Delayed Progression of a Murine Retrovirus-induced Acquired Immunodeficiency Syndrome in X-linked Immunodeficient Mice

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Summary

The murine acquired immunodeficiency syndrome (MAIDS) caused by defective LP-BM5 murine leukemia virus (MuLV) is a disease that shows severe immunodeficiency with abnormal lymphoproliferation, and hypergammaglobulinemia in susceptible C57BL/6 (B6) mice. To examine the cellular mechanisms of development of MAIDS, we injected LP-BM5 MuLV intraperitoneally into B6 mice bearing the X chromosome-linked immunodeficiency (*xid*). *xid* mice lack functionally mature B cells including Ly-1 B cells (also known as B-1 cells). All B6 mice died by 20 wk after LP-BM5 MuLV inoculation. In marked contrast, *xid* mice have continued to survive without any sign of MAIDS-related symptoms till at least 20 wk after the inoculation. The delayed progression of MAIDS in *xid* mice appears to depend on *xid* mutation, according to our experiments using both sexes of (B6.*xid* × B6)F_1 and (B6 × B6.*xid*)F_1 mice. Furthermore, Ly-1 B cells, enriched by a FACS®, were shown to integrate the defective genome and appeared to be a major virus-infected B cell population. Our data corroborate that Ly-1 B cells play an important role in the induction and progression of MAIDS.

The murine acquired immunodeficiency syndrome (MAIDS)[1] caused by a defective retrovirus (defective LP-BM5 murine leukemia virus [MuLV]) (1, 2), which encodes a gag fusion protein ($Pr60^{gag}$), is a disease that shows many similarities with human AIDS, in particular abnormal lymphoproliferation, polyclonal B cell activation, and severe immunodeficiency (3, 4). Previous studies have proven that complex cellular interactions between T and B cells are required for the induction and development of MAIDS. B cell abnormalities are shown in the presence of T cells of CD4 phenotype (5), whereas B cells are required for induction of phenotypic and functional T cell abnormalities in MAIDS (6). It is interesting that a recent study reported that the majority of cells infected with the defective LP-BM5 MuLV belongs to B cell lineages (7, 8), implying that B cells trigger the induction and development of MAIDS. The development of hypergammaglobulinemia in MAIDS also suggests that B cell activation and its differentiation is associated with the induction of MAIDS.

It still remains obscure, however, what kinds of B cell subsets are required for and involved in the induction and development of MAIDS. To examine these problems, we used the X-linked immunodeficient (*xid*) mice (9–12). The defects in *xid* mice, which show the impaired humoral immune response to type II T cell independent antigens (9, 10, 12), has been implicated in the arrest of functional maturation of B cells. In particular, the lack of Ly-1 B cells (also known as B-1 cells [13]) is noticed in *xid* mice (11).

Here we report that *xid* mice are resistant to MAIDS and Ly-1 B cells are a major virus-infected B cell population. Our data corroborate that Ly-1 B cells play an important role in the development of retrovirus-induced immunodeficiency, MAIDS.

[1] *Abbreviations used in this paper:* MAIDS, murine acquired immunodeficiency syndrome; MuLV, murine leukemia virus; *xid*, X-linked immunodeficiency.

Materials and Methods

Mice. B6.*xid* mice were kindly provided by Dr. Alfred Singer (National Institutes of Health (NIH), Bethesda, MD) through Dr. Toshiyuki Hamaoka (Osaka University Medical School, Osaka, Japan) and maintained in the animal facility in Kumamoto University under specific pathogen free conditions. It is unclear how far the *xid* gene was backcrossed onto the B6 background. But these mice show a defect in the response to TNP-Ficoll and the number of Ly-1^+ B cells (Hitoshi, Y., E. Sonoda, Y. Kikuchi, S. Yonehara, H. Nakauchi, and K. Takatsu, manuscript submitted for publication). B6 mice were purchased from Japan SLC Inc. (Hamamatsu, Japan).

