Virus–Lymphocyte Interactions during the Course of Immunosuppressive Virus Infection

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(Accepted 15 October 1986)

SUMMARY

Malignant rabbit fibroma virus (MV) is a lymphocytotropic leporipoxvirus which produces profound immunological dysfunction and lethal fibromyxosarcoma. We examined virus recovery from splenic lymphocytes as a function of time after inoculation in vivo, and correlated this with both immunological function and expression of virus-induced host suppressor activity. MV was most abundant in lymphocytes obtained 4 days following inoculation. At that time, immune function was relatively normal and host suppressor activity was not observed. By 7 days after infection, when active host immunosuppressor functions were observed, virus recovery was decreased. Eleven days post-inoculation host immune function began to recover despite increasing virus-induced tumours and developing opportunistic infection. Simultaneously, MV was no longer recoverable from spleen cells. Spleen cells from day 11 tumour-bearing rabbits did not support MV replication as efficiently as did normal or day 4 or 7 splenic lymphocytes, but they did not alter the ability of MV to grow in the latter cells. By fluorescence examination and cytofluorography, splenic lymphocytes bearing MV antigens were abundant 7 days after infection but disappeared by 11 days. This was temporally related to production of neutralizing antibody to MV, and development of virus-specific lymphocyte proliferative activity. The composition of splenic lymphocytes changed as well: the normal ratio of about 1:1 for B and T cells changed to 1:2 by day 7, and then inverted to almost 2:1 by day 11. Rabbits infected with MV thus appear to recover their immune function, concurrently eliminate virus-infected lymphocytes, and elaborate high titres of neutralizing serum antibodies despite progressive infections and tumour development.

INTRODUCTION

Malignant rabbit fibroma virus (MV) is a recombinant between Shope fibroma virus (SFV) and rabbit myxoma virus (Block et al., 1985). MV infection involves rapidly progressive disseminated tumours, secondary Gram-negative bacterial infection and profound immunological dysfunction (Strayer et al., 1983a, b, c). Death ensues within 12 to 18 days in all virus recipients even with inocula as small as 20 p.f.u.

Two basic mechanisms are responsible for the immunological consequences of MV infection in vivo and in vitro. First, virus infection of lymphocytes at high m.o.i. directly inhibits lymphocyte proliferation. In addition, MV also acts to induce host suppressor lymphocytes which are of T lymphocytic origin (Strayer et al., 1983c and unpublished results).

In our studies of the time course of MV infection and its relationship to immune dysfunction, we found that immunosuppression in MV-infected rabbits is not progressive. Peak immunological unresponsiveness is reached 7 days after MV inoculation. To our surprise, rabbits examined at later times showed either partial or complete recovery of immunological function (Strayer & Leibowitz, 1986). Spleen cells from such rabbits are also resistant to virus-
induced suppressor T lymphocytes present 7 days following MV inoculation. This immunological recovery occurs despite an increasing tumour burden and progressive Gram-negative infection.

To understand better how such rabbits recover their immune function, we studied MV infection of lymphocytes as a function of time after virus inoculation.

METHODS

Animals. Female adult New Zealand white rabbits were obtained from local suppliers. They were treated, housed, and sacrificed in accordance with accepted procedures.

Virus. The discovery, isolation, and growth characteristics of MV have been described already (Strayer et al., 1983a, b). MV and SFV are grown and titrated using RK-13 cells as outlined by Verna & Eylar (1962) and by Padgett et al. (1962). The procedures for MV growth in lymphoid cell populations are described elsewhere (Strayer et al., 1985a).

In all experiments the MV used was derived from stocks purified by two cycles of plaque-to-plaque purification. Rabbits received 1000 p.f.u. MV in 1 ml, administered intradermally in the hind leg.

