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Differential chemokine induction by the mouse adenovirus type-1 in the central nervous system of susceptible and resistant strains of mice

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Mouse adenovirus-type 1 (MAV-1) has recently been shown to cause a fatal hemorrhagic encephalopathy in certain strains of mice whereas other strains are resistant. Morbidity is associated with a productive infection of cerebrovascular endothelial cells, resulting in necrosis of the vasculature, infarction, hemorrhage and death within 4–6 days. Previous studies were not able to define a role for the innate or acquired immune response. In the current study we have addressed the effect of MAV-1 on chemokine and chemokine receptor expression in the central nervous system (CNS) and spleen of susceptible (C57BL/6) and resistant (BALB/c) strains of mice. Intra-peritoneal infection with MAV-1 in C57BL/6 animals resulted in early and prominent induction of IP-10/crg-2 in the spleen and CNS. Increased expression of MCP-1, MIP-1α, MIP-1β and RANTES was also noted in the CNS of MAV-1-infected C57BL/6 animals commencing around 72 h post-infection. In contrast, chemokine expression in BALB/c animals was more restricted with prominent upregulation only of MIP-2 in the CNS. *In situ* hybridization identified the vascular endothelium and CNS glia as the principal site of IP-10/crg-2 production in the C57BL/6 animals. The chemokine receptors CCR1–5 were upregulated in the CNS of both strains of mice. These data show that productive infection of the CNS with MAV-1 leads to the upregulation of a characteristic pattern of chemokines and their receptors, which may point to a role for these factors in disease pathogenesis.

Keywords: adenovirus; encephalopathy; chemokines; chemokine receptors

Introduction

Adenoviruses are nonenveloped, icosahedral double-stranded DNA viruses that have been isolated from a variety of different vertebrate species (Horowitz, 1996). In the mouse, they were first isolated in 1960 as a contaminant of Friend murine leukemia virus cultures and were subsequently found to be endemic in a number of mouse breeding colonies, as well as in wild mouse populations (Smith *et al*, 1986). Molecular characterization of mouse adenoviruses show that they have a genome of approximately 31.5 kb, with an overall genomic organization similar to human adenoviruses. The pathogenesis of mouse adenovirus type 1 (MAV-1) has been studied in mice of various ages, strains, and immunocompetencies. Early data indicated that MAV-1, while capable of infecting mice of any age, only causes morbidity and mortality in neonatal and suckling animals. Susceptibility to disease decreases rapidly to adult levels between 14 and 27 days of age. More recently, however, two reports have demonstrated that, in contrast to the earlier data, MAV-1 can cause significant disease in adult mice of certain strains (including C57BL/6, DBA and CD-1), whereas other strains (such as BALB/c) are resistant (Guida *et al*, 1995; Kring *et al*, 1995). Crosses between susceptible and resistant strains of mice revealed that the F1 progeny display an intermediate susceptibility (Guida *et al*, 1995). Susceptible animals infected with the virus develop a progressive paralysis accompanied by seizures leading to death between the 4th and 6th day after infection. Pathological analysis of the tissues show extensive petechial hemorrhages and associated areas of

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infarction within the central nervous system (CNS), and foci of cytolysis within the spleen. Analysis of viral E1A mRNA expression in tissues from affected mice reveals that the highest levels of MAV-1 E1A expression occurs in the brain and spinal cords with lesser amounts detected in the spleen, lung and heart. In contrast, in tissues taken from resistant BALB/c animals only low levels of MAV-1 E1A expression are detectable, predominantly in the heart (Guida *et al*, 1995).

Immunohistochemical and ultrastructural analyses have shown that the CNS microvasculature is the major site of viral replication within the brain (Kajon *et al*, 1998; Charles *et al*, 1998). Viral infection of CNS endothelial cells leads to a lytic infection with direct damage to the vascular architecture, as well as to secondary damage in the form of thrombus formation at sites distant from areas of active viral replication. In other organs in susceptible strains, MAV-1 also appears to display a predilection for endothelial cells, but infection at those sites is not associated with extensive viral replication or with gross and/or microscopic pathology. In resistant BALB/c mice, little involvement of the brain microvasculature is evident, indicating that in the susceptible strains infection of these cells is necessary and sufficient for the development of the hemorrhagic encephalopathy.

In studies that addressed the role of the host immune response in defining resistance and susceptibility to MAV-1, no obvious role for the innate (NK cells) or the acquired immune response could be defined (Charles *et al*, 1998). Analysis of the course of the disease in scid and *rag*$^{-/-}$ mice shows that the single factor that predicts resistance or susceptibility to disease is whether the mouse is of the BALB/c strain. C57BL/6 and BALB/c mice are known to differ in their genetic tendency to mount either a Th1 (C57BL/6) or a Th2 (BALB/c)-type cytokine response following infection with a number of different pathogenic organisms, but analysis of cytokine gene expression in the CNS of MAV-1 infected C57BL/6 and BALB/c mice failed to show any differences in the nature of the cytokines induced in the two groups of animals.

