The Role of Macrophages in Mice Infected with Murine Cytomegalovirus

By C. A. MIMS AND JANINE GOULD

Department of Microbiology, Guy's Hospital Medical School, London Bridge, London SE1 9RT, U.K.

(Accepted 18 May 1978)

SUMMARY

Quantitative studies were made of the infection of mouse peritoneal macrophages in vitro by cytomegalovirus, using virus assays and immunofluorescence. The efficiency of infection was low. Broth-induced peritoneal macrophages were about four times more resistant to infection than unstimulated macrophages and it was even more difficult to infect activated macrophages taken from mice 6 days after intravenous infection. Peritoneal macrophages (unstimulated) were infected at least 15 times more readily by tissue culture-passed (attenuated) virus than by salivary gland (virulent) virus, but macrophages prevented the spread of tissue culture virus to underlying susceptible mouse embryo fibroblasts, whereas they did so much less effectively with virulent salivary gland virus.

The pathogenesis of infection was studied in intact mice by immunofluorescence, and the observations paralleled the in vitro findings. When large doses of salivary gland virus were injected intravenously, infected Kupffer cells (liver macrophages) were occasionally seen and the inoculated virus directly infected large numbers of hepatic cells. In similar experiments with tissue culture-passed virus, there was initial infection of occasional Kupffer cells, which only rarely gave rise to infected hepatic cells. Differences in the extent of Kupffer cell infection by the two strains of virus were not detected in these experiments. Salivary gland virus also usually failed to infect splenic or lymph node macrophages. Occasional infected mononuclear cells were seen in the blood, lung and bone marrow, but were not identified. Infected cells were very rarely seen in the thymus, even in suckling mice.

INTRODUCTION

Macrophages have been shown to be key cells in the pathogenesis of many virus diseases, including those caused by viruses of the herpes group (Johnson, 1964; Hirsch et al. 1970). There have been two studies of the behaviour of cytomegalovirus (CMV) in mouse macrophages. In the first (Tegtmeyer & Craighead, 1968) unstimulated peritoneal macrophages from Swiss mice (Charles River strain) were shown to be readily infected by virus which had been passed four times in mouse embryo fibroblasts (MEF). The infection spread through the cell culture, and the macrophages developed intranuclear inclusions and yielded large amounts of virus. In the second study, using salivary gland-passaged and MEF-passaged virus (Selgrade & Osborn, 1974) in cultural macrophages from CBA/J and C57Bl/6 mice, together with in vivo investigations, it was concluded that macrophages were important cells in the pathogenesis of CMV infections in mice. However, during investigations in this laboratory, mouse macrophages were found to be infected only with low
efficiency by virus from salivary glands, and further studies of macrophage susceptibility are reported here.

METHODS

Virus. The strain of murine CMV used, kindly sent by Dr June Osborn, was originally derived from a chronically infected colony (McCordock & Smith, 1936), and during its long history had not undergone more than three passages in cell culture. It was further passed 1 to 4 times in three-week-old CD1 mice, harvesting salivary glands three weeks after intraperitoneal (i.p.) infection and is referred to as salivary gland virus. It was virulent as judged by its ability to kill 50% of three-week-old mice following i.p. injection of $10^3$ p.f.u. In certain experiments salivary gland virus was used after one passage in MEF and is referred to as TC1 CMV, or after eight passages in MEF (referred to as TC8 CMV). TC8 CMV was less lethal than salivary gland virus and failed to kill three-week-old mice, even when $10^6$–$10^7$ p.f.u. were injected (Osborn & Walker, 1970).

Mice. Specific pathogen-free outbred CD1 mice originally supplied by Olac Southern Ltd, Bicester, U.K. and bred in Guy's Hospital Medical School animal house were used in most experiments.

Assays. Samples were assayed for virus content in secondary or tertiary mouse embryo fibroblasts by two methods. First, a microplaque-forming technique in which dilutions of virus were mixed with a $2 \times 10^3$ ml cell suspension, portions of which were then added to ‘Microtitration’ plates (NUNC, Kamstrup, Denmark). Second, by a plaque-forming technique under carboxymethyl cellulose overlay in which 0.05 ml of virus dilutions were inoculated on to confluent mouse embryo fibroblast monolayers in ‘Multiwell’ dishes (Falcon Plasticware, Oxnard, California, U.S.A.). After 60 min adsorption at 36.5 °C, cultures were overlaid with Eagle’s medium containing a final concentration of 0.7% carboxymethyl cellulose (Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire, U.K.). With both methods of titration the cultures were drained after 4 days, stained with 0.005% crystal violet solution in saline containing 10% formalin and counted on a Carl-Zeiss Jena Dukomator projection microscope. Most titrations were made in ‘Multiwell’ dishes, but Microtitration plates were occasionally used for reasons of economy and convenience. Titres obtained in Microtitration plates were regularly found to be ten times lower and were multiplied by ten and expressed as p.f.u. per ml or g tissue in the ‘Multiwell’ system.

