Cytomegalovirus Infection

ABSTRACT

Host resistance to the development of metastatic lesions is complex and involves both lymphocyte and macrophage functions. Studies in both humans and animals have suggested that cytomegalovirus infection may alter these components of the defense mechanism of the host. In the present study, an experimental model was developed to determine whether cytomegalovirus infection would affect host resistance to the establishment of metastatic tumor nodules in the lungs of C3H mice after i.v. inoculation of a single-cell suspension of mammary tumor cells. The number of tumor nodules in the lungs, the lungs-heart/body weight ratio, and the mean day of death were determined in control animals inoculated i.v. with 10^6 mammary tumor cells and compared with groups of animals also receiving a sublethal i.p. inoculum of murine cytomegalovirus (MCMV) (10^5 plaque-forming units) either 3 days before, on the day of, or 10 or 13 days after tumor cell inoculation. The results suggest a biphasic effect of virus infection on tumor development in the lung. A preexisting or concurrent MCMV infection suppressed tumor growth and prolonged life, while a MCMV infection later in tumorigenesis enhanced tumor growth and shortened survival. These data suggest that MCMV modulates host resistance to the development of metastatic tumor nodules and that this experimental model may be utilized to investigate further the relationship between virus-induced alterations of host defense mechanisms and tumor growth.

INTRODUCTION

CMV is a common infection with worldwide endemicity. When acquired congenitally or after allograft transplantation, it may cause an especially serious problem (29). The widespread distribution of CMV infection may be at least partially attributed to its potential for latency and reactivation (3, 14, 29). It has been postulated that suppression of cell-mediated immunity during CMV infections may be potentiated during acute MCMV infection (4, 5, 19). While lymphocyte-mediated responses and resistance to secondary infections have been shown to be depressed by CMV infection, there have been few studies defining the effect of MCMV on the macrophage. Although various lymphocyte functions are depressed, recent studies suggest that macrophage function may be enhanced during a MCMV infection (11, 27).

Host resistance to the development of malignant tumors is a complex phenomenon which involves both lymphocyte (2, 6, 20) and macrophage functions (7, 8, 24). The capacity of CMV to modulate lymphocyte and reticuloendothelial cell functions, as well as to persist in the host in a latent form and to be reactivated at a later date, led us to postulate that infection with CMV may alter the resistance of the host to the development of malignant tumors. In order to test this hypothesis, we utilized the murine model developed by Hill and Bush (9). The effect of MCMV infection on the establishment and growth of pulmonary tumor nodules was determined in animals inoculated i.v. with a single-cell suspension of murine mammary tumor cells.

MATERIALS AND METHODS

Animals. Female C3H/He mice (8 to 10 weeks of age) used in this study came from a colony that has been inbred by L. A. Dethlefsen since 1958.

Virus. The Smith strain of MCMV was originally obtained from J. Osborn, University of Wisconsin Medical School, Madison, Wis. The preparation of both a salivary gland pool of MCMV and a normal salivary gland suspension in syngeneic mice, the exclusion of possible contamination of these preparations with lactic dehydrogenase virus and Mycoplasma, the assay used to titrate the virus preparation, the method utilized to study the pathogenesis of viral replication, and UV inactivation of MCMV have all been previously described (12, 13). The virus pool had a titer of 10^7 PFU/ml when assayed in secondary mouse embryo fibroblast cell culture. The experimental infec-
tion in mice was established by inoculating i.p. or i.v. either 10^5 or 10^6 PFU of MCMV diluted in MEM without fetal bovine serum or antibiotics. Virus infection was initiated at various times before, on the day of, or after i.v. tumor cell inoculation. Tumor control mice received a similar dilution of either normal salivary gland suspension or UV-inactivated MCMV.