LP-BM5 MuLV. An SC-1 clone chronically infected with LP-BM5 MuLV, termed G6 cell line, was kindly supplied by H. C. Morse III (NIH). Virus was prepared from the supernatant of G6 cells. A 24-h culture supernatant of G6 cells contained $\sim 5 \times 10^4$ PFU of ecotropic virus per ml, determined by the XC plaque assay described by Rowe et al. (14). The virus preparation was stored at −70°C until use.

Analyses of Development of MAIDS. B6 mice, B6.*xid* mice, (B6.*xid* × B6)F_1 mice and (B6 × B6.*xid*)F_1 mice were injected intraperitoneally with 10^4 PFU of LP-BM5 MuLV. At 7 wk after the injection, the spleen weight, the amount of serum IgM, and proliferative response of spleen cells to Con A were measured. The serum IgM level was determined by isotype-specific ELISA. Con A response was monitored by [^{3}H]thymidine incorporation of spleen cells (10^5) in the presence of Con A (2 μg/ml) for the last 12 h of 2-d culture.

Detection of the Defective LP-BM5 MuLV Genome by PCR. Template DNAs extracted from spleen cells (10^5) of the mice at 7 wk after the virus inoculation were amplified by PCR as described previously (15). The PCR primers were 5′-CCTCTTCCTTTATCGACACT-3′ and 5′-ATTAGGGGGGGAATAGCTCG-3′. These primers correspond to the sequences located in the defective LP-BM5 MuLV *gag*-encoded gene, p15 and p12, respectively. Template DNAs were added to a cocktail adjusted to final concentrations of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM deoxynucleoside triphosphate, 100 pM of each primer, and 2 U of Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany) in a total volume of 100 μl, and were subjected to 30 cycles of amplification. In each cycle of PCR, the mixture was denatured at 95°C for 1 min (5 min for the first cycle), annealed at 55°C for 3 min, and extended at 72°C for 1 min on a programmed temperature control system (Astec, Fukuoka, Japan). The PCR products (237 bp) were then analyzed by Southern blot hybridization with an internal probe, 5′-TGTCAAAGGGACCAGTTAAG-3′. The PCR can detect one infected cell among 10^4–10^5 cells. As control, DNA from mouse IL-5 receptor (IL-5R) α chain was amplified by PCR in the same methods. The primers used for PCR reaction were 5′-AAACCTTCGAATGAAACCA-3′ and 5′-TGGTATTAAAGGCGAGCTC-3′, and define fragments of 216 bp. These primers correspond to the sequences located in the cytoplasmic domain and 3′-uncoding region of IL-5R α chain, respectively (16).

Abs and Reagents. The following monoclonal Abs (mAbs) were used: RA3-6B2 mAb (rat IgG2a), which recognizes B220 (American Type Culture Collection, Rockville, MD); 53-7.3 mAb (rat IgG2a), which recognizes Ly-1 (American Type Culture Collection); 2.4G2 mAb (rat IgG1), which recognizes murine FcγR (American Type Culture Collection). Each mAb was purified from ascitic fluids with protein G-column (Pharmacia-LKB Laboratories, Uppsala, Sweden). PE-labeled streptavidin (PE-av) was purchased from Becton Dickinson & Co. (Mountain View, CA).

Staining and Sorting of Ly-1^+B220^+ Cells. Cells from inguinal LNs of B6 mice at 14 wk after the LP-BM5 MuLV inoculation were stained with biotinylated anti-Ly-1 mAb plus PE-av and FITC-labeled anti-B220 mAb (RA3-6B2). The stained cells were analyzed by a FACStar® (Becton Dickinson & Co.) and fractionated into Ly-1^-B220^-, Ly-1^+B220^-, Ly-1^-B220^+, Ly-1^+-B200^+ and unseparated. The cells (10^4) of sorted fractions were investigated for the detection of defective LP-BM5 MuLV genome and DNA from IL-5R α chain as described above.