Immunofluorescence. Immune serum was produced by infecting 3-week-old mice with $10^3$ to $10^4$ p.f.u. of salivary gland virus, followed by two fortnightly injections of virus. Mice were bled out 2 weeks after the last injection. The indirect staining method was used, with a fluorescein-conjugated goat anti-mouse globulin obtained from Nordic Immunological Laboratories, Maidenhead, U.K. Mouse tissues were frozen in liquid nitrogen and sections cut in a cryostat. Observations were made using a Leitz Orthoplan microscope equipped for epi-illumination, with HBO 200 lamp and water immersion objectives. Photographs were made on Kodak Tri-X-Pan film.

Macrophages. Peritoneal macrophages were obtained from unstimulated mice using 15% foetal calf serum in Eagle’s medium plus 5 units heparin/ml, placed in ring cultures (Cairns, 1960) thoroughly rinsed after 18 h to remove unattached cells, infected and re-fed. The culture medium, consisting of 15% foetal calf serum in Eagle’s medium, was changed every 3 days.

Activated macrophages were obtained from mice in the same way either 3 days after intraperitoneal injection of broth, or from mice infected intravenously with $10^6$ p.f.u. salivary gland CMV 6 days earlier.
Macrophages in murine CMV infection

RESULTS

Infection of macrophages in vitro

When salivary gland CMV was added to unstimulated peritoneal macrophages in rings, infected cells were seen by immunofluorescence by 24 h; these were more common by 36 h. CMV antigen could be convincingly demonstrated on the surface of infected macrophages by fluorescent antibody staining of living cells. At 42 h, but not at 36 h, a very occasional infected cell was seen to have 2 to 6 infected cells immediately adjacent to it. These cells were

Fig. 1. Infected peritoneal macrophages 6 days after addition of TC1 CMV. Intranuclear, perinuclear and cytoplasmic antigen is present and one multinucleate cell has formed. Magnification ×800. Fluorescent antibody stain.
Fig. 2. Characteristic focus of infection in peritoneal macrophages 48 h after addition of TCt CMV. Infected cells are separated by uninfected cells. Magnification ×200. Fluorescent antibody stain.

undoubtedly infected, but contained only small globules of antigen in the nucleus and small amounts of antigen (i.e. faint staining) in the cytoplasm. As the infection in these cells progressed the amount of virus antigen increased (Fig. 1), prominent antigen-containing intranuclear inclusions developed and after several days some of the macrophages had rounded up. The 'foci' of infection now seen were of remarkable appearance because uninfected cells separated the infected ones (Fig. 2). It was concluded that infection spread directly from initially infected to contiguous cells, and that these cells migrated, presumably in the early stages of the infection, so that by 3 to 4 days the foci were as described above. The further spread of infection from these foci was irregular, and although on occasions nearly all macrophages were eventually infected when cultures were maintained for more than 10 days, this was not always the case.
Macrophages in murine CMV infection

Fig. 3. Dose–response curve for infection of mouse macrophages by TC1 CMV. Each point is the mean number of foci from 4 to 6 ring cultures.

Table 1. Ability of different strains of CMV to infect mouse embryo fibroblasts (MEF) and mouse peritoneal macrophages (PM)

<table>
<thead>
<tr>
<th>Strain of virus</th>
<th>MEF log p.f.u./ml</th>
<th>PM log focus forming units*/ml</th>
<th>Calculated p.f.u. added per macrophage infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salivary gland stock</td>
<td>6.7</td>
<td>4.5</td>
<td>160</td>
</tr>
<tr>
<td>TC1 CMV (first tissue culture passage)</td>
<td>6.1</td>
<td>4.7</td>
<td>25</td>
</tr>
<tr>
<td>TC1 CMV (first tissue culture passage)†</td>
<td>4.6</td>
<td>2.8</td>
<td>40</td>
</tr>
<tr>
<td>TC8 CMV (eighth tissue culture passage)</td>
<td>5.3</td>
<td>4.2</td>
<td>10</td>
</tr>
</tbody>
</table>

* Mean count from at least four cultures, as determined by fluorescent antibody staining 48 h after infection.
† First tissue culture passage from a different salivary gland stock.

