Inhibition of the self-renewal capacity of blast progenitors from acute myeloblastic leukemia patients by site-selective 8-chloroadenosine 3',5'-cyclic monophosphate

(site-selective cAMP analogs/leukemic stem cells/clonogenic growth/cell differentiation)

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ABSTRACT   The physiologic balance between the two reg-
ulatory subunit isoforms, RI and RII, of cAMP-dependent
protein kinase is disrupted in cancer cells; growth arrest and
differentiation of malignant cells can be achieved when the
normal ratio of these intracellular signal transducers of cAMP
is restored by the use of site-selective cAMP analogs. In this
study we evaluated the effects of the site-selective cAMP analog
8-chloroadenosine 3',5'-cyclic monophosphate (8-Cl-cAMP)
on clonogenic growth of blast progenitors from 15 patients with
acute myeloblastic leukemia and 3 patients affected by ad-
vanced myelodysplastic syndrome. Leukemic blast progenitors
undergo terminal divisions, giving rise to colonies in methyl-
cellulose. The self-renewal capacity of blast progenitors is
conversely reflected in a secondary methylcellulose assay after
exponential growth of clonogenic cells in suspension cultures.
In all the samples tested, 8-Cl-cAMP, at micromolar concen-
trations (0.1–50 μM), suppressed in a dose-dependent manner
both primary colony formation in methylcellulose and the
recovery of clonogenic cells from suspension culture. Strik-
ingly, in the samples from the entire group of patients, 8-Cl-
cAMP was more effective in inhibiting the self-renewing clo-
nogenic cells than the terminally dividing blast cells (P =
0.005). In addition, in four out of six cases studied, 8-Cl-cAMP
was able to induce a morphologic and/or immunophenotypic
maturation of leukemic blasts. An evident reduction of RI levels
in fresh leukemic cells after exposure to 8-Cl-cAMP was also
detected. Our results showing that 8-Cl-cAMP is a powerful
inhibitor of clonogenic growth of leukemic blast progenitors by
primarily suppressing their self-renewal capacity indicate that
this site-selective cAMP analog represents a promising biolog-
ical agent for acute myeloblastic leukemia therapy in humans.

The progressive accumulation of leukemic cells in acute
myeloblastic leukemia (AML) is sustained by a small popu-
lation of leukemic blast progenitors (1, 2). Leukemic blast
progenitors, like normal hemopoietic stem cells, are able to
renew themselves and/or undergo terminal divisions giving
rise to nonrenewing end cells that, however, do not achieve
morphological maturity (2, 3). The self-renewal capacity of
blast progenitors has been shown to be highly correlated with
clinical outcome in AML (4–6).

Currently, however, no means are available to distinguish
between the "determined" and "self-renewing" blast cells
on morphological, phenotypical, or molecular grounds. Mc-
Culloch and associates (2, 7) have developed the only culture
method to date that is able to make an operational distinction
between the self-renewal and postdeterministic mitoses. The
culture method consists of two different assays: (*i*) direct
semisolid medium (methylcellulose) cloning and (*ii*) a 7-day
liquid culture, after which cells are replated in a methylcel-
lulose cloning assay. Conclusive evidence has been provided
that, during the liquid culture, self-renewing clonogenic cells
increase exponentially and are selected against determined
but still dividing blasts, so that they can be specifically
assayed in the subsequent cloning assay (8). Thus, in the
primary methylcellulose cloning, determined clonogenic
cells can be enumerated, while the secondary cloning fol-
lowing liquid culture allows the quantitations of self-renewing
clonogenic cells (8, 9). Most importantly, this method rep-
resents a predictive *in vitro* model for evaluating the thera-
peutic efficacy of anticancer agents in AML (1, 2, 6, 7). *In
vitro* evaluation of self-renewing clonogenic cells and their
inhibition by different drugs, as described by McCulloch and
associates (2, 4), have been significantly associated with the
clinical outcome and response to therapeutic agents admin-
istered *in vivo* to AML patients (2, 4, 6, 7). To eradicate
AML, it is therefore important to search for antitumor agents
that can inhibit not only terminal divisions, like most cyto-
toxic drugs (10, 11), but also can inhibit self-renewal of
leukemic blast progenitors.