Results

C57BL/6 (B6) mice are highly susceptible to LP-BM5 MuLV (this represents a mixture of the replication-defective virus and the ecotropic helper virus) and develop MAIDS in several weeks after viral infection (17, 18). We injected LP-BM5 MuLV intraperitoneally into B6 mice or B6 mice bearing the X chromosome-linked immunodeficiency (B6.*xid*). As shown in Fig. 1, all B6 mice died within 20 wk after LP-BM5 MuLV inoculation with changes characteristic of MAIDS. In marked contrast, B6.*xid* mice have continued to survive without any sign of MAIDS-related symptoms such as splenomegaly, lymphadenopathy, and hypergammaglobulinemia till at least 20 wk after the inoculation of LP-BM5 MuLV. Less than 30% of the virus-inoculated-B6.*xid* mice died with splenomegaly and lymphadenopathy 30 wk after the virus inoculation.

To investigate whether the delayed progression of MAIDS in B6.*xid* mice depends on *xid* mutation, both sexes of (B6.*xid* × B6)F_1 and (B6 × B6.*xid*)F_1 mice were inoculated with LP-BM5 MuLV. Since *xid* mutation is X-chromosomal recessive, (B6.*xid* × B6)F_1 male mice (*xid*/Y genotype) only express the defects of *xid* mutation. The extent of splenomegaly, elevation of serum IgM level, and impaired Con A-response were examined in each group of mice 7 wk after LP-BM5 MuLV inoculation. As shown in Table 1, B6.*xid* and the defective F_1 male mice were free of the MAIDS-related symptoms, whereas the other groups of mice showed the marked

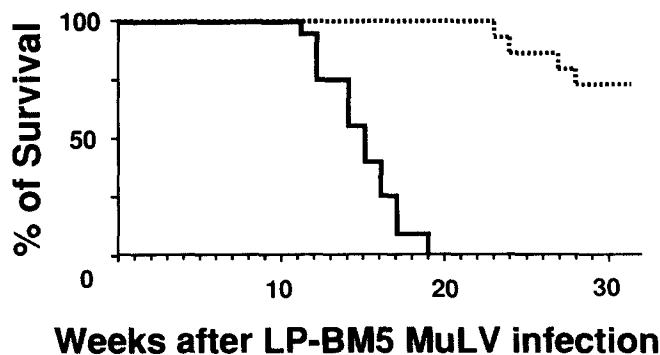

Figure 1. Survival of B6 (*solid line*) and B6.*xid* mice (*dotted line*) after the inoculation of LP-BM5 MuLV. B6 mice (n = 20) and B6.*xid* mice (n = 15) were injected intraperitoneally with LP-BM5 MuLV at 5–6 wk of age.

Table 1. *Delayed Progression of MAIDS in xid Mice*

Mice	Sex	Genotype	LP-BM5 infection	Spleen	IgM	Con A response
				mg	*μg/ml*	*cpm*
B6	F	X/X	–	81 ± 16	650 ± 300	40,635 ± 191
			+	593 ± 106	3,551 ± 1,432	1,187 ± 103
xid	F	*xid*/*xid*	–	63 ± 13	47 ± 14	ND
			+	67 ± 17	35 ± 11	ND
xid × B6	M	*xid*/Y	–	58 ± 18	34 ± 21	55,090 ± 1,731
			+	70 ± 20	67 ± 42	52,372 ± 2,035
xid × B6	F	*xid*/X	–	78 ± 10	325 ± 183	46,725 ± 630
			+	344 ± 51	1,022 ± 501	1,315 ± 197
B6 × *xid*	M	X/Y	–	63 ± 10	310 ± 106	38,252 ± 9,878
			+	578 ± 56	1,791 ± 322	769 ± 132
B6 × *xid*	F	X/*xid*	–	74 ± 10	263 ± 94	52,860 ± 9,516
			+	492 ± 77	1,739 ± 730	1,580 ± 482