The efficiency of infection of macrophages appeared to be low, and with input multiplicities of up to 100 p.f.u. of salivary gland virus per macrophage (see below), less than 5% of cells were infected after 3 days. A dose–response experiment was carried out with a strain of virus that had been passed once in tissue culture. Different amounts of virus were added to macrophages in rings, infected foci were counted by immunofluorescence at 48 or at 65 h and infectious foci produced were plotted against input virus on a log scale. The larger inocula were given by using virus that had been concentrated by centrifugation. A straight line dose–response curve was obtained (Fig. 3) and it can be seen that approximately 100 p.f.u. of this strain of virus were required to infect one macrophage.

A comparison of the ability of different strains of virus to infect macrophages was made by determining the number of foci produced in macrophage cultures at 48 h, and the titre obtained by plaque formation in MEF. It can be seen (Table 1) that compared with virulent
salivary gland virus, TC1 CMV has about five times the ability and TC8 CMV has about 15 times the ability to infect macrophages.

Immunofluorescent studies showed that peritoneal macrophages from 1- or 2-week-old mice showed similar susceptibility to those from older mice, but peritoneal macrophages from 3-day-old mice were 2 to 3 times as susceptible. Following the same inoculum of TC1 CMV, 3-day-old mouse macrophages showed 118 foci (mean per ring culture) at 60 h, compared with 32 foci in adult macrophages.

Attempts to achieve a high rate of macrophage infection at 48 h were unsuccessful and preliminary evidence indicated that yields per infected macrophage were very low, both for salivary gland CMV and TC8 CMV.

**Infection in activated macrophages**

Macrophages were obtained from mice 3 days after i.p. injection of broth, and also from mice infected intravenously with $10^5.4$ p.f.u. salivary gland CMV 6 days earlier. The macrophages from intravenously infected mice were not themselves infected, as detectable by fluorescent antibody staining, but were activated as judged by firm adherence to glass within half an hour. The next day, after thorough washing, $10^3$ p.f.u. of TC1 CMV was added to ring cultures of these macrophages and to those from unstimulated uninfected mice, either obtained at the same time, or maintained in vitro for 8 days. The number of infected cells in a standard number of microscope fields from five to six cultures of each type of macrophage was counted by immunofluorescence 48 h later. The mean numbers of infected cells were 909 (unstimulated), 1393 (unstimulated, 8 days in vitro), 235 (broth induced) and 145 (from infected mice). It is concluded that, compared with normal unstimulated macrophages, broth-induced macrophages were about four times more resistant and macrophages from infected mice nearly ten times more resistant to infection with CMV. There was, if anything, a slight increase in susceptibility when unstimulated macrophages were maintained for 8 days in vitro.

**Further in vitro experiments with macrophages**

The inefficiency of infection of peritoneal macrophages in vitro could theoretically be due to the presence of inhibitory substances in the culture medium. An experiment was therefore done to determine whether antiviral materials were liberated by unstimulated macrophages in vitro. Macrophages are known to release various materials, including lysozyme and lysosomal enzymes into the culture medium (Unanue, 1976). Fluid was taken from unstimulated peritoneal macrophages 72 h after initiation of cultures and tested for antiviral activity as in a neutralization test. The test fluid was incubated for 1 h at 37 °C with a standard dose of virus, then added to mouse embryo fibroblasts and plaques counted routinely. There was no reduction in plaque count with either salivary gland or TC8 virus.

Alveolar macrophages were obtained by irrigating the trachea with macrophage medium. These were shown by immunofluorescence to be infected by salivary gland CMV in vitro, but here too, only an occasional cell was infected.

Virulent salivary gland CMV initiated foci of hepatic cell infection in the mouse more readily than did TC8 CMV (see below). But the results of the in vitro experiments with macrophages suggest that salivary gland CMV would infect liver macrophages (Kupffer cells) less readily than TC8 CMV, and might therefore be expected to show less rather than greater capacity to initiate foci in hepatic cells. An experiment was done to test for the ability of the two virus strains to bypass a layer of macrophages and infect underlying susceptible cells. Ring cultures of confluent MEF were overlaid with $10^5$ peritoneal cells, and 18 h later
Macrophages in murine CMV infection

I49

Fig. 4. Mouse liver 24 h after intravenous injection of $10^4$ p.f.u. salivary gland CMV, to show extensive infection of hepatic cells. Infected Kupffer cells were uncommon. Magnification ×100.