Tumor. The transplanted tumors used in these studies were derived from spontaneous mammary tumors which arose in the breeding females used to maintain the C3H/He colony. The selection of the S102F line and its serial passage in the flanks of mice have been previously described (15). A modified lung colony assay after the technique of Hill and Bush (9) was utilized in these experiments. At the beginning of each experiment, S102F tumors which fit the growth kinetics pattern for this tumor line were excised and placed in ice-cold MEM. Viable tumor tissue was grossly dissected from necrotic tissue, washed, and then teased apart with forceps and an 18-gauge needle. The resulting cell suspension was gently vortexed and allowed to stand for 90 sec to remove large clumps of cells. After tumor cell viability determination by trypan blue dye exclusion, cells were centrifuged at 160 × g for 10 min and resuspended in MEM without fetal bovine serum or antibiotics at concentrations varying between 3.8 × 10^6 and 1.5 × 10^7 total cells per 0.5 ml (excluding erythrocytes). Viable tumor cells ranged from 15 to 23% in these experiments, and only occasionally were clumps of 3 to 8 cells seen microscopically. On the same day, all animals in a given experiment received 0.5 ml of a tumor cell suspension slowly injected i.v. into one of the lateral tail veins. Experimental groups were given injections in a random order, and cells were kept in suspension by a magnetic stirrer. Animals in the virus control groups, monitored for mortality due to virus infection, received 0.5 ml of a carrier medium by the same route.

Evaluation of Tumor Nodules. Twenty-five to 40 days after the inoculation of tumor cells, several mice in each experimental group were anesthetized with ether, weighed, and sacrificed by decapitation. Blood was allowed to drain out of the thoracic cavity, and the lungs, heart, and abdominal contents were removed. The lungs and heart were weighed either together or separately and then fixed for 24 hr in Bouin’s solution. The carcass weight was also determined. The number of tumor nodules in the lungs, the lungs-heart/body weight ratio, and the lungs/carcass weight ratios were based on the assumption that the tumor nodules present would increase the mass of the lungs. In other experiments, the heart was removed with the lungs for ease of dissection and has no bearing on these experiments. The fixed lungs and heart were photographed, and then histopathological sections of the lungs were made and stained with hematoxylin and eosin. Photomicrographs of these sections were taken at × 200 with a Zeiss Universal Photomicroscope. The animals in each experimental group not utilized in the above procedure were evaluated for mean day of death determinations.

Assay for Viral Replication within Tumor Nodules. Peripheral pulmonary tumor nodules were dissected from the lung tissue of mice infected with MCMV, and the nodules were either teased apart with an 18-gauge needle, homogenized as a 10% suspension, or homogenized as above and then frozen at −70°C. The resultant suspensions were overlayed on confluent monolayers of secondary mouse embryo fibroblasts and cultured for 14 to 21 days in MEM with 5% fetal bovine serum and antibiotics. Cultures were observed daily for MCMV cytopathic effect.

Statistics. In one experiment, the number of tumor cells injected versus the number of pulmonary tumor nodules or the lungs-heart/body weight ratio were evaluated by linear regression analysis. The mean numbers of tumor nodules, mean lungs-heart/body weight ratio, and mean day of death within each experiment were analyzed by the Mann-Whitney U test. Each experiment was analyzed individually to control for both the total number of tumor cells and the percentage of viable tumor cells injected. All values are expressed as a mean ± S.E. A p value of ≤ 0.05 was considered to be significant.

RESULTS

Evaluation of Parameters for Measurement of Host Response to Tumor Cell Inoculation. Two methods for determining the weight of tumor nodules developing in the lungs, the lungs-heart/body weight and the lungs/carcass weight ratios, were compared in order to establish the validity of the assay method for tumor growth. In normal animals, the lungs-heart/body weight ratio was 16.8 ± 2.0, and the lungs/carcass ratio was 13.5 ± 1.2; in a group of tumor-bearing animals, the lungs-heart/body weight ratio was 62.3 ± 12.2, and the lungs/carcass ratio was 78.0 ± 16.5. The weight of the heart did not vary from group to group, and thus its presence did not alter the results (data not presented). Because these ratios were not significantly different and because of the technical ease of dissection, the lungs-heart/body weight ratio was utilized in subsequent experiments. In another experiment, mice which were inoculated with serial dilution of a tumor cell suspension containing 20% viable cells were sacrificed 34 days later to determine the validity of 2 parameters for measurement of the host response to tumor cell inoculation (circles). Each point in Chart 1 represents the mean of 4 to 10 animals with the S.E. of the mean indicated by bars. The number of S102F tumor cells inoculated i.v. into mice was plotted against the number of lung colonies in Chart 1A. For the data indicated by the circles, the line of best fit calculated by linear regression analysis over the range of 3.8 × 10^5 to 1.5 × 10^6 S102F tumor cells inoculated had a slope of 7.99 ± 0.66. The number of S102F tumor cells inoculated versus the lungs-heart/body weight ratio is presented in Chart 1B. The line of best fit for the data indicated by the circles over the same range for this parameter had a slope of 2.41 ± 0.32. The data points indicated by the triangles are from 2 other experiments which were normalized to 20% tumor cell viability. Although these points were not included in the linear regression analyses, they are illustrated for completeness. The mean day of death was not evaluated in a similar manner in this experiment because of the limitation of the number of animals and tumor cells available.