Spleen cell cultures. Spleen and lymph node lymphocyte suspensions from MV-infected and normal rabbits were prepared according to conditions outlined by Redelman et al. (1976) and Sheppard et al. (1976). In brief, cells were suspended by passage through a steel mesh and cultured in 96-well microtitre plates (Costar) at a concentration of 10^6/well in 200 μl of RPMI 1640 (Gibco) supplemented with L-glutamine, antibiotics, 20 mM-HEPES, 5 × 10^-5 M-2-mercaptoethanol and 10% foetal calf serum (Hyclone). The procedures for measuring virus growth in these cultures have been described (Strayer et al., 1985a). Virus-specific lymphocyte proliferation assays were performed using SFV as the test antigen at a virus:lymphocyte ratio of 0.1. Twenty-four h incorporation of [3H]thymidine was measured 4 days after culture initiation (Skaletsky et al., 1984).

Antibody. Neutralizing antibody titres to MV were measured by incubation of serial twofold dilutions of serum samples with 100 p.f.u. MV for 2 h at 37 °C, and plating onto RK-13 cell monolayers in six-well cluster dishes. The neutralizing titres of the antisera are the reciprocals of the dilutions at which 50% MV neutralization occurred.

Immunofluorescence. Immunofluorescence analysis of spleen cell preparations was performed following cytocentrifugation. Hyperimmune rabbit anti-SFV Ig was used. This serum was absorbed with normal rabbit spleen cells prior to use. Ig preparations were made from whole antisera, and biotinylation was accomplished using standard procedures (Heitzmann & Richards, 1974). Slides were incubated with normal rabbit serum, followed by incubation with biotinylated anti-SFV, then FITC-avidin (Vector Laboratories, Burlingame, Ca., U.S.A.). Fluorescence was observed using a Leitz epifluorescence microscope with Ploem optics, and recorded on Tri-X film pushed for film development to ASA 1600. Staining for B lymphocytes was accomplished using FITC-goat anti-rabbit Ig (Miles Laboratories).

Cytofluorography. Cytofluorographic analysis was performed on a FACS II (Becton-Dickinson). Briefly, splenic lymphocytes from rabbits at various stages of virus infection were double-labelled by sequential treatment with normal rabbit serum, 9AE10 monoclonal antibody (see below), FITC–rabbit anti-mouse Ig (Miles Pharmaceuticals), hyperimmune rabbit anti-SFV–biotin, and avidin–phycocerythrin (Becton-Dickinson). Cells were fixed in phosphate-buffered saline containing 1% formalin for storage prior to analysis. Control cells received one of the following: normal rabbit serum alone, followed by the fluorochromes, the fluorochromes alone, or an unrelated hybridoma antibody followed by the fluorochromes. In all cases, the results were negative.

In determining the numbers of 'negative' and 'positive' cells by immunofluorescence using FACS, an arbitrary cutoff point was set at channel 10. All fluorescence intensity falling below that point was therefore defined as negative. Those cells fluorescing more brightly than that were defined as positive.

Monoclonal antibody. The mouse anti-rabbit T lymphocyte monoclonal antibody 9AE10 was the kind gift of Dr K. Knight of the University of Illinois. We used this reagent as an antibody recognizing T lymphocytes. 9AE10, as previously described by McNicholas et al. (1981), binds an antigen shared by T lymphocytes serving cytotoxic and helper functions. It does not recognize B lymphocytes or macrophages.

RESULTS

Virus recovery from spleen cells

We first assayed infectious MV in spleen cells of infected animals. Yields of such direct recovery attempts were reproducibly very low. When spleen cells from infected rabbits were cultured in vitro without lectin stimulation or added exogenous virus for 2 to 3 days, high yields of infectious MV were obtained. Using this technique, we examined the yield of MV from spleen cells of rabbits sacrificed 4, 7 or 11 days after inoculation of 1000 p.f.u. MV intradermally in the thigh (Fig. 1). Lymphocytes from the spleens of rabbits receiving MV 4 days previously...
Virus injection of lymphocytes

Fig. 1. Recovery of MV from spleen cells 4 (●), 7 (○) and 11 (■) days post-infection. Splenic lymphocytes from rabbits receiving 1000 p.f.u. MV intradermally were cultured in vitro. Samples were frozen and thawed and then assayed for infectious MV at daily intervals on RK-13 cell monolayers. Day 4 spleen cell time points are connected by a dashed line (--), day 7 by a dotted line (--), and day 11 by a solid line (---).

