RETROVIRAL INDUCTION OF ACUTE LYMPHOPROLIFERATIVE DISEASE AND PROFOUND IMMUNOSUPPRESSION IN ADULT C57BL/6 MICE

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The Laterjet-Duplan strain of radiation murine leukemia virus (MuLV)[1] differs from most other virus isolates in that it induces nonthymic rather than thymic lymphomas in mice (1). Haas and co-workers (2, 3) demonstrated that cell-free extracts produced from "reticulum cell neoplasms" (RCN) of mice infected with this agent contained a mixture of ecotropic, xenotropic, and mink cell focus (MCF)-inducing (4) MuLV. A cell line derived from bone marrow, termed RCN-BM5, has served as a source of these viruses in subsequent studies, which suggested that the lymphomagenic component in the virus mixture was an MCF-inducing MuLV (3, 5). Young adult mice infected with phenotypic mixtures of a nonpathogenic ecotropic virus and the MCF virus rapidly developed lymphadenopathy and splenomegaly, and died with massive enlargement of these tissues (2, 3, 5).

Although initial histologic studies of tissues from virus-infected mice yielded the diagnosis of RCN type B (2), according to Dunn's (6) classification of murine hematopoietic tumors, later studies (5) indicated that the mice developed a polyclonal proliferation of B cells that may not be truly malignant. The splenic and nodal lesions, while classified as polyclonal immunoblastic B cell lymphomas, could not be transplanted, contained diploid cells throughout the course of the disease, and were rarely invasive of nonlymphoid tissues. Since, by these criteria, the lesions induced by the viruses in RCN-BM5 are non-neoplastic, the term "reticulum cell neoplasm" appears to be inappropriate. To indicate the nonmalignant lymphoproliferative nature of this disease, we will refer to the MuLV mixture from the BM5 tissue culture line as LP (lymphoproliferative)-BM5.

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[1] *Abbreviations used in this paper:* AIDS, acquired immunodeficiency syndrome; BSA, bovine serum albumin; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; FACS, fluorescence-activated cell sorter; IL-2, interleukin 2; LPS, lipopolysaccharide; mAb, monoclonal antibody; MCF, mink cell focus–inducing virus; MLR, mixed lymphocyte reaction; MuLV, murine leukemia virus; PBS, phosphate-buffered saline; PFC, plaque-forming cell; PHA, phytohemagglutinin; RCN, reticulum cell neoplasm; RIA, radioimmunoassay; sIg, surface Ig; SRBC, sheep red blood cells; TNP, trinitrophenyl.

The current studies of LP-BM5 MuLV were initiated in our laboratories to determine the cellular mechanisms responsible for this unusual proliferation and polyclonal activation of B cells. We report here that injection of the virus mixture into adult C57BL/6 mice caused a rapid induction of cell proliferation, not only of B lymphocytes, but also of T lymphocytes and non-T, non-B lymphoid cells. All B lymphocytes showed evidence of polyclonal activation, and many were actively secreting IgM or IgG. There was a brief period of increased immune responsiveness, followed quickly by a lasting and profound immunosuppression that affected all measured aspects of cellular and humoral immunity. The mice died 4–6 mo after virus inoculation, and showed massive lymphadenopathy. We thus conclude that the LP-BM5 MuLV-induced disease is a new mouse model for the rapid induction of polyclonal lymphocyte proliferation and profound immunosuppression by retroviruses, and that the model may have considerable relevance for similar retrovirus-induced syndromes in humans.

Materials and Methods

Mice. C57BL/6NIcr mice of both sexes were used at 6–12 wk of age. Mice were reared either at the Laboratory Animal Facility of the Institute for Cancer Research or the Small Animal Section of the Division of Research Resources, National Institutes of Health. All animals were caged and handled in accord with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (Department of Health and Human Services publication 80-23).

Virus Passage. Subcultures of the bone marrow stromal cell line described previously (2) were obtained from Dr. Martin Haas, University of California at San Diego. This cell line was maintained by biweekly subculture in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum. Cell-free filtrates were prepared by collecting culture medium after 3–4 d of growth, centrifuging the culture fluids at 3,000 rpm for 10 min, and passing the supernatant through a 0.22 μm Millipore filter. 1 ml of the filtered supernatant was injected intraperitoneally into recipient mice within 1 h of preparation. These inocula contained 5×10^3–10^4 infectious ecotropic as well as MCF MuLV. All C57BL/6 mice injected in this way developed evidence of lymphoproliferative disease within 3 wk.

Antigens and Mitogens. The preparation of trinitrophenyl (TNP)-coupled lipopolysaccharide (LPS), *Brucella abortus* (*B. abortus*), and aminoethylcarbamylmethyl-Ficoll (Ficoll) have been described previously (7). Sheep red blood cells (SRBC) were obtained from a single animal (#190) at Colorado Serum Co., Denver, CO. Phytohemagglutinin (PHA) was obtained from Burroughs Wellcome, Beckenham, England, concanavalin A (Con A) from Pharmacia Fine Chemicals, Piscataway, NJ, and LPS 0111:B4 from Difco Laboratories, Inc., Detroit, MI. The preparation of affinity-purified goat anti-μ has been described previously (8).

Media and Culture Conditions. Most cultures of spleen or lymph node cells were performed at a cell density of 5×10^5 cells in 0.2 ml RPMI 1640 supplemented with 5% fetal bovine serum, 15 mM Hepes, 5×10^{-5} M 2-mercaptoethanol, and 50 μg/ml gentamicin. Mitogen responses, mixed lymphocyte reactions (MLR), and plaque forming cell (PFC) responses to T-independent antigens were performed in 0.2 ml microcultures in 96-well, flat-bottom trays (model 3596, Costar Packaging Corp., Cambridge, MA). In vitro responses to SRBC were performed in 1 ml cultures (3524 trays Costar Packaging Corp.) containing 5×10^6 cells/ml. In selected experiments, B cell survival was followed in serum-free Iscove's F12 medium (9).

