Induction of Interferon and Erythropoietic Differentiation in Cells Transformed by Friend Virus

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Received for publication 29 July 1975

Two lines of Friend virus (FV)-transformed mouse spleen cells have been analyzed in respect to their interferon production capacity: neither F4 cells, which liberate infectious FV when kept under tissue culture conditions, nor the thymidine kinase-deficient B8 cells, which do not produce significant amounts of FV, release detectable amounts of autogenous interferon into cell supernatants. However, interferon is produced in these cells in amounts comparable to that in L-929 cells when interferon induction is initiated with UV-inactivated Newcastle disease virus. Conversely poly(I)·poly(C), a potent interferon inducer in L-929 cells, proved ineffective in this capacity in F4 or B8 cells. When erythropoietic differentiation is induced in these cells by dimethyl sulfoxide, no autogenous interferon production occurs, but with NDV-induction a four- to fivefold increase of interferon production is observed. A similar elevation of interferon production is achieved during 5-bromodeoxyuridine stimulation of differentiation in the thymidine kinase-deficient B8 cells. The refractiveness against poly(I)·poly(C) displayed in unstimulated cells is not overcome at any stage of differentiation, indicating major differences of Newcastle disease virus and poly(I)·poly(C) induction mechanisms.

When transformed by Friend virus (FV), mouse spleen lymphocytes can be propagated under tissue culture conditions (9, 21). A cloned line (F4) of FV-transformed mouse spleen cells produces infectious virus (17) containing both components of the FV complex (8), the spleen focus-forming virus, and the murine leukemia virus-Friend (7, 20). Addition of dimethyl sulfoxide (ME₂SO) to the culture medium of these transformed cells induces their differentiation along the erythropoietic pathway (9, 17). The 5-bromo-2'-deoxyuridine (BrdU)-resistant cell-line B8 of the same origin likewise can be induced to differentiate by ME₂SO but also by high concentrations of BrdU (6, 16). This BrdU-induced hemoglobin synthesis in B8 cells (but not the ME₂SO induced) is accompanied by a transient burst in FV synthesis on the second day of BrdU treatment (18). Exogenous interferon inhibits this virus induction (Ostertag, Crozier, and Swetly, manuscript in preparation) as well as the FV release in F4 cells (22), demonstrating that these FV-transformed cells are sensitive to interferon. The synthesis of hemoglobin which is induced during the erythroid differentiation remains unaffected by the presence of interferon (22).
cells to synthesize and release interferon is investigated by using different inducers, Newcastle disease virus (NDV) or poly(I)·poly(C).

Erythropoietic differentiation, stimulated in F4 cells by addition of ME₂SO and in B8 cells by ME₂SO or by BrdU, occurs concurrently for more than 70% of the cells (6) and permits for the first time a study of interferon inducibility at the different stages of cellular differentiation.

MATERIALS AND METHODS

Cells and viruses. The FV-transformed cloned mouse spleen cell line F4 and the BrdU-resistant mutant B8 have been obtained from W. Ostertag, and their isolation and properties are published (6, 16, 21). The cells are cultured in plastic dishes in Basal Eagle medium supplemented with 15% fetal bovine serum, double amounts in amino acids and vitamins, and a fourfold amount of glutamine. Incubation is at 37 C in humidified air containing 5% CO_2.

NDV (California strain) grown in chicken eggs has been obtained from G. Bodo. Hemagglutination has been determined with chicken erythrocytes. For interferon induction UV-irradiated virus has been used. L-929 cells are kept in Basal Eagle medium containing 10% calf serum (inactivated for 30 min at 56 C). L-929 cells serve as controls in the interferon induction experiments with NDV and poly(I)·poly(C) and are used for the plaque reduction test of vesicular stomatitis virus.

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Vesicular stomatitis virus, strain Indiana, has been grown in chicken eggs and plaque assayed on L-929 cells.

