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B-cell activation following murine cytomegalovirus infection: implications for autoimmunity

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SUMMARY

Infection of susceptible mice with murine cytomegalovirus (MCMV) induces persistent inflammation, and the production of autoantibodies reactive with large numbers of proteins from all major organs. However the roles of polyclonal B-cell activation, autoreactive T-helper cells and host–virus cross-reactions in these phenomena have not been evaluated. The present study reveals six- to 20-fold increases in serum immunoglobulin levels in MCMV-infected BALB/c and CBA mice, with IgG3 and IgG2b most affected. Titres of antibodies reactive with autologous tissues and ovalbumin (OVA) also increased following MCMV infection, whilst responses to a synthetic antigen [polyvinyl pyrrolidone (PVP)] were unaffected or depressed. IgG2a was the isotype most affected in responses to OVA, MCMV antigens and autologous tissues, suggesting interferon-γ (IFN-γ) may contribute to responses induced in the presence of the relevant antigen. Increases in total and antigen-specific immunoglobulin levels were CD4 dependent, as they were reduced in infected mice depleted of these cells with anti-CD4 antibodies. Serological changes were preceded by B-cell expansion and activation evident from increased cell yields, frequencies of cells releasing immunoglobulin and proliferation of T-depleted spleen and lymph node preparations. Numbers of mature B cells and macrophages increased in the lymph nodes, but B-1a ($CD5^+Ig^+$) cell counts remained low. Alterations in the B-cell phenotypic profiles were more complex in the spleen, but correction for increased cell yields revealed increases in some subpopulations.

INTRODUCTION

Although most immunocompetent patients infected with cytomegalovirus (CMV) remain asymptomatic, hepatitis and carditis are reported, and CMV may initiate atherosclerosis in susceptible individuals.[1,2] CMV disease is also potentiated by extrinsic immunosuppression, so a high proportion of transplant recipients and acquired immune deficiency syndrome (AIDS) patients develop CMV pneumonitis, and other organs may be affected. Autoimmune mechanisms have been proposed to explain CMV-induced histopathological changes, because cells expressing viral antigens are rare and may not be detectable in tissues displaying widespread persistent monocytic infiltrates. The development of autoimmunity would also explain anti-smooth muscle antibodies found in sera from CMV patients.[3]

Ab, antibody; B-1a, $CD5^+$ B cell; B10, C57BL/10; CBA, CBA/CaH; d.p.m., disintegrations per minute; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; IL, interleukin; LD_{50}, 50% lethal dose; LPS, lipopolysaccharide; OVA, ovalbumin; PFU, plaque-forming units; PVP, polyvinyl pyrrolidone; SEM, standard error of mean; MCMV, murine cytomegalovirus.

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Immunological features of CMV-induced autoreactivity can be analysed in inbred mice infected with murine (M) CMV. BALB/c ($H\text{-}2^d$) mice are killed by moderate doses of MCMV, and at lower doses develop persistent carditis,[4] hepatitis (S. D. Olver and P. Price, in preparation) or pneumonitis.[5] Inflammatory T cells persist in affected tissues up to 8 weeks after infection, whilst cells expressing viral antigens are rare in relation to the severity of the inflammation, and disappear within 1 week. CBA mice survive challenge with doses of MCMV 20 times higher than are tolerated by BALB/c. This probably arises because $H\text{-}2^k$ cells are more resistant to MCMV *in vitro* than $H\text{-}2^d$ cells,[6] and because CBA mice mount more effective interferon (IFN) and natural killer (NK) cell responses.[7] However when given doses of virus adjusted for their resistance to lethal challenge, CBA mice exhibit severe hepatitis after 4 weeks with only partial resolution after 8 weeks (S. D. Olver and P. Price, in preparation).

Antibodies reactive with tissues from uninfected syngeneic hosts have been demonstrated in sera from MCMV-infected mice.[8] Although antigenic cross-reactions between host and viral proteins have since been described,[9,10] the large number of peptides from normal mouse tissues recognized by Western blotting with sera from infected mice[8] suggests that autoantibody production may result from polyclonal B-cell activation.

