

Effect of adoptive transfer of CD4, CD8 and B cells on recovery from MHV3-induced immunodeficiencies

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SUMMARY

A chronic viral infection can occur when the host fails to mount an effective immune response to clear the virus. Mouse hepatitis virus type 3 (MHV3) appears to be an excellent model for the study of the relationship between viral-induced immunodeficiency and chronic disease development. (C57BL/6 × A/J)F1 mice surviving acute hepatitis develop a chronic disease characterized by T- and B-cell immunodeficiencies, viral persistence in various organs including the brain, spleen and thymus, and death within 3 months postinfection (p.i.). We have reported that T- or B-cell deficiencies, observed in MHV3 chronically infected (C57BL/6 × A/J)F1 mice, can be partially or totally thwarted by adoptive transfer of CD4⁺, CD8⁺ and/or B cells, at 15 days p.i. in mice surviving the acute phase of the disease. Adoptive transfer of syngeneic CD4⁺ and/or CD8⁺ allowed a partial restoration of the T-cell deficiencies, as characterized by thymic atrophy, decrease in splenic T cells, and in all thymocyte subpopulations. B-cell immunodeficiency, as defined by a decrease in splenic B cells, as well as in the bone marrow pre-B- and B-cell compartments, and the occurrence of abnormally larger forms of bone marrow pre-B and B cells, were partially thwarted by B-cell treatment only. Splenic B cells and the bone marrow B-cell compartment, respectively, returned partially or totally to normal values, whereas the pre-B-cell compartment remained depleted in infected mice treated with B cells. Levels of all immunoglobulin classes returned to normal values in MHV3 chronically infected mice when treated with CD4⁺ in combination with CD8⁺ cells. All T- and/or B-cell treatments, however, were sufficient to thwart the process of the chronic disease, and favoured the survival of mice for up to 6 months p.i.

INTRODUCTION

Chronic viral infection of various organs, including the central nervous system (CNS), usually represents a complication of an acute systemic disease. The infection persists when the host immune system fails to clear the virus. Coronavirus infections are interesting models to analyse the mechanisms involved in immune-mediated viral persistency leading to chronic neurological diseases. Coronaviruses are ubiquitous pathogens in mouse colonies, and are known to induce various pathologies such as hepatitis or enteritis, and acute or chronic neurological diseases in surviving animals.¹ In human, coronaviruses are leading upper respiratory tract pathogens, are associated with gastroenteritis, and have also been isolated from multiple sclerosis patients.^{2–4} Functional or cellular immunodeficiency has been reported in virulent mouse hepatitis virus type 3 (MHV3) or MHV-JHM-infected mice.^{5–7}

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MHV3 serotype appears to be an excellent model to study the relationship between viral-induced immunodeficiency and the development of a chronic disease, since humoral and cellular immunodeficiencies occur in chronically MHV3-infected mice. Susceptible C57BL/6 mice develop an acute hepatitis and die within three days of infection, while virus-resistant A/J mice develop only a subclinical infection, with viral clearance occurring within 7 days postinfection (p.i.).⁸ Mice resulting from a cross-breeding between susceptible C57BL/6 and resistant A/J mice, (C57BL/6 × A/J)F1, show a partial resistance to the acute disease. The surviving animals develop a chronic disease characterized by a wasting syndrome including weight loss, alopecia, and oily hair. In later stages, the mice develop incoordination and hindlimb paralysis, and eventually die within three months postinfection.⁹ We have previously observed significant decreases of thymocytes, splenic cells, and macrophages in chronically infected (C57BL/6 × A/J) F1 mice.¹⁰ Low antibody titres against MHV3 virus, mostly immunoglobulin M (IgM), were found for up to 60 to 70 days postinfection.⁵ In spite of the production of anti-MHV3 antibodies, all immunoglobulin types gradually decreased during the first three months, followed by a return to normal

values in the paralysed animals.⁵ Primary and secondary antibody responses were also markedly diminished in both paralysed and non-paralysed mice. Splenic T and B cells were depleted as early as 48 hr p.i., and maintained at low levels for up to three months until death of mice. Such deficiencies resulted from thymic depletion in all T-cell subpopulations, and in B lymphocytes only in the bone marrow of MHV3-infected mice. In addition, abnormal forms of pre-B or B cells, leading to cell lysis, were generated during the chronic phase of the disease.¹¹ MHV3 viruses were occasionally isolated from the brain, liver, spleen, and lymph nodes of chronically infected mice.^{9,12} In consequence, the cellular and humoral immunodeficiency observed in chronically infected (C57BL/6 × A/J) F1 mice results primarily from depletion of T- and B-cell subpopulations in the lymphoid organs during the acute phase of the disease. The decrease in T and/or B cells may favour viral persistency by abrogating T-cell-dependent immune mechanisms involved in the viral elimination process, and thus lead to the development of a chronic disease, and persistency of the immunodeficiency.¹¹ It was reported by Boespflug *et al.*¹³ that use of cyclosporine, a well-known immunomodulating agent, increased the percentage of death in the acute phase of the disease, when administered during the first week of infection. We have no information on the respective roles of CD4⁺ or CD8⁺ T cell, or B-cell depletions in the process of the chronic disease.

We postulate, therefore, that adoptive transfer of syngeneic CD4⁺, CD8⁺ or B cells to MHV3-infected (C57BL/6 × A/J) F1 mice surviving the acute phase of the disease, may thwart T- or B-cell immunodeficiencies. We report here that T-cell deficiencies and levels of immunoglobulins are partially or totally restored by administration of CD4⁺ and CD8⁺, whereas B-cell treatment is sufficient to improve levels of bone marrow or splenic B cells, only. On other hand, T- and/or B-cell treatments favor the survival of chronically infected mice for up to 6 months p.i.

MATERIALS AND METHODS

Mice

(C57BL/6 × A/J) F1 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Before being used, the animals were tested for the presence of anti-MHV antibodies by enzyme-linked immunosorbent assay (ELISA) using a MHV3 preparation as antigen. During experiments, the animals were housed in a sterile atmosphere (Forma Scientific, Marietta, OH).

Cells

L2 cells, a continuous mouse fibroblast cell line, were grown in Eagle's minimal essential medium (MEM) with glutamine (2 mM), 5% fetal calf serum (FCS), and antibiotics (penicillin, 100 U/ml, and streptomycin, 100 mg/ml) (Gibco Laboratories, Grand Island, NY). L2 cells were used for propagating and titrating the viruses.

