ENDOGENOUS RETROVIRUS EXPRESSION IN STIMULATED MURINE LYMPHOCYTES

Identification of a New Locus Controlling Mitogen Induction of a Defective Virus

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Hybridization data indicate that germ line DNA from all mouse strains contains numerous endogenous retroviruses (1). In vivo expression of viral proteins and complete infectious viruses is variable and both mouse strain and tissue specific (2). Studies of the control of endogenous virus expression might be expected to provide insight into both the role of endogenous viruses in leukemogenesis and gene regulation in eukaryotic cells.

A number of in vitro systems for induction of endogenous viruses have been described (reviewed in reference 3). The best studied of these systems is the addition of the halogenated pyrimidine derivatives bromodeoxyuridine (BrdU)[1] and iododeoxyuridine (IdU) to mouse fibroblasts (4), treatments that have been shown to induce both ecotropic and xenotropic viruses. Induction is apparently dependent on BrdU or IdU incorporation into cellular DNA (5). It has been shown that ecotropic virus inducibility maps to different chromosomes in different strains of mice (6–8). In contrast, xenotropic virus induction is controlled by an allelic locus on chromosome 1 in several strains of mice (9, 10).

We are interested in the control of retroviral expression in lymphocytes, the cells normally transformed by C-type viruses. It has been shown that lymphoid cells activated in a graft versus host reaction produce endogenous virus probably solely of xenotropic host range (11). Similarly, it has been found that the activation of fresh, resting splenocytes in vitro with some B cell mitogens results in xenotropic virus induction (12, 13). Only B cell mitogens, such as lipopolysaccharide (LPS) and lipoprotein (LP), which are capable of stimulating B cells to differentiate into immunoglobulin-secreting plasma cells, induced virus; mitogens that stimulate only T cells did not (14, 15). If BrdU was added to cultures treated with inducing mitogens, a 5 to 15-fold increase in virus production as measured by reverse transcriptase was observed (14, 15). In contrast, BrdU addition to cells stimulated with noninducing mitogens did not result in virus release (14) even though BrdU becomes incorporated (16). Ecotropic virus production by mitogen-stimulated lymphocytes was not observed

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[1] *Abbreviations used in this paper:* BrdU, 5-bromo-2′-deoxyuridine; Con A, concanavalin A; FFU, focus-forming unit; IdU, 5-iodo-2′-deoxyuridine; LP, lipoprotein *E. coli*; LPS, lipopolysaccharide *E. coli* (0111:B4); TMP, thymidine monophosphate.

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(13), except with mouse strains such as AKR which spontaneously produce ecotropic virus.[2]

The differential effects of BrdU treatment on fibroblasts, B cells, and T cells suggest that virus inducibility is dependent on the cell differentiation stage. The observation that BrdU appears to amplify xenotropic virus production from B cells rather than inducing it as in fibroblasts raises the question whether the mechanism of BrdU action on xenotropic virus expression is the same in B cells and fibroblasts. To examine this question we have taken a genetic approach to ask whether LPS and BrdU plus LPS in fact induce one and the same virus, measured both in infectivity assays and by reverse transcriptase, and whether this is the same virus induced from fibroblasts. Furthermore, we have asked whether additional proviruses are induced. The results presented here confirm the results of Kozak and Rowe (10) that infectious xenotropic virus induction by BrdU plus LPS from spleen cells is under the control of the same gene as in fibroblasts. In addition they show that induction by LPS, LP, LP/BrdU, and Con A/BrdU are controlled by the same genetic locus and that LPS induces the expression in lymphocytes of a second, independently segregating, endogenous genome coding for an apparently defective virus.

Materials and Methods

Mice. 1–2-mo old BALB/cTif, 129/RrJ (hereafter referred to as BALB/c and 129, respectively), and hybrid mice came from the Sisseln Tierfarm AG, Switzerland.

Spleen Cell Cultures. Spleen cells were cultured for 3 d as previously described (15) at 2 or 2.5 × 10^6 cells/ml in RPMI 1640 medium (Gibco Biocult, Glasgow, United Kingdom (UK)) supplemented with 20 mM Hepes (Microbiological Associates, Walkersville, MD), 100 IU/ml penicillin, 100 μg/ml streptomycin, 1.6 mM glutamine and, in most experiments, 8% fetal calf serum (batch K763101S; Gibco Biocult). LPS-W from *Escherichia coli* 0111:B4 (Difco Laboratories, Detroit, MI), LP (a generous gift of Dr. L. Tarcsay, Ciba-Geigy Ltd., Basel, Switzerland), or concanavalin A (Con A) (Miles-Yeda, Elkhart, IN) were added at the start of culturing to concentrations of 16, 80, and 4 μg/ml, respectively. BrdU (Calbiochem-Behring Corp., San Diego, CA) was routinely added to a concentration of 5 μg/ml 24 h later. Most batches of fetal calf serum (including K763101S) are weakly mitogenic and thus are inducers of xenotropic virus as measured by co-cultivation (17); however, serum also appears to stabilize induced virus (data not shown). Thus, in experiments to detect xenotropic virus by co-cultivation (see below), serum was omitted from spleen culture medium whereas in other experiments it was included.

