Murine Cytomegalovirus Infection Model in Balb/c Mice

3. Immunoglobulin Production during Infection

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In mice infected with a lethal dose of murine cytomegalovirus (MCMV) the serum immunogloblin (Ig) levels and the Ig-bearing cells in the spleen dropped to barely detectable levels 2 days after infection. In mice with acute but non-lethal MCMV infection, the serum IgM was twice and the IgG 32 times that of the uninfected controls by Day 8 of infection; the numbers of spleen cells bearing IgM and the IgG subclasses (IgG1, IgG2a, IgG2b, IgG3) were also greatly increased. In the asymptotically infected group, serum IgM remained unchanged but the IgG increased to 16 times that of uninfected controls by Day 11 of infection; the numbers of spleen cells bearing IgM and IgG subclasses were also increased, although to a lesser extent than in the acute, non-lethally infected mice. In the latter two groups, serum IgA and IgA-bearing cells in the spleen did not alter significantly. Complement-requiring neutralizing antibodies to MCMV were detected 8 days post infection.

(Key Words: murine cytomegalovirus, immunoglobulin, antibody response)

INTRODUCTION

The interaction between cytomegalovirus (CMV) and the host immune system is a complex one. The effects of CMV infection on the humoral immune system of the host can be summarized as follows: 1) CMV suppresses the humoral response of the host to independent antigens such as sheep red blood cells, according to studies done in both conventional and germfree mice (6, 12, 24). 2) CMV infection elicits a predictable humoral immune response specific to CMV. In human infection, the different immunoglobulin subclasses against CMV have been detected by a variety of methods and in various clinical situations (8, 9, 11, 29, 30); in the murine system, neutralizing antibodies (3, 25, 26), complement fixation antibodies (6), and antibody-dependent cell-mediated cytoltoxicity (18) have been demonstrated. However, the degree of protection afforded by the specific anti-CMV antibodies is controversial as the virus is often detectable in various anatomic sites in the presence of the neutralizing antibodies. 3) In addition to the specific anti-CMV response, an increase in antibodies unrelated to the viral antigens have been reported in humans (2, 15); and autoantibodies to liver-specific lipoprotein (4), nuclear antigen, DNA and nucleoprotein, as well as immune complexes (21) have been reported in mice. 4) The possibility that CMV is a B-cell activator has been suggested by in vitro studies using human CMV (13, 28), although as yet this phenomenon has not been studied in vivo.

To explain these varied and somehow controversial humoral activities during CMV infection, it seemed necessary to examine the dynamics of immunoglobulin production during the course of infection. We have previously established models of lethal, acute non-lethal, and asymptomatic murine CMV (MCMV) infections (17). Using these models, the changes in serum immunoglobulin (Ig) levels during the
3 forms of infection were examined. The serum Ig levels were correlated with the changes in Ig-bearing cells in the spleen.

MATERIALS AND METHODS

Mice:
Four-week-old male Balb/cA Jc1 mice were purchased from CLEA, Tokyo. Animal experiments were carried out according to the Guideline for Animal Experimentation of the Tokai University School of Medicine.

Virus:
The preparation of the Smith strain of MCMV was as described previously (16). Briefly, MCMV was serially passaged in ICR mice by intraperitoneal injection of salivary extracts prepared from mice infected 2 weeks previously with MCMV. The salivary gland extract from the 11th serial passage was used as virus stock for infection purposes. The stock titer was 5 × 10⁸ plaque-forming units (pfu) per ml.

Experimental design:
Three groups of 4-week-old mice were infected intraperitoneally with an inoculum of 0.1 ml containing 2 × 10⁸, 2 × 10⁹ or 2 × 10¹ pfu of MCMV. These 3 doses produced lethal, acute non-lethal, and asymptomatic infections, respectively, as described previously (17). Control mice were inoculated with appropriately diluted salivary gland extract from normal mice. At stated time intervals, 2 mice from each experimental or control group were sacrificed. Heart blood was collected and serum was separated and stored at −80°C. The spleen was removed and preserved in 10% formalin. Paraffin sections were prepared by routine laboratory procedures. One set was stained by haematoxylin and eosin and another processed for peroxidase-labelled antibody staining for immunoglobulins.