Reagents. ME$_2$SO (purchased from Merck, Darmstadt; gas chromatography grade) is added without treatment to the culture media where indicated at concentrations between 140 to 250 mM. BrdU has been purchased from Sigma Chemical Co., St. Louis. It is dissolved in 0.2 N NaOH at 20 mg/ml. The final concentration after adding it to B8 cells for stimulation of erythroid differentiation is 0.8 mM; the final concentration to suppress erythroid differentiation in F4 cells is 25 μM.

Poly(I)·poly(C) has been purchased from Miles Laboratories; DEAE-dextran was from Pharmacia; and neomycin was from C. H. Boehringer, Ingelheim.

Stimulation of erythropoietic differentiation and hemoglobin synthesis. To cells growing at a density of 0.5×10^6 to 10^6 cells/ml, ME$_2$SO is added on day 1 at a concentration of 1%. Cells are then transferred in daily intervals to fresh medium containing ME$_2$SO at concentrations: 1.25% on day 2, 1.5% on day 3, and 1.75% on days 4 and 5. The cell density throughout the stimulation period is maintained at 10^6 to 1.5×10^6 cells/ml. The BrdU stimulation of differentiation in BrdU-resistant B8 cells is carried out in cells which have been maintained for a period of 8 weeks in medium without BrdU. Cells are plated then at a density of 10^6 cells/ml and exposed to medium with 0.8 mM BrdU with daily changes of medium through a 5-day stimulation period. The percentage of cells involved in erythroid differentiation and synthesizing hemoglobin has been determined by methanol fixation of cells on slides and staining with benzidine-Wright-Giemsa. A minimum of 10^3 cells has been scored for the ratio of benzidine reactive cells to viable cells in culture.

Production of interferon in the FV-transformed cells. Production of interferon in F4 and B8 cells has been assayed in supernatants of cultures kept either without ME$_2$SO or in presence of ME$_2$SO. Medium which has supported cellular growth for at least 24 h has been centrifuged to remove cells (800 × g, 10 min), and cell debris and supernatant virus (82,000 × g, 50 min) were adjusted to pH 2.5 with 1 N HCl and dialyzed against 0.1 M glycin-hydrochloride buffer, pH 2.5, for 5 days at 4 C. After adjustment to neutral pH, the amount of interferon has been determined by its capacity to inhibit the cytopathic effect of vesicular stomatitis virus in L-929 mouse cells. One unit of interferon is defined as the reciprocal of the highest dilution of a sample that reduces vesicular stomatitis virus plaque formation in a L-929 cell culture by 50%. The plaque assay has been standardized with an NIH reference standard mouse interferon preparation.

Induction of interferon production. Two types of interferon-inducing agents have been applied: UV-inactivated NDV and poly(I)·poly(C) with DEAE-dextran and neomycin.

The NDV induction of interferon has been carried out by removing the medium from cells and exposing them to aliquots of 0.6 hemagglutinating units of NDV per 10^6 cells for 1 h at 37 C, in a volume of 0.5 ml of phosphate-buffered saline. For poly(I)·poly(C) in-

duction of interferon, a spectrum of inducer concentrations ranging from 0.5 to 40 μg of poly(I)·poly(C), 10 to 500 μg of DEAE-dextran, and 300 μg of neomycin in phosphate-buffered saline per 10^6 cells has been applied. Mouse L-929 cells serve as controls in the interferon induction experiments. In these cells interferon induction is optimal when 10^6 cells are exposed to 4 μg of poly(I)·poly(C), 100 μg of DEAE-dextran, and 300 μg of neomycin for 30 min at 25 C. For the poly(I)·poly(C) induction, the spleen cells are grown as described above and centrifuged at 1,000 × g for 5 min, and the resulting cell pellets are exposed to various concentrations of poly(I)·poly(C) at 25 C for 30 min. Controls are obtained by incubating cells in phosphate-buffered saline without inducers.

After the incubation period the cells are washed four times with medium without serum and further incubated at 37 C in serum-containing medium with the appropriate amounts of ME$_2$SO or BrdU for interferon induction in differentiating cells. Cell-free supernatants are tested for interferon after 24 h as described above. Cell counts and benzidine staining of cells are carried out to relate interferon production to cell number and to cell differentiation.

