Coevolution of Host and Virus: Cellular Localization of Virus in Myxoma Virus Infection of Resistant and Susceptible European Rabbits

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Received April 24, 2000; returned to author for revision May 23, 2000; accepted July 6, 2000

The coevolution of myxoma virus and the wild European rabbit in Australia and the development of resistance to myxomatosis in wild rabbits have been well described. However, the mechanism of resistance to myxomatosis in wild rabbits is not understood. To determine the basis of resistance, the pathogenesis of the virulent standard laboratory strain (SLS) and the attenuated Uriarra (Ur) strain of myxoma virus were examined in Australian wild rabbits that have been naturally selected in the field for resistance to myxomatosis and in laboratory rabbits which have never been selected for resistance. Virus was localized in tissue sections by immunofluorescence. In all cases virus antigen was initially present in dendritic cells of the dermis before localizing predominantly to the epidermis by Day 6. Antigen-containing cells were detected in the lymph nodes by 24 h after inoculation. Virus replication occurred predominantly in T lymphocytes of the paracortex but SLS also replicated in germinal centers. SLS replication induced loss of most lymphocytes from the lymph nodes of susceptible rabbits. Apoptosis of lymphocytes within the lymph nodes was a major feature of all infections. These apoptotic cells did not contain detectable viral antigen but were often adjacent to infected cells. Ongoing apoptosis of lymphocytes within lymph nodes was also a feature of the recovery phase when very few or no virus-infected cells could be detected. Differences between virulent and attenuated viruses in the wild and laboratory rabbits were predominantly in the degree of tissue pathology in the draining lymph node and distal lymph node and in the type of inflammatory responses, particularly in the skin. SLS infection of laboratory rabbits was associated with a very mild inflammatory response, often distant from the site of virus replication and comprised predominantly of neutrophils. In contrast, Ur-infected rabbits and SLS-infected wild rabbits had an intense inflammatory response adjacent to the site of virus replication and this was comprised predominantly of mononuclear cells. Both the initial infection of dendritic cells and the ongoing destruction of lymphocytes provide obvious mechanisms for the suppression of the immune response by myxoma virus.

Key Words: myxoma virus; myxomatosis; disease resistance; rabbit; virulence; Serp-2.

INTRODUCTION

Adaptation to a new host may drive the evolution of both an emerging pathogen and its new host. One of the best-studied examples of host-virus coevolution is the release of myxoma virus for biological control of the wild European rabbit population of Australia and the subsequent selection for rabbits with resistance to myxomatosis and attenuated strains of virus. The coevolution of myxoma virus and the European rabbit in Australia was extensively studied as it occurred and has formed the basis for conceptual and mathematical models of infectious disease evolution (Anderson and May, 1982; Fenner, 1983). However, remarkably little is understood about either the mechanism of resistance in the rabbit or the attenuation of the virus (reviewed by Kerr and Best, 1998).

Myxoma virus is a poxvirus (genus: leporipoxvirus). Two subtypes of the virus have been described. The south American type is found in Sylvilagus brasiliensis (forest rabbits) and the Californian type in S. bachmani (brush rabbits) (Fenner and Ross, 1994). In its normal host species, myxoma virus replication induces a cutaneous fibroma at the site of inoculation. The virus is probably not disseminated systemically. Myxoma virus is spread by biting arthropods such as fleas and mosquitoes which pick up the virus as they probe through the skin lesion. Transmission is passive as myxoma virus does not replicate within the vector. High virus titers in the skin are thus critical for transmission (Fenner and Ross, 1994). A virulent strain of myxoma virus from Brazil (the standard laboratory strain, SLS) was released in Australia in 1950.

In the European rabbit (Oryctolagus cuniculus), myxoma virus causes the lethal disease myxomatosis characterized by systemic spread of the virus and the development of secondary skin lesions (myxomas). Following intradermal inoculation, myxoma virus replicates in the skin at the inoculation site and spreads from there to the
draining lymph node. The virus replicates to high titers in the lymph node and spreads in infected leukocytes to distal tissues such as the spleen, testis, lung, and mucocutaneous sites, such as the nose and conjunctivae, and skin (Fenner and Woodroffe, 1953; Best and Kerr, 2000). The major sites of pathology are the lymphoid tissues and skin, although some alterations occur in most organs (Hurst, 1937). The main pathological features are cellular proliferation and cellular necrosis. An unusual feature of the pathology of myxoma virus infection is the proliferation of cells in the walls of the small blood vessels and the appearance of large stellate “myxoma cells” (Hurst, 1937).

To study the mechanism of resistance to myxoma virus, we have used the original SLS (standard laboratory strain) myxoma virus and an attenuated field strain (Ur-arrua, Ur) to infect unselected laboratory rabbits (to represent the original host susceptibility) and naturally selected wild resistant rabbits. We have killed rabbits from each of the four groups at 12 and 24 h and 2, 4, 6, and 10 days after infection and from three of the groups at 15 and 20 days after infection. Thus we have directly compared virulent and attenuated virus strains in resistant and susceptible outbred rabbits. This paper describes which cells virus was infecting, the clearance of virus from the tissues, cell death, and inflammatory responses. Histopathological differences between the virulent and attenuated viruses and the wild and laboratory rabbits are described. The spread of virus and replication of these strains of myxoma virus in resistant and susceptible rabbits was described in a previous paper (Best and Kerr, 2000).

