Reactivation of Persistent Papovavirus K Infection in Immunosuppressed Mice

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Papovavirus K (K virus) is a murine papovavirus that produces a fatal interstitial pneumonia in newborn mice and a clinically inapparent infection in older animals. The present study was conducted to determine whether the virus produces latent infection in animals surviving acute infection and whether the infection can be reactivated by immunosuppression. Mice were inoculated by the oral route with 100 newborn mouse 50% lethal doses at 12 days of age and followed for 8 months by using immunofluorescence staining. Cells positive for K virus capsid antigen were found in lungs, livers, kidneys, intestines, and brains for 6 months, but not thereafter. Organs examined at 8 months were negative for virus by tissue culture assay, mouse inoculation, explantation, and cocultivation. Immunosuppression of the remaining animals with 8 weekly injections of cyclophosphamide (150 mg/kg) resulted in the reappearance of viral antigen and infectious virus in multiple organs including brains. The highest titers of virus were present in kidneys. One animal sacrificed after 42 days of immunosuppression was found to have a small pulmonary adenoma or alveologenic carcinoma, but efforts to explant this tumor into tissue culture were unsuccessful. The present study demonstrates that K virus produces a latent infection that is reactivated by immunosuppression, and our results raise questions as to whether reactivated infection may occasionally be associated with the development of neoplasia.

Papovaviruses are ubiquitous human agents. By age 6, over 60% of children have antibodies to papovaviruses JC and BK (JC and BK viruses, respectively) and by late adulthood the prevalence of antibodies to these viruses has risen to over 70% (3, 4, 24). These agents do not cause clinically obvious illness in immunologically normal patients. Approximately one-third of immunosuppressed patients shed papovaviruses in their urine, however, as do 2 to 3% of pregnant women (8, 13, 19). In rare immunocompromised patients JC virus is associated with a fatal demyelinating infection of the central nervous system, progressive multifocal leukoencephalopathy (PML) (18), and BK virus is associated with severe nephritis or urethritis (7, 20).

Although reactivation of latent papovavirus infection is a common occurrence under conditions of immunosuppression and may result in severe illness or death, little is known about the course or distribution of this reactivated infection, and it is not known whether these agents involve only urinary epithelia or whether reactivation of latent BK or JC virus infection results in more extensive systemic involvement. Similarly little is known about the biological behavior of papovaviruses of other species during persistence or immunosuppression; simian virus 40 and polyomavirus are known to be present in renal tissue of their natural hosts (1, 6, 16, 21), and reactivation of polyoma virus infection in mouse renal tissue is known to occur during pregnancy (16). Detailed studies of persistent or reactivated infection with either agent have not been reported, however. The present study was conducted to determine whether K virus, a murine papovavirus, causes persistent infection in its natural host and whether reactivation of this persistent infection occurs during immunosuppression.

MATERIALS AND METHODS

Virus. K virus obtained from the American Type Culture Collection was inoculated intracranially into newborn outbred Swiss mice. A 10% (wt/vol) extract prepared from

pooled lungs, livers, kidneys, and spleens of moribund infected animals was used as a crude virus stock (9). Purified virus was prepared by banding concentrated suspensions of infected lungs and livers in 1.33 g of CsCl per ml with a Beckman SW65 rotor at 50,000 rpm for 24 h as previously described (9).

Antisera. Antibody to crude virus stock was obtained from adult mice given six weekly intraperitoneal injections of 10⁶ newborn mouse 50% lethal doses of K virus. Antiserum to K virus V antigen was prepared by inoculating rabbits at weekly intervals with UV-inactivated, gradient-purified virus in Freund complete adjuvant until their sera produced bright nuclear fluorescence when used to overlay K virus-infected cells (9, 10).