Lymphoid cells were obtained from groups of three mice. The mice were killed by cervical dislocation, and the spleen, thymus or femurs were collected. The non-adherent splenic cells were collected according to the previously described method.¹⁴ The thymuses were teased apart in Hanks' balanced salt solution (HBSS) with 10% FCS at room temperature, by

using needles. Femoral shafts were flushed four times with 1 ml cold (4°) RPMI-1640, supplemented with 10% FCS. Large particles were removed by sedimentation on a cushion of 1 ml of FCS for 5 min at 4°. The cell suspension was then centrifuged and resuspended in 1 ml of RPMI-1640 supplemented with 10% FCS. Cell preparations were electronically counted (Coulter Counter, Coulter Electronics, Hialeah, FL) and cell viability, ranging from 90 to 100%, was assayed by the trypan blue exclusion test.

Virus

Pathogenic L2-MHV3 is a cloned substrain of MHV3 produced in L2 cells. YAC-MHV3 virus was a non-pathogenic variant isolated from chronically infected YAC cells, and inducing protective immune responses against challenge with L2-MHV3 virus.¹⁵ The viruses were passaged into L2 cells before use and their pathogenic properties were verified regularly.

T- and B-cell purification procedures

T- and B-cell purification was done by panning according to Wysocki and Sato's method¹⁶ with some modifications. Before purification, adherent monocytes were removed by incubating the thymic or bone marrow cell suspensions (supplemented with 20% FCS), respectively, in a petri dish for 1 hr at 37° with 5% CO₂. After incubation, the dish was thoroughly washed with HBSS. The non-adherent cell suspension (10⁷ cells/ml) was pipetted into an anti-Thy1.2 or anti- μ chain (Jackson Immunoresearch, Mississauga, Ontario, Canada) coated plastic dish and incubated twice at room temperature for 30 min each time. Non-adherent cells were aspirated off the dish and the remaining adherent cell layer was washed four times with Ca-Mg-free phosphate-buffered saline (PBS) and then scraped free with a policeman. Immunofluorescence examinations revealed that the final cell populations contained around 97% of Thy1.1+ and 94% of surface μ (s μ ⁺) cells, respectively. CD4⁺ or CD8⁺ cells were purified by cytotoxicity with anti-CD8 or anti-CD4 antibodies, respectively in presence of rabbit complement (C') (Cedarlane, Hornby, Ontario, Canada); adherent cells were scraped off, centrifuged at 200 g at 4° for 10 min and resuspended in a RPMI-1640 medium. An equal volume of anti-CD4 or CD8 antibodies was added, followed by incubation at 4° for 1 hr. The cells were then centrifuged, resuspended in 150 μ l of optimal dilution of C', and then incubated at 37° for 1 hr. The cells were washed in RPMI-1640 medium, centrifuged, and resuspended at a concentration of 2 × 10⁷/ml. Immunofluorescence examinations revealed that the final cell populations contained around 94% of CD4⁺ or CD8⁺ cells and did not contain any B lymphocytes.

Adoptive transfer of lymphoid cells

(C57BL/6 × A/J)F1 mice were injected i.p. with 1000 TCID₅₀ of L2-MHV3. Mock-infected mice received a similar volume of PBS. At 15 days p.i., mice surviving to the acute phase of the disease and mock-infected mice were separated in groups of three. Each group was i.v. injected with 2 × 10⁶ cells (100 μ l) of one of following cell suspensions: CD4⁺, CD8⁺ or B cells alone, CD4⁺ in combination with CD8, or CD4⁺ in combination with CD8⁺ and B cells. Control groups of mice infected or mock-infected received a similar volume of RPMI-1640. At various times post-treatment (0 to 90 days), groups of

three mice were killed by cervical dislocation and the following parameters were recorded: (1) survival rate, (2) percentages of splenic T and B cells, (3) percentages, absolute numbers, and diameters of bone marrow pre-B and B cells, (4) percentages and absolute numbers of thymic T-cell subpopulations, and (5) determination of concentrations of immunoglobulin classes (IgG1, IgG2a, IgG2b, IgM, IgA) using an ELISA test (Bio-Rad Laboratories, Richmond, VA) and expressed as a percentage of a standardized antibody solution.

Immunofluorescence labellings

T- or B-cell labellings. To detect splenic T or B lymphocytes, direct immunofluorescence assays with fluorescein isothiocyanate (FITC) anti-Thy 1.2 (Miles Scientific, Naperville, IL), or anti- μ chain antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were performed on cytocentrifuged cell preparations. T- or B-cell subpopulations were subsequently identified by double immunofluorescent labelling.

Double labelling of μ -chains. Immunofluorescent labelling was performed as described by Park & Osmond.¹⁷ Briefly, bone marrow samples ($100\ \mu\text{l}$ of 4×10^7 nucleated cells/ml suspension) were incubated for 30 min on ice with an optimal dilution of FITC anti- μ -chains (Cappel Biochemical, Malvern, PA) for $s\mu$ labelling. The cells were washed twice by centrifugation through FCS at $200\ g$ at 4° for 7 min. Cells were resuspended in PBS with 5% bovine serum albumin (BSA), cytocentrifuged, and fixed in acetic acid-methanol solution. Cytoplasmic μ ($c\mu$) chains were labelled with an optimal dilution of tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti- μ chain antibodies (Cappel Biochemical) on the cell spots, incubated for 2 hr at room temperature in a humidified chamber, then washed four times, and mounted as described below.

Double labelling of 14.8 and μ -chains. Bone marrow samples ($100\ \mu\text{l}$ of 4×10^7 nucleated cells/ml suspension) were incubated with rat monoclonal antibody (mAb) 14.8 (obtained from Dr D.G. Osmond, Department of Anatomy, McGill University, Montréal, Québec Canada) for 30 min on ice, then washed, and an optimal dilution of FITC anti-rat IgG (Cappel Biochemical) was added to the cells. The cells were washed twice by centrifugation through FCS at $200\ g$ at 4° for 7 min. Cells were resuspended in PBS with 5% BSA, cytocentrifuged, and fixed in acetic acid-methanol solution. $c\mu$ -chains were labelled with an optimal dilution of TRITC-conjugated anti- μ chain antibodies (Cappel Biochemical) as above.

Double labelling of CD4 and CD8. Thymic cells were incubated with FITC rat mAb anti-CD4 (Dimension Laboratories, Mississauga, Ontario, Canada) at 4° for 30 min, then washed, and resuspended in $100\ \mu\text{l}$ of RPMI-1640 with 10% FCS. Mouse mAb anti-CD8 (Dimension Laboratories) was then added to the cell suspension and incubated for another 30 min at 4° . TRITC anti-mouse IgG antiserum (Cappel Biomedical, Malvern, PA) was added and incubated for 30 min at 4° , cytocentrifuged after washings, fixed as above, and kept overnight in PBS.