Despite numerous reports documenting autoimmune sequela of other viral infections (for review, see ref. 11), the contributions of polyclonal activation, antigen and cognate help have not been assessed. This report describes increases in serum immunoglobulin (Ig) and autoantibody levels following infection with MCMV, and compares the isotype profiles with those of antibodies reactive with other antigens encountered before infection. Populations of B cells and macrophages are also described, including B-1a ($CD5^+$ B) cells which produce natural antibodies and mediate autoimmune conditions such as rheumatoid arthritis.[12]

MATERIALS AND METHODS

Experimental animals and MCMV infection

Specific pathogen-free mice obtained from the Animal Resources Centre (Murdoch, Western Australia) were maintained under minimal disease conditions. Serological screening established that the holding centre remained free of mouse hepatitis virus, Sendai virus and *Mycoplasma pulmonis*. At 8–10 weeks of age, mice were infected i.p. with MCMV prepared as a salivary gland homogenate.[4] The strain of MCMV [designated K181 (Perth)] was obtained from Dr D. Lang (Durham, NC) as the Smith strain. However recent restriction enzyme digest analyses of the viral DNA revealed patterns similar to K181.[13] Unless specified otherwise, BALB/c mice received 0·3 times the amount of virus required to induce 50% mortality (0·3 BALB/c LD_{50} MCMV). C57BL/10 (B10) and CBA/CaH (CBA) mice received three- and 20-fold greater doses of virus, respectively, as this corresponds to 0·3 LD_{50} in these strains.[4] This approach ensures that all mice experience similar levels of viral replication and disease. A dose of 0·3 BALB/c LD_{50} typically contains $2{\cdot}5 \times 10^4$ plaque forming units (PFU) MCMV.

Proliferation of freshly isolated cells

Suspensions of spleen or lymph node (axial and scapular) cells from three to five mice were adjusted to $2{\cdot}5 \times 10^6$ cells/ml in HEPES-buffered RPMI-1640 with 5×10^{-4} M 2-mercaptoethanol and 0·5% normal mouse serum.[7] These were cultured for 24 hr with 0·5 μCi [^{3}H]thymidine (Amersham International, Amersham, U.K.) per 0·2 ml aliquot, harvested and the incorporated radioactivity was assessed by liquid scintillation counting.

Aliquots of each preparation were depleted of T cells prior to culture by incubation (30 min on ice) in pooled supernatants from Rl-172 and 31M hybridomas, containing rat IgM recognizing L3T4 and Lyt-2 antigens. Guinea-pig complement diluted in HEPES-buffered RPMI containing 0·2 mg/ml deoxyribonuclease (DNase) (Sigma Chemical Co., St Louis, MO) was added for 30 min at 37°, and the cells were resuspended in their original volume so that the concentration of non-T cells was unchanged. T-cell depletion inhibited concanavalin A (Con A)-induced proliferation by 99% relative to unseparated preparations.

Leucocyte phenotypic profiles

For single-colour cytofluorometry, cells were incubated on ice with culture supernatants containing rat monoclonal antibodies recognizing Mac-1 (M1/70 15.11.5), IgM (331.12), B220 (RA3-6B2) or F4/80, followed by fluorescein-conjugated goat anti-rat Ig (Silenus, Australia; mouse Ig-adsorbed). Cells expressing surface Ig were detected with biotinylated sheep anti-mouse Ig and streptavidin–phycoerythrin (Amersham).

To detect co-expression of IgM and IgD, cells were treated sequentially with 331.12 supernatant, fluorescein-conjugated anti-rat Ig, biotinylated AMS.15.1 antibody and streptavidin–phycoerythrin. To enumerate B-1a ($CD5^+$ B) cells, preparations were reacted with anti-Lyt-1 antibodies (53.7.8), followed sequentially by fluorescein-conjugated goat anti-rat Ig, biotinylated sheep anti-mouse Ig and streptavidin–phycoerythrin.

Positive cells were detected by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA) and analysed with the programme LYSYS. Single-colour analyses were gated between discrete peaks on histogram plots. IgD^+ IgM^+ and $CD5^+$ Ig^{high} cells were enumerated using log:log contour plots divided into regions containing distinct subpopulations. Gates and regions were held constant for all equivalent assays.

Enumeration of antibody-secreting cells

Using a modified 'ELISA spot' technique,[14] 24-well plates were coated (24 hr, 4°) with the Ig fraction of a sheep antiserum reactive with all classes of mouse Ig, generously provided by Dr G. A. Stewart (Perth, Australia). Diluted single-cell suspensions were applied to the coated plates for 12 hr at 37° without disturbance. Antibody bound adjacent to plasma cells was detected with phosphatase-conjugated goat anti-mouse Ig (Tago Inc., Burlingame, CA) and dianisidine substrate (Sigma).

