Expression of Defective Virus and Cytokine Genes in Murine AIDS

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A syndrome characterized by severe immunodeficiency and lymphoproliferation develops in susceptible strains of mice infected with a mixture of murine leukemia viruses (MuLVs) designated LP-BM5 MuLV. The etiologic agent in this mixture has been shown to be a replication-defective virus (BM5d) with a 4.8-kb genome that required replication-competent helper viruses, primarily ecotropic (BM5e), for cell-to-cell spread in the host. In the present study, we studied the expression of BM5d and BM5e in tissues of infected mice at various times after inoculation in relation to the expression of cytokine genes that may contribute to the pathogenesis of this disorder. Northern (RNA) analysis of total RNA showed that BM5d was expressed at significant levels in lymphoid tissues within 1 week of infection and that the levels of expression increased with time after inoculation. By 16 weeks postinfection, BM5d was expressed in all tissues examined. Expression of BM5e was relatively more restricted to lymphoid tissues and was detected at lower levels than expression of BM5d at early times after infection, but this virus was expressed in all tissues by 16 weeks. Infection with the virus mixture was associated with constitutive expression of tumor necrosis factor in all tissues examined and of interleukin-1 (IL-1) in lymphoid tissues within 1 week of infection, and at later times with widespread expression of these cytokines and gamma interferon. Also, the levels of interferon regulatory factor 1 mRNA were significantly increased in all infected tissues during the infection. In contrast, expression of IL-3, IL-4, IL-5, and IL-6 was not detectable by Northern analysis of the respective mRNAs in any infected tissue at early or late times postinfection.

C57BL/6 mice inoculated with the mixture of murine leukemia viruses (MuLVs) called LP-BM5 MuLV (ecotropic [eco], mink cell focus-forming (MCF), and defective viruses) develop an immunodeficiency syndrome which in many ways resembles that seen in humans infected with human immunodeficiency virus type 1 (HIV-1) (9, 19). The progression of the disease is characterized by an early polyclonal proliferation of T and B cells, hypergammaglobulinemia, enhanced expression of gamma interferon (IFN- γ), and finally, the development of B-cell lymphoma (20, 22). Since many of these symptoms, including the development of B-cell lymphoma, are dependent on the presence of functioning B and T cells (4, 20), it is likely that overproduction or dysregulated synthesis of certain cytokines is responsible for some of these changes.

Recent studies have identified a replication-defective virus as the etiologic agent of this murine AIDS (MAIDS) (2, 7). Molecular cloning and sequencing of the defective virus (BM5d and Du5H MuLV isolates) showed that its genome contain a conserved *gag* region with a novel sequence encoding the p12 protein, while major portions of the genes for *pol* and *env* are deleted (4b, 11). By analogy with the other defective viruses in the MuLV system, it can be expected that BM5e and MCF viruses function as helpers for the spread of BM5d virus, although Huang et al. (11) have recently shown that mice infected by the Du5H defective virus in the absence of helper virus also developed MAIDS.

The current studies were undertaken to analyze the expression and distribution of BM5d and BM5e in different organs of infected mice at early and late times postinfection. To determine whether infection with LP-BM5 MuLV leads

to the induction of expression of cytokine genes, we examined the expression of a number of cytokine genes in tissues of infected mice. The results of this study show that BM5d was expressed at high levels in tissues of infected mice at 1 week after inoculation and that expression increased with time. Infection with the mixed virus preparation resulted in constitutive expression of interleukin-1 (IL-1), tumor necrosis factor (TNF), and IFN- γ , whereas expression of IL-3, IL-4, IL-5, and IL-6 was not observed at either early or late times postinfection.

MATERIALS AND METHODS

Mice and viruses. C57BL/6 mice were obtained from Jackson Laboratory, Bar Harbor, Maine. Virus stocks of the LP-BM5 MuLV mixture were prepared as cell-free supernatants from the G6 clone of SC-1 cells as described previously (20). Mice were inoculated intraperitoneally with 0.1 ml of the virus stock, and virus expression was determined at 1, 3, or 16 weeks postinoculation. The virus stock contained a mixture of LP-BM5 eco (BM5e), MCF, and replication-defective (BM5d) viruses (7). The LP-BM5 pool contained $10^{5.0}$ PFU of BM5e and $10^{1.0}$ focus-forming units of MCF MuLV per 0.2 ml. Control mice were age-matched littermates. Generally, groups of three to six mice were used at each time point for analysis.