Previous studies have shown that site-selective cAMP
analogs are able to induce potent growth inhibition and
differentiation in a broad spectrum of cancer cells (12),
including human leukemia cells (13, 14). Site-selective cAMP
analogs bind selectively to sites present on each of the two
regulatory subunits of the cAMP-dependent protein kinases,
type I (RI) and type II (RII) (15–17), respectively. Inhibition
of cancer cell growth by 8-chloroadenosine 3',5'-cyclic
monophosphate (8-Cl-cAMP), the most potent site-selective
cAMP analog, is accompanied by a reduction in the levels of
the RI cAMP receptor protein and an increase in RII levels
(13, 14, 18). Accordingly, an accumulation of the RI subunit
has been associated with growth stimulation and neoplastic
transformation, whereas an increase in the RII subunit has
been related to growth arrest and cell differentiation (12, 19,
20). Site-selective cAMP analogs represent a promising ad-
ditional class of antitumor agents; however, their prospective
impact in the therapy of human leukemias remains to be fully

Abbreviations: RI and RII, regulatory subunits of cAMP-dependent
protein kinases types I and II, respectively; 8-Cl-cAMP, 8-chloro-
adenosine 3',5'-cyclic monophosphate; AML, acute myeloblastic
leukemia; FAB, French–American–British classification of AML;
PEmc, plating efficiency in methylcellulose; PEs, plating efficiency
after suspension culture; 5637-CM, medium conditioned by the 5637
cell line; D_{10} and D_{50}, the doses required to reduce survival to 10%
and 50% of the control, respectively.
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elucidated. In the present study we have analyzed the effects of 8-Cl-cAMP on blast progenitors from AML patients.

MATERIALS AND METHODS

Leukemic Cells. Peripheral blood samples were obtained after informed consent from 15 patients with AML at the time of diagnosis. Bone marrow samples from 3 patients with myelodysplastic syndrome were also studied. The diagnosis was made according to the French–American–British (FAB) criteria (21) (see Table 1) and confirmed by cytochemistry and flow cytometry immunophenotyping. Ficoll/Hypaque-isolated blast cells were T-cell-depleted as described (22) and either cultured immediately or cryopreserved in liquid nitrogen.

Culture Methods. Methylcellulose clonogenic assays and suspension cultures were performed according to McCulloch (2), Wang and McCulloch (10), and Nara *et al.* (23). Cells (1 × 10^6/ml) were incubated for 48 hr in Dulbecco's modified Eagle's medium containing 20% (vol/vol) heat-inactivated fetal calf serum and 10% medium conditioned by the 5637 cell line (5637-CM) (24) as a source of growth factors. All samples showed >90% blast cells following preculture, except for those from patients 1, 7, and 16, in which 63%, 68%, and 70% blasts were detected, respectively. For the methylcellulose blast colony assay (10, 25), 2 × 10^5 cells were suspended in 1 ml of Dulbecco's modified Eagle's medium containing 20% fetal calf serum, 10% 5637-CM, and 0.8% methylcellulose along with increasing concentrations (1–50 μM) of 8-Cl-cAMP (Division of Cancer Treatment, National Cancer Institute, National Institutes of Health) and cultured in 100-μl aliquots in 96-well flat-bottomed microplates. After 7 days of incubation, aggregates with >20 cells were scored as colonies, and the results were expressed as a plating efficiency in methylcellulose (PEmc). Eight to 10 replicates were used to determine PEmc.

For suspension cultures, cells (2 × 10^5/ml) were incubated in the presence of 10% 5637-CM and increasing concentrations of 8-Cl-cAMP in six-well microplates. After 7 days, nonadherent cells were harvested, counted, washed twice in Dulbecco's modified Eagle's medium to remove 8-Cl-cAMP, and replated in analog-free methylcellulose as described above. Colonies grown in this assay yielded a plating efficiency after suspension culture (PEs). The number of self-renewing clonogenic cells was expressed as the number of colonies (PEs) adjusted for the number of nonadherent cells present in 1 ml of suspension culture (clonogenic cell recovery) (2, 10, 23).

Simple negative survival curves were computed by linear regression by comparing the number of clonogenic cells, derived from suspension culture or methylcellulose culture at each dose of 8-Cl-cAMP, with controls. From these curves, the doses required to reduce survival to 10% (D_{10}) and 50% (D_{50}) of the control, respectively, were obtained.

Survival curves were also obtained from experiments performed either without growth factors or by using a serum-free medium (26) or medium supplemented with serum-free 5637-CM (27). The effects of such culture manipulations were inferred from the multiple parameters obtained from methylcellulose or suspension cultures and were conveniently integrated in a star diagram (28, 29).