RESULTS
Clinical disease in laboratory and wild rabbits infected with SLS and Ur

The clinical disease in laboratory and wild rabbits infected with SLS or Ur has been described in detail elsewhere (Best and Kerr, 2000). Laboratory rabbits inoculated with SLS developed severe myxomatosis. These rabbits were not kept for longer than 10 days as previous experience showed that they would all die between 10 and 14 days after inoculation (Robinson et al., 1999). Wild rabbits inoculated with SLS also developed severe myxomatosis but the clinical signs were delayed compared to laboratory rabbits and the animals recovered rapidly starting around Days 12 to 15. Laboratory rabbits inoculated with the attenuated Ur strain of myxoma virus developed quite severe clinical myxomatosis with the peak clinical signs between Days 12 and 15. They began to recover between Days 15 and 20. Wild rabbits inoculated with Ur had a very mild infection characterized by a primary lesion at the inoculation site and mild conjunctival swelling and mild anogenital inflammation.

The main differences in histopathology and virus localization between the laboratory and the wild rabbits infected with virulent SLS or attenuated Ur strains of myxoma virus are summarized in Table 1 and are described in detail below. The histology of SLS infection has been previously described (Hurst, 1937) and is only briefly described here to highlight differences between the infections.

Virus replication in dermis and epidermis

Following intradermal inoculation, myxoma virus replication was detected by immunofluorescence in the dermis at the inoculation site at 24 h but not at 12 h after intradermal inoculation. The infected cells were elongated, had prominent cell processes, and were located either at the epidermal-dermal junction or deeper in the dermis. At 2 and 4 days after infection the dermis was packed with virus-infected cells (Fig. 1A). These cells stained positively for MHC-II. In uninfected rabbits, MHC-II-positive cells were located predominantly at the junction of the epidermis and dermis (Fig. 1B). However at 4 days after infection MHC-II-positive cells were largely in the dermis (Fig. 1C). Smaller mononuclear cells, probably lymphocytes, within the dermis were also positive for virus in wild rabbits infected with SLS but not in the other infections. At Day 6 in all infections, virus was no longer present in MHC-II-positive cells but was concentrated in the epidermis, within round cells in the dermis and for SLS-infected laboratory rabbits only, large rectangular cells proximal to dermal blood vessels (see below in Fig. 3D). Also in SLS-infected laboratory rabbits, virus was present within the subdermal muscle layers but this was not the case in wild rabbits. By 10 days after infection, the majority of cells staining positive for virus were in the epidermis, including the cells of the hair follicles, rather than the dermis (Fig. 1D).

The colocalization of virus with MHC-II antigen-positive cells was confirmed by double staining (Fig. 2).

In all infections with SLS or Ur in wild or laboratory rabbits, virus replication initially occurred within MHC-II-positive cells in the skin. However, there were clear differences between wild and laboratory rabbits and SLS and Ur in the extent and timing of subsequent pathology and particularly in the inflammatory responses in the dermis and epidermis. These differences could be seen as early as Day 4 when there was a mononuclear cell infiltrate in the dermis of all infections, but in SLS-infected laboratory rabbits the cells were confined to the lower dermis. By Day 6 the dermis of the inoculation site of Ur in laboratory rabbits and both viruses in wild rabbits contained large numbers of macrophages, lymphocytes, and plasma cells concentrated within the upper dermis with aggregates of cells at the epidermal/dermal junction directly below areas of epidermal pathology that stained positively for virus. The cells of this inflammatory response were similar in Ur-infected laboratory rabbits and...
SLS- and Ur-infected wild rabbits but there were always more mononuclear cells in the dermis of wild rabbits. In contrast, inflammatory cells in the dermis of SLS-infected laboratory rabbits were predominantly polymorphs with some lymphocytes and macrophages. These cells were frequently karyorrhectic or apoptotic and were aggregated deep in the dermis adjacent to the superficial muscle. Myxoma virus was detected by immunofluorescence at this location in laboratory rabbits infected with either SLS or Ur but not in wild rabbits.

Differences in the inflammatory response and pathology between infections were marked by 10 days after infection. At this time, the histopathology of the skin from Ur-infected laboratory rabbits or from SLS-infected wild rabbits resembled that from SLS-infected laboratory rabbits at Day 6. In SLS-infected laboratory rabbits at Day 10, the epidermis was thickened and contained vesicles containing fibrin and polymorphs. There was only a weak cellular inflammatory response in the dermis comprised mostly of polymorphs (Fig. 3A). This is in contrast with wild rabbits (Fig. 3B) where there was a pronounced cellular response in the dermis and the inflammatory cells were predominantly lymphocytes rather than polymorphs. A similar cellular response occurred in the dermis of laboratory rabbits infected with Ur (Fig. 3C). By Day 15 the pathology in the skin of Ur-infected laboratory rabbits or the SLS-infected wild rabbits resembled that of SLS-infected laboratory rabbits at Day 10.

