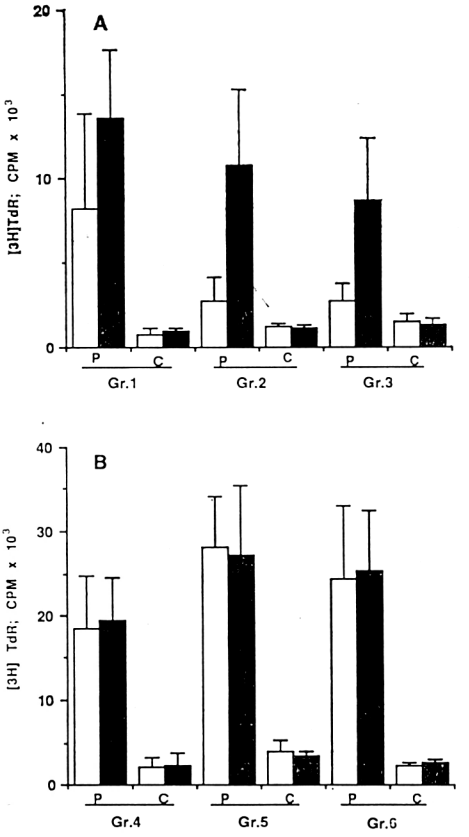


Fig. 4
Antigen-driven splenocyte proliferation, as appraised by ^{3}H-Thymidine incorporation (in cpm ± standard deviation) (Pool of 3 independent experiments) Groups and symbols as in Figs 2 and 3.

production. In the case of the mice primed with live MHV3, addition of MHV3 antigen to the cultured cells resulted in significyntly higher IL-2 production than in the case of the non-primed donors (Fig. 3A). In contrast, in the case of mice primed with killed virus + lipo-somes, IL-2 production was always higher than in the case of the non-primed mice, whether or not MHV3 antigen had been added to the cultures. No genetic effect could be observed (Fig. 3B). The results presented were pooled from three experiments.

Antigen-driven splenocyte proliferations

Other aliquots of splenocytes, from the same donors as those which generated the results shown in Figs 2 and 3, were cultured for 96 hr, and cell proliferations were assessed. In the case of donors primed with live virus, the splenocyte proliferation levels were slightly increased in comparison with those exhibited by splenocytes from non-primed donors. When MHV3 antigen was added to cultures, this resulted in $\sim$ 2—3 fold ^{3}H-TdR incorporation increases (Fig. 4A). In contrast, in the case of donors primed with killed

virus + liposomes, addition of MHV3 antigen to cultures did not bring about significant changes in the proliferation levels, which appeared in all cases manyfold higher than that of the non-primed controls (Fig. 4B). Again no clearcut difference, from one group to another, was observed for a given priming protocol.

Discussion

A clearcut correlation between cellular antivirus reactions, especially delayed type hypersensitivity (DTH), and virus-induced murine demyelating disease, has been observed in the case of the Theiler virus (Clatch *et al.*, 1986). In the present work, we looked for a correlation between anti-virus cellular reactions and the MHV3-induced paralysis. Unfortunatly, our killed virus preparations (with or without liposomes added) did not seem to be sufficiently immunogenic to permit in vivo detection of DTH as measured by foot pad swelling (not shown). Perhaps this could have resulted from an effect of U.V. irradiation on MHV3 antigens. Therefore, we limited this study to some in vitro aspects of the anti-MHV3 cellular reaction, in conditions whereby our virus preparations were found antigenic. This was done with groups of mice whose genomes exhibited three different levels of resistance to MHV3-induced paralysis, depending on H-2 gene or genes.

A class I gene, H-2D, had been shown to be associated with resistance to Theiler virus-induced paralysis, as well as with DTH against virus antigens (Clatch *et al.*, 1986). In another experimental model, myelin basic protein (MBP)-induced paralysis, resistance appeared to be controlled by I-A (a class II gene of the H-2 complex), and anti I-A monoclonal antibody was able to interfere with MBP-specific IL-2 production (Fritz *et al.*, 1985). In the present system, different H-2 haplotypes were studied in association with either the A/J (groups 1, 2 and 3) or the C3H (groups 4, 5 and 6) genome. None of the experiments performed permitted to reveal strong or significant influence of H-2 gene(s) on the different components of the immune reactions studied, and, therefore, none of these components could be associated with either higher or lower resistance to paralysis.

However, in all cases, the mode of presentation of the in vivo priming material appeared to be of utmost importance for the response pattern observed. This strong influence of antigen presentation for priming was observed both with the F_1 hybrid mice and with C3H mice. We confirmed, in the case of killed MHV3, that adding antigenically inert liposomes to viral preparation permitted higher IL-2 production, provided that optimal dose or schedule were used. This had previously been observed with various rabies virus derived preparations (Mansour *et al.*, 1988). An apparently comparable adjuvant-like effect of liposome addition to priming antigen had already been reported by Van Rooijen and Van Nieuwmegen (1977), and these authors later found that this effect resulted from the adsorption of the antigen on the liposome surface (Van Rooijen and Nieumegen, 1980).

In the case of the mice primed with killed MHV3 plus liposomes, an unexpectedly high response to *in vivo* priming only was observed in the case of

all the immune reaction components studied, i. e., IL-1 and IL-2 productions, cell proliferation. With the exception of IL-1 production, these high responses were undistinguishable from the antigen-specific responses obtained when an antigenic preparation was added to the cultured cells, to boost the "primed" cells. These "primary" responses seemed to be a direct consequence of in vivo reaction, since they were sensitive to the action of indomethacin on the primed mice (Fig. 1B). Indomethacin can inhibit the synthesis, by adherent cells, of PGE, a suppressor of IL-2 production (Rappaport and Dodge, 1982). Thus, the increase of IL-2 production we observed after indomethacin treatment could be interpreted as abrogation, exerted by the drug, of PGE production by certain adherent monocytes (Leclerc *et al.* 1984). One of these "primary" responses, reflecting true immune events occuring *in vivo*, has been formally described in the case of mice intrasplenically injected with sheep red blood cells (Spitz *et al.*, 1985). We occasionally observed such kind of effect, after intraperitoneal immunizations with various rabies virus-derived preparations, but the exact conditions required for its occurrence were not clear (Oth *et al.*, 1987; Mansour *et al.*, 1988).

On the contrary, when anti-MHV3 priming was performed with live MHV3 infections, the "primary" responses were either barely or not observed. Significant antigen-specific "secondary" responses were observed in the wells where killed MHV3 preparations have been added to the cultured splenic (IL-2 and proliferation responses) or peritoneal (IL-1 response) cells. The apparent existence of an antigen-specific IL-1, or IL-1 like, response has been reported previously by a few authors (Chao *et al.*, 1977) and raises the question of the mechanism by which a cell population which consist mainly of phagocytes could specifically respond to a second stimulus with the same antigen. Interleukin-1 is known as a major mediator of inflammation (Billiau, 1988) and inflammation is considered a very important factor in paralysing disease (Billiau *et al.*, 1988). In the present case, the absence of difference in IL-1 production observed among the mice of different genomes suggests that it is not at this level that the differential susceptibilities to MHV3-induced paralysis is likely to reside. Also, MHV3-induced IL-2 production, as well as splenocyte proliferation, were similar in mice of all the genomes studied. Clearly other effectors, both cellular and molecular, must be considered for explaining the difference of susceptibility to paralysis exhibited by these different but closely related strains of mice.

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