Proc. Nat. Acad. Sci. USA
Vol. 72, No. 2, pp. 535–538, February 1975

Induction of Endogenous Murine C-Type Virus in Spleen Cell Cultures Treated with Mitogens and 5-Bromo-2′-Deoxyuridine

(lipopolysaccharide/concanavalin A/endogenous virus/leukemia)

CHRISTOPH MORONI*‡, GEBHARD SCHUMANN†, MARJORIE ROBERT-GUROFF*§, ELSI R. SUTER†, AND DORIS MARTIN*

* Friedrich Miescher-Institut, Basel, Switzerland; and † Research Department, Pharmaceuticals Division, CIBA-GEIGY Limited, Basel, Switzerland

Communicated by V. Prelog, November 21, 1974

ABSTRACT **In short-term cultures of BALB/c spleen cells, treatment with a combination of 5-bromo-2′-deoxyuridine (BrdU) and either lipopolysaccharide W. *Escherichia coli* or concanavalin A resulted in release of C-type virus into the medium. Only lipopolysaccharide induced virus release when given alone. This could be potentiated by a combined treatment with BrdU.**

In contrast, phytohemagglutinin at mitogenic concentration had no effect with or without BrdU, suggesting that inducibility may vary between various mitogen-responsive spleen cell populations.

In AKR mice, spontaneous virus release was detectable in nonstimulated spleen cell cultures. This could be potentiated by lipopolysaccharide, whereas no further increase occurred upon additional BrdU treatment.

The induced viruses had C-type characteristics in that they contained reverse transcriptase that could be distinguished from cellular enzymes by template-primer preference experiments. Furthermore, the enzyme activities were particle-associated, banding in isopycnic sucrose gradients at 1.15–1.17g/cm³. The presence of C-type viruses was confirmed by electron microscopy.

C-type viruses induce tumors predominantly in cells of the lympho-reticular system (1). However, most of the available methods for *in vitro* induction of endogenous viruses have been developed for fibroblasts (2–5). The most efficient induction occurs with bromodeoxyuridine (BrdU) (4,5), whereby the rapidly dividing fibroblasts incorporate this thymidine analogue into DNA (6), thus presumably altering the binding of regulatory protein to DNA (7) and resulting in the induction of endogenous C-type virus.

In lymphoid cells, virus induction has been reported to occur in the graft-versus-host reaction (8) or in its *in vitro* correlate, the mixed lymphocyte reaction (8). Recently, it was shown that induction of viral antigens, but not of full infective particles, occurs upon *in vitro* cultivation of murine lymphoid cells (9).

In attempting to develop efficient *in vitro* methods for virus induction in lymphoid cells, we examined the combined effect of mitogens and BrdU. The rationale was to induce DNA synthesis with mitogen, thereby initiating incorporation of BrdU and resultant virus induction. We report here that in combination with BrdU, either lipopolysaccharide (LPS) or concanavalin A (Con A) can induce virus¶ release in cultures of mouse spleen cells, while phytohemagglutinin-P (PHA) does not. LPS, unlike the other mitogens, was by itself sufficient for induction, a finding we have reported elsewhere (Ch. Moroni and G. Schumann, submitted for publication).

MATERIALS AND METHODS

Spleen Cell Cultures were prepared from 6- to 8-week old male BALB/c mice (Bomholdgard, Denmark) under aseptic conditions as described (10). One-milliliter cultures contained 2×10^6 viable nucleated spleen cells in loosely capped disposable 12 × 75-mm tubes (Falcon). Incubation was at 37° and 8% CO_2 in air. Culture medium RPMI 1640 (Microbiological Assoc.) containing 8% fetal bovine serum (Rehatuin, Reheis), penicillin (50 IU/ml, Hoechst), and streptomycin (50 μg/ml, Novo) was changed every 24 hr. The removed medium was centrifuged at 1000 × *g* for 10 min and kept at −20° until used. One-milliliter samples were thawed at 30°, centrifuged at 35,000 × *g* for 60 min at 4°, and resuspended in 1/10–1/50 of the original volume in buffer, pH 7.9, containing 10 mM Tris·HCl, 20 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 50% (v/v) glycerol. During the first 24 hr, cultures contained 16 μg of LPS W. from *Escherichia coli* O111:B4 (Difco), or 4 μg of Con A (Calbiochem) or 10 μg of PHA (Difco) or no mitogen. During the second 24 hr, some cultures contained 5 μg of BrdU. In preliminary experiments the indicated mitogen concentrations were found to induce maximum DNA synthesis. We observed no differences in the total number of viable cells between the various groups.