Cell numbers are determined by counting the viable cells after trypan blue staining in a hemocytometer.

RESULTS

No autogenous interferon production occurs in FV-transformed cells. The aim of this part is a correlation between continuous release of infectious FV from the transformed cells and production of autogenous interferon. Cells of line F4 produce 10^4 spleen focus-forming units/10^6 cells and 10^4 to 10^5 PFU/10^6 cells of murine leukemia virus as tested in the XC assay. On the other hand the BrdU-resistant B-8 cells produce only 1 to 5 spleen focus-forming units/10^6 cells and no detectable leukemia virus component in the XC-test (1, 15, 19).

Both types of cells are plated either under conditions of exponential cell growth (0.5×10^6 to 1.5×10^6 cells/ml) or under conditions of low mitotic activity (2.5×10^6 to 3×10^6 cells/ml). Supernatant medium is tested after 24 h of incubation for interferon as described above. No interferon activity has been found (Table 1) in the supernatants of growing or resting cells, nor in FV releasing or non-producer cells.

ME$_2$SO or BrdU stimulation of cell differentiation does not lead to autogenous interferon production. When F4 cells or B8 cells are exposed to 1 to 2% ME$_2$SO for 4 to 5 days, more than 70% of the cells react with benzidine, indicating their erythroid character, and 20 to 30% of the soluble cytoplasmic protein fraction constitutes hemoglobin (17, 22). Parallel to the progress in cell differentiation a reduction in the

TABLE 1. *Autogenous interferon production in F4 and B8 cells either unstimulated or at various stages of erythroid differentiation and the interferon induction by NDV and poly(I)·poly(C)*

Differentiation stimulating agent	Interferon inducer	Interferon production (U/10^6 cells) in cell line:		
		F4	B8	L-929
None	None	<10	<10	<10
ME$_2$SO (0.14 to 0.25 M) (1 to 5 days)	None	<10	<10	NT[a]
BrdU (10^{-3} M) (1 to 5 days)	None	NT	<10	NT
None	Poly(I)·poly(C)	<10	<10	5,500
None	NDV	5,250	4,600	9,450
ME$_2$SO (0.14 to 0.25 M) (1 to 5 days)	Poly(I)·poly(C)	<10	<10	NT
BrdU (10^{-3} M) (1 to 5 days)	Poly(I)·poly(C)	NT	<10	NT

[a] NT, Not tested.

uptake of low-molecular-weight metabolites and a decrease in DNA, RNA, and protein synthesis are observed (6). FV synthesis increases 10-fold in F4 cells in the early stages of stimulation and decreases again from day 3 on (22). The BrdU stimulation of B8 cells leads to a lower level of hemoglobin synthesis (5% of soluble cytoplasmic proteins and about 40% of benzidine-positive cells), to a similar reduction of membrane permeability and macromolecular synthesis and to a transient induction of FV synthesis on day 2 to 3. With this diversity of events preceding the onset of differentiation, it seemed worthwhile to look for autogenous interferon induction during the course of ME$_2$SO or BrdU treatment. Supernatants harvested in daily intervals after stimulation of differentiation have been assayed for interferon activity. Neither stimulation of F4 or B8 cells with ME$_2$SO nor of B8 cells with BrdU leads at any stage of this differentiation period to detectable autogenous interferon production (Table 1).

