Relationship between the Production of Murine Cytomegalovirus and Interferon in Macrophages

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SUMMARY

Macrophages (Mφ) harvested from the peritoneal cavities of mice after thioglycolate stimulation could be infected with murine cytomegalovirus (MCMV), although the efficiency of infection was low. Sequential measurements of interferon (IFN) production by virus-infected Mφ were performed in an attempt to explain the characteristics of MCMV infection in the cell cultures. Infected Mφ produced moderate amounts of IFN, which was completely neutralized by anti-IFN-α/β serum. The IFN was detectable in cultures as early as 8 h after infection and was produced only by exposing Mφ to infectious virus. Production increased until 48 to 72 h and preceded virus production, which was initially detected 72 h after infection. Treatment of the Mφ cultures with anti-IFN-α/β resulted not only in a marked increase in virus production, as well as a shortening of the long eclipse period of MCMV infection, but also induced increases in the number of Mφ releasing MCMV (VR-Mφ). Thus, the IFN produced in MCMV-infected Mφ (MCMV-Mφ IFN) appeared to suppress the production and spread of MCMV. The increase in the number of VR-Mφ observed was more resistant to anti-IFN-α/β treatment than the production of infectious virus. The antiviral effect of MCMV-Mφ IFN on MCMV infection in mouse embryo fibroblasts was similar to that induced by IFN-α/β. Therefore, MCMV-Mφ IFN appeared to be more active in protecting against the spread of cell-free MCMV than of cell-associated virus. These differences in sensitivity to IFN action suggest that Mφ may have a role in the latency of MCMV and that their production of IFN may facilitate the generation of latent infection.

INTRODUCTION

Macrophages (Mφ) are recognized as permissive for cytomegalovirus (CMV) infection (Brautigam et al., 1979; Drew et al., 1979; Mims & Gould, 1978; Selgrade & Osborn, 1974; Shanley & Pesanti, 1983; Tegtmeier & Craighead, 1968) and are key elements in its pathogenesis (Booss, 1980; Brautigam et al., 1979; Jordan & Mar, 1982; Katzenstein et al., 1983; Mims & Gould, 1978). However, reports concerning the amount of infectious virus which is produced by infected Mφ have varied. Tegtmeier & Craighead (1968) and Brautigam et al. (1979) demonstrated that infected murine peritoneal Mφ produce extracellular virus at concentrations 10- to 100-fold higher than mouse embryonic fibroblasts (MEF) infected with the same concentration of virus. In contrast, Selgrade & Osborn (1974) reported that only small amounts of virus, at least 10-fold lower than the initial inoculum, could be detected in the extracellular media of infected Mφ cultures. Other studies demonstrated that although Mφ can be infected and produce murine CMV (MCMV), they are relatively resistant to this infection.

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(Drew et al., 1979; Mims & Gould, 1978). Virus production appeared to be slower and less efficient in Mφ than in a permissive cell line such as MEF. The differing results reported in these studies could be due to alterations in the functional state of the Mφ examined, which could suppress the production of virus by Mφ (Glasgow & Habel, 1963; Nugent & Pesanti, 1979; Selgrade & Osborn, 1974; Shanley & Pesanti, 1982; Wildy et al., 1982). Therefore, understanding the factors that regulate virus production early after MCMV infection of Mφ in vitro may provide insight into the susceptibility of Mφ to MCMV infection.

It has been shown that Mφ inhibit viral replication of herpes simplex virus in vitro; however, mechanisms for this protective effect have not been determined (Morahan et al., 1980; Morse & Morahan, 1981; Stevens & Cook, 1971). With regard to MCMV, studies of the inhibition of virus replication by Mφ have been reported by Mims & Gould (1978) and Shanley & Pesanti (1983). These studies revealed that Mφ inhibited the viral replication of adjacent susceptible cells, but no inhibitory substances could be detected in the cultures. Therefore, the slower replication of MCMV in Mφ was not felt to be due to the secretion of antiviral factors from the cells. In these studies, intact resident Mφ known to be deficient in production of various monokines including lysozyme, lysosomal enzyme and interferon (IFN) were utilized. Since activated and virus-infected Mφ (Azuma, 1976; Ettensohn & Roberts, 1984; Glasgow & Habel, 1963; Lagwinska et al., 1975; Tàlas et al., 1973; Tsukui, 1977) have been shown to produce substantial amounts of IFN in vitro, in order to understand the mechanism of the decreased production and delayed spread of MCMV in Mφ cultures, the antiviral functions in MCMV-infected Mφ cultures should be examined. Preliminary experiments in this laboratory revealed that MCMV-infected Mφ inhibited vesicular stomatitis virus (VSV) production by L-929 cells and that the inhibition was eliminated by anti-IFN-α/β. The present study was designed to determine whether or not MCMV-infected Mφ produced antiviral substances, to characterize them, and to determine the kinetics of production and how they participated in virus production.