Assessment of total immunoglobulin levels by ELISA

Sera were titrated in 96-well plates coated with anti-mouse Ig reagents. Total IgG levels were obtained from plates coated with the fractionated sheep antiserum described above, and developed with alkaline phosphatase-conjugated goat anti-mouse Ig (Tago) in the presence of excess monoclonal murine IgM. Plates coated with the sheep antiserum were also used to detect IgM in test sera, by reaction with phosphatase-conjugated goat anti-mouse IgM (μ chain; Tago). Subclasses of IgG were titrated using plates coated with goat antisera specific for murine IgG1, IgG2a, IgG2b or IgG3 (Sigma), and visualized with phosphatase-conjugated goat anti-mouse Ig. The specificity of isotype-specific reagents was verified by titration of monoclonal antibodies of known Ig isotype. Test sera and phosphatase conjugates were each incubated on the plates for 2 hr at 37°, then dinitrophenyl phosphate substrate (Sigma) was added and optical densities (OD) were recorded at 405 nm.[15] Normal mouse serum from a standard pool was titrated on each plate and assigned a value of 1000 Ig units. Semi-logarithmic standard curves were then used to calculate the unit value of each sample. Data are presented as the geometric mean unit values for each experimental group divided by the geometric mean unit values for uninfected syngeneic controls. Statistical evaluation was by Student's *t*-tests applied to the unit values after logarithmic transformation. The absolute levels of Ig in the standard serum were obtained later by titration in parallel with a suspension of known concentration (Sigma).

Antigen-specific antibody

To titrate antibodies reactive with ovalbumin (OVA), polyvinyl pyrrolidone (PVP) or B-lactoglobulin, sera were diluted in 96-well plates coated with the required antigen (Sigma; 50 μg/ml). Bound antibody was detected with the unconjugated isotype-

specific goat anti-mouse Ig reagents described above, followed by alkaline phosphatase-conjugated rabbit anti-goat immunoglobulin (Cappel, Westchester, PA). Responses to MCMV antigens were assessed in the same manner, using plates coated with MCMV propagated in susceptible fibroblasts and purified by ultracentrifugation.[13] Results were calculated as above, using sera from hyperimmunized mice as standards.

Detection of autoantibodies

Autoantibody was detected by incubation of test sera (diluted 1 in 10) on histological sections from paraffin-embedded composite blocks of tissues from uninfected syngeneic mice. Background staining was eliminated by preincubation of the sections with hydrogen peroxide (1% in H_2O) and skim milk [2% in phosphate-buffered saline (PBS)]. Bound antibody was visualized using isotype-specific goat anti-mouse Ig reagents (as above), followed by peroxidase-conjugated rabbit anti-goat Ig (Nordic, Tilberg, The Netherlands) and 3'3'-diaminobenzidine substrate[4,9] applied for 4 min ± 10 seconds. Mounted sections were coded and the intensity of staining was scored macroscopically by three independent observers, who recorded concordant results in all studies. To eliminate day-to-day variation, scores were averaged and scaled so that sera from normal mice yielded values of 1·0 U. Replicate runs were then used to calculate 95% confidence limits based on Student's *t*-test.

Depletion of T cells in vivo

Mice were injected i.p. with monoclonal antibodies recognizing L3T4 or Lyt-2 determinants, as purified ascites fluids from YTS-169.4 or YTS-191.1 hybridoma cells (respectively) provided by Dr A. A. Scalzo (Perth, Australia). These were administered on days −2, 0, 4 and 8,[16] relative to the time of MCMV infection, and mice were killed on day 11. Lymph node preparations from individual mice were screened for residual T cells using fluorescein-conjugated anti-Lyt-2 and phycoerythrin-conjugated L3T4 reagents (Becton Dickinson).

Table 1. Assessment of lymphoproliferation in freshly isolated cells

		[^{3}H]thymidine incorporation	
	Cell yield* ($\times 10^7$)	Unseparated cells	T-depleted cells
Spleen			
Uninfected	3·8	2·9 ± 0·6	1·0 ± 0·2
Day 11	7·1	6·2 ± 0·8	3·5 ± 0·3
Lymph nodes			
Uninfected	0·5	2·4 ± 0·5	0·5 ± 0·2
Day 11	1·1	4·8 ± 0·6	1·8 ± 0·1

Lymphoproliferation assessed in pooled preparations from groups of five BALB/c mice cultured with [^{3}H]thymidine for 24 hr (mean d.p.m. $\times 10^{-3}$ ± SEM).

