Regulation of Transferrin Receptor Expression on Human Leukemic Cells during Proliferation and Induction of Differentiation

EFFECTS OF GALLIUM AND DIMETHYLSULFOXIDE

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ABSTRACT The association of transferrin receptor expression with cellular proliferation has been studied extensively, but a number of events have not been defined. We therefore assayed receptor on promyelocytic leukemia (HL-60) cells at early times after exposure to a stimulus for proliferation (subculture), as well as agents that either induce differentiation (dimethylsulfoxide [DMSO]) or inhibit iron uptake (transferrin-gallium). Within 4 h after subculture, we found that a significant increase in total cellular immunoreactive receptor occurred that preceded by 8 h the increase in cell-surface transferrin binding. Automated fluorocytometric analysis of cells in an immunofluorescent assay indicated that increased surface receptor density appeared on cells in the S, G_2, and M phases of the cell cycle. DMSO-treated cells proliferated at the same rate as untreated (control) cells for the first 72 h, but as early as 12 h after treatment transferrin receptor was significantly decreased (65% of control cells). Further decreases occurred at later time points until transferrin receptor was undetectable after 7 d, when proliferation had ceased, cells were arrested in G_1 phase of the cell cycle, and myeloid differentiation occurred. After exposure to transferrin-gallium, proliferation ceased, but cells exhibited increased surface receptor and were arrested at S phase of the cell cycle without associated myeloid differentiation.

We conclude that events preceding cell division provide the regulatory stimulus for the synthesis and subsequent appearance of the transferrin receptor on the cell surface. Additionally, decreased receptor expression may be important in causing cessation of proliferation and/or differentiation. Finally, the way in which gallium salts are currently being investigated as chemotherapeutic agents should be reevaluated in light of our findings concerning transferrin-gallium effects on cellular proliferation.

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INTRODUCTION

Cell surface receptors for transferrin, the major plasma iron transport protein (1), were initially characterized on cells with high iron requirements including hemoglobin-producing cells and placental cells (2–6). More recently, the finding of high densities of transferrin receptors on cells that would appear to require less iron, including proliferating nonhemoglobin-producing cells (7–9), has generated considerable interest. These studies have indicated that transferrin-binding sites markedly increase soon after these cells are exposed to tissue culture conditions that stimulate proliferation (7, 8, 10, 11). Furthermore, studies using immunoassay techniques as well as transferrin binding studies have indicated that this increase in transferrin binding is due to an actual increase in total cellular immunoreactive receptor molecules, and is not due to a change in receptor affinity for transferrin or receptor availability (12, 13). These findings may have functional significance, since previous studies have demonstrated that the addition of transferrin to serum-free media is a requirement for cell growth (14–16), and an important recent study has indicated that a specific monoclonal antibody that blocks transferrin binding causes arrest of cellular proliferation (17).

Nevertheless, although these previous studies have suggested that the presence of the transferrin receptor represents a marker for proliferation and that it prob-

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ably has functional importance, extensive studies have not been performed to determine the early events associated with expression of the receptor on actively proliferating cells, nor has receptor density on subpopulations of cells in different phases of the cell cycle been measured. These studies are important, since they may not only further elucidate the functional requirements for transferrin binding during proliferation, but they may also better define the role that variation in expression of transferrin receptor on the cell plays in the regulation of proliferation. Additionally, since recent in vitro studies indicate that induction of cell differentiation may initially require decreased cellular proliferation (18), studies of regulation of transferrin receptor density soon after exposure to an inducer of differentiation may indicate whether receptor expression plays a role in this process.

We have, therefore, studied the well-described human promyelocytic leukemic cell line (HL-60) (19) at various stages of cellular proliferation and during dimethylsulfoxide (DMSO)[1] induction of differentiation (20, 21), in order to measure changes in transferrin receptor by immunoassay techniques and transferrin-binding studies. Our studies indicate that events preceding cell division provide the regulatory stimulus for the synthesis and subsequent appearance of the transferrin receptor on the cell surface. The peak of surface receptor density appears on cells at a stage that occurs just before cell division. We also show that relative transferrin receptor density decreases at a very early stage after induction of differentiation, even before proliferation has decreased and subsequent differentiation occurs. These latter changes, however, appear to be somewhat different from the events associated with the addition of specific agents that block either transferrin-mediated iron uptake (i.e., gallium salts or transferrin-gallium) or transferrin binding (i.e., monoclonal antibodies) and inhibit cellular proliferation resulting in arrest of cells at a diffent phase of the cell cycle without induction of myeloid differentiation.

METHODS

Human apotransferrin, nitroblue tetrazolium (NBT) and DMSO were obtained from Sigma Chemical Co., St. Louis, MO. Gallium nitrate was obtained from Alfa Products, Thiokol/Ventron Div., Danvers, MA.

^{125}I-Sodium iodide and $^{59}FeCl_3$ were obtained from New England Nuclear, Boston, MA, and Amersham Corp., Arlington Heights, IL, respectively.

The method of Bates and Schlambach (22) was followed, with either $FeCl_3$ or gallium nitrate to saturate transferrin with nonradioactive iron or gallium (23), respectively. A similar method was used for saturation of apotransferrin with ^{59}Fe (12). Iodination of transferrin-^{59}Fe and the human transferrin receptor was performed by previously described methods (12).