METHODS

Mice. Inbred BALB/c mice were obtained from Jackson Laboratories, Bar Harbor, Me., U.S.A. All were females aged 6 to 8 weeks. Mice were injected intraperitoneally with 1 ml of 3% thiglycollate broth (Eiken Chemical Co., Tokyo, Japan) to induce thiglycollate-elicited Mφ (TG-Mφ) 4 days later.

Virus. The Smith strain of MCMV, originally obtained from Dr J. D. Shanley (University of Connecticut, Storrs, Conn., U.S.A.), was maintained by animal passage and prepared as a 10% (w/v) salivary gland homogenate. This virus was plaque-purified twice in secondary MEF monolayers from BALB/c mice and a single virus stock prepared during the third passage in MEF from tissue culture supernatants (Shanley & Pesanti, 1982). The virus stock, stored at −70 °C in the presence of 10% foetal calf serum (FCS) and 10% DMSO, had a titre of 5 × 10^6 p.f.u. per ml. For use, the stock virus was diluted with RPMI-1640 containing 2% FCS, 2 mM-L-glutamine, 0.07% sodium bicarbonate and 100 units (U)/100 µg/ml of penicillin/streptomycin (culture medium).

Collection of Mφ. TG-Mφ were harvested by peritoneal lavage with 5 ml Hank's balanced salt solution containing 100 U/100 µg per ml of penicillin/streptomycin, washed twice and suspended in culture medium at a concentration of 1 × 10^6 per ml (Mφ suspension). The proportion of the cells that were Mφ was evaluated by a phagocytosis assay using latex and yeast. One ml of particles at 1 × 10^7 per ml in RPMI-1640 supplemented with 10% mouse serum was added to Mφ cultures. After 2 h cultivation, only the cells that ingested more than 10 latex particles and four yeast particles were scored as phagocytic cells. More than 98% of the adherent cells were classified as mononuclear phagocytes. To identify the MCMV-infected cells as Mφ, infected, adherent cells that phagocytosed latex and yeast particles were fixed with cold acetone for 15 min and then virus antigen in the cells was detected by indirect immunofluorescence staining as described below. Results from Mφ cultures were compared with MCMV-infected MEF.

Virus infection. The Mφ were infected with MCMV using two techniques to determine whether susceptibility to the virus differed. In the first technique, 1 ml of Mφ suspension was washed in 15 ml tubes (Corning) and resuspended in 0.5 ml of culture medium. The cell suspensions were then infected at an m.o.i. of 0.001 to 10 p.f.u. of MCMV per cell for 90 min at 4 °C (suspension infection; SI). The cells were then washed three times, mixed together and adjusted to a concentration of 1 × 10^6 cells/ml with fresh culture medium. One ml of infected Mφ was plated into 24-well culture plates (Costar), and incubated at 37 °C for 2 h to allow adherent cells to attach. The adherent cells were washed free of non-adherent cells and then refed with 1 ml of fresh culture medium. In the second technique, 1 ml of Mφ suspension was allowed to adhere to culture plates as described above. After
washing out non-adherent cells, the adherent Mφ were infected at the same m.o.i. as that used for the SI (adhesion
infection; AI). Thereafter, Mφ cultures were washed three times and refed with 1 ml of fresh culture medium. A
known permissive cell line, MEF, was also infected with the same amounts of MCMV as Mφ cultures to compare
virus production.

Production of hyperimmune serum to MCMV. Antibody to MCMV (MCMV-immune serum) was produced by
inoculating BALB/c mice intravenously with a 10% (w/v) salivary gland homogenate containing 10^8 p.f.u. of virus
(Shanley et al., 1981). After 3 weeks, the mice were killed and bled. The pooled serum was heat-inactivated at
56 °C for 30 min, and frozen at −70 °C. A 1:20 dilution of MCMV-immune serum inhibited over 99.9% of 1 × 10^3
p.f.u./ml of MCMV by a direct neutralization test. Pooled serum from mice previously inoculated with a
homogenate of normal salivary glands served as negative control serum (normal serum).