* Yields of viable cells per donor prior to culture.

Table 2. Enumeration of antibody-secreting cells

		Day		
	Uninfected	8	13	22
Spleen	24	140	48	16
Lymph nodes	< 1	60	12	< 1

Antibody-secreting cells were detected in preparations from groups of three to five BALB/c mice by ELISA spot assay (mean value/10^6 cells).

RESULTS

Lymphoproliferation and immunoglobulin secretion

Total numbers of cells present in the spleens and peripheral lymph nodes of BALB/c mice increased after MCMV infection (Table 1). These preparations exhibited increased incorporation of [^{3}H]thymidine when cultured for 24 hr without *in vitro* stimulation. Increased [^{3}H]thymidine incorporation was also evident after the depletion of T cells *in vitro*. Similar results were obtained with cells from CBA and B10 mice given 20- and threefold higher doses of virus (strain-adjusted 0.3LD50 MCMV; data not shown). The findings suggest an increase in B-cell proliferation *in vivo* after MCMV infection, contributing to increased cell yields.

MCMV also increased the proportions of cells in the spleens and lymph nodes secreting antibody. Values peaked on day 8 and remained higher than uninfected mice on day 13 (Table 2).

Phenotypic profiles of spleen and lymph node cells

Infection of BALB/c mice increased the proportions of spleen and lymph node cells expressing moderate levels of immunoglobulin (Ig^{mod}). The percentage of cells expressing high levels of immunoglobulin (Ig^{high}) also increased in the lymph nodes, where there is little viral replication or histological change.[7] These changes were maintained from 4 to 30 days after infection (Table 3), so further studies were limited to day 11. On day 11, MCMV increased the proportion of IgM^+IgD^- cells in spleen preparations, but the proportion of cells expressing the pre-B and B-cell marker, B220, declined. However correction for the increased cell yields demonstrates that total numbers of $B220^+$ cells were maintained or increased. As expression of B220 may decline when B cells are activated, MCMV may activate splenic B cells more rapidly than the resting B cells can be replaced, and activation may precede IgD expression. In contrast, all categories of conventional B cells were expanded in the lymph nodes, suggesting activation concurrent with recruitment and proliferation. There was no change in the proportions of $CD5^+Ig^{high}$ cells in the spleen, but small increases were noted in the lymph nodes. Macrophage numbers increased two- to threefold at both sites, when assessed by expression of Mac-1 (Table 3) or F4/80. Values peaked 8–14 days after infection, and returned to normal by day 30 (data not shown). Phenotypic changes were similar in lymph node preparations from BALB/c mice given 0·1–0·5 LD_{50} MCMV, but the higher dose caused splenic atrophy.

CBA mice given a strain-adjusted dose of MCMV (Table 3) exhibited changes similar to BALB/c mice, with increased

Table 3. Frequencies of B cells and macrophages in lymphoid tissues

	BALB/c		CBA	
	Uninfected	Day 11	Uninfected	Day 11
Spleen				
Ig^{high}	14	13	24	16
Ig^{mod}	7	13*	8	12*
$CD5^+Ig^{high}$	1·0	0·8	2·8	2·5
$B220^+$	31	27	37	23
IgM^+IgD^+	14	16	26	14
IgM^+IgD^-	4	14*	8	11
$Mac\text{-}1^+$	4	8*	4	8*
Lymph nodes				
Ig^{high}	2	8*	3	6*
Ig^{mod}	2	5*	2	5*
$CD5^+Ig^{high}$	0·7	1·4*	1·0	3·4*
$B220^+$	13	19*	7	12*
IgM^+IgD^+	6	10*	5	9*
IgM^+IgD^-	1·3	10*	5	14*
Mac-1+	1	3*	0·6	2*

Phenotypic profiles were assessed by flow cytometry, using preparations from groups of three to five mice given 0·3 LD_{50} MCMV (mouse strain-adjusted) on day 0.

All assessments were replicated in three to six separate studies, and those showing clear increases in every study are marked with an asterisk (*).

proportions of Ig^{high}, $CD5^+Ig^{high}$, IgM^+, IgD^+ and $B220^+$ cells in peripheral lymph nodes. The splenic Ig^{mod} population increased in infected CBA mice, but the proportions of cells expressing other markers declined. Limited studies of CBA mice given 0·3 BALB/c LD_{50} MCMV (20-fold less virus) revealed similar changes in lymph nodes, but clearer increases in splenic Ig^+ cells (data not shown).

