Role of interferon and 2’,5’-oligoadenylate synthetase in erythroid differentiation of Friend leukemia cells. Studies with interferon-sensitive and -resistant variants.

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It has been suggested that the interferon (IFN)-induced 2',5'-oligoadenylate (2-5A) synthetase, which polymerizes ATP into a series of 2',5'-linked oligomers with the general formula pppA(2'p5'p)n, plays a general role in cell growth and terminal differentiation. For instance, an increase in 2-5A synthetase activity has been described during dimethyl sulfoxide (Me2SO)-induced erythroid differentiation of Friend leukemia cells (FLC).

2-5A synthetase has been measured in two Friend leukemia cell sublines by various techniques including a radioimmunoassay of its products which would detect 10^-18 mol of 2-5A cores. Although cells of both sublines fully differentiate (as measured by benzidine staining), only in one subline was there an increase in 2-5A synthetase activity upon treatment with Me2SO. Hexamethylenebisacetamide, another potent agent of differentiation in this system, did not increase 2-5A synthetase activity in either of these two sublines. An IFN-resistant FLC variant differentiated normally upon treatment with Me2SO or hexamethylenebisacetamide while it was noninducible for 2-5A synthetase activity by exogenous IFN or by the inducers themselves. A similar situation has been observed with regard to the level of phosphorylation of the IFN-induced M5 = 67,000 protein band. In addition, treatment of IFN-sensitive and resistant FLC sublines with mouse aIFN antiserum did not affect differentiation. Even though we have duplicated previous findings on the increase of 2-5A synthetase activity in Me2SO-induced FLC, the lack of an increase with other inducers or other sublines indicates that there is no causal relationship between the enzyme activation and FLC differentiation.

Besides their antiviral activity, interferon(s) can serve as pleiotropic effector molecules in a variety of systems, including cells differentiating in vitro (Rossi et al., 1980, 1982). Extensive studies have revealed the major role played by two antiviral activity, interferon(s) can serve as pleiotropic effector molecules in a variety of systems, including cells differentiating in vitro (Rossi et al., 1980, 1982). Extensive studies have revealed the major role played by two

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## Role of Interferon and 2',5'-Oligoadenylate Synthetase in Erythroid Differentiation of Friend Leukemia Cells

**STUDIES WITH INTERFERON-SENSITIVE AND -RESISTANT VARIANTS**

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tained from C. Friend, Mount Sinai Medical Center, New York, were independently passed for several years in the laboratories of L. Harel (Institut de Recherches Scientifiques sur le Cancer (IRSC) Villejuif) or G. B. Rossi. They will be referred hereafter as 745A1 and 745A4, respectively. Cells of these sublines and of 745A5 1C1, an IFN-resistant subline derived from 745A4 (Affabris et al., 1982) were grown in RPMI 1640 medium supplemented with 5% (v/v) fetal calf serum. Moloney sarcoma virus-transformed C243-3 murine fibroblasts, obtained from L. Gresser (IRSC, Villejuif), were grown in MEM supplemented with 10% (v/v) NBS. L929 cells were grown in MEM supplemented with 5% (v/v) fetal calf serum.

3. Vesicular stomatitis virus (Indiana strain) stocks, obtained by infecting L929 cell monolayers with low multiplicity of infection (0.01–0.1 plaque-forming unit/cell) were titrated by plaque assay on the same cells. Titers ranged between 10^8 and 10^9 plaque-forming units/ml. Newcastle Disease virus, strain F, stocks were prepared from the same cell cultures and kept frozen at -70 °C. Protein concentration was determined by the method of Whitaker and Granum (1980).

RESULTS

2-5A Synthetase Activity in Several Growing and Differentiating FLC Sublines Treated with Me2SO—Cells of two FLC sublines (745A5 and 745A4) and of one IFN-resistant variant (745A5 1C1) were seeded at 10^6 cells/ml in medium supplemented with Me2SO and were grown for 6 days under stationary conditions. As shown in Fig. 1A, the three growth curves were similar. Under these conditions, erythroid differentiation, as measured by hemoglobin-containing (benzidine-positive) cells, attained nearly 100% in all three lines (Fig. 1B). In accordance with published data (Kimchi, 1981), 2-5A synthetase activity increased in parallel with the accumulation of benzidine-positive cells in the 745A5 subline. On the contrary, neither the wild type 745A5 subline nor the IFN-resistant variant derived therefrom, exhibited any detectable enzyme activity (Fig. 1C). It was verified that 745A5 cells responded to exogenous mouse dIFN with the expected elevation in 2-5A synthetase activity (as shown by Affabris et al., 1983) while 745A5 1C1 cells did not respond to such treatment (data not shown), as previously described (Affabris et al., 1983).
As 2-5A synthetase levels have been reported to be related to growth conditions (Krishnan and Baglioni, 1980; Creasey et al., 1980), two additional protocols were also followed. 745A~ cells seeded at 5 x 10^5/ml in MeSO-rich medium were counted daily and diluted in MeSO-containing medium to the initial concentration for 8 days. Alternatively, cells of the same subline were seeded at 10^5/ml in the same medium, counted 3 days later (when cell saturation density had not yet been attained), diluted to 2 x 10^5/ml in MeSO-rich medium, and grown for 8 days, essentially as described by Friedman-Einat et al. (1982). Although the percentage of benzidine-positive cells remained almost 100% in all these conditions, no increase in 2-5A synthetase activity could be detected (data not shown). Likewise, no increase in 2-5A synthetase was observed when cells were diluted in MeSO-free medium (data not shown).

