Comparison of the Pathogenesis of Murine Cytomegalovirus in Lung and Liver Following Intraperitoneal or Intratracheal Infection

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(Accepted 1 December 1983)

SUMMARY

This study compares the pathogenesis of murine cytomegalovirus (MCMV) infections following intraperitoneal (i.p.) and intratracheal (i.t.) inoculation. No deaths were seen in mice given 10^6 p.f.u. MCMV i.t., whereas 52% mortality occurred among animals given this dose i.p. This difference in mortality was not due to different effects on the lung, since virus titres in this organ on progressive days post-infection were similar for the two routes of inoculation and similar, minor histopathological changes were observed. In contrast, virus titres in the livers of mice inoculated i.p. were 100-fold higher than for those inoculated i.t., and histopathological changes were noticeably greater in the i.p. group. This suggests that the mortality seen following i.p. inoculation may have been due, at least in part, to effects of viral infection on liver function. Parallels between various forms of human cytomegalovirus infections and the types of infections seen following i.t. and i.p. infection with MCMV were observed.

INTRODUCTION

Human cytomegalovirus (HCMV) infections occur in several different forms, the most serious being congenital and perinatal infection and infection in individuals who are immunosuppressed. Less serious forms include post-perfusion syndrome, infectious mononucleosis and the vast majority of infections which are subclinical (for reviews, see Wright, 1973; Gold & Nankervis, 1976). Pneumonia, the most life-threatening aspect of infection in immunosuppressed patients (Abdallah et al., 1976; Betts & Hanshaw, 1977; Neiman et al., 1977), is also a feature of congenital infection (Gold & Nankervis, 1976). Infection of the liver with hepatosplenomegaly, alterations in liver functions, and sometimes jaundice has been demonstrated in varying degrees of severity in all forms of clinically apparent disease (Stern, 1972).

Murine cytomegalovirus (MCMV) has been used extensively as an experimental model to study the pathogenesis of cytomegalovirus infections (for review, see Osborn, 1982). Because HCMV pneumonia is a serious problem for human transplant patients, several investigators have studied the effects of MCMV on the lung (Brody & Craighead, 1974; Brody 1978; Jordan, 1978; Rose et al., 1982; Shanley et al., 1982; Quinnan et al., 1982). The more recent of these studies have initiated infection via the respiratory route because epidemiology suggests that droplet transmission may frequently be the route of entry for human virus (Gold & Nankervis, 1976). These studies have reported widely differing effects in immunologically competent mice ranging from lethal infection to minor histological changes.

The purpose of the present study was to resolve some of the discrepancies between previous studies involving the respiratory route of infection and to compare directly infections initiated by this intratracheal (i.t.) route with those initiated by the intraperitoneal (i.p.) route, which has been used in the vast majority of MCMV studies. The involvement of the liver and lung in these
infections was examined and provides insight into the contribution of infection in these two organs to morbidity and mortality. The data suggest possible parallels between various forms of the human infection and MCMV infections initiated by these two routes.

METHODS

Virus. The Smith strain of MCMV was obtained from the American Type Culture Collection and was passaged four or five times through mouse salivary glands. Virulent virus pools (Selgrade et al., 1981), which were used throughout this study, were obtained by making a 10% (w/v) extract of salivary glands removed from mice 2 to 3 weeks post-infection. These pools ranged in titre from $2 \times 10^7$ to $4 \times 10^7$ p.f.u./ml. Procedures for producing virus pools and quantifying virus in various target organs using a standard plaque assay have been described previously (Selgrade et al., 1981, 1982).

Mice. Outbred CD-1 female mice (Charles River Breeding Laboratories, Kingston, N.Y., U.S.A.) were obtained at 30 days of age and were 6 to 8 weeks old at the time of use. Animals sampled on arrival were free of Sendai virus, pneumonia virus of mice, MCMV, mouse hepatitis virus, and a number of other murine viruses, ectoparasites, endoparasites, mycoplasmas and salmonella. Also, there was no evidence of these agents in sentinel mice housed in the same room as test animals and monitored for such infections throughout the study. Routine histopathology of sentinel animals as well as animals sampled on arrival showed no pulmonary or hepatic abnormalities.

Histopathology. Lungs were fixed in situ by inserting a catheter into the trachea and filling the lungs with fixative (0.103 M-glutaraldehyde, 0.1125 M-sodium cacodylate) at a hydrostatic pressure of 20 cm. Lungs were allowed to fix for 30 min and then removed from the carcass and trimmed of extraneous tissues. Livers were removed, cut in small pieces, and fixed in 10% phosphate-buffered formalin. Haematoxylin and eosin-stained tissue sections were obtained by standard techniques for histopathological evaluation. Slides, which were coded such that the pathologist who evaluated them was not biased, were scored for histopathological lesions on a scale of 0 to 5.