Changes in serum immunoglobulin levels

IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 levels were assessed in sera from mice of several strains sampled 4–35 days after infection. Representative results obtained with BALB/c, CBA and B10 mice are presented in Table 4, as ratios of the unit values recorded in sera from infected and uninfected mice. IgG levels rose by day 14, remained high until day 30 and then declined. This is 7–14 days after the decrease in antibody-secreting cells (Table 2). IgM levels in sera from B10 (Table 4) and BALB/c (see Table 6) mice increased 7–11 days after infection.

Increases in serum IgG were statistically significant in BALB/c and CBA mice given 0·3 BALB/c LD_{50} MCMV and CBA mice given 0·3 CBA LD_{50} MCMV. Substantial increases were also recorded when 0·3 BALB/c LD_{50} MCMV was given to A/J and BALB.B mice, which are more susceptible than BALB/c, and C3H/HeJ and BALB.K mice which are as resistant as CBA (data not shown). Hence Ig levels do not correlate with H-2^k-mediated resistance to MCMV, or with input virus dose (relative to LD_{50}). However increases in serum IgG were smaller in B10 mice (Table 4), as in B10.BR and B10.D2 (data not shown).

IgG3 levels showed the greatest *relative* increases in most studies, whilst IgG2b exhibited significant but slightly smaller rises, and IgG2a and IgG1 were less affected. Our standard serum pool contained 280, 20, 150 and 50 μg/ml IgG1, IgG2a, IgG2b, IgG3, respectively, when titrated against commercial Ig standards. There was some variation between mouse strains, but levels of IgG2b in sera from uninfected mice were always higher than IgG2a or IgG3. This is consistent with published Ig levels[17] and suggests that IgG2b exhibits the largest *absolute* rises after MCMV infection.

Antigen specificity of the immunoglobulin present after infection

MCMV-specific antibody

Pooled sera collected from BALB/c mice 19 days after infection were assayed for antibody of each subclass reactive with tissue culture-derived MCMV. The relative amounts of antibody of each subclass were calculated from the dilutions of serum yielding OD of 1·0. Ratios of IgG1: IgG2a: IgG2b: IgG3 were 1: 144: 11: 7, respectively. Hence the response to MCMV antigens is predominantly IgG2a.

Autoantibody

Sera from infected BALB/c and CBA mice were applied to tissue sections from uninfected syngeneic donors and binding was visualized by immunoperoxidase staining. The colour reaction was minimal in sections treated with control sera, but intense with sera from mice infected for 12–28 days. Normal spleen, muscle, kidney, liver and heart yielded similar results, and all cells in the sections were stained to some degree. MCMV induced IgG2a autoantibody in CBA sera, with smaller but significant increases in IgG2b and IgG3 (Table 5). BALB/c mice exhibited significant IgG2a and IgG2b autoantibody responses.

Responses to other antigens

BALB/c mice were immunized with 10 μg OVA or 1 μg PVP[15] and infected with MCMV 9 days later. Adjuvants were avoided to prevent non-specific mitogenesis. Total and antigen-specific Ig titres were measured 7 or 14 days after infection, and ratios of ELISA values from infected and uninfected mice were calculated (Table 6).

Antibody reactive with PVP cannot be detected in unimmunized mice, but mice immunized with PVP displayed vigorous antigen-specific responses.[15] These ongoing responses were reproducibly depressed 14 days after MCMV infection, even though total IgG levels increase (as in Table 4). Anti-PVP IgM levels rose slightly 7 days after infection, in parallel with total IgM.

IgG and IgM reactive with OVA were detectable in sera from uninfected mice and increased after infection, irrespective of whether the mice were immunized with OVA. Antibody reactive with OVA in unimmunized mice may reflect oral priming, as sheep proteins are present in mouse chow. This could also explain the antibodies to bovine β-lactoglobulin found in the same sera (data not shown). Alternatively OVA and β-lactoglobulin may cross-react with murine proteins, since MCMV induces autoantibodies. Cross-reactions between OVA and MCMV were not evident when sera from uninfected OVA-immune mice were screened for antibodies to MCMV.