Fluorescent antibody stain.

the cultures were gently rinsed and 0.1 ml of salivary gland or TC8 CMV was added to the cultures without disturbing the macrophage layer. At the time of adding virus more than half the MEF area was covered by macrophages, with a mean of more than one macrophage per fibroblast. Forty-eight hours later the fluid was removed, the cultures dried and fixed and foci of infection in the MEF layer counted after fluorescent antibody staining. In each instance three to four replicate cultures were counted, and foci in TC8 CMV-infected cultures were reduced from 29 (mean) in controls to 2 in the presence of macrophages. In cultures infected with salivary gland virus counts were 82 (mean) without macrophages, and 43 (mean) in the presence of macrophages. With both strains of virus, foci were generally smaller in the presence of macrophages. It was not possible to identify and count infected macrophages separately from infected MEF. A similar result was obtained in another experiment. Thus, macrophages exposed to attenuated CMV prevent infection of underlying cells whereas those exposed to virulent salivary gland CMV do so much less effectively.

Infection of macrophages in vivo

Liver

When newborn mice were infected i.p. with salivary gland virus the liver was infected, partly directly from the peritoneal cavity, because many of the foci appeared in the subcapsular region. When 3-week-old or older mice were infected i.p., liver foci were established
by blood borne virus as shown by the random distribution of lesions in lobules, but these foci were common only when larger doses of virus were given. In adult (8 weeks or older) mice they were not seen except when very large doses (> $10^5$ p.f.u.) were injected. When $10^6$ p.f.u. salivary gland virus were injected by the intravenous route into adult mice extensive infection occurred in the liver and mice died after 5 to 7 days with severe liver necrosis and liver titres of up to $10^7$ p.f.u./g. Infection in hepatic cells was initiated within 24 h, but infected Kupffer cells were not often seen at this stage (Fig. 4). At 48 h there were infected Kupffer cells and numerous hepatic cell foci; by 72 h the infection in the liver had become almost confluent. In contrast to the results with salivary gland virus, intravenous injections of $10^6$ p.f.u. of TC8 CMV produced small numbers of infected Kupffer cells after what is assumed to be a single cycle of growth at 24 h, but hepatic cells were not involved. At 48 and 72 h infected hepatic cells were seen only rarely (Fig. 5) and infection in the liver remained at a very low level.

Thus, intravenously injected salivary gland virus infected hepatic cells without the need for prior growth in Kupffer cells (Mims, 1964) and the infection then extended to involve most of the liver. With tissue culture virus, on the other hand, Kupffer cells were involved during the first cycle of growth, but the infection rarely spread to hepatic cells.

**Spleen**

Adult mice were infected i.p. with $10^5$-9 p.f.u. salivary gland virus and spleens examined at intervals by immunofluorescence. Infection was initiated in perifollicular regions of the spleen by 2 to 3 days and then spread into the follicles and into the red pulp. Peak spleen titres, at 3 days after infection, were $10^5-5$ p.f.u./g. A characteristic necrosis took place, some-
times involving the entire spleen (Mims & Gould, 1978). The initially infected cells appeared
not to be macrophages and did not take up carbon, although occasional macrophages were
infected at a later stage. TC8 CMV gave less infection in the spleen, and necrosis was not
seen when as much as \(10^6\) p.f.u. were injected.

*Lymph nodes, thymus, blood and bone marrow*

Lymph nodes remained almost uninfected after i.p. or intravenous inoculations of salivary
gland virus. Also, after extensive local growth of virus in injected footpads or in salivary
glands, no more than a very occasional infected large mononuclear cell was seen in the
draining lymph node (popliteal or cervical). In the thymus, infected mononuclear cells were
only rarely seen, even when 48-h-old mice were infected. Thymus titres 7 days after i.p.
infection of 48-h-old mice with \(10^4\) p.f.u. salivary gland CMV were \(10^4\) p.f.u./g.

In the blood, circulating mononuclear cells were seen to be infected 5 days after i.p. injec-
tion of salivary gland virus into newborn or 3-week-old mice but infected cells were not seen
by immunofluorescence at 10, 14 or 21 days. Infectivity titres were up to \(10^5\) p.f.u. per \(10^6\)
buffy coat leukocytes at 4 to 5 days after infection of adult mice but virus was not detectable
subsequently. Virus was not usually recoverable from serum or plasma.

Occasional unidentified mononuclear cells were seen to be infected in bone marrow smears
and infectivity titres in marrow were low \((10^5\) p.f.u./\(10^6\) cells).