Reverse Transcriptase Assay. Reverse transcriptase activity was measured as previously described (18). Briefly, duplicate aliquots of virus concentrated by ultracentrifugation from spleen cell culture supernatants, were incubated in 40 mM Tris HCl, pH 7.9, 60 mM KCl, 1 mM dithiothreitol, 1.5 mM manganese II acetate, 0.2 mM EDTA, 0.1% Nonidet P-40, 9 μM thymidine triphosphate, 1 μM methyl-[^{3}H]thymidine triphosphate (50 Ci/mmol; Radiochemical Centre, Amersham, UK), and 20 μg/ml oligo(dT)-poly(rA) as template primer, for 60 min at 30°C. In one set of experiments 20 μg/ml oligo(dT)-poly(dA) was used as a negative control for the polymerase activity. Trichloroacetic acid-precipitable counts were determined with 7,500 dpm corresponding to the incorporation of 1 pmol thymidine monophosphate (TMP). Values for reverse transcriptase are given as picomoles TMP incorporated by virus concentrated from 1 ml culture supernatant.

Antibody inhibition of reverse transcriptase activity was measured as previously described (19). In brief, virus-containing samples were incubated for 15 min at 4°C with 0.5% Nonidet P-40 in 80 mM Tris-HCl, 300 mM KCl and 20 mg/ml bovine serum albumin, pH 7.7. Then control or immune sera (antiserum against reverse transcriptase purified from Rauscher leukemia virus was a generous gift of Dr. R. C. Gallo, National Cancer Institute) at an appropriate dilution in 30 mM Tris was added and the incubation continued overnight.

[2] J. P. Stoye and C. Moroni. Manuscript submitted for publication.

Reverse transcriptase activity was then determined as described above.

Detection of Xenotropic Virus. Xenotropic virus was assayed using the mink S^+L^- cell line of Peebles (20). Mink cell lines were grown in Dulbecco's medium containing 10% fetal calf serum. 1 ml of culture supernatant or 1 ml medium with 1 to 2 × 10^6 3-d cultured spleen cells were added to 30% confluent S^+L^- cells in 6-cm plates and incubated for 2 h in the presence of 15 μg/ml polybrene (Aldrich-Europe, Beerse, Belgium). Dishes were then washed twice, removing almost all the spleen cells, and cultured for 5 d with one medium change. Xenotropic virus-induced foci were counted on unfixed cell preparations.

3-d spleen cell cultures contain a mixture of viable and nonviable cells. In some experiments viable cells, purified by flotation on bovine serum albumin gradients (21), were used for infection; data from these experiments is given as foci per 10^6 cells. The presence of dead cells did not affect the assay and since both the proportion and absolute numbers of viable (~25% of the starting cells) and nonviable spleen cells remained relatively constant from culture to culture, viable cells were not purified in the genetics experiments. Data from these experiments are expressed as foci per 5 × 10^6 starting cells (the number of cells present at the start of the 3-d spleen cultures).

Xenotropic virus production by stimulated lymphocytes was also detected following virus amplification by co-cultivation with mink CCL-64 cells. 5 × 10^5 stimulated spleen cells in 100 μl medium containing 15 μg/ml polybrene were added to CCL-64 cells growing in flat-bottomed microtiter plates (plated 1 d previously at 2 × 10^3 cells/well). After 2 h incubation the medium was removed, the plates vortexed, and the wells washed with 100 μl medium. The medium was changed on days 1 and 3 after infection, and on day 4 the cells were trypsinized and replated in 60 or 100-mm plates. The CCL-64 cells were grown to confluence and the medium changed. The next day 1-ml samples of supernatant were assayed in the S^+L^- assay for focus-forming units (FFU).

Pep-3 Isozyme Analysis. Kidney extracts were run on starch gels (Electrostarch Co., Madison, WI.) with a Tris-EDTA-borate pH 8.6 buffer for 16 h at 300 V as previously described (22). Gels were stained with l-leucyl alanine as substrate. Before analysis, kidneys were stored at −70°C and extracts were prepared by homogenization in 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 20 mM KCl, 10% glycerol, 0.5% Nonidet P-40, followed by centrifugation at 18,000 rpm for 30 min at 4°C in a Sorval SS-34 rotor.