Assays. Proliferation was determined by measuring the incorporation of [^{3}H]thymidine into DNA following a 4 h pulse after 3–5 d of culture. Cytotoxic T lymphocyte (CTL) effector cells were generated in mixed lymphocyte cultures, and their activity was quantitated using ^{51}Cr-labeled, concanavalin A (Con A)-stimulated lymphoblast targets, as

described (10). PFC assays were performed using SRBC, TNP-coupled SRBC (11), or protein A–coupled SRBC (12) as indicator cells. Assays were done after 3–5 d of culture. Rabbit anti-μ serum was used to develop protein A–PFC (12). Serum Ig isotype determinations were carried out using a competitive radioimmunoassay (RIA). Briefly, flexible polyvinylchloride microtiter tray wells (Dynatech Labs, Alexandria, VA) were coated with a purified hybridoma protein of the appropriate isotype, diluted in phosphate buffered saline (PBS) containing 0.01% bovine serum albumin (BSA). The plates were blocked by the addition of 1% BSA in PBS, and an appropriate dilution of affinity-purified rabbit anti-isotype antibodies were added. After fixation in 0.0025% glutaraldehyde, serial dilutions of serum from normal or LP-BM5–infected mice were assayed for their ability to inhibit the binding of ^{125}I-labelled monoclonal antibodies (mAb) of the appropriate isotype. Standard inhibition curves were constructed by competitive binding of ^{125}I-labeled proteins against the same, unlabelled Ig. 50% inhibition of binding was seen at 0.2–0.6 μg/ml of reference Ig.

Flow Cytometry Analysis. Cell size and immunofluorescence were analyzed using a Becton-Dickinson FACS II (fluorescence-activated cell sorter). Blast cells were defined as those with a forward light scatter signal above channel 650, when the unimodal peak of small lymphocytes was at channel 400, the amplifier gain at 4, and the argon laser output at 400 mW. Fluorescein emission was excited at 488 nm and collected at 520–530 nm. Fluorescent signals were logarithmically amplified before analysis. The percentage of cells in replicative cycle was estimated by staining of ethanol-fixed cell suspensions with chromamycin A3 (13).

Cell Separation. In some experiments, T lymphocytes were depleted by treatment with anti-Thy-1 mAb (clone 13-4) (14) and anti-Ly-2 mAb (clone 3.168) (15) followed by complement-mediated lysis using rabbit low-Tox M (Accurate Chemical Company, Westbury, NY). Spleen cells were size-fractionated by passage over a discontinuous Percoll gradient (15) for the type of experiment illustrated in Table III.

Results

Phenotypic Changes in Lymphoid and Nonlymphoid Cells Induced by LP-BM5 MuLV. Injection of the LP-BM5 virus mixture into adult C57BL/6 mice resulted in the rapid and reproducible appearance of gross spleen and lymph node enlargement. We examined the cellular composition of these enlarged lymphoid organs using mAb against lineage-specific determinants (17). The results of such studies on spleen cell populations are summarized in Tables I and II. Within 1 wk of LP-BM5 injection, the number of spleen cells almost doubled. As shown

TABLE I

Changes in C57BL/6 Splenic Lymphocyte Populations Induced by LP-BM5 MuLV

Time postinfection	Blast cells* ($\times 10^{-6}$)	Proportion of blast cells	Median cell size‡	T cells ($\times 10^{-6}$)	Thy-1$^+$ cells	B cells ($\times 10^{-6}$)	sIg$^+$ cells
wk		%			%		%
1	59 ± 5§	29 ± 3	332 ± 7	47 ± 4	23 ± 3	120 ± 10	59 ± 2
2	96 ± 16	36 ± 3	343 ± 9	59 ± 10	22 ± 7	165 ± 28	62 ± 1
3	105 ± 7	39 ± 4	353 ± 12	35 ± 3	13 ± 4	153 ± 11	57 ± 3
4	136 ± 26	41 ± 4	396 ± 6	43 ± 8	13 ± 4	176 ± 34	53 ± 5
Control values	34 ± 3	29 ± 3	325 ± 13	40 ± 4	34 ± 2	62 ± 6	53 ± 5

* Blast cell number measured by increase in light-scatter signal on FACS.

‡ Median channel number of light-scatter signal on FACS. Increasing numbers indicate increasing volume of blast cells.

§ Underlined values are significantly different from controls at $P < 0.05$.

TABLE II

Surface Phenotype of Spleen Cells From C57BL/6 Mice Infected With LP-BM5 MuLV

Mouse	Time post-infection	T antigens				Common B antigens			Specific B antigens		Macrophage antigen MAC-1	Common T-B antigens		Granulocyte antigen 8C5
		Thy-1	Ly-2	Ly-1	sIgM	Lyb-8	Ly-5 (B220)	ThB	sIgG2	sIgG1		Ly-24	Ly-5	
	wk													
204	4	28	—	—	—	—	36	29	44	—	30	91	86	5
217	4	21	—	—	—	—	25	14	—	—	38	86	75	7
404	12	17	10	22	8	—	12	8	3	—	21	91	54	17
407	13	24	12	33	18	—	25	20	8	2	20	92	67	13
477	14	8	5	41	29	—	38	23	43	3	17	—	75	7
506	15	24	9	28	17	37	36	27	24	6	19	—	80	—
234	16	20	12	—	21	—	15	9	24	2	27	89	63	13
235	16	28	8	—	15	—	16	11	31	1	19	91	75	7
284	16	23	10	—	49	—	18	11	24	2	18	92	63	12
285	18	35	6	—	35	—	18	13	31	1	11	94	75	6
291	18	15	—	14	8	7	4	3	24	3	1	79	51	13
292	18	8	—	10	30	50	28	59	69	7	1	88	75	4
398	18	16	6	34	17	—	12	7	4	1	21	—	79	17
Normal C57BL/6		31 ± 4	15 ± 2	39 ± 4	56 ± 4	57 ± 5	56 ± 4	56 ± 4	5 ± 2	1 ± 1	5 ± 3	80 ± 10	97 ± 1	2 ± 1

The origins of the mAb used in these studies are given in (17). Numbers in table represent percentage of cells positive for the named antigen.

in Table I, this increase was mainly due to an increase in the number of surface Ig–positive (sIg^+) B lymphocytes. The number of T cells did not increase significantly, so the fractional percentage of T cells decreased. There was an increase in blast cells, as measured by light scatter. These large cells were predominately B cells, but some T cells and some non-T, non-B cells were also included in the blast cell fraction. With increasing time after virus infection, B cells and blast cells continued to increase in number. Mature T and B cells became a smaller total fraction of the spleen, as 34% of the spleen cells were neither Thy-1^+ nor sIg^+ by 4 wk post-infection. A more complete surface phenotype profile of these cells is shown in Table II.

