JOURNAL OF VIROLOGY, Nov. 1987, p. 3602–3606
0022-538X/87/113602-05$02.00/0

Vol. 61, No. 11

Functional Changes in Murine Macrophages Infected with Cytomegalovirus Relate to H-2-Determined Sensitivity to Infection

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Received 28 April 1987/Accepted 6 August 1987

Peritoneal macrophages were infected with murine cytomegalovirus in vitro, and indices of infection and macrophage function were monitored over 4 days. When the cells were assessed for the expression of viral antigen or for cytopathic effects, infection was found to be solely determined by the H-2 phenotype. Less than 10% of the macrophages from resistant *H-2*^{k} strains were affected, whereas 90% of *H-2*^{d} cells and approximately 80% of *H-2*^{b} and *H-2*^{a} cells became infected. Similar trends were demonstrable by the measurement of viral DNA. In *H-2*^{a} cells (B10.A), *D*^{d} conferred sensitivity despite the resistant K and class II phenotype. The findings suggest a critical association between the class I antigens and an early stage in the infectious process. Indices of infection were paralleled by a loss of Fc receptor expression and optimal colloidal gold uptake, whereas most cells remained trypan blue negative, retained dehydrogenase and acid phosphatase activities, and did not release infectious virus during the period of study. This is consistent with a role for macrophages in the persistence of cytomegalovirus in the host.

Cytomegalovirus is an important cause of disease in immunosuppressed adults and affects a significant number of newborn infants (13). Among healthy individuals, the seropositivity rate varies widely and is influenced by both socioeconomic status and racial background (13, 14). Under these circumstances, it would be helpful to establish which individuals are genetically predisposed to infection and whether these differences are related to the susceptibility of individual cells or to the host response.

The genetic basis for the sensitivity of inbred mice to lethal infection with the murine analog of cytomegalovirus (MCMV) involves the *H-2* complex and background genes (3). *H-2*^{d} mice are sensitive to MCMV, and *H-2*^{b} mice with the same genetic background show only slight resistance (1.3-fold), while *H-2*^{k} mice are up to 10-fold more resistant, as assessed by the relative 50% lethal dose and virus titers. In addition, H-2-matched strains with BALB/c, C57BL, and CBA genetic backgrounds (respectively) show progressively increasing resistance to lethal infection with MCMV. We have established that resistance associated with the non-*H-2* genetic background has a leukocyte-mediated component which involves natural killer cells, T cells, and probably inflammatory macrophages (1, 19; C. Lawson, J. E. Grundy, and G. R. Shellam, J. Gen. Virol., in press; P. Price, J. G. Winter, and G. R. Shellam, J. Gen. Virol., in press).

Factors determining resistance at the level of individual cells have been studied using mouse embryo fibroblasts (MEFs) and tracheal ring cultures (5, 12), demonstrating patterns of resistance similar to those seen in vivo, influenced by both the background and *H-2* genotype. However, the differences were frequently smaller than those seen in vivo, and it is questionable whether embryo fibroblasts are a relevant indicator for the sensitivity of adult cells.

The present study uses peritoneal macrophages to investigate the sensitivity of cells from inbred and H-2 congenic mice. These cells are ideal because they do not replicate, so the percentage of infected cells can be determined after various periods of culture. Infection was assessed by the expression of viral antigen, DNA, and cytopathic effects (CPE), minimizing the variation associated with the efficiency with which infection proceeds to the level of virus release. To achieve high-level infection of susceptible cells, the virus and cells were coincubated under a centrifugal acceleration of 700 × *g* (8). Before selecting this technique, we established that H-2-determined resistance of MEFs was maintained when the level of infection in cells of both strains was increased by centrifugal enhancement.