Preparation and analysis of RNA. Organs were excised from mice and frozen immediately in liquid nitrogen, and total RNA from the frozen tissues was processed as described previously (22). Each RNA preparation was prepared from a pool of organs from three to six mice. RNA (10 or 15 μ g) was electrophoresed through 0.8% agarose-formaldehyde gels and transferred onto nitrocellulose membranes. The membranes were prehybridized and hybridized with either ³²P-labeled riboprobes or DNA probes, and the filters were washed as described previously (24).

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Probes. The RNA probes for murine IFN- α , IFN- β , and IFN- γ were prepared as described previously (22). The murine IFN regulatory factor 1 (IRF-1) probe was constructed by inserting the 1,024-bp *Xba*I-*Pvu*II fragment of the pCMIRS plasmid (17) into the Sp64 vector; TNF, IL-1 β , and IL-6 probes were described recently (13, 14). The murine IL-2 probe was prepared by inserting the 0.6-kb cDNA clone into the pGEM1 vector. The IL-3 probe represented a 400-bp *Xba*I-*Hind*III fragment of murine IL-3 cDNA cloned into pSP64 vector; the IL-4 riboprobe was prepared by inserting a 373-bp *Hinc*II fragment of murine IL-4 cDNA into the pGEM3 vector. Murine IL-5 was represented by a 1.7-kb full-length IL-5 cDNA cloned into the *Bam*HI site of pBR322. The IL-3, IL-4, IL-5, and IL-6 probes used were able to detect the respective mRNAs in D10 cells that produce these interleukins constitutively. The eco-specific probe (6) and MCF-xenotropic (xeno) virus-specific probes were described before (5, 6); the defective virus-specific probe was represented by a 100-bp sequence from the *gag* p12 region of BM5d (4a). This fragment was cloned into the pGEM3 vector by using *Sal*I and *Xba*I sites and subsequently used as the template in randomly primed transcription. This probe was similar to the Du30 probe used by Aziz et al. (2).

RESULTS

Detection of replication-defective and eco LP-BM5 viruses in organs of infected mice.

To determine how development of MAIDS relates to expression of BM5d in various organs of diseased mice, we analyzed the levels of viral RNA encoded by the BM5d genome. RNA extracted from various organs of mice at 1, 3, and 16 weeks postinfection was analyzed by Northern (RNA) hybridization for the presence of the mRNA species corresponding to the transcript of the defective virus (2). The results showed (Fig. 1) that the 4.8-kb transcript was readily detected in spleen, lymph nodes, thymus, and kidneys by 1 week postinfection. The levels of the transcripts in these tissues increased with time, and by 3 weeks postinfection, the defective virus could be detected in lung as well. Sixteen weeks after infection, transcripts of BM5d virus could be detected in all organs examined, including the liver (on prolonged exposure). In addition to the major 4.8-kb transcript, the probe was seen to react with the 8.0- and 5.6-kb RNA species upon prolonged exposure. This most likely reflects cross-reactivity of the probe with transcripts from endogenous viral sequences, since identical weakly hybridizing 8.0-kb and strongly hybridizing 5.6-kb transcripts could be seen, after a long exposure, in RNA isolated from spleen and lymph nodes of uninfected mice hybridized with either BM5d or MCF/xeno virus-specific probe (Fig. 2). However, neither 4.8-kb mRNA nor ecovirus-specific transcripts could be detected in RNA from uninfected mice (Fig. 2).

Our results further show that the relative levels of the 4.8-kb transcripts in different organs changed with the time after infection. At 1 and 3 weeks postinfection, the levels of 4.8-kb message were substantially higher in both spleen and lymph nodes than in thymus. At 16 weeks, however, the highest levels of BM5d mRNA were found in thymus. Note that at 16 weeks postinfection, the tissue recovered from the mediastinum contains a mixture of residual thymus and adjacent lymph nodes and that the thymus recovered from mice infected with the LP-BM5 virus mixture is often heavily infiltrated by B-lineage cells of immunoblastic and plasmacytoid morphologies (10). Note that in other studies