As the lymphoproliferative disease induced by LP-BM5 MuLV progressed, the cell populations involved in the disease became more pleimorphic, and individual animals showed unique splenic cellular compositions. The fractional percentage of Thy-1^+ T cells varied considerably, but seldom reached normal ranges. There was a significant increase in granulocytes (identified by the 8C5 mAb) in all infected mice. Mac-1^+ cells increased in number in most mice, but since it has been suggested that Mac-1 may be expressed on early B-lineage cells as well as macrophages (17), the identity of the Mac-1^+ cells was ambiguous. Many of the virus-infected mice had expanded numbers of B-lineage cells, including Lyb-8^+, Ly-5 $(B220)^+$, and ThB^+ cells, in excess of $sIgM^+$ B cells. This apparent discrepancy in the expression of B cell markers is probably due to reduced sIg expression concomitant with maintenance of other markers on Ig-secreting immunoblasts. This view was supported by the observation of a spontaneous fivefold increase in IgM-secreting cells (see below). The increase in IgG2-bearing cells may be due to cytophilic binding to Fc receptors on macrophages or B cells. Some mice have a significant increase in Ly-1^+ cells compared to Thy-1^+ T cells, (e.g. mice 398 and 477 in Table II). This suggests that the Ly-1^+ B cell subpopulation (18) had expanded in those animals. Finally, some splenic cells in some mice were untyped by our reagents; we do not know their identity.

Lymph node and peripheral blood lymphocytes were also studied, and showed similar changes of T and B cell populations (data not shown). The ratio of Ly-1^+/Ly-2^+ cells in peripheral blood from virus-infected mice was not substantially different from normal controls, although the percentage of recirculating Thy-1^+ T cells was diminished by virus infection.

The rapid increase in lymphoid mass and the appearance of large numbers of blast cells (Table I) could be due to either the stable accumulation of nondividing blast cells, or the continued accretion of cells in cycle. To discriminate between these alternatives, spleen cells from normal or LP-BM5-infected mice were tested simultaneously for surface markers and DNA content (see Materials and Methods). Spleen cells were either directly stained with chromamycin A3 (13), anti-Thy-1, or anti-Ig, or they were first size-fractionated on a discontinuous Percoll gradient, then stained. Table III shows the results of a representative experiment. There was a threefold increase in the fraction of splenocytes in active cycle after LP-BM5 infection. While most of the normal splenocytes in cell cycle were intermediate- to large-sized, substantial numbers of small-sized cells from virus-infected mice were in cell cycle. Note also that most T and B cells were larger (and less dense) than normal following virus infection, and that among small,

TABLE III

LP-BM5 MuLV Induces Mitosis in T Lymphocytes, B Lymphocytes and Non-T, Non-B Cells of All Size Ranges

Size range*	Source	Cells in S or G_2/M phase	T cells in fraction	B cells in fraction
			%	
All cells (unfractionated)				
	Normal	8.0	35.6	56.8
	LP-BM5-infected	24.4	22.5	45.9
Large (1.062)‡				
	Normal	11.2	27.3	54.2
	LP-BM5-infected	28.7	29.1	36.3
Intermediate (1.074)				
	Normal	7.7	20.7	69.5
	LP-BM5-infected	30.9	25.1	30.4
Small (1.086)				
	Normal	3.7	64.6	18.4
	LP-BM5-infected	19.4	26.6	8.6

* Spleen cells from normal C57BL/6 mice or C57BL/6 mice 6 wk post–LP-BM5 infection were size-fractionated on discontinuous Percoll gradients and stained with chromamycin A3, anti-Thy-1, or anti-Ig to determine the percentage of cells in cycle, and the percentage of T cells and B cells in each size fraction.

‡ Density of cells collected at Percoll gradient interfaces.

high-density splenocytes, more cells were in cycle than could be accounted for by B lymphocytes. We conclude that LP-BM5 induced widespread lymphoproliferative disease, and that few, if any, normal lymphoid cells escaped involvement in the proliferative process.

Our data show that fewer mature sIg^+ B cells are involved in the lymphoproliferative disease than reported previously (5). One explanation for this discrepancy is that the profound hypergammaglobulinemia (see below and reference 5) leads to acquisition of cytophilic IgM by non-B cells. This hypothesis was tested by trypsin "stripping" of sIg and evaluation of sIgM density after 24 h of culture to allow sIg reexpression. As shown in Fig. 1, untreated spleen cells from LP-BM5-infected mice had few $sIgM^-$ cells. Instead, a population of cells with normal sIgM density, and a diffuse population with low sIgM density was seen. Following trypsin treatment and culture, the population with low sIgM density disappeared, for the most part, and only the normal sIgM density cells remained. Data not shown demonstrated that trypsin treatment effectively removed sIgM and sIgD from all B cells. We have thus scored as B cells only those lymphoid cells with normal ranges of sIg expression.

LP-BM5 Infection Rapidly Causes B Cell Polyclonal Activation and Hypergammaglobulinemia. Previous studies (5) demonstrated that serum IgM and IgG2a levels were elevated by 3 wk after virus inoculation, and that there were only modest elevations of other Ig isotypes during the course of the disease (5). Our data (Table IV) confirmed the elevation in serum IgM and the lack of significant increase in serum IgA levels. IgG3 levels, which were not examined in the previous study, were also significantly elevated. In addition, IgG1 levels were increased 1 wk after infection. These results for IgG1 levels contrast with those reported previously (5). The discrepancy between our results and the previous

