Proliferation and Differentiation Requirements for the Induction of Two Retroviral Loci during B-Cell Activation

By JONATHAN P. STOYE† AND CHRISTOPH MORONI*
Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland

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SUMMARY

Mitogen treatment of murine (BALB/c) B-cells induces two different endogenous retroviruses involving two unlinked, presumably proviral, loci Bxv-1 and Bdv-1. To determine the usefulness of these loci as genetic markers for B-cell differentiation their expression was studied under conditions that interfered with B-cell proliferation and differentiation into IgM-secreting plaque-forming cells (p.f.c.). Maximum production of both viruses followed peak DNA synthesis by an interval of about 18 h. Treatments that blocked DNA synthesis or killed proliferating cells inhibited virus production. Addition of BUdR to mitogen-stimulated cultures selectively induced Bxv-1 while inhibiting the generation of p.f.c. Both effects require BUdR incorporation into the DNA of proliferating cells. 5-Azacytidine induced Bxv-1-dependent virus production without inhibiting terminal B-cell differentiation. Pretreatment of mitogen-stimulated B-cells with anti-mouse IgM serum decreased both virus production and generation of p.f.c., but had little effect on DNA synthesis. Experiments using a mitogenic F(ab′)2 preparation of anti-IgM in the presence and absence of lymphokines also suggested that the generation of p.f.c. and Bxv-1-dependent virus production are linked phenomena. The data imply that Bxv-1- and Bdv-1-dependent virus production require DNA synthesis and cell proliferation and, at least for Bxv-1, B-cell differentiation. It is proposed that the induction of these loci reflects the involvement of neighbouring DNA sequences in B-cell proliferation or differentiation.

INTRODUCTION

Numerous endogenous retroviruses are present in the germ line of all strains of mice (Coffin, 1982). Studies of spontaneous and induced activation of endogenous proviruses have shown that their expression is both strain- and tissue- or cell type-dependent (Strand et al., 1974; Coffin, 1982). Addition of 5-iododeoxyuridine or 5-bromodeoxyuridine (BUdR) to fibroblasts or mitogen-stimulated B-lymphocytes from BALB/c mice results in xenotropic virus (BXV) induction (Lowy et al., 1971; Moroni et al., 1975; Kozak & Rowe, 1980; Stoye & Moroni, 1983). In contrast, BUdR-treated concanavalin A (Con A)-stimulated T-cells do not produce BXV (Moroni et al., 1980a, b). BXV induction from BALB/c fibroblasts and B-cells is controlled by the same genetic locus, Bxv-1, presumably the BXV provirus, which maps on mouse chromosome 1 (Kozak & Rowe, 1980). We and others have shown that some B-cell mitogens such as lipopolysaccharide (LPS) are capable of inducing very low levels of BXV in the absence of BUdR (Moroni & Schumann, 1975; Greenberger et al., 1975; Stoye & Moroni, 1983). In addition, we have recently demonstrated that a second, apparently defective virus, termed BDV, is induced from LPS-stimulated lymphocytes (Stoye & Moroni, 1983). Induction is controlled by the genetic locus Bdv-1, which segregates independently from Bxv-1 in crosses between BALB/c and 129 mice.

† Present address: Cancer Research Center, Tufts University School of Medicine, 136 Harrison Avenue, Boston, Mass. 02111, U.S.A.
Bdv-1 is induced primarily by LPS with BUdR having little effect on its expression; by contrast, only low levels of Bxv-1 are expressed following LPS stimulation; however, BUdR addition results in a 100-fold increase in the number of BXV-producing cells (Stoye & Moroni, 1983). Thus, measurement of induced reverse transcriptase activity in LPS-stimulated cultures reflects Bdv-1 expression, but in LPS/BUdR-treated cultures it reflects mainly Bxv-1 expression. As BDV appears to be defective, only Bxv-1 expression will be measured in infectivity assays. Consequently, we can study the expression of these two loci independently in stimulated BALB/c lymphocyte cultures, depending on the virus assay.