Table 1. Proliferative responses of spleen cells from normal and MV tumour-bearing rabbits*

<table>
<thead>
<tr>
<th>Spleen cells</th>
<th>Background</th>
<th>Con A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.45 ± 0.05</td>
<td>4.32 ± 0.04</td>
</tr>
<tr>
<td>Day 7 MV</td>
<td>3.37 ± 0.03</td>
<td>3.89 ± 0.01</td>
</tr>
<tr>
<td>Day 11 MV</td>
<td>3.10 ± 0.18</td>
<td>4.51 ± 0.09</td>
</tr>
</tbody>
</table>

* Spleen cells from normal rabbits and rabbits taken 7 and 11 days after infection with MV were cultured with and without Con A for 3 days. Twenty-four h lymphocyte proliferation was measured as incorporation of [3H]thymidine. Results shown are averages from three rabbits per experimental group.

† log_{10} C.p.m. [3H]thymidine ± S.D.

routinely produced the highest quantities of MV after culture. Recipients of MV 7 days previously gave comparable but slightly lower amounts of virus. For day 4 and day 7 rabbits, these virus growth curves were similar: virus recovery increased approximately 10-fold daily. However, on culturing spleen cells taken 11 days after in vivo infection we detected no virus.

Immune function

The ability of lymphocytes from these different experimental groups to proliferate in response to the T lymphocyte mitogen concanavalin A (Con A) is shown in Table 1. [3H]Thymidine incorporation was measured over 24 h. Spleen cells from day 4 MV-infected and normal rabbits responded comparably. Those from rabbits sacrificed 7 days after inoculation showed depressed responsiveness. By 11 days, Con A responses had significantly recovered, though the extent of this recovery varied from individual to individual.

Virus growth in spleen cells taken at different times post-infection

As spleen cells from rabbits 11 days after inoculation with MV appeared to contain little or no replicating virus in vivo, we determined whether these cells supported replication of MV added in vitro. Spleen cells from normal or MV-infected rabbits were cultured with added MV and without mitogens. Live virus recovery was determined on sequential days. The results (Fig. 2) showed that spleen cells from rabbits at day 11 of infection supported some MV growth, though to a lesser degree than those from normal or day 7 rabbits. MV recovery from day 7 rabbits in these experiments was, of course, influenced by endogenous MV replication resulting from the in vivo infection.
Fig. 2. Growth of exogenously added virus in spleen cells from MV-infected rabbits at different times after infection. Spleen cells from normal rabbits or infected rabbits at 7 or 11 days were cultured without (a) or with MV at m.o.i. 0.001 (b) or 0.01 (c). Representative samples were harvested daily, frozen and thawed and assayed for infectious MV on RK-13 cells. Day 7 time points (○) are connected by a dashed line (--), day 11 (○) by a dotted line (...) and normal (■) by a solid line (---).

Fig. 3. Replication of MV in mixed lymphocyte cultures. Spleen cells from normal rabbits, and from rabbits given MV 7 or 11 days previously were cultured together and separately. In each case, 10⁵ cells of each type were added per well (200 μl) in 96-well cluster dishes. Cultures were assayed daily for viable MV recovery following freezing and thawing. MV recoveries from day 7 spleen cells alone (○) and from day 11 spleen cells (○) are shown by a solid line (---), from day 7 + day 11 spleen cells (■) by a dashed line (---), and from day 7 + normal spleen cells (□) by a dotted line (-----).

Effects of day 11 spleen cells on virus growth in day 7 spleen cells

The low rate of replication of added MV in cultures of day 11 spleen cells could reflect developed resistance of these cells to MV, loss of a cell population particularly supportive of MV growth, or the development of active antagonists to MV replication, either viral [defective interfering (DI) particles], cellular or humoral. The latter possibility was examined by mixing spleen cells from rabbits taken 4, 7 or 11 days after infection. Normal rabbit spleen cells served as controls. As above, virus recovery was determined daily following culture initiation. Neither lectins nor added virus was placed in these cultures. Fig. 3 shows that adding day 11 spleen cells did not alter the permissiveness of day 7 lymphocytes for MV replication. When normal lymphocytes were added to cultures of day 7 spleen cells MV recovery was slightly but not greatly increased at all time points.