Table 4. Subclass profiles of serum immunoglobulin after MCMV infection

	IgG†	IgG1	IgG2a	IgG2b	IgG3	IgM
BALB/c	7·6*	1·4	2·4*	3·7*	7·3*	1·1
CBA	22*	1·2	2·9*	16*	10*	0·7
CBA (high dose)‡	14*	2·8	1·9	4·4*	10*	0·4*
B10	2·8	1·0	0·6	1·2	4·1*	0·3*
B10 (day 11)	1·1	0·6	2·0	—	3·8*	4·7*

Ig levels in individual sera from groups of three to six mice killed 19–25 days after infection with 0·3 BALB/c LD_{50} MCMV were assessed by ELISA in arbitrary units relative to a standard serum pool and presented as ratios calculated by the formula

$$\frac{\text{geometric mean Ig U in MCMV-infected mice}}{\text{geometric mean Ig U in control mice}}.$$

* Significant difference between infected and control mice (Student's *t*-test, $P<0·05$).

† All subclasses of IgG.

‡ 0·3 CBA LD_{50} MCMV.

Table 5. Subclass profile of antibodies reactive with normal syngeneic tissue

	BALB/c	CBA
IgM	1·3±0·1	1·2±0·1
IgG1	1·2±0·1	1·4±0·4
IgG2a	1·5±0·2*	3·3±0·9*
IgG2b	1·6±0·2*	1·5±0·2*
IgG3	1·2±0·1	1·6±0·2*

Sera pooled from groups of three infected (days 12–20) or three control mice were applied to normal tissue sections and developed with immunoperoxidase reagents under standardized conditions. Ratios of the colour intensitities recorded with sera from infected and uninfected mice are presented as mean (±SEM) from six separate studies.

* Significant difference between infected and uninfected mice (Student's *t*-test, $P<0·05$).

MCMV infection increased the levels of all isotypes of OVA-specific antibody (Table 7). However the subclass profile was similar to the autoantibody and MCMV-specific responses, rather than total Ig, as IgM and IgG2a increased more markedly than IgG3 and IgG2b.

The effects of *in vivo* T-cell depletion using monoclonal antibodies

CBA mice treated with monoclonal antibodies reactive with L3T4 and Lyt-2 determinants were infected with MCMV and assayed on day 11, together with uninfected and untreated controls (Table 8). Virus titres assesed in the salivary glands demonstrated that L3T4 cells were most critical for the control of infection in that organ, as described previously.[18] The percentages of lymph node cells bearing L3T4 or Lyt-2 antigens were reduced from 52±1·0 to 1·2±0·1 and 29±0·7 to 0·6±0·1 in infected mice treated with these antibodies (mean±SEM, respectively). Lymph node preparations from infected mice treated with both antibodies displayed a 5·8-fold reduction in total cell number relative to other groups and comprised 8·2±4·4% $L3T4^+$ cells and 0·5±0·1% $Lyt\text{-}2^+$ cells. The efficiency of T-cell depletion was similar in uninfected mice.

Depletion of $L3T4^+$ cells reduced MCMV-induced increases in total serum IgG, with IgG3 most affected. L3T4 depletion also prevented increases in levels of antibodies reactive with OVA, MCMV antigens or syngeneic tissues after MCMV infection. In these responses IgG2a was most clearly affected by MCMV in the non-depleted mice, and was the only isotype blocked by depletion of L3T4 cells in all replicate studies. Depletion of Lyt-2 cells did not prevent the elevation of total IgG or antigen-specific antibody by MCMV, irrespective of the isotype measured. Similar results were obtained in a single study of BALB/c mice (data not shown).

DISCUSSION

Serum IgG levels increased following MCMV infection of BALB/c, CBA, C3H/HeJ, A/J, BALB.B and BALB.K mice, and to a lesser extent in B10, B10.A and B10.D2 mice (Table 4; P. Price, S. D. Olver, A. E. Gibbons and G. R. Shellam, unpublished results). Hence the effects of MCMV on immunoglobulin levels are not affected by $H\text{-}2^k$-mediated resistance to MCMV,[6] but may be reduced by genes in the B10 genetic background. This would be consistent with earlier studies in which autoantibodies were demonstrated less frequently in B10 sera than BALB/c or C3H/HeJ,[8] and B10 sera had lower levels of anti-myosin antibodies.[19] Mechanisms underlying the low polyclonal and autoimmune responses in B10 mice will be investigated further as they may explain the failure of B10 mice to develop persistent carditis, pneumonitis and hepatitis (refs 4,5; S. D. Olver and P. Price, in preparation).