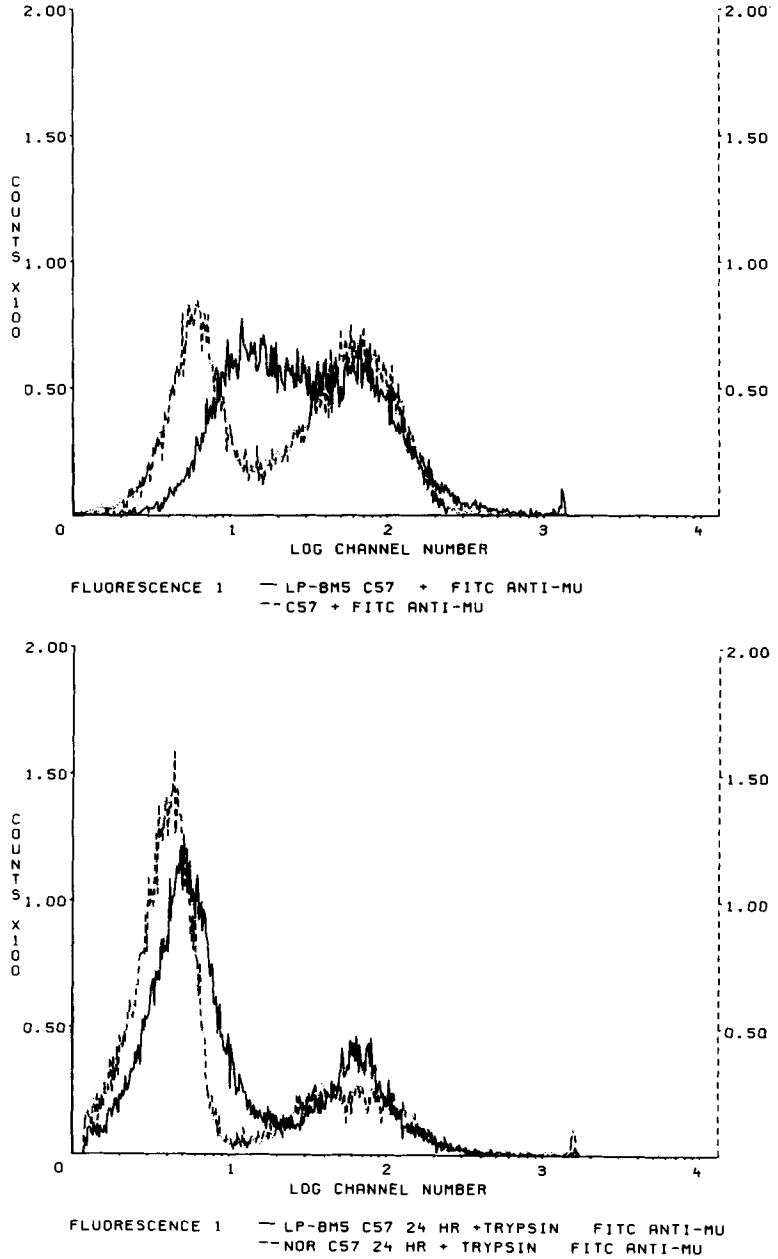

FIGURE 1. FACS histograms of spleen cells from LP-BM5 MuLV-infected (——) or normal (– – –) C57BL/6 mice stained with FITC-conjugated anti-μ antibodies. In the top panel is shown the sIgM density of freshly prepared spleen cells, while the lower panel displays the sIgM density following trypsin "stripping" of sIg and 24 h of culture to allow resynthesis. Note that the low-intensity sIgM staining of spleen cells from LP-BM5-infected mice is mainly due to reaction with cytophilic IgM.

TABLE IV
Ig Levels in LP-BM5 MuLV-infected C57BL/6 Mice

Strain and history of mice	IgM	IgG3	IgG1	IgA
		μg/ml		
Normal C57BL/6 (n = 3)	350 ± 36	485 ± 164	192 ± 26	87 ± 13
LP-BM5–infected C57BL/6				
1 wk postinfection (n = 3)	1,554 ± 1,070	998 ± 39	518 ± 136	ND
2 wk postinfection (n = 3)	1,681 ± 379	630 ± 157	2,335 ± 271	54 ± 20
3 wk postinfection (n = 3)	2,310 ± 420	1,040 ± 197	1,019 ± 162	97 ± 8

Concentrations of each isotype were determined in a competitive RIA with an mAb standard as described in Materials and Methods. Results are mean ±SEM. ND, not determined.

ones may reflect differences in assay techniques (see Materials and Methods), or differences in the mice used.

As a further measure of polyclonal activation, spleen cells for LP-BM5-infected mice were examined for IgM-secreting cells by the protein A–PFC assay (12). Within 1 wk of infection, the number of IgM-secreting cells had increased threefold over normal, and by 3 wk there was a 10-fold increase in IgM-secreting cells (data not shown). We conclude that viral infection led to an elevation in all Ig classes except IgA (or IgE, which has yet to be measured).

Immunosuppressive Effects of LP-BM5 Infection. To determine whether the lymphoproliferative disease and polyclonal B cell activation induced by LP-BM5 MuLV adversely affected immune responses, the immunocompetence of C57BL/6 mice that had been infected with this virus mixture was examined in a series of in vivo and in vitro assays of immunity.

The data presented in Table V demonstrate that by 4 wk following LP-BM5 infection, C57BL/6 mice were profoundly unresponsive to SRBC, a T cell–dependent antigen. At this time, the spleens of infected mice had increased in size two- to threefold, and contained large numbers of IgM-secreting cells. However, immunization of the mice in vivo, or of their spleen cells in vitro resulted in no SRBC-specific PFC response. The few SRBC-specific PFC that were seen in the virus-infected mice appear to be due to polyclonal B cell activation, since their numbers were unaffected by SRBC immunization. It can also be seen that a mixture of normal and virus-infected spleen cells gave an in vitro anti-SRBC response intermediate between that seen with either population alone. This result suggests that active suppression of responsiveness by T cells or continued viral replication was unlikely. This type of mixing experiment was extended to responses to mitogens and T-independent antigens (see below), and in no instance did we observe evidence of active suppression of the response generated by the normal spleen cell component.

We next examined the proliferative responses of spleen cells from LP-BM5-infected mice to T or B cell–specific mitogens. The response to PHA, Con A, LPS, and anti-μ were suppressed in both spleen and lymph node lymphocytes by 4 wk postinfection (data not shown). The time course of the onset of this suppression in spleen cells is shown in Fig. 2. The response to all four mitogens declined within 2–3 wk of infection. PHA and LPS responses diminished sooner

TABLE V

Infection of C57BL/6 Mice With LP-BM5 MuLV Depresses In Vivo and In Vitro Antibody Responses to SRBC

Mouse cells used	PFC/10^6 cells		PFC/spleen	
	SRBC	IgM	SRBC	IgM
In vivo				
LP-BM5 MuLV-infected C57BL/6 (SRBC-immunized)	<1	672 ± 13	220 ± 49	355,000 ± 21,850
LP-BM5 MuLV-infected C57BL/6 (unimmunized)	<1	795 ± 51	277 ± 58	419,000 ± 10,030
Normal C57BL/6 (SRBC-immunized)	184 ± 35	413 ± 23	35,117 ± 4,606	81,500 ± 9,262
In vitro			SRBC PFC/culture‡	
LP-BM5 MuLV-infected C57BL/6 (5×10^6)			0	
Normal C57BL/6 (5×10^6)			$440 \overset{\times}{\div} 1.03$	
LP-BM5 MuLV-infected and normal spleen cells, 1:1 mixture (5×10^6 total)			$247 \overset{\times}{\div} 1.02$	

* Numbers are the mean ±SEM of PFC determined using three mice per group.