Immunofluorescence studies

The striking decline in recoverable MV from day 7 to day 11, the extant but decreased ability of day 11 lymphocytes to support MV replication, and the evidence of parallel recovery of immune function (Strayer & Leibowitz, 1986) suggested that lymphocytes capable of replicating infectious MV had been eliminated from the spleen. However, the possibility that MV infection persisted in a non-replicating form could not be ruled out. We therefore examined spleen cell preparations from rabbits on days 7 and 11 by immunofluorescence to determine whether we
could detect virus antigens in those spleens. Spleen cell suspensions were prepared using a cytocentrifuge, and stained with biotin-conjugated rabbit anti-SFV followed by avidin–FITC. At 7 days, positive staining for MV was observed evenly distributed in the cytoplasm of small cells resembling lymphocytes (Fig. 4a). When, instead of immunofluorescence, immunoperoxidase staining was performed to visualize virus antigen-containing cells, the majority of these appeared to be medium- to small-sized lymphocytes (data not shown). About one-third of day 7 spleen cells stained positively for MV antigen in this fashion.

By day 11, however, very few small lymphocytes demonstrated MV antigen. To the extent that positive staining was observed, very large cells containing large oval positively staining bodies were noted. These were most consistent with macrophages. The large positively staining bodies in the cytoplasm suggested cells of smaller dimensions, possibly phagocytosed lymphocytes.

Cytofluorographic analysis of spleen cells from normal, day 7 and day 11 spleen cells confirmed the suggestion that virus antigen-bearing lymphocytes were not common in day 11 spleens. Suspensions of spleen cells from normal rabbits and 7 and 11 days rabbits were stained as described in Methods with 9AE10 (mouse anti-rabbit T lymphocyte monoclonal antibody), and anti-virus antibody (Fig. 5). The former is shown as the abscissa for FITC and the latter on the ordinate for phycoerythrin. There was some ‘background’ leakage of FITC fluorescence into the phycoerythrin range in normal (i.e. virus-negative) controls. This is a frequent finding in cytofluorographic preparations, and has been observed consistently by us as well. We corrected for this in calculating percentages of virus antigen-positive cells. Nonetheless, day 7 spleen cells stained immediately ex vivo showed a large number (23%) of cell surface virus antigen-positive cells, mostly of T lymphoid lineage. By day 11 positive cell surface staining for MV was at background levels (0% positive cells).
Table 2. Cell surface markers on splenic lymphocytes during the course of MV infection*

<table>
<thead>
<tr>
<th>Rabbits</th>
<th>Anti-Ig</th>
<th>9AE10</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>43</td>
<td>41</td>
</tr>
<tr>
<td>Day 7 MV</td>
<td>31</td>
<td>63</td>
</tr>
<tr>
<td>Day 9 MV</td>
<td>59</td>
<td>43</td>
</tr>
<tr>
<td>Day 11 MV</td>
<td>68</td>
<td>41</td>
</tr>
</tbody>
</table>

* Spleen cells from normal and days 7, 9 and 11 MV tumour-bearing rabbits were analysed separately by FACS for the presence of surface Ig using FITC anti-rabbit Ig, and for T cell markers using 9AE10 followed by FITC anti-mouse Ig. Using channel 10 as the cutoff between positive and negative cell populations, the peaks of fluorescence activity were integrated from channel 10 downwards (negative) and from channel 10 upwards (positive). The overlap in anti-Ig positive cells and T lymphocytes in some groups may reflect the arbitrariness of this cutoff.

Lymphocyte populations

To determine whether the lymphocyte populations of the spleen were altered during the course of MV infection, we analysed spleen cells from normal rabbits and rabbits infected with MV 7, 9 and 11 days previously. These cells were stained separately either for B lymphocytes (FITC-anti-Ig) or for T lymphocytes (9AE10 followed by FITC-anti-mouse Ig) as indicated above, and analysed by cytofluorography. Using criteria described in Methods, we found (Table 2) that a dramatic change in lymphocyte population distribution occurred in the course of infection. Normal spleen cells were evenly divided between B and T lymphocytes. Midway through MV infection, spleens appeared to contain a preponderance of T lymphocytes (T:B ratio approx. 2). However, by 11 days, a majority of B lymphocytes had emerged (T:B approx. 0.5). In the course of this process, the number of non-marking cells decreased. Some overlap existed between lymphocyte populations scoring for B and T markers, and probably reflects the arbitrary nature of setting the lower exclusion border (channel 10).