B6 mice, B6.*xid* mice, (B6.*xid* × B6)F_1 mice, and (B6 × B6.*xid*)F_1 mice were injected intraperitoneally with LP-BM5 MuLV. At 7 wk after the injection, the spleen weight, the amount of serum IgM and proliferative response of spleen cells to Con A were measured. The serum IgM level was determined by isotype-specific ELISA. Con A response was monitored by [^{3}H]thymidine incorporation of spleen cells (10^5) in the presence of Con A (2 μg/ml) during the 12 h after 2-d culture. Data were expressed as mean ± SD of five mice.

symptoms. These results further supported the notion that *xid* mutation is responsible for the delayed progression of MAIDS.

We then investigated the integration of the defective LP-BM5 MuLV genome in spleen cells (Table 1). The defective LP-BM5 MuLV has a long open reading frame encoding a putative *gag* precursor protein. A *gag* protein, *gag* p12, was the most divergent in comparison with that of nondefective MuLV (2). The unique DNA sequence encoding the *gag* p12 protein of defective LP-BM5 MuLV was detected by PCR and Southern blot hybridization. As shown in Fig. 2, PCR product of the defective LP-BM5 MuLV gene was not detected in B6.*xid* and the F_1 male mice 7 wk after LP-BM5 MuLV inoculation, whereas the PCR product was clearly shown in the other groups of mice. Since the defective LP-BM5 MuLV has proven to be the disease (MAIDS)-causing virus, it is conceivable that mice bearing the *xid* mutation are resistant to the defective virus infection. Several B6.*xid* mice showed splenomegaly and lymphadenopathy 30 wk after the virus inoculation (Fig. 1). These mice showed the integration of the defective LP-BM5 MuLV genome in cells of the enlarged spleen (data not shown). In addition, we could detect a weak expression of the defective LP-BM5 MuLV genome without remarkable MAIDS-related symptoms 20 wk after virus inoculation in B6.*xid* mice tested (data not shown).

An apparent correlation between the *xid* mutation and re-

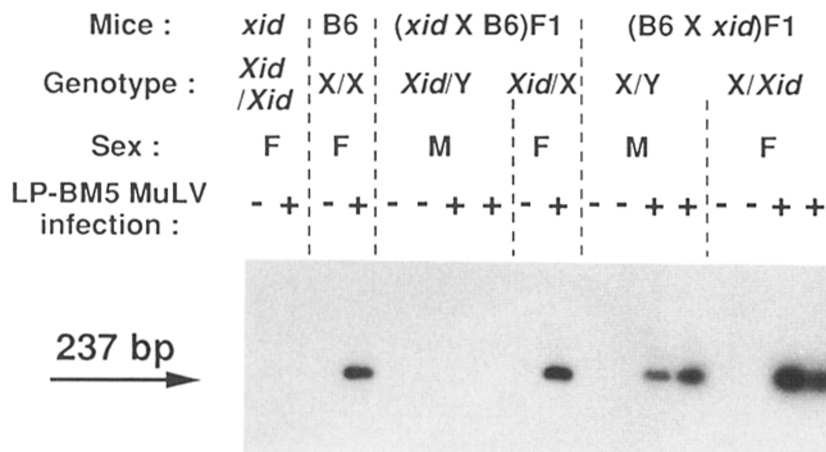

Figure 2. Detection of the defective LP-BM5 MuLV genome by PCR. The template DNAs were extracted from spleen cells (10^6) of B6 mice, B6.*xid* mice (*xid*), (*xid* × B6)F_1 mice, and (B6 × *xid*)F_1 mice 7 wk after the virus inoculation, and were amplified by PCR. The PCR products (237 bp) were then analyzed by Southern blot hybridization.