Morphological Studies and Cell Surface Antigen Analysis. In selected cases (cases 6, 9, 11, 12, 17, and 18), blast cell morphology and immunophenotypic changes were assessed by May–Grunwald–Giemsa staining of cytospin preparations and flow cytometry analysis of 7-day-old liquid cultures performed in the presence of 8-Cl-cAMP (1–50 μM). Modulation of cell surface antigens (CD11b/Mo1, CD13/My7, CD14/My4, CD33/My9, CD71/anti-transferrin receptor,

and CD15/OKM15) was analyzed by a FACScan cytofluorograph (Becton Dickinson) as described (30).

Photoaffinity Labeling of cAMP Receptor Proteins. Preparation of cell extracts and photoactivated incorporation of 8-azidoadenosine 3′,5′-cyclic [^32P]monophosphate followed the method previously described (13, 18). The samples containing 25–50 μg of protein were subjected to electrophoresis in 0.05% SDS/12% polyacrylamide gels (18), and the separated proteins were transferred to nitrocellulose sheets (18). The sheets were air dried and exposed to Kodak XAR film overnight at −20°C.

Statistical Analysis. Mean values, standard errors (SE), and 95% confidence intervals for the means were computed (31). Statistical differences between means were tested by Student's *t* test (31). D_{10} and D_{50} values for 8-Cl-cAMP were determined from the slope of negative exponential dose–response survival curves computed by linear regression and were compared as described (31).

RESULTS

Effects of 8-Cl-cAMP on Self-Renewal and Terminal Divisions of Leukemic Blast Progenitors. Representative dose–response survival curves of leukemic blast progenitors are shown in Fig. 1. Exposure to 8-Cl-cAMP (0.1–50 μM) resulted in a dose-dependent inhibition of blast cells both in the primary methylcellulose cloning and the secondary cloning after suspension culture. Survival curves are depicted by standard slope parameters D_{10} and D_{50}, which represent the concentration of 8-Cl-cAMP required to reduce survival to 10% and 50% of controls, respectively (Table 1). In blast cells from all patients, 8-Cl-cAMP showed a striking inhibitory effect on colony growth after suspension culture. D_{10} and D_{50} mean values in methylcellulose were higher ($P = 0.005$) than in suspension (Table 1). According to their patterns of response to 8-Cl-cAMP, we divided our samples into three subgroups operationally termed A, B, and C (Fig. 1). These subgroups were mathematically defined by statistically significant differences between D_{10} (or D_{50}) mean values in methylcellulose and in suspension. In each of the three groups, 95% confidence intervals were never overlapping (data not shown). These subsets, however, did not correlate with the FAB classification or with the clinical state of patients and more likely reflect a biological heterogeneity within the AML group (Table 1) (2). In the first subset (group A; patients 1, 6, 10, 13, and 15), clonogenic cells were strongly suppressed in suspension compared with methylcellulose cultures; they exhibited the maximal difference between D_{10} (or D_{50}) mean values in methylcellulose and in suspension. In the second group (group B; patients 2, 3, 4, 14, and 17), both clonogenic cells in suspension and in methyl-
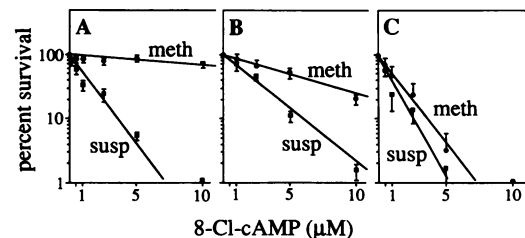

FIG. 1. Representative dose–survival curves of leukemic blast progenitors exposed to increasing concentrations of 8-Cl-cAMP in methylcellulose (●) and suspension (■) cultures from patients 6 (*A*), 2 (*B*), and 9 (*C*). The negative exponential dose–response survival curves are drawn by linear regression and may be described by a single slope parameter [i.e., the D_{10} or D_{50} value (see *Materials and Methods*)]. Comparisons between linear regression curves obtained in methylcellulose (meth) and in suspension (susp) cultures reached statistical significance ($P < 0.05$) in *A* and *B*.