The HL-60 (human promyelocytic leukemia) cells were obtained from the laboratory of Dr. Michael Glode, University of Colorado Medical School. Cells were grown in RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) containing 10% fetal calf serum in a 7.5% CO_2 atmosphere. For all experiments, cells grown to confluence were subcultured at a density of 5×10^5/ml and reached a maximum density (confluence) of $\sim 2.2 \times 10^6$ cells/ml in 72 h. This rate of proliferation is similar to that reported recently by others (24). Studies using DMSO were carried out by plating cells in a manner identical to that described above, except that DMSO was added to the media. Cells with normal growth kinetics in serum-supplemented media reached confluence at 72 h and maintained >95% viability over the next 24 h, but significant cell death occurred after this period. Therefore, cells were seldom exposed continuously to the different agents for longer than 3 d, and unless specifically stated they were recultured at 3-d intervals at a concentration of 5×10^5 cells/ml in fresh media containing the different agents until a desired time of exposure had been reached. Aliquots of cells at different time points (after subculture in fresh media) were removed for assays and transferred to 15-ml polystyrene tubes and centrifuged at 200 *g* for 10 min in a Beckman TJ-6 centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The supernatant was aspirated and the pellet resuspended in ice-cold 10 mM KPO_4, pH 7.5, 150 mM NaCl (PBS) and bovine serum albumin (BSA), 1 mg/ml. All cell counts were performed in triplicate with a hemacytometer.

Transferrin-binding studies were performed by incubating varying concentrations of ^{125}I-transferrin-^{59}Fe (5,000 ^{125}I cpm/mg protein) with 0.5×10^6 cells in a total incubation volume of 500 μl PBS and 1 mg/ml BSA. Incubations were carried out for 4 h at 4°C. The incubations for binding studies were performed at 4°C to insure that internalization of radioactive transferrin was not included in the calculations (25, 26). The 4-h incubation period was chosen, since binding of ^{125}I-transferrin to the cells had reached saturation by this time. After the incubation, 1 ml of ice-cold PBS with BSA was added to each tube and after centrifugation at 500 *g* at 4°C, the supernatant was aspirated, and the pellet was counted for radioactivity. Specific binding was determined by subtracting the radioactivity in an identical sample that contained 1,000-fold excess of nonradioactive transferrin. The results were plotted and maximal transferrin binding was calculated according to the method of Scatchard (27).

Total cellular immunoreactive transferrin receptor protein was determined in a double-antibody precipitation radioimmunoassay as previously described (12). Although maximal transferrin binding and immunoreactive receptor was expressed as nanograms per 10^6 cells, in initial experiments we determined cellular protein concentration and found that the concentration of ~90 μg protein/10^6 cells did not change significantly for cells at different stages of growth or under various media conditions described below.

For studies using varying concentrations of apotransferrin and other forms of transferrin (see above), we used serum-free media supplemented with various concentrations of transferrin and 5 μg/ml of insulin (defined media), which has been noted to support growth of HL-60 cells (16). Although cell proliferation in defined media was somewhat slower than in serum-supplemented media, there was a pre-

[1] *Abbreviations used in this paper:* DMSO, dimethylsulfoxide; MII, median immunofluorescence intensity; NBT, nitroblue tetrazolium.

dictable rate of cell growth similar to the rate previously described (16). Moreover, as reported (16), cells exposed to DMSO in defined media also exhibited myeloid differentiation. Serum-free media lacking either transferrin, insulin, or both failed to support cell growth and no effect on cellular differentiation was noted.

Cell proliferation was assessed by performing cell counts, as well as by analyzing DNA distribution (see below). Differentiation of a population of cells was assessed using NBT reduction (24, 28), as well as morphologic criteria to identify metamyelocytes and more mature cells (20).

Cell surface transferrin receptor was assayed by a double-antibody immunofluorescence technique (12, 29). Approximately 1–3 million HL-60 cells were washed with ice-cold PBS, resuspended in 100 μl of PBS, and first incubated with 100 μl of polyclonal rabbit anti-human transferrin receptor antisera (6) at 4°C for 1 h. The cells were then washed by centrifugation to remove unbound antibody and resuspended in 100 μl of PBS followed by the addition of 100 μl of the second antibody, goat anti-rabbit IgG labeled with fluorescein isothiocyanate (Gibco Laboratories, Grand Island, NY) with the second incubation performed at 4°C for 1 h. Some cell populations were analyzed separately for cellular DNA by the propidium iodide staining technique (30). For this technique, cells were fixed in 70% ethanol and washed with PBS; then the cell pellet was resuspended in RNase (Sigma Chemical Co.) (1 mg/ml) and incubated for 30 min at 37°C. After this, the cells were again washed twice and resuspended in 1 ml of propidium iodide (10 mg/ml in PBS) for 20–30 min at 4°C.

For some experiments, the same cells were stained for both immunofluorescent cell surface transferrin receptor and cellular DNA. After immunofluorescent staining as described above, cells were fixed by a 10-s exposure to 1% paraformaldehyde, immediately diluted 1:5 with PBS and then washed twice by centrifugation at 4°C. Cells were then resuspended in 1 ml of RNase and incubated at 4°C for 1 h, washed free of RNase, and stained with propidium iodide as described above.

Samples of cells were then run through a fluorescence-activated cell sorter, the FACS IV flow cytometer (Becton-Dickinson FACS Systems, Sunnyvale, CA), with a 50-μm nozzle tip. An argon-ion laser operating at 488 nm with a 5-W intensity was used as an excitation source. Red fluorescence (DNA stain) and green fluorescence (surface receptor immunofluorescence) were detected at 550–590 and 510–520 nm, respectively. In the experiments shown (Figs. 2–4), ~25,000 cells were analyzed for each cell sample. Although a previous study had indicated that changes in cell surface area during cellular proliferation do not appreciably affect calculation of binding sites expressed per cell (31), each cell sample was analyzed for forward-scatter distribution and the integrated curve (histogram) obtained was an indicator of cell volume (30), so that we also could estimate surface area (29). Simultaneously, another histogram plotted fluorescence intensity on the x-axis vs. the number of cells with a given fluorescent intensity on the y-axis. For immunofluorescence studies defining surface transferrin receptor (green fluorescence), an automated analysis of each cell population calculated a point on the x-axis that represented the median immunofluorescence intensity (MII) of the cell population. This point could not be assigned a specific unit quantity, but when two samples (one being a control sample) were analyzed at the same time, the test sample could be expressed as a percentage of control. This analysis was possible, since under most conditions described above, including confluent cells, enough surface transferrin receptor was expressed so that the specific immunofluorescent polyclonal antibody technique was sensitive enough to detect >90% of cells as fluorescent positive when compared with fluorescence detected when control rabbit serum was substituted for antitransferrin receptor antibody as the first antibody.