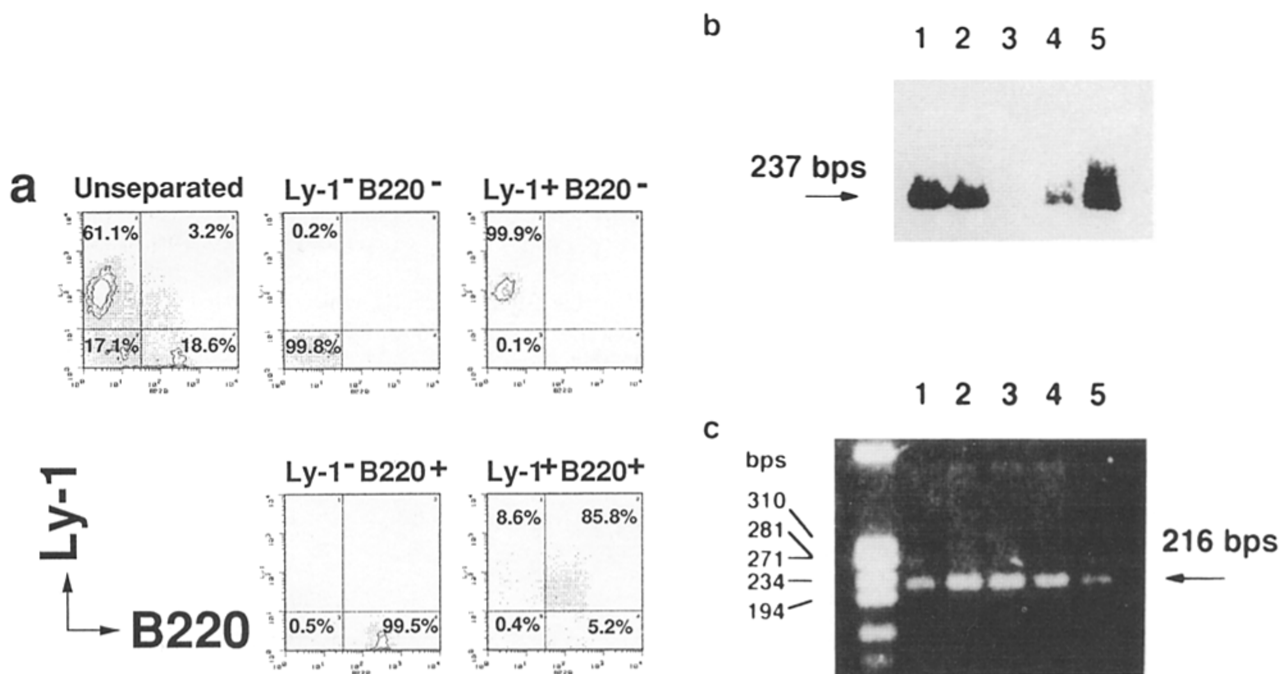

Figure 3. Detection of the defective LP-BM5 MuLV genome in Ly-1 B cells by PCR and Southern blot hybridization. (*a*) Cells from inguinal LN of B6 mice 14 wk after the LP-BM5 MuLV inoculation were stained with biotinylated anti-Ly-1 mAb plus PE-labeled streptavidin and FITC-labeled anti-B220 mAb. The stained cells were analyzed by a FACStar® and fractionated into Ly-1$^-$B220$^-$, Ly-1$^+$B220$^-$, Ly-1$^-$B220$^+$, Ly-1$^+$B220$^+$, and unseparated. Percentages of stained cells in the gated windows of each fraction were shown in a corner of each window. (*b*) The cells (10^4) of sorted fractions were investigated for the detection of defective LP-BM5 MuLV genome as described in Materials and Methods. (Lane *1*) Unseparated cells; (lane *2*) Ly-1$^-$B220$^-$ cells; (lane *3*) Ly-1$^+$B220$^-$ cells; (lane *4*) Ly-1$^-$B220$^+$ cells; (lane *5*) Ly-1$^+$B220$^+$ cells. (*c*) The cells (10^4) of sorted fractions were investigated for the detection of DNA from IL-5 receptor α chain as described in Materials and Methods. (Lane *1*) Unseparated cells; (lane *2*) Ly-1$^-$B220$^-$ cells; (lane *3*) Ly-1$^+$B220$^-$ cells; (lane *4*) Ly-1$^-$B220$^+$ cells; (lane *5*) Ly-1$^+$B220$^+$ cells.