[³H]Thymidine Incorporation. DNA synthesis in the cultured cells was determined by measuring acid-precipitable [³H]thymidine incorporation, following published procedures (10).

RNA-Dependent DNA Polymerase (Reverse Transcriptase) Assay (11). The 100-μl reaction mixture contained 10–50 μl of sample; 40 mM Tris·HCl, pH 7.9; 60 mM KCl; 1 mM

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; LPS, lipopolysaccharide W. *E. coli* 0111:B4; Con A, concanavalin A; PHA, phytohemagglutinin-P; RLV, Rauscher leukemia virus; $A_n \cdot dT_{12-18}$, poly(A)·oligo(dT).

‡ Address reprint requests to this author: Friedrich Miescher-Institut, P.O. Box 273, Ch-4002 Basel, Switzerland.

§ Present address: Bionetics Research Laboratory, Bethesda, Md.

¶ We use the term virus although the infectivity of these particles has not yet been shown.

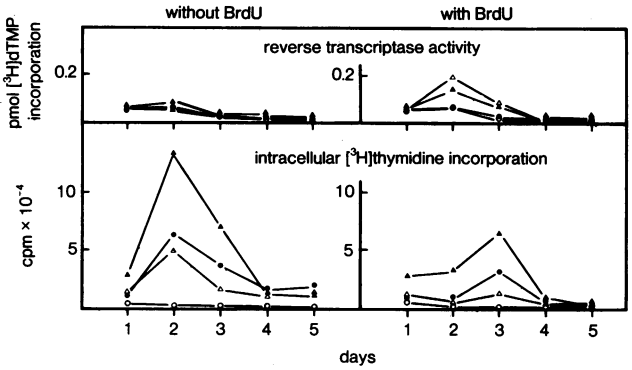

FIG. 1. Rate of virus release and DNA synthesis in BALB/c spleen cell cultures. One-milliliter cultures, containing 2 × 10^6 cells, were treated from day 0 to 1 with 16 μg of LPS (△), 4 μg of Con A (▲), 10 μg of PHA (●) or with no addition for control (○). Half of the cultures (right panels) were treated in addition with 5 μg of BrdU from day 1 to 2. Medium was replaced daily and processed for determination of reverse transcriptase activity (upper panels) by measuring the incorporation of [^{3}H]dTMP during 60 min with $A_n \cdot dT_{12-18}$ as template-primer. Samples corresponding to 0.2 ml of culture medium were analyzed. Mean values obtained from three cultures are expressed in pmol of dTMP incorporated per 0.2 ml of culture medium. Acid precipitable intracellular [^{3}H]thymidine incorporation (lower panels) during the 16-hr interval before harvest was determined in parallel cultures. Mean values obtained from two cultures are given.

dithiothreitol; 1.5 mM Mn(II) acetate; 0.2 mM EDTA; 0.1% Nonidet P-40; 10 μM [^{3}H]dTTP (Amersham, 7500 dpm/pmol); and 20 μg/ml of $A_n \cdot dT_{12-18}$ or 20 μg/ml of $dA_n \cdot dT_{12-18}$ or 20 μg/ml of dT_{12-18}. Annealing of dT_{12-18} (Collaborative Research) to A_n or dA_n (Miles) was performed in equimolar amounts, in buffer, pH 7.1, containing 0.01 M Tris·HCl and 0.1 M NaCl by incubation for 30 min at 37°. The annealed mixture was cooled at room temperature and stored at −20°. The enzyme reaction mixture was incubated at 30° and stopped at indicated times by the addition of excess 10% trichloroacetic acid containing 0.02 M Na pyrophosphate. Following 30 min of incubation in ice, acid-precipitable radioactivity was washed on GF/C glass fiber filters (Whatman) with cold 5% trichloroacetic acid and processed for liquid scintillation counting by standard procedures. Values from nonincubated samples or from samples lacking enzyme were included for background determination and the obtained values, about 300 dpm (= 0.05 pmol) were subtracted from the experimental values.