### TABLE 1

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<td><strong>Distal skin</strong></td>
<td>Virus initially replicated within MHC-II-positive dendritic cells of the dermis. Then moved to epidermal cells. Some myxoma cells present. Polymorphs localized to deep dermis.</td>
<td>Virus initially replicated within MHC-II-positive cells in the upper dermis, proximal to blood vessels. Scattered distribution. Mononuclear cells infiltrated throughout dermis. Virus not detected by immunofluorescence after Day 10.</td>
<td>Virus initially replicated within MHC-II-positive cells in the upper dermis, proximal to blood vessel. Scattered distribution. Intense mononuclear cell infiltration at epidermal/dermal junction. Virus still present in epidermis at Days 15 and 20.</td>
<td>Virus not detected by immunofluorescence.</td>
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Virus was able to persist in the skin in laboratory rabbits infected with Ur and in wild rabbits infected with SLS or Ur. At Day 15, virus was present in the epidermis although there were fewer infected cells compared to Day 10. The epithelial cells of the external root sheath of the hair follicles were strongly positive for virus. At this stage many epidermal cells were necrotic and large vesicles had formed within the epidermis giving a similar appearance to SLS at Day 10. Fragmented cell debris was common within the epidermis and a scab was forming on the surface. At Day 20, there was a thick scab over the inoculation site and virus was present only in the basal layer of the epidermis and in the epithelial cells of the hair follicles. There was a massive aggregation of inflammatory cells at the margins of the scab and within the dermis the density and organization of collagen fibers had increased as had fibroblast numbers. Extravasated red blood cells were a prominent feature.

A further difference in SLS-infected laboratory rabbits from the other infections was the appearance of large cells in the walls of small blood vessels, which were disrupted with extravasated red cells leaking into the surrounding tissues. These cells appeared to be migrating into the surrounding tissues of the dermis where they were frequently surrounded by polymorphs (Figs. 3Di and 3Dii). Virus antigen was present in endothelial cells of blood vessels and within the large migrating cells clustered around these vessels (Fig. 3Diii). These cells are presumably the “myxoma cells” described by Hurst (1937), although whether they are all derived from the one progenitor type has not been determined. These cells did not stain for MHC-II and neither did endothelial cells. Endothelial cells were enlarged in the other infections but in general no further changes were detected and there was no virus detected within the endothelial cells in these infections. Large stellate myxoma cells were also uncommon in the other infections.

**Virus replication in the lymph node draining the inoculation site**

We had previously demonstrated that the popliteal lymph node predominantly drained the foot inoculation site (Best and Kerr, 2000). SLS in laboratory or wild rabbits or Ur in laboratory rabbits was detected in the draining lymph node at 24 h after infection. Virus was present in the lymph node at 24 h after infection. Virus was present in the lymph node at 24 h after infection.
After infection, large elongate cells within the wall of the capsule stained positive for virus in laboratory rabbits infected with SLS (Fig. 4F) but this was not a feature of other infections. During infection of the draining lymph node SLS infections had consistently greater numbers of virus-infected cells in the node than were present in the other infections and these were evenly distributed throughout the cortex of the node and within the follicles. In draining lymph nodes from the other infections there were areas with high densities of infected cells but also other areas with few or no infected cells and virus-infected cells were not normally found within the follicles.

In all infections, virus replication predominantly occurred in the T cell zone of the lymph node. In SLS-infected laboratory rabbits this was associated with dramatic depletion of the lymphocytes, proliferation of reticular cells, and an influx of inflammatory cells. There was also an initial lymphocyte depletion in wild rabbits infected with SLS. Swelling of the endothelial cells of small blood vessels was present in both wild and laboratory rabbits infected with SLS by Day 4. Stellate myxoma cells were also a feature of lymph nodes from SLS-infected laboratory and wild rabbits. By Day 6 after infection, similar pathological changes were occurring in the paracortex and germinal centers of both wild and laboratory rabbits infected with SLS and there were high numbers of neutrophils in the subcapsular sinus and the surrounding connective tissue. Depletion of lymphocytes was more localized in wild rabbits and the extent of lymphocyte loss varied greatly between rabbits. Figure 5A shows the loss of lymphocytes from a laboratory rabbit lymph node infected with SLS (compare with Fig. 5C which is similar to a normal lymph node). In addition, there has been an influx of polymorphs and there are changes in the walls of small blood vessels similar to those described in the dermis. Figure 5B depicts the same time point in a severely affected wild rabbit and shows that while there has been a large-scale loss of lymphocytes there are still lymphocytes present and there are far fewer polymorphs than in the laboratory rabbits infected with SLS. Lymph nodes in wild rabbits were particularly variable at Day 10 with pathology ranging from extensive lymphocyte depletion and reticular cell proliferation to relatively normal tissue. In laboratory rabbits infected with Ur, lymphocyte depletion was not observed and there were few polymorphs (Fig. 5C). By Day 15, the lymph nodes of wild rabbits inoculated with SLS were well populated with lymphocytes (Fig. 5D) and virus could not be detected by immunofluorescence. In Ur-infected laboratory rabbits, virus was only present in a few lymphocytes at Day 15 and was not present at Day 20. In Ur-infected wild rabbits, viral antigen was not present after Day 2.
Virus replication in the contralateral lymph node

Virus was detected at Day 4 in the contralateral node for both laboratory and wild rabbits infected with SLS. At Day 6 virus was detected in cells migrating from blood vessels into the contralateral lymph node and in cells migrating within the subcapsular sinus of the lymph node (Fig. 6). This suggests that this node is infected both by virus-infected lymphocytes from the bloodstream.
and by virus-infected cells draining from other sites such as the skin. Viral antigen was detected in the contralateral node in Ur-infected laboratory rabbits consistently from Day 6 and was cleared between 10 and 15 days after infection. Viral antigen was only detected in the contralateral lymph node of wild rabbits infected with Ur at Day 10.