sistance to MAIDS development was found in this study. Since lack of Ly-1 B cells in *xid* mice has been noticed, the question was whether the lack of Ly-1 B cells is responsible for the delayed progression of MAIDS in *xid* mice. In fact, the B6.*xid* mice have few Ly-1 B cells in the peritoneal cavity and spleen (data not shown). To address whether Ly-1 B cells are the target cells of defective LP-BM5 MuLV, we enriched Ly-1 B cells by FACStar® from LN cells of B6 mice infected with LP-BM5 MuLV, and analyzed the integration of the defective genome in Ly-1 B cells by the same method used in Fig. 2. Fig. 3 *a* shows FACS® profiles and the purity in each sorted fraction. Fig. 3 *b* clearly showed that the defective genome was detected in unfractionated cells (lane *1*), Ly-1$^+$B220$^+$ cells (Ly-1 B cells) (lane *5*), and Ly-1$^-$B220$^-$ cells (lane *2*), and slightly in Ly-1$^-$B220$^+$ cells (lane *4*), whereas the genome was not found in Ly-1$^+$B220$^-$ cells (T cells) (lane *3*). In contrast, DNA from IL-5R α chain was similarly detected in reaction performed with materials from unfractionated cells (lane *1*), Ly-1$^-$B220$^-$ cells (lane *2*), Ly-1$^+$B220$^-$ cells (T cells) (lane *3*), Ly-1$^-$B220$^+$ cells (lane *4*), and Ly-1$^+$B220$^+$ cells (Ly-1 B cells) (lane *5*) (Fig. 3 *c*). These results indicated that Ly-1 B cells were infected with the defective LP-BM5 MuLV.

Discussion

We clearly demonstrated that B6.*xid* mice did not show any signs of MAIDS-associated symptoms 20 wk after the LP-BM5 MuLV infection, although B6 mice show the symptoms within 7 wk after the MuLV infection at the latest. The defective LP-BM5 MuLV was not detected using PCR and Southern blot hybridization in B6.*xid* mice 7 wk after inoculation. These results suggest that *xid* mutation affects the induction and development of MAIDS. In addition, the cell sorting experiments suggest that Ly-1 B cells are the target cell population for the defective LP-BM5 MuLV. In light of our finding, we can hypothesize that Ly-1 B cells play an important role in the induction and progression of MAIDS and the lack of Ly-1 B cells in B6.*xid* mice may account for the delayed progression of LP-BM5 MuLV infection in *xid* mice.

Ly-1 B cells, which are characterized by the expression of surface markers such as Ly-1$^+$, sIgM^{bright}, sIgD^{dull}, B220^{dull}, and Mac-1$^+$, constitute a distinct B cell lineage and differ in their functional properties from conventional B cells (11, 19). Huang reported that the majority of the LP-BM5 MuLV-infected cells are B220^{dull} positive, not B220^{bright} cells (8). Their B220^{dull} cells may be Ly-1 B cells. And it has been reported that all B-lineage cells established in culture from LP-BM5 MuLV-infected mice are Ly-1-positive (20), and these cells show the expression of multiple copies of the defective virus (21). These reports may support our data that Ly-1 B cells are target cells for the defective LP-BM5 MuLV.