Induction of interferon production succeeds with UV-inactivated NDV but not with poly(I)·poly(C). To relate the failure of interferon production in the FV releasing cells, either to a defect caused by the inability of the transformed cells to synthesize interferon or to a defect of FV to induce it, the following experiment has been performed. F4 or B8 cells are exposed to either of two established interferon inducers: UV-inactivated NDV and the double-stranded polynucleotide poly(I).poly(C). This second inducer is ineffective by itself in another mouse cell line (L-929); however, in the presence of DEAE-dextran (5) and neomycin (2), it

induces interferon production in L-929 cells to a level of 5,000 U/10^6 cells. A cell toxic effect is associated with this interferon induction. The results are summarized in Table 1 and represent data from five experiments. For induction of interferon in F4 and B8 cells, the concentration of poly(I)·poly(C) has been varied between 0.5 and 40 µg per 10^6 cells. Neither of the applied concentrations, without or in combination with various amounts of DEAE-dextran and with neomycin, have resulted in an induction of interferon or in significant cell toxicity. In contrast to this inability of poly(I)·poly(C) to induce interferon in the FV-transformed spleen cells, UV-inactivated NDV induces about 5,000 U of interferon/10^6 cells. No significant difference in the interferon production has been found between the FV releasing F4 cells and the B8 cells, which do not produce virus.

The amount of interferon induced by NDV depends on the stage of erythropoietic cell differentiation. Not only hemoglobin synthesis increases but also the release of FV from F4 cells goes up more than 10-fold when ME$_2$SO is added and cells begin to differentiate (22). However, when F4 cells are exposed to 2×10^{-5} M BrdU for 2 days before and then during the 5 days of ME$_2$SO treatment, no significant effect on cell growth and viability occurs, but hemoglobin synthesis is inhibited (16). Interferon may be seen also as a specialized cellular product, and thus it appeared interesting to follow the capacity of the cell to respond to NDV induction of interferon at different stages of the erythropoietic differentiation.

The F4 cells have been treated either with 2×10^{-5} M BrdU for 2 days and then exposed to ME$_2$SO and BrdU, or without pretreatment exposed to ME$_2$SO. In daily intervals, UV-irradiated NDV was added to aliquots of 2×10^7 cells, incubation was carried on for 24 h in ME$_2$SO-containing medium with or without BrdU, and cell virus-free supernatants were assayed for interferon. The percentage of cells involved in erythroid differentiation is determined by benzidine staining. The results are presented in Fig. 1. Interferon production increases fourfold on day 3 of the stimulation period. This time coincides with a strong increase in hemoglobin synthesis in these cells. During the late phase of stimulation, the interferon production decreases again to a level below that of unstimulated cells. The optimum of interferon production fluctuated slightly between days 2 and 3. Treatment of cells with BrdU, which inhibits the hemoglobin synthesis (Fig. 1, lower panel), keeps the NDV-induced interferon production on the level of un-
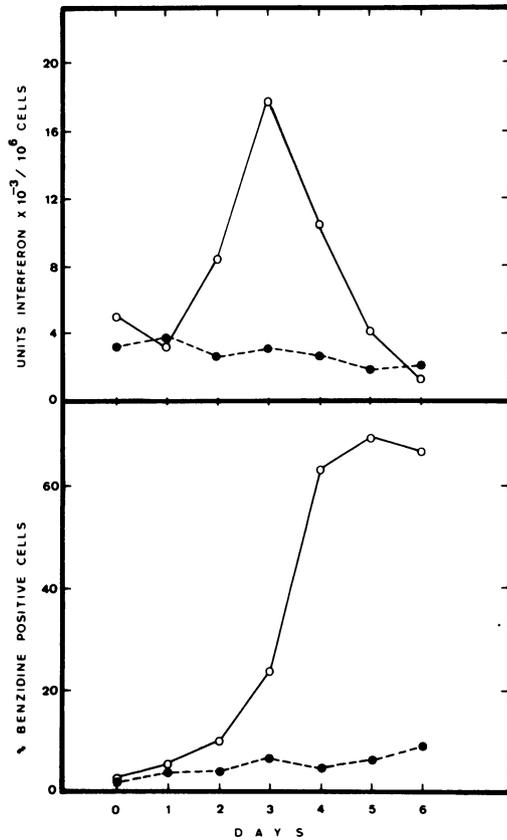
FIG. 1. *NDV induction of interferon in differentiating F4 cells. F4 cells were stimulated by ME₂SO as described, and aliquots of cells were exposed to 0.6 hemagglutinating units of UV-inactivated NDV. After 24 h, interferon activity was determined in the cell supernatants. (Upper panel) Units of interferon produced by 10⁶ cells at different times after ME₂SO stimulation. (Lower panel) Percentage of cells in erythropoietic differentiation as determined by benzidine staining. Symbols: O, F4 cells stimulated by ME₂SO; ●, F4 cells treated with 2 × 10⁻⁵ M BrdU for 2 days and then exposed to ME₂SO for 6 days in the presence of 2 × 10⁻⁵ M BrdU.*