Time Schedule of Inoculations. A time line indicating the sequence of the various inoculations is shown in Chart 2. Mammary tumor cells were injected i.v. on Day 0 in all experiments. In the experiment reported in detail below, 10^6 S102F tumor cells were injected in a suspension containing 20% viable cells, or 2.0 × 10^6 viable cells/mouse. As indicated in Line A, a sublethal dose of MCMV, 1 × 10^5 PFU/mouse, was injected i.p. either 3 days before (−3), on the day of (0), or 10 or 13 days after tumor cell inoculation (+10 or +13). All mice,
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except those utilized for mean day of death determinations, were sacrificed 34 days after tumor cell inoculation. In other experiments, schematically outlined in Line B, virus infection was initiated either 21 or 7 days before or 3 or 7 days after tumor cell inoculation.

MCMV Alteration of Response to Tumor Cells. Fig. 1A shows a pair of normal mouse lungs and heart on the left. A histopathological section of these lungs removed from a normal, noninfected mouse is shown in Fig. 1B. Notice the open alveolar spaces and the thin alveolar septae. The lungs and heart removed from animals infected 3 days prior to sacrifice with 10^8 PFU of MCMV appeared grossly similar (data not shown). A section of the lungs from a mouse infected with 10^8 PFU of MCMV 3 days prior to sacrifice is shown in Fig. 1C. In this section, the alveoli are approximately the same size, but the alveolar septae have become swollen and slightly more cellular. There is no consolidation or edema in the alveoli. On the right side of Fig. 1A, the tumor nodules in the periphery of the lungs (arrow) of tumor control mice (S102F 0) receiving 10^5 S102F tumor cells are depicted. A histopathological section of these lungs is shown in Fig. 1D. This adenocarcinoma (7) typically grows by expansion and compression of the surrounding alveoli, not by infiltration. The cellular infiltrate causing a thickening of the alveolar septae and the edema are a result of the tumor growth. Large numbers of histiocytes, or tissue macrophages, surround the tumor nodule (arrows). The effect of MCMV infection initiated on the same day as tumor cell inoculation is shown in Fig. 2A. There is a striking reduction in the number of tumor nodules in these lungs. Histopathological sections of these lungs (Fig. 2B) reveal less cellular infiltration in the alveolar septae, less consolidation or edema in the alveoli, and fewer histiocytes, or tissue macrophages, surrounding the tumor nodules (7). This same protective effect was observed when MCMV was inoculated 3 days prior to tumor cells (data not shown). In contrast, the lungs and hearts that were removed from mice infected with MCMV 10 days after tumor cell inoculation are presented in Fig. 3A. Since virus infection was not initiated until 10 days after tumor cell inoculation, it was assumed that an equal number of tumor cells became established and initiated growth in the lungs of these mice as in those of the tumor control mice, yet there is a striking increase in the number of peripheral tumor nodules seen in the lungs of these animals. Although the histopathological sections of these lungs (Fig. 3B) reveal a tremendous infiltration of inflammatory cells near the tumor nodule (7) and massive edema in the alveoli, more tumor nodules were apparent and they appeared larger. When virus was inoculated 13 days after tumor cells, a similar enhancement of tumor growth was observed (data not shown).

