

## Mouse Cytomegalovirus Is Infectious for Rats and Alters Lymphocyte Subsets and Spleen Cell Proliferation

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With 2 Figures

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### Summary

The Smith strain of mouse cytomegalovirus (MCMV) was infectious for infant and mature DA strain laboratory rats as judged by development of neutralizing antibodies and specific spleen cell proliferation on stimulation with MCMV antigen. An i. p. inoculum of  $10^6$  PFU of MCMV was fatal for more than two-thirds of infant mice (1–7 days of age), and disseminated viral infection was documented by isolation of virus from body organs. In contrast, weanling and adult rats did not become ill as a result of infection with a larger inoculum of  $10^7$  PFU. However, these older MCMV infected rats did show transient reversals of T helper/suppressor cell ratios and alterations of immune cell function as detected by *in vitro* spleen cell proliferation assays. Seven days after MCMV infection, there was a generalized increase in  $^3\text{H}$ -thymidine incorporation by spleen cells in both resting (unstimulated) cultures and cultures exposed to mitogens (Con A, PHA, LPS) and to MCMV antigen. At 14 days, the spleen cell proliferation in the unstimulated cultures returned to normal but was depressed compared to controls in response to Con A. These observations show that laboratory rats are susceptible to MCMV infection and that asymptomatic infection may occur and cause transient alterations in lymphocyte subsets and in their reactivity to mitogens.

### Introduction

Although natural cytomegalovirus infections have been described in many species of laboratory and domestic animals, it is generally believed

that these viruses are quite species specific in terms of their infectivity and pathogenicity (RAPP, 1983). Recently, PRISCOTT and TYRRELL (1982) reported that the mouse cytomegalovirus (MCMV) was infectious by the intracranial route for newborn Sprague-Dawley rats. Because acute MCMV infections of mice have been associated with transient alterations in immune functions (HAMILTON, 1983), we assessed the infectivity and pathogenicity of MCMV for several strains of newborn and mature laboratory rats. In the following report, we describe a transient effect of MCMV infections on rat T lymphocyte helper/suppressor cell ratios and on spleen cell proliferation following stimulation with mitogens and MCMV antigen.

## Materials and Methods

### *Virus*

The Smith strain of MCMV was obtained from Kelsey, who has maintained its pathogenicity by serial passage in Swiss-Webster mice (KELSEY *et al.*, 1977). The virus pool was prepared by inoculating three week old Swiss-Webster mice intraperitoneally (i. p.) with  $10^4$  PFU of salivary gland pool of virus and harvesting salivary glands from survivors 14 days after inoculation. Salivary glands were minced and disrupted by homogenization and a 10 percent suspension was made in Eagle's minimum essential medium (MEM) with 10 percent heat inactivated fetal bovine serum (GIBCO). The suspension was then clarified by centrifugation (1200 rpm, 15 minutes) and stored at  $-80^{\circ}\text{C}$ . This pool contained  $8.5 \times 10^8$  PFU/ml infectious virus by plaque assay on mouse embryo fibroblast cell cultures. The final inoculum was found to be free of contamination with mycoplasmas by culture and fluorescent antibody staining. All experimentally infected animals received 0.1 or 0.2 ml of a  $10^{-1}$  dilution of virus in MEM by i. p. injection.

### *Rats*

DA, Wistar-Firth (WF) and August 2880 n (Aug) strain rats were obtained from our own breeding colonies. Periodic testing of these colonies for antibodies (Microbiological Associates, Bethesda, MD) indicated that they were free of infections with the following specific pathogens: rat parvoviruses H 1 and Kilham, sialodacryoadenitis virus, Reo Virus 3, rat corona virus, GDV-II, Sendai, LCM virus and mouse adenovirus. Some animals had antibodies to pneumonia virus of mice.

### *Virus Isolation*

Organs and tissues from rats sacrificed by  $\text{CO}_2$  were homogenized, freeze thawed once, and diluted 1 : 5 in MEM before inoculation onto monolayers of mouse embryo fibroblasts (MEF). Cell cultures were maintained at  $37^{\circ}\text{C}$  in MEM with 10 percent fetal bovine serum, penicillin 100 units/ml and streptomycin 25  $\mu\text{g}/\text{ml}$ . Negative cell cultures were blind passed if tissue toxicity was apparent or after 14 days if negative. MCMV was identified by the appearance of typical cytopathology. In some instances, spleen tissue slices were maintained in explant culture before passage to MEFs according to the method of JORDAN and MAR (1982).