Table 1. Clinical characteristic and response to 8-Cl-cAMP of blast cell progenitors from patients with AML and myelodysplastic syndrome

Patient no.	Diagnosis (FAB)	Blasts at diagnosis, %	D_{10}, μM		D_{50}, μM	
			MC	Suspension	MC	Suspension
1	RAEB-T	25	100.00	35.95	40.00	10.95
2	M1	90	16.81	5.94	4.91	1.83
3	M2	54	31.66	17.33	9.34	5.19
4	M1	85	22.52	9.55	6.7	3.0
5	M0	90	8.15	10.15	2.00	2.75
6	M2	63	65.00	3.50	19.40	1.10
7	RAEB-T	27	0.50	—	0.10	—
8	M4	40	7.82	—	2.31	—
9	M1	95	3.64	2.50	1.10	0.69
10	M1	92	37.37	0.03	10.71	0.01
11	M4	94	2.14	0.88	0.65	0.27
12	M5	51	9.63	2.74	2.82	0.83
13	M5	90	89.00	4.00	26.60	1.80
14	M0	80	50.00	28.55	15.00	8.43
15	M2	86	38.55	3.74	11.41	1.06
16	RAEB-T	25	1.30	6.50	0.44	1.80
17	M2	95	27.85	2.50	8.25	0.75
18	M4	93	6.65	—	2.00	—

A statistical analysis was performed, excluding patients 7, 8, and 18. In the whole group of patients, D_{10} mean values ± SE in methylcellulose and in suspension culture were 33.6 ± 7.9 μM and 8.9 ± 2.7 μM, respectively ($P = 0.005$). Similarly, D_{50} mean values ± SE were 10.6 ± 2.8 μM in methylcellulose and 2.7 ± 0.8 μM in suspension ($P = 0.005$). RAEB-T, refractory anemia with excess of blasts; MC, methylcellulose culture. Data are expressed as the mean of 8–10 replicate microwells. —, No growth was observed both in controls and in 8-Cl-cAMP-treated dishes after suspension culture.

cellulose cultures were inhibited by 8-Cl-cAMP, but a more marked effect in suspension was observed. 8-Cl-cAMP dose–survival curves of blast progenitors from these two groups were similar to those obtained with ara-C, a cytotoxic drug previously shown primarily to suppress self-renewal of AML progenitors (7). In the third group (group C; patients 5, 9, 11, 12, and 16), clonogenic cells recovered in suspension or grown in semisolid medium were similarly suppressed, as described for the anticancer drugs adriamycin and 4-hydro-peroxycyclophosphamide (7, 11). Finally, in two patients from this group, culture conditions favoring self-renewal rather than terminal divisions resulted in an increased sensitivity of leukemic progenitors to 8-Cl-cAMP in suspension (see below).

Effects of Culture Conditions Favoring Self-Renewal or Terminal Divisions of Leukemic Blast Progenitors on Sensitivity to 8-Cl-cAMP. Star diagrams and 8-Cl-cAMP dose–survival curves after suspension culture of blast cells from two patients cultured in conditions favoring self-renewal or terminal divisions are shown in Fig. 2. In case 9 (Fig. 2A Inset), serum-containing medium strongly enhanced blasts' self-renewal, as evidenced by the star diagram displaying high values for the self-renewal-related parameters PEs and clonogenic cell recovery. Conversely, in serum-free cultures, self-renewal-related functions were decreased and terminal divisions were favored, as shown by an increased PEmc. Dose–survival curves (Fig. 2A) showed that blast cells from patient 9 were more sensitive to 8-Cl-cAMP under culture conditions where self-renewal mitoses are favored [serum-containing medium (SCM) vs. serum-free medium (SFM)] ($P < 0.001$). For blast cells from patient 5 (Fig. 2B Inset), analysis of star diagrams showed that growth in the absence of growth factors was characterized by higher self-renewal values (PEs and clonogenic cell recovery) compared with cultures with serum-free 5637-CM in which a low PEs was detected. Again, survival curves (Fig. 2B) were consistent with the star diagrams, and blasts appeared more sensitive to 8-Cl-cAMP in suspension culture in the absence of growth factors [no growth factors (GFs) vs. 5637-CM] ($P < 0.001$).

Effects of 8-Cl-cAMP on Morphology and Surface Phenotype of Fresh AML Cells. To investigate the relationship between inhibition of colony growth and the capability of 8-Cl-cAMP to promote leukemia cell differentiation, experiments were performed using blast cells from selected patients (patients 6, 9, 11, 12, 17, and 18) exhibiting a different
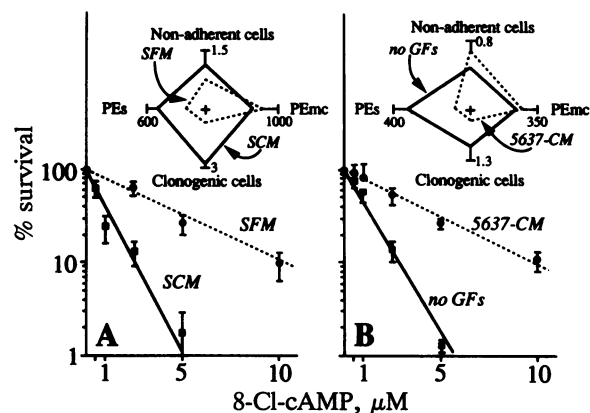