Slide mounting. Slides were mounted on a medium containing 90% glycerol in PBS, pH 8.0, and 0.1% *p*-phenylenediamine (Fisher Scientific Co., Montréal, Québec, Canada) A fluorescence microscope (Leitz Dialux 22, Midland, Ontario, Canada) equipped with a mercury lamp and phase contrast optics was used. Slides were examined for $c\mu^+ s\mu^-$ and $c\mu^+ s\mu^+$ B lineage or $CD4^+ CD8^+$, $CD4^- CD8^+$ or $CD4^+ CD8^-$ T-cell

subpopulations. The diameter of at least 100 cells of each B-lineage subpopulation was measured using a calibrated micrometer scale. The percentage of labelled cells was determined by counting a total of 1000 cells. The absolute numbers were calculated by the percentage of positive labelled cells and the total bone marrow or thymus count.

Statistical analysis

Statistical analysis of survival rates in each group of treated mice was performed using the χ^2 test. Percentages and absolute numbers were evaluated by a Wilcoxon Mann-Whitney *U*-test and Student's *t*-test, respectively.

RESULTS

Survival rates of MHV3 chronically infected (C57BL/5 \times A/J)F1 mice treated with $CD4^+$, $CD8^+$ or B cells alone or in combination

The survival rate analysis of MHV3 infected (C57BL/6 \times A/J)F1 mice indicated that 57% mice survived the acute phase of the disease, as measured at 15 days p.i. Neurological signs, however, gradually occurred in these mice from within the next two weeks until up to 90 days (Fig. 1). To determine the protective role of lymphoid cells in development of the chronic disease, groups of 30 mice that survived the acute phase of the disease, were intravenously (i.v.) injected with thymocytes or splenic lymphoid cells collected from YAC-MHV3-immunized mice (1000 TCID₅₀ of virus, and lymphoid organs collected at 7 days p.i.) or from non-immunized mice, at 15 days post-infection. We observed that thymocytes or splenic cells from immunized as well as non-immunized mice protected the chronically infected mice against the occurrence of paralysis or death for more than 6 months after infection (results not shown). These results indicate that the adoptive transfer of syngeneic lymphoid cells from non-immunized mice was efficient to thwart the onset of the chronic disease, suggesting that these cells may sufficiently compensate for the T- or B-cell losses occurring during the acute phase of the disease. To determine the role of $CD4^+$, $CD8^+$ or B cells in the protection against chronic disease development, we analysed the survival rate and the occurrence of paralysis in groups of surviving mice treated with $CD4^+$, $CD8^+$ or B cells, alone or in combination. As shown in Table 1, all treatments thwarted the onset of

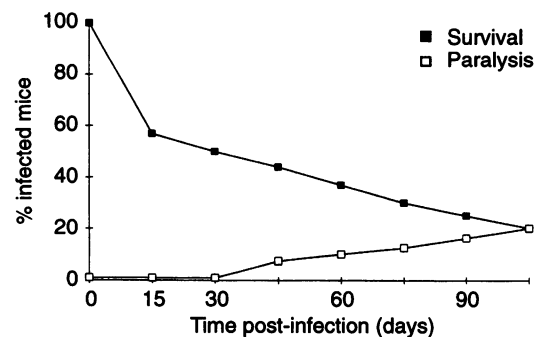


Figure 1. Survival rate (■) and occurrence of paralysis (□) in L2-MHV3-infected (C57BL/6 \times A/J)F1 mice, at various times post-infection.

Table 1. Survival rate of (C57BL/6 × A/J) F1 mice chronically infected with MHV3, and treated with CD4⁺ or CD8⁺ or B cells, alone or in combination, at 15 days post-infection

Cell treatment	Time post-treatment (days)				
	0	15	30	60	90
None	17/30*	15/30	13/30	10/30	7/30
CD4 + CD8	20/30	20/20	17/17	14/14	11/11
CD4	21/30	21/21	18/18	15/15	12/12
CD8	21/30	21/21	18/18	15/15	12/12
B	18/30	18/18	15/15	12/12	9/9
B + CD4 + CD8	20/30	20/20	17/17	14/14	11/11

*No. of surviving mice / no. of MHV3-infected mice. Surviving mice at 15 days p.i. were injected i.v. with 2×10^6 cells in each treatment. At various times after cell treatment, three mice were killed for analysis of lymphoid cell subpopulations. Posterior paresis and paralysis until death occurred in some infected untreated mice. No neurological signs were observed in all other groups of treated mice.

paralysis and death for up to 90 days after the adoptive transfer.

Effect of adoptive transfer of CD4⁺, CD8⁺ or B cells alone or in combination on splenic T and B lymphocytes in MHV3 chronically infected (C57BL/6 × A/J)F1 mice

We have previously reported that, either in the acute or chronic phase of the disease induced by MHV3 in (C57BL/6 × A/J)F1 mice, the splenic T and B cells were depleted as early as 48 hr p.i., and maintained at low levels for up to 3 months until death of the mice.¹¹ To verify the role of splenic lymphoid cell depletions in the chronic disease process, we injected CD4⁺ or CD8⁺ T cells, or B-cell subpopulations, alone or in combination in surviving mice at 15 days p.i., and analysed the percentages of splenic T (Thy1.1⁺) or B ($s\mu^+$) cells in treated or untreated mice. As shown in Fig. 2, the number of T cells transiently increased in mice treated with CD4⁺ or CD8⁺ cells, alone or in combination ($p \leq 0.05$). Splenic B cells increased only after adoptive transfer of B cells. Similarly, adoptive transfer of CD4⁺, CD8⁺, and B cells together induced an increase of splenic T and B cells ($p \leq 0.05$). Such increases, however, did not reach normal values for splenic T- and B-cell populations, as seen in mock-infected mice, and gradually decreased during the next 3 months. Analysis of splenic CD4/CD8 ratios did not show significant alterations in spite of a small decrease in untreated–infected mice (results not shown).