Inoculation of animals. Pregnant outbred Swiss mice were demonstrated to be free of antibody to K virus. The offspring of these animals were inoculated by the oral route with 10² newborn mouse 50% lethal doses of K virus at 6 to 12 days of age. These animals were initially studied 4, 7, 10, 14, 21, 28, and 56 days after inoculation by immunofluorescent staining, histopathology, and virus assay as previously reported (10). Individual animals were then sacrificed at monthly intervals for 7 months. Sera from these animals were assayed for anti-K virus antibody by hemagglutination inhibition methods, and organs were examined by immunofluorescence staining for the presence of K virus antigen. At 8 months after inoculation 4 animals were sacrificed. Organs from each of these animals were examined for viral antigen by fluorescent antibody methods, and attempts were made to detect persistence of K virus infection with virus assay, animal inoculation, explantation, and cocultivation with mouse embryo cells. Throughout the experiment, all animals were maintained in isolation from other mice or laboratory animals.

Immunosuppression. All animals exhibiting high serum titers of anti-K virus hemagglutination inhibition activity 8 months after inoculation were immunosuppressed with subcutaneous injections of cyclophosphamide (150 mg/kg) given at weekly intervals for 8 weeks. Animals were sacrificed in

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groups of 4 after 7, 14, 21, 28, 42, and 56 days of immunosuppression. Suppression of cell-mediated immune response to viral infection by this regimen of cyclophosphamide was confirmed through experiments similar to those reported by MacFarland et al. (15); these studies demonstrated that cyclophosphamide at this dosage abolished the characteristic lymphocytic response to acute Sindbis virus infection in mice. The effect of this immunosuppressive regimen on humoral immune response was studied in mice given intraperitoneal injections of sheep erythrocytes. Cyclophosphamide, at the dosage employed in the present study, prevented development of antibody response to sheep erythrocytes in immunologically naive animals, but did not cause significant reduction in antibody titers once antibody had developed (Greenlee and Dodd, unpublished observations). Studies by Mokhtarian and Shah (17) and from this laboratory (Greenlee; unpublished observations) have demonstrated that immunosuppression of adult mice with cyclophosphamide at this dosage converts the usually inapparent infection caused by K virus in mature animals into a lethal systemic infection similar to that in newborn mice.

Fluorescent antibody studies. Organs from immunosuppressed animals were studied after 7, 14, 21, 28, 42, and 56 days of immunosuppression. Cryostat sections of infected organs or cover slip cultures of infected mouse embryo cells were fixed in cold acetone and overlaid with K virus antiserum followed by fluorescein-conjugated anti-mouse or anti-rabbit globulin (9). Appropriate organs from uninfected normal or immunosuppressed adult outbred Swiss mice were used as negative controls. Normal mouse or rabbit sera and commercially obtained mouse antipolyoma serum (Microbiological Associates) served as serum controls.

Assay of infected organs. Organs were ground and sonicated at 10% (wt/vol) suspensions in Hanks balanced salt solution. Serial 10-fold dilutions of these suspensions were inoculated onto cover slip cultures of mouse embryo cells as previously described (11). Cover slips were examined by immunofluorescent staining 14 days after inoculation. The infectivity of the suspensions (50% tissue culture infective dose) was calculated as previously described (11) using the final dilution that produced specific fluorescence as the endpoint. Pooled organs from three to five animals were studied 8 months after inoculation before beginning immunosuppression and after 7, 14, 28, and 56 days of immunosuppression.

Attempts to recover virus by animal inoculation. Newborn litters of outbred Swiss mice without antibody to K virus were inoculated intracranially with 10% (wt/vol) suspensions of organs and were observed for 21 days. Two litters were used for pooled suspensions of each organ. Animals were studied 14 and 21 days after inoculation for the presence of hemagglutination inhibition antibody to K virus, and frozen sections of livers and lungs were stained for viral antigen by the indirect fluorescent antibody method.