Activation of B- or T-lymphocytes has been shown to be a multistep process, the cells becoming responsive to both proliferation and differentiation stimulating factors (Andersson et al., 1980; Larsson et al., 1980; Parker et al., 1980; Palacios, 1982). LPS is apparently capable of stimulating B-cells in all these steps simultaneously and polyclonally; thus, induction of Bxv-1 and Bdv-1 takes place against a background of B-cell proliferation and differentiation into immunoglobulin-secreting cells. Since neither locus becomes expressed or can be induced from T-cells, molecular studies of Bxv-1 and Bdv-1 expression might provide insight into B-cell activation. Before attempting such studies, we wanted to examine more closely the relationship between the expression of the two loci and B-cell proliferation and differentiation. We describe here a series of experiments in which the effect of interfering with cell proliferation (measured by $[^{3}H]$thymidine incorporation) or differentiation (quantified by the number of IgM-secreting cells) on virus induction (detected by reverse transcriptase and infectivity assays) was examined. We present evidence that both proliferation and differentiation are required for endogenous retrovirus induction and suggest that provirus induction reflects the expression of neighbouring genes involved in these processes.

**METHODS**

*Animals.* One- to 2-month-old BALB/c mice came from the Sisseln Tierfarm, Switzerland and were used within 2 months of delivery.

*Lymphocyte cultures.* Spleen or thymus cell suspensions were prepared as previously described (Schumann & Moroni, 1976) and cultured for 3 days in RPMI 1640 medium (Gibco) supplemented with 8% foetal calf serum (Gibco batches L 465001S or K 763101S), 20 mM-HEPES, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1 mM-glutamine. In the majority of experiments, cells were cultured at $2 \times 10^{6}$ viable, nucleated cells/ml. LPS-W from *Escherichia coli* 0111:B4 (Difco) or Con A (Miles-Yeda) was added at the start of culturing to concentrations of 16 and 4 µg/ml respectively. In some experiments BUdR, hydroxyurea, thymidine or deoxycytidine (all from Calbiochem) was added to the cultures at times and concentrations described below. Lymphocytes at $2 \times 10^5$ cells/ml culture medium containing $2 \times 10^{-5}$ M-2-mercaptoethanol were also stimulated with mitogenic F(ab')$_2$, preparations of rabbit anti-mouse IgM sera plus supernatants from 24 h cultured Con A-stimulated spleen cells prepared as described by Parker et al. (1980). Rabbit anti-mouse IgM sera were obtained from rabbits hyper-immunized with MOPC 104E protein emulsified in complete Freund's adjuvant. F(ab')$_2$ fractions were prepared by affinity purification of IgG on Protein A-Sepharose, pepsin digestion and G-100 chromatography (Parker et al., 1980). Spleen cells were routinely cultured for 3 days before quantifying virus induction and B-cell differentiation.

*Tritiated nucleotide incorporation.* One ml spleen cell cultures were pulsed with 1 µCi [6-3H]thymidine (25 Ci/mmol, Amersham) for 6 h. The cells were then pelleted, supernatants discarded and the cells resuspended in 10% TCA, 20 mM-sodium pyrophosphate. After standing on ice for 2 h, samples were filtered and counted in a liquid scintillation counter. Alternatively, TCA-precipitable radioactivity in 200 µl cultures was determined after collection of samples with a semi-automated multiple sample harvester. Triplicate determinations were performed, and standard deviations were less than 15% of the mean.

*Quantification of IgM-secreting cells.* IgM-secreting cells (p.f.c.) were detected by the plaque assay of Gronowicz et al. (1976). Briefly, cultured spleen cells were mixed with rabbit anti-mouse IgM, guinea-pig complement (Gibco), Protein A-coupled sheep red blood cells and then plated in soft agar. After incubation for 4 to 6 h at 37°C, plaques of lysed red blood cells were counted. Results are expressed as p.f.c./10^6 cells or p.f.c./10^6 cells at the start of spleen cell culturing. Routinely, quadruplicate determinations were performed. IgM represents the majority of antibody secreted in 3-day LPS-stimulated spleen cell cultures (Andersson et al., 1978).