Table 1 summarizes the quantitative data obtained in this experiment. Each data point represents the mean of 4 to 6 animals. When 10^5 S102F tumor cells (20% viability) were inoculated i.v. in each animal, tumor control mice (S102F 0) developed a mean of 54.5 ± 5.5 tumor nodules in the lungs,

Chart 1. Linear regression analyses of the number of S102F mammary tumor cells injected versus the number of resultant lung colonies (A) and the lungs-heart/body weight ratio (B). S102F tumor cells were inoculated i.v.; 34 days later mice were sacrificed, and the above parameters were determined. Linear regression analyses were calculated for data from one experiment (Ø). Data from other experiments (©) are added for completeness but were not used in calculations.
had a mean lungs-heart/body weight ratio of 29.6 ± 1.1 g/kg, indicating the increased weight of the tumor tissue in the lungs of these mice as compared with normal, noninfected age-matched control mice, and had a mean day of death of 38.5 ± 3.8 days. Mice did not die from MCMV infection alone. Initiation of a MCMV infection on the same day as tumor cell inoculation resulted in striking protection. The number of tumor nodules in the lungs of each animal was significantly reduced, the lungs-heart/body weight ratio was decreased, and the mean day of death was also significantly increased. As shown previously (Fig. 2B), a moderate inflammatory response and little edema were observed in the lungs of these mice. A similar protective effect was observed when virus was inoculated 3 days prior to tumor cells. In other experiments, when MCMV infection was initiated 7 days before tumor cell inoculation, a similar but not as striking protective effect was observed. If, however, virus was injected 21 days prior to or 3 days after tumor cell inoculation, no protective effect was observed, and the development of tumor nodules in the lungs of virus-infected animals appeared to be similar to that in control animals. To observe the effects of viral infection initiated late in tumorigenesis, virus was inoculated after tumor cells in other experimental groups. When MCMV infection was initiated 10 days after tumor cell inoculation, the mice were found to have a significant increase in the mean number of tumor nodules in the lungs of each animal and in the lungs-heart/body weight ratio, as well as a significantly reduced mean day of death. As shown previously (Fig. 3B), the enhanced development of tumor nodules was associated with large numbers of inflammatory cells present in the areas surrounding the growing tumors and with a massive edematous response. A similar effect was observed if viral infection was initiated 13 days after tumor cell inoculation. In other experiments, when virus was inoculated 7 days after tumor cells, the lungs showed a similar, although not statistically significant, enhancement of tumor growth.

Additional experiments were designed to characterize this disease process further. When tumor control mice were challenged i.p. with normal salivary gland preparation, as in the experiment reported above, or with UV-inactivated MCMV, there were no significant differences observed in: (a) the number of tumor nodules in the lungs of each animal; (b) the lungs-heart/body weight ratio; or (c) the mean day of death. In addition, the previously reported pattern of pathogenesis of MCMV replication (12) was found to be identical in tumor-bearing and non-tumor-bearing control animals, regardless of the time of initiation of MCMV infection relative to tumor cell challenge. Finally, MCMV could not be isolated from tumor nodules in any experimental group by either cocultivation techniques, homogenization, or homogenization followed by freeze-thaw treatment utilizing our regular assay system for MCMV in mouse embryo fibroblast cells (data not presented).

**DISCUSSION**

CMV alters lymphocyte and macrophage functions involved in host resistance to viral, bacterial, and fungal infections. Many of these same lymphocytic and reticuloendothelial cell functions are also implicated in the response of the host to the development of malignant tumors. We postulated, therefore, that a MCMV infection could alter the host defense mechanisms involved in resisting a tumor challenge and allow increased tumor growth. The data presented in this paper support the hypothesis of altered host response due to virus infection, but suggest a biphasic effect of MCMV infection on tumor development. A preexisting or concurrent MCMV infection suppressed tumor growth, while virus infection later in tumorigenesis resulted in enhanced tumor growth. In addition, both the number of tumor nodules in the lungs and the lungs-heart/body weight ratio were shown to be valid parameters for measurement of tumor growth and thus indirectly of the host's capacity to control tumor growth. When large numbers of tumor
nODULES AND TUMORS