FIG. 2. Dose–survival curves for recovery of clonogenic cells from suspension cultures of blast progenitors from patients 9 (A) and 5 (B) exposed to 8-Cl-cAMP under different growth conditions. Dashed lines and circles refer to experiments carried out in serum-free medium (SFM) in A or in the presence of 5637-CM in B; continuous lines and squares indicate experiments performed in serum-containing medium (SCM) in A and in the absence of any source of growth factors (no GFs) in B. (Insets) For each panel, two superimposed star diagrams are included; each axis of the star diagram is scaled for one of the growth parameters that characterize blast population: the upper vertical axis represents the nonadherent cell number × 10^{-6} per ml after suspension culture, the lower vertical axis represents clonogenic cell recovery (cell number × 10^{-3} per ml) from liquid cultures, the right horizontal axis is PEmc (colonies per 2 × 10^5 cells), and the left horizontal axis is PEs (colonies per 2 × 10^5 cells). According to Miyauchi *et al.* (28, 29), axes have been chosen so that a movement to the left and down indicates a shift toward self-renewal mitoses while a movement to the right and up suggests that terminal divisions are favored.

sensitivity to 8-Cl-cAMP. Blasts from patients 6, 9, 11, and 18, exposed for 7 days to 8-Cl-cAMP (1–50 μM), did not show any significant morphological changes when compared with controls. In contrast, the blast cells from two patients (patients 12 and 17) were induced to differentiate by 8-Cl-cAMP as shown by morphological changes consistent with granulocytic (case 17; Fig. 3 *A–D*) or monocytic (case 12; Fig. 3 *E* and *F*) differentiation and by a striking upregulation of the mature granulocytic (CD15) and monocytic (CD14) antigens (Fig. 3 *G* and *H*). A significant reduction in the expression of the early myeloid antigen CD33 and of the proliferation-associated transferrin receptor (CD71) was also observed in both cases. Interestingly, in cells from patients 9 and 18, exposure to 8-Cl-cAMP, although not inducing a morphological maturation, resulted in a significant downregulation of CD33 and CD71 antigens.

Effects of 8-Cl-cAMP and RI and RII cAMP Receptor Levels of Fresh Blast Cells from AML Patients. To examine whether the growth inhibitory effect of 8-Cl-cAMP observed in the AML blast cells correlates with the modulation of cAMP receptor proteins (RI and RII) in the leukemia cells, we determined the receptor levels in these cells by photoaffinity labeling with 8-azidoadenosine 3′,5′-cyclic [^{32}P]monophos-

FIG. 4. Effect of 8-Cl-cAMP on cAMP receptor levels of fresh blast cells from three AML patients: patient 1 (RAEB-T), patient 2 (FAB-M1), and patient 3 (FAB-M2). Preparation of cell extracts and photoaffinity labeling with 8-azidoadenosine 3′,5′-cyclic [^{32}P]monophosphate followed the methods described in *Materials and Methods.* Lanes 1, 3, and 5, untreated control cells (patients 1–3); lanes 2, 4, and 6, cells treated for 3 days with 5 μM 8-Cl-cAMP (patients 1–3); lanes RI_α and RII_α, rabbit skeletal muscle RI_α and bovine heart muscle RII_α, respectively.