Measurement of immunoglobulins:
The levels of IgM, IgG, and IgA in serum were determined by the agar gel precipitation method as described by Hashimoto et al (10). Plates containing 0.9% Agarose (Wako Pure Chemical Industries, Ltd., Osaka, Tokyo) in barbital buffer, pH 8.6, were used. Two-fold serial dilutions of the sera from mice were made and tested with rabbit anti-mouse IgM, IgG and IgA (Bionetics Co., Kensington) Immunoglobulin titers were expressed as the reciprocal of the highest dilution giving precipitation reactions.

Immunoglobulin (Ig)-bearing cells:
An indirect peroxidase-labeled antibody test for IgM, IgA and the subclasses of IgG were carried out on the spleen sections according to Nakane and Pierce (20). Rabbit antiserum specific for mouse IgM, IgA, and IgG1, 2a, 2b and 3, and a peroxidase-labeled goat anti-rabbit antiserum were purchased from Bionetics Co. The paraffin sections were dewaxed and treated with hydrogen peroxide and diluted normal goat serum to eliminate nonspecific staining. The treated sections were stained with the respective antisera for 1 hr at room temperature. The optimal titers for use were found to be 1:200 for anti-IgM and anti-IgA and 1:25 for the anti-IgG subclasses. The goat anti-rabbit peroxidase-labeled IgG antibody was then applied and incubated at room temperature for 1 hr. The reaction was developed with 20 mg diaminobenzidine and 17 μl hydrogen peroxide in 100 ml of 0.05M Tris buffer adjusted to pH 7.6. Each test slide was accompanied by a negative control in which buffer was substituted for the rabbit anti-immunoglobulin serum. In view of the uneven distribution of the Ig-bearing cells, the number of stained cells in the whole spleen section was counted under low-power objective. For each spleen, two sections for each immunoglobulin were prepared and counted and the results averaged.

Neutralizing antibodies to MCMV:
The MCMV stock used for infection was passaged once in mouse embryonic fibroblasts (MEF). When the cell sheet showed maximum cytopathic effect, it was freeze-thawed three times to release the virus, and the cell debris was removed by centrifugation. The virus suspension was assayed for MCMV and adjusted to contain 4000 pfu/ml in growth medium (Eagle’s minimum essential medium supplemented with 10% fetal calf serum). The test sera were inactivated at 56°C for 30 min and serial twofold dilutions were made. To 0.2 ml of the neat or diluted serum was added 0.2 ml of the virus suspension, and the mixture incubated at 37°C for 1 hr. After 0.05 ml of cold guinea pig
complement diluted 1:2 was added, the mixture was again incubated at 37°C for 1 hr. One-tenth ml from each dilution was then layered on MEF monolayers in 96-well culture plates and allowed to adsorb for 1 hr at 37°C. Then the cell sheet was overlayed with Eagle's minimum essential medium containing 2% fetal calf serum, L-glutamine, penicillin, streptomycin and 0.75% methylcellulose. The cultures were incubated for four days at 37°C and the number of plaques counted. The neutralizing antibody titer was defined as the reciprocal of the highest dilution of serum resulting in a 50% reduction in the number of plaques.

RESULTS

Staining of spleen sections:
The histopathological findings of this study resembled those of previous studies, and have been described in detail (17). With the peroxidase-labelled antibody technique, the IgM-bearing cells in the paraffin sections were stained satisfactorily. Non-specific staining was not detected on the negative control slides. The majority of the stained cells showed the typical morphology of plasma cells (Fig. 1).

Changes in IgM levels during infection:
The serum IgM levels and the numbers of IgM-bearing cells per spleen section are shown in Fig. 2. In the group inoculated with the lethal dose of $2 \times 10^6$ pfu, no mice survived more than 6 days. Serum IgM dropped to barely detectable levels and the number of IgM-bearing cells was greatly diminished by day 4 post-infection.

In the group which received $2 \times 10^5$ pfu, the mice became visibly ill but all survived (the acute, non-lethal infection). The number of IgM-bearing cells in the spleen had increased by day 4, peaked on day 6 and afterwards declined, but was still in excess of the controls 17 days after infection. The serum IgM level was definitely raised on day 8, compared with the uninfected controls, and appeared to decline from day 14.

The group of mice inoculated with $2 \times 10^4$ pfu developed an asymptomatic infection. There was a slight rise in IgM-bearing cells in the spleen by day 4 which returned to normal on day 8. The serum IgM level remained the same as the uninfected controls all through the experimental period.