The striking reduction in tumor growth observed when mice were concurrently infected with MCMV is presumably a result of the viral infection or of the response of the host to it, since the pathogenesis of viral replication was unchanged in these tumor-bearing animals and UV-inactivated MCMV did not elicit similar protection. It seems unlikely that during MCMV replication in the lungs of infected mice virus also replicates in and destroys tumor cells and developing nodules. This assumption is based on the facts that virus could not be isolated by the techniques of tissue cocultivation or homogenization, and that the development of tumor nodules was not inhibited by MCMV infection initiated 10 or 13 days after tumor cell inoculation. It was observed that mice acutely infected with MCMV developed a smaller inflammatory response and that fewer tissue macrophages on histiocytes were noted by the techniques of tissue cocultivation or homogenization, and failure of the nodule to progress. It is not possible to determine the inflammatory cell content of the tumor nodules by histopathological section, and studies similar to those of Russell et al. (24) are planned to evaluate this possibility further. Based on the hypothesis that host macrophages might be stimulated to be tumoricidal by MCMV infection, studies were undertaken in our laboratory which showed that peritoneal macrophages were activated, as measured by several parameters including cytotoxicity to S102F cells, within 3 days after initiation of MCMV infection (27). Since MCMV replicates in the lungs of infected mice (12), it seems reasonable to postulate that alveolar macrophages may be activated and thus participate in the capacity of the host to reject tumor cell challenge.

In contrast to the reduction noted above, MCMV infection later in tumorigenesis was shown to enhance tumor growth and subsequent death of the animals. At this time in the development of tumor nodules, it may be postulated that MCMV suppressed the normal response of the host to the tumor cell challenge and allowed increased tumor growth compared to control animals. This is in contrast to the work by Milas et al. (16) who used Corynebacterium granulosum to stimulate the defense mechanism of the host and subsequently suggested that the response of the host in the first few days (less than 7) was critical for the establishment of pulmonary tumor nodules. Histopathologically, it was observed that the MCMV-infected mice had greater numbers of chronic inflammatory cells surrounding the pulmonary tumor nodules even in the face of enhanced tumor growth. These results suggest that the local lung environment may have an adverse effect on lymphocyte and macrophage function once tumor nodules have been established in the lung.

In summary, we have established an experimental system that provides an excellent model in which to study host defense mechanisms to tumor growth under normal conditions and under conditions of altered host defense mechanisms caused by a common viral infection. These data suggest that an acute, preexisting, or concurrent CMV infection may suppress tumor growth and prolong life, while a CMV infection later in tumorigenesis may enhance tumor growth and cause subsequent death. Finally, this model may be used to investigate further the interaction between alteration of host resistance by a viral infection and subsequent tumor growth.

REFERENCES


Fig. 1.  A, lungs and heart from a normal, noninfected mouse (left) and a tumor control mouse which received $10^6$ S102F tumor cells i.v. 34 days prior to sacrifice (S102F 0, right). Arrow, tumor nodules. B, histopathological section of lungs from normal mouse depicted in A. H & E, $\times$ 200. C, histopathological section of lungs from a mouse infected 3 days prior to sacrifice with $10^5$ PFU of MCMV i.p. H & E, $\times$ 200. D, histopathological section of the lungs from S102F tumor cell-inoculated mouse depicted in A showing tumor nodule (T) and histiocytic infiltrate (arrows). H & E, $\times$ 200.
Fig. 2. A, lungs and heart from a tumor control mouse which received $10^6$ S102F tumor cells i.v. 34 days prior to sacrifice (S102F, left) and similarly inoculated mouse infected with $10^5$ PFU of MCMV i.p. on the same day as tumor cell challenge (right). B, histopathological section from the S102F, MCMV 0 lungs depicted in A. T, tumor nodule. H & E, × 200.

Fig. 3. A, lungs and heart from a tumor control mouse which received $10^6$ S102F tumor cells i.v. 34 days prior to sacrifice (S102F, left) and a similarly inoculated mouse infected with $10^5$ PFU of MCMV i.p. 10 days after tumor cell challenge (right). B, histopathological section from the S102F, MCMV +10 lungs depicted in A. T, tumor nodule; arrow, histioyte. H & E, × 200.
Alteration of Murine Mammary Tumor Metastasis and Growth by Cytomegalovirus Infection

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