### *Neutralizing Antibody*

The guinea pig complement assisted virus neutralizing antibody assay as described by STADLER and EHRENSBERGER (1980) was used.

*Peripheral Blood T Lymphocyte Surface Antigen Analysis*

Peripheral blood was obtained by retro-orbital bleeding and diluted with two parts PBS before layering over Ficoll-Hypaque solution (S. G. 1.077). After centrifugation, the mononuclear cell-rich fraction was washed and resuspended in PBS containing 2 percent FBS and 0.1 percent sodium azide to give a final concentration of  $10^7$  cells/ml. This suspension contained 95 percent lymphocytes and <5 percent monocytes and neutrophils.

Mouse monoclonal antibodies to rat T lymphocyte surface antigens OX 8, which identifies suppressor cells, and W 3/25, which identifies helper cells (BRIDEAU *et al.*, 1980) were obtained from Accurate Chemical (Westbury, N.Y.). Both monoclonals were diluted 1/20 in PBS and reacted with equal volumes of cell suspensions for 30 minutes at 0° C. The cells were washed and then reacted with an equal volume of 1/20 dilution of fluorescein conjugated F(ab)<sub>2</sub> fragment goat anti-mouse IgG (Cooper Biomedical) for 30 min at 0° C, the cells were washed and resuspended to a concentration of  $10^5$  cells/ml in 1 percent paraformaldehyde. The labeled cell suspensions were stored at 4° C for no more than 48 hours until counted in a fluorescence activated cell sorter (Becton-Dickenson FACS III). The results were calculated from a minimum of 10,000 cells counted for each monoclonal antibody.

*Proliferative Response of Spleen Cells to Mitogens and MCMV Antigens*

Spleen cell suspensions were treated with Tris-ammonium chloride buffer to lyse erythrocytes and then washed three times with RPMI-1640. Cells were cultured in 96 well flat bottomed microtiter plates and  $2.2 \times 10^5$  cells were added to each well in RPMI-1640 supplemented with L-glutamine (2 mM), 10 mM Hepes buffer, 50  $\mu$ /ml penicillin, 0.05 mg/ml streptomycin,  $2 \times 10^{-2}$  mM  $\beta$ -mercaptoethanol and 10 percent fresh rat serum (from same rat strain and matched according to sex).

*E. Coli* lipopolysaccharide (LPS, Sigma) was added at a concentration of 12.5  $\mu$ g/ml, phytohemagglutinin (PHA, Wellcome Ltd.) was added at a concentration of 4  $\mu$ g/ml and concanavalin A (Con A, Sigma) was added at a concentration of 2.5  $\mu$ g/ml to spleen cell cultures and plates were incubated for 72 hours at 37° C. Antigen stimulation was with the inoculum strain of MCMV ( $10^7$  PFU/ml) passaged once in DA rat embryo fibroblasts (REF), and heat inactivated at 56° C/60 minutes. Similarly treated REF's served as controls. The incubation period for the antigen and LPS stimulations was 5 days. <sup>3</sup>H-thymidine obtained from New England Nuclear was added to each well (1  $\mu$ Ci) 24 hours before termination of the culture. Cells were harvested on filter discs with a Beckman 12 channel harvester and <sup>3</sup>H-thymidine uptake was measured in a Beckman liquid scintillation counter.

Each sample was tested in triplicate or quadruplicate and replicates were screened for aberrant values using the Outlier test (GRUBBS, 1975) before calculating mean cpm uptake for each test. Approximately 10 percent of determinations were discarded by this method. Statistical analysis for differences was done by the Student's t-test with two degrees of freedom.