Results

Detection and Quantitation of Xenotropic Virus Production by Stimulated Lymphocytes. In previous studies (13, 17, 23) co-cultivation techniques have been used to investigate the host range of the endogenous virus induced from lymphocytes. A more rapid alternative method for the detection of xenotropic virus is to use mink S^+L^- cells (20), in which xenotropic virus infection leads to sarcoma virus rescue and focus formation. To investigate the practicability of using this detection method in genetic studies, different quantities of viable 3-d LPS- and BrdU plus LPS-stimulated BALB/c spleen cells were tested for focus induction on S^+L^- cells. Table I shows that BrdU plus LPS-treated cells induce about 100 foci per 10^6 cells whereas LPS-stimulated cells give only about one focus per 10^6 cells. Focus induction by stimulated spleen cells follows one hit kinetics (data not shown). Addition of LPS-stimulated cells to BrdU plus LPS-treated cells does not cause inhibition of focus induction (data not shown). Thus, the S^+L^- assay offers a rapid (5 d) method for the detection of xenotropic virus induction by LPS/BrdU but does not provide a useful method for following induction by LPS alone. Detection of xenotropic virus induction by LPS requires co-cultivation of stimulated spleen cells with a susceptible cell line before assaying for xenotropic virus by the S^+L^- assay. After one passage with CCL-64 cells, between 100 and 400 FFU/ml were detected. No xenotropic virus was found in the supernatants of CCL-64 cells co-cultivated with LPS- or LPS-BrdU-treated 129 lymphocytes even after long-term

TABLE I

*Xenotropic Virus Infectious Centers in LPS and LPS/BrdU-treated Spleen Cell Cultures**

Experiment	LPS-treated cells		LPS/BrdU-treated cells	
	Foci/10^6 cells	Frequency	Foci/10^6 cells	Frequency
1	0.5	$1/2 \times 10^6$	125	$1/8 \times 10^3$
2	0.5	$1/2 \times 10^6$	48	$1/2 \times 10^4$
3	1.7	$1/6 \times 10^5$	154	$1/7 \times 10^3$
Average	0.9	$1/1 \times 10^6$	109	$1/9 \times 10^3$

* In each experiment viable cells were purified from 3-d cultures, the number of infectious centers on S^+L^- cells determined at five different spleen cell concentrations, and the number of S^+L^- foci per 10^6 spleen cells calculated and averaged.

TABLE II

*Ratio of Infectious Xenotropic Virus to Reverse Transcriptase Activity in BALB/c Spleen Cell Culture Supernatants**

	Reverse transcriptase	Xenotropic virus	Xenotropic virus / Reverse transcriptase
	pmol/ml	*FFU/ml*	
Control	0.04 ± 0.01	0	0
Control/BrdU	0.14 ± 0.04	7.1 ± 2.9	51
LPS	0.12 ± 0.06	0.4 ± 0.1	3
LPS/BrdU	1.72 ± 0.38	99.9 ± 29.9	58

* Values shown are mean ± SD from three experiments. In each experiment cell-free supernatants from 3-d cultures were assayed for reverse transcriptase (duplicate 10-ml samples) and xenotropic virus in the S^+L^- assay (8 1-ml samples).

passaging.

Infectious Xenotropic Virus in BALB/c Spleen Cell Culture Supernatants. The observation that BrdU increased 100-fold the number of xenotropic virus infectious centers in LPS-stimulated cultures (Table I) but only amplified reverse transcriptase production by a factor of 5–15 (18 and see below) suggested the possible presence of a reverse transcriptase-positive but noninfectious virus in LPS supernatants. Comparison of infectious virus and reverse transcriptase activity in culture supernatants provided further evidence for this possibility. Control/BrdU and LPS-treated spleen cells produced similar amounts of reverse transcriptase activity but the former cultures had >15 times as much infectious virus as measured in the S^+L^- assay (Table II). The fetal calf serum in the spleen cell cultures was weakly mitogenic, presumably permitting some BrdU incorporation and virus induction in the absence of LPS (17). The ratio of infectious virus to reverse transcriptase in both control/BrdU and LPS/BrdU was similar even though much more reverse transcriptase activity was detected in the latter cultures.

Genetic Control of Xenotropic Virus Induction from Lymphocytes. Virus inducible BALB/c mice and noninducible 129 mice were crossed and spleen cells from 20 of the progeny tested separately for xenotropic virus induction by BrdU plus LPS. Stimulated spleen cells from all these mice gave results indistinguishable from BALB/c, i.e., LPS/BrdU-treated cells induced ~100 foci on S^+L^- cells per 10^6 spleen cells. Inducibility was therefore the dominant phenotype and no gene dosage effect was observed.

F_1 mice were backcrossed to 129 mice and 229 of the progeny tested for induction with BrdU plus LPS. Half of these mice proved inducible, which indicates that one gene controls induction by BrdU plus LPS. A similar conclusion was reached from an analysis of 64 F_2 mice, where 77% of the mice were found inducible. No difference was observed with respect to inducibility between male and female backcross mice and the direction of the crosses was also without effect. Data on induction by BrdU plus LPS with spleen cells from individual mice is summarized in Table III.