‡ Numbers are the mean $\overset{\times}{\div}$SE factor of five replicate cultures containing 5×10^6 cells each.

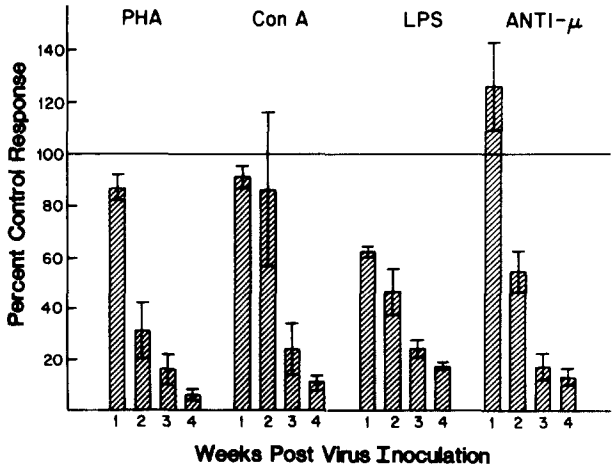

FIGURE 2. Proliferative responses of spleen cells from LP-BM5 MuLV-infected C57BL/6 mice were compared to responses of age- and sex-matched controls at weekly intervals following virus injection. Cells were stimulated with PHA, Con A, LPS, or goat anti-μ, and uptake of [^{3}H]thymidine was determined after 3 d of culture. Data were normalized to the control response for each mitogen for the duration of the experiment. Vertical bars represent the standard error of the mean.

than Con A or anti-μ responses. There was a brief but significant increase in the anti-μ–stimulated proliferative response of B cells 1 wk after infection with LP-BM5. The anti-μ response of lymph node B cells was still elevated 2 wk after infection, while LPS-stimulated mitogenesis diminished to 20% of control values (data not shown). Thereafter, the proliferative responses of lymph node cells declined in parallel with splenic responses.

In the same experiment, we examined the in vitro antibody response of spleen cells from normal or virus-infected mice to the T-independent antigens TNP-LPS, TNP-*B. abortus*, and TNP-Ficoll. By 2 wk after virus infection, the response to all three antigens was totally absent. However, as shown in Table VI, there was a temporary elevation of PFC responses to TNP-*B. abortus* and TNP-Ficoll 1 wk after virus inoculation. These increases were more apparent than real, however, since it is clear that a large number of both TNP-specific and polyclonal IgM PFC were generated independently of antigen addition. The polyclonal activation induced by LP-BM5, thus, seems to initially augment antigen-driven immune responses, and then rapidly preempt or suppress them.

To further investigate the unresponsiveness of spleen cells from virus-infected mice, we studied the survival of IgM-secreting cells and total B cells in culture, following exposure to LPS. Serum-free medium (9) was used for this experiment rather than FCS-supplemented RPMI 1640, so that the only stimulus to B cells was the added LPS. Fig. 3, *A* and *B* show that the (initially higher) number of IgM-secreting splenic B cells in T cell–depleted cultures from virus-infected mice declined rapidly, with no response to LPS. By comparison, normal T-depleted spleen cells responded to LPS with a burst of IgM-secreting PFC. Total B cell survival (Fig. 3*A*) was similar between virus-infected and control groups for the first 2 d of culture, but thereafter, normal B cells increased in number, while B cells from virus-infected mice remained nearly constant in number. The failure of B lymphocytes from LP-BM5-infected mice to respond to mitogens or antigens in culture is, therefore, apparently not due to their inability to survive in short term culture, but instead must be related to some intrinsic defect in B cell activation induced by the virus. In contrast to the results of these short term

TABLE VI

Antibody Responses In Vitro Are Elevated 1 wk After LP-BM5 MuLV Infection

Antigen	PFC/10^6 cells			
	Normal spleen		LP-BM5–infected spleen	
	TNP	IgM	TNP	IgM
—	0	4,500	70*	11,200
TNP-LPS	108	10,100	124	12,800
TNP-*B. abortus*	82	6,100	158	7,100
TNP-Ficoll	150	4,600	274	11,000

* Underlined values are significantly greater than corresponding control groups. PFC assays were performed after 4 d of culture, and numbers are the geometric mean of five replicate cultures per group.

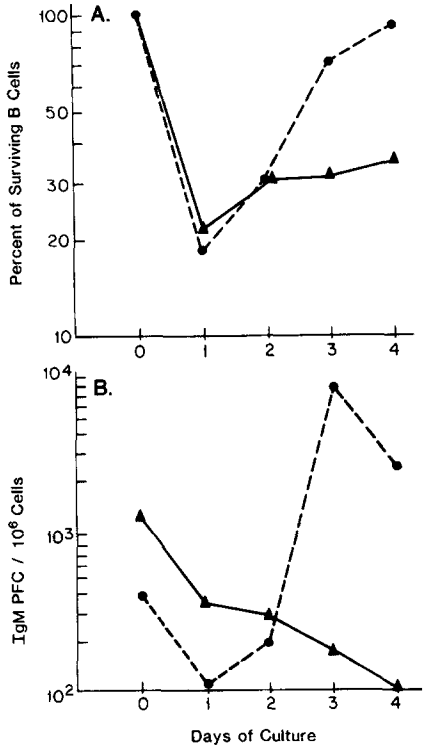

FIGURE 3. The survival (*A*) and IgM PFC response (*B*) of splenic B lymphocytes from LP-BM5-infected (▲——▲) or normal (●– – –●) C57BL/6 mice stimulated with LPS and cultured for 4 d in serum-free Iscove's/F12 medium. IgM-secreting cells were detected by the protein A–PFC assay (12). PFC numbers are per 10^6 cells initially cultured. Standard errors (not shown) were <10% of the mean.

experiments, B lymphocytes from virus-infected mice do not survive well in long-term culture (see below).