When >10% of cells were immunofluorescent negative, such as with continued DMSO exposure, calculation of MII could be assigned a percentage of control expressed as the maximum value possible (e.g., <20% of control). Measurement of surface transferrin receptor by this method was useful, since only a few cells were needed for an accurate analysis and, if appropriate controls were analyzed at the same time, a large number of samples could be studied. Moreover, as with measurements we have previously described (12, 29) while studying the above cell system, the presence of transferrin bound to receptor or the addition of 50 μg human transferrin to the incubation mixture had no effect on fluorescence intensity. Thus, this method was particularly useful in measuring surface transferrin receptor under conditions in which varying concentrations and forms of transferrin were added to the cells in culture.

When samples were analyzed for DNA content, histograms represented the red fluorescence intensity (DNA content) on the x-axis and the number of cells with certain red fluorescence intensity on the y-axis. Automated analysis of subpopulations of cells with differences in DNA content permitted calculation of percentages of cells in G_1, S, or G_2 and M phases of the cell cycle. Although the automated technique could not separate cells in either G_2 or M phases of the cell cycle, when a number of samples were analyzed morphologically, it was found that even at the 24-h point after subculture, when the greatest percentage of cells were in the active phases of proliferation, the cells that were morphologically in mitosis represented only about one-third of cells in G_2 and M and ~8% of the total cell population, which as a mitotic index agrees with studies performed by others (24).

In certain samples that had been stained for both immunofluorescent surface receptor and cellular DNA, two histograms were obtained simultaneously, one representing DNA distribution and another representing surface receptor fluorescence. Cells in different phases of the cell cycle (as evidenced by different amounts of DNA) were studied by using the capabilities of the FACS IV to gate a desired subpopulation of cells. The corresponding median intensity of surface fluorescence could then be determined for that particular subpopulation of cells (G_1, S, or G_2 and M).

Cells fixed and stained for DNA after immunofluorescent surface receptor staining showed a slight increase in green fluorescent-negative cells (~15%) as compared with cells stained for immunofluorescent receptor alone. We surmised that these negative cells had been randomly damaged by the fixation process, since, when these cells were discounted, the histogram obtained showed an identical intensity of surface fluorescence as cells stained for immunofluorescence alone. Therefore, it appeared that the DNA staining per se did not alter surface receptor fluorescent intensity.

RESULTS

We had previously shown that actively proliferating cells have higher numbers of both transferrin-binding sites and total cellular immunoreactive transferrin receptor than nonproliferating or confluent cells (12).

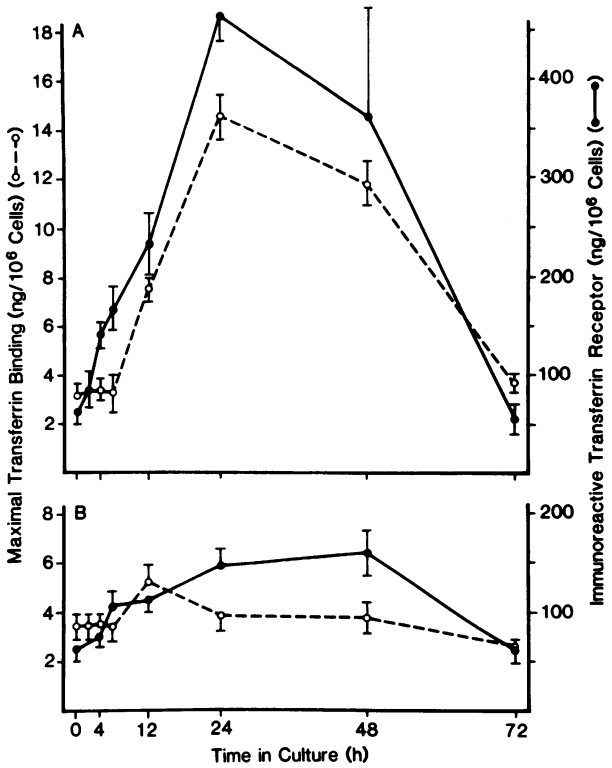

FIGURE 1 (*A*) Confluent HL-60 cells were subcultured in fresh medium at 5×10^5 cells/ml and assayed for both transferrin binding and total cellular immunoreactive receptor (12) at different time points after subculture. Maximal transferrin binding was determined by using the calculation defined by Scatchard (27). All points represent the mean of three experiments±SEM. (*B*) Assays of HL-60 cells grown under the same conditions as defined above, except the medium contained 1% DMSO. Cell counts at various time points after subculture were identical for both DMSO-treated cells and untreated cells (see Fig. 2).

The results we obtained (Fig. 1 *A*) extend these previous observations by measuring changes in total cellular immunoreactive transferrin receptor and maximal transferrin binding at early time points after confluent HL-60 leukemic cells (2.2×10^6 cells/ml) are subcultured at low density (5×10^5 cells/ml) to stimulate proliferation. An increase in maximal transferrin binding was evident at 12 h after subculture (Fig. 1 *A*), reached a maximum at 24 h, and returned to base-line levels at 72 h, when the cells became confluent. Total cellular immunoreactive receptor was also at its highest level at 24 h after subculture, but significant increases in immunoreactive receptor were measured as early as 4 h after subculture (Fig. 1 *A*), suggesting that a stimulus for proliferation results in synthesis of new transferrin receptor molecules, which precedes by a number of hours an increased expression in surface transferrin receptor as measured by maximal transferrin binding. Further evidence that this early increase in immunoreactive transferrin receptor represents synthesis of new receptor molecules was provided by the fact that cells subcultured in media containing cyclohexamide (20 μg/ml) for 12 h showed neither the early increase in immunoreactive transferrin receptor nor the later increase in transferrin binding. The delay in the expression of newly synthesized transferrin receptor on the cell surface agrees with previous synthetic studies (32), which demonstrated rapid synthesis of an immature molecule, which was converted a number of hours later into the mature receptor protein. According to previously defined calculations (12), the data shown in Fig. 1 *A* indicate that transferrin-binding sites increase from 26,000 sites/cell in confluent cells to 114,000 sites/cell at maximal transferrin binding at 24 h, whereas total cellular immunoreactive receptor molecules increase from 214,000 molecules/cell at confluence to 1,660,000 molecules/cell at the highest density of receptor.