Preparation of IFN and anti-IFN serum. Mouse IFN-α/β, and anti-IFN-α/β and -γ antisera were kindly supplied
by Dr G. J. Stanton (University of Texas Medical Branch). The IFN-α/β (5 × 10^5 U/ml) was induced in L-cells
with Newcastle disease virus (NDV). Anti-IFN-α/β and -γ antisera were prepared in rabbits and neutralized 5 × 10^5
U of IFN-α/β and 8 × 10^3 U of IFN-γ, respectively. IFN was produced by Mφ cultures (MCMV-Mφ IFN)
48 h after AI at an m.o.i. of 2. Pooled supernatants were concentrated by polyethylene glycol precipitation
(Yamaguchi et al., 1984) and allowed to stand at pH 2.0 for 24 h at 4 °C to inactivate virus and then dialysed
against three changes of culture medium for 48 h at 4 °C. The activity of this preparation of MCMV-Mφ IFN was
320 U/ml.

Production of IFN and MCMV. Infected Mφ cultures were maintained in 1 ml of culture medium for 7 days after
infection. At intervals, the fluid in individual plates was collected and titrated for IFN and the amount of virus
released. At the same intervals, Mφ monolayers were also assayed for Mφ releasing MCMV (VR-Mφ) and Mφ
expressing viral antigens (VI-Mφ). In some experiments, to determine the relationship between MCMV
replication and IFN production, various amounts of either MCMV-immune serum or anti-IFN-α/β were added to the
cultures.

Determination of virus titre in culture. The amount of virus released from infected Mφ was determined by plaque
assay in MEF (Bixler & Boos, 1981). Briefly, 1 × 10^6 BALB/c MEF per ml were seeded in 24-well culture plates
with 1 ml of Eagle’s MEM containing 10% FCS, 2 mm-L-glutamine, 0.07% sodium bicarbonate and 100 U/100 μg
per ml of penicillin/streptomycin (growth medium). After incubation in 5% CO₂ for 3 days at 37 °C, the confluent
monolayers were infected by decanting the medium and adding 0.2 ml per well of 10-fold dilutions of Mφ culture
supernatants. After a 1 h adsorption period, the inocula were decanted and 1 ml of growth medium containing a
final concentration of 0.5% CM-cellulose (MC-growth medium) was overlaid on cultures. After incubation in 5%
CO₂ for 5 days at 37 °C, the monolayers were stained with crystal violet and the mean number of virus plaques
observed in triplicate wells was calculated. The virus titre was expressed as log_{10} p.f.u. per ml.

Infectious centre (IC) assay for VR-Mφ. At certain intervals, MCMV-infected Mφ cultures were treated with
MCMV-immune serum for 15 min at 4 °C to neutralize cell-free virus. After washing twice with culture medium,
adenoid cells were scraped from duplicate wells with rubber policemen and the number of viable cells was
counted. They were then added to MEF monolayers containing 0.2 ml of culture medium in triplicate in 10-fold
dilutions. The cells were allowed to adhere for 1 h and the cultures were washed once with 1 ml of growth medium
and refed with 1 ml of MC-growth medium (Brautigam et al., 1979; Selgrade & Osborn, 1974). After incubation in
5% CO₂ for 5 days at 37 °C, the plates were stained and IC number was counted. The number of VR-Mφ was
expressed as the number of IC per 10^6 cells.

Indirect immunofluorescence (IF) assay for VI-Mφ. The number of VI-Mφ was determined by indirect IF staining
(Shanley et al., 1979; Shanley & Pesanti, 1982). Infected Mφ were cultured on Lab-Tek tissue culture chamber
slides (Miles Laboratories). At certain intervals the cells were fixed in cold acetone for 15 min, dried and stored at
4 °C until use. When assayed, slides were rinsed in phosphate-buffered saline (PBS) and incubated for 30 min at
37 °C with a 1:40 dilution of the MCMV-immune serum which had been determined to produce the highest
immunofluorescence. After two washes in PBS for 10 min, slides were stained for 30 min at 37 °C using fluor-
escein-conjugated rabbit anti-mouse globulin (Cappel Laboratories). Infected and uninfected MEF were used as
positive and negative controls respectively. Five-hundred cells in every specimen were counted using a Zeiss
fluorescence microscope III-RS and the number of VI-Mφ was defined as the percentage of the total cells counted.