It was noted in earlier studies [7,20] that lymph node cells from MCMV-infected BALB/c or CBA mice display enhanced proliferative responses when stimulated *in vitro* with bacterial lipopolysaccharide (LPS), whilst spleen preparations comprise more infected cells and exhibit depressed responses at high virus

Table 6. Effects of MCMV on antibodies reactive with test antigens

Interval between MCMV infection and sampling (days)	Anti-PVP		Anti-OVA		Total Ig	
	IgG	IgM	IgG	IgM	IgG	IgM
Mice preimmunized with PVP						
7	0·9	1·7	—	—	0·9	2·2
14	0·4*	0·3*	3·5*	2·6*	5·6*	0·3*
Mice preimmunized with OVA						
7	—	—	3·4*	4·4*	—	—
14	—	—	3·5*	3·1*	—	—

Groups of 12 BALB/c mice were immunized with 1 μg PVP or 10 μg OVA, and six of each group were infected with MCMV 9 days later. All mice were bled after a further 7 or 14 days, and results are expressed as ratios of the ELISA unit values of sera from infected and uninfected mice (see Table 4).

* Significant difference between infected and uninfected mice (Student's *t*-test, $P < 0{\cdot}05$).

Table 7. Subclass profile of OVA-specific antibody in infected mice

	IgG†	IgG1	IgG2a	IgG2b	IgG3	IgM
Total immunoglobulin	6·9*	2·6	2·8*	5·2*	5·6*	1·4
OVA-specific antibody	4·9*	1·9*	6·5*	2·4*	2·2*	6·4*

Groups of 10 BALB/c mice were primed with 10 μg OVA, five of each group were infected with MCMV 8 days later, and all mice were bled after a further 8 days. Results are expressed as ratios of ELISA unit values recorded in sera from infected and uninfected mice (see Table 4).

* Significant difference between infected and uninfected mice (Student's *t*-test, $P < 0{\cdot}05$).

† All classes of IgG.

Table 8. MCMV-induced serological changes in mice depleted of L3T4 or Lyt-2 cells

	Total Ig		Anti-OVA		Anti-MCMV		Autoantibody		MCMV load
	IgG†	IgG3	IgG†	IgG2a	IgG†	IgG2a	IgG2a	IgG3	PFU × 10^{-6} ± SEM‡
No Ab	28·5	7·4	1·3	3·4	25	102	2·8	1·4	0·06 ± 0·01
Anti-L3T4	2·2	1·2	0·4	0·8	2·3	2·5	1·2	1·2	7·44 ± 2·39
Anti-Lyt-2	11·2	6·6	2·3	4·4	55	> 100	2·6	1·9	0·10 ± 0·06
Both Ab	1·1	1·2	0·6	1·9	2·5	0·6	1·0	0·8	11·05 ± 1·03

Groups of seven CBA mice were treated with monoclonal antibodies to L3T4 or Lyt-2 determinants, four mice from each group were infected with MCMV and all mice were assessed on day 11. Total Ig, anti-OVA and anti-MCMV levels are presented as ratios of ELISA units from infected and uninfected mice (see Table 4) and autoantibody levels as ratios of staining scores obtained using tissue sections (see Table 5).

† All classes of IgG.

‡ Plaque-forming units per salivary gland.

loads. The changes can now be associated with the expansion of all B-cell populations assessed in peripheral lymph nodes and alterations in the phenotypic profiles of B cells in the spleen. Increased frequencies of cells releasing immunoglobulin were demonstrated in the spleen and peripheral lymph nodes after infection (Table 2), in addition to increases in total cell yields (Table 1). The cell yields may be increased by B-cell proliferation *in vivo*, as [^{3}H]thymidine incorporation by freshly isolated cells was increased by MCMV. This was evident after the depletion of T cells *in vitro*.

B-cell proliferation, phenotypic shifts and enhanced antibody secretion are unlikely to be direct consequences of MCMV replication in the individual B cells, as only one to 10 lymph node cells per million are productively infected.[7] A more probable scenario is that the phenomena are driven by macrokines. For example, interleukin-1 (IL-1) may induce B-cell proliferation directly or via IL-6.[21] The numbers of macrophages in lymphoid tissues increase after MCMV infection (Table 3) and IL-1 production by peritoneal macrophages is enhanced.[20] The latter was demonstrated in populations where only 0·1% of cells were productively infected, and so is not a consequence of infection of the macrophages.