## Results

MCMV was infectious and lethal for more than 65 percent of one to seven day old DA and WF strain rats inoculated i. p. with  $5 \times 10^5$  to  $8 \times 10^6$  PFU of virus (Table 1). This mortality was greater than the 33 percent mortality seen in four day old rats after injection of normal mouse salivary gland and greater than the 26 percent two-week mortality of newborn DA rats followed at the same time in our breeding colony. Illness was first notable by four days and mortality was seldom detected after 14 days. Virus was isolated

Table 1. *Outcome of MCMV infection of rats*

Age	Rat. Strain	Inoculum (PFU) i. p.	Number	Mortality	Virus Isolated/ No Cult	Antibody Response (>4 ×)
1-3 days	DA	$8 \times 10^6$	13	70%	5/7	3/3
3 days	DA	$3 \times 10^6$	18	100%	1/1	N.D.
4 days	WF	$5 \times 10^6$	7	85%	2/2	N.D.
6 days	DA	$5 \times 10^6$	7	85%	2/2	N.D.
7 days	DA	$5 \times 10^5$	3	66%	N.D.	N.D.
9 days	DA	$5 \times 10^4$	6	0	N.D.	5/6
6 wk	DA	$3.5 \times 10^6$	12	16%	2/4	N.D.
8 wk	DA	$2 \times 10^7$	20	0	1/20	20/20
8 mo	DA	$3.6 \times 10^7$	4	0	0/4	4/4
	WF	$3.6 \times 10^7$	4	0	0/4	4/4
	Aug	$3.6 \times 10^7$	4	0	0/4	4/4
Controls						
8 mo	WF	Heat inact. virus	2	0	N.D.	0/2
4 days	DA	Mouse saliv. gland	15	33%	N.D.	N.D.
Newborn- 14 days	DA	Breeding colony	165	26%	N.D.	N.D.

from internal organs of 10 of 12 rats that appeared moribund or died three to 14 days after virus inoculation. Kidneys and lungs were most often positive (>75 percent), while virus was grown from 37 percent of spleens and only 17 percent of livers.

Mortality was only 16 percent in six week old animals inoculated with MCMV and none of the rats died that were inoculated at 8 or more weeks of age even though they received higher titers of 2 to  $3.6 \times 10^7$  PFU of virus. None of these animals appeared clinically ill, however, total body weights in the MCMV infected animals were transiently lower than those in control rats. Virus was isolated from spleen and lung tissues of 2 of 4 six-week-old rats cultured 7 to 14 days after inoculation and from the lung and spleen tissues of 1/20 8-week-old rats cultured four weeks after inoculation. Virus could not be detected by culture in salivary gland or other body organs of older animals when cultured 28 days after inoculation.

MCMV at an inoculum of  $10^5$  or greater was infectious for all older animals as judged by the appearance at two to eight weeks after inoculation of serum neutralizing antibody at titers of 1/32-1/256. Control animals receiving heat inactivated ( $56^\circ\text{C}$ , 1 hour) MCMV inoculum ( $3.6 \times 10^7$  PFU virus antigen equivalence) did not develop detectable MCMV neutralizing antibodies. Infectivity of MCMV for weanling and adult rats was also indicated by the appearance of high levels of spleen cell proliferation in response to stimulation with MCMV antigens (Fig. 2).

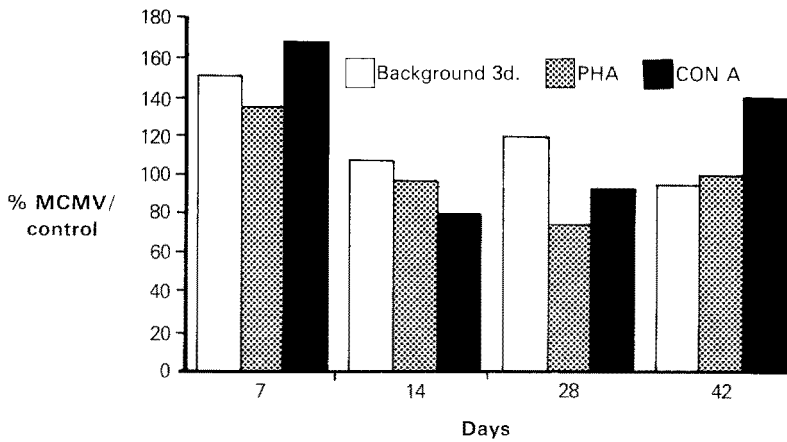


Fig. 1. Uptake of <sup>3</sup>H-thymidine by spleen cells from 40 MCMV infected rats expressed as percent of uptake by 27 control (non-infected) animals. Data are compiled from six separate experiments. Incubation period was three days for background spleen cell cultures and in the presence of the mitogens PHA and Con A

The proliferative responses of spleen cells to mitogens and MCMV antigen in MCMV infected as compared to control animals are presented in Table 2 and Fig. 1 and 2. These figures represent a compilation of findings from six separate experiments which utilized a total of 40 MCMV infected and 27 control rats.