Changes in IgG levels:
The levels of serum IgG and the numbers of cells in the spleen bearing the IgG subclasses are shown in Fig. 3. In the uninfected control group, there was a slight increase in the serum IgG level during the experimental period. In
Fig. 2  Serum IgM levels and numbers of IgM-bearing cells in the spleens of mice during MCMV infection. Each point represents the average of readings from two mice. Control mice received appropriately diluted extracts of normal salivary glands. * No mice survived for further sampling.
Immunoglobulin production in MCMV infection – 15

![Graphs showing IgG production and spleen cell numbers during MCMV infection](image)

Fig. 3 Serum IgG levels and numbers of spleen cells bearing IgG subclasses during MCMV infection. [IgG1, IgG2a, IgG2b, IgG3. Each point represents the average of readings from two mice. Control mice received appropriately diluted extracts of normal salivary glands. * No mice survived for further sampling.}
the spleens of the control mice, most of the IgG-bearing cells were found to carry the subclass IgG2a, with the other subclasses present in minute numbers.

In the group with the lethal infection, serum IgG levels dropped to barely detectable by day 2, with the accompanying disappearance of IgG-bearing cells in the spleen.

In the acute, non-lethal infection group, cells bearing the IgG subclasses in the spleen showed an increase as early as day 2 and peaked on day 8. Thereafter, although the numbers diminished sharply, the counts for all four subclasses were still raised even at the end of the experimental period with the counts for IgG1, IgG2b, and IgG3 being considerably higher than those of the uninfected controls. There was a slight lag between the increase of IgG-bearing cells in the spleen and the rise in serum IgG titer. A small drop was observed on day 2 followed by a rather steep increase up to a titer of $2^4$ by day 11, thirty two times greater than that of the controls. Thereafter, the high IgG level persisted.

In the group with the asymptomatic infection, the magnitude of increase in spleen cells bearing the IgG subclasses was not as dramatic as in the acute, non-lethal infection group. A gradual increase to a peak at day 11 was observed, followed by an even slower decline. The proportion of cells bearing the four IgG subclasses remained more or less the same as that of the controls throughout. The serum IgG level also rose gradually, reached its high of $2^7$ on day 11, and thereafter plateaued.

**IgA levels:**

The serum IgA levels and the numbers of IgA-bearing cells in the spleens are shown in Fig. 4. In the lethally infected group, the serum IgA and number of IgA-bearing cells in the spleen rapidly dropped to undetectable levels, as was the case with the other Ig classes.

In the group with the acute but non-lethal infection, the number of IgA-bearing cells in the spleen showed a marked increase on day 6, but the pattern of change in numbers was irregular. In the group with the asymptomatic infection, a significant increase in IgA-bearing cells was observed on day 4. In these latter two groups of mice, the increase in IgA-bearing cells in the spleen was not accompanied by an increase in serum IgA levels, as compared with the uninfected controls.

**Neutralizing antibody to MCMV:**

The titers of complement-requiring neutralizing antibodies to MCMV are shown in Fig. 5. These antibodies were not detected in the lethally-infected group.

In the acute (non-lethal) and asymptomatic groups, specific antibody was detected on day 6 and rose slowly, plateauing around day 11. The titers reached were $2^4$ in the former, $2^3$ in the latter.

**DISCUSSION**

We have examined the changes in serum Ig levels, and, in a semi-quantitative way, studied the fluctuations in Ig-bearing cells in the spleen during the course of 3 types of MCMV infections in mice. Non-lethal MCMV infections, both the acute and asymptomatic, resulted in marked increases in IgG- and IgM-bearing cells in the spleen, which was followed closely by increases in serum Ig levels, 16-32 times that of uninfected controls for IgG and less markedly for IgM. Furthermore, the majority of the Ig-bearing cells morphologically resembled Ig-secreting plasma cells. It is clear that MCMV infection has an activating effect on the humoral system. Although specific anti-MCMV antibody was detected during the course of infection, it alone can hardly account for the huge increase in the total Ig level. Comparable findings were seen in adenovirus infections in mice, in which hardly any changes occurred in serum IgM and IgG levels, even though neutralizing antibodies rose to a titer of $2^4$ (27).