stimulated cells throughout the period of treatment.

The production of interferon after NDV induction in BrdU-resistant B8 cells has been investigated under the following conditions: treatment for 5 days with (i) ME₂SO, 1 to 2%; (ii) BrdU, 0.8×10^{-3} M; (iii) ME₂SO, 1 to 2%, and BrdU, 0.8×10^{-3} M. UV-inactivated NDV is added at daily intervals to aliquots of cells, and the interferon activity is determined in the cell supernatants 24 h thereafter.

Stimulation with ME₂SO leads to differentia-

tion of more than 60% of the cells in culture and to patterns of interferon synthesis similar to that in ME₂SO-treated F4 cells (Fig. 2A). Interferon production rises to 16,000 U/10⁶ cells on days 3 and 4 of stimulation. Under these conditions of stimulation, no FV induction occurs in the B8 cells (18). When stimulation of hemoglobin synthesis is carried out with 0.8×10^{-3} M BrdU in the absence of ME₂SO (Fig. 2b), 40% of cells are benzidine reactive and the overall rate of hemoglobin synthesis is 5% of the soluble cytoplasmic protein fraction (16). Increase over the basic level of interferon production during BrdU stimulation is lower (threefold increase on day 3) than in B8 cells stimulated by ME₂SO. When ME₂SO and BrdU were added together (Fig. 2C), both the interferon inducibility and the hemoglobin synthesis were higher than with either of the stimulating agents alone, indicating an additive effect.

Attempts to induce interferon by poly(I)·poly(C) in F4 and B8 cells at different stages of erythropoietic differentiation have been unsuccessful (Table 1).
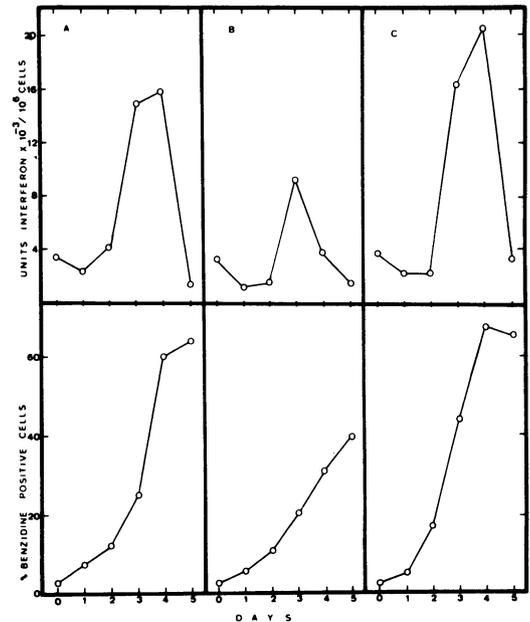

FIG. 2. *NDV induction of interferon in BrdU-resistant B8 cells during erythropoietic differentiation. Stimulation of differentiation by ME₂SO treatment (A), by treatment with 0.8×10^{-3} M BrdU (B), and by treatment with ME₂SO and 0.8×10^{-3} M BrdU (C). (Upper panels) Interferon induced by NDV in relation to time of stimulation. (Lower panels) Percentage of cells involved in differentiation as indicated by benzidine staining.*

DISCUSSION

The aim of this study is the control of interferon induction in cells undergoing differentiation. In this specific situation differentiation results in the induction of a specialized cellular protein (hemoglobin) and a parallel decrease in DNA, RNA, and overall protein synthesis.