This system provides a marker for the sensitivity of other cells from the same adult donor. It is also the first controlled comparative study of the functional capacity of susceptible macrophages infected with MCMV and of resistant macrophages activated by contact with the virus. Furthermore, as sensitivity to infection correlated solely with the H-2 phenotype of the macrophages, we were able to analyze the roles of the *K* and *D* loci and the class II region. This led to the hypothesis that the class I major histocompatibility complex molecules may determine viral entry to the cell.

MATERIALS AND METHODS

Animals and virus stocks. Female BALB/c, BALB.K, BALB.B, B10.D2, B10.BR, B10, B10A, and CBA mice were obtained from the Animal Resources Centre, Murdoch, West Australia at 8 to 10 weeks of age, maintained under minimal disease conditions, and used within 3 weeks.

MCMV was maintained by salivary gland passage as described previously (3). Tissue culture-derived MCMV, used throughout this study, was produced in MEFs inoculated with salivary gland virus 3 days before collection of the virus-containing supernatant. For one experiment, MCMV was inactivated by UV irradiation (2 min, 800 $\mu W/cm^2$) immediately before use and a total loss of infectivity was confirmed by plaque assay in MEFs.

Collection and infection of cells. Peritoneal cells (PCs) were collected as pooled preparations and were concentrated by centrifugation (15). They were placed into wells of 24-well Costar tissue culture plates at approximately 5×10^6 cells in 0.3 ml of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic

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acid (HEPES)-buffered RPMI 1640 containing 2% fetal calf serum (FCS). These wells had been coated with FCS overnight and washed with saline immediately before use. An equal volume of MCMV or medium was added to selected wells with the PCs, and the plates were centrifuged at 700 × *g* (Beckman J6-B) at room temperature for 30 min. They were then incubated at 37°C for 90 min before being resuspended by vigorous pipetting with cold medium and dispensed at 5×10^5 cells per well into 24-well plates containing alcohol-washed glass cover slips. The cells were maintained in culture in HEPES-RPMI 1640 supplemented with 5×10^{-5} M 2-mercaptoethanol and 0.5% normal mouse serum only, to avoid macrophage activation (15). To study the inhibition of MCMV infection by blocking the H-2 antigens on the cell surface, 5×10^6 macrophages resuspended in 0.25 ml of medium were incubated for 1 h at 37°C with an equal volume of anti-K^bD^b murine hybridoma ascites (Australian monoclonal development) at a dilution of 1:50, before the addition of 0.25 ml of MCMV, for centrifugation by the standard protocol.

Enumeration of infected cells. Productively infected cells were enumerated by a reverse plaque assay (20). Cells expressing viral antigens were detected in acetone-fixed cover slip preparations (85% acetone, 0°C, 5 min) by immunofluorescence. Cover slips were treated with normal goat serum and then rabbit anti-MCMV antibody which was raised by immunization, without adjuvant, with virulent MCMV which had been purified on a sucrose density gradient. This antiserum was adsorbed with pooled murine peritoneal, spleen, thymus, and lymph node cells and yielded negligible fluorescence with uninfected cells. Positive cells were visualized with fluorescein-conjugated goat anti-rabbit immunoglobulin (Cappel). Preliminary time course studies with these reagents demonstrated minimal viral antigen expression on day 2 and peak levels on days 3 and 4 postinfection.

MCMV DNA was detected by hybridization to a biotinylated probe prepared by J. B. Hudson (University of British Columbia, Vancouver, British Columbia, Canada) from a *Hin*dIII fragment of MCMV cloned in pACY184. Infected macrophages were maintained for 2 days in the usual medium in FCS-precoated wells of 6-well culture trays (Costar) and harvested by vigorous pipetting. Cell counts confirmed that the recovery was not influenced by the donor strain or MCMV infection. Preparations were centrifuged, suspended in 20 to 50 μl of distilled water (approximately 5×10^4 cells per μl), and stored at −70°C until use. Viral DNA was visualized in a spot hybridization assay (2) by the Blu-Gene detection system (Bethesda Research Laboratories, Inc.). The hybridization membranes were photographed, and the negatives were scanned by microdensitometry. The areas under the peaks were calculated and normalized relative to the value obtained with cells from BALB/c mice. No reaction between this probe and cellular DNA was evident either by Southern hybridization or in preliminary experiments with the present detection system using DNA extracted from control and infected BALB/c and BALB.K macrophages in place of lysed whole cells.