*Reverse transcriptase assay.* Virus was concentrated by ultracentrifugation and reverse transcriptase activity measured as previously described (Stoye & Moroni, 1983). Values for reverse transcriptase activity are given as pmol TMP incorporated by virus concentrated from 1 ml culture supernatant.
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Detection of infectious xenotropic virus.
Xenotropic virus production by LPS-stimulated spleen cells was detected by overnight co-cultivation of stimulated spleen cells with mink CCL-64 cells in the presence of 15 μg/ml Polybrene (Aldrich) followed by repeated passaging of the mink cells and assaying cell-free supernatants for reverse transcriptase activity (Monckton & Moroni, 1980). Alternatively, xenotropic virus-producing LPS/BUdR-treated spleen cells were quantified using the mink F 648.1 S+L- cell line (Peebles, 1975) as previously described (Stoye & Moroni, 1983). Triplicate determinations were performed and expressed as loci/10^6 initial spleen cells.

RESULTS

Lymphocyte proliferation and virus induction

To examine the temporal link between cell proliferation and endogenous virus induction in stimulated lymphocytes the following experimental approach was undertaken. First, the temporal sequence of events was monitored by measuring [3H]thymidine incorporation into cellular DNA and reverse transcriptase activity in culture supernatants at frequent intervals following LPS stimulation. Second, knowing the temporal relationship between DNA synthesis and virus production, the effect on virus production of blocking DNA synthesis with hydroxyurea was measured. Third, the hot thymidine suicide technique (Melchers & Andersson, 1974) was used to kill proliferating cells and infectious virus production was measured. Together, these experiments should show whether the stimulation of cell proliferation is required for virus induction and, if so, whether DNA synthesis is necessary before, or concomitant with, virus production. Incorporation of [3H]thymidine and reverse transcriptase activity were measured every 6 h for 78 h following mitogen stimulation (Fig. 1). The maximum mitogen-induced [3H]thymidine incorporation occurred 42 to 48 h after LPS stimulation (Fig. 1 a). During the first 18 h of culture, high levels of [3H]thymidine incorporation were observed in both control and LPS-stimulated cultures, presumably due to the presence of lymphocytes activated in vivo within the spleen. Little detectable virus was released into supernatant within the first 30 h of culture. Then both LPS- and LPS/BUdR-stimulated cultures started to release increasing amounts of virus (Fig. 1 b, c). As previously shown (Moroni et al., 1975), BUdR addition led to a very strong stimulation of reverse transcriptase activity. Whereas LPS/BUdR-induced activity reached a sharp maximum value approximately 18 h after maximum [3H]-thymidine incorporation, LPS-stimulated cells showed a less well-defined time of maximum virus production, releasing similar amounts of virus for a longer time than LPS/BUdR-treated cells. The fixed time interval observed in three repeat experiments suggests a strong link between the two phenomena. We therefore examined the possible effect of inhibiting DNA synthesis on virus production. Hydroxyurea has been reported to give almost complete inhibition of DNA synthesis with very little effect on RNA synthesis in LPS-treated spleen cells (Janossy et al., 1976). Virus production, was measured between 62 and 66 h after LPS stimulation in the presence of 100 μg/ml hydroxyurea added either at 38 h after LPS stimulation, i.e. before maximum DNA synthesis (compare Fig. 1), or just prior (at 62 h) to the 4 h measurement period. In both cases, addition of this concentration of hydroxyurea was sufficient to block thymidine incorporation almost completely (data not shown). Virus induction by both LPS and LPS/BUdR was almost completely blocked in cultures given 100 μg/ml hydroxyurea at 38 h (Table 1). Addition of hydroxyurea at 62 h had little effect on virus production (Table 1). Similar results were seen in two repeat experiments. These data imply that concomitant DNA synthesis is not necessary for virus production, but suggest that prior DNA synthesis is required.

Analogous results were obtained following colcemid treatment. Addition at 36 h inhibited virus release measured at 66 h; however, addition at 62 h had little effect (data not shown). We conclude that not only DNA synthesis but also cell division are required for virus production, a finding consistent with the observed time interval between maximum DNA synthesis and maximum virus production.