HL-60 cells cultured in 1.25% DMSO exhibit an early decrease in cellular proliferation, followed by differentiation of cells to more mature myeloid forms (20). In addition to these effects, we found that cells exposed to 1.25% DMSO, in agreement with other studies (21, 33), showed an associated decrease in surface transferrin receptor. Moreover, when cells were exposed to only 1% DMSO, there was a clear dissociation between relative decreases in transferrin receptor density and decreased cellular proliferation (Fig. 1 *B*). Despite an identical rate of proliferation compared with control cells for the first 72 h (see below), DMSO-treated cells did not show the expected increase in transferrin receptor during active cellular proliferation. Thus, although a small increase in both immunoreactive transferrin receptor and transferrin binding is noted in treated cells at 12 h after subculture, this increase is significantly lower than that seen with untreated cells at the same time point. Furthermore, whereas control cells show even further increases in both immunoreactive transferrin receptor and maximal transferrin binding at 24 h, DMSO-treated cells showed a plateau in both values (Fig. 1 *B*), even though proliferation continued at the same rate. A decreased affinity of transferrin binding to the cell surface receptor does not appear to be a significant factor, since Scatchard analysis of transferrin binding to control cells and DMSO-treated cells at different time points resulted in similar association constants (K_a = 2–5 $\times 10^8$ M^{-1}).

To obtain a better definition of the effect of proliferation and DMSO treatment on transferrin receptor density, we measured surface transferrin receptor using the immunofluorescence technique and an automated fluorocytometer. The results of these analyses

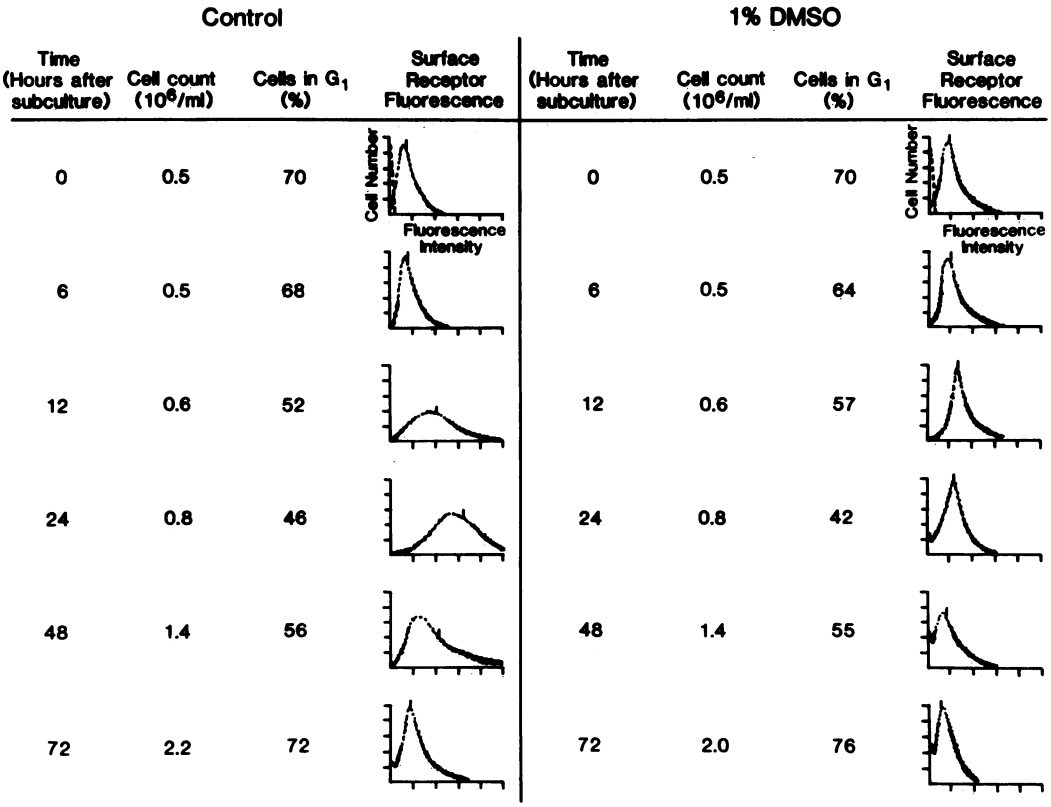

FIGURE 2 Comparison of rate of proliferation and surface transferrin receptor using an automated immunofluorescence technique for both untreated (control) cells and 1% DMSO-treated cells at various time points after subculture. A representative experiment is shown. Cells in G_1 were determined by fluorescent staining for DNA (Methods). Immunofluorescent histograms shown were traced directly from polaroid photographs of curves projected on FACS IV screen. Vertical dash on each histogram represents MII of each cell population, with increased shift to right indicative of higher MII. The broken line projected on both 0-h histograms represents the negative control (Results) and any cells to the left of the line are therefore designated fluorescent negative. For the 12-h sample, cell counts ranged from 0.53 to 0.57 $\times$ 10^6/ml for three experiments and were not significantly greater than the 0-h sample, but have been corrected to the nearest tenth in the figure.

on both control and DMSO-treated cells at various time points after subculture are shown in Fig. 2. As noted by the cell counts at each time period after subculture, there is no difference in the rate of proliferation between control cells and DMSO-treated cells. Additionally, analyses of DNA content of the cells at various time points after subculture also show that a similar number of cells are in the G_1 phase of the cell cycle for both control and DMSO-treated cells. For three different experiments, cell counts as well as the percentage of cells in G_1 varied <10% for all time points for both control and DMSO-treated cells.