Measurement of CPE and macrophage functional markers. Fc receptors (FcR) were enumerated by using a rosetting assay in which sheep erythrocytes sensitized with rabbit hemolysin were overlaid onto PCs adhering to glass cover slips (16). Colloidal gold uptake was determined on parallel cover slips after a 60-min incubation at 37°C with the gold preparation, followed by acetone fixation and hematoxylin counterstain (Price et al., in press). To count the cells showing CPE, 50 μl of acridine orange (0.1 mg/ml) was added to each well and the cover slips were inverted onto microscope slides and counted under fluorescent illumination within a few minutes. Uninfected cells showed spreading, green nuclei and red cytoplasmic granules. Infected cells were round and mottled green and orange, suggesting a loss of internal organization. This method of staining yielded results similar to those obtained with fixed preparations but with less ambiguity. For all of these assays, at least 200 cells were counted from several regions on two separate cover slips and subjected to a chi-square (χ^2) analysis.

The proportion of macrophages able to exclude trypan blue was determined by adding 0.1 ml of 0.2% trypan blue to selected wells and then inverting the cover slips onto microscope slides and counting within 5 min. Metabolic integrity was determined by three biochemical markers of activated macrophages. Infected and control PCs were diluted serially from 10^6 cells per well in 96-well Falcon tissue culture plates and maintained in the same way as the cover slip cultures. Plates were centrifuged briefly on the day of assay, and the medium was replaced with the appropriate reagents. Acid phosphatase levels were determined on cells lysed by freezing and thawing in the trays in distilled water, followed by 2 h of incubation with dinitrophenol phosphate in acetate buffer, pH 5 (15). The ability to reduce (3-[4,5-dimethylthiazol-2-yl]-diphenyl-2,5-tetrazolium bromide (MTT) and Nitro Blue Tetrazolium (NBT) to their formazan bases was measured as described previously (11, 17) and quantified in terms of the amount of formazan product formed. NBT reduction was carried out in the presence of phorbol myristate acetate as recommended for the measurement of oxidative burst activity (17). Reactions were read colorimetrically on a Titertek Multiscan spectrophotometer. The statistical analyses were based on triplicate titrations from pooled preparations (as used throughout this study) and do not reflect individual donors.

Histochemical assessment of NBT reductase activity was performed with the reagents described above, followed by acetone fixation and hematoxylin counterstain. Slides were stained for acid phosphatase activity with naphthol AS-BI phosphate and hexazo-*p*-rosaniline (9).

RESULTS AND DISCUSSION

Peritoneal cells collected from BALB/c, BALB.B, BALB.K, B10.D2, B10, B10.BR, B10.A, and CBA mice were infected as described and maintained in culture for 4 days. Reverse plaque assays were set up on day 0, and a range of determinants of infection and functional capacity was studied each day. Results obtained on day 3 are presented in Table 1. On days 1 and 2, a steady progression to the day 3 values was observed, and on day 4, the results were essentially constant. This is shown in more detail in Table 2. MCMV DNA was detected by hybridization in cells harvested on day 2. The results presented in Table 1 are a compilation of a number of experiments, as the levels of infection achieved were highly reproducible.