FIG. 1. Expression of a BM5d virus in organs of mice infected with LP-BM5 MuLV. RNA isolated from various tissues at different times postinfection was analyzed by Northern blot analysis with the 32 P-labeled defective virus-specific probe as described in Materials and Methods. Each lane contained 15 μ g of a total RNA. Lanes: 1, spleen; 2, lung; 3, liver; 4, lymph nodes; 5, thymus, and 6, kidney. (a) One week postinfection with LP-BM5 MuLV (6-h exposure); (b) 3 weeks postinfection (6-h exposure); (c) 16 weeks postinfection (30-min exposure). The membranes were also rehybridized with β -actin; a representative result is shown (bottom).

of mRNA from tissues of mice infected for 1 to 5 weeks, transcripts of BM5d were not readily detectable in kidney samples but, consistent with the data in Fig. 1, were abundant in preparations from spleen (data not shown).

While mice infected with BM5e alone or with BM5e and MCF viruses do not develop MAIDS (28), cell-to-cell spread of the BM5d genome requires the presence of replication-competent MuLV. To evaluate the tissue distribution of BM5e expression in relation to expression of BM5d, we analyzed RNA prepared from tissues at various times after infection by Northern hybridization with an eco virus-specific probe (Fig. 3). At 1 week postinfection, faint bands corresponding to genomic and *env* transcripts of BM5e were detectable in RNA from spleen and lymph nodes only on prolonged exposures, and no infectious virus (less than 100 PFU/10⁷ cells) could be recovered from these tissues. By 3 weeks, BM5e transcripts were readily detectable in RNA prepared from spleen, lymph nodes, and thymus, with higher levels present in thymus than in spleen or lymph nodes. At 16 weeks postinfection, eco viral transcripts were detected

FIG. 2. Hybridization of RNA isolated from organs of uninfected C57BL/6 mice with *eco*, MCF/xeno, and BM5d (De) virus probes. RNA was isolated from different organs of C57BL/6 mice, and 15 μ g was analyzed by Northern hybridization with *eco*, MCF/xeno, and BM5d virus 32 P-labeled probes. Lanes: 1, spleen; 2, lung; 3, liver.

in all tissues examined, including the liver (on prolonged exposures).

These results indicated that both the BM5d and BM5e viruses were expressed in lymphoid tissues soon after infection and that the progression of disease, which culminates with the development of B-cell lymphoma at about 16 weeks after the infection, was associated with increased transcription of the BM5d genome in lymphoid tissues. The quantitative difference found in the expression of BM5d and BM5e at the early stages of infection in different tissues suggests that the mechanisms controlling the transcription of these two viruses are not identical despite the fact that sequences in their long terminal repeats are almost identical (4b).

Alterations in expression of cytokine genes. In previous studies, we showed that infection of mice with LP-BM5 MuLV markedly affected the expression of IFN genes and IL-2 (18, 22). Induction of IFN- α and - β expression by inoculation of infected mice with Newcastle disease virus was completely inhibited, while IFN- γ was expressed constitutively soon after infection with the mixed LP-BM5 virus preparation (22). In addition, the ability of spleen cells to produce IL-2 in response to mitogenic and antigenic stimuli was progressively impaired with time after infection (18). We extended these studies to evaluate the effects of infection on expression of IL-1 β , IL-3, IL-4, IL-5, IL-6, and TNF.

Analyses of total RNA prepared from various tissues showed that none of these genes were expressed constitutively in tissues from uninfected mice (data not shown) but that at 1 week postinfection, transcripts for IL-1 β were present in spleen and lymph nodes and that TNF mRNA could be detected in all tissues examined (Fig. 4A). At later times after infection (3 and 16 weeks), expression of IL-1 β among the tissues was more widespread (Fig. 4B and 5, respectively). In addition, constitutive expression of IFN- γ was detected in spleen at 5 weeks postinfection (data not shown) and in most tissues at 16 weeks (Fig. 5). In contrast, transcripts for IL-3, IL-4, IL-5, and IL-6 were not detected in total RNA prepared from infected mice at times ranging from 1 to 16 weeks postinfection (data not shown).