The pathology in the contralateral lymph node of SLS-infected laboratory rabbits was similar to that in the draining node. There was a generalized depletion of lymphocytes from the paracortex, a proliferation of reticular cells, and an influx of polymorphs. This was in contrast to the SLS-infected wild rabbits, which had very minor pathological changes in the contralateral lymph node during infection. Similarly, neither wild nor laboratory rabbits infected with Ur had significant pathological changes in the contralateral nodes, which were well populated with lymphocytes and had active germinal center formation.

A further obvious difference between the infections was the development of germinal centers in the lymph nodes. In Ur-infected rabbits, at 4 days after infection there was a large number of follicles developing into germinal centers, up to 40 per tissue section. This was much greater than for SLS-infected rabbits where a maximum of 8 developing follicles was found. In SLS-infected wild rabbits the formation of germinal centers was limited in the draining lymph node but in the contralateral node germinal center formation was similar although not quite as extensive as it was in the Ur-infected rabbits. Germinal center formation seemed to be inversely related to virus replication as one Ur-infected rabbit had no development of germinal centers in the contralateral node at Day 6 but it had more virus-infected cells than any of the other rabbits.

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**FIG. 4.** Immunofluorescence staining of myxoma virus infection of lymph nodes. (A) SLS infection of laboratory rabbit: draining lymph node Day 2 after infection. Infected cells (arrowed) directly under the subcapsular sinus (S). P, paracortex; C, connective tissue around the lymph node. Scale bar, 50 μm. (B) SLS infection of laboratory rabbit: draining lymph node Day 2 after infection. Lines of infected cells in the paracortex (P). Scale bar, 150 μm. (C) SLS-infected wild rabbit: draining lymph node Day 6. Generalized infection of the paracortex (P). Scale bar, 200 μm. (D) Ur-infected laboratory rabbit: contralateral lymph node Day 4. GC, germinal center. Scale bar, 80 μm. (E) SLS-infected lymphocyte high magnification. Scale bar, 8 μm. (F) SLS-infected laboratory rabbit: Day 4. High magnification of infected cell in lymph node capsule. Scale bar, 20 μm.
Virus replication in the spleen

The spleen was not a major site of myxoma virus replication in any infection as determined by the number of virus-positive cells. Viral antigen was detected within lymphocytes and macrophages in the spleen of laboratory rabbits infected with either SLS or Ur at Day 6 but was not detected at any time point in wild rabbits.

The pathological changes in the spleen resembled those in the lymph nodes but were not as severe. There was a general depletion of lymphocytes and proliferation and degeneration of reticular cells, with a limited influx of...
inflammatory cells. The pathology was most severe in spleens of SLS-infected laboratory rabbits. At 10 days these were depleted of lymphocytes and engorged with red cells so that the red and white pulp were not clearly defined. There was an inflammatory influx of neutrophils and many macrophages contained cellular debris within their cytoplasm. Germinal centers were not defined. In contrast, in spleens of wild rabbits infected with SLS, at the same time point, there was some depletion of lymphocytes and infiltration of neutrophils. However, the red and white pulp were clearly defined and there were well-defined germinal centers. Spleens from laboratory rabbits infected with Ur contained multiple large germinal centers with no evidence of lymphocyte depletion from the white pulp.

Virus replication in distal skin

SLS was first detected by immunofluorescence in distal skin in MHC-II-positive cells of the dermis at 4 days after infection in one of two laboratory and in one of two wild rabbits. At 6 days after infection virus was detected in the distal skin of both laboratory rabbits. The infected cells in the dermis were MHC-II positive and were evenly distributed in the dermis. By 10 days after infection SLS was predominantly infecting cells in the epidermis of laboratory rabbits with few infected cells in the dermis. Pathological changes resembled those in the primary inoculation site at 6 days after infection. There were inflammatory cells, predominantly polymorphs, within the deeper layers of the dermis and some stellate myxoma cells were present.

In Ur-infected laboratory rabbits, virus was first detected in the distal skin at 6 days after infection. In wild rabbits infected with SLS or laboratory rabbits infected with Ur, cells positive for virus were predominantly distributed in the upper dermis and proximal to blood vessels and did not have the uniform distribution in the dermis observed in SLS at the distal skin site or in the other infections at the primary inoculation site. There were also far fewer MHC-II-positive cells. At 10 days after infection SLS was only present in the epidermis in one of three wild rabbits and in the others was restricted to the dermis. At 15 days after infection one of two of the SLS-infected wild rabbits had a secondary lesion at the distal skin site and virus was present in the epidermis of both rabbits. However, it was restricted to short stretches of 30 or so cells. At 20 days after infection two of three SLS-infected wild rabbits had some virus present in the epidermis. In all three rabbits, the dermis contained numerous mononuclear inflammatory cells particularly concentrated at the epidermal/dermal junction and within the deep dermis. Virus was not detected by immunofluorescence in the epidermis of the Ur-infected laboratory or wild rabbits at Days 15 or 20. The most striking difference between laboratory rabbits infected with Ur or wild rabbits infected with SLS and laboratory rabbits infected with SLS was the infiltration of mononuclear cells throughout the dermis particularly by 10 days after infection. This was most prominent in SLS-infected wild rabbits.