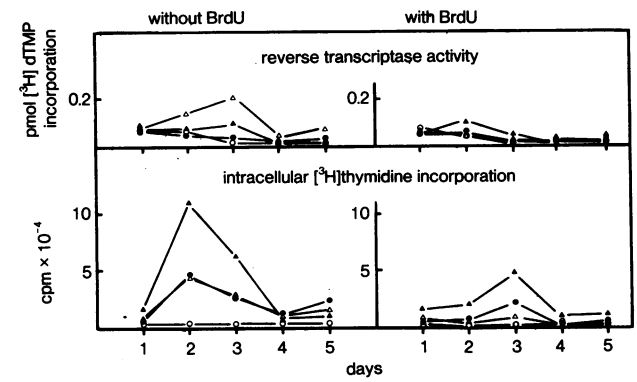

FIG. 2. Rate of virus release and DNA synthesis in AKR spleen cell cultures. Same conditions as in Fig. 1. LPS (△), Con A (▲), PHA (●), control (○).

TABLE 1. *Template-primer requirements for [^{3}H]dTMP polymerization activity*

Source of enzyme	Template-primer $A_n \cdot dT_{12-18}$	$dA_n \cdot dT_{12-18}$	dT_{12-18}
Supernatant of LPS/BrdU-stimulated BALB/c spleen cell culture*	3.39	0.07	0.05
Supernatant of Con A/BrdU-stimulated BALB/c spleen cell culture*	1.79	0.10	0.06
Supernatant of LPS-stimulated BALB/c spleen cell culture*	0.79	0.08	0.05
Supernatant of unstimulated AKR spleen cell culture*	4.31	0.05	<0.01
Supernatant of LPS-stimulated AKR spleen cell culture*	50.84	0.12	0.1)
Homogenate of BALB/c spleen cells†	0.14	0.83	0.07
Rauscher leukemia virus‡	9.87	0.06	0.01

Incubation was 90 min. Mean values of triplicate determinations are expressed in pmol [^{3}H]dTMP incorporated.

* Supernatants obtained from days 2 to 4.

† BALB/c spleen cell suspension was treated in a glass hand homogenizer and cleared by centrifugation.

‡ Purified from JLS-V5 culture medium (19).

Electron Microscopy. Mouse spleen cells were prefixed in culture medium by adding equal volumes of 3% glutaraldehyde in sodium phosphate buffer, pH 7.4, with 0.01% $CaCl_2$. Fifteen minutes later the cells were centrifuged at 200 × *g* for 10 min, the pellet was resuspended in fresh identical fixative, and fixation was continued for another hour. After four washings with phosphate buffer containing 4% sucrose, the cells were postfixed 1 hr in 1% phosphate-buffered osmium tetroxide at pH 7.4. The cell pellets were dehydrated with acetone and embedded in Araldite. Ultrathin sections were cut on a Reichert Ultratome and examined in a Siemens Elmiscop I.

RESULTS

Induction Studies with Mitogens and BrdU. To test whether mitogen-stimulated spleen cells would express C-type virus after BrdU incorporation, BALB/c spleen cell cultures stimulated with LPS, Con A, or PHA and control cultures were incubated with and without BrdU. Samples prepared from supernatants containing material released during 24-hr intervals were assayed for reverse transcriptase activity (Fig. 1, upper panels). In parallel cultures DNA synthesis was monitored by determining the intracellular incorporation of [^{3}H]thymidine (Fig. 1, lower panels). Reverse transcriptase activity was detected in cultures treated with the combination LPS/BrdU and, to a lesser extent, Con A/BrdU. The activity was maximum on day 2. PHA, although inducing greater DNA synthesis than LPS, induced no enzyme activity. BrdU caused some inhibition of DNA synthesis.