Explantation and cocultivation studies. Lungs, livers, spleens, kidneys, brains, and intestines were trypsinized and seeded onto cover slips. Trypsinized organs for cocultivation studies were mixed with equal numbers of mouse embryo cells (11) and similarly seeded onto cover slips. A minimum of 12 cover slip cultures of each explanted or cocultivated organ preparation was examined by indirect fluorescent antibody staining for viral antigen 14 and 21 days after inoculation.

Histological studies. Organs fixed in Bouin solution were embedded in glycol methacrylate (JB-4; Polyscience Corp.), sectioned at 2 μ m, and stained with hematoxylin-eosin or a

modified Lendrum stain (9). Organs were examined before immunosuppression and after 7, 21, 28, and 56 days of immunosuppressive therapy. Organs from cyclophosphamide-treated, uninfected adult mice were used as controls.

Hemagglutination determinations. Doubling dilutions of each sample were prepared in phosphate-buffered saline. An equal volume of 0.5% sheep erythrocytes was mixed with each dilution, and samples were incubated at 4°C for 4 h.

Hemagglutination inhibition studies. Sera from individual pregnant mice or pooled sera from animals at each point in the study (four animals at each point) were incubated with receptor-destroying enzyme (Microbiological Associates) and, after the addition of sodium citrate, were heated at 56°C for 30 min. Phosphate-buffered saline was then added to produce a 1:10 dilution of the original serum sample. Doubling dilutions of this material were incubated with 4 hemagglutinating units of K virus for 30 min at room temperature, mixed with an equal volume of a 0.5% suspension of sheep erythrocytes, and incubated at 4°C for 4 h (10).

RESULTS

Clinical observations. Acute K virus infection in these older suckling animals was clinically asymptomatic, as previously described (10). Animals remained healthy throughout the 8-month period of observation before immunosuppression. Several animals in the K virus-infected group died of intercurrent bacterial infections during the period of immunosuppression, but respiratory distress suggestive of K virus pneumonia was not observed, nor did animals develop behavioral abnormalities suggestive of central nervous system involvement. One animal studied after 42 days of immunosuppressive therapy had a small pulmonary nodule. Tumors were not found in other immunosuppressed or control animals.

Fluorescent antibody studies before immunosuppression. Lungs, livers, intestines, and brains continued to exhibit small numbers of positive cells until 4 months after inoculation, and extremely rare positive cells were found in lungs, livers, intestines, and brains 6 months after inoculation, but not at 7 months. All sections examined 8 months after inoculation were negative.

Attempts to detect virus in animals before immunosuppression. Virus assay of suspensions of individual organs in mouse embryo cells was negative. Litters of newborn mice inoculated with these suspensions remained healthy and did not develop antibody to K virus; the lungs and livers of these animals were negative for K virus antigen. Attempts to recover virus by explanation or cocultivation methods were unsuccessful.

Fluorescent antibody studies of immunosuppressed animals. By 7 days after initiation of immunosuppressive therapy rare positive cells were observed along hepatic sinusoids, splenic sinuses, and capillaries of intestinal villi. The numbers of positive cells increased slightly by day 14 of immunosuppression, and at day 14 positive cells could also be identified in lungs, kidneys, and brains. Scattered, positive cells were observed in these organs at days 21, 28, 42, and 56 (Fig. 1 through 3). Fluorescent antibody stains of the lung nodule from the animal taken at day 42 of immunosuppression were negative.

Virus assay of organs during immunosuppressive therapy. Low levels of infectivity ($<10^2$ 50% tissue culture infective doses) were present in all organs by day 7 after initiation of immunosuppressive therapy (Table 1). The levels of virus remained essentially constant in all organs except kidneys, where infectivity titers rose rapidly until day 14 of immuno-

FIG. 1. Fluorescent antibody stain of lung from mouse taken 9 months after inoculation with K virus and after 28 days of immunosuppression. Cells exhibiting fluorescence specific for K virus antigen are present in alveolar walls ($\times 325$).

suppression and fell slowly thereafter. Attempts to grow cells from the lung nodule for virus assay and other studies were unsuccessful.