Upon adequate stimulation, interferon is produced as well in highly differentiated cells of reticuloendothelial origin with no capacity of further proliferation, like leukocytes (10, 11), as in cells with a low degree of differentiation and infinite proliferative capacity, like continuous cell lines (4). Spleen cells have been discussed repeatedly as possible in vivo target cells for interferon induction (13, 14). Production of interferon may be viewed as a cellular response to an external stimulus and represents a process analogous to enzyme induction and thus differentiation. Inhibition by actinomycin D of interferon formation (12) is in line with this view. ME_2SO, which induces hemoglobin formation, has no stimulating effect on the interferon production. This implies that induction of cell differentiation and presence of FV are not sufficient for interferon induction.

UV-inactivated NDV induces around 5,000 U of interferon in 10^6 F4 or B8 cells.

When cells stimulated with ME_2SO for 2 to 3 days are challenged with UV-NDV, the fourfold amount of interferon activity is released. This effect may indicate either a higher potential of differentiating cells to produce interferon or simply an increased receptiveness of ME_2SO-exposed cells to the viral interferon inducer. To distinguish between the two possibilities, ME_2SO stimulation of hemoglobin synthesis has been inhibited in BrdU-sensitive F4 cells by a low dose of BrdU, which does not affect cell proliferation (16). Under these condition the increase in interferon induction is not observed, which indicates that the mere presence of ME_2SO does not facilitate interferon induction but that cells had to evolve in their differentiation.

In BrdU-resistant B8 cells hemoglobin production can be induced by high concentrations of BrdU also in the absence of ME_2SO. The two- to threefold increase of interferon inducibility when cells had been stimulated for 3 days with BrdU gives further support to the concept of linkage between interferon production and the state of cell differentiation. At the final stage of erythropoiesis interferon inducibility regresses.

A special property of these transformed spleen cells seems to be the refractiveness of interferon production mechanism to poly(I)·poly(C) induction. A wide variety of reaction conditions as well as the presence of DEAE-dextran and/or neomycin did not lead to measurable interferon induction.

When differentiating cells are challenged with poly(I)·poly(C) under similar conditions where NDV-mediated induction is elevated, no release of interferon is observed. That these cells remain refractive to the polynucleotide inducer may reflect either a property of the cell membrane (nucleolytic activity) or a fundamental difference in the induction mechanisms by NDV and poly(I)·poly(C) (3), the latter being inactive here.

LITERATURE CITED

1. Axelrad, A. A., and R. A. Steeves. 1964. Assay for Friend leukemia virus: rapid quantitative method based on enumeration of macroscopic spleen foci in mice. Virology 24:513-518.
2. Billiau, A., C. E. Buckler, F. Dianzani, C. Uhlendorf, and S. Baron. 1969. Induction of the interferon mechanism by singlestranded RNA: potentiation by polybasic substances. Proc. Soc. Exp. Biol. Med. 132:790-796.
3. Burke, D. C. 1973. The mechanisms of interferon formation, p. 107-133. In N. B. Finter (ed.), Interferons and interferon inducers. North-Holland Publishing Co., Amsterdam.
4. Cantell, K., and K. Paucker. 1963. Quantitative studies on viral interference in suspended L cells. Virology 21:11-18.
5. Dianzani, F., P. Cantagalli, S. Gagnoni, and G. Rita. 1968. Effect of DEAE-dextran on production of interferon induced by synthetic double-stranded RNA in L-cell cultures. Proc. Soc. Exp. Biol. Med. 128:708-710.
6. Dube, S. K., G. Gaedicke, N. Kluge, B. I. Weimann, H. Melderis, G. Steinheider, T. Crozier, H. Beckmann, and W. Ostertag. 1974. Haemoglobin-synthesizing mouse and human erythroleukemic cell lines as model systems for the study of differentiation and control of gene expression, p. 103-139. In W. Nakahara, T. Ono, T. Sugimura, and H. Sugano (ed.), Differentiation and control of malignancy of tumor cells. University Park Press, Baltimore.
7. Dube, S. K., I. B. Pragnell, N. Kluge, G. Gaedicke, G. Steinheider, and W. Ostertag. 1975. Induction of endogenous and of spleen focus forming viruses during dimethylsulfoxide-induced differentiation of mouse erythroleukemia cells transformed by spleen focus forming virus. Proc. Natl. Acad. Sci. U.S.A. 72:1863-1867.
8. Eckner, R. I., and R. A. Steeves. 1972. A classification of the murine leukemia viruses. J. Exp. Med. 136:832-850.
9. Friend, C., W. Scher, I. G. Holland, and T. Sato. 1971. Hemoglobin synthesis in murine virus-induced leukemic cells in vitro: stimulation of erythroid differentiation by dimethylsulfoxide. Proc. Natl. Acad. Sci. U.S.A. 68:378-382.
10. Glasgow, L. A., and K. Habel. 1963. Interferon produc-