Since even very low levels of 2-5A synthetase activity could suffice to activate RNase L and therefore be biologically meaningful, these negative data have been re-assessed with a radioimmunological assay of 2-5A cores (Cailla et al., 1982a). The cell-free products of 2-5A synthetase were dephosphorylated with alkaline phosphatase and quantified using monoclonal antibodies specifically detecting as low as nanomoles of 2-5A cores (Cailla et al., 1982b). Fig. 2 shows the linear conversion of ATP into 2-5A expected for an enzymatic assay. The use of appropriate dilutions allowed detection of both the induction of 2-5A synthetase activity upon exogenous IFN treatment of 745A~ cells (Fig. 2A) and a low but measurable basal 2-5A synthetase activity in cell-free extracts prepared from untreated cells (Fig. 2B). Similar low levels of 2-5A synthetase activity were detected in extracts of IFN-resistant variants of 745A~ cells (Cailla et al., 1982b). Fig. 2 shows the linear conversion of ATP into 2-5A expected for an enzymatic assay.
2-5A Synthetase and Differentiation in Friend Cells

Fig. 3. Autoradiography of SDS-polyacrylamide gel electrophoresis of protein kinase assay mixtures. 745A~ cells were seeded at 10^5/ml and grown in the presence of 1.5% (v/v) Me_2SO, 5 ml HMBA, or 200 units/ml of IFN. After 3 days, cells were diluted to 2 x 10^5/ml with medium containing the same inducer and grown for an additional 4 days. Cell extracts were prepared, as described under "Experimental Procedures," 3, 5, and 7 days after cell seeding. Percentages of B' cells were 49, 85, and 89 for Me_2SO-treated cells and 80, 97, and 99 for HMBA-treated cells, when measured 3, 5, and 7 days after seeding, respectively. The patterns of phosphorylation of the cell extracts, in the absence (-) or presence (+) of poly(rI)-poly(rC), were analyzed by polyacrylamide gel electrophoresis as described in the text. Assay mixture applied to each lane were as follows: a, 745A~ (3 days); b, 745A~ + Me_2SO (3 days); c, 745A~ + HMBA (3 days); d, 745A~ + αIFN (3 days); e, 745A~ (5 days); f, 745A~ + Me_2SO (7 days); g, 745A~ + HMBA (5 days); h, 745A~ (7 days); i, 745A~ + Me_2SO (7 days); l, 745A~ + HMBA (7 days); m, L929 + αIFN (1 day). The indicated molecular weights correspond to those of bovine serum albumin (68,000) and chymotrypsinogen (25,000).

Fig. 4. Effects of αIFN antiserum on growth and differentiation of FLC. 745A~ cells were seeded at 10^5/ml in the presence of 1.5% (v/v) Me_2SO with (O) or without (●) sheep antiserum against murine αIFN. Cell growth (A), cell differentiation determined by the percentage of B' cells (B), or by spectrophotometric determination of hemoglobin content (C). Differentiation of untreated cells with (△) or without (●) antiserum was taken as a control. 2-5A synthetase activity was measured as an internal control, in extracts prepared, as described under "Experimental Procedures," from cells treated with Me_2SO, Me_2SO + IFN (320 units/ml) and Me_2SO + IFN (320 units/ml) + antiserum. The obtained values were <1, 40, and <1 nmol of ATP transformed/h/mg of protein, respectively.

FLC—If IFN were released during FLC differentiation, as recently proposed (Friedman-Einat et al., 1982), it should increase the double-stranded RNA-activated protein kinase activity. Extracts were thus prepared from FLC induced to differentiate with either HMBA or Me_2SO, for increasing periods of time and incubate with γ-32P-labeled ATP with or without double-stranded RNA. None of these conditions led to an increased level of phosphorylation of the M_r = 67,000 protein band in 745A~ and 745A~ 1C11 sublines (Fig. 3) and in 745A~ up to day 6 (data not shown). On the contrary, extracts prepared from exogenous IFN-treated FLC did exhibit the characteristic double-stranded RNA-dependent increase of the M_r = 67,000 band phosphorylation, although less pronounced than for IFN-treated L929 cells taken as a positive reference (Fig. 3).