As these reverse transcriptase measurements quantify mainly BDV induction in LPS cultures, we took a separate approach to the question of whether DNA synthesis is also required for BXV induction, namely the hot thymidine suicide technique. Cells proliferating in the presence of high concentrations of radioactive thymidine of high specific activity incorporate sufficient
Fig. 1. Time course of DNA synthesis and virus production expressed as 6 hourly measurements of 
\[ ^3H \] thymidine incorporation and reverse transcriptase production respectively. Values are plotted in
the middle of each 6 h period. (a) \[ ^3H \] thymidine incorporation in control (\( \triangle \)) and LPS-stimulated (\( \bigcirc \))
cultures; (b) reverse transcriptase released into culture supernatants by control (\( \triangle \)) and LPS-stimulated
(\( \bigcirc \)) cultures; (c) reverse transcriptase in control/BUdR (\( \triangle \)) and LPS/BUdR (\( \bigcirc \)) cultures. The arrow in
(b) and (c) marks the time of maximum DNA synthesis.

Fig. 2. Thymidine suicide experiment. (a) CCL-64 cells infected with serial 10-fold dilutions of
xenotropic virus passaged twice weekly and reverse transcriptase activity in the supernatants measured.
Values are plotted as pmol/ml culture supernatant. Virus was diluted 1/10 (\( \bigtriangleup \)), 1/100 (\( \bigcirc \)),
1/1000 (\( \bullet \)) and 1/10000 (\( \bigtriangleup \)). (b) CCL-64 cells co-cultivated with LPS-treated spleen cells (\( \bigtriangleup \)), with LPS
+ 20 \( \mu \)Ci/ml \[ ^3H \] thymidine-treated spleen cells (\( \bullet \)) and with LPS + 20 \( \mu \)Ci/ml \[ ^3H \] thymidine + 100
\( \mu \)g/ml thymidine (\( \bigcirc \)).

| Table 1. Effect of hydroxyurea on induction of reverse transcriptase* |
|-----------------------------|-----------------------------|-----------------------------|
| No addition | Hydroxyurea at 38 h | Hydroxyurea at 62 h |
| LPS | 0-25 | 0-01 | 0-21 |
| LPS/BUdR | 2-21 | 0-19 | 2-07 |

* Spleen cells were cultured for 66 h, prior to determination of reverse transcriptase activity. Hydroxyurea was
added at the times indicated to a final concentration of 100 \( \mu \)g/ml. Reverse transcriptase was measured as pmol
TMP/ml culture supernatant.

quantities of radioactivity to receive a lethal dose of irradiation, resulting in rapid cell death.
This technique has been used to show that the generation of IgM-secreting cells in LPS-
stimulated cultures requires cell proliferation (Melchers & Andersson, 1974). Xenotropic virus
production was measured by co-cultivation with CCL-64 cells followed by reverse transcriptase
assays (Monckton & Moroni, 1980). To demonstrate that this technique can be used at least
semiquantitatively, CCL-64 cells were infected with serial 10-fold dilutions of infectious
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xenotropic virus. The mink cells were passaged twice weekly and reverse transcriptase activity in the supernatants monitored. The time of appearance of reverse transcriptase in the cultures infected with different dilutions of virus was dependent on the virus dilution with a difference of approximately two passages between each 10-fold dilution (Fig. 2a). Four groups of five 1 ml spleen cell cultures were set up, three with LPS and one without. One day later [3H]thymidine (27 Ci/mmol) was added, to a final concentration of 20 μCi/ml, to two of the LPS groups. Excess unlabelled thymidine (100 μg/ml) was also added to one of these groups. Two days later, cells from these groups were co-cultivated overnight with CCL-64 cells, the cultures passaged twice weekly and the appearance of reverse transcriptase activity in mink cell culture supernatants was followed. Treatment of spleen cells with 20 μCi/ml [3H]thymidine delayed the appearance of reverse transcriptase activity by four passages, i.e. reduced virus production considerably (Fig. 2b). Addition of excess unlabelled thymidine reversed this effect. Mink cells co-cultivated with the control unstimulated lymphocytes did not produce any virus even after 10 passages (not shown).

These experiments demonstrated that inhibiting cell proliferation or killing proliferating cells resulted in inhibition of endogenous virus production measured either by reverse transcriptase or by infectivity assays. These findings imply that LPS induction of both BXV and BDV is dependent on the stimulation of cell proliferation.