Assay and characterization of IFN. The IFN activity in Mφ culture supernatants was assayed by plaque
reduction in L-cells using VSV (Grundy et al., 1982; Lagwinska et al., 1975). Briefly, 50 μl of serial twofold
dilutions of the samples were added to each well of 96-well culture plates (Costar) with 50 μl of 1 × 10^5 L-929 cells.
After incubation for 24 h, the confluent monolayers were drained and inoculated with VSV at 30 p.f.u. per well.
After adsorption for 2 h, the wells were decanted and 100 μl of MC-growth medium was overlaid. The monolayers
were stained with crystal violet 40 to 48 h after virus inoculation and the number of plaques was counted. The units
of IFN were expressed as the reciprocal of the dilution that produced a 50% reduction in plaques when compared
to control cultures. The MCMV-Mφ IFN was characterized by stability at pH 2.0 for 14 h at 4 °C, inactivation at
56 °C for 45 min, and neutralization by anti-IFN-α/β and -γ antisera. The characterization using anti-IFNs
were performed in L-929 cells after a 2 h incubation at 37 °C of appropriate dilutions of anti-IFNs and IFN preparations previously titrated (Sergieiscu, 1983).

Induction of antiviral activity by IFNs. The sensitivity of MCMV to inhibition by MCMV-Mφ IFN was compared to sensitivity to IFN-α/β. The MEF monolayers were treated with MCMV-Mφ IFN (1 to 100 U/ml), and IFN-α/β (1 to 1000 U/ml) for 24 h before and after infection with 0.2 ml of MCMV at a concentration of 2 × 10^2 p.f.u./ml. Cultures were then replenished with 1 ml of MC-growth medium and the percentage reduction of plaques was plotted against the IFN concentration on a logarithmic scale to determine the amount of IFN that produced a 50% reduction in plaques.

RESULTS

Susceptibility of Mφ to MCMV infection

When peritoneal cells that adhered to plastic tissue culture plates were infected with MCMV at an m.o.i. of 1, approximately 12% of the adherent cells expressed MCMV antigen and 0.7% of the cells produced infectious virus. More than 98% of the virus-infected, adherent cells were extremely phagocytic for latex or yeast particles. Since MEF with virus antigen phagocytosed only a few particles, the phagocytic ability distinguished the Mφ from MEF and eliminated the possibility that infection of MEF accounted for the production of MCMV in the cultures. Next, the efficiency of MCMV infection was compared between the SI and AI methods. In both, VI-Mφ and VR-Mφ were identified 48 h after infection by IF or IC assays, respectively, and the results were plotted against m.o.i.s of 0.01 to 10 p.f.u. of virus per Mφ using a logarithmic scale. A straight line dose-response curve was observed with both infection methods (Fig. 1). The amount of virus required to produce one VI-Mφ was approximately 28 p.f.u. with SI and 16 p.f.u. with AI. To produce one VR-Mφ, 24 to 37 times more virus was required than was required to produce one VI-Mφ. This indicated that the adhesion-infected Mφ produced both virus antigen and infectious virus more efficiently than the suspension-infected Mφ. Therefore, the AI method was utilized in the majority of the subsequent experiments. Next, the production of infectious virus and VR-cells was compared between Mφ and MEF by 7 days after infection (Fig. 2). The cells were infected with MCMV at an m.o.i. of 1. In MEF cultures, production of infectious virus was detectable by 24 h, and the virus yield reached 10^5-6 p.f.u./ml 72 h after infection. The percentage of VR-cells increased from 0.8 to 73.5% during the first 3 days. In contrast, infectious virus was not demonstrated in Mφ cultures at 48 h. The virus production, which was initially detected at 72 h, increased slowly until it was 10^3-5 p.f.u./ml by 7 days. The number of VR-Mφ observed had significantly increased by 72 h after infection; however, they increased in number much more slowly than in the MEF cultures. A small number of Mφ, approximately 10%, compared with over 98% of MEF, produced infectious virus late after the infection. This indicated that the rate of virus replication and dissemination in Mφ is lower than in MEF; however, Mφ could be significantly infected with MCMV.