*Lungs*

Infected mononuclear cells were sometimes seen in interstitial tissues in the lung 1 to 3
weeks after i.p. infection of 3-week-old mice and more commonly when large doses of virus
were given intravenously to adult mice. Lung titres were usually low \((10^5\) p.f.u./g), although
as high as \(10^6\) p.f.u./g at 3 to 5 days after large intravenous injections of virus.

Materials are known to be taken up into alveolar macrophages following intranasal (i.n.)
inoculations. Salivary gland CMV \((10^5\) p.f.u.) was therefore given i.n. to adult mice and
\(10^9\) p.f.u. i.n. to 1-week-old mice. Lungs were examined by immunofluorescence one week
later. In 1-week-old mice there were very occasional infected mononuclear cells in either
alveolar or interstitial tissues, but in adults neither alveolar macrophages nor any other type
of cell was infected.

**DISCUSSION**

Both the *in vitro* and *in vivo* results reported here show that the macrophages of CD1
mice are productively infected with low efficiency by CMV which has been harvested routinely
from salivary glands. This is not in conflict with the report of Selgrade & Osborn (1974) who
used infectious centre assays to show that only a small proportion of peritoneal macrophages
from Swiss mice were infected with salivary gland virus *in vitro* after 3 days, the number
increasing to \(50\%\) by 9 days.

A striking finding in the present study was the four- to fivefold increase in the suscepti-
bility of macrophages to CMV after only one passage in MEF and the 15-fold increase by the
eightth passage. This is in agreement with the findings of Tegtmeyer & Craighead (1968) who
used CMV after four passages in MEF and were able to infect all macrophages within 6 days,
as judged by the presence of intranuclear inclusions. The increase in the ability of tissue
culture passed virus to infect macrophages *in vitro* occurs in parallel with a decrease in
pathogenicity. This was unexpected, because the evidence from other virus infections,
including ectromelia (Roberts, 1964) and mouse hepatitis (Allison & Mallucci, 1965) shows
that virulent strains of virus infect macrophages more readily than attenuated strains.
Experiments in intact animals showed that macrophages in lymph nodes and spleen were not often infected by salivary gland virus. Kupffer cells were not easily infected by either strain of virus, at least in the early stages of liver invasion after intravenous infection. There are more than $10^8$ Kupffer cells in the mouse liver and even if all the $10^6$ p.f.u. CMV injected had been taken up randomly by these cells, the input multiplicity of infection would have been less than one, an amount that fails to infect peritoneal macrophages in vitro. The virulent salivary gland virus, however, was able to infect hepatic cells directly without the need for a preliminary cycle of growth in neighbouring Kupffer cells, so that antigen-containing hepatic cells were visible after a single cycle of growth at 24 h. When this occurred on a large enough scale it soon gave rise to confluent involvement of the liver. Kupffer cells were infected at later stages, perhaps because large enough concentrations of virus were available locally from infected hepatic cells. Similar patterns of growth in the liver have been described for a number of other viruses (Mims, 1964). The attenuated TC8 CMV strain, in contrast, gave no more than a very occasional small hepatic cell focus, and this occurred as a sequel to infection of Kupffer cells. These in vivo patterns of liver growth were reflected in the experiment where susceptible MEF were overlaid with macrophages. The macrophages protected MEF from infection with TC8 CMV, but did so far less effectively in the case of salivary gland CMV. Foci of infection were counted by immunofluorescence at 48 h, so that the experiment tested the ability of macrophages to control the initiation of infection in underlying MEF rather than to control the development and final size of foci. The yield from infected macrophages appeared to be low, but unfortunately it was not possible to obtain reliable data. A similar restriction of virus spread is seen in adult mouse macrophages infected with herpes simplex virus (Johnson, 1964). Infected macrophages from suckling mice are less able to restrict the spread of virus to neighbouring cells and this helps account for the greater virulence of herpes simplex infection in suckling mice.

The macrophage can perhaps be regarded as a relatively insusceptible cell which does not often undergo productive infection in vivo with either strain of CMV. Mouse embryo fibroblasts are ten times as susceptible (Table 1) and yields from infected macrophages are probably low. The role of the macrophage in the pathogenesis of murine CMV is probably to restrict virus replication and differences in the susceptibility of macrophages to infection, although they can be demonstrated, do not explain differences in the virulence of virus strains. The evidence obtained here suggests rather that differences in virulence may be associated with differences in the ability of macrophages to contain, control and inactivate virus after phagocytosis.

The authors would like to thank Cecilia Callis for expert technical assistance. The work described here was carried out with the help of a Project Grant (No. G972/751/C) from the Medical Research Council.

REFERENCES


Macrophages in murine CMV infection


(Received 8 February 1978)