LPS-stimulated spleen cells from a number of backcross mice were also tested for induction by co-cultivation with CCL-64 cells followed by assaying for xenotropic virus in the S^+L^- assay to examine whether LPS induction was under the same genetic control as LPS/BrdU. A near-perfect correlation was observed between induction with LPS/BrdU and by LPS alone (Table IV). Similar results were

TABLE III

*One Gene Controls LPS/BrdU Induction in BALB/c × 129 Mice**

	Number positive/ number tested	Percent positive
BALB/c	35/35	100
129	0/20	0
(BALB/c × 129)F_1	20/20	100
(BALB/c × 129)F_1 × 129	129/229	53
(BALB/c × 129)F_1 × (BALB/c × 129)F_1	49/64	77

* Spleen cells from 3-d cultured LPS/BrdU-treated cultures were tested for focus induction in the S^+L^- assay.

TABLE IV

*Correlation of Inducibility with LPS/BrdU and Other Induction Protocols in (BALB/c × 129)F_1 × 129 Mice**

Induction method	LPS/BrdU-inducible mice (number inducible/number tested)	LPS/BrdU-noninducible mice (number inducible/number tested)
LPS	59/61	0/55
LP	28/31	0/30
LP/BrdU	11/11	0/19
Con A/BrdU	10/10	0/10

* Spleen cells from 3-d cultures were tested for xenotropic virus induction by the S^+L^- assay (:PS/BrdU, LP/BrdU, Con A/BrdU) or by co-cultivation (LPS, LP).

TABLE V

*Linkage Between LPS/BrdU Induction and Pep-3 in (BALB/c × 129)F_1 × 129 Mice**

Nonrecombinant phenotype‡		Recombinant phenotype		Recombinants/ total	Percent recombinants
$V^+Pep\text{-}3^{a/b}$	$V^-Pep\text{-}3^b$	$V^+Pep\text{-}3^b$	$V^-Pep\text{-}3^{a/b}$		
51	42	9	16	25/118	21

* 3-d LPS/BrdU-treated spleen cells were tested for focus induction on S^+L^- cells. Kidney extracts from these mice were tested by starch gel electrophoresis for their Pep-3 phenotype.
‡ V^+, virus inducible; V^-, noninducible.

obtained with a second B cell mitogen, LP (Table IV). Furthermore, induction with LP/BrdU and Con A/BrdU using the direct S^+L^- focus induction test correlated with induction by LPS/BrdU (Table IV). Thus, it appears that induction of xenotropic virus from lymphocytes by a number of different treatments is under identical genetic control.

To examine whether this inducibility gene, like the fibroblast gene, is located on chromosome 1, kidney extracts from these mice were tested by starch gel electrophoresis for the isozymes of Pep-3. Linkage was observed between the BALB Pep-3 isozyme and virus inducibility with a recombination frequency of ~21% (Table V).

Comparison of Reverse Transcriptase and Xenotropic Virus Induction in First Backcross Mice. In a number of experiments, each with 16 backcross mice, supernatants from LPS/BrdU-stimulated spleen cell cultures were tested for reverse transcriptase activity in addition to the routine infectious center assay for xenotropic virus. The reverse transcriptase values from cultures scoring positive in the S^+L^- test (i.e., producing xenotropic virus) were averaged and this value corresponds to 100% on the abscissa of Fig. 1. Values from individual first backcross mice were normalized and are expressed as percentages of this 100% value, plotted as a histogram (Fig. 1, C and D). Values from 129 (Fig. 1 A) and BALB/c mice (Fig. 1 B) are shown for comparison. The BALB/c values show a distribution around 100%, the 129 values scatter around 2–