Cell death in lymph nodes and spleen

Depletion of lymphocytes was a major feature of SLS infection of lymph nodes of laboratory and wild rabbits. To investigate the role of apoptosis in cell death in the lymph nodes, sections were stained by TUNEL reaction. Large numbers of positively stained cells were evenly distributed across the paracortex of the draining lymph nodes from all myxoma virus-infected rabbits whereas in uninfected control sections apoptotic cells were far fewer and tended to be clustered in germinal centers (Fig. 7). The numbers of apoptotic cells were compared between infections and are shown in Fig. 8. Apoptosis was a major feature
of SLS-infected laboratory rabbits by 4 days after infection and in the other groups by 6 days after infection and was present even when most virus had been cleared from the nodes at Days 15 and 20.

There were only small numbers of apoptotic cells in the spleen compared to the lymph nodes (Fig. 8C). However, in SLS-infected laboratory rabbits by 10 days after infection there were substantially less apoptotic cells in the spleens than in the other groups.

Double staining of sections for apoptosis and virus suggested that the apoptotic cells were not virus infected (data not shown). This was confirmed by examining serial sections stained either for virus or for apoptosis. Apoptotic cells were often adjacent to or close by virus-infected cells but did not contain viral antigen (Figs. 9A and 9B). In SLS-infected laboratory rabbits, virus-infected and apoptotic cells were a feature of germinal centers as well as the paracortical cells (Figs. 9C and 9D). Widespread apoptosis also occurred in the absence of detectable virus-infected cells (Figs. 9E and 9F). An increased number of apoptotic cells in germinal centers was seen in other infections but these were confined to the outer edges of the dark zone and did not involve the entire germinal center.

Sequence analysis of the Serp 2 gene from SLS and Ur

It has been proposed that the serine proteinase inhibitor Serp 2 (myxoma virus gene M151R (Cameron et al., 1999)) encoded by myxoma virus may have a role in preventing lymphocyte apoptosis in lymph nodes (Mes-sud-Petit et al., 1998). These authors observed apoptosis in lymphocytes in lymph nodes from laboratory rabbits infected with Serp 2 knock-out virus but not from rabbits infected with the wild-type T1 French strain of myxoma virus. As we had shown large-scale apoptosis in lymph nodes during infections with both SLS and Ur, this suggested that there may have been sequence differences or even loss of the Serp 2 gene in the SLS virus released into Australia. To examine this, the Serp 2 genes were amplified by PCR from Lausanne (French strains of myxoma virus are derived from Lausanne (Fenner and Ratcliffe, 1965)), SLS, and Ur and sequenced. A complete coding sequence was present for Serp 2 in all three viruses (GenBank Accession Nos.: 325366, 325375, and 325377). There were four nucleotide differences in the sequences we obtained compared to the sequence published by Petit et al. (1996); all of these
would lead to amino acid changes: Thr (72)-Lys; Val (76)-Ala; Asp (127)-Ala; Ile (142)-Phe. However, the sequences we obtained were identical to that published for Serp 2 (M151R) from the Lausanne strain by Cameron et al. (1999). These amino acid changes are not within the C-terminal region of the protein where the active center for serine proteinase binding is believed to be (Petit et al., 1996). Thus while we have not done a functional analysis of the Serp 2 protein from these viruses, based on the sequencing studies we do not believe that the apoptosis in lymph nodes of rabbits infected with either SLS or Ur was due to significant mutations in the Serp 2 gene.

DISCUSSION

Resistance to myxoma virus represents a gradation in clinical and pathological effects which depends on the virulence of the virus and the resistance of the rabbit. In the early stages of infection, SLS in laboratory rabbits is the most virulent, followed by SLS in wild rabbits and then Ur in laboratory rabbits. Ur in wild rabbits is quite innocuous. However, wild rabbits recovered from infection with SLS somewhat earlier than laboratory rabbits infected with Ur. This suggests that resistance cannot be simply modeled by examining the pathogenesis of attenuated virus strains in laboratory rabbits.

There were three important features of myxoma virus pathogenesis observed. First, the virus replicated in cells mediating the antiviral immune response. Initially virus replication occurred in MHC-II-positive dendritic-like cells in the dermis. These are probably dermal dendritic cells and Langerhan's cells, based on their location, morphology, and MHC-II staining, but this has not formally been demonstrated because of a lack of markers for rabbit dendritic cells. These cells are also likely to be responsible for transporting virus to the draining lymph node. Within the lymph node, the virus infected lymphocytes in the T cell zone. These infected lymphocytes may be responsible for transporting virus around the body.

The second feature was the depletion of lymphocytes from the lymph nodes of SLS-infected rabbits and the induction of widespread apoptosis in lymphocytes of all infected rabbits. Infection with Ur did not cause lymphocyte depletion but was still associated with widespread lymphocyte apoptosis.

The third feature was the difference in the inflammatory response between laboratory and wild rabbits and between virulent and attenuated viruses. In laboratory rabbits infected with SLS, the predominant inflammatory cells were polymorphs. However, mononuclear cells comprised a large part of the cellular response in wild rabbits and in Ur infections.