Histology. Animals examined before the initiation of immunosuppressive therapy were histologically normal. Lungs, livers, brains, and intestines of immunosuppressed animals remained histologically normal, except in animals developing bacterial infections, where typical abscess formation was observed. Spleens showed depletion of lymphoid cells; similar lymphocyte depletion was also present in spleens of uninfected, immunosuppressed control animals. Kidneys from animals examined after 7 and 21 days of immunosuppression contained occasional cells with swollen nuclei within glomeruli and renal tubules, and sections of kidneys after 21, 28, and 56 days of immunosuppression showed parenchymal infiltrates of mononuclear cells. Definite intranuclear inclusions were not found in any organ during the period of immunosuppression. Histological examination of the lung nodule from the animal taken after 42 days of immunosuppression showed it to have a well-developed acinar pattern (Fig. 4). Nuclei were uniform in size, without increased chromatin. Rare mitotic figures were present, but vascular invasion was not observed. The tumor was thus histologically classifiable as an alveologenic carcinoma (23).

Hemagglutination inhibition studies. Animals were negative for hemagglutination inhibition antibody to K virus 7 and 14 days after inoculation. Titers were first detected 21 days after inoculation and rose steadily to a level of 1:10,240 by 2 months after inoculation. The titers were as follows: 7 days, <10 ; 14 days, <10 ; 21 days, 160; 1 month, 2,560; 2 months, 10,240. Titers of pooled sera at 4, 6, 7, and 8 months after inoculation remained 1:10,240. Titers of individual animals, including those taken for fluorescent antibody studies and studies to detect viral infectivity, 8 months after

inoculation and before initiation of immunosuppressive therapy ranged between 1:5,120 and 1:20,480. Titers of anti-K virus hemagglutination inhibition antibody during the period of immunosuppression were 1:5,120 to 1:10,240. The titers were as follows: 0 days, 10,240; 7 days, 5,120; 14 days, 10,240; 21 days, 5,120; 28 days, 10,240; 56 days, 10,240. Thus, although an antibody rise was not observed, previous levels of antiviral antibody were maintained throughout the period of immunosuppression.

DISCUSSION

K virus was initially identified by its ability to produce fatal interstitial pneumonia in newborn mice (14). The virus has subsequently been shown, in older animals, to cause a protracted infection whose distribution is determined by the age and immunological status of the animal at the time of inoculation (10, 17). The present study indicates that K virus persists within its host and can be reactivated by immunosuppression to reappear within multiple organs almost simultaneously. The absence of detectable viral antigen or infectious virus in previously infected animals before immunosuppressive therapy suggests that viral persistence may involve establishment of a latent infection. The continued presence of high titers of antiviral antibody over a period of several months raises questions as to whether this latent infection is absolute or whether lytic infection may continue in cells too few in number and too widely scattered to be detected by the immunofluorescence and virological methods employed in the present study. Although the cell populations involved in K virus persistence and reactivation could not be identified histologically, K virus infects predominantly vascular endothelia during acute infection, and the widespread reappearance of positive cells and viral infectivity during immunosuppression is consistent with reactivation of virus latent within this cell population. Additional experi-

FIG. 2. Fluorescent antibody stain of intestine from animal taken 9 months after inoculation with K virus and after 28 days of immunosuppression, showing a focal area of K virus fluorescence within intestinal villi ($\times 325$).

FIG. 3. Fluorescent antibody stain of section of brain from mouse taken 9 months after inoculation with K virus and after 28 days of immunosuppression. Fluorescence specific for K virus is present in a cell within cerebral parenchyma ($\times 450$).

ments employing in situ hybridization methods and immunoenzymatic staining for early and late viral proteins are planned to identify cell populations involved in latent and productive K virus infection, as are studies to determine whether K virus DNA persists in host cells as episomes or in integrated form.