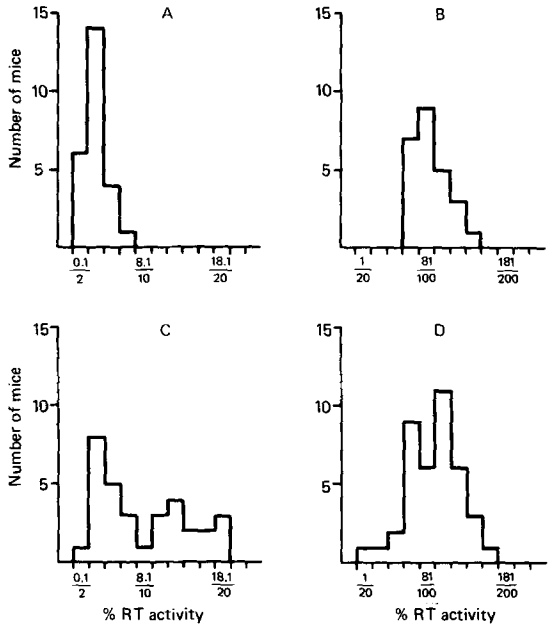

FIG. 1. Reverse transcriptase (RT) induction in BALB/c, 129 and (BALB/c × 129)F_1 × 129 mice. Virus induction was measured both by reverse transcriptase and by S^+L^- focus induction in LPS/BrdU-treated spleen cell cultures from ~90 backcross mice. Reverse transcriptase values were normalized as described in the text and histograms plotted to illustrate the distribution of the reverse transcriptase values from backcross mice. Backcross mice scoring negative (C) and positive (D) for infectious xenotropic virus in the S^+L^- assay. Values from 25 129 (A) and 25 BALB/c (B) mice are shown for comparison. Note the different scales in A and C compared to B and D. The 100% value in the abscissa corresponds to the averaged value from all backcross mice scoring positive in the S^+L^- test (i.e., producers of xenotropic virus). The units on the abscissa represent intervals, e.g., (A) 14 mice showed values between 2.1 and 4.0 of the normalized 100% value.

4%. Backcross mice scoring positive in the S^+L^- assay have, as expected, high levels of reverse transcriptase activity (Fig. 1 D). Mice scoring negative in the S^+L^- assay show a biphasic distribution and appear to fall into one of two equally sized categories (Fig 1 C), one with background levels of incorporation comparable to 129 mice, a second with between 10 and 20% of the reverse transcriptase activity seen with xenotropic virus-positive mice. This finding, suggesting the presence of an independently segregating gene for a defective retrovirus, is consistent with the proposition that two retroviruses are induced from BALB/c mice.

Characterization of Reverse Transcriptase Activity Induced in First Backcross Mice. To confirm the retroviral specificity of the polymerase activity identifying the putative defective retrovirus, concentrated supernatants were assayed in the presence of anti-reverse transcriptase and with two different template primers, oligo(dT)-poly(rA) and digo(dT)-poly(dA). One 5-ml LPS/BrdU-treated and one 20-ml LPS- or LPS/BrdU-treated culture were set up from spleen cell suspensions of 32 individual first backcross mice. 3 d after culture initiation the cells and supernatants from the 5-ml cultures were taken for determination of infectious centers and reverse transcriptase. The medium from the 20-ml cultures was harvested and stored at −70°C. On the basis of the results from analysis of the 5-ml cultures, nine supernatants were then chosen for further analysis; virus was concentrated by ultracentrifugation and the reverse transcriptase activity analysed. The data from the preliminary analysis of the nine mice chosen for further analysis are summarized in Table VI. The first three cultures were positive for xenotropic virus and reverse transcriptase; the next three apparently had reverse transcriptase activity but no xenotropic virus and the last three had no signs of virus induction. Concentrated supernatants from the 20-ml cultures of these nine mice were then assayed after incubation with control serum and anti-reverse transcriptase serum as well as with the two different template primers oligo(dT)-poly(rA) and oligo(dT)-poly(dA) (Table VII). Supernatants from cultured spleen cells of mice 1 and 2, positive for xenotropic virus induction by LPS/BrdU, contained an activity that was inhibited by anti-reverse transcriptase and preferred oligo(dT)-poly(rA) to

TABLE VI

*Virus Induction in Nine (BALB/c × 129)F_1 × 129 Mice**

Animal	Xenotropic virus (Foci/5 × 10^6 starting cells)	Reverse transcriptase‡
1	>100	117
2	>100	121
3	>100	68
4	0	32
5	0	22
6	0	16
7	0	6
8	0	3
9	0	6

* Characterization of virus induction from LPS/BrdU-treated cultures of nine individual mice from which the reverse transcriptase was further examined (see Table VII).

‡ Expressed as a percentage of the average value from cultures scoring positive in the S^+L^- assay.

oligo(dT)-poly(dA). Similar qualitative results were obtained with the supernatants of lymphocytes from mice 4, 5, and 6 even though no xenotropic virus was induced from these cells. No significant difference was observed between mouse 4 and mice 5 and 6 even though the supernatant from mouse 4 came from LPS/BrdU-treated cells whereas 5 and 6 came from LPS-stimulated cells. One LPS supernatant showed no reverse transcriptase activity even though LPS/BrdU induced xenotropic virus (mouse 3). No reverse transcriptase activity was detected in the supernatants of stimulated cells from mice 7, 8, and 9. These data confirm that both induced polymerases have the properties of reverse transcriptase, supporting the argument that two different retroviruses are induced. We propose naming the locus that controls defective virus induction Bdv-1 by analogy with the locus controlling BALB/c xenotropic virus, Bxv-1 (10). The two viruses will now be referred to as Bxv and Bdv. The data in Table VII also provide support for the hypothesis that LPS and BrdU might exert differential effects on the two genes, with LPS inducing primarily the noninfectious virus (compare mouse 3 with mouse 5 or 6) and BrdU acting primarily on the infectious virus (compare mice 1 and 3 with 4 and 5). This hypothesis was further examined using second backcross mice, where we asked whether the two phenotypes bred true (i.e., low or no reverse transcriptase with LPS, but xenotropic virus-associated reverse transcriptase with LPS/BrdU [Bxv], and intermediate levels of reverse transcriptase with both LPS and LPS/BrdU in the absence of xenotropic virus [Bdv]).