In further studies, spleen or lymph node cells from C57BL/6 mice (infected 6 wk previously with LP-BM5 MuLV) were stimulated in a one-way MLR with allogeneic BALB/c spleen cells, or with syngeneic spleen cells from normal or virus-infected mice. We also compared the ability of normal and virus-infected C57BL/6 spleen cells to stimulate MLR by BALB/c spleen cells. The results of one such experiment are shown in Table VII. Spleen cells from virus-infected mice were unresponsive in the MLR, while lymph node cells generated a very meager response. In addition, the ability of irradiated spleen cells from LP-BM5-infected mice to function as stimulators for an MLR by BALB/c spleen cells was diminished by ~50%. In additional studies (not shown), we examined the ability of spleen cells from LP-BM5-infected mice to respond in MLR at 2, 3, and 4 wk postinfection. We found modest elevation of MLR in some mice at 2 wk after viral infection, but all mice showed decreased MLR by 3 wk, and no detectable MLR by 4 wk postinfection.

The ability of spleen cells to generate CTL in MLR was also studied. Spleen cells from normal C57BL/6 mice or C57BL/6 mice infected with LP-BM5

TABLE VII
LP-BM5 MuLV Infection Depresses Allogeneic-MLR

Responder cells	Stimulator cells (given 1,500 rad)	[^{3}H]Thymidine incorporation*
		cpm × 10^{-3}
Normal C57BL/6 spleen (H-2^b)	C57BL/6 spleen (H-2^b)	13.8
	LP-BM5–infected C57BL/6 spleen (H-2^b)	4.8
	BALB/c spleen (H-2^d)	94.7
LP-BM5–infected C57BL/6 spleen	C57BL/6 spleen (H-2^b)	1.6
	LP-BM5–infected C57BL/6 spleen (H-2^b)	2.0
	BALB/c spleen (H-2^d)	2.0
LP-BM5–infected C57BL/6 lymph node	C57BL/6 spleen (H-2^b)	2.1
	LP-BM5–infected C57BL/6 spleen (H-2^b)	1.1
	BALB/c spleen (H-2^d)	7.1
Normal BALB/spleen (H-2^d)	C57BL/6 spleen (H-2^b)	37.5
	LP-BM5–infected C57BL/6 spleen (H-2^b)	19.9
	BALB/c spleen (H-2^d)	4.9
	C$_3$H spleen (H-2^k)	43.0

* [^{3}H]Thymidine incorporation following a 4-h pulse on the fifth day of culture. Standard errors (not shown) were ±20% of the mean.

TABLE VIII
CTL Activity of LP-BM5 MuLV–infected C57BL/6 (H-2^b) Spleen Cells After In Vitro Stimulation With BALB/c (H-2^d) Cells

Time postinfection	CTL activity* against target cells		
	H-2^d	H-2^k	H-2^b
wk			
3	1,092 ± 323	101 ± 46	7 ± 7
7	29‡	0	0
16	8	0	0
Normal	545 ± 257	34 ± 25	0

* In CTL lytic units, calculated as described in 10.
‡ Only one of three mice gave a weak CTL response.

MuLV 3, 7, or 16 wk previously were stimulated for 5 d in culture with H-2^d BALB/c cells. The recovered cells were assayed for their ability to lyse ^{51}Cr-labeled Con A-induced blasts of H-2^d, H-2^k, or H-2^b origin. Assays were set up at multiple effector/target ratios, and the net CTL activity calculated as previously described (10). At 3 wk postinfection, there was an elevation of CTL activity (Table VIII), followed by a virtual disappearance of CTL activity 7 wk after LP-BM5 infection. The elevated CTL activity early after infection was also manifested as enhanced crossreactive killing of H-2^k targets following H-2^d stimulation.

Taken together, these results show that T cell function is rapidly impaired following LP-BM5 MuLV infection, and that T cell mitogen responses are lost

before MLR, and MLR before CTL responses. As with some B cell responses, it also appears that the virus mixture can temporarily elevate some T cell responses early in the course of the lymphoproliferative disease.

Mechanisms of Immunosuppression in LP-BM5-induced Lymphoproliferative Disease. Injection of the LP-BM5 MuLV mixture induces expansion of the B lymphocyte lineage, T cell blast formation, and profound suppression of humoral and cellular immunity. One possible explanation for the ablation of T and B lymphocyte function is that the LP-BM5 virus mixture is directly immunosuppressive. For example, the p15E viral envelope protein of several mammalian retroviruses has been reported to suppress in vitro lymphocyte function (19–22). Such a direct immunosuppressive mechanism seems unlikely, since a mixture of normal and virus-infected spleen cells should have contained replicating virus (R. Yetter and H. C. Morse, unpublished observations), yet the expected response of the normal spleen cell component was unimpaired (Table VI). We nonetheless chose to address this question directly by adding virus-containing culture supernatants to normal C57BL/6 spleen cells. As shown in Table IX, there was only marginal suppression of B cell proliferative responses to LPS and anti-μ at the highest concentration of virus, and no suppression of T cell mitogenesis. Virus-containing supernatants failed to suppress in vitro PFC responses to SRBC, TNP-LPS, TNP-*B. abortus*, or TNP-Ficoll (data not shown). It thus seems unlikely that the LP-BM5 MuLV mixture is directly immunosuppressive for T or B cell function during short term in vitro experiments.

An alternative explanation for the immunosuppressive effects of LP-BM5 is that the viral infection leads to the generation of activated suppressor T cells. In an attempt to rule out T cell–mediated suppression, we eliminated T cells from LP-BM5-infected spleen cells by two rounds of treatment with anti-Thy-1 plus anti-Ly-2 plus complement, to see if the remaining B cells recovered function. These T-depleted spleen cells still were unable to respond to B cell mitogens or T-independent antigens (data not shown), so lack of B cell function is unlikely to be due to T suppressor activity.