Production of IFN by MCMV-infected Mφ

Since the previous experiments had demonstrated that MCMV infection of Mφ was characterized by delayed as well as decreased virus replication when compared to MEF, the production of IFN from infected Mφ was examined. Both the SI and AI methods were used and the results were compared (Fig. 3). The Mφ were infected with MCMV at an m.o.i. of 1 and IFN production was determined periodically during the course of infection. IFN activity was initially detected in the fluid 8 h after infection, and the titres increased until 48 to 72 h when the highest titres (AI, 98 to 133 U/ml; SI, 53 to 82 U/ml) were observed. Thereafter, IFN production gradually decreased. To determine the amount of virus required for IFN production, Mφ were infected by the AI method with MCMV at an m.o.i. of between 0.0001 and 1.0 (Table 1). Production of IFN was detected in Mφ cultures infected at an m.o.i. of 0.01 or greater. In the cultures producing IFN, VI-Mφ and VR-Mφ were always detected. The amount of IFN produced depended on increases in the number of VI-Mφ and VR-Mφ. Heat-killed virus or virus inactivated with MCMV-immune serum neither produced VI-Mφ or VR-Mφ nor induced IFN. This suggested that Mφ must be infected with MCMV before they produce IFN. When
MCMV infection of macrophages

Fig. 1. Dose–response curve of MCMV infection in Mφ by the AI and SI methods. The number of VI-Mφ and VR-Mφ were measured by IF and IC assays, respectively, 48 h after infection with various m.o.i. of MCMV per cell. O, VI-Mφ SI method; ●, VR-Mφ SI method; △, VI-Mφ AI method; ▲, VR-Mφ AI method. Each determination represents the geometric mean per 10^5 cells from three experiments.

Fig. 2. Comparison of MCMV infection in Mφ (○) and MEF (●) cultures. The cells were infected with MCMV at an m.o.i. of 1 per cell and the production of infectious virus (a) and VR-cells (b) were determined. Each determination represents the geometric mean from three experiments.

Table 1. Interferon production by Mφ infected with MCMV*

<table>
<thead>
<tr>
<th>Virus added</th>
<th>VI-Mφ (%)</th>
<th>VR-Mφ (IC/well)</th>
<th>IFN (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>≤0:2</td>
<td>≤5:0</td>
<td>≤4:0</td>
</tr>
<tr>
<td>10^{-1}</td>
<td>11-9</td>
<td>3246-0</td>
<td>135-5</td>
</tr>
<tr>
<td>10^{-2}</td>
<td>3-2</td>
<td>557-6</td>
<td>36-5</td>
</tr>
<tr>
<td>10^{-3}</td>
<td>0-4</td>
<td>101-8</td>
<td>5-8</td>
</tr>
<tr>
<td>10^{-4}</td>
<td>≤0:2</td>
<td>6-7</td>
<td>≤4-0</td>
</tr>
<tr>
<td>Heat-killed virus†</td>
<td>10^0</td>
<td>≤0:2</td>
<td>≤5-0</td>
</tr>
<tr>
<td>MCMV-immune serum-treated virus‡</td>
<td>10^0</td>
<td>≤0:2</td>
<td>≤5-0</td>
</tr>
</tbody>
</table>

* Mφ were infected by the AI method. VI-Mφ and VR-Mφ were determined by the same methods as described in Methods. Values represent the mean of at least four experimental determinations.
† Prepared by inactivation at 56 °C for 45 min.
‡ Pretreated with MCMV-immune serum for 1 h at 4 °C.
MEF were infected with the same dose of MCMV, the amount of IFN produced was always much less than in Mφ cultures, although the kinetics in both cultures were similar.

**Characterization of MCMV-Mφ IFN**

The physicochemical and serological properties of the MCMV-Mφ IFN were determined and compared to IFN-α/β and IFN-γ. Exposure of 0.2 mg of trypsin per ml for 1 h at 37 °C completely destroyed the antiviral activity. The amount of IFN remaining after various treatments is shown in Table 2. The MCMV-Mφ IFN was stable at pH 2.0 for 12 h at 4 °C, and relatively labile when heated for 45 min at 56 °C. Anti-IFN-α/β treatment completely neutralized the MCMV-Mφ IFN activity detected, but no significant decrease in the IFN titre was detected after exposure to anti-IFN-γ. These properties of the MCMV-Mφ IFN are consistent with IFN-α/β.