When the same experiment was performed with spleen cells from the AKR strain, known to harbor C-type viruses

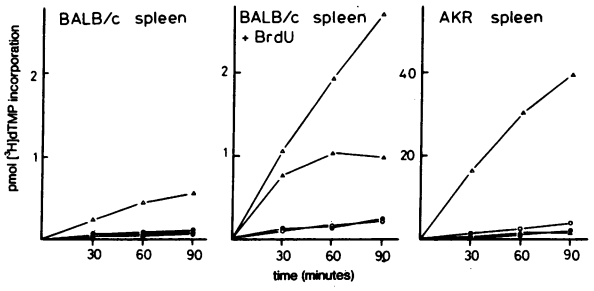

FIG. 3. Kinetics of reverse transcriptase activity. Cultures were treated with mitogens with and without BrdU as indicated in Fig. 1. LPS (△), Con A (▲), PHA (●), no addition (○). Samples originated from pooled day 2 to 4 supernatants which were 50-fold concentrated by centrifugation. Twenty-microliter aliquots were assayed for reverse transcriptase by measuring [³H]dTMP polymerization activity for the indicated times using $A_n \cdot dT_{12-18}$ as template-primer. Mean values of duplicate determinations are expressed in pmol of dTMP incorporated. Note scale difference in right panel.

throughout life (12), elevated enzyme activity was found in the absence of BrdU in LPS-stimulated cultures (Fig. 2, upper panel). Activity was first detected on day 2 and was maximum on day 3. As in BALB/c, LPS had the lowest mitogenic activity (Fig. 2, lower panel).

Since the induced enzyme activities present in 1-ml cultures were low, the findings had to be corroborated by studying the kinetics of the enzyme reaction in more concentrated samples (Fig. 3). With BALB/c cells, as expected, most activity was detected in samples originating from LPS/BrdU- and Con A/BrdU-treated cultures (Fig. 3, middle), while the kinetics revealed that samples originating from cultures treated with LPS alone contained activity too. Con A and PHA alone showed control values (Fig. 3, left). The small increase with time observed with the samples from BALB/c cultures treated with BrdU alone (Fig. 3, middle), may indicate that this drug was inductive by itself. However, the activity was too small to be analyzed further. PHA, with and without BrdU, did not increase the activity over control values.

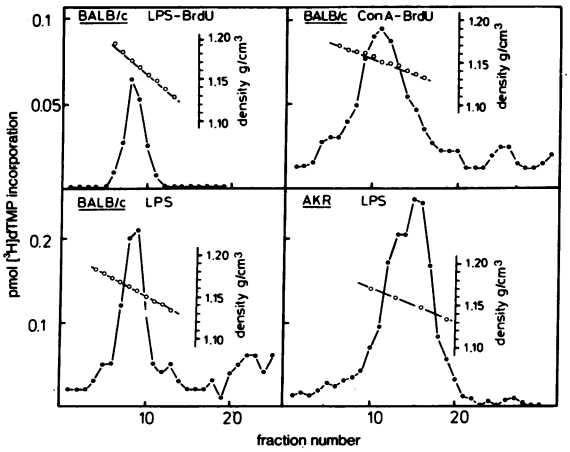

FIG. 4. Isopycnic sucrose density gradient centrifugation. Pellets prepared from pooled day 2 to 4 culture supernatants were resuspended in phosphate-buffered saline pH 7.4, layered over 15–60% (w/v) sucrose gradients, and centrifuged in a Beckman SW 65 rotor at 35,000 rpm for 16 hr at 4°. Three-drop fractions were collected from the bottom and 50-μl fractions were assayed for [³H]dTMP polymerization activity with $A_n \cdot dT_{12-18}$ as template-primer. Incubation was 90 min. Density determinations of selected fractions were performed with an Abbé refractomer and calibration data. ●, Reverse transcriptase activity; ○, density.

Samples prepared from unstimulated AKR cultures (Fig. 3, right; note scale difference) showed considerable activity, comparable to LPS/BrdU-treated BALB/c cells. Nevertheless, addition of LPS resulted in a 10-fold enhancement. Con A and PHA appeared to be slightly inhibitory.

Characterization of Enzyme Activity. Pooled day 2 to 4 supernatants from BALB/c cultures stimulated with LPS/BrdU, Con A/BrdU, or LPS and from LPS-stimulated AKR cultures were assayed for reverse transcriptase activity after isopycnic sucrose gradient centrifugation (Fig. 4). Major peaks occurred at 1.15–1.17 g/cm³, the density characteristic for C-type viruses.