As shown in Fig. 2, the histograms obtained for control cells by the immunofluorescence technique as a measure of immunoreactive surface transferrin receptor first showed a significant increase in MII (measured as a shift to the right in the relative scale shown on the x-axis) at 12 h after subculture, a result that agrees with measurements of surface transferrin receptor expressed as maximal transferrin binding (Fig. 1 *A*). Also as seen with measurements of maximal transferrin binding, the peak increase in mean cellular surface transferrin receptor by the immunofluorescence technique is seen at 24 h. At 48 h, there is a decrease in the MII, which decreases even further to base line at 72 h, when the cells become confluent.

Forward scatter determinations, which are representative of cell volume (34, 35), showed that at time periods where cells expressed the highest number of receptor, there was <5% change in the median scatter distribution as compared with confluent cells (data not shown), indicating that an increase in surface area can-

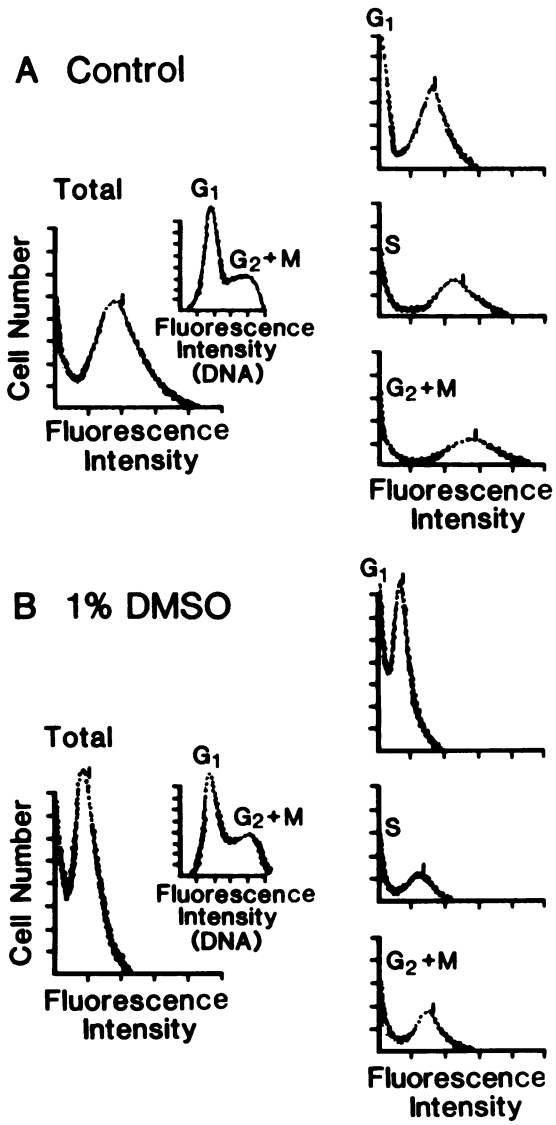

FIGURE 3 Histograms obtained from 24-h postsubculture cells analyzed with both fluorescent staining for DNA as well as the immunofluorescence assay for surface transferrin receptor. Note that, compared with the 24-h samples shown in Fig. 2, the immunofluorescence histogram has been purposely attenuated along the x-axis, so that the entire histogram obtained for each subpopulation of cells can be compared with others. MII in these experiments discounts fluorescent-negative cells (see Methods for details). (*A*) Control cell population with immunofluorescence histogram to the left representative of the total cell population analyzed, whereas *inset* represents fluorescent stain for DNA with diploid amount of DNA designated as G_1, twice that amount designated as G_2 and M, and intermediate DNA content indicative of cells in S phase. As indicated on the right side of the figure, cells in different phases of the cell cycle were then analyzed separately for immunofluorescence intensity. Note the marked shift in MII for subpopulations of cells in active phases of the cell cycle. (*B*) Same measurements as shown in *A*, except cells have been exposed to 1% DMSO. Note that there is marked attenuation of MII of this cell population as compared with control cells, even though DNA distribution is almost identical. DMSO-treated cells in active phases of proliferation also have higher MII as compared with cells in G_1 phase.

not account for the marked increase in transferrin receptor seen during proliferation.

As expected, cells cultured in 1% DMSO as compared with control cells showed a much lower median intensity of immunofluorescence at all time points starting at 12 h after subculture (Fig. 2). MII of treated cells at 24 and 48 h was 35 and 38% of control values, respectively, a result that agrees well with comparison of maximal transferrin binding between the two groups of cells (Fig. 1). At all time points, forward scatter histograms showed essentially identical patterns as control cells (data not shown). At 72 h, DMSO-treated cells become confluent, but, using the automated immunofluorescence assay, we found that these confluent cells had decreased immunofluorescence compared with control cells, with the median fluorescence somewhat <40% of control, since ~15% of cells exhibited negative fluorescence.

We suspected that subpopulations of cells with high surface transferrin receptor might represent cells in the active phases of the cell cycle, since these highly fluorescent cells were seen at time points preceding and during active proliferation (Fig. 2). Fig. 3 *A* shows that cells in S or G_2 and M have a markedly increased MII as compared with cells in G_1 phase of the cell cycle. Analysis of forward scatter distribution indicated that cells in S phase showed about a 5% increase in median scatter distribution as compared with cells in G_1, whereas cells in G_2 and M had about a 20% comparative increase in scatter, although this latter increase may overestimate surface area, since cells in M do not have a spherical shape (30). Therefore, increased surface area cannot totally explain the 75% increase in MII seen in cells in S phase or the 90% increase seen in cells in G_2 and M phase. These results suggest, therefore, that the increased expression of transferrin receptor is found on the population of cells in active stages of proliferation. Moreover, analyses of different subpopulations of cells at various time points after subculture showed that control cells in the G_1 phase of the cell cycle also expressed higher densities of surface transferrin receptor at 24 and 48 h as compared with confluent cells (this data can be extrapolated from Fig. 2), indicating that expression of relatively high densities of receptors are also present on some cells in G_1 phase during active cellular proliferation.