Approximately 90% of cells from the two *H-2*^{d} strains showed CPE, and most displayed viral antigen. There was only a slight reduction in these levels in *H-2*^{b} strains (χ^2, $P <$ 0.025 to 0.01 for CPE), while less than 10% of cells from *H-2*^{k} strains were infected. These trends are qualitatively similar to the effects of the *H-2* complex on resistance to MCMV in vivo and in MEFs (3–6, 12) but are more clearly pronounced than in any previous studies and for the first time show no association with non-*H-2* genes. Furthermore, the loss of

TABLE 1. Percentage of peritoneal macrophages infected with MCMV or expressing functional markers 3 days after in vitro infection

Donor strain	*H-2* genotype				CPE (%)	MCMV antigen (%)	MCMV virus release (%)	MCMV DNA[a] (%)	FcR[b] (%)	Colloidal gold uptake[c] (%)
	K	*IA*	*IE*	*D*						
BALB/c	*d*	*d*	*d*	*d*	95	76	12	100	7	16
BALB.B	*b*	*b*	*b*	*b*	80	40	11	74	18	20
BALB.K	*k*	*k*	*k*	*k*	10	5	1.0	4	91	66
CBA	*k*	*k*	*k*	*k*	<10	7	1.9	0	93	61
B10.D2	*d*	*d*	*d*	*d*	89	70	21	270	8	15
B10	*b*	*b*	*b*	*b*	76	68	10	36	18	18
B10.BR	*k*	*k*	*k*	*k*	6	4	1.4	4	93	74
B10.A	*k*	*k*	*k*	*d*	82	50	30	250	6	20
B10[d]	*b*	*b*	*b*	*b*	9	34	2.3		70	75

[a] Mean intensity of dot blots normalized relative to BALB/c cells (see Materials and Methods). Control cells gave zero readings.
[b] Control cells were 90 to 100% positive.
[c] Control cells were 60 to 80% positive.
[d] Macrophages incubated with anti-K^bD^b ascites before MCMV.

FcR expression mirrored the development of CPE precisely, involving 90% of *H-2*^{d} cells and a slightly lower percentage of *H-2*^{b} cells (χ^2, $P < 0.05$). The ability of the cells from the various strains to take up colloidal gold after exposure to MCMV followed the pattern observed with FcR expression. However, the differences between strains were slightly lower because macrophages not exposed to MCMV were only 60 to 80% positive by this assay. These functional changes both showed no association with non-*H-2* genes (χ^2, $P > 0.05$) and could be used as indices of infection.

The proportion of cells from each strain releasing infectious virus followed the same trends as the other assays but

TABLE 2. Functional and metabolic changes in peritoneal macrophages resulting from MCMV infection

Donor, virus, and day postinfection	FcR (%)	Colloidal gold uptake[a] (%)	Trypan blue exclusion (%)	NBT reduction (μg/10^6 PCs)	MTT reduction (μg/10^6 PCs)	Acid phosphatase (ng/10^6 PCs)
BALB/c						
Control						
1	86	0, 56				
2	91	0, 84	98	8.4	23	
3	93	3, 83	97	6.9	23	79
4	81	2, 82	100	3.2		
MCMV						
1	28	0, 29				
2	12	22, 22	90	6.0	24	
3	7	39, 28	99	4.8[c]	19	96
4	4	16, 29	93	4.2		
UV-inactivated MCMV						
1	99	NT[b]				
2	100	NT	NT	NT	NT	
3	99	NT	NT	NT	NT	NT
4	97	2, 74	NT	NT		
BALB.K						
Control						
1	NT	NT				
2	97	5, 75	77	NT	NT	
3	NT	6, 76	100	9.5	24	128
4	85	NT	96	NT		
MCMV						
1	NT	NT				
2	90	6, 75	83	NT	NT	
3	93	7, 90	100	14.2[c]	25	180[c]
4	89	NT	99	NT		

[a] The first value shown is the low-level uptake (typically by round cells), while the second value is the high-level uptake (typically by healthy, spread macrophages).
[b] NT, Not tested.
[c] Statistically significant effect of MCMV (*t* test, $P < 0.05$).

at a much lower level (Table 1). Furthermore, these data may reflect virus released by cells other than macrophages in the PC preparations, as these cannot be distinguished morphologically in the reverse plaque assay. A contribution from other cell types may explain the higher variation between experiments with this assay (particularly with BALB/c mice), as genetically determined differences in resistance to MCMV are less pronounced in fibroblasts and epithelial cells. In these cells, the effects of interferon on infection are known to be genetically determined (6). The nature of the block in the replicative cycle in macrophages after viral antigen expression may be associated with interferon present in the virus preparation, because exposure to the neat virus preparation inhibited productive infection while inducing maximal CPE and loss of FcR (unpublished observation).