Antibody-secreting cells and virus induction

We have previously shown that not all lymphocyte mitogens promote BUdR induction of BXV-1 (Moroni et al., 1980b). All inducing mitogens, however, share the property of stimulating the appearance of immunoglobulin-secreting B-cells (Moroni et al., 1980b). One explanation for this finding would be that it is the antibody-secreting cells themselves that produce virus. However, it has been reported that BUdR reduces the number of IgM-secreting cells in LPS cultures without affecting cell proliferation (Janossy et al., 1976). Hence, we examined whether concentrations of BUdR required to amplify virus production also inhibited the formation of p.f.c. Reverse transcriptase and p.f.c. were measured in spleen cell cultures given increasing doses of BUdR 24 h after LPS stimulation. The concentration of BUdR (5 μg/ml) giving maximal virus amplification also gave full inhibition of p.f.c. (Fig. 3). Similar results were obtained in three repeat experiments. BUdR did not affect the number of viable cells in the cultures (data not shown). These results indicate that the generation of antibody-secreting cells was not necessary for BXV induction by BUdR. Furthermore, the mirror symmetry of the curves for inhibition and amplification suggested a possible link between the two phenomena, that blocking B-cell maturation was actually required for BUdR induction, perhaps by arresting maturation in a phase of high virus production. The effect of adding increasing amounts of thymidine to LPS- and LPS/BUdR-treated cultures on virus production and the number of p.f.c. was next measured. Fig. 4 shows that BUdR inhibition of p.f.c. and amplification of virus could be reversed by addition of similar concentrations of thymidine; thus, the two phenomena could not be dissociated. The concentrations of thymidine used did not affect either reverse transcriptase release or the number of p.f.c. in LPS-stimulated cultures.

To examine the link between BXV amplification and p.f.c. inhibition further, we asked whether 5-azacytidine, a virus inducer in fibroblast cultures (Niwa & Sugahara, 1981; Groudine et al., 1981), was also active on lymphocytes and if so, what, if any, effect might this drug have on B-cell differentiation. The data presented in Fig. 5 show that this drug is indeed an inducer of BXV-1, with an efficiency similar to that of BUdR. Combining the two drugs did not result in higher BXV production than seen with BUdR alone, implying that both drugs are acting on the same level, presumably transcription (Razin & Riggs, 1980). Next, the effects of BUdR and 5-azacytidine on the number of S^+L^- foci and IgM-secreting p.f.c. were compared. The data shown in Table 2 indicate that although 5-azacytidine and BUdR induced similar numbers of BXV-producing cells, they have different effects on the number of p.f.c. In contrast to BUdR, there was no inhibition of B-cell differentiation with 5-azacytidine. This demonstrates that BXV production in LPS-stimulated cultures can occur independently of a late block of B-cell differentiation.
Fig. 3. BUdR dose-response curve. Reverse transcriptase (■) and the number of p.f.c. (○) were determined 66 h after LPS stimulation in cultures treated with various doses of BUdR 24 h after stimulation. The values of IgM-secreting cells are expressed as p.f.c./10^4 stimulated spleen cells.

Fig. 4. Thymidine competition of BUdR virus amplification and of p.f.c. inhibition. Reverse transcriptase activity and the number of p.f.c./10^4 stimulated spleen cells were determined (at 66 h) in LPS- and LPS/BUdR-treated cultures following the addition of various concentrations of thymidine simultaneously with BUdR. △, LPS-induced reverse transcriptase; ▲, LPS/BUdR-induced reverse transcriptase; ○, LPS-induced p.f.c.; ●, LPS/BUdR-induced p.f.c.

Fig. 5. Effect of 5-azacytidine on activated B-cells. LPS-stimulated spleen cells were assayed for the production of endogenous xenotropic virus by the mink S^+/L^- assay. Data are expressed as S^+/L^- foci/10^6 cells at the start of lymphocyte culture. Cultures contained the indicated concentrations of 5-azacytidine with (●) or without (○) BUdR at 5 μg/ml.
Table 2. Comparison of 5-azacytidine and BUdR effects*

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Control</th>
<th>LPS</th>
<th>LPS/BUdR</th>
<th>LPS/5-ac</th>
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<tr>
<td></td>
<td>S+L− foci/10^6 viable cells</td>
<td>p.f.c./10^6 viable cells</td>
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</tr>
<tr>
<td>Expt. 1</td>
<td>0</td>
<td>280</td>
<td>5120</td>
<td>490</td>
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<td>Expt. 3</td>
<td>0</td>
<td>0</td>
<td>12160</td>
<td>1320</td>
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</table>

* Spleen cell cultures contained, as indicated: LPS (16 μg/ml); BUdR (5 μg/ml); 5-azacytidine (5-ac) (3 μg/ml). The latter two drugs were added 24 h after culture initiation. Medium was changed on day 2. On day 3 viable spleen cells were counted and the data are expressed in terms of S+L− foci or p.f.c./10^6 viable 3-day cultured lymphocytes.