In addition to cytokines, recent studies have shown that genetic differences in chemokines and/or their receptors may underlie susceptibility or resistance to certain viral infections. Chemokines are low molecular weight secreted proteins that function principally as chemoattractants, mediating the recruitment of specific subsets of leukocytes to sites of inflammation (Baggiolini, 1998). They are subdivided into families that reflect the degree of homology between the different members, with positioning of four conserved cysteines defining the two major groups. The CXC chemokines (α-chemokines) have been shown to be primarily involved in forming a chemotactic gradient for polymorphonuclear cells, and the CC chemokines (β-chemokines) for mononuclear cells. Chemokines may also function as anti-viral agents since certain viruses, such as HIV-1, use chemokine receptors as co-receptors for viral entry (D'Souza and Harden, 1996). To address potential differences in chemokine expression in mice that are resistant or susceptible to MAV-1, we have used a multiprobe ribonuclease protection assay (RPA) to analyze mRNA expression in MAV-1 infected mice. The results show distinct differences in the kinetics and pattern of response of chemokine gene expression in the two groups of animals.

Results

Kinetics of chemokine mRNA expression in the spleen and CNS in BALB/c and C57BL/6 mice

To determine whether infection with MAV-1 leads to the induction of chemokine mRNA expression at sites of viral replication, we used a multiprobe RPA system (PharMingen RiboQuant®, La Jolla, CA, USA) that permits the simultaneous analysis of the chemokines lymphotactin, regulation on activation normal T cell expressed (RANTES), eotaxin, macrophage inflammatory protein 1α and 1β (MIP-1α and MIP-1β), macrophage inflammatory protein-2 (MIP-2), interferon-γ inducible protein-10/cytokine regulated gene-2 (IP-10/crg-2), monocyte chemotactic protein-1 (MCP-1), and T-cell activation-3 (TCA-3). Total RNA was prepared from the CNS and spleen at 0, 12, 24, 48, 72 and 96 h post-i.p. infection with 500 p.f.u. MAV-1, or following mock infection with L929 culture supernatant. Representative autoradiograms from the spleen and CNS for RNA extracted at the 72 h and 96 h time-point for BALB/c and C57BL/6 mice are shown in Figure 1A and B. Cumulative data, expressed as a ratio of the band of interest to the sum of the signals for mL32 and GAPDH, are shown in Figure 2.

In spleen samples from naive, mock, or MAV-1 infected BALB/c or C57BL/6 mice a prominent band for RANTES was detected. Differences between the two strains were noted in that in the spleens from MAV-1 BALB/c mice a prominent signal for MCP-1 mRNA was detected, whereas in the spleens from the infected C57BL/6 animals a prominent signal for IP-10/crg-2 mRNA was observed (Figures 1A and 2A). The relative levels of these chemokines in control tissues did not change at any of the time-points tested (data not shown). In MAV-1 infected BALB/c mice, kinetic studies showed no change in the relative levels of mRNA for the chemokines present in the spleen at 12 and 24 h post-infection (p.i.) (data not shown). However, at 48 h post-infection (p.i.), a marked increase in the levels of MCP-1 mRNA was detected and a weak signal for MIP-1β was first observed (data not shown). At 72 and 96 h post-infection, all of the chemokines showed an increased signal with the most promi-