The limited course of reactivated K virus infection in immunosuppressed mice is strikingly different from that of acute K virus infection in adult mice treated with cyclophosphamide, where the virus produces an overwhelming pneumonia (17; Greenlee, unpublished data). Recovery from K virus infection has been shown to depend heavily on the development of circulating antiviral antibody (10, 17), and the high levels of antibody maintained by the animals in the present study may have played a major role in limiting the spread of infection.

The presence of a lung tumor in one mouse with reactivated K virus infection suggests that K virus may occasionally cause both productive and transforming infectious events within the same organ of its natural host. This possibility is of considerable interest in view of the fact that human PML involves lytic infection of oligodendrocytes and apparent transformation of astrocytes (2). In addition, two cases of PML have been associated with multifocal astroglial tumors whose distribution corresponded to areas of demyelination (4, 22). The ability of K virus to produce cell transformation has been demonstrated in previous studies by Takemoto and Fabisch (25). These workers reported that cells containing viral antigen and exhibiting features consistent with cell transformation can be grown in vitro from lungs of mice dying of K virus pneumonia. In more recent investigations we have found that K virus produces both persistent infection and transformation of mouse glial cells inoculated in vitro (Greenlee et al., *J. Gen. Virol.*, in press). The tumor observed in the present study, however, an alveologenic carcinoma, is a common, spontaneous tumor in both wild and laboratory mice (23), and, in unpublished studies, we have been unable to induce tumors in mice or hamsters with K virus during observation periods as long as 18 months. Unfortunately, explant cultures of the tumor detected in the present study failed to grow, sera containing high titers of antibody against K virus T antibody were not available for

TABLE 1. Infectivity content of organs from mice immunosuppressed 8 months after inoculation with K virus

Days of immunosuppression	Infectivity content ^a the following organs:					
	Lung	Liver	Spleen	Kidney	Gut	Brain
7	$<10^{2.0b}$	$10^{3.5}$	$<10^{2.0}$	$10^{3.2}$	$10^{2.2}$	$10^{2.2}$
14	$10^{2.4}$	$10^{3.3}$	$10^{2.4}$	$>10^{6.0}$	$10^{2.4}$	$10^{2.9}$
28	$10^{3.2}$	$10^{2.1}$		$10^{4.9}$	$10^{2.7}$	$10^{2.2}$
56	$10^{2.0}$	$10^{2.0}$		$10^{2.2}$	$<10^{2.0}$	$10^{2.4}$

^a The data are presented as 50% tissue culture infective dose.

^b Specific fluorescence was identified in cultures inoculated with undiluted material only.

immunofluorescence staining of frozen sections, and the residual portion of the tumor did not contain sufficient material for hybridization studies to determine the presence of the K virus genome. The question of whether the tumor observed in the present study was virus induced thus remains unanswered.

The ability of K virus to involve multiple organs during reactivated infection raises questions as to whether similar widespread involvement might occur during reactivation of JC or BK virus infection in humans. Recent studies applying DNA hybridization methods to autopsy material (5) have shown that JC or BK virus (or both) genetic material can frequently be detected in kidneys, but not brains, of immunologically normal persons. Similar studies by Grinnell et al. (12) have shown that in adult PML patients, JC viral DNA is often present in kidneys, but is not usually detectable in other extraneural organs. These data suggest that latent and reactivated infection with the human papovaviruses may be more limited in distribution than is K virus infection. In one adult PML patient, however, Grinnell et al. detected JC virus DNA in lung tissue, and JC virus DNA has been found in all extraneural organs examined from two children developing PML in the setting of severe combined immunodef-

FIG. 4. Hematoxylin-eosin stain of a lung tumor from an animal examined after 42 days of immunosuppressive therapy ($\times 325$).

ciency (12). These data suggest that the distribution of JC virus during both acute and reactivated infection in humans may, like K virus infection in mice, be determined by the maturity or integrity of host immune defenses at the time of primary infection. K virus infection of mice may thus serve as a useful animal model for the study of papovavirus persistence and reactivation and may provide information applicable to the pathogenesis of PML.

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