In parallel experiments we examined the effect of 5-azacytidine on virus expression in T-lymphocytes which are non-inducible with BUdR (Moroni *et al.*, 1980a, b). Neither BXV nor BDV could be induced from Con A-stimulated thymocytes by 5-azacytidine (data not shown), a finding which further underscores the importance of the lymphocyte differentiation stage on virus inducibility.

**Stimulation of lymphocyte differentiation and virus induction**

Since virus production does not appear to be dependent on the appearance of antibody-secreting cells, we wanted to examine whether the induction process is at all dependent on mitogen-induced differentiation or whether stimulation of proliferation in a given subset of lymphocytes is sufficient. A system in which it appeared possible to examine differentiation independently from proliferation involved using anti-mouse immunoglobulin-stimulated LPS-stimulated lymphocytes. Certain batches of anti-mouse immunoglobulin sera have been shown to block the appearance of IgM-secreting cells without affecting LPS-induced [3H]thymidine incorporation (Andersson *et al.*, 1974). We tested sera from several rabbits which had been immunized with mouse immunoglobulin for this property. Treatment of spleen cells prior to LPS stimulation with one of these sera resulted in a 90% reduction in the number of p.f.c, but only inhibited thymidine incorporation by 15% (data not shown). The effect of this serum on LPS and LPS/BUdR induction, measured by the reverse transcriptase assay, was then examined (Table 3a). Virus induction by LPS/BUdR was inhibited about 70% by anti-mouse IgM. However, virus induction by LPS was only inhibited by around 30%. One explanation for the differential effect of anti-IgM serum on LPS and LPS/BUdR induction as measured by reverse transcriptase activity might be that the serum treatment inhibits BXV but not BDV induction. To test this possibility we measured the number of BXV-producing cells in LPS/BUdR cultures treated with anti-IgM using an infectious centre assay (Table 3b). If anti-IgM acts only to inhibit BXV induction one would expect an almost complete inhibition of BXV production rather than the 70% inhibition observed in reverse transcriptase assays. However, this proved not to be the case, since again we observed 70% reduction in the number of BXV-producing cells. Nevertheless, these experiments with anti-IgM suggest that virus induction, at least of Bxv-1, is linked to and dependent on mitogen-induced differentiation.

To examine further the relationship between virus induction and cell differentiation we tested whether mitogenic F(ab')2 preparations of anti-IgM serum could induce virus production in the presence of BUdR. It has been shown that F(ab')2 anti-IgM preparations induce proliferation, as measured by thymidine uptake, of B-cells but the appearance of IgM-secreting cells is
Table 3. *Effect of anti-IgM on virus induction*  

(a) Inhibition of induced reverse transcriptase†

<table>
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<th>Expt. 3</th>
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<tbody>
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(b) Inhibition of Bxv-1 induction‡

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<td>265</td>
</tr>
<tr>
<td>LPS/BUDR + anti-IgM</td>
<td>3</td>
<td>42</td>
<td>91</td>
</tr>
</tbody>
</table>

* Spleen cells were treated with rabbit serum for 60 min on ice prior to LPS addition.
† Expressed as pmol/ml culture supernatant.
‡ S+L⁻ foci/10⁶ initial spleen cells.