Seven days after MCMV infection, a consistently greater proliferation of spleen cells from MCMV infected rats as compared to control animals was seen with all mitogens and also in unstimulated background cultures (Table 2 and Fig. 1 and 2). These differences ranged from 134 percent to 168 percent

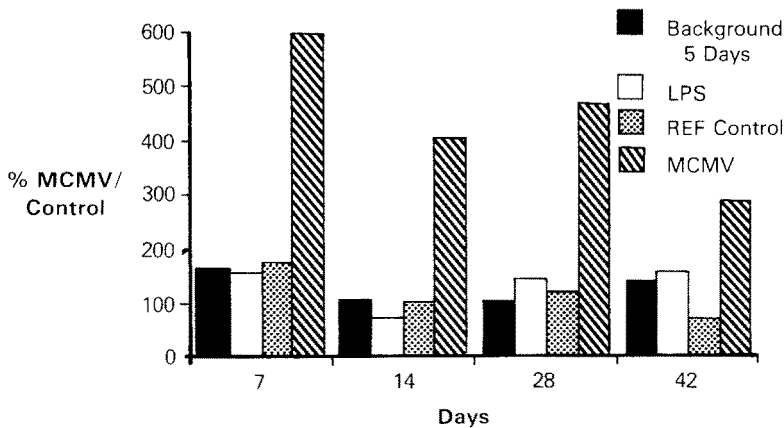


Fig. 2 Uptake of <sup>3</sup>H-thymidine by spleen cells from 40 MCMV infected rats expressed as percent of uptake by 27 control (non-infected) animals. Data are compiled from six separate experiments. Incubation period was five days for background spleen cell cultures and in the presence of LPS, REF cell controls and MCMV antigen

Table 2. Incorporation of  $^3\text{H}$  Thymidine by spleen cells following stimulation with indicated mitogen or antigen

	Mean counts per minute $\pm$ S.E.						REF cell control
	Back- ground (3 days)	PHA	CON A	Back- ground (5 day)	LPS	MCMV	
Exp. no. II							
7 days, MCMV (N=6)	1,665 $\pm$ 184	169,993* $\pm$ 6,450	209,957* $\pm$ 13,839	2,115* $\pm$ 226	— —	7,766* $\pm$ 2,788	4,118* $\pm$ 327
Normal (N=7)	1,127 $\pm$ 184	82,731 $\pm$ 5,467	162,429 $\pm$ 9,147	1,373 $\pm$ 220	— —	959 $\pm$ 207	1,640 $\pm$ 352
Exp. no. IV							
14 days, MCMV (N=4)	1,321 $\pm$ 189	136,013 $\pm$ 15,332	198,448* $\pm$ 15,031	1,589 $\pm$ 297	1,547 $\pm$ 78	8,256 $\pm$ 3,261	1,718 $\pm$ 93
Normal (N=4)	1,104 $\pm$ 180	139,475 $\pm$ 14,119	343,395 $\pm$ 50,423	2,120 $\pm$ 663	2,198 $\pm$ 350	1,496 $\pm$ 97	1,390 $\pm$ 203
Exp. no. VI							
42 day, MCMV (N=4)	1,027 $\pm$ 117	118,010 $\pm$ 21,845	242,125 $\pm$ 18,611	1,609 $\pm$ 234	1,423 $\pm$ 290	3,096* $\pm$ 897	1,574 $\pm$ 247
Normal (N=4)	1,076 $\pm$ 259	118,320 $\pm$ 8,609	173,023 $\pm$ 26,783	1,131 $\pm$ 56	901 $\pm$ 160	1,067 $\pm$ 172	1,093 $\pm$ 197

\* MCMV infected rats vs normal rats  $p \leq 0.05$  by t test

of control values, and were highest for Con A. When spleen cell proliferation response to mitogens was assessed 14, 28 and 42 days after MCMV infection, the acute (7 day) stimulating effect of MCMV was gone. There was a transient inhibition (80 percent of control,  $p < 0.05$ ) of spleen cell responsiveness to Con A at 14 days following MCMV infection. The greatest difference between MCMV infected and control animals was seen in response to MCMV antigen (Fig. 2). This difference was greatest at 7 days (597 percent of control), and although it had declined to 300 percent at 42 days, the difference was still statistically significant ( $p < 0.05$ ) (Table 2).