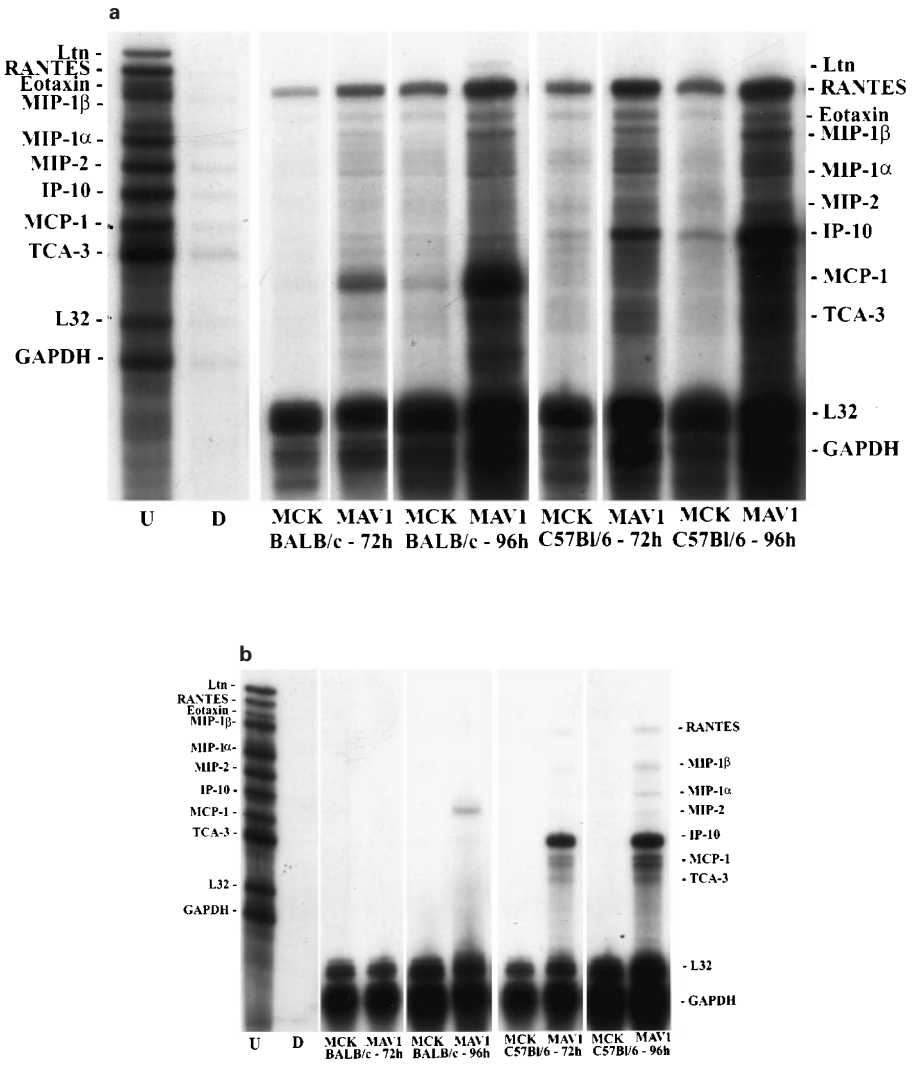

Figure 1 Chemokine mRNA expression in BALB/c and C57BL/6 mice using RPA: Spleens (**A**) and CNS (**B**) were harvested from saline-perfused animals at 72 and 96 h following mock-infection (MCK) or i.p. infection with MAV-1 (MAV1), RNA isolated and an RPA analysis performed using the mCK-5 probe set (PharMingen Riboquant®). The first two lanes on the left are the controls and show the undigested probe (U) and digested, unprotected (D) samples. The position of the protected bands for each chemokine species is indicated on the right. The data for the 72 and 96 h assay for the resistant BALB/c strain are shown in the left hand panels and for the susceptible C57BL/6 strain in the right hand panels. Representative data from one of two experiments are shown.

nent increase noted for MCP-1. In MAV-infected C57BL/6 animals, spleen samples harvested at 12 h p.i. showed a clear increase in the signal for IP-10/crg-2 whereas the levels for all of the other chemokines remained unchanged (data not shown). Between 24 and 48 h p.i. increased expression of IP-10/crg-2 was observed, but no changes were noted in signals for any of the other chemokines at these time-points (data not shown). In the samples harvested at 72 and 96 h p.i. all of the chemokine mRNAs were detected. A marked induction of IP-10/crg-2 was observed, and increases in MCP-1, MIP-1β MIP-1α and MIP-2 were also present (see Figures 1A and 2A).

In the CNS of naive BALB/c or C57BL/6 mice, or mice that had been mock-infected with L929 supernatants, no protected fragments for chemokine mRNAs were observed at any of the time-points tested (see Figure 1B for data at 72 and 96 h p.i. and Figure 2B for the 96 h time-point). Following i.p. infection with MAV-1, CNS samples isolated from BALB/c mice showed no signal for any of the chemokines tested until 96 h p.i. when a clear signal for MIP-2 was observed (Figures 1B and 2A), and a very low signal for IP-10/crg-2. No other chemokines were detected in these samples. In the CNS of MAV-1-infected C57BL/6 mice, trace levels of IP-10/crg-2 were noted at 12 h p.i. after a

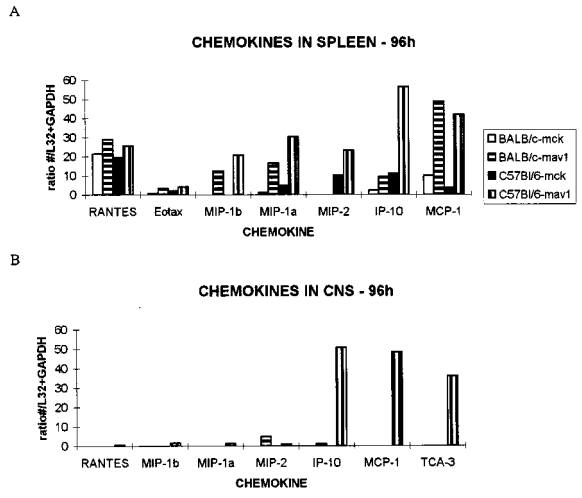

Figure 2 Quantitation of chemokine mRNA expression in BALB/c and C57BL/6 mice at 96 h: the intensity of the signals for the protected bands in the 96 h samples from the spleen (**A**) and CNS (**B**) were determined by phosphoimaging the gels. Data are expressed as a ratio of the band of interest to the sum of the signals for L32 and GAPDH. Pooled data from two experiments are shown. Differences between the samples were less than 10%.

prolonged exposure time and at 48 h p.i. a strong induction of IP-10/crg-2 was observed (data not shown). IP-10/crg-2 expression continued to increase, such that at 72 and 96 h p.i. this chemokine remained the most prominently expressed. MCP-1 and TCA-3 were also increased, but less than IP-10/crg-2. Small amounts of RANTES, MIP-1β, and MIP-1α were also observed (Figures 1B and 2B).