Table 4. *Induction of Bxv-1 by anti-IgM plus Con A supernatant*  

<table>
<thead>
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<th>S+L⁻ foci/10⁶ cells</th>
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<tr>
<td>Con A-SN‡</td>
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<tr>
<td>F(ab')₂ anti-IgM† + Con A-SN‡</td>
<td>34</td>
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</table>

* Spleen cells were cultured in the presence of BUdR (5 μg/ml) at 2 × 10⁵/ml in flat-bottomed microtitre wells in medium containing 2 × 10⁻⁵ M-2-mercaptoethanol for 4 days before determining BXV.
† 100 μg/ml.
‡ Cultures contained 50% conditioned medium from 24 h Con A-stimulated lymphocytes cultured at 10⁷ cells/ml.

dependent on the addition of T-cell factors, supplied by supernatants of Con A-stimulated spleen cells (Parker et al., 1980). We tested several F(ab')₂ preparations of rabbit anti-mouse IgM for mitogenicity and selected the most active for further study. An appropriate concentration of this preparation stimulated thymidine incorporation to a level around 45% of that seen with LPS. However, it did not induce p.f.c unless conditioned medium from 24 h Con A-stimulated cells was added which induced a p.f.c. response of about 35% compared to LPS (data not shown). The Con A supernatant alone was inactive. When Bxv-1 induction by BUdR was measured, using the S+L⁻ infectious centre assay, virus-producing cells were only seen upon the addition of Con A supernatants to F(ab')₂-stimulated cultures (Table 4).

Taken together, these results imply that virus induction is dependent on the stimulation of B-cell differentiation but that terminal differentiation to p.f.c. is not necessary.

**DISCUSSION**

Several lines of evidence indicate that mitogen stimulation of lymphocyte proliferation is one prerequisite for LPS and LPS/BUdR induction of BXV and BDV. First, blocking DNA synthesis with hydroxyurea or cell division with colcemid inhibits BXV and BDV production. Second, a strain of mice, C3H/HeJ, which is not mitogenically stimulated by LPS does not yield virus after LPS addition, whereas other B-cell mitogens do induce reverse transcriptase activity (Moroni & Schumann, 1976). Third, killing proliferating cells with a pulse of hot thymidine also results in reduced levels of BXV production. Furthermore, the thymidine competition experiment suggests that BUdR incorporation into cellular DNA is required for Bxv-1 induction from B-cells in a manner analogous to BUdR induction of endogenous viruses from fibroblasts (Teich
et al., 1973; Besmer et al., 1975). Taken together, these experiments demonstrate a requirement for the stimulation of cell proliferation in Bxv-I and BdV-1 induction.

Cell proliferation might be involved in virus induction by LPS in two different ways. It is possible that LPS induction of endogenous virus production represents nothing more than an amplification of spontaneous virus expression involving either an increase in the number of pre-existing virus-producing cells due to the stimulation of cell proliferation or by facilitating ecotropic virus spread (Stoye & Moroni, 1984). This seems unlikely since immunofluorescence studies using a broadly reactive anti-Friend leukaemia virus serum have detected no viral antigen-positive cells in 1-day- compared to 5% in 3-day-stimulated BALB/c lymphocytes (Jongstra, 1981). In addition, no xenotropic virus could be detected in control cultures tested by co-cultivation for xenotropic virus. Furthermore, no ecotropic viruses (Stoye & Moroni, 1984) are expressed in BALB/c spleen cell cultures. More likely, LPS activation of B-cells leads to expression of the retrovirus structural genes. In other words, retrovirus expression is a consequence of B-cell activation. LPS stimulation of cultures apparently plays an additional role in BUdR-treated cultures, that of promoting BUdR incorporation, which leads to increased retrovirus RNA synthesis (DeLamarter et al., 1981).

Stimulation of lymphocyte proliferation by itself does not appear sufficient for virus induction. Stimulation by mitogens which do not lead to the appearance of antibody-secreting cells does not result in virus release. Certain kinds of lymphocytes, for example T-cells, appear refractory to virus induction by BUdR or 5-azacytidine. Additionally, virus production was inhibited in cultures in which LPS-induced differentiation was inhibited by anti-IgM. The correlation between virus induction and the appearance of antibody-secreting cells suggests that virus production is a programmed event occurring within a fraction of differentiating B-cells and that these cells, as they differentiate, are permissive to the action of BUdR and 5-azacytidine.