As shown in Fig. 3 *B*, analyses of the cell population cultured in DMSO for 24 h showed a marked decrease in surface transferrin receptor. However, analyses of subpopulations of cells in different phases of the cell cycle, although MII was always lower than the control cell sample, still showed that treated cells in active stages of proliferation had higher densities of surface transferrin receptor as compared with cells in G_1 phase.

To study further the effect of DMSO, we analyzed the DMSO-treated cells grown beyond 72 h. Previous studies have exposed cells to DMSO continuously for up to 6 d without subculture. However, to assess the prolonged effect of DMSO on transferrin receptor density and resultant decreases in cellular proliferation, cells continuously exposed to 1% DMSO were subcultured in fresh media every 3 d at lower density (5×10^5 cells/ml) to stimulate proliferation (Table I). Although 1% DMSO-treated cells proliferated at the same rate as control cells for the first 72 h (Fig. 2), when the treated cells were recultured, a definite decrease in the rate of proliferation was noted during the next 3 d (days 4–6 of DMSO exposure), as compared with control cells, which always proliferated at the same rate (Fig. 2). Not only is the cell number less at days 4, 5, and 6, the percentage of cells in G_1 at these time points also is greater, indicating that fewer cells are entering the active phases of the cell cycle. Significantly, the surface transferrin receptor on these recultured cells decreases even further when compared with control cells (Table I) and total cellular immunoreactive receptor was undetectable (data not shown). When cells are recultured a second time 6 d after exposure to DMSO, no proliferation occurs over the succeeding 24 h. At this time, essentially all of the cells are arrested in the G_1 phase of the cell cycle and both transferrin binding and immunofluorescence are at undetectable levels. Also, as noted in Table I, significant differentiation occurs beginning at day 4 after recultured cells are exposed to DMSO. The degree of differentiation correlates directly with the decrease in proliferation and is coincidental with the loss of transferrin receptor.

Our results suggested that the attenuation of transferrin receptor density with DMSO treatment results in eventual arrest of cellular proliferation with associated myeloid differentiation. We therefore attempted to determine the effect of other agents that might specifically affect transferrin receptor density and result in either arrested proliferation and/or differentiation. One such agent chosen was gallium, a relatively nontoxic metallic cation that avidly binds to transferrin (23). Previous studies have indicated that gallium salts inhibited growth of some cells in tissue culture (36, 37), and we also found that the addition of various concentrations of gallium nitrate to HL-60 cells resulted in a dose-related inhibition of proliferation. For example, a concentration of 9 μg/ml of gallium in serum-supplemented media resulted in cessation of proliferation 2 d after subculture with >95% of cells still viable. However, for better control of the amount of gallium and transferrin present, cells were cultured in serum-free media with different forms of transferrin. When transferrin-iron was added at a concentration of either 25 μg/ml (Table II) or up to 150 μg/ml (data not shown), the rate of proliferation was identical to that seen with 25 μg/ml apotransferrin (16; see Methods). When cells exposed to the different forms of transferrin in serum-free media were analyzed for transferrin receptor in the automated immunofluorescence assay, there was, as expected, a threefold increase in MII at day 1 after subculture as compared with confluent cells. Moreover, in this sensitive assay, transferrin receptor expression showed a

TABLE I

Effect of Prolonged DMSO Exposure on Transferrin Receptor Density, Cell Proliferation, and Differentiation

Day,* in DMSO	Cell count	Cells in G_1	Maximum transferrin binding	MII‡	Differentiation	
					NBT§	Morphologically
	$\times 10^6$/ml	%	ng/10^6 cells	% control	%	
4	0.7	78	0.7	<25	21	10
5	1.0	82	0.5	<20	30	25
6	1.2	90	0.5	<5	42	35
7	0.5	95	<0.3	<5	51	40

All values are mean of two experiments.

* Cells were replated at a density of 0.5×10^6/ml after the first 3 d of exposure to DMSO and again on day 6.

‡ MII expressed as percentage of value obtained from control cell sample at same time point after subculture.

§ NBT-positive cells.

TABLE II

Effect of Transferrin-Iron and Transferrin-Gallium on Cell Proliferation and Immunofluorescent Surface Transferrin Receptor Expression

Day*	Transferrin-Fe (25 μg/ml)			Transferrin-gallium (25 μg/ml)			Transferrin-gallium (150 μg/ml)		
	Cell count	Cells in G_1	MII‡	Cell count	Cells in G_1	MII	Cell count	Cells in G_1	MII
	$\times 10^6$/ml	%	% control	$\times 10^6$/ml	%	% control	$\times 10^6$/ml	%	% control
1	0.7	61	74	0.7	60	123	0.5	61	167
2	1.2	71	77	1.1	70	197	0.6	47	259
3	1.8	78	83	1.7	75	277	0.7	36	400
4	0.7	55	78	0.7	57	240	—	—	—
5	1.1	65	83	0.8	38	280	—	—	—
6	1.8	80	84	1.0	38	420	—	—	—

All values are average of two experiments.

* Cells were plated at a density of 5×10^5/ml on day 0 and replated at the same density on day 3.