These data show, therefore, that striking differences were observed in chemokine expression both in the spleen and CNS between the susceptible C57BL/6 animals and the resistant BALB/c animals. There was prominent upregulation of IP-10/crg-2 in the spleen and CNS of MAV-1-infected C57BL/6 animals, in contrast to MCP-1 in the spleen and MIP-2 in the CNS of MAV-1-infected BALB/c animals. These data also suggest that these differences reflect an underlying predisposition towards the expression of IP-10/crg-2 in C57BL/6 mice and MCP-1 in BALB/c mice.

Chemokine receptor expression in the spleen and CNS of C57BL/6 and BALB/c mice infected with MAV-1

PharMingen Riboquant® MCR-5 probe set was used to analyze the expression of the chemokine receptors CCR1–CCR5. CCR1 functions as a receptor for RANTES, MIP-1α, MCP-2, MCP-3; CCR2 for MCP-1, 2, 3 and 4; CCR3 for eotaxin, eotaxin-2, RANTES, MCP-2, 3 and 4; CCR4 for TARC, RANTES, MIP-1α and MCP-2; and CCR5 for RANTES, MIP-1α and MIP-1β (Baggiolini, 1998). In spleen cells from naive BALB/c and C57BL/6 animals, all of the CC-chemokine receptors represented on this probe set were observed. Mock-infection with L929 supernatant did not change this expression at any of the time-points tested (Figure 3A and data not shown).

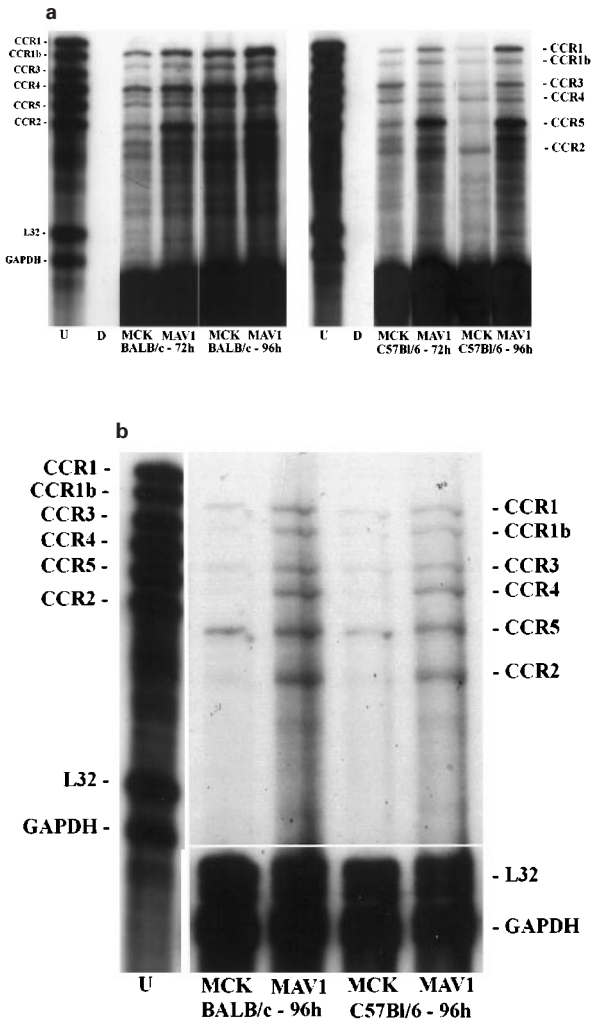

Figure 3 Chemokine receptor mRNA expression in BALB/c and C57BL/6 mice: RNA was extracted from the spleens (**A**) and CNS (**B**) of mock-infected (MCK) or MAV-1 (MAV1) infected animals and the presence of mRNA for chemokine receptors determined at 72 and 96 h for the spleen (**A**) and 96 h for the CNS (**B**) using the mCR5 riboquant probe. The bands for the undigested (U) and the digested probes (D) are as marked. Representative data from one of two experiments are shown.

In the spleens of BALB/c mice infected with MAV-1, there was significant upregulation of CCR1 and CCR5 by 72 h p.i. A similar pattern was noted at 96 h p.i. In the spleen of C57BL/6 animals infected with MAV-1, there was striking upregulation of CCR-5 and a smaller change CCR-1 commencing at 72 h p.i. but increasing through 96 h p.i. Interestingly, in these samples the band for CCR2, which increased at 72 h, had decreased by 96 h and another lower molecular weight band appeared.