TABLE IX

Addition of LP-BM5 MuLV Directly to Normal C57BL/6 Spleen Cells In Vitro Does Not Induce Immunosuppression

Treatment of C57BL/6 spleen cells	[^{3}H]Thymidine incorporation in response to mitogen*				
	0	LPS	anti-μ	PHA	Con A
			cpm $\times 10^{-3}$		
control medium (virus cleared)	11.8 $\overset{\times}{\div}$ 1.16	186.6 $\overset{\times}{\div}$ 1.03	68.7 $\overset{\times}{\div}$ 1.03	211.4 $\overset{\times}{\div}$ 1.01	264.2 $\overset{\times}{\div}$ 1.03
50% LP-BM5 supernatant	8.4 $\overset{\times}{\div}$ 1.05	169.7 $\overset{\times}{\div}$ 1.02	57.0 $\overset{\times}{\div}$ 1.02	226.5 $\overset{\times}{\div}$ 1.03	276.5 $\overset{\times}{\div}$ 1.03
25% LP-BM5 supernatant	9.2 $\overset{\times}{\div}$ 1.04	174.2 $\overset{\times}{\div}$ 1.06	64.6 $\overset{\times}{\div}$ 1.04	178.9 $\overset{\times}{\div}$ 1.13	256.4 $\overset{\times}{\div}$ 1.01
12.5% LP-BM5 supernatant	10.0 $\overset{\times}{\div}$ 1.06	180.4 $\overset{\times}{\div}$ 1.05	47.7 $\overset{\times}{\div}$ 1.08	202.4 $\overset{\times}{\div}$ 1.11	277.7 $\overset{\times}{\div}$ 1.01

* [^{3}H]Thymidine incorporated into DNA following a 4-h pulse. The geometric mean $\overset{\times}{\div}$SE factor of five replicate cultures is shown.

T suppressor cells could still have a more limited role in LP-BM5-induced immunosuppression in that they might decrease T helper cell function in virus-infected mice. We thus used nylon wool–passed T cells from normal or virus-infected mice to supply helper function for T-depleted spleen cells from either normal or LP-BM5-infected mice. A representative experiment is shown in Table X. T-depleted spleen cells (B cells plus macrophages) were prepared from normal C57BL/6 mice, and C57BL/6 mice 6 wk postinfection, as noted above. Nylon wool–passed spleen cells were used as a source of T cells, but note that the recovery of T cells from spleens of LP-BM5-infected mice was substantially less than that from normal spleens. Two points emerged from this experiment. First, normal T cells were unable to reconstitute either the SRBC-specific response or the polyclonal response of LP-BM5-infected B cells and macrophages. Second, the small number of nylon wool–passed T cells recovered from virus-infected mice, when mixed with normal T-depleted spleen cells, yielded meager helper activity for the SRBC response, but did support an elevated polyclonal IgM response (1,407 vs. 690 IgM PFC per 10^6 cells). These studies thus show that B cells from LP-BM5-infected mice are unresponsive to normal T cell–inductive signals. In addition, there is the suggestion that some T cells from virus-infected mice have an enhanced capacity for the polyclonal activation of normal B lymphocytes.

In a final attempt to demonstrate the extent of virus-induced immunoincompetence, we added exogenous IL-2-containing medium to determine if it could reverse the functional defects induced by LP-BM5 infection. As shown in Table XI, the addition of IL-2-containing Con A supernatant from normal spleen cells failed to restore the proliferative response of spleen cells from LP-BM5-infected mice to T or B cell mitogens. In similar experiments (not shown), Con A supernatants were unable to restore in vitro PFC responses of LP-BM5 spleen cells. Thus, immunosuppression induced by the LP-BM5 virus mixture is not readily reversible.

LP-BM5 MuLV Infection Does Not Lead to Immortalized B Lymphocytes. We

TABLE X

LP-BM5 MuLV Infection Makes B Cells Unresponsive to Normal T Cell Help

Source of B cells and macrophages	PFC* against	Source of T cells‡	
		Normal mice	LP-BM5–infected mice
Normal mice	SRBC	78	12
	IgM	690	1,407§
LP-BM5–infected	SRBC	0	0
	IgM	40	25

* Numbers are PFC/10^6 B cells and macrophages, and represent the geometric mean of five replicate cultures per group.

‡ 2×10^6 nylon wool–passed T cells were added to 5×10^6 T-depleted spleen cells (B cells plus macrophages) and SRBC- and IgM-specific PFC determined after 4 d of culture.

§ Mean is significantly higher than corresponding group with normal T cells ($P < 0.01$).

TABLE XI

Interleukin 2 (IL-2)-containing Con A Supernatant Fails to Reverse Immunosuppression in LP-BM5 MuLV–infected C57BL/6 Mice

Cells	Con A supernatant*	[^{3}H]Thymidine incorporation in response to mitogen‡			
		0	PHA	Con A	LPS
		cpm × 10^{-3}			
LP-BM5–infected spleen	−	1.2 ×/÷ 1.13	1.6 ×/÷ 1.09	3.3 ×/÷ 1.05	11.8 ×/÷ 1.03
	+	3.0 ×/÷ 1.05	3.0 ×/÷ 1.07	5.0 ×/÷ 1.03	5.1 ×/÷ 1.19
Normal spleen	−	4.2 ×/÷ 1.17	104.3 ×/÷ 1.09	128.0 ×/÷ 1.06	122.4 ×/÷ 1.06
	+	7.2 ×/÷ 1.15	107.7 ×/÷ 1.05	132.2 ×/÷ 1.03	58.9 ×/÷ 1.10

* Con A supernatant was shown to contain IL-2 by its ability to support the proliferation of the IL-2-dependent HT-2 line. Con A supernatant was added up to 50% in this experiment. Residual Con A activity was blocked by the addition of 20 mM α-methylmannoside.

‡ [^{3}H]Thymidine incorporation expressed as geometric mean ×/÷ SE.

isolated spleen cells, lymph node cells, and bone marrow cells from LP-BM5-infected C57BL/6 mice and placed them in tissue culture under a wide variety of culture conditions. In no case did mature sIg$^+$ B lymphocytes survive for more than 7–8 d. In contrast, normal C57BL/6 B lymphocytes were able to survive for up to 6 wk in culture. Only the bone marrow cultures became established lines; these lines were identical to the ones previously established (2) in that they contained a predominant stromal cell component and continued to replicate LP-BM5 MuLV. We also added LP-BM5 directly to long-term cultures established from normal lymphoid tissue. The addition of LP-BM5 was without effect on spleen and lymph node cultures, but normal bone marrow could be infected in vitro with LP-BM5 to yield lines that were morphologically indistinguishable from in vivo–derived bone marrow lines, and which continued to replicate virus. LP-BM5 MuLV, then, does not lead to the immortalization of any lymphoid cells, and its role in promoting the survival of bone marrow stromal cell elements is obscure.