Differential Regulation of Bxv-1 and Bdv-1 in Second Backcross Mice. 16 male (BALB/c × 129)F_1 × 129 mice were each mated with 4 female 129 mice and then spleen cultures from the males were tested for induction with LPS and LPS/BrdU. Two sets of litters were selected for further study on the basis of their fathers' phenotype, one the offspring of mouse 4 that apparently carried only Bxv-1, the other of mouse 14 which carried only Bdv-1. A number of the progeny of these two mice were analyzed for induction of reverse transcriptase activity by LPS and LPS/BrdU and also xenotropic virus by LPS/BrdU. Six out of nine of the tested progeny of mouse 4 (Bxv^+) were positive for xenotropic virus induction with LPS/BrdU and showed high

TABLE VII

*Characterization of Polymerase Activity in (BALB/c × 129)F_1 × 129 Mice**

Animal	Polymerase induced with:	Antisera:	pmol TMP incorporated/ml			
			—	—	Anti-reverse transcriptase	Control serum
		Template primer:	dT-dA	dT-rA	dT-rA	dT-rA
1	LPS/BrdU		0.14	2.42	0.15	5.70
2	LPS/BrdU		0.15	3.10	0.20	7.19
3	LPS		0.04	0.12	0.07	0.22
4	LPS/BrdU		0.04	0.49	0.06	0.91
5	LPS		0.09	0.40	0.05	0.63
6	LPS		0.06	0.40	0.08	0.94
7	LPS/BrdU		0.06	0.09	0.06	0.14
8	LPS		0.05	0.08	0.08	0.12
9	LPS		0.05	0.08	0.06	0.12
Rauscher Leukemia Virus			0.28	2.51	0.26	4.12

* Supernatant from LPS- or LPS/BrdU-treated spleen cell cultures were tested for polymerase activity with different template primers and in the presence of anti-reverse transcriptase and control serum.

levels of reverse transcriptase with LPS/BrdU, but very little with LPS alone (Table VIII). None of the LPS/BrdU-treated spleen cell cultures, from progeny of mouse 14 (Bdv^+), gave foci on S^+L^- cells, but in about half of the mice tested induction of reverse transcriptase was observed with LPS alone. Weak amplification (less than twofold) was seen with BrdU (Table IX). The average reverse transcriptase values in cultures for mice inducible for Bxv and Bdv are plotted in Fig. 2 and indicate that the two genes are differentially affected by LPS and LPS/BrdU.

TABLE VIII

*Bxv Induction from Second Backcross Mice**

Mouse	Reverse transcriptase			Xenotropic virus (foci induced by LPS/BrdU cells)‡
	Control	LPS	LPS/BrdU	
		pmol/ml		
1	0.02	0.04	0.05	0
2	NM§	0.05	0.89	+
3	0.04	0.07	0.85	+
4	0.02	NM	0.05	0
5	0.02	0.05	1.35	+
6	0.03	0.06	1.27	+
7	0.04	0.06	1.37	+
8	0.03	0.07	1.52	+
9	0.03	0.03	0.07	0

* Progeny of a cross between 129 and (BALB/c × 129)F_1 × 129. The first backcross mouse was typed Bxv^+ and Bdv^- by comparison of virus induction by LPS and LPS/BrdU.
‡ + indicates >50 foci.
§ Not measured

TABLE IX

*Bdv Induction From Second Backcross Mice**

Mouse	Reverse transcriptase			Xenotropic virus (foci induced by LPS/BrdU cells)
	Control	LPS	LPS/BrdU	
		pmol/ml		
1	0.03	0.20	0.36	0
2	0.03	0.03	0.08	0
3	NM‡	0.04	0.07	0
4	0.03	0.04	0.10	0
5	0.06	0.32	0.54	0
6	0.05	0.23	0.44	0
7	0.08	0.28	0.54	0
8	0.03	0.08	0.13	0
9	0.02	0.02	0.02	0
10	0.04	0.24	0.39	0
11	0.02	0.03	0.08	0
12	0.05	0.37	0.64	0

* Progeny of a cross between 129 and (BALB/c × 129)F_1 × 129. The first backcross mouse was typed Bxv^- and Bdv^+ by comparison of virus induction by LPS and LPS/BrdU.
‡ Not measured.