Effect of adoptive transfer of CD4⁺, CD8⁺ or B cells alone or in combination on the thymus in MHV3 chronically-infected (C57BL/6 × A/J)F1 mice

The most prominent feature of the acute MHV3 disease in (C57BL/6 × A/J) F1 mice is a thymic atrophy, occurring as early as 48 hr p.i.¹¹ (C57BL/6 × A/J)F1 mice were infected with MHV3 virus and surviving mice were treated with CD4⁺, CD8⁺ or B cells, alone or in combination, as previously described. Gross examination revealed that thymuses from all treated mice were larger than those of infected–treated mice,

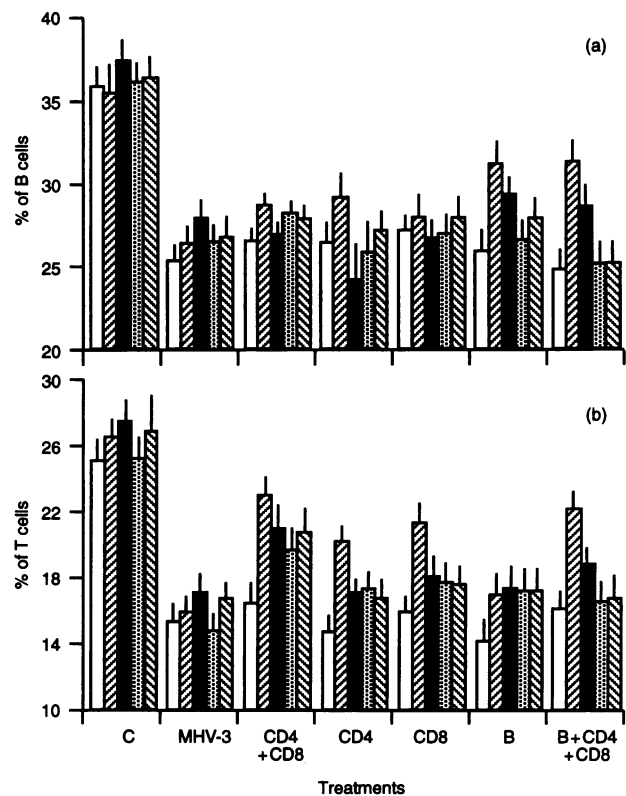


Figure 2. Percentages of T (Thy1.1⁺) or B (sIg^+) cells in spleen from mock-infected, untreated L2-MHV3-infected, or CD4⁺, CD8⁺ or/and B cells treated L2-MHV3-infected (C57BL/6 × A/J)F1 mice, at 0 (□), 15 (▨), 30 (■), 60 (▤), or 90 (▥) days post-treatment (p.t.). Groups of thirty mice were infected i.p. with 100 TCID₅₀ of viruses. Mock-infected mice received i.p. a similar volume of PBS. Mice surviving the acute phase of the disease were injected i.v. with 2×10^6 cells from CD4⁺, CD8⁺, and/or B-cell preparations. At various times p.t., groups of three mice were killed, and spleen collected. Splenic cells were electronically counted, labelled with FITC anti-Thy1.1 or FITC anti- Ig antibodies, and analysed. Results are expressed as mean \pm SD.

but remained smaller than those from mock-infected mice. Percentages of T cells transiently increased for 2 to 4 weeks following adoptive transfer of CD4⁺ or CD8⁺, alone or in combination (Fig. 3) ($p \leq 0.05$). In spite of such increases, thymocyte levels remained lower than in mock-infected mice. All thymocyte subpopulations (CD4⁺ CD8⁺, CD4⁺ CD8⁻ or CD4⁻ CD8⁺) similarly increased in CD4⁺, CD8⁺ or in combination with or without B-cell-treated mice, reflecting the reappearance of a normal T lymphopoiesis (results not shown).

Effect of adoptive transfer of CD4⁺, CD8⁺ or B cells alone or in combination on bone marrow pre-B or B lymphocyte compartments in MHV3 chronically infected (C57BL/6 × A/J)F1 mice

We have previously demonstrated that bone marrow B-cell maturation process was particularly affected during MHV3 infection in (C57BL/6 × A/J)F1 mice.¹¹ Pre-B ($c\mu^+ s\mu^-$) and B ($c\mu^+ s\mu^+$) cell compartments were depleted whereas more immature cells, as precursor cells (TdT⁺) or pro-B (B220⁺ $c\mu^-$) cells were not affected. To verify if the administration of

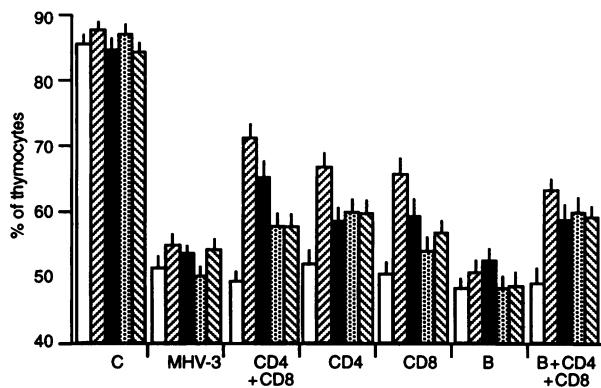


Figure 3. Percentages of thymocytes (Thy1.1⁺) in thymus from mock-infected, untreated L2-MHV3-infected, or CD4⁺, CD8⁺ or/and B cells treated L2-MHV3-infected (C57BL/6 × A/J)F₁ mice, at 0 (□), 15 (▨), 30 (■), 60 (▩), or 90 (⊞) days p.t. Groups of thirty mice were infected i.p. with 100 TCID₅₀ of viruses. Mock-infected mice received i.p. a similar volume of PBS. Mice surviving the acute phase of the disease were injected i.v. with 2 × 10⁶ cells from CD4⁺, CD8⁺, and/or B-cell preparations. At various times p.t., groups of three mice were killed, and thymuses collected. Thymic cells were electronically counted, labelled with FITC anti-Thy1.1, and analysed. Results are expressed as mean ± SD.

T- or B-cell populations can re-establish normal B lymphopoiesis in the bone marrow of chronically infected mice, groups of infected mice were treated with the T- or B-cell preparations, and pro-B-, pre-B- and B-cell populations were analysed at various times post-treatment. Figure 4 shows that the B-cell compartment returned to normal values, for up to 3 months, after administration of B cells alone or in combination with CD4⁺ and CD8⁺ cells ($p \leq 0.05$). All treatments tested, however, did not alter the pre-B-cell deficiency. No disturbance in the pro-B-cell compartment was seen in bone marrow from infected-treated mice, as well in that of as infected mice treated with the T- or B-cell populations (results not shown). These results suggest that the B-cell maturation process is not completely re-established in spite of increase of bone marrow B cells.