Procedure for inoculations. Mice were inoculated either i.p. or i.t. with doses of MCMV ranging at 10-fold intervals from $10^2$ to $10^6$ p.f.u. I.p. inoculations were performed by injecting 0.2 ml of diluted virus into the left lower quadrant of the abdomen (well below the liver and away from the midline to avoid the bladder). Halothane anaesthesia was used for i.t. inoculations which were performed by passing 0.05 ml of virus via a 24-gauge intragastric needle through the mouth and epiglottis into the trachea. This procedure is described in detail by Hatch et al. (1981), who demonstrated that approximately 70% of radioactivity labelled protein injected by this method was recovered from lung and trachea, 7% remained in the head, and the remainder was found in the stomach. In our hands, Indian ink injected by this method was found in the lungs in 96% of the mice tested. In some cases animals observed for mortality were inoculated i.p. with 150 mg cyclophosphamide (Cytoxan; Mead Johnson & Co., Evansville, Ind., U.S.A.) per kg 2, 3 or 4 days post-infection.

RESULTS

Incidence of mortality following i.t. and i.p. inoculation

Table 1 shows a comparison of mortalities observed during a 21 day period following infection with either $10^5$ or $10^6$ p.f.u. MCMV given either i.t. or i.p. Very few deaths occurred following i.t. infection with $10^6$ p.f.u., the highest dose that could be delivered due to virus yields and the small size of the inoculum. In contrast, deaths occurred in 52% of mice given this same dose i.p. In an effort to increase the incidence of mortality, infected mice were treated with a single, Table 1. Comparison of mortalities following murine cytomegalovirus infection by intraperitoneal (i.p.) and intratracheal (i.t.) routes

<table>
<thead>
<tr>
<th>Dose and route of infection</th>
<th>MCMV alone</th>
<th>MCMV + 150 mg/kg cyclophosphamide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 days*</td>
<td>3 days</td>
</tr>
<tr>
<td>i.t. $10^6$†</td>
<td>2/68 (3%)†</td>
<td>0/15 (0%)</td>
</tr>
<tr>
<td>i.t. $10^5$</td>
<td>0/72 (0%)</td>
<td>0/15 (0%)</td>
</tr>
<tr>
<td>i.p. $10^6$</td>
<td>14/27 (52%)</td>
<td>ND§</td>
</tr>
<tr>
<td>i.p. $10^5$</td>
<td>5/36 (14%)</td>
<td>12/15 (80%)</td>
</tr>
</tbody>
</table>

* No. of days post-infection when cyclophosphamide was given.
† Dose in p.f.u./mouse.
‡ No. dead/total (% mortality).
§ ND, Not done.
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Immunosuppressive dose (150 mg/kg) of cyclophosphamide (CY) 2, 3 or 4 days after infection. Previous studies have shown that these treatment regimens produced maximum enhancement of infection following i.p. inoculation (Selgrade et al., 1982). Enhanced mortality was not seen in mice given CY 2 or 3 days after i.t. inoculation, and mice treated 4 days post-infection showed only a slight increase in mortality over those not receiving CY. In contrast, all three treatments resulted in enhanced mortality in mice infected by the i.p. route. No deaths were observed in control animals given appropriately diluted uninfected salivary gland extract either with or without subsequent CY treatment.

Comparison of virus titres in various organs

In an effort to account for the differences in mortality seen following i.t. and i.p. infection, a comparison was made of virus titres in lungs, livers, spleens and salivary glands of animals infected either i.t. or i.p. with various doses of virus. Fig. 1 to 4 show the course of infection in these key target organs. Data were collected at intervals during the first 2 weeks of infection, since the deaths shown in Table 1 routinely occurred within this time period. The lowest dose shown for a given inoculation route was the lowest dose that produced detectable infection in the indicated organ.

Fig. 1. Comparison of virus titres in the lung following intraperitoneal (a) and intratracheal (b) inoculation of doses of murine cytomegalovirus ranging from $10^3$ to $10^6$ p.f.u. Each point represents the mean ± the standard error for three to seven mice.
Fig. 2. Comparison of virus titres in the liver following intraperitoneal (a) or intratracheal (b) inoculation of doses of murine cytomegalovirus ranging from $10^4$ to $10^6$ p.f.u. Each point represents the mean ± the standard error for three to seven mice.