Discussion

The disease induced by the injection of the LP-BM5 mixture of ecotropic and MCF MuLV into adult C57BL/6 mice has several features which make it unique among retroviral infections of mice. First, the LP-BM5 MuLV–induced disease seems clearly to be a lymphoproliferative process, as first postulated by Pattengale et al. (5), and not a malignant B cell lymphoma. There was no evidence from our studies or from the earlier studies that B cells from LP-BM5-infected mice were transformed for malignant growth in vitro or in vivo. We have provided direct evidence (Table III) for the expansion of lymphocytes, macrophages, and myeloid elements during the course of the disease, and we have shown that both T and, to a greater extent, B lymphocytes are actively proliferating, so that the lymphoproliferative nature of the disease is thus confirmed. A second unique feature was that adult mice were susceptible to the lymphoproliferative disease;

neonatal injection of MuLV was not required for disease induction. Third, major manifestations of the disease process, namely lymphoproliferation and immunosuppression, began with virtually no latency period. Fourth, the degree of immunosuppression was remarkable for a MuLV infection, with virtually no immune function detectable by 4 wk postinfection. Fifth, the mechanism of immunosuppression may be unique, particularly within the B cell lineage, where most cells were involved in a polyclonal activation process with eventual maturation to antibody-secreting plasmablasts. Finally, the mechanism of immunosuppression may depend upon a T cell–dependent pathway of B lymphocyte activation, since C57BL/6 *nu/nu* mice developed a much-attentuated form of the disease, with a longer latency period, increased IgM but not IgG levels, and preservation of some normal B cell functions (D. E. Mosier, unpublished observations).

The major novel observations we present here relate to the rapid onset and profound consequences of LP-BM5-induced immunosuppression. We have demonstrated a rapid loss of T lymphocyte blastogenic responses to mitogens and alloantigens, a somewhat delayed loss (following an initial increase) in cytotoxic T cell generation, and a loss of helper T cell function for antibody production in vitro. There is a concomitant loss of B lymphocyte function, with diminished proliferative responses to B cell mitogens, and inability to generate specific antibody responses to T-dependent or T-independent antigens. We found no evidence that the LP-BM5 MuLV mixture was directly immunosuppressive in vitro (Table X), nor was there any suggestion that T suppressor activity was enhanced by virus infection. In view of the evidence that most or all mature lymphocytes are involved in the LP-BM5-induced lymphoproliferative process, the simplest explanation for the profound immunosuppression is that specific immune responses are preempted by polyclonal lymphocyte activation. The preliminary experiments in C57BL/6 *nu/nu* mice cited above suggested that functional T cells were required for the full development of the LP-BM5-induced disease, so the polyclonal activation of B lymphocytes may represent a collaborative process between T and B cells.

The virus mixture that induces this immunosuppressive lymphoproliferative disease (which we have renamed the LP-BM5 isolate, but which was referred to as RCN-BM5 in previous publications) was derived from bone marrow stromal cell cultures established by Haas and Meshorer (2) from virus-inoculated C57BL/6 mice. The in vivo–passaged MuLV used to inoculate the donor of the bone marrow cell line was a splenic cell–free extract of the viral agent described by Laterjet and Duplan (1). This viral agent was originally derived from C57BL/6 mice that had received fractionated, low-dose irradiation (23), but it was unique in that it caused RCN instead of the more common thymic lymphomas. It has been reported (3) that both the LP-MB5 MuLV mixture and a biologically cloned dualtropic MuLV [termed M(BC4)] could induce lymphoproliferative disease, but it now seems likely that the ecotropic/MCF MuLV mixture is required for disease induction (M. Haas, personal communications, and R. A. Yetter and H. C. Morse, unpublished observations). While much work remains to be done on the interaction of MuLV during the induction of this lymphoproliferative disease, it may be extremely important for understanding murine or human disease to

realize that ongoing interactions among retroviruses may increase their immunosuppressive and lymphoproliferative effects.

Several recent studies (19–22, 24) have suggested that retroviral structural components (particularly p15E) may be directly immunosuppressive. Since LP-BM5 appears not to be directly immunosuppressive in vitro, p15E-mediated suppression seems unlikely. It should also be emphasized that the degree and rapidity of onset of the immunosuppression induced in mice by the LP-BM5 MuLV mixture is much more striking than that induced by Friend, Moloney, or Rauscher MuLV (25, 26), where p15E is thought to play an active role (24).

The lymphoproliferative disease induced by LP-BM5 MuLV resembles, in some respects, two human disease states. The first is the X-linked lymphoproliferative syndrome described by Purtilo et al. (27). This disease appears to be the result of Epstein-Barr virus infection of genetically immunodeficient individuals, who then develop a fatal expansion of polyclonally activated B lymphocytes. The histopathological features of this disease are similar to the LP-BM5 MuLV–induced disease, but the biology of LP-BM5 infection differs, in that an immunosuppressive virus infects a normal host, as opposed to an already immunocompromised host. The second human disease which the lymphoproliferative state induced by the LP-BM5 MuLV mixture resembles is the early phases of the acquired immunodeficiency syndrome (AIDS) when lymphadenopathy (28), hypergammaglobulinemia, and polyclonal B cell activation (29) are prominent features. Despite the profound immunosuppression associated with LP-BM5 infection, most mice die of respiratory failure due to mediastinal lymph node enlargement, and not of intercurrent infections. Perhaps the latter stages of LP-BM5 induced disease would more resemble AIDS if the mice were exposed to the range of pathogens associated with the human population at risk for AIDS. Despite this divergence in the clinical course between AIDS and LP-BM5-induced lymphoproliferation, the early similarities in the two diseases and the common retroviral etiology suggest that a further understanding of the biology of the murine disease will be enlightening.

Summary

We have shown that a mixture of murine leukemia viruses (MuLV) causes the acute onset of lymphoproliferation and immunosuppression when injected into adult C57BL/6 mice. The ecotropic/MCF (mink cell focus–inducing) mixture of MuLV stimulates polyclonal B lymphocyte proliferation and differentiation to antibody-secreting cells. Serum Ig levels are elevated for all isotypes except IgA. The viral infection leads to a rapid decline in T lymphocyte responses to mitogens and alloantigens, as well as a decrease in helper cell activity. Specific antibody responses to both T-dependent and T-independent antigens are impaired, and the response of B lymphocytes to mitogens is abolished. The profound immunosuppression seems to be due to the MuLV-induced polyclonal activation of lymphocytes. No active suppression of normal lymphocyte responses by cells from virus-infected mice was observed. The disease induced by the LP-BM5 MuLV isolate thus seems a promising model for the study of lymphocyte activation and the mechanisms of retrovirus-induced immunosupppression.

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