Consistent with other indices of nonproductive infection, viral DNA expression was maximal in $H\text{-}2^d$ cells. Lower levels were detected in $H\text{-}2^b$ cells, and none was detected in $H\text{-}2^k$. B10.D2 and B10.A cells exhibited higher levels of MCMV DNA than BALB/c, but this may represent experimental variation, as the B10 congenic strains were only included in this assay on one occasion. It may also reflect greater DNA replication per cell but not a greater number of cells infected, as approximately 90% of BALB/c macrophages developed CPE. Despite these considerations, the results attribute the effects of the H-2 phenotype on CPE, viral antigen, FcR, and colloidal gold uptake to a stage in the replicative cycle before viral DNA synthesis.

Cells from B10.A mice (K^k, $I\text{-}A^k$, $I\text{-}E^k$, D^d, and L^d) were highly sensitive to infection, as assessed by all parameters described in Table 1, in contrast to PCs from B10.BR mice (K^k, $I\text{-}A^k$, $I\text{-}E^k$, D^k, and L^k). This indicates that the D^d or L^d locus of the class I region conferred sensitivity, independent of the K or class II phenotype. The latter is expected since the level of infection achieved in $H\text{-}2^d$ and $H\text{-}2^b$ cells was greater than the level of *Ia* expression (10 to 17% for BALB/c macrophages in our laboratory). The simplest mechanism by which D^d or L^d could confer sensitivity, rather than K^k conferring resistance, is an association between D^d or L^d and the MCMV receptor. Recent studies (10) have shown that human cytomegalovirus exists bound to β2-microglobulin in vivo and in culture supernatants containing FCS. This protein is normally in equilibrium with the class I heavy chain antigens. If MCMV were to exhibit similar binding to β2-microglobulin in culture fluids, the class I antigens may be critical for the binding of MCMV before entry into the cell. In support of this possibility, kinetic studies in our laboratory have established that the infection of MEFs with MCMV involves a rapid and reversible temperature-independent binding phase, followed by slower irreversible binding, possibly associated with uptake by the cell (P. D. Hodgkin, manuscript in preparation). The possibility that H-2 class I molecules may be involved in the binding of the virus is now being investigated by using anti-class I monoclonal antibodies to block the infection. A pilot study presented as the final line of Table 1 achieved 80% protection from MCMV infection in B10 macrophages pretreated with anti-K^bD^b hybridoma ascites. A range of anti-$H\text{-}2^d$ and anti-$H\text{-}2^b$ reagents have been prepared and standardized for more extensive blocking studies to evaluate the roles of the D, L, and K antigens in MCMV infection. The possibility that the infectibility of cells with the various phenotypes relates to their affinity for β2-microglobulin also warrants consideration, as Schmidt et al. (18) showed that D^b, D^k, and K^k exchanged native β2-microglobulin with labeled human β2-microglobulin at much higher levels than D^d did, while K^b and K^d had intermediate affinity. High-level exchange suggests low-affinity binding, consistent with our finding that all cells bearing the latter three phenotypes were sensitive to MCMV, despite coexpression of K^k (B10.A) or D^b (B10). The affinity of mouse β2-microglobulin for the class I antigens and the ability of antibodies to β2-microglobulin to block infection are now being studied.