Fig. 1 shows that virus titres in the lungs peaked sooner following i.t. infection than following i.p. infection. However, a dose for dose comparison shows little difference in the magnitude of infections initiated by these two routes. In contrast, at a given dose, Fig. 2 and 3 show a much more vigorous infection of liver and spleen due to i.p. as compared to i.t. infection. In fact, levels of infection following i.t. infection with $10^6$ p.f.u. are similar to those following i.p. infection with 100-fold less virus. While i.p. inoculation of $10^2$ p.f.u. resulted in detectable spleen infections, spleen infections following doses below $10^4$ p.f.u. i.t. were negligible (mean values < $10^{2.1}$ p.f.u./g tissue). Similarly, infection of the liver was detectable in animals given $10^4$ p.f.u. i.p. but was negligible (mean values < $10^{2.2}$ p.f.u./g tissue) in animals given less than $10^6$ p.f.u. i.t. Infection of the salivary glands (Fig. 4) was relatively constant regardless of dose or route of infection with the possible exception of the two lowest doses of virus given i.t. Because the sensitivity of the plaque assay is such that virus titres less than 100 p.f.u. per g of tissue (log 2.00) cannot be detected, we assumed the worst case and averaged a value of 1.99 into the mean log_{10} p.f.u./g tissue for mice that had no detectable level of virus in an organ at the time of assay. Only three of 98 mice infected i.p. and four of 102 mice infected i.t. were found to be negative for all four organs tested. These cases all occurred 3 or 6 days post-infection and, with one exception, in mice which had received $10^2$ or $10^3$ p.f.u.

Effect of route of inoculation on pathological changes in lung and liver

Of the four organs under study, effects on lung and/or liver were deemed most likely to contribute to mortalities. Therefore, an attempt was made to determine the effect of route of infection on the weights of these organs and on histopathological changes following doses of $10^4$ and $10^6$ p.f.u. compared to uninfected controls. Fig. 5 shows the effects of infection on the lung, liver and body weights expressed as percentage of control value. Statistical analyses were performed using organ weights alone (as in Fig. 5) and organ to body weight ratios (not shown).
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Results were analysed by analysis of variance and the route by day interaction was subtested using T tests with a Bonferroni correction. By 10 days post-infection lung weights in mice infected i.p. had increased by 60% over control whereas only a 30% increase was seen in mice infected i.t. Lung weights were significantly higher \( (P < 0.05) \) 10 and 13 days after i.p. infection compared to i.t. infection. When adjusted for body weight, a significant difference was also noted 6 days post-infection. I.p. infection caused a 40 to 60% increase in liver weight (depending on dose) compared again to a 20% increase following i.t. infection. Increases in liver weights 3, 10 and 13 days after i.p. infection were significantly higher \( (P < 0.05) \) than those following i.t. infection. When adjusted for body weight, significant differences occurred 6, 10 and 13 days post-infection. Increases in organ weights occurred at times when body weights were either slightly depressed or normal when compared to controls.
Fig. 4. Comparison of virus titres in the salivary glands following intraperitoneal (a) or intratracheal (b) inoculation of doses of MCMV ranging from $10^2$ to $10^6$ p.f.u. Each point represents the average for three to seven mice. Standard errors have been omitted from the graph for simplicity. For i.p. infections standard errors ranged from 0.08 to 0.58 (average 0.26). For i.t. infections standard errors ranged from 0.06 to 1.12 (average 0.43).

Fig. 5. Changes in lung (a), liver (b) and body weight (c) following infection with $10^6$ p.f.u. i.t. (○), $10^5$ p.f.u. i.t. (●), $10^6$ p.f.u. i.p. (△) or $10^5$ p.f.u. i.p. (▲). Each point was obtained by dividing weights for individual infected mice by the mean weight of four to seven control mice, multiplying by 100 and averaging values so obtained from four to seven infected mice.
Fig. 6. Photomicrographs of haematoxylin and eosin-stained lung sections taken from mice 6 days after i.p. (a) or i.t. (b) infection with 10⁶ p.f.u. murine cytomegalovirus. Bar markers represent 100 μm. Minimal multifocal interstitial pneumonitis with some alveolar wall thickening was observed in both treatment groups.

Livers and lungs from three mice per inoculation group (controls, i.t. 10⁶, i.t. 10⁵, i.p. 10⁶, i.p. 10⁵) were removed at 6 and 10 days post-infection and processed for histological examination. No histological lesions were present in either lungs or livers taken from control animals which had been treated i.t. with appropriately diluted uninfected salivary gland extract. Pulmonary lesions were most consistently noted and to a greater degree in animals given 10⁶ p.f.u. as compared to those given 10⁵ p.f.u. regardless of route of infection. With one exception, animals given the higher dose showed minimal multifocal interstitial pneumonitis with some thickening of alveolar walls. One animal in the i.t. group showed a somewhat more prominent lesion which was scored as moderate. Scattered neutrophils and mononuclear macrophages were observed in the alveolar lumina. These changes appeared greatest on day 6 after i.t. infection and were of about the same magnitude 6 and 10 days after i.p. infection. Fig. 6 shows that histopathological changes seen in groups of animals given 10⁶ p.f.u. either i.t. or i.p. were minor and that there was no difference between the two inoculation groups. In neither case did the histopathological changes observed appear to be severe enough to account for deaths.