phate followed by SDS/PAGE. As shown in Fig. 4, untreated control cells from three AML patients each exhibited a major band of 48-kDa, which comigrated with rabbit skeletal muscle RI_α, that we refer to as RI_α. Minor bands of 65-, 44-, and 37-kDa proteins were also identified; the 44- and 37-kDa bands, which could be displaced by the addition of a 1000-fold excess of unlabeled cAMP, may represent proteolytically degraded cAMP-binding proteins, whereas the 65-kDa protein band, which shows partial displacement by excess cAMP and comigrates with bovine serum albumin, exhibits nonspecific cAMP binding. Treatment of these cells with 8-Cl-cAMP (5 μM) for 3 days resulted in an almost complete abolishment of RI_α and a decrease in the minor cAMP-binding proteins. However, a decrease was not observed in the 65-kDa protein, the nonspecific binding protein (Fig. 4). RII cAMP-binding protein was not detected in either untreated control cells or in cells treated with 8-Cl-cAMP (Fig. 4).
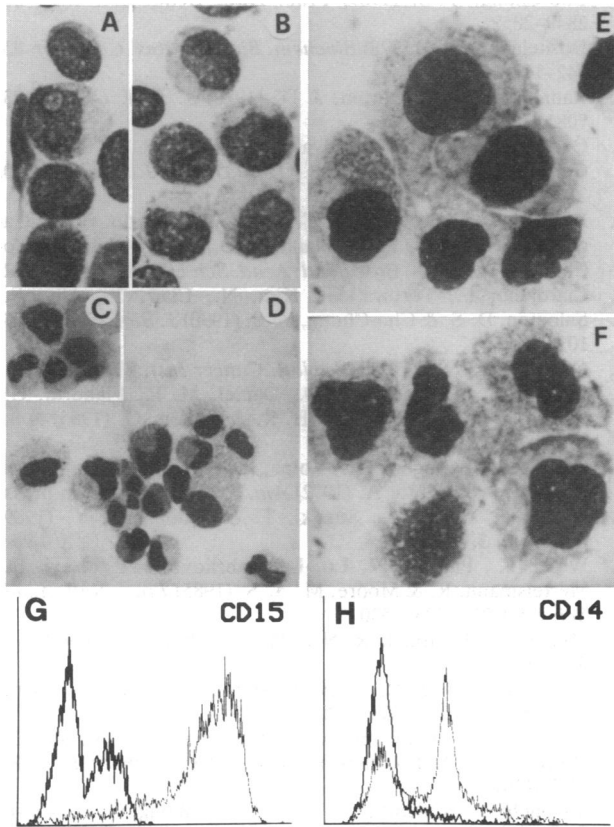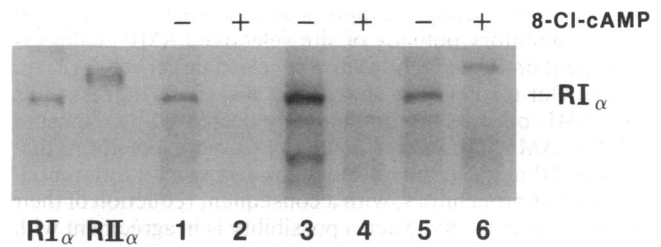
DISCUSSION

In the present study, we examined the ability of a site-selective cAMP analog, 8-Cl-cAMP, to affect both terminal divisions (clonogenic growth in methylcellulose) and self-renewal capacity (clonogenic growth after suspension culture) of AML blast cell progenitors. 8-Cl-cAMP was highly effective in suppressing leukemic blasts' colony formation in a dose-dependent manner. In addition, in 10 of 15 samples, 8-Cl-cAMP was more effective in inhibiting clonogenic cells grown in suspension than primary colonies grown in methylcellulose. From these data, it can be concluded that 8-Cl-cAMP is a powerful inhibitor of blast cell growth, primarily affecting its self-renewal capacity.

The mechanisms underlying the preferential suppression of the self-renewal capacity by 8-Cl-cAMP remain to be determined, and at least two possibilities must be considered. One is that self-renewal of blast progenitors is directly suppressed by 8-Cl-cAMP. High levels of RI cAMP receptor proteins have been associated with active cell growth, neoplastic transformation, and early stages of differentiation (12, 20, 32, 33), whereas a RI decrease and/or increase in RII correlate with growth arrest and maturation in a variety of cell types, including human leukemic cells (13, 14). It is conceivable that an actively self-renewing subpopulation of blast progenitors may display higher levels of RI subunits compared with blasts committed to terminal divisions, resulting in greater sensitivity to 8-Cl-cAMP. Whether changes in culture microenvironment that modify the balance between self-renewal and terminal divisions would alter RI and RII relative levels in fresh AML blasts remains to be determined. The marked alteration in sensitivity of leukemic cells to 8-Cl-cAMP that we observed after manipulation of culture conditions in two

FIG. 3. Morphological and immunophenotypical changes induced by 8-Cl-cAMP on fresh AML cells. (*A–D*) Blast cells from patient 17 (FAB-M2) cultured for 7 days in the absence (*A* and *B*) or in the presence (*C* and *D*) of 20 μM 8-Cl-cAMP. (*E* and *F*) Blast cells from patient 12 (FAB-M5) cultured for 7 days in the absence (*E*) or in the presence (*F*) of 20 μM 8-Cl-cAMP. Cytospin preparations of cultured cells were stained with May–Grunwald–Giemsa for morphological assessment. (*A* and *B*, ×950; *C* and *D*, ×475; *E* and *F*, ×1190.) (*G* and *H*) Overlay histograms of fluorescence intensity showing the upregulation of differentiation antigens CD15 (*G*) and CD14 (*H*) on blast cells from patients 17 (*G*) and 12 (*H*) following exposure for 7 days to 20 μM 8-Cl-cAMP. The *x* axis indicates decades of log fluorescence intensity, and the y-axis indicates the number of cells. Flow cytometry data were generated by means of a LYSIS II software (Becton Dickinson).