Several characteristics of Ly-1 B cells could contribute to the development of MAIDS. First, we notice that Ly-1 B cells have a self-renewal ability (22). Huang et al. (8, 23) reported

the induction of MAIDS using a helper-free stock of the defective virus that cannot replicate on its own. The expansion of target cells should be necessary for the development of MAIDS in their system. Cheung et al. (24) reported that the levels of transcripts encoded by ecotropic LP-BM5 MuLV (helper virus) were lower than those of defective LP-BM5 MuLV. Their findings also suggest that cell populations expressing defective LP-BM5 MuLV may be amplified, in part, by cell division, as well as by continuing infection of naive cells. The virus-infected Ly-1 B cells could expand themselves and develop MAIDS. Second, O'Garra et al. (25) reported that Ly-1 B cells are the main source of B cell–derived IL-10, which suppresses cytokine production by Th1-type $CD4^+$ T cells and macrophages (26, 27) and has B cell stimulatory activity (28). In fact, Gazzinelli et al. (29) reported that IL-10 is produced during progression of MAIDS. LP-BM5 MuLV–infected Ly-1 B cells may be responsible for producing IL-10, which could provoke persistent activation of Th2-type T cells, downregulate the production of Th1-derived cytokines, impair $CD8^+$ T cell function, and lead to chronic B cell activation in LP-BM5 MuLV infection. Third, Van de Velde et al. (30) demonstrated that Ly-1 (CD5) and Lyb-2 (CD72), the activated B cell surface proteins are involved in the B cell activation process as a pair of interacting receptors. It has been postulated that virus-infected Ly-1 B cells help the activation of conventional B cells in the development of MAIDS (30) in which Ly-1/Lyb-2 interaction may be involved. Collectively, Ly-1 B cells seem to be very important for the rapid development of MAIDS.

It is intriguing that <30% of the virus-inoculated-B6.*xid* mice develop MAIDS 30 wk after the virus inoculation. These data suggest that the development of MAIDS depend on not only Ly-1 B cells but also other target cells of LP-BM5 MuLV.

It is interesting that B6.*xid* mice had a few Ly-1 B sister cells (known as B-1b cells [13]) that share several functional features of Ly-1 B cells such as surface phenotypes, an unusual IgM/IgD ratio, certain specificities to antigen, and distinctive anatomical localization, though we do not show the data. These cells in *xid* mice may play the same role as for $Ly\text{-}1^+$ B cells in normal mice, but because of their low frequency, the progression of MAIDS is delayed. It was reported that macrophages from mice with MAIDS express viral RNA coded by ecotropic and defective LP-BM5 MuLV (31). Our experiments indicated that $Ly\text{-}1^-B220^-$ cells were other target cells of LP-BM5 MuLV. These cell populations may include macrophages. The development of MAIDS in *xid* mice, though delayed, may be induced by the virus-infected macrophages. Recent studies demonstrated the presence of transplantable T-lymphoid cells, which integrated the defective viral genome, in C57BL/6 mice infected with LP-BM5 MuLV (4, 32). These cells may also be involved in the development of MAIDS. But we could not detect the defective virus in the $Ly\text{-}1^+B220^-$ cell population, which corresponds to T lymphoid cells (Fig. 3). This discrepancy raises the possibilities that the transplantable T-lymphoid cells may be $Ly\text{-}1^-$ cells or the frequency of the T-lymphoid cells infected with the defective virus may be too low, i.e., below 1/10,000, to detect the virus genome from $Ly\text{-}1^+B220^-$ cells.

Failure to exhibit disease in *xid* mice was due to a block in virus replication (Fig. 2). The inability to exhibit integration of the defective genome in disease raises the possibility that there may have been a block to spread of helper virus and impaired helper restriction may contribute to the delayed progression of MAIDS in *xid* mice. Ly-1 B cells may contribute to the replication of helper virus, although we do not have the data about the level of helper virus in Ly-1 B cells. The kinetics of appearance of the defective virus and helper virus in *xid* mice would be of interest.

In conclusion, we provide evidence in the present study that Ly-1 B cells are required for the progression of MAIDS, although the involvement of Ly-1 B cells may not be sufficient. The cellular mechanisms for the induction of a mouse retrovirus-induced immunodeficiency have not been fully covered and need further investigation, our findings would provide a clue to approach this important issue and help in elucidating the pathogenesis of retrovirus-induced immunodeficiency syndromes including AIDS.

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