Neutralizing antibody

Sera from MV-infected rabbits were used to determine the time course of neutralizing antibody production. Normal rabbits and those at 4, 7 and 11 days post-infection were tested.
Virus infection of lymphocytes

Serum neutralizing antibody titres were determined as described above and are shown as the means of from two (day 4) to eight (day 7) samples from different rabbits (Fig. 6). Little neutralizing antibody to MV was observed at day 4; low levels were observed by day 7, and very high titres were present by day 11.

**Lymphocyte response to virus antigen**

We also determined the development of cell-mediated immunity to MV. Spleen cells were cultured in vitro with live MV or SFV and 24 h incorporation of $[^3]H$ thymidine was measured. Fig. 7 shows that normal and day 7 cells were incapable of mounting proliferative responses to any of the virus preparations, as reported by Skaletsky *et al.* (1984). By day 11, however, lymphocyte responsiveness to SFV had developed. While such responsiveness is resistant to the tumour-induced immunosuppressive factor elaborated by T cells in response to MV (Strayer & Leibowitz, 1986), active infectious MV remained capable of preventing such proliferation (data not shown).

**DISCUSSION**

The course of a virus infection depends upon interplay of many factors, involving both host resistance and intrinsic viral pathogenicity. However, only a small number of outcomes is possible. A virus infection may resolve, usually leaving the host with lasting residual immunity of variable duration. Inappropriate or ineffectual host responses may occur, resulting either in latent virus infection (Fujinami & Oldstone, 1984) or transformation. Finally, the infection may pursue a more or less progressive course, causing death or debilitation of the host.

Few investigators have studied immune function in fulminant lethal virus infection. Examinations of virus infection of lymphocytes and its relation to both clinical course and immunological function in rapidly progressive virus infections have not been reported. This is one such study. The impetus for our work here was the observation that immune function in MV-infected rabbits reached a trough at about 7 days and rose thereafter. Normal or near normal responsiveness to mitogens was observed by day 11. Other data from our laboratory indicate that this recovery involved generation of active resistance to host T suppressor mechanisms (Strayer & Leibowitz, 1986).

In the accompanying report, we document the growth potential of MV on different lymphoid populations and detail factors involved in the genesis of this potential. In this report, we set out to determine the time course of MV replication in and recovery from lymphocytes exposed to virus *in vivo*. 

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**Fig. 6.** Time course of neutralizing antibody response to MV. Normal rabbit sera and sera from rabbits 4, 7 and 11 days after MV infection were analysed for their ability to neutralize infectious MV as described in Methods. Titres for each point are expressed as the geometric mean of titres from samples from different rabbits: six normal rabbits, two day 4 rabbits, five day 7 rabbits and three day 11 rabbits.

**Fig. 7.** Lymphocyte responses to MV antigens as a function of time post-inoculation. Spleen cells from rabbits 7 and 11 days after MV inoculation, and also from normal rabbits, were incubated with Con A (5 µg/ml), or SFV at m.o.i. 0-1 ($2 \times 10^4$ MV/2 x $10^4$ spleen cells/200 µl). Twenty-four h incorporation of $[^3]H$ thymidine was measured; standard error is less than 0-10 log$_{10}$ units in all cases. Background, ■. + SFV, □. + Con A.
MV increases 10-fold in titre daily when spleen cells taken from animals 4 and 7 days after infection are assayed in culture. Such replication is undetectable by 11 days. Lack of virus recovery from lymphoid populations coincides with returning immune function. While this process occurs, virus-laden tumour masses enlarge throughout the superficial body parts (ears, snout, etc.), and Gram-negative infection continues (Strayer & Sell, 1983).