**Effect of MCMV-Mφ IFN on the production and spread of infectious virus**

To determine the effect of MCMV-Mφ IFN on virus production, Mφ were infected by the AI method with MCMV at an m.o.i. of 1 and treated with enough anti-IFN-α/β to inhibit completely the IFN produced endogenously after infection. As shown in Fig. 4(a), MCMV-Mφ
**MCMV infection of macrophages**

**Table 2. Properties of MCMV-Mφ IFN**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MCMV-Mφ IFN</th>
<th>IFN-α/β</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>84.0</td>
<td>92.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Physicochemical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin (37 °C 1 h)</td>
<td>&lt;4.0</td>
<td>&gt;95.2</td>
<td>&gt;95.7</td>
</tr>
<tr>
<td>Heat (56 °C 45 min)</td>
<td>&lt;4.0</td>
<td>&gt;95.2</td>
<td>8.4</td>
</tr>
<tr>
<td>Acid (pH 2.0 12 h)</td>
<td>80.0</td>
<td>48</td>
<td>86.0</td>
</tr>
<tr>
<td>Serological</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-IFN-α/β serum</td>
<td>&lt;4.0</td>
<td>&gt;95.2</td>
<td>87.0</td>
</tr>
<tr>
<td>Anti-IFN-γ serum</td>
<td>82.0</td>
<td>2.4</td>
<td>90.4</td>
</tr>
</tbody>
</table>

*Titres represent the mean of at least two experimental determinations and are presented as the percentage reduction of residual titres against that of each control.

IFN was completely inhibited by the addition of anti-IFN-α/β for 3 days after infection. However, a small amount of IFN activity was subsequently detected. As can be seen in Fig. 4(b), in cultures not treated with anti-IFN-α/β (control cultures), virus was produced by 72 h after infection and the titres increased slowly until 5 days when the peak yield was observed. In contrast, earlier virus production was detected in cultures treated with anti-IFN-α/β (anti-IFN cultures) 48 h after infection. Virus production in the cultures increased rapidly until day 5. In addition, the amounts of infectious virus were significantly greater in anti-IFN cultures than in control cultures at all intervals after 2 days of infection. These findings suggested that the long eclipse period of MCMV infection in Mφ might be due to MCMV-Mφ IFN-mediated delay since treatment with anti-IFN-α/β was followed by more rapidly appearing and enhanced production of virus.

In order to assess the effect of MCMV-Mφ IFN on the intercellular spread of MCMV, the number of VR-Mφ capable of producing IC was determined in control and anti-IFN cultures and compared to virus production (Fig. 4 c). Initially, 0-63% of the Mφ released infectious virus and this proportion increased gradually to 12-6% by day 7. In comparison, Mφ monolayers in anti-IFN-α/β cultures contained a significant number of VR-Mφ by day 3 and 39-8% of Mφ produced IC on day 7. These results indicated that the MCMV-Mφ IFN could inhibit the intercellular spread of MCMV. The number of VR-Mφ increased by 3-2-fold of that of the control cultures after anti-IFN-α/β treatment (Fig. 4 c). As described above, when the effect of anti-IFN-α/β on MCMV infection of Mφ was evaluated on day 5 after infection, the production of infectious virus was 19-9 times higher than that of control cultures (10^4.5 compared to 10^3.2 p.f.u./ml) (Fig. 4 b). Therefore, one Mφ in anti-IFN cultures was estimated to produce 6-5-fold more virus than one in control cultures (0-78 compared to 0-12 p.f.u./VR-Mφ). These data suggested that MCMV-Mφ IFN prevented the extracellular spread more efficiently than the intercellular spread of MCMV.

**Antiviral activity of MCMV-Mφ IFN**

To examine whether MCMV-Mφ IFN induced resistance of MEF to MCMV infection, different dilutions of the IFN preparation were added to MEF cultures and the inhibition of MCMV infection was compared to that produced by IFN-α/β. The results, as shown in Fig. 5, demonstrated a dose-dependent effect of the IFN which reduced the number of plaques produced by MCMV infection. A straight line dose–response curve was obtained with each IFN preparation when they were added before infection. The titre of IFNs required to induce a 50% reduction was approximately 145 U/ml for MCMV-Mφ IFN and 175 U/ml for IFN-α/β. Thus, the MCMV-Mφ IFN preparation protected MEF from infection with MCMV in the same range as observed with IFN-α/β. This antiviral activity was completely prevented by treatment with trypsin or anti-IFN-α/β serum, indicating that the MCMV-Mφ IFN had produced the inhibition of MCMV infection. However, treatment of MEF with IFNs at similar doses after infection was not protective. A higher dose of IFN-α/β did confer slight protection.
Fig. 5. The antiviral activity of MCMV-Mφ IFN on MCMV infection of MEF monolayers. The MEF were exposed to IFNs for 24 h before (open symbols) and after (closed symbols) virus infection. The antiviral activity of IFNs was defined as the percentage reduction of plaque number compared to cultures without IFN. ○ and ●, MCMV-Mφ IFN; △ and ▲, IFN-α/β. Each determination represents the mean percentage reduction from at least three experiments.