tion by mouse leukocytes in vitro and in vivo. J. Exp. Med. 117:149–156.

11. Gresser, I. 1961. Production of interferon by suspensions of human leukocytes. 1961. Proc. Soc. Exp. Biol. Med. 108:799–803.

12. Heller, E. 1963. Enhancement of Chikungunya virus replication and inhibition of interferon production by actinomycin D. Virology 21:652–663.

13. Kono, Y., and M. Ho. 1965. The role of the reticuloendothelial system in interferon formation in the rabbit. Virology 25:162–166.

14. Osborn, J. E., and D. L. Walker. 1969. The role of individual spleen cells in the interferon response of the intact mouse. Proc. Natl. Acad. Sci. U.S.A. 62:1038–1045.

15. Ostertag, W., T. Cole, T. Crozier, G. Gaedicke, J. Kind, N. Kluge, J. C. Krieg, G. Roesler, G. Steinheider, B. I. Weimann, and S. K. Dube. 1974. Viral involvement in the differentiation of erythroleukemic mouse and human cells, p. 493–521. In W. Nakahara, F. Ono, T. Sugimura, and H. Sugano (ed.), Differentiation and control of malignancy of tumor cells. University Park Press, Baltimore.

16. Ostertag, W., T. Crozier, N. Kluge, H. Melderis, and S. Dube. 1973. The action of 5-bromodeoxyuridine on the induction of hemoglobin synthesis in mouse leukemia cells sensitive and resistant to 5-BUdR. Nature (Lon-don) New Biol. 243:203–205.

17. Ostertag, W., H. Melderis, G. Steinheider, N. Kluge, and S. K. Dube. 1972. Synthesis of mouse haemoglobin and globin mRNA in leukemic cell cultures. Nature (London) New Biol. 239:231–234.

18. Ostertag, W., G. Roesler, C. J. Krieg, J. Kind, T. Cole, T. Crozier, G. Gaedicke, G. Steinheider, N. Kluge, and S. Dube. 1974. Induction of endogenous virus and of thymidine kinase by bromodeoxyuridine in cell cultures transformed by Friend virus. Proc. Natl. Acad. Sci. U.S.A. 71:4980–4985.

19. Rowe, W. P., W. E. Pugh, and I. W. Hartley. 1970. Plaque assay techniques for murine leukemia viruses. Virology 42:1136–1139.

20. Steeves, R. A. 1975. Spleen focus forming virus in Friend and Rauscher Leukemia virus preparations. J. Natl. Cancer Inst. 54:289–297.

21. Steinheider, G., H. Melderis, and W. Ostertag. 1972. Mammalian embryonic hemoglobins, p. 225–235. In H. Martin and L. Novicki (ed.), Synthesis, structure and function of hemoglobin. J. F. Lehmann's Verlag, Munich.

22. Swetly, P., and W. Ostertag. 1974. Friend virus release and induction of hemoglobin synthesis in erythroleukemic cells respond differently to interferon. Nature (London) 251:642–644.