The possibility that mouse salivary gland tissue rather than MCMV might be responsible for the observed changes was examined in a separate experiment. Seven days after injection of normal mouse salivary gland tissue, there was a moderate (150 percent) but not statistically significant increase in the five day background proliferation response as compared to controls, while responses of spleen cells to Con A and PHA were 1 percent and 15 percent less than control values.

MCMV infection of DA strain rats was associated with a reversal of lymphocyte T helper/suppressor cell ratios (Table 3). Prior to inoculation, Th/Ts ratios were in the range of 1.7 for both groups. In the MCMV infected

Table 3. Rat peripheral blood lymphocyte subsets following infection with MCMV

		Mononuclear Cells/ml × 10 <sup>6</sup> ± SD	Percent cells (+ SD) reacting with monoclonal antibody		
			W3/25	0X8	W3/25/0X8
Day 0					
MCMV	(N=5)	7.1 ± 1.6	56.2 ± 1.8	32.8 ± 2.7	1.72 ± 0.16
Control**	(N=2)	5.3 ± 2.5	55.5 ± 0.7	33.0 ± 2.8	1.70 ± 0.14
Day 7					
MCMV	(N=5)	11.6 ± 2.2*	52.6 ± 5.4*	63.2 ± 4.3*	0.81 ± 0.13*
Control	(N=2)	4.3 ± 1.3	66 ± 0	54 ± 1.4	1.2 ± 0
Day 14					
MCMV	(N=5)	11.4 ± 1.4	35.8 ± 7.0	41.8 ± 3.0	0.88 ± 0.2
Control	(N=2)	8.4 ± 3.7	35.5 ± 3.5	35.5 ± 0.7	1.0 ± 0.14

\* Significant difference between MCMV and control, P < 0.05 by Students T-test

\*\* Controls were injected with normal mouse salivary gland

rats, ratios fell to .84 and .87 at 7 and 14 days. There was a smaller decline in the Th/Ts ratio to the level of 1.1–1.2 in the animals receiving normal salivary gland. The greater decline in Th/Ts ratios in the MCMV infected animals was significantly different from that of controls at 7 days. MCMV infection was associated with increases in total mononuclear cell counts at 7 and 14 days, suggesting that the reversed Th/Ts ratios were due to increases in total numbers of circulating suppressor cells.

### Discussion

Mouse cytomegalovirus (MCMV) was found to be infectious by the I. P. route for infants and adults of three strains of laboratory rats as judged by virus isolation, neutralizing antibody response and development of a high level of spleen cell reactivity to MCMV antigen. Mortality following MCMV infection was more than 66 percent in young infant rats, while weanling and adult rats survived the infection without apparent illness. This decrease in mortality as animals became weanlings has been described for mice infected with MCMV (BOSS and WHELOCK, 1975, MANNINI and MEDEARIS, 1961).

Marked specificity of cytomegaloviruses for the host of origin has been a general characteristic of this group of viruses (RAPP, 1983). Although cytomegaloviruses will occasionally grow in cell cultures derived from different species (KIM and RAPP, 1971), infectivity and pathogenicity for other species is rare. MCMV appears to represent an exception to this rule. BRUGGEMAN *et al.* (1982) reported that MCMV would grow on rat cells and PRISCOTT and TYRRELL (1982) recently reported that the Osborn strain of MCMV was infectious for infant Sprague-Dawley rats when given by the I. C. route, causing 23 percent mortality. The greater mortality seen in our study was probably related to our use of a larger inoculum of virus (10<sup>6</sup> vs

10<sup>3</sup>), although virus and rat strain differences are probably also important. Our observations indicate that susceptibility of laboratory rats to MCMV infection is not strain specific, and that infection can occur after i. p., as well as, i. c. inoculation.

Increased uptake of <sup>3</sup>H-thymidine by spleen cells from rats seven days after infection with MCMV was seen in background unstimulated cultures and in cultures stimulated with the mitogens PHA, Con A and LPS. Animals receiving injections of normal mouse salivary gland also exhibited increased spleen cell uptake in resting unstimulated cultures, however, no appreciable differences were seen between spleen cells from salivary gland injected and control animals in response to stimulation with mitogens or MCMV antigen. These results indicate that there was a general stimulatory effect of MCMV infection that was in addition to a lesser effect seen in response to injection of salivary gland tissue.