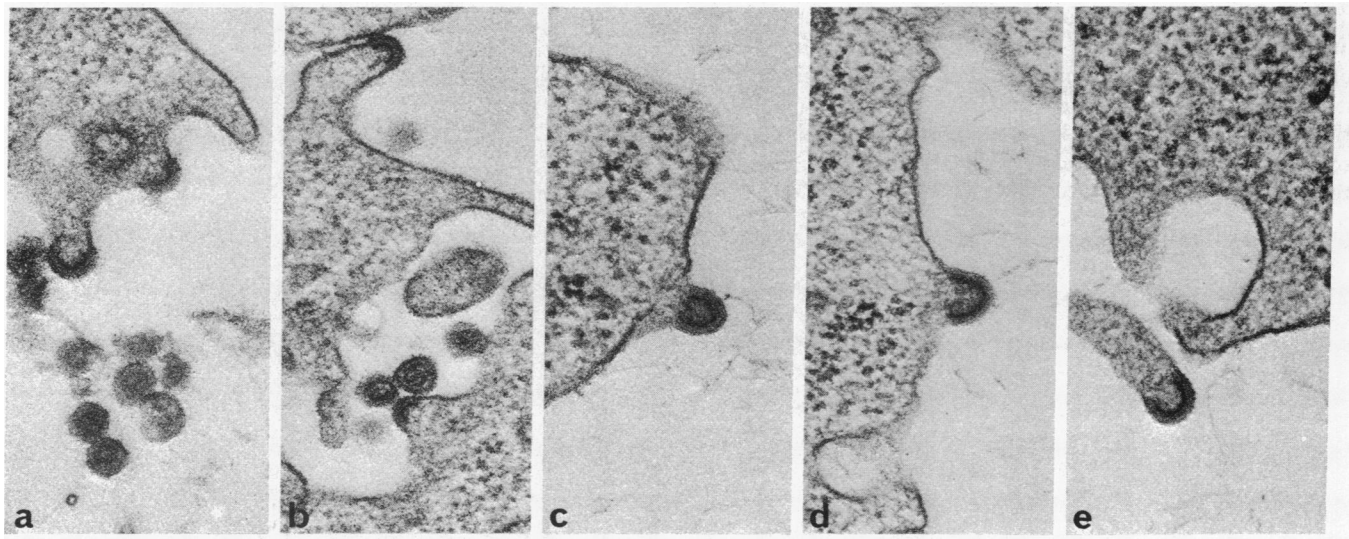

FIG. 5. Electron microscopy. Free mature C-type viruses and particles budding from the membrane of LPS-stimulated AKR cells (a), and unstimulated AKR cells (b). Typical C-type particles budding from the membrane of BALB/c cells stimulated with LPS/BrdU (c), with Con A/BrdU (d), and with LPS (e). Magnification: 65,000×.

Some cellular DNA polymerases and related enzymes, such as terminal nucleotidyl transferase (13), have been reported to have some dTMP polymerization activity when incubated with $A_n \cdot dT_{12-18}$. They can be distinguished from viral reverse transcriptase by template-primer preference experiments (14–16). We therefore compared the activities of the induced enzymes to activities present in a homogenate from nonstimulated BALB/c spleen cells (Table 1). Rauscher leukemia virus (RLV) was included as a positive control. The induced enzymes, just as RLV reverse transcriptase, preferred $A_n \cdot dT_{12-18}$ over $dA_n \cdot dT_{12-18}$ and had very little activity with dT_{12-18}. In contrast, cellular enzymes preferred $dA_n \cdot dT_{12-18}$ and had considerable activity when tested with dT_{12-18}.

Ultrastructure of Induced Spleen Cell Cultures. Among spleen cells cultured for 3 days and which had released reverse transcriptase activity in parallel cultures, typical free as well as budding C-type virus particles were observed. They were most numerous in LPS-stimulated AKR cultures (Fig. 5a), as well as in nonstimulated AKR cultures (Fig. 5b). Amongst BALB/c cultures treated with LPS/BrdU (Fig. 5c), Con A/BrdU (Fig. 5d), and LPS (Fig. 5e) the frequency of free and budding particles appeared to be decreasing in that order.