Several mechanisms of virus-induced polyclonal B-cell activation have been proposed, and can be broadly categorized as either T cell-independent or T cell-dependent. In the first category, the virus acts as a direct B cell mitogen. The classical example is the Epstein-Barr virus which specifically infects (14) and transforms (5, 22) B cells, and the subsequent immortalization of the B cells results in polyclonal activation. The human immunodeficiency virus (HIV), which has an affinity to T4-bearing cells (mainly the T helper/inducer population), has also been reported to be a T cell-independent polyclonal B cell activator (23). In the case of HIV, however, direct interaction be-
Fig. 4 Serum IgA levels and numbers of IgA-bearing cells in the spleens of mice during MCMV infection. Each point represents the average of readings from two mice. Control mice received appropriately diluted extracts of normal salivary glands. *No mice survived for further sampling.
Fig. 5  Titers of complement-requiring neutralizing antibodies to MCMV during infection. Each point is the average obtained from two mice. Control mice received appropriately diluted extracts of normal salivary glands. *No mice survived for further sampling.
between the virus and B cells, but not infection, seems to be responsible. In the second category, two types of T cell-dependent B-cell activation have been proposed. The virus may inactivate or infect suppressor T cells, thereby boosting B-cell activation, as postulated for the vesicular stomatitis virus-induced augmentation of antibody production (19). Alternatively, polyclonal B-cell activation may be mediated by virus-specific helper T cells, as in the case of the mouse lymphocytic choriomeningitis virus (1). With CMV, there have been two in vitro studies (13, 28) which showed that human CMV was a B-cell activator, and that infection of the target cells was not required for activation. However, discrepancies exist in these two papers over T cell dependency and the requirement of previous exposure of the B cells to CMV. Therefore the exact mechanism remains to be elucidated. Our present study demonstrates that activation of immunoglobulin production does take place in vivo and that this phenomenon starts before peak virus replication and coincides with an increase in cellularity of the spleen (17). Our system offers a convenient tool for detailed studies on the mechanism of polyclonal B cell activation and its relationship to the infection process.

The humoral response to sheep red blood cells (SRBC) was found to be temporarily depressed in MCMV-infected mice (6, 12, 24). Tazume et al further demonstrated that this also occurred in germfree mice (24). The depression was greatest at peak virus replication. We have observed that increased immunoglobulin production also started around this time. Thus, the depression of the primary humoral response could possibly be explained by a feedback regulation of the host in response to the non-specific activation of the humoral system. However, this cannot explain why the specific response to MCMV can proceed while the response ineness to other antigens is impaired. In addition, the mechanism of the depression of the primary antibody response to SRBC and other antigens is still not fully understood. All of these items require further investigation.

The increased humoral activities observed in this study could in part explain the clinical observations of increased autoimmune antibodies in patients with CMV (15). Autoantibodies to LPS were also demonstrated in mice with severe CMV infections (4), but the situation in milder infections has not yet been examined. In this study, although the acute (non-lethal) and asymptomatic groups exhibited marked increases in serum IgG levels, the acutely infected group showed a much more dramatic increase in the number of Ig-bearing cells than did the asymptomatic, mildly infected mice. Furthermore, the proportion of the IgG subclasses was altered in the former but not in the latter group. Coutelier et al showed that different types of antigenic stimuli caused different patterns of distribution of the IgG subclasses in mice (7). Therefore, the mode of activation of the humoral response may be different in the two groups infected with MCMV and whether it has any relationship to the development of autoantibodies remains to be determined.

In the lethally-infected group, both serum Ig levels and Ig-bearing cells in the spleen dropped to very low levels early in the infection. We have shown previously that the inflammatory response and white-cell counts were also severely depleted in this group (17). The defense mechanisms in the lethal infections seemed to be in complete breakdown. In the acute (non-lethal) and asymptomatic groups, the pattern of changes in Ig-bearing cells in the spleen bore some resemblance to the "class-switch" in antibody production: there was an initial increase in IgM which soon declined, to be replaced with a more drastic IgG increase. The serum Ig levels too, followed this pattern in general, but the increase in IgM level was not detectable in the asymptomatic group, which may be due to insensitivity in detection or the method of sampling. Unlike the changes in IgG and IgM, there was no fixed pattern with IgA. This could be due to the fact that the intraperitoneal route of infection is unsuitable for the study of IgA changes.

REFERENCES
20—Wai Chi Teresa LEUNG et al.