Histopathological changes in the liver were minimal in animals inoculated i.t. and ranged from minimal to moderately severe in mice infected i.p. The differences between animals given 10⁵ p.f.u. and those given 10⁶ p.f.u. and between those sacrificed on day 6 and day 10 were not notable in either treatment group. Liver changes in both inoculation groups included multifocal subacute hepatitis and increased mitosis. In addition, sections from mice inoculated i.p. showed periportal lymphocytic infiltration, diffuse hepatocellular hypertrophy, and megakaryocytic hyperplasia. There was a good deal of variation in the severity and frequency of hepatic lesions among animals in the same treatment group, and again none of the lesions appeared severe enough to cause death.

DISCUSSION

We were unable to produce a lethal infection in mice given MCMV i.t. whereas mortalities were easily obtainable in mice infected i.p. This difference did not seem to be due to different effects on the lung since similar infections occurred in this organ regardless of route of infection. In contrast, virus titres in the liver differed markedly depending on the route of infection. Although histopathological changes in the liver did not appear to be serious enough to cause death, it is conceivable that the difference in mortality following i.p. and i.t. infection is at least partially due to differences in the involvement of the liver. Lymphocytic choriomeningitis virus has been shown to turn off the 'differential' or 'luxury' functions necessary to keep the cells alive and morphologically normal (Oldstone et al., 1982). MCMV may similarly disrupt liver function without producing major histopathological changes.

Although the spleen is not essential to the survival of mice, the fact that virus titres in this
organ were much lower following i.t. infection may be pertinent. Katzenstein et al. (1983) recently showed that significantly more splenectomized mice survived an i.p. infection and had less virus in the liver than sham-operated, MCMV-infected littermates. From this and other observations, they concluded that early replication of MCMV in splenic macrophages augmented virus-induced hepatic injury even though histopathological changes following infection were similar in splenectomized and sham-operated mice. Thus, differences between virus titres in livers following i.p. and i.t. infection may be influenced by differences in virus titres in the spleen.

Our data are consistent with those of Shanley et al. (1982), who reported similar virus titres in lung, liver and salivary gland of BALB/c mice following intranasal (i.n.) infection. As in our studies no deaths occurred in immunologically competent mice infected i.n., and few or no histopathological abnormalities were observed. These authors were also unable to enhance mortality with cyclophosphamide. In contrast, Rose et al. (1982) reported extensive histopathological changes in the lung and 46% mortality following i.t. infection of CD-1 mice with 10^6 p.f.u. virulent MCMV. Virus titres in the lung were similar to those reported here. The liver was not examined. Quinnan et al. (1982) also described histopathological changes in the lungs of C57Bl/HeN mice which were more severe than those we observed. One possible explanation for discrepancies in the various studies of MCMV respiratory infection could be the age of mice used, since resistance to MCMV increases with age (Selgrade & Osborn, 1974). We recently tried to repeat the work of Rose et al. (1982) by infecting 26-day-old female mice with 10^6 p.f.u. i.t. We observed only 13% mortality (unpublished observation) which suggests that age differences do not entirely explain the discrepancies between the various studies. Since CD-1 mice from the same source were used in this study and that of Rose et al. (1982), differences do not seem to be attributable to the strain of mice. No other explanations are readily apparent. Following i.n. MCMV infection, Jordan (1978) observed a much more lethal infection than any of the other studies reported. His results seem to be due to the particular strain of mice he used. Unfortunately, since this strain is no longer available the reason for their exceptional sensitivity to MCMV remains a mystery (Shanley et al., 1982).

It appears that the advantage of using the i.t. route is not a greater ability to produce pneumonia but rather the ability to infect the lung without severely infecting the liver. In this regard infection established by the i.t. route may be more representative of the subclinical infection, which is thought to be frequently established by droplet transmission, in humans (Gold & Nankervis, 1976). In our mouse i.t. model a respiratory infection was established in the lungs and a persistent infection occurred in the salivary glands but the disease was relatively benign. I.p. infection of mice resulted in disease which was more like the widely disseminated congenital infection (Weller & Hanshaw, 1962), mononucleosis and post-perfusion type syndromes, and those instances where the virus had been associated clinically with liver disease (Stern, 1972; Hanshaw et al., 1965; Toghill et al., 1969).

We thank Margaret Grady for statistical analyses and Experimental Pathology Laboratories, Inc. for preparation and preliminary analysis of histopathological samples.

REFERENCES


(Received 19 August 1983)