There are several possibilities to explain the disappearance of recoverable virus from day 11 MV spleen cells. A population of virus-susceptible cells may be destroyed. While alterations in total lymphocyte viability are not observed with MV (Strayer et al., 1985b), the loss of a critical population that is small in numbers but disproportionately susceptible to MV might not be noted. To some extent this may occur, as day 11 lymphocytes do not permit MV to replicate as well as do normal lymphocytes. This explanation is at best incomplete, however, as MV does replicate to some extent in day 11 spleen cells.

DI particles are incomplete viruses capable of preventing replication of whole virus in target cells (Holland et al., 1980). DI particles infecting lymphocytes could inhibit wild-type virus replication, and might be responsible for the apparent loss of MV from spleen cells examined 11 days after MV infection in vivo. DI particles have been described for almost every type of virus. They require intact virus for their own replication (Perrault, 1981; Nayak et al., 1985). We examined this possibility in two ways. By immunofluorescence, we found that few or no lymphocytes from day 11 MV spleens express cell surface or cytoplasmic viral antigens. In addition, mixing lymphocytes from day 7 and day 11 MV-infected rabbits did not alter MV replication in the former cells. While these findings do not completely rule out the possibility of DI particles, they suggest that such particles are not involved in the refractoriness of day 11 MV spleen cells to MV replication. These same experiments suggest that soluble virus inhibitors such as interferon are not involved. In other experiments we have not found evidence for interferon in our system (Strayer et al., 1983a).

The most likely explanation, we feel, for the disappearance of recoverable MV from spleen cells by 11 days, is that virus-infected cells, comprising a large proportion of the day 7 spleen cell population, have been eliminated. This elimination coincides with the development of very high titres of neutralizing antibody and of proliferative reactivity to virus antigens. We also note what appears to be phagocytosis of MV antigen-positive cells.

In experimental and clinical systems studied by others, recovery from virus infection appears to be quite complex. Cellular contributions may derive from cytotoxic T lymphocytes (St. Geme et al., 1965; McMichael & Hildreth, 1982), natural killer cells (Habu et al., 1981, 1984; Bukowski et al., 1983; Biron et al., 1984), killer cells (Perlmann, 1984), and macrophages (Mogensen, 1979).

In addition, antibody has been shown to protect animals from viruses in a number of different ways (Sissons & Oldstone, 1980; Bartholomew et al., 1978; Welsh, 1977; Hirsch et al., 1980; Hicks et al., 1978).

The role of antibody in the elimination of infectious MV from lymphocytes is not definitively determined. Interestingly, we have noted that during the recovery phase of the immune response, T lymphocytes in the spleen are outnumbered by B cells 2:1. This contrasts with splenic lymphocyte composition earlier in infection, when T cells predominate. Such findings are consistent with our previously reported observations that lymph nodes taken early in MV infection demonstrate loss of germinal centre activity and expansion of the diffuse cortex (Strayer et al., 1983b). This expansion consists almost entirely of T lymphocytes (Strayer & Sell, 1983). Reagents to subtype T lymphocytes in rabbits are not as yet available. However, the peak of immunosuppressive activity in spleen cells is observed on day 7, and the predominance of T cells in the spleen at that time may reflect this suppressive function.

We cannot, on the basis of the in vitro data presented here, assign relative weights to the contributions of T cytotoxic or natural killer cells, or antibody, to the development of resistance to MV in vivo. The dramatic differences between MV recovery from lymphocytes exposed to MV in vivo and the growth of MV in the same cells when placed in culture indicate that the host defence mechanisms responsible for dealing with MV infection are quite potent, and comprise more than the cell populations present in the spleen at the time of sacrifice. In addition, the fact
that such recovery does occur suggests that mechanisms to prolong the life of MV-infected rabbits, such as treatment of the secondary bacterial infections, could provide sufficient time for the recovering host immune system to eliminate the tumours and resolve the virus infection.

We appreciate the technical assistance of Mr Jan Dombrowski, Mr Kenneth Korber, Mrs Mani Sastry and Mrs Jan Hurt. Dr Dorothy Lewis and Mr Rocco Carbone helped us with the cytofluorography. We also acknowledge the generous support of Drs Charles A. Janeway, Jr and Stewart Sell. This work has been supported by grant number IM-358 from the American Cancer Society.

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*(Received 12 August 1986)*