These data suggest that there is an alternatively spliced form of CCR2 that is expressed in spleen cells in the MAV-1-infected C57BL/6 mice, and data would be consistent with previous observations of multiply spliced variants of CCR2 associated with activation (Polentarutti *et al*, 1997). In the CNS of normal BALB/c and C57BL/6 animals, only very low levels of mRNA for chemokine receptors were expressed, the most abundant of which was CCR5. Mock infection with L929 supernatant did not change this expression at any of the time-points tested (Figure 3B). Following i.p. infection with MAV-1 in both BALB/c and C57BL/6 mice, no signal for chemokine receptors was noted in the CNS prior to 72 h, when all of the receptors became evident, with CCR-1, CCR-2, CCR-3 and CCR-5 being the most prominently expressed (data not shown). These levels increased at 96 h p.i. when all of the chemokine receptors were induced with equal intensity (Figure 3B). Thus, no striking differences between C57BL/6 and BALB/c animals were noted in the CNS in the expression of chemokine receptors in either naive mice, or in mice infected with MAV-1.

In situ *hybridization for IP-10/crg-2*

In situ hybridization was used to identify the cellular sites of IP-10/crg-2 mRNA production within the CNS of MAV-1 infected BALB/c and C57BL/6 mice. Digoxigenin labeled anti-sense IP-10/crg-2 RNA failed to hybridize with anything within the CNS of uninfected BALB/c, uninfected C57BL/6 mice, or MAV-1 infected BALB/c mice (data not shown). Specific hybridization was observed within cells having the morphology and localization of vascular endothelium in the CNS of MAV-1 infected C57BL/6 mice at 72 and 96 h p.i. (Figure 4A and B). Additionally, signal was detectable in perivascular glial cells in areas of high vascular pathology in the CNS of MAV-1 infected C57BL/6 mice at 96 h p.i. (Figure 4C). Little hybridization was observed using digoxigenin labeled sense RNA within the CNS of MAV-1 infected C57BL/6 mice (Figure 4D), nor was any hybridization observed in uninfected C57BL/6, uninfected BALB/c, nor MAV-1 BALB/c mice at any time point (data not shown).

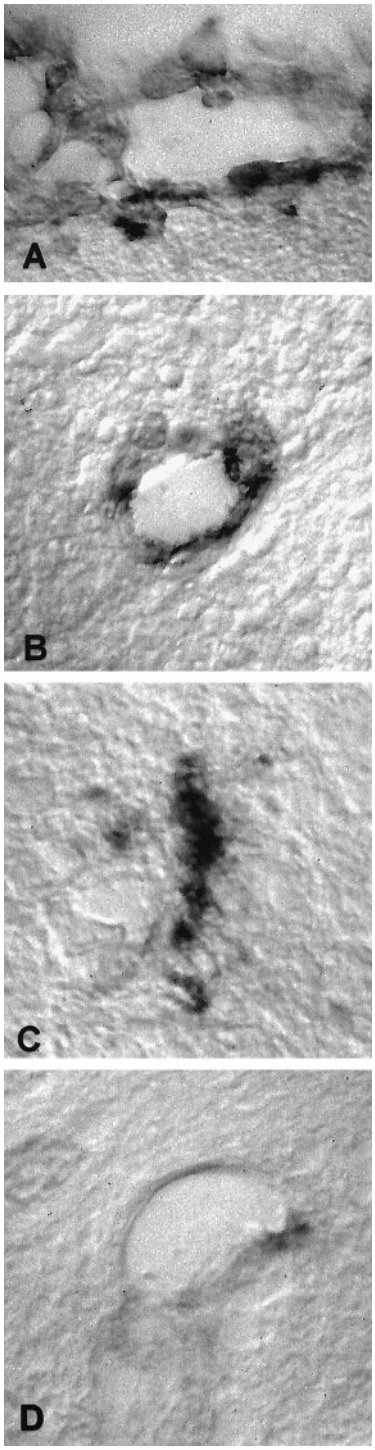

Figure 4 *In situ* hybridization for IP-10/crg-2 in the CNS of MAV-1 infected C57BL/6 mice: Sections of the brain from C57BL/6 mice that had been infected 72 or 96 h previously with MAV-1 were reacted with a digoxigenin-labeled RNA probe complementary to the crg-2 ORF. The sense probe was used as a negative control. 3,3′-Diaminobenzidine was used as the chromagen. In tissues labeled with the antisense probe, the reaction product could be observed associated with the meningeal vessels (**A**), vessels within the parenchyma (**B**) and perivascular glial cells (**C**). Tissues reacted with the sense probe are shown in (**D**). Original magnification × 480.

Discussion

Previous studies from this and another laboratory have shown that adult BALB/c and C57BL/6 mice differ in their susceptibility to MAV-1, with disease course leading to survival in the former and rapid death in the latter (Kajon *et al*, 1998; Charles *et al*, 1998). Mortality was associated with productive infection of cerebrovascular endothelial cells, resulting in direct necrosis of the vasculature and ischemic damage to the CNS parenchyma. No role for the

innate or acquired immune response in defining susceptibility to viral challenge could be identified (Charles *et al*, 1998). In the current study, we have addressed the effect of MAV-1 on chemokine and chemokine receptor expression in the spleen and CNS of adult C57BL/6 and BALB/c animals. The results showed that i.p. infection with MAV-1 in C57BL/6 animals resulted in prominent induction of IP-10/crg-2 in the spleen and CNS, whereas in BALB/c mice prominent induction of MCP-1 was observed in the spleen and MIP-2 in the CNS. Only slight induction of IP-10/crg-2 was detected in either organ of BALB/c mice. These differences in chemokine induction were evident as early as 12 h post-infection and were most marked once the C57BL/6 animals became moribund. *In situ* hybridization identified the vascular endothelium as the principal site of IP-10/crg-2 production in the CNS of C57BL/6 animals.