AML samples strongly supports such a view (Fig. 2). The growth-inhibitory potency of site-selective cAMP analogs is dependent on RI and RII relative levels in target cells (12, 20, 32, 33). Our results have shown that RI_α levels in fresh cells from AML patients are dramatically decreased by exposure to 8-Cl-cAMP. Another explanation for our results is that 8-Cl-cAMP might induce terminal divisions and differentiation of blast progenitors, with a consequent reduction of their self-renewal capacity. Such a possibility is in agreement with the demonstration that site-selective cAMP analogs, including 8-Cl-cAMP, are able to induce morphologic and/or functional differentiation of the human leukemic cell lines HL60 (13) and K562 (14). In the present study, blast cells from two of six patients were induced to differentiate by 8-Cl-cAMP, as evaluated by morphological and immunophenotypical changes, and in two other cases cell surface modifications compatible with a shift toward a mature phenotype were obtained. In operational terms, therefore, 8-Cl-cAMP, like any biological agent able to induce the loss of self-renewal in leukemic cells (without causing acute cell killing), can be considered a "differentiation inducer" even if not all mature phenotypic markers are acquired as a result of its action. As a matter of fact, the majority of so-called differentiation inducers, including those currently adopted in clinical trials, can cause a cytological and phenotypical maturation in a relatively low percentage of fresh AML cells *in vitro* (34) while inducing an almost total abolishment of the clonogenic capacity of the whole cell population. Thus, the noncytologically mature cells are, notwithstanding, "functionally differentiated" as long as they lack the stem cell property of self-renewal. From our data it clearly appears that 8-Cl-cAMP induced functional differentiation (i.e., the loss of self-renewal) in almost all blast cell progenitors in all the samples; in addition, it further pushed the differentiation program (i.e., acquisition of cytological and/or surface markers) in four of six samples tested (i.e., 66%). On the other hand, it is most probable that AML cells are rendered by 8-Cl-cAMP sensitive to other differentiation signals (i.e., hemopoietic growth factors) and that the combination of more than one factor is required to bring about the entire differentiation program in AML cells.

The preferential inhibition of the self-renewal capacity of AML progenitors by 8-Cl-cAMP demonstrated by us in the present study suggest another approach in AML therapy. Self-renewal capacity of leukemic cells is strictly correlated with the clinical outcome in AML (4–6); patients in which blast progenitors display a high self-renewal capacity *in vitro* have a low probability of achieving complete remission and show a low survival rate (4–6). The availability of biological agents inhibiting the self-renewal capacity of leukemic stem cells provides a very useful tool for AML therapy, since a cure in these patients may be achieved only if blasts' self-renewal is completely abolished. We have shown here that 8-Cl-cAMP, at micromolar concentrations, provokes a drastic suppression of AML clonogenic cells by primarily affecting their self-renewal capacity. Our data strongly indicate that 8-Cl-cAMP may represent a promising additional agent for AML therapy in humans.