If myxoma virus is replicating within dendritic cells, which are responsible for inducing a large part of the adaptive immune response (Banchereau and Steinman, 1998), this could have several effects. First, it may inhibit antigen presentation. Myxoma virus downregulates MHC-I molecules on the surface of infected cells (Boshkov et al., 1992; Zuniga et al., 1999) and thus may reduce antigen presentation to cytotoxic T lymphocytes (CTLs). In addition, binding proteins that inhibit IFN-γ (Upton et al., 1992), TNF (Upton et al., 1991), and chemokines (Graham et al., 1997; Lalani et al., 1997, 1998) are secreted from myxoma virus-infected cells. These proteins might also prevent the development of a type-1 cytokine milieu at the site of inoculation and within the dendritic cell-lymphocyte microenvironment of the lymph node and thus prevent an effective antiviral immune response developing (Karupiah, 1998). By inhibiting chemokines the virus may prevent the influx of lymphocytes and other antiviral effector cells to the target tissues. Other virus proteins such as the secreted Serp 1 (Macen et al., 1993) and membrane-localized M11L (Graham et al., 1992) would prevent the influx of lymphocytes and other antiviral effector cells to the target tissues. Other virus proteins such as the secreted Serp 1 (Macen et al., 1993) and membrane-localized M11L (Graham et al., 1992)
have also been shown to significantly downregulate the inflammatory response at the inoculation site. Production of these inhibitory mediators of the immune response by virus-infected dendritic cells could inhibit innate and adaptive antiviral immune responses. Infection of dendritic cells in vitro by the orthopoxvirus vaccinia virus downregulated activation and maturation of these cells although vaccinia was unable to productively infect the cells and only early proteins were expressed (Englemayer et al., 1999). Similarly measles virus infection of dendritic cells suppresses both survival and function of these cells (Fugier-Vivier et al., 1997; Grosjean et al., 1997).

Both the virulent SLS and the attenuated Ur replicated in MHC-II-positive dendritic-like cells. Thus cell tropism was not responsible for the attenuated phenotype of the Ur strain. In contrast, tropism for dendritic cells is a function of virulence in mice infected with virulent lymphocytic choriomeningitis virus (Borrow et al., 1995). Similarly SLS replicated in MHC-II-positive dendritic-like
cells in both wild (resistant) and laboratory (susceptible) rabbits. Thus resistance to myxoma virus was not because these cells were resistant to virus infection.

As well as infecting MHC-II-positive cells, myxoma virus infected lymphocytes in the T cell zone of the lymph nodes and virulent virus induced significant depletion of lymphocytes from infected laboratory rabbits. As a model for the depletion of lymphocytes in infected rabbits we suggest that infection of cells with virulent SLS induces lymphocyte death and an ongoing depletion of lymphocytes from the node. There is a failure to repopulate the node. In the attenuated Ur infections, there is an influx of lymphocytes into the node and local proliferation as part of an active immune response; many of these cells undergo apoptosis, however, the influx and proliferation of cells more than compensate for the loss. Infection of wild rabbits with SLS causes ongoing cell death and depletion of lymphocytes but there is also an influx and proliferation of lymphocytes and between 10 and 15 days the influx outweighs the cell death and the nodes are repopulated with cells.

Apoptotic cells were predominantly within the T cell zone of the lymph node, where the virus was replicating, although in SLS infection a considerable amount of apoptosis was also observed within follicles and these follicles also stained positively for virus. As far as we could show, using serial sections and double staining, cells containing viral antigen did not undergo apoptosis but cells adjacent to infected cells did. In addition, apoptotic cells continued to be present in high numbers long after virus-infected cells could no longer be found. Apoptosis in bystander lymphocytes has been described for virus infections such as HIV-1 and SIV-1 both in vitro and in vivo (Finkel et al., 1995; Herbein et al., 1998b) and HHV-6 in vitro (Inoue et al., 1997). Several explanations are possible for the apoptosis observed in myxoma virus-infected lymph nodes. First, cells infected with myxoma virus could be primed to rapidly undergo apoptosis before the expression of late proteins detected by immunostaining. Therefore these cells would not stain positively for myxoma virus antigen. Myxoma virus encodes at least four proteins which prevent apoptosis of infected T cells in vitro (McFadden and Barry, 1998; Nash et al., 1999); however, in vivo there may be significant external signals in the form of cytokines or ligand interactions influencing the cells. Secondly, some antigen-specific cells may be partially activated by presentation of viral antigens by dendritic cells but failure of second signals may induce apoptosis. Thirdly, virus-infected cells may produce a signal or signals which induce apoptosis in neighboring cells in vivo (Herbein et al., 1998a). The continuation of apoptosis in lymph nodes beyond the period when virus could be detected may be due to a very active immune response with many activated or inappropriately activated cells being deleted (Walsh and McNally, 1999).

In resistant rabbits, or infections with attenuated virus, there was still dissemination of virus from the skin and draining lymph node to the distant tissues such as lymph nodes and skin. However, both viral replication as seen by immunofluorescence and tissue pathology were constrained in distal tissues and differences in virus titers were seen as early as 4 days after infection (Best and Kerr, 2000). In particular titers of SLS in the draining lymph node were 10- to 100-fold lower in wild rabbits than laboratory rabbits. Based on a model for resistance to ectromelia virus in mice (Karupiah, 1998), we suggest that infection of resistant rabbits with SLS or susceptible rabbits with Ur leads to a type 1 cytokine response with high IFN-γ levels, an NK cell response, and the development of an appropriate antiviral CTL response. This immune response controls virus replication in both draining lymph node and distal sites but is not sufficient to control virus replication at the primary inoculation site or to prevent virus spreading to distal sites. In contrast, this model suggests that infection of susceptible rabbits with SLS leads to a type 2 immune cytokine response dominated by IL-4 and IL-10 and failure to control the virus.