The second aim of this study was to characterize the functional changes occurring in infected macrophages from a susceptible strain (BALB/c). Table 2 shows the development of functional changes with time after infection. Exposure to live virus resulted in a progressive loss of FcR and a rapid loss of high-level uptake of colloidal gold, while UV-inactivated virus either did not affect or increased these activities. Increases have also been seen in PCs from BALB.K or other $H\text{-}2^k$ strains and are thought to reflect macrophage activation. Hence, loss of FcR expression, colloidal gold uptake (Table 2) and the development of CPE result only from active infection.

The number of cells remaining on cover slip cultures from BALB/c mice after 4 days was not influenced by MCMV infection (unpublished data), and the cells remained viable by trypan blue exclusion while showing distinct CPE (Table 2). This contrasts with fibroblast cultures in which the most infected cells detach from the cover slips and are lost over this period. Closer analysis of macrophage cover slip cultures treated with colloidal gold revealed that infected cells lost the ability to take up large amounts of gold as they took on the distinctive round shape of infected cells. However, after 2 days of culture, many of these cells took up gold at a level below that normally scored as positive, indicating residual functional capacity.

Acid phosphatase levels (Table 2) were enhanced in cells from BALB/c and BALB.K mice, indicating increased macrophage activation (11), although the effect was clearest and only reached statistical significance in BALB.K cells (*t* test, $P < 0.01$). Histochemical staining showed that BALB.K cells were 100% positive for this marker at all times, but the intensity of staining increased after exposure to MCMV. The proportion of BALB/c cells staining for acid phosphatase remained about 63% after infection, and many cells showing the round morphology indicative of infection (90% of the population) stained positive. The variation in the intensity of staining for this enzyme increased after infection, consistent with concurrent activation and depression, as suggested by the results obtained with low-level colloidal gold uptake.

Although MTT conversion is generally used as a measure of viable cell number (11) while NBT is used to measure superoxide production (17), both reflect mitochondrial dehydrogenase activity. Over the course of several studies of the ability of BALB/c PCs to reduce NBT, the maximum depression was 30% of the control value, recorded on day 2 (Table 2). Exposure of BALB.K PCs to MCMV under the same conditions increased their ability to reduce NBT, demonstrating macrophage activation (17). Slight depression of the ability to reduce MTT was observed occasionally in BALB/c cells only, but the changes were not significant. Although activation of the few uninfected cells may have masked a loss of dehydrogenase activity arising from infection, this cannot explain the present result, because 85% of cells from an infected BALB/c preparation showing high-level CPE and fixed after exposure to NBT showed crystals of formazan. Corresponding uninfected populations were 88% formazan positive. Hence, both dehydrogenase and acid phosphatase activities were substantially retained in

preparations in which 80 to 90% of the cells exhibited CPE and viral antigen.

The conclusion from this study is that the infection of macrophages with MCMV in vitro is solely determined by the H-2 phenotype, which exerts its effects at a stage of the replicative cycle before viral DNA replication, such as the binding or uptake of virus. However, in the majority of infected cells, replication is blocked after the expression of viral antigens 3 days after infection. Such cells show a complete loss of FcR expression and reduced ability to take up colloidal gold but remain metabolically active for several days and may exhibit some indices of macrophage activation. This is consistent with the hypothesis that macrophages infected by MCMV in vivo may contribute to its spread and serve as a reservoir of persistent virus (7). This system for the infection of macrophages in vitro can be applied to the study of human blood monocytes as a determinant of sensitivity to cytomegalovirus infection, as well as contributing to our understanding of the cytomegalovirus receptor and the role of macrophages in the persistence and pathogenicity of this virus.

ACKNOWLEDGMENTS

We thank A. Scalzo and P. D. Hodgkin for advice throughout this project, Sandra Pemba for technical assistance, and R. I. Price for the optical scanning and computations for the quantitation of MCMV DNA.

The project was supported by the National Health and Medical Research Council of Australia, Woden, Australia.

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