DISCUSSION

We describe here a simple method for the induction of endogenous C-type virus in spleen cell cultures. The viruses show C-type characteristics by electron microscopy. They are released from the cells into the supernatant, have a density of 1.15–1.17 g/cm^3 (Fig. 4), and contain the enzyme reverse transcriptase. This viral enzyme was clearly distinguished from cellular enzymes present in spleen cell homogenates by template-primer preference experiments (Table 1).

Our initial suggestion, namely that a mitogen in combination with BrdU, but not alone, might induce virus release was confirmed with Con A. Surprisingly, however, LPS by itself also induced virus release which could still be potentiated about 5-fold by combination with BrdU. In contrast, PHA, while stimulating more DNA synthesis than LPS, never induced virus with or without BrdU. This did not seem to be due to inhibition of virus release brought about by PHA-mediated cell agglutination, since PHA did not reduce the activity induced by LPS when the two were added in combination (unpublished results). It, therefore, seems that those cells responsive to the PHA mitogenic signal are not the cells that release virus.

The mitogens used here are known to affect different cell populations (17, 18), which may explain their differential inducing capacities. Recently, we confirmed this hypothesis, finding that induction depends on the presence of B-cells (bone-marrow-derived) (unpublished results).

In the experiments reported here, we observed differences between the high leukemia strain AKR and the low leukemia strain BALB/c. As expected, unstimulated AKR cells, but not BALB/c cells, released virus. A potentiation of virus release was found with LPS in AKR, but in contrast to BALB/c, no further increase occurred by combined treatment with BrdU. Rather, BrdU was inhibitory for AKR virus release and accordingly, in contrast to BALB/c, no virus was induced by Con A/BrdU. We have no explanation for these differences. In both strains, PHA and Con A given alone had no effect.

1. Gross, L. (1970) *Oncogenic Viruses*, Pergamon Press, New York and London).
2. Aaronson, S. A., Hartley, J. W. & Todaro, G. J. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 87–94.
3. Rowe, W. P., Hartley, J. W., Lander, M. R., Pugh, W. E. & Teich, N. (1971) *Virology* **46**, 866–876.
4. Lowy, D. R., Rowe, W. P., Teich, N. & Hartley, J. W. (1971) *Science* **174**, 155–156.
5. Aaronson, S. A., Todaro, G. J. & Scolnick, E. M. (1971) *Science* **174**, 157–159.
6. Teich, N., Lowy, D. R., Hartley, J. W. & Rowe, W. P. (1973) *Virology* **51**, 163–173.
7. Lin, S.-Y. & Riggs, A. D. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2574–2576.
8. Hirsch, M. S., Phillips, S. M., Solnik, C., Black, P. H., Schwartz, R. S. & Carpenter, C. B. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1069–1072.
9. Lonai, P., Declève, A. & Kaplan, H. S. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 2008–2012.
10. Schumann, G., Schnebli, H. P. & Dukor, P. (1973) *Int. Arch. Allergy* **45**, 331–340.
11. Wu, A. M., Ting, R. C., Paran, M. & Gallo, R. C. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3820–3824.
12. Hartley, J. W., Rowe, W. P., Capps, W. I. & Huebner, R. J. (1969) *J. Virol.* **3**, 126–132.
13. McCaffrey, R., Smoler, D. F. & Baltimore, D. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 521–525.
14. Baltimore, D. & Smoler, D. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1507–1511.
15. Goodman, N. C. & Spiegelman, S. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2203–2206.
16. Robert, M. S., Smith, R. G., Gallo, R. C., Sarin, P. S. & Abrell, J. W. (1972) *Science* **176**, 798–800.
17. Janossy, G. & Greaves, M. F. (1972) *Clin. Exp. Immunol.* **10**, 525–536.
18. Gery, I., Krüger, J. & Spiesel, S. Z. (1972) *J. Immunol.* **108**, 1088–1091.
19. Moroni, C. (1972) *Virology* **47**, 1–7.