The inflammatory response in wild rabbits was dominated by mononuclear cells (lymphocytes and monocytes) and occurred close to where the virus was replicating whereas in domestic rabbits infected with SLS it was predominantly neutrophils and was often some distance from where the main virus replication was occurring. The T1 (M001L/R) chemokine-binding protein of myxoma virus inhibits the migration of mononuclear cells in vitro by binding β chemokines but does not inhibit the migration of neutrophils stimulated by α chemokines (Lalani et al., 1998). This raises the possibility that part of the genetic resistance and attenuation phenotype may be due to an enhanced β chemokine response. The recent demonstration that the chemokine receptor proteins may be involved in cell entry by myxoma virus (Lalani et al., 1999) provides a potential mechanism for resistance by which expression of elevated levels of chemokines by wild rabbits could block receptor binding sites.

MATERIALS AND METHODS

Viruses

The Standard Laboratory Strain of myxoma virus (SLS) used in this study was derived from a freeze-dried rabbit tissue stock prepared by Professor Frank Fenner (John Curtin School of Medical Research, Canberra, Australia) in 1953. This was passaged twice in RK13 cells and twice in rabbits. The virus is of grade 1 virulence (Fenner and Marshall, 1957) killing 100% of infected laboratory rabbits with an average survival time of <13 days and this was confirmed prior to this study (Robinson et al., 1999). The Uriarra (Ur) strain of myxoma virus was derived from the Uriarra/2/53-1 isolate (Mykytowycz, 1953) by Russell and
Rabbits were randomly allocated to killing times prior to inoculation and killed by an intravenous overdose of barbiturate. For wild rabbits infected with Ur were killed at each of 12 and 24 h and 2, 4, 6, and 10 days after inoculation. For wild rabbit infections with SLS or Ur, 2 rabbits were killed at 12 and 24 h and 2 and 4 days after infection and 3 rabbits at 6, 10, 15, and 20 days after inoculation. Rabbits were randomly allocated to killing times prior to inoculation and killed by an intravenous overdose of barbiturate.

Rabbit breeding and housing

All animal experiments followed CSIRO/NHMRC guidelines for animal usage and were approved by the CSIRO Wildlife and Ecology animal experimentation ethics committee.

Laboratory rabbits were bred at the CSIRO Wildlife and Ecology animal facility and were an outbred line of predominantly New Zealand White stock. Wild rabbits were bred in floor pens in the same animal facility; breeding stock was derived from rabbits captured as kittens in the Canberra district. The animals used for experiments were one to two generations removed from the wild. There was no selection for resistance to myxomatosis in the breeding facility. Only male rabbits were used in the experiments and all rabbits used were more than 4 months old.

Infections and monitoring of rabbits

Laboratory rabbits were inoculated with 100 PFU of either SLS (12 rabbits) or Ur (16 rabbits) intradermally into the dorsum of the left hind foot. Wild rabbits were inoculated in the same way but 20 rabbits were inoculated with each virus. Two laboratory rabbits infected with SLS were killed at each of 12 and 24 h and 2, 4, 6, and 10 days after inoculation. Two laboratory rabbits infected with Ur were killed at each of 12 and 24 h and 2, 4, 6, 10, 15, and 20 days after inoculation. For wild rabbit infections with SLS or Ur, 2 rabbits were killed at 12 and 24 h and 2 and 4 days after infection and 3 rabbits at 6, 10, 15, and 20 days after inoculation. Rabbits were randomly allocated to killing times prior to inoculation and killed by an intravenous overdose of barbiturate.

Tissue collection and processing

At autopsy the skin inoculation site, the left popliteal lymph node (draining lymph node), right popliteal lymph node (contralateral node), skin from the equivalent site on the right hind foot to the inoculation site (distal skin), and spleen were collected. Tissue portions were fixed in 10% buffered formalin and embedded in paraffin wax for histological processing. Four 5-μm sections were cut from each tissue at intervals of 500 μm. Sections were stained with hematoxylin and eosin. The remainder of each tissue was used to determine virus titers. These titers have been described elsewhere (Best and Kerr, 2000).

Tissue slices for frozen sections were covered in OCT embedding fluid (Tissue Tek), frozen in liquid nitrogen, and stored at −70°C. Cryostat sections were cut at 4–7 μm onto poly-l-lysine-treated slides, fixed in formal-calcium (10% v/v formalin, 10% v/v 1 M CaCl2, 80% v/v H2O), dipped in cold acetone, immersed in chloroform:methaceton (1:1 v/v) for 5 min at −20°C, dipped in cold acetone, washed twice in PBS, and stored at −70°C.