We have previously reported that pre-B and B cells were permissive to a productive viral replication leading to generation of typical larger forms of these cells leading to cell lysis.^{11,15} To verify if B-lineage cells produced in treated mice still contained virus-induced larger cells, we analysed the cell diameter distribution of B (Fig. 5) or pre-B (Fig. 6) cells. Abnormally larger forms of B cells, as seen in MHV3 infected mice (11 to 18 μm) strongly decreased in mice treated with B cells or B cells with CD4⁺ and CD8⁺ T cells, whereas no effect was seen with T-cell treatments alone. Rare larger cells, however, were still present in mice treated with B cells, with or without T cells. On the other hand, larger forms of pre-B cells persisted in all treated mice. Such larger cells were not found in the pro-B-cell population from untreated-infected or treated-infected mice (results not shown).

Effect of adoptive transfer of CD4⁺, CD8⁺ or B cells alone or in combination on immunoglobulin class levels in MHV3 chronically infected (C57BL/6 × A/J)F₁ mice

It was previously reported that an humoral immunodeficiency occurred in MHV3-chronically infected (C57BL/6 × A/J)F₁

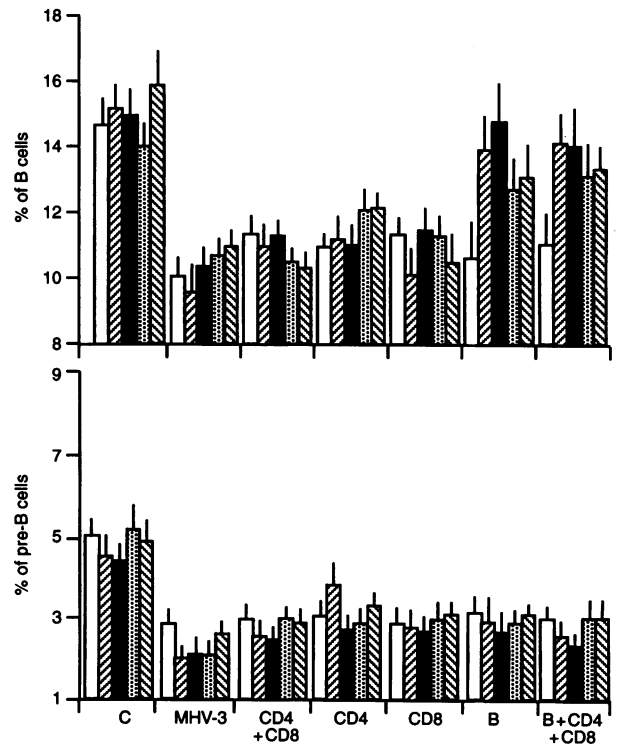


Figure 4. Percentages of pre-B ($c\mu^+ s\mu^-$) or B ($c\mu^+ s\mu^+$) cells in bone marrow from mock-infected, untreated L2-MHV3-infected, or CD4⁺, CD8⁺ or/and B cells treated L2-MHV3-infected (C57BL/6 × A/J)F₁ mice, at 0 (□), 15 (▨), 30 (■), 60 (▩), or 90 (⊞) days p.t. Groups of thirty mice were infected i.p. with 100 TCID₅₀ of viruses. Mock-infected mice received i.p. a similar volume of PBS. Mice surviving the acute phase of the disease were injected i.v. with 2 × 10⁶ cells from CD4⁺, CD8⁺ and/or B-cell preparations. At various times p.t., groups of three mice were killed, and bone marrow collected. Bone marrow cells were electronically counted, double-labelled with FITC anti- $s\mu$ chains, and TRITC- $c\mu$ chains, and analysed. Results are expressed as mean ± SD.

mice, and was characterized by a decrease in all immunoglobulin (Ig) classes, in spite of the production of anti-MHV3 antibodies.⁵ To verify if adoptive transfer of T or B cells can restore the ability of mice to produce immunoglobulins, levels of IgG1, IgG2a, IgG2b, IgG3, IgG, and IgA were analysed in mock-infected, untreated-infected and treated-infected mice at various times post-treatment. Figure 7 shows that CD4⁺ treatment transiently increased Ig levels, whereas they decreased in CD8⁺-treated mice. The combination of CD4⁺ and CD8⁺ cells, however, restored Ig levels to normal values for up to 90 days after treatment. Adoptive transfer of B cells, however, did not prevent immunoglobulin deficiencies. In addition, immunoglobulin levels did not increase when T cells were added to the B-cell preparation.

DISCUSSION

In the present paper, we have shown that T- or B-cell deficiencies, observed in MHV3 chronically infected (C57BL/6 × A/J)F₁ mice, can be partially or totally thwarted by the adoptive transfer of CD4⁺, CD8⁺ and/or B cells, at 15 days p.i. Adoptive transfer of syngeneic CD4⁺ and/or CD8⁺ allowed a