Effects of IFN Antiserum on FLC Erythroid Differentiation—Neutralization of IFN effects with a specific antiserum should provide an alternative approach to the elucidation of the role of IFN-induced enzymes in erythroid cell differentiation, if indeed any increase in their activity resulted from IFN release. FLC were treated with an antiserum raised against an unfractionated preparation of mouse αIFN at doses shown in preliminary experiments to completely inhibit exogenous IFN-induced 2-5A synthetase activity. As described in Fig. 4, such a treatment did not significantly affect FLC growth (A) or differentiation induced by Me_2SO as measured by the increase in benzidine-positive cells (B) or by spectrophotometric determination of hemoglobin content (C). IFN was included in parallel experiments as a control of antiserum efficacy, as described in Fig. 4.

DISCUSSION

The terminal differentiation of one FLC subline is accompanied by an increase of the IFN-induced 2-5A synthetase, as originally described by Kimchi (1981). However, the data presented in this paper do not support a general role of 2-5A synthetase induction in the differentiation of erythroid cells, as: 1) an increase in 2-5A synthetase activity following Me_2SO treatment is not observed in a closely related but independently passed FLC subline; and 2) HMBA does not modulate 2-5A synthetase activity even in the FLC subline (745A~) which shows an increase of 2-5A synthetase activity following Me_2SO treatment.

These data are in keeping with the absence of any detectable increase in 2-5A synthetase activity in five of five of the IFN-resistant variants isolated in this laboratory (Affabris et al., 1983) while all of them respond to inducers of differentiation.

3 Unpublished observations.
indistinguishably from their wild type parent.

The observed differences in the ability of Me₆SO and HMBA to increase 2-5A synthetase activity in at least one FLC subline is not surprising. It has been shown that differentiation inducers such as Me₆SO or Na butyrate increase 2-5A synthetase activity in several unrelated lines such as C243 aurine fibroblasts (Besançon et al., 1981) or HeLa cells, which are not known to express markers specific to a given differentiation lineage.

Since subnanomolar amounts of 2-5A suffice to activate RNase L (Lengyel, 1981), conventional assays for measuring 2-5A synthetase activity might not have been sensitive enough to detect low amounts of 2-5A products still of possible biological significance. Indeed, a recently developed sensitive radioimmunoassay of 2-5A (Cailla et al., 1982a) did allow us to measure previously undetectable basal levels of 2-5A synthetase activity in all three sublines. Yet, Me₆SO was unable to modulate these levels in 745AR and the IFN-resistant variant cells. We cannot exclude, however, a role of the 2-5A synthetase recently detected in the nuclei (Nilsen et al., 1981) in the differentiation process of FLC, as experimental procedures conventionally used to assay 2-5A synthetase do not lead to a major disruption of the nucleus. Likewise, a localized activation of the 2-5A system (as hypothesized by Nilsen and Baglioni, 1979) in the intact differentiating FLC could have escaped our detection and yet play a significant biological role.

2-5A synthetase induction could merely reflect the excretion of IFN, as reported by Friedman-Einat et al. (1982). There are examples of excretion of minute amounts of IFN undetectable by its biological assay. As we could not detect any IFN level in the supernatants of differentiating FLC, it would neutralize any IFN produced under these circumstances. As the differentiation process was not affected by this treatment, we can exclude a role of "spontaneously" produced αIFN in the course of Me₆SO-induced hemoglobinization. In this respect, it is not surprising that protein kinase is not induced by Me₆SO or HMBA under these experimental conditions in 745AR sublines. Likewise, IFN antisera suppresses the increased level of 2-5A synthetase activity accompanying mouse myeloid leukemia cell differentiation without preventing differentiation itself (Schawa et al., 1981). This does not exclude, however, a role of IFN and of the 2-5A system in the regulation of growth and differentiation in other systems such as rat liver regenerating after partial hepatectomy (Etienne-Smeakens et al., 1981) or lymphocyte activation (Kimchi, 1981).

Considering the presence of an elevated 2-5A synthetase activity reported in mammalian reticulocytes (Hovanessian and Kerr, 1978) and the data reported in this paper, we might hypothesize that any such increase takes place very late in the process of erythroid cell differentiation, e.g., at a stage preceding closely the extrusion of the nucleus. Differences in FLC sublines would then clearly explain why only some of them (see the data reported here for 745A₅ and those reported by Kimchi (1981)) do reach this stage while others do not (see data reported here for 745A₅ and for HMBA-induced 745A₅). Whatever the case, the 2-5A systems per se (i.e., independently from exogenous IFN addition) would not play any role either in the commitment of FLC to terminal differentiation or in the hemoglobinization process which is fully expressed in this in vitro system (Cioe et al., 1978).

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