Human CMV can induce polyclonal B-cell expansion directly *in vitro*. This does not require infectious virus and may be T-cell independent.[22] MCMV grown in fibroblasts and inactivated by ultraviolet radiation stimulates. [^{3}H]thymidine incorporation by normal spleen cells two- to four-fold, relative to unstimulated cultures (P. Price, S. D. Olver, A. E. Gibbons and G. R. Shellam, unpublished results). Hence B-cell activation could result from a direct effect of free virus on B cells or macrophages without viral replication. Stimulation of cellular metabolism has been demonstrated in fibroblasts treated with human CMV. It involves activation of cellular protein kinases, without the expression of immediate early antigens.[23]

B-1a cells are implicated in several autoimmune conditions[12] and produce IgM or IgG3. They are self-maintaining independent of the bone marow, which is atrophied in infected mice (A. E. Gibbons and P. Price, in preparation). MCMV probably did not increase frequencies of $CD5^+$ Ig^{high} cells enough to affect serum Ig levels (Table 3), but induced a population of $CD5^+$ Ig^{mod} cells which warrant further investigation.

The progressive decreases in serum IgM and the changes in the phenotypic profiles of the B cells suggest polyclonal maturation of conventional B cells favouring IgG production. In general IgG3 levels were the most markedly elevated 14 or more days after infection, whilst IgG2b, IgG2a and IgG1 exhibited progressively smaller increases (Table 4). This pattern of isotype switching is observed in LPS-stimulated cells in limiting dilution cultures. The isotypes showing the clearest increases (IgG3 and IgG2b, respectively) are encoded by C-region genes immediately downstream from $C\mu$ and $C\delta$.[24] Although the phenomenon is independent of T cells *in vitro*, it may be influenced by T cells *in vivo* via the induction of macrokines, explaining the reduced effects of MCMV on total Ig levels in mice depleted of $L3T4^+$ cells (Table 8). Subsequent studies will address the role of IL-6, as IL-6 production by monocytes is implicated in the B-cell hyperplasia seen in human immunodeficiency virus (HIV) patients and in several autoimmune disorders.[25] IL-6 promotes the differentiation of B cells into plasma cells, without inducing isotype switching directly.

A simpler explanation for the increase in total serum Ig is antibody to the virus itself, as proposed for HIV-induced hypergammaglobulinaemia.[26] However primary MCMV-specific antibody is mostly IgG2a, with some IgM, IgG2b and IgG3. The preponderance of IgG2a does not favour a major contribution of MCMV-specific antibody to the increased total Ig levels, which involved IgG3 (largest relative change) and IgG2b (largest absolute amount). Furthermore, B10 mice showed a smaller increase in total IgG than CBA mice (Table 4), but develop higher MCMV-specific humoral responses.[27]

Previous studies show that IFN-γ selectively induces IgG2a, whilst IL-4 potentiates IgG1 *in vitro*.[28] IgG1 levels increase in mice infected with intestinal helminths,[29] whilst lactate dehydrogenase virus can increase serum IgG2a levels 50-fold.[30] Both are clearly distinct from the effects of MCMV. However IgG2a was evident in the responses of MCMV-infected mice to self-antigens, OVA and MCMV, suggesting a role for IFN-γ (consistent with the effects of L3T4 depletion). These responses may require antigenic stimulation of B cells via their surface Ig, plus specific T-cell help. The need for antigenic stimulation would explain the absence of a potentiated response to the synthetic antigen, PVP (Table 6). These results would be consistent with the hypothesis that the development of a normal B-cell repertoire able to respond rapidly to environmental pathogens involves positive selection for cells expressing Ig reactive with antigens encountered in the periphery, with autoreactivity limited by the absence of autoreactive helper T cells.[31] Thus mechanisms preventing the deletion of autoreactive T-cell clones in the thymus or activating anergic clones in the periphery may lead to the production of autoantibody. It may be relevant that MCMV infection induces severe thymic atrophy, with the same genetic distribution as immunoglobulin changes described herein (P. Price, S. D. Olver, A. E. Gibbons and G. R. Shellan, in preparation).

In conclusion, MCMV-induced increases in serum immunoglobulin may arise from the expansion and activation of conventional B cells in the lymph nodes and possibly the spleen. This may be driven by macrokines up-regulated by factor(s) released from $L3T4^+$ cells, as it does not perturb patterns of isotype switching seen in isolated B cells. In addition, autoantibody production may be further facilitated by the presence of antigen. It requires $L3T4^+$ cells and includes IgG2a, as do the responses to MCMV itself and the common protein antigen, OVA. The model will now be used to clarify the role of the subversion of T-cell tolerance in the generation of autoimmunity and persistent inflammation following CMV infection.

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