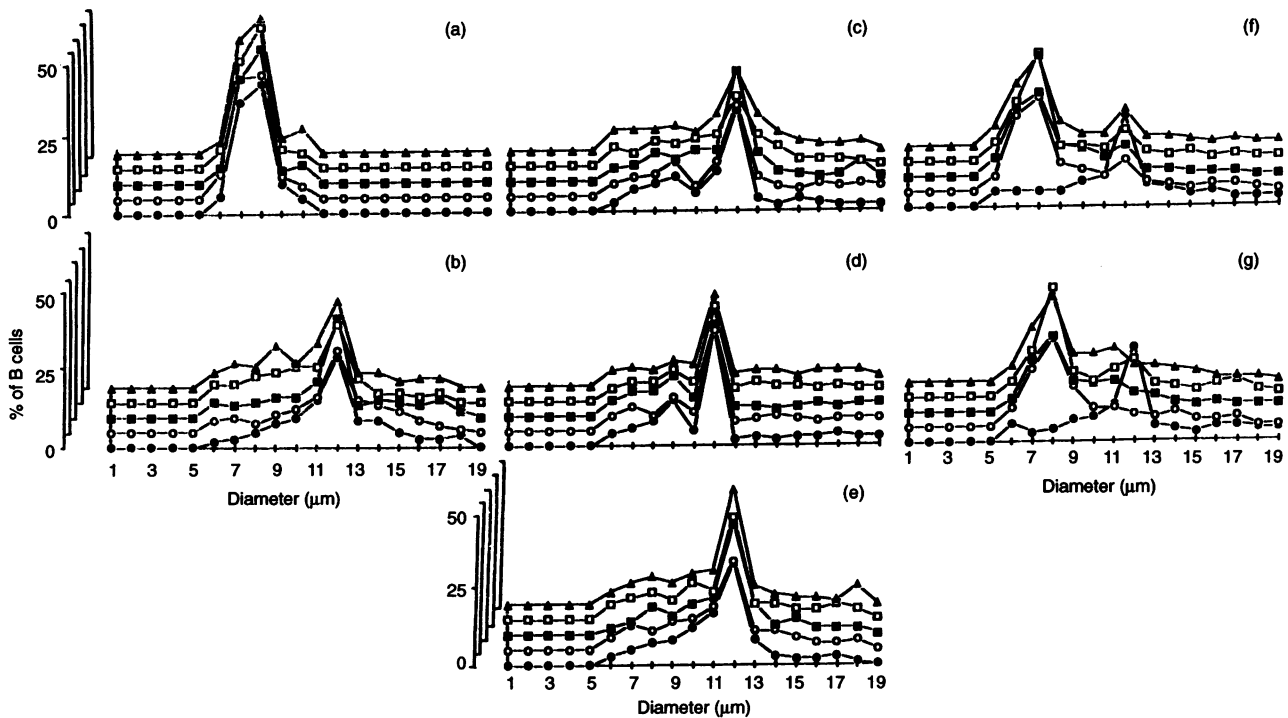


Figure 5. Size distribution of B ($c\mu^+ s\mu^+$) cells in bone marrow from mock-infected, untreated L2-MHV3-infected, or CD4⁺, CD8⁺ or/and B cells treated L2-MHV3-infected (C57BL/6 \times A/J)F1 mice, at 0 (\bullet), 15 (\circ), 30 (\blacksquare), 60 (\square), or 90 (\blacktriangle) days p.t. Groups of thirty mice were infected i.p. with 100 TCID₅₀ of viruses (b). Mock-infected mice received i.p. a similar volume of PBS (a). Mice surviving the acute phase of the disease were injected i.v. with 2×10^6 cells from CD4⁺ (c), CD8⁺ (d), CD4⁺ with CD8⁺ (e), B (f), and B with CD4⁺ and CD8⁺ (g). At various times p.t., groups of three mice were killed, and bone marrow collected. Bone marrow cells were electronically counted, double-labelled with FITC anti- $s\mu$ chains, and TRITC- $c\mu$ chains, and analysed. The diameter of at least 100 cells was measured using a calibrated micrometer scale (standard deviation < 3%).

partial restoration of the T-cell populations to normal values, from a deficiency characterized by thymic atrophy, decrease in splenic T cells, and in all thymocyte subpopulations. B-cell immunodeficiency, as defined by a decrease in splenic B cells, bone marrow pre-B- and B-cell compartments, and the occurrence of abnormally larger forms of bone marrow pre-B and B cells, were partially corrected by B-cell treatment only. Splenic B cells or bone marrow B-cell compartment returned partially or totally, respectively, to normal levels, whereas the pre-B-cell compartment remained depleted in infected-treated mice. Levels of all immunoglobulin classes returned to normal values in L2-MHV3 chronically infected mice when treated with CD4⁺ in combination with CD8⁺ cells. All T- and/or B-cell treatments, however, were sufficient to prevent the chronic disease, and allowed the mice to survive for up to 6 months p.i.

Our work mainly shows that the administration of T and B cells from naive syngeneic mice, and not only from immunized mice, is sufficient to prevent the onset of chronic viral disease, suggesting that the decrease in T- or B-cell subpopulations in the first days p.i. plays a major role in the occurrence of the chronic disease rather than the inability of residual cells to mount an immune response. Leray *et al.*⁵ have observed that T-cell functions on residual lymphocytes were normal, and that T cells, B cells, and macrophages, originating from MHV3-infected, paralysed mice, had the ability to fully reconstitute immune responses in lethally irradiated syngeneic animals. We have shown that adoptive transfer at 15 days p.i. of various T-

or B-cell subpopulations can restore partially or totally the cellular immunodeficiencies in the thymus, spleen or bone marrow cell compartments. Dupuy *et al.*¹⁸ have reported that irradiated mice can be protected against acute MHV3 infection by an adoptive transfer of splenic cells, bone marrow cells, and peritoneal macrophages, suggesting that disease may be thwarted by efficient immune-mediated viral clearance mechanisms. It is postulated that viral persistency may be related to a loss of T or B cells, and to the inability of chronically infected mice to produce new mature T cells, because of thymic destruction, or to regenerate normal bone marrow B cells during the chronic disease. Adoptive transfer of such cell populations at a crucial time in the development of the chronic disease (15 days p.i) may allow a compensation in of such cell losses and gradually favour the activity of immune-mediated viral clearance mechanisms.

The chronic disease induced by MHV3 in (C57BL/6 \times A/J) F1 mice is characterized by an acute phase in which a sublethal hepatitis occurs. However, neuropathological lesions were found at 7 days p.i. and consisted of an important meningeal infiltration of polymorphonuclear and mononuclear cells, as well as perivascular infiltrations of mononuclear cells.¹⁹ At 14 days p.i., the infiltrates contain small lymphocytes, plasmocytes, and macrophages. After this time, diffuse meningeal infiltrate progressively decreased, and inflammatory lesions disappeared. These inflammatory responses can normally disappear after viral clearance in the brain. Viral persistency,