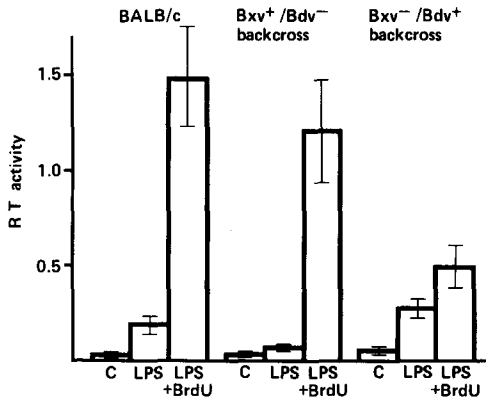

FIG. 2. Differential effect of LPS and BrdU on Bxv-1 and Bdv-1 expression. 3-d values for reverse transcriptase (RT) activity were measured in control, LPS-, and LPS/BrdU-treated cultures from BALB/c mice and second backcross mice carrying either the Bxv-1 or the Bdv-1 locus. Mean values and standard deviations from independent cultures of at least five mice are shown.

Discussion

In this paper we present data indicating that at least two different endogenous C-type viruses are induced in stimulated BALB/c lymphocytes. One of these is xenotropic in host range and its induction is controlled by the gene locus designated Bxv-1 (9, 10). The other virus, Bdv, is apparently defective; lengthy co-cultivations of BALB/c lymphocytes with mouse SC-1 cells have not yielded ecotropic virus. In addition, similar co-cultivations with CCL-64 cells of lymphocytes from (BALB/c × 129) F_1 × 129 mice containing Bdv-1 but not Bxv-1 did not result in xenotropic virus replication (data not shown). We have not yet succeeded in mapping Bdv-1. The locus is not sex linked and does not appear to segregate with coat color (data not shown). The two genes Bdv-1 and Bxv-1 appear to be differently regulated since expression of Bdv is primarily affected by LPS and that of Bxv by BrdU (Fig. 2). Stimulated 129 lymphocytes also release a defective virus particle characterized by the presence of *gag* and *env* antigens, which can be distinguished from Bdv by the absence of reverse transcriptase activity (24). Whether BALB/c lymphocytes release a similar particle remains to be determined.

Our results with the inducible xenotropic virus confirm those of Kozak and Rowe (9, 10). They showed that an allelic site mapping ~20 centiMorgans from Pep-3 on mouse chromosome 1 controlled xenotropic virus induction from both fibroblasts (9) and lymphocytes (10). We also show that, in all probability, a number of other induction protocols (LPS, LP, LP/BrdU, and Con A/BrdU) induce the same gene (Table IV). Testing statistically insufficient numbers (5×10^5) of LPS- and LP-stimulated spleen cells probably accounts for the mice that were inducible by LPS/BrdU but not by LPS or LP since more recent limiting dilution co-cultivation experiments using the same protocol indicate that only 1 to 2×10^5 LPS-stimulated (and 1 to 2×10^3 LPS/BrdU-treated) BALB/c spleen cells produce Bxv (Stoye, unpublished data); hence some cultures with 5×10^5 cells would contain no cells producing Bxv.

By analogy with other systems in which endogenous retrovirus expression has been studied it seems likely that the Bxv-1 locus represents a complete xenotropic provirus (9, 10, 25) and that strains of mice from which xenotropic virus cannot regularly be

induced, such as NFS/N and 129, lack this gene. We have recently tested an alternative possibility, that the defective virus induced from 129 mice (24) might be the result of faulty, host cell-controlled virus maturation in superinfection experiments. We showed that both ecotropic and xenotropic (introduced as a pseudotype virus with an ecotropic virus coat) viruses replicate equally well in lymphocytes from BALB/c and 129. Furthermore, we could demonstrate, by the appearance of pseudotype viruses, that ecotropic virus was replicating in the cells producing Bxv.[2] These findings further support the hypothesis that Bxv induction is controlled by the presence or absence of a complete provirus.

Only a limited number of the total set of endogenous mouse retroviruses have been characterized. For instance germ line DNA contains upwards of 20 endogenous provirus (26), but only 2, the replication-competent ecotropic and xenotropic viruses that can be induced by BrdU from fibroblasts, have been extensively studied. However, understanding the structure and expression of other endogenous retroviruses is likely to be of importance in the study of the retrovirally induced leukemias characterizing certain strains of mice such as AKR, C58, and HRS. The causative agents of disease in these mice are, in all probability, retroviruses of dual tropic host range (27) formed by recombination between two or more endogenous viruses. One parental virus is clearly the ecotropic virus. However, RNA fingerprinting (28) and restriction enzyme analysis (29) rule out any replication-competent xenotropic virus yet isolated, such as Bxv, as the other parent. Recombinant dual tropic viruses most likely are formed after infection of a proliferating cell expressing a defective virus containing an *env* gene specifying dual tropism. Both Bdv and the 129 virus are presumably expressed in vivo by antigenically stimulated proliferating B cells and appear to be potential candidates for defective parental viruses. To test this hypothesis, sufficient quantities of either viral RNA or protein must be obtained for biochemical analysis. Such studies might also be expected to reveal why Bdv and the 129 virus are defective.