DISCUSSION

The ability of MCMV to infect Mφ has been demonstrated previously; however, assessments of the efficiency of virus infection in vitro have produced variable results (Brautigam et al., 1979; Mims & Gould, 1978; Selgrade & Osborn, 1974; Shanley & Pesanti, 1983; Tegtmeyer & Craighead, 1968). In order to determine the reasons for this variability, two different methods of virus infection of Mφ were utilized. At various doses of virus, the AI method consistently produced more VI-Mφ and VR-Mφ than did the SI method. These results suggest that the conditions under which Mφ are cultured influence their susceptibility to infection with MCMV. However, there was no significant difference in production of infectious virus between these two methods on day 5 after infection.

The production of MCMV-infected Mφ has been reported to be closely associated with the m.o.i. of virus (Mims & Gould, 1978; Shanley & Pesanti, 1982). In the present study, infection at an m.o.i. greater than 0.01 per cell consistently induced VI-Mφ and VR-Mφ. In addition, when Mφ were exposed to MCMV at a lower m.o.i. (0.0001 per cell), VI-Mφ and VR-Mφ were not induced. Since VR-Mφ are able to produce MCMV and are detectable at an m.o.i. of 0.001 per cell, it would seem that an m.o.i. of at least 0.001 per cell was required for productive infection of Mφ using the AI method. The MCMV infection of Mφ was also affected by the number of cells per well in which subconfluent or barely confluent cultures were most susceptible to MCMV (data not shown). Therefore, it would appear that differences in susceptibility to MCMV infection of Mφ can be explained by culture conditions, the m.o.i. of the virus and the number of cells cultured at the time of infection.

By comparison with MEF cultures, the infection of Mφ with MCMV was characterized by a delayed time course, lower yield of virus and slower dissemination of virus (Fig. 2). Similar patterns of virus infection have been described by Shanley & Pesanti (1983). They suggested that virus production in Mφ could be influenced by inhibitors that were released in the cultures. However, no inhibitors of MCMV replication have been detectable in uninfected Mφ cultures (Mims & Gould, 1978; Vaczi et al., 1966). Other viruses such as NDV (Isaacs & Baron, 1960; Tfilas et al., 1973), lactate dehydrogenase-elevating virus (Lagwinska et al., 1975) and influenza virus (Ettenson & Roberts, 1984; Tsukui, 1977) have been shown to be able to infect mouse Mφ.
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and induce IFN production. Because of the findings with these other viruses, studies designed to elucidate the mechanisms regulating the delay and the limited production of MCMV after infection focused on IFN production by MCMV-infected Mϕ. The data from the present study demonstrated that Mϕ cultures infected with MCMV produced moderate amounts of IFN. The peak of production always preceded the increases in VI-Mϕ, VR-Mϕ and infectious virus. When MCMV was inactivated by heat treatment or MCMV-immune serum, virus did not induce Mϕ to produce IFN. These results indicate that MCMV-infected Mϕ synthesize IFN in the early phase as a result of exposure to infectious virus. The AI method resulted in better IFN production than the SI method. These differences in IFN production could be explained by the differences in the relative susceptibility to infection between the two methods since the AI method produced VI-Mϕ and VR-Mϕ in higher numbers than the SI method. The neutralization of MCMV-Mϕ IFN by anti-IFNs indicated that the IFN was IFN-α/β. These results are similar to studies which examined the ability of MCMV to induce IFN in MEF cultures (Henson & Smith, 1964; Oie et al., 1975), and confirm that murine Mϕ could synthesize IFN-α/β in response to exposure to viruses in vitro (Azuma, 1976; Lagwinski et al., 1975; Tálas et al., 1973; Tsukui, 1977).