1. McCulloch, E. A. (1986) *J. Cell. Physiol. Suppl.* **4**, 27–33.
2. McCulloch, E. A. (1990) in *New Approaches to the Treatment of Leukemia*, European School of Oncology Monographs, ed. Freireich, E. J. (Springer, Berlin), pp. 87–116.
3. Buick, R. N., Chang, L. J.-A., Messner, H. A., Curtis, J. E. & McCulloch, E. A. (1981) *Cancer Res.* **41**, 4849–4852.
4. McCulloch, E. A., Curtis, J. E., Messner, H. A., Senn, J. S. & Germanson, T. P. (1982) *Blood* **59**, 601–608.
5. Curtis, J. E., Messner, H. A., Hasselback, R., Elhakim, T. M. & McCulloch, E. A. (1984) *J. Clin. Oncol.* **2**, 253–259.
6. Nara, N., Suzuki, T., Nagata, K., Yamashita, Y., Murohashi, I. & Adachi, Y. (1988) *Br. J. Haematol.* **70**, 187–191.
7. Nara, N., Curtis, J. E., Senn, J. S., Trichler, D. L. & McCulloch, E. A. (1986) *Blood* **67**, 762–769.
8. Nara, N. & McCulloch, E. A. (1985) *Blood* **65**, 1484–1493.
9. Buick, R. N., Minden, M. B. & McCulloch, E. A. (1979) *Blood* **54**, 95–104.
10. Wang, C. & McCulloch, E. A. (1987) *Blood* **69**, 553–559.
11. Nara, N., Suzuki, T., Yamashita, Y., Murohashi, I. & Aoki, N. (1988) *Cancer Res.* **48**, 2348–2352.
12. Cho-Chung, Y. S. (1990) *Cancer Res.* **50**, 7093–7100.
13. Tortora, G., Tagliaferri, P., Clair, T., Colamonici, O., Neckers, L. M., Robins, R. K. & Cho-Chung, Y. S. (1988) *Blood* **71**, 230–233.
14. Tortora, G., Clair, T., Katsaros, D., Ally, S., Colamonici, O., Neckers, L. M., Tagliaferri, P., Jahnsen, T., Robins, R. K. & Cho-Chung, Y. S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2849–2852.
15. Døskeland, S. O. (1978) *Biochem. Biophys. Res. Commun.* **83**, 542–549.
16. Rannels, S. R. & Corbin, J. D. (1980) *J. Biol. Chem.* **255**, 7085–7088.
17. Ogreid, D., Ekanger, R., Suva, R. H., Miller, J. P., Sturm, P., Corbin, J. D. & Døskeland, S. O. (1985) *Eur. J. Biochem.* **150**, 219–227.
18. Ally, S., Tortora, G., Clair, T., Frieco, D., Merlo, G., Katsaros, D., Ogreid, D., Døskeland, S. O., Jahnsen, T. & Cho-Chung, Y. S. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6319–6322.
19. Ciardiello, F., Tortora, G., Kim, N., Clair, T., Ally, S., Salomon, D. S. & Cho-Chung, Y. S. (1990) *J. Biol. Chem.* **265**, 1016–1020.
20. Cho-Chung, Y. S. (1989) *J. Natl. Cancer Inst.* **81**, 982–987.
21. Bennett, J. M., Catovsky, D., Daniel, M. T., Flandrin, G., Galton, D. A. G., Gralnick, H. R. & Sultan, C. (1985) *Ann. Intern. Med.* **103**, 620–625.
22. Carbone, A., Gloghini, A., Volpe, R., Attadia, V., Gattei, V., Zagonel, V. & Pinto, A. (1992) *Am. J. Clin. Pathol.*, in press.
23. Nara, N., Tohda, S., Suzuki, T. & Yamashita, Y. (1989) *Leukemia* **3**, 572–577.
24. Welte, K., Platzer, E., Lu, L., Gabrilove, J. L., Levi, E., Mertelsmann, R. & Moore, M. A. S. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1526–1530.
25. Minden, M. D., Buick, R. N. & McCulloch, E. A. (1979) *Blood* **54**, 186–195.
26. Salem, M., Delwel, R., Touw, I., Mahmoud, L. & Lowenberg, B. (1988) *Leukemia Res.* **12**, 157–165.
27. Lu, L., Welte, K., Gabrilove, J. L., Hangoc, G., Bruno, E., Hoffman, R. & Broxmeyer, H. E. (1986) *Cancer Res.* **46**, 4357–4361.
28. Miyauchi, J., Kelleher, C., Yang, Y. C., Wong, G. C., Clark, S. C., Minden, M. D., Minkin, S. & McCulloch, E. A. (1987) *Blood* **70**, 657–663.
29. Miyauchi, J., Kelleher, C. A., Wang, C., Minkin, S. & McCulloch, E. A. (1989) *Blood* **73**, 1272–1278.
30. Pinto, A., Colletta, G., Del Vecchio, L., Rosati, R., Attadia, V., Cimino, R. & Colombatti, A. (1987) *Blood* **70**, 1450–1457.
31. Armitage, P. & Berry, G. (1987) *Statistical Methods in Medical Research* (Blackwell, Oxford).
32. Cho-Chung, Y. S., Clair, T., Tortora, G., Yokozaki, H. & Pepe, S. (1991) *Life Sci.* **48**, 1123–1132.
33. Cho-Chung, Y. S., Clair, T., Tortora, G. & Yokozaki, H. (1991) *Pharmacol. Ther.* **50**, 1–33.
34. Koeffler, H. P. (1983) *Blood* **62**, 709–721.