Immunostaining for myxoma virus localization

Frozen tissue sections were blocked overnight in 3% BSA w/v in PBS at room temperature and washed with 1% BSA/PBS. Sections were placed in a humidified container and covered with 1:200 dilution of mouse anti-myxoma virus monoclonal antibody (clone 3B6E4; Fountain et al., 1997) in 1% BSA/PBS. In immunoblots, using concentrated virus as antigen, this antibody binds to an approximately 42-kDa late protein that, in infected RK13 cells, is not expressed at 2 or 4 h after infection but is strongly expressed from 6 h after infection (P. J. Kerr, unpublished). Two controls were used for each section: normal mouse ascites fluid and no primary antibody. Incubation was for 2 h at 37°C followed by three washes with 0.01% v/v Tween 20 in PBS. Goat anti-mouse Ig conjugated to FITC (Boehringer Mannheim), 75 μl of a 1:50 dilution in 1% BSA/PBS was added, and sections were incubated in the dark for 60 min at 37°C. After washing in TWEEN/PBS sections were mounted in Anti-fade (Molecular Probes, OR) and visualized using a confocal microscope. Staining for rabbit MHC-II followed the same protocol using the mouse anti-rabbit monoclonal antibody 2C4 (MHC II R-DQ) at a 1:10 dilution (Serotec, NC) (Robbins, 1989). It was passaged an unknown number of times in CV-1 cells before we obtained it and subsequently was passaged twice in Sirc cells and twice in rabbits. This virus is of grade 5 virulence and over 95% of laboratory rabbits recover from infection but all develop clinically severe myxomatosis (Fountain et al., 1997; Kerr unpublished). The Lausanne (Brazil/Campinas 1949) strain of myxoma virus was derived by two rabbit passages from a commercial stock produced in 1973 by the Commonwealth Serum Laboratories, Melbourne, Australia. Virus stocks of SLS or Ur were prepared as testis homogenates from laboratory rabbits. Titers were determined by plaque assay on Vero cell monolayers.

The Lausanne (Brazil/Campinas 1949) strain of myxoma virus was derived by two rabbit passages from a commercial stock produced in 1973 by the Commonwealth Serum Laboratories, Melbourne, Australia. Virus stocks of SLS or Ur were prepared as testis homogenates from laboratory rabbits. Titers were determined by plaque assay on Vero cell monolayers.
above. For each section used in double staining the section cut immediately before or after it was used for single staining for myxoma virus or MHC-II.

TUNEL reaction

Tissues were fixed in 10% buffered formalin, paraffin embedded, and sectioned onto 0.1% poly-L-lysine-coated slides. The sections were dewaxed with xylene and then passed through a graded ethanol series to water. Sections were then soaked in 2× SSC for 20 min at 80°C soaked in water for 10 min at room temperature, and immersed in prewarmed 0.1 M Tris/EDTA at 42°C for 2 min. Sections were placed in an humidified container and covered with 100 µl of 0.1 mg/ml proteinase K in 0.1 M Tris/EDTA for 10 min at 37°C followed by a rinse in water. Sections were blocked in 1% BSA (w/v) in PBS for 5 min at room temperature and then permeabilized for 2 min at 4°C in 0.1% (w/v) sodium citrate, 1:1000 Triton X-100 followed by two washes with PBS. Sections were returned to the humidified container and covered with 50 µl of terminal transferase reaction mix (31 µl water, 10 µl 5× TdT buffer, 2 µl CoCl₂, 3 µl 1 mM dATP, 2 µl, 1:100 dUTP-FITC, 1 µl TdT (all reagents from Boehringer Mannheim), for 1 h at 37°C. The reaction was stopped by the addition of 50 µl 0.05 M EDTA. Reaction mix without terminal transferase was used as a negative control. Positive controls of rabbit ovary and negative controls of rabbit liver were used in each reaction set. To provide background controls, sections of lymph nodes from 11 individual uninfected rabbits were stained for apoptosis by TUNEL reaction. Sections were mounted using anti-fade and viewed using a confocal microscope. To quantitate the number of apoptotic cells, a grid was superimposed on the X10 field of view and the number of cells stained was counted. Counting was segregated into different zones of the lymphoid tissue. Four fields of view were used for each section of lymph node and five for each section of spleen. Three different sections were used from each tissue for each rabbit. The number of positive cells was expressed as mean +/- standard error and included all counts for a group of infected rabbits killed at the same time point.

Staining sections for myxoma virus and apoptosis

Double staining was done by first staining frozen sections for myxoma virus as described above but using a sheep-anti-mouse Ig rhodamine conjugate. Sections were then processed for the TUNEL reaction as described above but omitting the 2× SSC treatment, proteinase K treatment, and permeabilization treatments as the tissue had already been permeabilized during fixation. To confirm that signal for virus or apoptosis was not being lost during the double labeling, sequential serial sections were stained for either myxoma virus or with the TUNEL reaction.

Imaging and section analysis

For each rabbit at least three sections were examined from every tissue for histology and immunofluorescence staining for virus.

PCR amplification and sequence analysis of the Serp 2 gene

The Serp 2 gene was amplified by polymerase chain reaction (PCR) from RK13 cells infected with either Lu, SLS, or Ur using oligonucleotide primers designed from the published sequence (Petit et al., 1996): 5’-AGAG-TATAATGTATAAGTGCC-3’; 5’-CTAACGGATACGAGGAG-3’ Cloning into pGem T easy (Promega) and sequence analysis by Big Dye terminator cycle sequencing (PE Biosystems, CA) followed the manufacturer’s protocols.

ACKNOWLEDGMENTS

We thank Dr. Peter Janssens and Dr. Tony Robinson for many helpful discussions. Louise Silvers provided technical assistance. Part of this study was funded by a grant from the Foundation for Rabbit Free Australia (formerly the Anti Rabbit Research Foundation of Australia).

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