A central question that arises from these studies is whether the observed differences in chemokine expression simply reflect differences in the host response to a productive, lethal viral infection in one mouse strain (C57BL/6) and a non-fatal viral infection in the other (BALB/c), or whether differential chemokine expression contributes to the pathogenic process. Studies in other systems have suggested that both possibilities could be operative in these animals.

IP-10/crg-2 is a member of the CXC family of chemokines that was first identified as a product of IFNγ-activated macrophages (Luster *et al*, 1985; Vanguri and Farber, 1990). Although IFNγ is the most potent inducer of IP-10/crg-2 in cells of the monocyte/macrophage series, particularly in cells of human origin, IP-10/crg-2 can also be induced by IFNα/β, TNFα, and LPS. It has been suggested that the effects of LPS are mediated through the induction of interferons, which then activate the interferon-stimulated response element (ISRE) in the IP-10/crg-2 promoter. In contrast to most members of the CXC family of chemokines, IP-10/crg-2 is selectively chemotactic for activated memory T cells, but has no effect on resting T cells. This selectivity is mediated through interaction with the CXCR3 chemokine receptor, which is expressed almost exclusively by effector T cells (Qin *et al*, 1998; Loetscher *et al*, 1996).

The induction of IP-10/crg-2 in the CNS has been noted in a number of different infectious and inflammatory disorders. In monkeys infected with simian immunodeficiency virus, IP-10/crg-2 was prominently induced in the CNS of animals with encephalitis (Sasseville *et al*, 1996). During the course of lymphocytic choriomeningitis virus (LCMV) in mice, IP-10/crg-2 was expressed early and abundantly in the CNS of affected animals, with expression localized primarily to resident cells of the CNS (Asensio and Campbell, 1997). LCMV infection of mice in which the gene for IFNγ had been inactivated reduced this expression, and none was observed in LCMV-infected nude mice, indicating that viral infection *per se* was not sufficient to stimulate expression. Conversely, Newcastle disease virus (NDV) has been shown to stimulate the upregulation of IP-10/crg-2 mRNA with kinetics consistent with direct viral modulation (Vanguri and Farber, 1994). This effect was found to be independent of viral replication, as upregulation was observed even upon treatment with inactivated virus. Studies in patients with viral meningitis also strongly implicated IP-10/crg-2, along with MCP-1, as major mediators of extravasation of activated T cells and monocytes into the CSF compartment (Lahrtz *et al*, 1997). Strong induction of IP-10/crg-2 has also been noted in the CNS of animals sensitized to develop experimental autoimmune-encephalomyelitis (EAE), a model for multiple sclerosis (Ransohoff *et al*, 1993). A role for this chemokine in disease progression was suggested following the observation that clinical severity of disease was reduced in animals in which phosphodiester antisense oligonucleotides against *crg-2* mRNA were injected intracerebrally (Wojcik *et al*, 1996). Production of IP-10/crg-2 in EAE was localized predominantly to astrocytes (Ransohoff *et al*, 1993). *In vitro* studies have shown that IFNγ is a potent inducer of IP-10/crg-2 in astrocytes and microglia (Vanguri and Farber, 1994). All of these studies implicate IP-10/crg-2 expression as an early response gene to viral infection or inflammation in the CNS.

In addition to its chemotactic activity, IP-10/crg-2 promotes the adhesion of activated lymphocytes to endothelial cells (Taub *et al*, 1993), antagonizes the effects of the growth factors rSCF and IGF-II for hematopoietic progenitors, and inhibits recombinant basic FGF-induced angiogenesis (Angiolillo *et al*, 1995). It has been suggested that these inhibitory effects of IP-10/crg-2 on hematopoiesis and angiogenesis may be mediated by blocking or altering growth-factor interactions with glycosaminoglycans (Luster *et al*, 1995). IP-10/crg-2 has also been shown to be involved in a variety of models of vascular pathology. IP-10/crg-2 is upregulated following ischemia-associated with vascular thrombosis, and is potently induced by balloon angioplasty (Wang *et al*, 1996). It is upregulated during the course of experimental atherosclerosis and is present in human aortic atherosclerotic lesions. IP-10/crg-2 also appears to be able to inhibit tumor cell growth by effects on the vasculature that result in ischemic necrosis and thrombosis (Strieter *et al*, 1995), as well as by enhancing NK-cell-mediated killing of tumor cell targets (Taub *et al*, 1995). Thus, IP-10/crg-2 appears to have direct effects on endothelial cells that are distinct from its effects as a chemotactic factor, and could, therefore, play a direct role in the induction of the cerebral vascular necrosis and thrombosis observed in the MAV-1-infected C57BL/6 mice.