Bxv-1 and Bdv-1 might provide useful models for studying two aspects of gene regulation: activation in differentiating cells and comparison of the control of gene expression in different cell types. We have previously shown a correlation between xenotropic virus inducibility and cell differentiation stage; BrdU treatment of mitogen-stimulated B cells and fibroblasts but not T cells leads to xenotropic virus induction (14). Since endogenous virus expression has been correlated with undermethylation of cellular DNA (30) we have examined the effect of 5-azacytidine, an inhibitor of DNA methylation (31) and an endogenous retrovirus inducer (32), on the expression of Bdv and Bxv in B and T cells. 5-azacytidine shows the same effect as BrdU on Bxv-1 expression, inducing from B but not T cells, and has little effect on Bdv-1 (Stoye, unpublished data), indicating that although 5-azacytidine can induce gene expression, other differentiation-specific factors are also important.

LPS shows contrasting effects on the expression of Bdv-1 and Bxv-1. It appears to act directly on Bdv-1, with BrdU having little effect on its expression (Fig. 2). This suggests that Bdv-1 is expressed during the normal pathway of B cell differentiation. In this respect it resembles other antigens of endogenous retroviruses which have been detected on murine lymphocytes at different stages of differentiation, such as G_{IX} on some thymocytes (33), and gp85 on many classes of activated lymphocytes (34).

In contrast, LPS primarily appears to act indirectly on Bxv-1 expression. However,

LPS is a very poor inducer of Bxv-1 compared with BrdU (Table I). LPS treatment is required for efficient BrdU induction. One reason for the LPS requirement is the stimulation of cell proliferation permitting BrdU incorporation into cellular DNA. However, it is probable that LPS stimulation of cell differentiation is also required for virus induction, since the inhibiting of cell differentiation with anti-IgM without affecting proliferation (35) inhibits Bxv production (Stoye and Moroni, manuscript in preparation). In addition we have shown that BrdU does not induce from spleen cells treated with mitogenic $F(ab')_2$ fragments of rabbit anti-mouse IgM unless a differentiation-inducing Con A supernatant (36) is added (Stoye and Moroni, manuscript in preparation). Thus inducibility appears controlled by the differentiation stage, most probably at the chromosomal level. LPS-induced differentiation might then be envisaged as causing chromosomal changes that allow BrdU and 5-azacytidine induction.

. A number of factors complicate the study of the molecular basis of Bdv-1 and Bxv-1 activation. One of these is the number of related, cross-hybridizing proviruses in the mouse genome. To meet this problem we are currently attempting to generate plasmids carrying DNA sequences for specific integrated proviruses and their host flanking sequences; the latter might provide the necessary probes for studying gene activation. In addition they will permit us to resolve the question whether Bxv-1 and Bdv-1 represent proviral genomes. We are also continuing to breed Bxv-1 and Bdv-1 independently into a 129 background to study their expression and possible role in leukaemogenesis. Another problem in the study of these loci is the low number of virus-producing cells and the transient nature of primary lymphocyte cultures. One solution would be mitogen- and/or growth factor-dependent cell lines. This is already feasible with T cells (see reference 37) but not for B cells. Virally transformed or tumor cell lines such as the LPS-responsive BCL_1 line (38) might provide the necessary systems.

Why should proviral antigens be expressed during normal lymphocyte differentiation? We have suggested that virus or virus-related proteins might play a functional role in the immune system (13, 39). This possibility seems unlikely, however, since endogenous virus-free chickens have been bred (25). A more likely possibility is that the endogenous viruses found to be activated may be integrated in chromosomal regions that are activated during specific differentiation events. Study of the molecular basis of retroviral activation might provide clues towards differentiation-specific transcriptional activation of otherwise undefinable regions of the mouse genome.

Summary

Germ line DNA from all strains of mice contains numerous endogenous retroviruses. One of these viruses, a virus with xenotropic host range is induced from lymphocytes of most strains by treatment with B cell mitogens. Virus induction is amplified by 5-bromo-2′-deoxyuridine (BrdU) treatment. We report here studies of the genetic control of retrovirus induction from lymphocytes in crosses between BALB/cTif mice and noninducible 129/Rrj mice. We identify a novel locus, Bdv-1, which controls the expression of a reverse transcriptase-positive, defective retrovirus in BALB/cTif lymphocytes. In addition, we confirm previous reports that xenotropic virus is controlled by a locus, Bxv-1, mapping to chromosome 1. The two loci are nonlinked and respond differently to inducing stimuli. Bxv-1 is induced mainly by BrdU and

only marginally by mitogen; in contrast, Bdv-1 is induced by mitogen and BrdU has little effect. The induction of these two loci is discussed with respect to B cell differentiation.

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