It remains unclear precisely which subtypes of B-cells produce BXV and BDV. Two observations suggest that terminally differentiated antibody-secreting cells might be involved: the correlation between a mitogen's ability to induce virus and the appearance of p.f.c (Moroni et al., 1980b), and the finding that antibody-secreting cells can be specifically killed by antiviral sera plus complement (Wecker et al., 1977). However, two lines of evidence argue against this possibility. First, the number of BXV-producing cells even in LPS/BUdR-treated cultures is much lower than the number of IgM-secreting cells (Table 2; Stoye & Moroni, 1983). Second is the finding that BUdR amplifies BXV production while at the same time inhibiting p.f.c. generation. Similarly, BUdR has no effect on BDV production (Stoye & Moroni, 1983).

Proliferation and differentiation of lymphocytes are closely linked phenomena; thus, it is difficult to study differentiation independently from proliferation. In the blocking experiments reported here, inhibition of differentiation was always accompanied by some inhibition of cell proliferation. We obtained similar results when we compared virus induction in cells from male and female (CBA/N × BALB/c) F1 mice. CBA/N mice carry a recessive X-chromosome linked defect in B-cell maturation (Scher et al., 1975); thus, the female progeny of the cross inherit a normal X-chromosome from their BALB/c parent whereas the males do not. Levels of reverse transcriptase and the number of IgM-secreting cells were much lower (15%) in males compared to their female littermates; however, [3H]thymidine incorporation was also reduced, though to a lesser extent (50%) (Stoye & Moroni, 1981). However, the most straightforward explanation of the data in the systems studied is that BXV induction is dependent on processes occurring during late B-cell differentiation, but before the generation of p.f.c., as exemplified by the BUdR effects presented in Fig. 3. A similar conclusion has been reached by other workers who examined viral antigen expression by LPS-stimulated lymphocytes treated with anti-IgM (Alberto et al., 1982).

What factors control virus production and how might they be altered during B-cell differentiation? Previous studies have implicated provirus transcription in virus expression (Besmer et al., 1975; Thomson et al., 1980; DeLamarter et al., 1981). It has been suggested that alterations in DNA methylation might provide a means of controlling gene expression (Razin & Riggs, 1980). Consistent with this idea, an inverse relationship has been observed between the level of
proviral DNA methylation and virus expression (Niwa & Sugahara, 1981; Groudine et al., 1981; Conklin et al., 1982). It should be noted, however, that studies of the oestrogen responsive chicken gene vitellogenin have suggested that oestrogen treatment results in a specific demethylation even in a tissue which does not express the vitellogenin gene (Wilks et al., 1982). Changes in chromatin structure which generate nuclease-hypersensitive sites are thought to be of greater importance in regulating the expression of the vitellogenin gene (Burch & Weintraub, 1983). The observation that 5-azacytidine amplifies Bxv-l expression in LPS-stimulated B-cells suggests a role for methylation in controlling virus expression in lymphocytes. However, methylation does not appear to be the only factor of importance, since 5-azacytidine does not induce BXV from Con A-stimulated thymocytes. Thus, it appears likely that developmentally regulated cis-acting sequences play a role in controlling provirus expression as has also been suggested by studies of gp70 expression in different tissues of 129 mice (Elder et al., 1977). The most likely possibility is that low levels of Bxv-l and Bdv-l are expressed in differentiating lymphocytes because they are integrated within transcriptionally active regions of the mouse genome. Although still largely suppressed by factors such as methylation of proviral DNA, which could be abrogated by treatment with 5-azacytidine or possibly BUdR, sufficient leakiness would exist to permit virus expression in occasional cells. Such a two level control over expression might explain why neither BUdR nor 5-azacytidine will induce BXV from T-cells. Precedent for the control of virus expression by flanking sequences comes from studies of Mov expression (Jaenisch et al., 1981) and from transfection studies (Cooper & Silverman, 1978; Copeland & Cooper, 1979). Recent studies using recombinant inbred strains of mice have placed a variety of endogenous viruses in the vicinity of lymphocyte differentiation antigens (Blatt et al., 1983; Meruelo et al., 1983). By molecularly cloning the integrated proviruses of BXV and BDV, both of which are expressed in differentiating B-cells, we expect the flanking sequences to contain interesting probes for studying the expression of genes important for B-cell development in addition to facilitating molecular studies on the control of the retrovirus sequences. We are currently attempting to obtain such clones.

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REFERENCES


Virus induction from B-cells


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