Our observations of MAV-1-induced IP-10/crg-2 upregulation may, therefore, be explained by three possible mechanisms: (1) MAV-1 infection of vascular endothelial cells may directly stimulate the expression of IP-10/crg-2, similar to what has been noted for NDV (Vanguri and Farber, 1994); (2) type I interferons in response to viral infection may lead to IP-10/crg-2 induction in cells adjacent to MAV-1 infected cells, and (3) damage to the vascular endothelial cells secondary to MAV-1 infection (necrosis, thrombosis, or ischemia) may lead to IP-10/crg-2 induction. The kinetics of IP-10/crg-2 of MAV-1-infected mice implicate all three mechanisms, with early IP-10/crg-2 expression being mediated by the first and second mechanisms, and the late, high-level expression by the third.

Increased expression of MCP-1, MIP-1α, MIP-1β, and RANTES was also noted in the CNS of MAV-1-infected C57BL/6 animals. MCP-1 is a potent chemokine and activating factor for cells of the monocyte/macrophage series and would thus be expected to target the endogenous microglia of the CNS and blood-borne macrophages. The activation and recruitment of these two cell types in response to tissue damage within the CNS is thought to reflect the nature and extent of the injury. When tissue damage is relatively restricted and is present at the cellular level, then activation of microglia is the characteristic response. However, when tissue damage is more extensive and involves disruption of the blood-brain barrier, influx of circulating monocytes occurs. This differential response has come to be known as the 'graded response' hypothesis (Dickson *et al*, 1991). Although mononuclear cell infiltration of the CNS was not a prominent feature of the MAV-1-induced encephalopathy in the C57BL/6 mice, we did note a marked and widespread activation of microglia (Charles *et al*, 1998). Microglia also acquired a 'streaming' morphology, suggesting active migration of these cells through the CNS parenchyma. An increased activation state of microglia was further supported in this study by the observation that the chemokine receptor CCR-2, which is the receptor for MCP-1, was upregulated in the CNS at 96 h p.i. MCP-1 was originally cloned from a human glioma cell line (Kuratsu *et al*, 1989), but is also known to be secreted by activated macrophages, smooth muscle cells, astrocytes and endothelial cells. MIP-1α and MIP-1β are also products of activated macrophages/microglia and function to attract different populations of T cells. MIP-1α is also a potent chemoattractant for NK cells. Although we did not observe many T cells in these lesions, the presence of RANTES, as well as cytokines such as IFNγ (Charles *et al*, 1998), would be indicative of some lymphocyte entry into the CNS compartment.

One interesting aspect of MAV-1 disease in mice is the relatively minor accumulation of inflammatory cells in the damaged, and virally infected areas of the CNS. While the time course of the disease is rapid, the fact that detectable levels of the IP-10 were present within 1 day after infection would lead one to expect a substantial infiltration of mononuclear cells by the fourth and final day of the disease. The fact that this does not occur to any great extent implies a defect in the ability of IP-10 to recruit this population of cells in the setting of MAV-1 disease in the susceptible C57BL/6 mouse. Chemotaxis, however, is dependent upon the establishment of an appropriate gradient for sensitive cells to follow, a process that may be impossible if the chemokine is being secreted directly into the blood stream from vascular endothelial cells. In at least one other model system, secretion of IL-8 by vascular endothelium was shown to inhibit chemotaxis for precisely this reason (Hechtman *et al*, 1991).

In contrast to the data obtained in the C57BL/6 mice, upregulation of chemokines in the CNS of BALB/c mice was restricted to MIP-2. MIP-2 is a chemoattractant for neutrophils and injection of recombinant MIP-2 into the CNS initiates a florid polymorphonuclear response (Bell *et al*, 1996). Expression of MIP-2 would be indicative of some type of traumatic insult to the CNS in these animals. Viral recovery studies with MAV-1 indicated that essentially no infectious virus was detectable in the brain or spinal cord of BALB/c mice; however, a low level of expression of E1A message was found by 96 h p.i.(Guida *et al*, 1995). Thus the induction of MIP-2 could represent an early response to viral replication. In this regard it is interesting to note that transient expression of MIP-2 was noted in the CNS of C57BL/6 animals 48 h p.i. In the spleen there was upregulation of most chemokines, with marked upregulation of MCP-1, MIP-1α and MIP-1β. As noted above, MCP-1 acts as chemoattractant for monocytes, but is also a potent degranulating agent for mast cells and an activating factor for macrophages and could, therefore, play a role in viral clearance. MIP-1α and MIP-1β are chemoattractants for activated T cells as well as NK cells and could, therefore, also contribute to host defense in this mouse strain. Data from the scid mouse has shown that the host immune response in BALB/c mice is necessary for prevention of disseminated systemic disease (Charles *et al*, 1998).