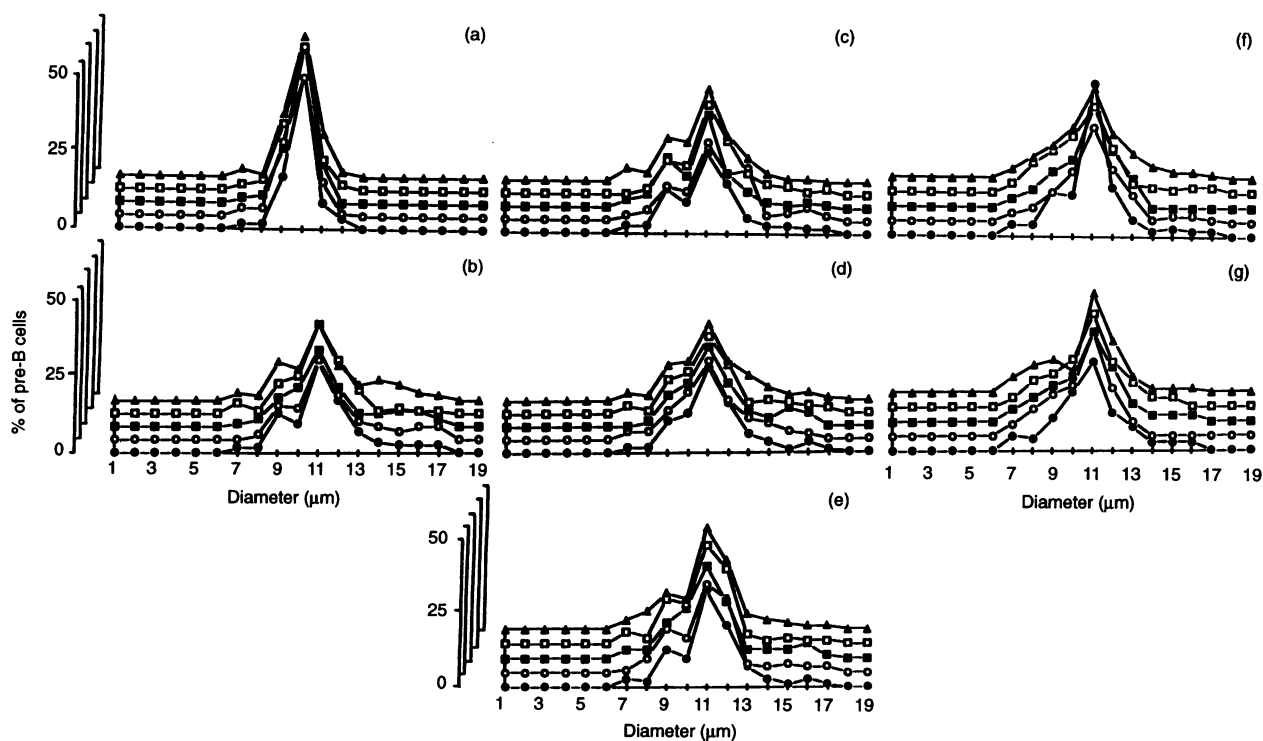


Figure 6. Size distribution of pre-B ($c\mu^+s\mu^-$) cells in bone marrow from mock-infected, untreated L2-MHV3-infected, or $CD4^+$, $CD8^+$ or/and B cells treated L2-MHV3-infected (C57BL/6 \times A/J)F1 mice, at 0 (\bullet), 15 (\circ), 30 (\blacksquare), 60 (\square), or 90 (\blacktriangle) days p.t. Groups of thirty mice were infected i.p. with 100 TCID₅₀ of viruses (b). Mock-infected mice received i.p. a similar volume of PBS (a). Mice surviving the acute phase of the disease were injected i.v. with 2×10^6 cells from $CD4^+$ (c), $CD8^+$ (d), $CD4^+$ with $CD8^+$ (e), B (f), and B with $CD4^+$ and $CD8^+$ (g). At various times p.t., groups of three mice were killed, and bone marrow collected. Bone marrow cells were electronically counted, double-labelled with FITC anti- $s\mu$ chains, and TRITC- $c\mu$ chains, and analysed. The diameter of at least 100 cells was measured using a calibrated micrometer scale (standard deviation < 3%).

however, is associated with the pathogenic process of the chronic disease, indicating a failure in the immune-mediated virus elimination process.⁹ Immunodeficiencies, occurring as soon as 48 hr p.i. and maintained until the death of the animals, may explain the disappearance of inflammatory cells in the brain after 15 days p.i.¹¹

Splenic depletion of T or B cells following the acute phase of the disease induced by MHV3 in (C57BL/6 \times A/J)F1 mice may result from a direct lytic effect of viral replication or from the inability of thymus or bone marrow to produce mature T or B cells. We have previously reported that B cells were permissive to viral replication,^{11,15} but that T cells were not.^{11,20} Thymic atrophy in such mice primarily results from the depletion of all thymocyte subpopulations without regard to the cell maturation state.¹¹ Accumulated data show that the most important factor involved in the differentiation and maturation of T cells is direct contact between the thymocyte and the nonlymphoid thymic constituents. We have previously reported that thymic stromal cells from (C57BL/6 \times A/J)F1 mice were permissive to viral replication, leading to cell lysis, and the thymic atrophy reflected the disappearance of stromal cells involved in the thymocyte maturation process.¹¹ In addition, a loss of normal thymic architecture was observed in MHV-3 infected C57BL/6 mice.²⁰ We can expect that higher levels of splenic or thymic T cells following adoptive transfer of $CD4^+$ or $CD8^+$, or in combination in MHV3-infected (C57BL/6 \times A/J)F1 mice result from partial repair of the thymic architecture, including thymic

stromal cells, thereby allowing some occurrence of normal T lymphopoiesis. This explanation supposes that infectious virus has been cleared in the mice, and/or newly formed stromal cells are not permissive to viral replication. The first hypothesis is in agreement with the fact that a few infectious viral particles were normally detected in the thymus, spleen or brain of chronically infected mice,⁹ but were not detected *in vitro*, except from the brain.¹²

It is possible that the $CD4^+$ and $CD8^+$ adoptive transfer could be sufficient to clear the residual infectious viruses in the mice. Sussman *et al.*²¹ have demonstrated that clearance of the neurotropic MHV-JHM strain from the CNS in acutely infected mice was under control of $CD4^+$ cells, acting as a helper, and of $CD8^+$ cells that recognize viral antigens at the cell surface. Since depletion of $CD4^+$ or $CD8^+$ cells occurred as early as 48 hr p.i. in MHV3-infected (C57BL/6 \times A/J)F1 mice, the immune-mediated viral clearance mechanisms were impaired, favouring viral persistency. Adoptive transfer of $CD4^+$ and $CD8^+$ permitted a sufficient restoration of the level of splenic or thymic T-cell subpopulations involved in the viral clearance. However, $CD4^+$ or $CD8^+$ treatment alone cannot cause a recovery for a long period of time, as seen when the two cell populations were given together, suggesting that infectious viruses were still produced, even at low titres, in site protected from immune cells, such as the brain. We have observed that inflammatory cells transiently increased in the brain of such treated mice (results not shown). It was recently reported that