It has been reported that the susceptibility of tissues to virus infection was associated with the production of IFN, and with enhanced susceptibility being related to a relative failure of IFN production (Isaacs & Baron, 1960; Vaczi et al., 1966). IFN has also been implicated as a possible determinant of the susceptibility of mice to infection with viruses (Chong et al., 1983; Grundy et al., 1982; Sawicki, 1961; Stebbing et al., 1978). Therefore, we attempted to elucidate whether MCMV-Mϕ IFN was instrumental in limiting MCMV multiplication in Mϕ. Since MCMV-Mϕ produced IFN-α/β as described, anti-IFN-α/β was utilized to inhibit the activity completely. The results showed that this treatment greatly enhanced virus production and resulted in the earlier production of virus (Fig. 4b). The growth of MCMV has been characterized by a long eclipse period after infection in MEF (Henson & Smith, 1964; Oie et al., 1975) and in Mϕ (Brautigam et al., 1979; Shanley & Pesanti, 1983; Tegtmeyer & Craighead, 1968). Some have hypothesized that this period may be due to an IFN-mediated delay of virus replication in the MCMV-MEF system (Oie et al., 1975). The results of the present study utilizing treatment with anti-IFN-α/β demonstrated that this appeared to be operative in the MCMV-Mϕ system. The rapid increase of virus production early in anti-IFN-α/β cultures appeared to be due to increased virus production by individual VR-Mϕ rather than an increased number of them, because they did not significantly increase in the anti-IFN-α/β cultures until 2 days after infection. In CMV-infected cells, virus particles have been shown to be engulfed by lysosome-like structures in the cytoplasm after release from the nucleus (McGavran & Smith, 1965; Tegtmeyer & Craighead, 1968). Since IFN involves producing activated Mϕ, in which phagocytic activity was augmented, and tumoricidal and microbicidal activity were enhanced (Schultz et al., 1978; Schultz, 1980), it would seem possible that the limited ability of Mϕ to support MCMV infection might be due to production of MCMV-Mϕ IFN which could regulate virus spread by the activation of cellular functions.

On the other hand, it has recently been reported that the infectivity of MEF-passaged MCMV was closely associated with the density of Fc receptors on Mϕ (Inada et al., 1985; Inada & Mims, 1985). Since it has been demonstrated that IFNs enhance Fc receptor expression of Mϕ (Vogel et al., 1983; Fertsch & Vogel, 1984), it might also be interpreted that the limited dissemination of MCMV in Mϕ cultures could be due to the dynamic alteration of Fc receptors on Mϕ by IFN action.

Treatment of Mϕ with anti-IFN-α/β resulted in significant increases of VR-Mϕ 3 days after infection. However, the enhancement in the number of VR-Mϕ was much less than that of infectious virus (Fig. 4b, c). Lang et al. (1969) reported that cell-associated CMV was highly resistant to the antiviral action of IFN, whereas cell-free CMV was very sensitive. Viruses are known to spread at least by three different routes (Lodmell et al., 1978): (i) to nearby or distant cells by the extracellular route, (ii) to adjacent cells by passing through an intercellular bridge and (iii) to progeny cells via the viral genome. With the culture conditions utilized in the present study, Mϕ were adjacent to one another, and MCMV released from VR-Mϕ could have spread...
by both the extracellular route and the intercellular bridge. In the former the virus could not reach another cell without being exposed to IFN, but in the latter the virus could be transferred without being exposed. The production of cell-free virus requires extracellular spread. VR-Mϕ could be infected via cell-associated virus and spread intercellularly. Therefore, the observation of differences between the production of infectious virus and VR-Mϕ in this system may be explained by the difference in sensitivity to IFN of these different routes of virus infection.

Since it was demonstrated that the MCMV-Mϕ IFN produced the restriction of MCMV infection in Mϕ cultures, whether this IFN could produce an antiviral state in MEF was determined. Pretreatment of MEF with various concentrations of MCMV-Mϕ IFN or IFN-α/β resulted in resistance to virus infection. The amount of MCMV-Mϕ IFN required to produce a 50% reduction in the number of plaques closely approximated that of IFN-α/β. In contrast, treatment of MEF with less than 100 U/ml of IFN after infection with MCMV did not produce any inhibition. Thus, a significant antiviral effect was produced when MCMV-Mϕ IFN was utilized with a low dose of MCMV before infection.

Despite the evidence that Mϕ could represent a reservoir of MCMV during latent infection (Booss, 1980; Brautigam et al., 1979; Jordan & Mar, 1982; Katzenstein et al., 1983; Mims & Gould, 1978), direct experimental proof has not been available. The data from the present studies suggest that IFN may be important in limiting the spread of both inter- and extracellular MCMV by Mϕ, enabling them to become a potential reservoir for the maintenance of latent virus. Further definition of the production of IFN and elucidation of its actions in the MCMV-Mϕ system might provide a valuable insight into virus latency in Mϕ as well as information which might prove useful in investigations of the reactivation of latent virus.

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