Studies of IRF-1 expression. In further studies, we evaluated the expression of IRF-1, a factor that was shown to play a role in the inducible expression of IFN- α and - β (8, 16), to determine whether alterations in the regulated expression of this gene could be related to the impaired expression of IFN- α and - β observed in mice infected with the LP-BM5

FIG. 3. Expression of nonpathogenic BM5e virus in organs of mice infected with LP-BM5 MuLV. RNA (15 μ g) identical to that analyzed in Fig. 1 was hybridized with *eco* virus-specific probe. Lanes: 1, spleen; 2, lung; 3, liver; 4, lymph nodes; 5, thymus; and 6, kidney. (a) One week postinfection with LP-BM5 MuLV (26-day exposure); (b) 3 weeks postinfection (2-day exposure); (c) 16 weeks postinfection (8-h exposure). The same membranes were stripped and hybridized with β -actin; a representative result is shown (bottom).

virus mixture (22). While low levels of IRF-1 mRNA were detected in tissues of normal mice (data not shown), the levels of IRF-1 mRNA in spleen, lung, lymph nodes, and thymus were significantly increased at 1 week postinfection with LP-BM5 MuLV and remained increased during the infection (Fig. 3B and 4).

DISCUSSION

The results presented in this work demonstrate that infection of adult C57BL/6 mice with LP-BM5 MuLV results in rapid expression of the BM5d virus in lymphoid organs. The 4.8-kb transcript coded by the BM5d virus could be detected in spleen, lymph nodes, and thymus as early as 1 week postinfection, and the levels of this RNA increased with time postinoculation (Fig. 1), suggesting that expression of the defective virus is directly related to the progression of disease. Analyses of the distribution of viral transcripts showed that spleen and lymph nodes were primary sites of BM5d virus expression soon after infection but that with

FIG. 4. Detection of IL-1 β , TNF, and IRF-1 mRNAs in organs of mice infected with LP-BM5 MuLV. RNA (15 μ g) identical to that analyzed in Fig. 1 and 3 was hybridized with the respective 32 P-labeled cytokine probe. Lanes: 1, spleen; 2, lung; 3, liver; 4, lymph node; 5, thymus; 6, kidney. (A) Organs were excised from mice 1 week postinfection with LP-BM5 MuLV; (B) 3 weeks postinfection. Time of exposure of the films was 4 days for IL-1 β , 3 days for TNF, and 16h for IRF-1. A duplicate membrane was hybridized with β -actin, and a representative result is shown (bottom).

time, the BM5d transcript could also be detected in nonlymphoid tissues and at the high levels in the thymus.

There was some cross-reactivity between the BM5d-specific probe used and viral transcripts encoded by the endogenous retroviruses present in C57BL/6 mice. When RNA from uninfected animals was analyzed by hybridization with either MCF/xeno virus-specific probe (5) or BM5d probe, transcripts of identical sizes were detected (Fig. 2).

FIG. 5. Detection of IFN- γ , IL-1 β , TNF, and IRF-1 mRNAs in organs of mouse at 16 weeks postinfection with LP-BM5 MuLV. Each lane contained 10 μ g of total organ RNA. Lanes: 1, spleen; 2, lung; 3, liver; 4, lymph nodes; 5, thymus; and 6, kidney. Films were exposed for 16 h for IFN- γ , 5 days for IL-1 β , 2 days for TNF, and 8 h for IRF-1. Duplicated membrane were hybridized with β -actin (bottom).

These transcripts were detected only after prolonged exposure, and none corresponded to the 4.8-kb mRNA of BM5d. It is possible that these endogenous sequences contributed to the formation of BM5d by recombination with BM5e.

It is noteworthy that the levels of transcripts encoded by BM5e in tissues of infected mice (Fig. 3) were lower than those of BM5d (Fig. 1). Since cell-to-cell spread of BM5d is dependent on helper MuLV, this dissociation suggests that cell populations expressing BM5d are amplified, in part, by cell division as well as by continuing infection of naive cells. This view is consistent with the observations that tissues in mice infected with helper-free defective virus had greatly expanded populations of cells expressing the defective genome (11).