An unexpected finding that emanated from these studies was the observation in normal naive animals that the levels of mRNA for IP-10/crg-2 were greater than those for MCP-1 in the spleens of C57BL/6 mice whereas in the spleens of the BALB/c mice the reverse was observed. Infection with MAV-1 then appeared to selectively upregulate IP-10/crg-2 or MCP-1 in these two strains of mice. The fact that IP-10/crg-2 can be detected in normal spleen tissues, where it was localized to stromal cells, has been noted previously (Amichay *et al*, 1996; Gattass *et al*, 1994). It has been speculated that a role for

constitutive expression of certain chemokines could be the establishment and maintenance of groups of cells that form functionally distinct units (Baggiolini, 1998), but how IP-10/crg-2 might function in this regard is not known at the present time. It is possible, therefore, that a predisposition towards the expression of different types of chemokines may in some way affect the ability of MAV-1 to infect these two strains of mice. A significant role for chemokines and chemokine receptors in the ability of HIV-1 to infect different types of cells has now been well established, but at the present time little is known about the receptor or mechanism of entry for MAV-1. In humans, a common receptor for Coxsackie B and the fibers of adenoviruses 2 and 5 has recently been identified (Bergelson *et al*, 1997), but it is known that adenoviral entry is also facilitated by interactions between the viral penton base protein and cell surface integrins (Wickham *et al*, 1995). Chemokines bind to cell surface integrins and could, therefore, influence the nature of the extracellular matrix in different organs. With the recent availability of targeted gene mutations in chemokines and chemokine receptors that have been backcrossed into specific strains of mice, it should be possible to define more precisely in future experiments the role of these molecules in the pathobiology of MAV-1 encephalopathy.

Materials and methods

Cells and virus

Murine L929 (L cells) were obtained from the American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 μg streptomycin and 50 U penicillin per ml. For production of virus, the concentration of FCS was reduced to 3%. MAV-1 was grown in L cells, and titer was determined by plaque assay on L cells as previously described (Guida *et al*, 1995). Purified virus was prepared by infection of L cells in suspension culture at a MOI of 0.01. After 5 days in culture, cells were pelleted and virus liberated from cells by freeze/thaw and Freon extraction. Virus was purified from the aqueous phase of the Freon extraction by two rounds of equilibrium centrifugation in continuous CsCl gradients. Purified virus was stored at -70°C in 50% glycerol. Extracts of uninfected L929 cells were prepared in parallel.

Mice

Five- or six-week-old female C57BL/6 and BALB/c mice were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). Mice were maintained in microisolator cages with food and water *ad libitum* on a 12 h light/dark cycle. All animal experimentation was performed in accordance with NIH guidelines, and animal protocols were approved by the animal care and use committee of the Albert Einstein College of Medicine.

Viral infections

Mice were inoculated by intraperitoneal injection of 5×10^3 p.f.u. of MAV-1 (strain FL, ATCC) or mock-infected with an equal volume of L929 cell extract. At various time-points after infection, mice were deeply anesthetized with sodium pentobarbital (Nembutal, Abbot Laboratories, North Chicago, IL, USA), and perfused transcardially with phosphate buffered saline (PBS). Tissues were immersion fixed in Trump's fixative and embedded in paraffin for light microscopy and *in situ* hybridization, or homogenized in Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) for ribonuclease protection assay. For each time-point in each experiment, two normal naive animals, two animals that had been mock-infected with L929 supernatant and two MAV-1 infected animals were studied.

Ribonuclease protection assay

Ribonuclease protection assay (RPA) using Riboquant (PharMingen, Burlingame, CA, USA) probe sets and the RPA II RPA kit (Ambion, Austin, TX, USA) were performed essentially as described (Hobbs *et al*, 1993). Briefly, ^{32}P-UTP-labeled anti-sense RNA transcripts for chemokines, and chemokine receptors were generated using Riboquant probe template sets (mCK-5 and mCR-5) and T7 RNA polymerase. Hybridizations were performed according to the manufacturer's instructions using 20 μg total RNA from each sample for 20 h at 43°C. Single-stranded RNA was digested with an RN'ase A/T1 mixture, and the hybrids were analyzed on 8% denaturing urea/polyacrylamide gels. Protected fragments were visualized by autoradiography, and quantitated by phosphoimaging using a Storm 860 scanner and the ImageQuant v3.01 software package (Molecular Dynamics, San Francisco, CA, USA).

In situ *hybridization*

The probe for *crg-2* was kindly supplied by Dr I Campbell (The Scripps Research Institute, La Jolla, CA, USA) and tissues were prepared for *in situ* hybridization essentially as described (Charles *et al*, 1997). *In vitro* Sp6 (sense) or T7 (anti-sense) polymerase transcription reactions containing digoxigenin-11-UTP (Boehringer-Mannheim) produced an 871 nt digoxigenin-labeled RNA probe complementary to the crg-2 ORF. The sense probe was used as a negative control. Probes were hybridized to 3 μm paraffin sections that had been mounted on aminopropyl triethoxysilane-coated slides (ProbeOn Plus, Fisher Scientific). Hybridization and wash conditions were as previously described (37). Slides were reacted with a horseradish peroxidase-labeled sheep anti-digoxigenin

Fab fragment, and hybridization was detected by reaction with diaminobenzidine as substrate (Pierce, Rockford, IL, USA).

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