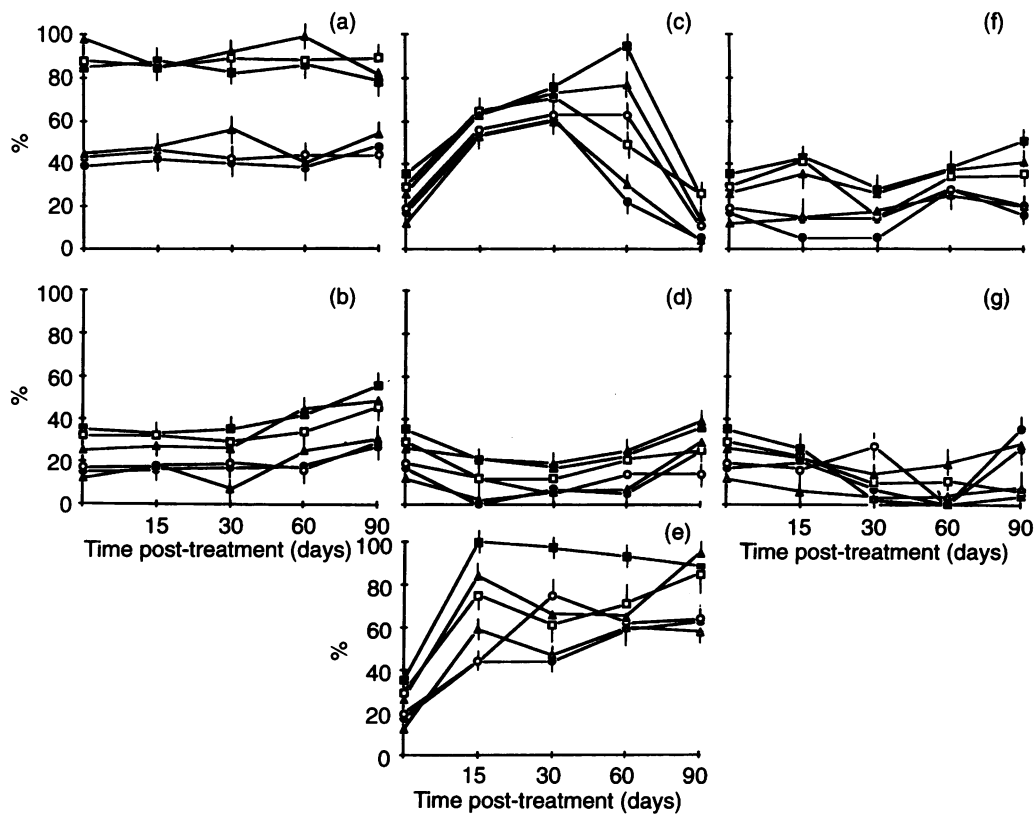


Figure 7. Levels of immunoglobulins IgG1 (●), IgG2a (○), IgG2b (■), IgG3 (□), IgM (▲), or IgA (△) in serum from mock-infected, untreated L2-MHV3-infected, or CD4⁺, CD8⁺ or/and B cells treated L2-MHV3-infected (C57BL/6 × A/J)F1 mice, at various times p.t. Groups of thirty mice were infected i.p. with 100 TCID₅₀ of viruses (b). Mock-infected mice received i.p. a similar volume of PBS (a). Mice surviving the acute phase of the disease were injected i.v. with 2×10^6 cells from CD4⁺ (c), CD8⁺ (d), CD4⁺ with CD8⁺ (e), B (f), and B with CD4⁺ and CD8⁺ (g).

the majority of CD4⁺ T lymphocytes entering the MHV-JHM-infected CNS in the course of infection are primed cells that neither proliferate *ex vivo* nor can be stimulated to proliferation by viral antigens or mitogens *in vitro*, whereas T lymphocytes from lymph nodes of the same animals revealed a strong proliferative response.²² In addition, a CD4⁺ cell subset secreting interferon- γ plays an important role in preventing neurological disease progression in persistently MHV-JHM-infected C57BL/6 mice²³ but do not inhibit viral replication.²⁴ On the other hand, cytotoxic T cells were found in the CNS of such mice, but their presence did not prevent the demyelinating process.²⁵ These cells were efficient in reducing viral replication in ependymal cells, astrocytes, and microglia, but not in oligodendrocytes.²⁶ We can postulate that adoptive transfer of CD4⁺ and CD8⁺ in MHV3-infected (C57BL/6 × A/J)F1 mice may only allow restoration of inflammatory cell responses in brain, favouring immune-mediated viral clearance mechanisms. In spite of partial efficiency of CD4⁺ or CD8⁺ alone, viral replication may be delayed, permitting a transient recovery of T-cell subsets in lymphoid organs, as seen in the thymus or spleen.

On the other hand, humoral immunodeficiency, characterized by a decrease in all immunoglobulin classes has been thwarted by adoptive transfer of CD4⁺ and CD8⁺, but not by administration of B cells. These results indicate that hypoglobulinaemia was due to T-cell immunodeficiency occurring

during the first days p.i., and was not caused by functional depression of B cells. Leray *et al.* have previously observed that chronically infected mice cannot mount an immune response against T-dependent or T-independent antigens.⁵ Our results show that restoration of higher levels of splenic or thymic CD4⁺ or CD8⁺ cells permit the production of all immunoglobulin classes for a longer period of time than seen after transfer of CD4⁺ cells only. All immunoglobulin classes, however, decreased after administration of CD8⁺ cells, suggesting that either suppressor cells were present in CD8⁺ cell preparation or that CD4⁺ cells are essential for immunoglobulin production stimulation. Since the residual B cells remained functional in chronically infected mice,⁵ we postulate that the transfer of CD4⁺ can stimulate B cells to produce immunoglobulins. The transient production of antibodies, however, may be explained by the permissivity of pre-B or B cells to viral replication leading to cell lysis, as previously reported.^{11,15} Addition of CD8⁺ cells to CD4⁺ cells may permit the destruction of infected cells by cytotoxic T cells, favouring the stimulation of non-infected newly produced B cells. Figure 4 shows that abnormally formed B cells in bone marrow, resulting from viral infection, disappeared after the transfer of CD4⁺, CD8⁺ and B cells. Otherwise, administration of B cells alone was sufficient to thwart production of such larger B cells. This observation may be explained by the specific cytotoxic property of B cells.^{27,28} Splenic B cells from uninfected mice can selectively lyse MHV-

infected cells by the interaction of viral surface glycoprotein on the target cell surface with receptors on the splenocyte membrane. We can expect that adoptive B cells can lyse infected target cells, including bone marrow B cells, thereby explaining the disappearance of abnormal larger B cells. In spite of the cytotoxic activity of adoptive B cells, abnormal larger pre-B cells remained in bone marrow, indicating that viral infection was not cleared. Newly formed pre-B or B cells may still be infected by residual virus in the bone marrow, rendering them incapable of immunoglobulin production, as seen in B-cell-treated mice. In addition, adoptive transfer of B cells provide fresh target cells for the persistent MHV3, explaining the weak efficiency of B-cell transfer in restoring normal B lymphopoiesis and humoral immune responses. Casebolt *et al.*²⁹ have shown that MHV infection can alter the microenvironment of Peyer's patches, then inhibiting the production of antibodies by local B cells. In addition, humoral immunity did not totally protect against MHV acute or chronic diseases, as shown by the adoptive transfer of immunoglobulins^{8,30} or monoclonal antibodies.^{31,32}

The cytoimmunotherapy approach used in this work suggests that the host may thwart the virus-induced chronic disease by more than one immune mechanisms. Further work is in progress to understand the mechanisms involved in the protecting effect of T- or B-cell subpopulations in chronic neurological disease.

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