‡ MII expressed as percentage of control cells grown in 25 μg/ml apotransferrin at same day after culture.

slight down-regulation in transferrin-iron-supplemented cells as compared with apotransferrin-supplemented cells for each day after subculture (Table II), in spite of the same rate of proliferation. As with gallium nitrate, the addition of transferrin-gallium to the serum-free media resulted in a dose-related decrease in proliferation (Table II). Thus, when 150 μg/ml of transferrin-gallium was added to the media (0.25 μg/ml gallium), cells ceased proliferation by day 2 after subculture. It was noteworthy that cells supplemented with transferrin-gallium showed a significant up-regulation in surface transferrin receptor (Table II), in spite of decreased proliferation. Furthermore, DNA analyses of cells exposed to transferrin-gallium (Table II, Fig. 4) showed a decrease of cells in the G_1 phase of the cell cycle and indicated there was arrest of cells in the S phase, and to a lesser extent G_2 and M phases. Exposure of cells to a lower dose of transferrin-gallium (25 μg/ml) also led to eventual growth arrest, but only after the cells have been replated in fresh media after the first 72 h and continuously exposed to the transferrin-gallium for 6 d (Table II). Again, analyses of surface transferrin receptor showed up-regulation of receptor compared with the base-line values obtained with addition of apotransferrin. This up-regulation increased even further as proliferation decreased.

Additionally, although DNA analyses for the first 3 d of exposure to the low dose gallium was similar to that seen with transferrin-iron additions, when proliferation decreased at later time points, arrest of cells in the S phase of the cell cycle became evident (Fig. 4). Significantly, when growth was arrested with either dose of transferrin-gallium, there was no increase in cellular differentiation as compared with control cells. Thus, as opposed to treatment with DMSO, inhibition of cellular proliferation with transferrin-gallium results in no significant myeloid differentiation and is associated with arrest of cells in the S phase of the cell cycle.

It is of interest that the effect of gallium on cellular proliferation is similar to the effect caused by inhibi-

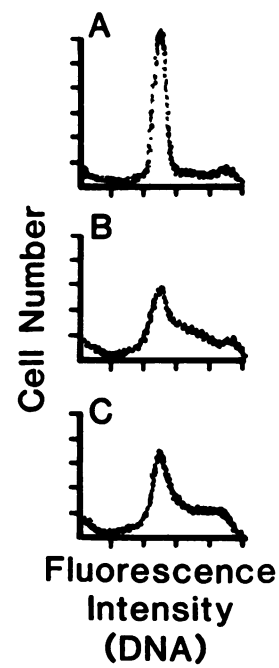

FIGURE 4 DNA analyses of cells 3 d after subculture in serum-free media with different forms of transferrin. (*A*) 25 μg/ml transferrin-iron: G_1, 78%; S, 14%; G_2 and M, 8%. (*B*) 150 μg/ml transferrin-gallium: G_1, 31%; S, 59%; G_2 and M, 10%. (*C*) 25 μg/ml transferrin-gallium: G_1, 40%; S, 48%; G_2 and M, 12%. In *C*, cells had been recultured 3 d previously but had been continuously exposed for 6 d to the same concentration of transferrin-gallium, when, therefore, proliferation had ceased.

tion of transferrin binding with a monoclonal antibody (17). Recently, we have developed a monoclonal antibody raised against purified human transferrin receptor that inhibits transferrin binding to HL-60 cells.[2] In preliminary experiments, we found that the addition of unpurified monoclonal antibody (designated TRA7) contained in hybridoma supernatants resulted in a dose-related inhibition of cellular proliferation. Cells grown in a 40% concentration of supernatant containing antibody showed a slight decrease in proliferation and undetectable transferrin binding by 3 d after subculture. When cells were recultured at day 3 in the same concentration of antibody, proliferation ceased. Immunofluorescence analyses of surface transferrin receptor at this time showed that >50% of the cells were immunofluorescence negative and DNA analyses revealed that the cells were arrested in the S phase of the cell cycle, agreeing with the previous observations (17). Additionally, as with exposure to transferrin-gallium, monoclonal antibody treatment did not result in increased differentiation of HL-60 cells as compared with control conditions.

DISCUSSION

Previous studies have demonstrated that actively proliferating nonmalignant and malignant cells express a marked increase in density of transferrin receptors as compared with nonproliferating cells (7, 8, 12, 33, 38, 39). The present manuscript further studies this association by presenting data that indicate that at early time points after cells are subcultured at low density to stimulate proliferation, synthesis of transferrin receptor occurs as early as 4 h after subculture and a number of hours later this newly synthesized receptor is expressed as increased surface transferrin receptor.[3] Additionally, analyses of a 12-h sample after subculture indicates that the marked increase in surface receptor appears before significant increases in cell proliferation begins. Furthermore, when cells in different phases of the cell cycle were separately analyzed for immunofluorescent surface transferrin receptor, the cells in the active phases of the cell cycle expressed the higher density of transferrin receptor. This result appeared to be most convincing for cells in the S phase of the cell cycle, since these cells had almost similar cell volumes as cells in the G_1 phase of the cell cycle, despite a 75% increase in median immunofluorescent intensity. These latter results lend support to earlier studies, which had suggested that a functional requirement for transferrin occurs after the S phase of the cell cycle (40), and a more recent study that demonstrates that cells exposed to a monoclonal antibody that blocked transferrin binding resulted in decreased proliferation and arrest of cells in the S phase (17). Thus, our studies indicate that the increased density of receptor on cells in the active phases of proliferation may participate in one or more required events that occur before cell division. When active proliferation begins, for example, at 24 h after subculture, it also appeared that cells in the G_1 phase of the cell cycle had significantly increased receptor density as compared with G_1 cells at confluency. This finding is not surprising since the half-life of newly synthesized receptor has been determined to be as long as 60 h (32) and, thus, the increased receptor found on cells in active phases of the cell cycle should still result in higher receptor density on daughter cells shortly after division, particularly if synthesis of receptor continues. The higher density of receptor on cells in G_1 at this time period may represent a mechanism by which HL-60 cells show an increased rate of proliferation some time after subculture, as evidenced by the S-shaped growth curves obtained by us and others (24).