Expression of BM5d in various tissues was associated with constitutive expression of IFN- γ , IL-1, and TNF but not other cytokines (Table 1). Transcripts of IL-1 and TNF, two inflammatory cytokines produced by macrophages as well as by other cell types, were readily detectable in tissues of infected mice (Fig. 4). Whether induction of these cytokines is mediated by IFN- γ , a potent activator of macro-

TABLE 1. Summary of cytokine mRNA expression in various organs from a C57BL/6 mouse infected with LP-BM5 MuLV for 16 weeks

Cytokine	mRNA expression in:					
	Spleen	Lung	Liver	Lymph nodes	Thymus	Kidney
IFN- α	—	—	—	—	—	—
IFN- β	—	—	—	—	—	—
IFN- γ	+	+	—	+	+	—
IL-1 β	+	+	+	+	+	+
TNF	+	+	+	+	+	+
IL-3	—	—	—	—	—	—
IL-4	—	—	—	—	—	—
IL-5	—	—	—	—	—	—
IL-6	—	—	—	—	—	—
IRF-1	+	+	±	+	+	+

phages, or reflects a direct effect of BM5d on infected cells remains to be determined. The possibility that constitutive expression of IFN- γ is central to the development of MAIDS appears unlikely since development of lymphoproliferation and immunodeficiency in infected mice treated with neutralizing monoclonal antibody to this cytokine was not detectable altered (14a).

In view of the massive proliferation and activation to immunoglobulin secretion of B cells that characterizes MAIDS, it was expected that infection might also induce expression of B-cell activation-differentiation factors such as IL-4, IL-5, and IL-6. Expression of these genes was not detected in total RNAs prepared from various tissues through 16 weeks of infection (Table 1), but the sensitivity of this assay system may not be high enough to detect transcripts sufficient to produce biologically significant levels of these lymphokines. This view is supported by the findings that in comparison with peritoneal macrophages from uninfected mice, cells from infected mice produced enhanced levels of IL-6 following stimulation with lipopolysaccharide or Newcastle disease virus in the absence of detectable message in total RNA preparations (7a).

The lack of expression of any known IFN- α genes in the infected organs confirms our previous observations *in vitro* that the retroviral infection does not lead to activation of IFN- α and - β genes (7a, 7b). Similarly, we were not able to detect the presence of a biologically active IFN- α/β in the sera of mice with AIDS (data not shown). An acid-labile IFN- α has been detected in sera of patients with autoimmune disease or in the late stages of AIDS (23, 26). Although acid-labile IFN was suggested to play a significant role in the development of AIDS (25), little is known about the nature of cells which produce this protein and about its gene(s). In view of the absence of expression of IFN- α and - β genes, increased expression of the nuclear factor IRF-1 in tissues of infected mice was an unexpected finding. IRF-1 was shown to be one of the transcriptional factors mediating induction of IFN- α and - β genes that by itself can be induced both by Newcastle disease virus and IFN (8, 16); whether it plays a role in the induction of acid-labile IFN- α as well is not known. Our results indicate that this factor can also be induced by retroviral infection *in vivo* in both infected tissues (Fig. 4 and 5) and peritoneal macrophages isolated from LP-BM5-infected mice (7a). The discordant expression of IRF-1 and IFN genes in infected mice confirms our recent data (13) and data of others (21, 29) which show that the function of IRF-1 may be more general and not limited to the IFN system.

These findings extend the similarities reported for mice infected with LP-BM5 MuLV and humans infected with HIV-1, with reports of the human disorder describing increased levels of circulating IFN- γ , IL-1, TNF, and IL-6 (3, 12, 25). It has been suggested that cytokine induction during HIV infection is triggered by the interaction of HIV gp120 with CD4 molecules that serve as virus receptors on the surfaces of T cells and macrophages (15). It remains to be determined whether similar mechanisms contribute to altered cytokine gene regulation in MAIDS (1). However, the observation that MAIDS develops in the absence of reinfection (11) suggests otherwise. These findings were interpreted as an indication that the defective virus is oncogenic and that immunodeficiency develops as a paraneoplastic syndrome. We favor a model in which the Pr60^{gag} product of BM5d expressed on the surface of infected B cells (4a, 10a) stimulates polyclonal activation of helper T cells, resulting in production of lymphokines that secondarily activate other T

cells, B cells, and macrophages. In this model, neoplasia is a consequence rather than a cause of immunodeficiency. Studies designed to discriminate among these possibilities are in progress.

Our data also demonstrate distinct differences between the retrovirus-induced immunodeficiency syndromes of mouse and human in that high-level expression of the etiologic virus can be detected throughout the course of MAIDS (Fig. 1), with latent infection being a prominent feature of HIV in AIDS (27).

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