A similar immuno-stimulatory effect of MCMV infection of mice has been described by others. BOSS and WHEELock (1975) tested spleen cells collected four days after non-lethal infection of weanling or adult mice with MCMV and detected a moderate increase in uptake of <sup>3</sup>H-thymidine by unstimulated cultures and a slight depression of response to Con A. A larger increase (three-fold) in uptake of <sup>3</sup>H-thymidine by unstimulated spleen cells collected 5–15 days after MCMV infection was reported by HOWARD *et al.* (1978).

The early (seven day) stimulatory effect of MCMV infection on rat spleen cells was generally gone by 14 days, and in one experiment, a depressive effect was detected in response to stimulation by Con A. These changes were smaller in magnitude than the depressive effect on the response to Con A that BOSS and WHEELock (1975) saw in mice following infection with MCMV. In an earlier study, HOWARD *et al.* (1974) reported depressed lymphocyte proliferation in mixed lymphocyte cultures following MCMV infection of mice. Cytomegalovirus infections of humans have also been associated with depression of lymphocyte reactivity to stimulation with the mitogens Con A and PHA (RINALDO *et al.*, 1980).

The greatest differences between MCMV infected and control animals were seen in response of spleen cells to stimulation with MCMV antigen. This four- to six-fold increase is consistent with a vigorous immune response to cytomegalovirus infection. Our failure to detect a response to MCMV antigen in control rats injected with mouse salivary gland is further evidence for the specificity of the spleen cell response to MCMV antigen. The lymphocyte response to MCMV antigen was greatest seven days after infection, and a decline in responsiveness to MCMV antigen was seen after the first week, most notably at 14 days. The possibility that active CMV infection may decrease the magnitude of the cell-mediated immune response to CMV antigens has been reported to occur in children and adults with CMV infection (REYNOLDS *et al.*, 1979, LEVIN *et al.*, 1979).



Rats infected with MCMV consistently exhibited an acute reversal of lymphocyte T helper/suppressor ratios, the effect being greatest 7 days after infection. Human cytomegalovirus infections have been associated with similar reversals of Th/Ts ratios (RINALDO *et al.*, 1983) and SELL *et al.* (1985) found reversals of Th/Ts ratios in mice tested 1, 3, 5, and 9 days after infection with MCMV. The general increase in total mononuclear cells observed following MCMV infection suggests that the reversal of Th/Ts ratios in the MCMV-rat model are due primarily to an increase in the Ts population. The marked reversals seen following CMV infections in humans have been associated primarily with increases in Ts lymphocytes and to a lesser degree with decreases in total Th cells (CARNEY *et al.*, 1981). SELL *et al.* (1985) observed that reversed Th/Ts ratios in MCMV infected mice were due to decreased Th cell numbers on days 3 and 5 and due to increased Ts cell numbers on day 9.

It is tempting to try and correlate the observed changes in Th/Ts ratios with changes in lymphocyte reactivity to mitogens and antigens as has been done in the human CMV system (CARNEY *et al.*, 1981). The early (7 day) increase in reactivity of spleen cells following MCMV infection does not seem consistent with an increase in Ts lymphocytes. This discrepancy could be related to the presence of circulating immunostimulating lymphokines shortly after infection and to a relatively delayed appearance of functional Ts cells. Our monoclonal antibodies to the Ts cell subset measure surface antigen markers only and not functional activity. The suppression of responsiveness of spleen cells to Con A at 14 days correlates with an increase in functional Ts cell activity at that time and is consistent with the observations of CARNEY *et al.* (1981) in humans.

Rats injected with normal rat salivary gland tissue had changes in lymphocyte Th/Ts ratios that were similar in direction, but lesser in magnitude, to the changes we observed following MCMV infection. Similar changes thought to be related to the non-specific effects of bleeding or stress were described by Ross *et al.* (1984).

Our observations indicate that several strains of laboratory rats are susceptible to MCMV infection and that infection is associated with changes in lymphocyte T helper/suppressor cell ratios and in spleen cell reactivity to mitogens. Investigators studying immune functions in laboratory rats should be aware that their results can be altered by subclinical laboratory acquired MCMV infection. Experimental MCMV infections in laboratory mice have been very useful in exploring the effect of viral infections on immune functions (HAMILTON, 1983), and our observations suggest that MCMV infection of the laboratory rat may also be a useful model for similar studies.

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