Since it is not known how the increase in density in transferrin receptor functions as an event leading to cell division, we studied several agents that might modulate transferrin receptor density. The first agent chosen, DMSO, has been studied extensively in the HL-60 cell system, and although the mechanism of action has not been elucidated, cells exposed to DMSO show decreased proliferation followed by terminal myeloid differentiation. Although previous reports have indicated that surface transferrin receptor density on HL-60 cells decreases during DMSO treatment (21, 33) and that this decrease may precede by 24 h the decrease in cellular proliferation (33), these measurements have not been compared with changes in untreated cells during different stages of cellular proliferation. We found that a specific concentration of DMSO (1% wt/vol) allowed for an identical rate of proliferation for the first 3 d of treatment as compared with untreated cells. Within 12 h after subculture, DMSO-treated cells showed a marked attenuation of transferrin receptor with further attenuation at later time points. Continued DMSO treatment, even when a stimulus for proliferation was provided by subculturing cells in fresh media, resulted in cessation of proliferation, arrest of cells in G_1 phase of the cell cycle, followed by significant myeloid differentiation.

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[2] Miller, Y. E., C. R. Chitambar, and P. A. Seligman. Unpublished observations.

[3] Recent studies performed in collaboration with Dr. Joel E. Levine, using an ultrastructural immunocytochemical technique demonstrate that within 4 h after subculture, immunologic transferrin receptor is found in rough endoplasmic reticulum synthetic sites whereas it is not found on these sites in confluent cells.

These results suggest that although DMSO treatment results in attenuation of transferrin receptor expression, the density of receptor present may allow for a finite number of cell divisions to occur, but the further decrease in receptor expression may be at least one factor responsible for cessation of proliferation. The attenuation of expression of transferrin receptor caused by DMSO appears to be complex and may be due to one or more of the following factors: (*a*) decreased receptor synthesis, although the decrease in synthesis may be somewhat delayed, since DMSO treatment does allow for any early increase in transferrin receptor density (Fig. 2), whereas cyclohexamide completely inhibits any increase in receptor; (*b*) decreased availability of functional receptor, although measurements of total cellular immunoreactive receptor in treated cells are proportionately decreased, suggesting that any change in receptor must cause marked immunologic differences; (*c*) increased receptor degradation, a factor that may be significant, since control cells initially treated with DMSO at highest receptor density (24-h control sample) measured 24 h after treatment have even less surface transferrin binding (60% of 48-h control sample) than a similar group of cells treated with cyclohexamide (85% of control sample).

In additional experiments, we studied the effect of agents that we hypothesized would be more specific in affecting transferrin receptor expression. Previous studies have shown that radioactive gallium was taken up avidly by certain tumors (41), and the use of gallium salts as an antitumor agent had been suggested a number of years ago (42). More recent studies suggest that the addition of transferrin potentiates gallium salt-mediated arrest of cellular proliferation (36), an effect that may be related to inhibition of iron uptake (37). We used a well controlled serum-free system to demonstrate that cells exposed to transferrin-gallium showed a dose-related decrease in cellular proliferation and an associated up-regulation of surface transferrin receptor. The fact that this effect can be inhibited by either the addition of transferrin-iron or iron salts to the media adds further proof that increased transferrin receptor density during proliferation functions to provide iron to proliferating cells.[4] Thus, our experiments showing similar rates of proliferation using either apotransferrin or transferrin-iron may be deceiving, since iron present even in trace amounts in media may be enough to support a kinetically active iron pool necessary for cell division. The effect of gallium on arrested proliferation, can be distinguished from the DMSO effect by at least two aspects: (*a*) Transferrin-gallium treatment is associated with an increase in transferrin receptor density suggesting up-regulation of receptor, whereas DMSO treatment results in decreased transferrin receptor. (*b*) Arrested proliferation with DMSO results in accumulation of cells in the G_1 phase of the cell cycle, whereas transferrin-gallium, as with our preliminary study using blocking monoclonal antibody and the studies of others (17), results in arrest of cells in active phases of the cell cycle (particularly S phase). This latter effect may be related to the fact that DMSO-treated cells precede through the stages of myeloid differentiation and gallium-treated cells do not show significant increases in differentiation even though growth arrest occurs. Thus, although there is some controversy as to whether differentiation is linked to cessation of proliferation and arrest of cells in a specific phase of the cell cycle (24, 43), the above data support the recent hypotheses that cells must be arrested in a specific stage of G_1 to differentiate (18). These data additionally indicate that, although modulation of receptor density, with agents such as DMSO or phorbol esters (44), may be associated with differentiation, the specifics of this association are presently unknown.

It appears, therefore, that the modulation of transferrin receptor associated with DMSO exposure is associated with other events besides simply an inhibition of iron uptake or transferrin binding to cells, since arrest of cells occurs at a different stage in the cell cycle and differentiation begins. However, it is also clear that agents that affect iron uptake may be useful in further studies to better define cellular proliferation and additionally may be useful as chemotherapeutic agents. Significantly, since our in vitro studies show that transferrin-gallium results in marked inhibition of cellular proliferation, reevaluation of the use of gallium salts as a chemotherapeutic modality should recognize gallium interactions with transferrin, as has been suggested by clinical studies at other institutions (45).

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REFERENCES

1. Aisen, P., and E. B. Brown. 1975. Structure and function of transferrin. *Prog. Hematol.* 9:25–56.

[4] Chitambar, C. R., and P. A. Seligman. Unpublished observations.

2. Jandl, J. H., and J. H. Katz. 1963. The plasma-to-cell cycle of transferrin. *J. Clin. Invest.* 42:314–326.
3. Van Bockxmeer, F. M., and E. H. Morgan. 1979. Transferrin receptors during rabbit reticulocyte maturation. *Biochim. Biophys. Acta.* 584:76–83.
4. Seligman, P. A., R. B. Schleicher, and R. H. Allen. 1979. Isolation and characterization of the transferrin receptor from human placenta. *J. Biol. Chem.* 254:9943–9946.
5. Wada, H. G., P. E. Hass, and H. H. Sussman. 1979. Transferrin receptor on human placental brush border membranes. *J. Biol. Chem.* 254:12629–12635.
6. Loh, T. T., D. A. Higuchi, F. M. van Bockxmeer, C. H. Smith, and E. B. Brown. 1980. Transferrin receptors on the human placental microvillous membrane. *J. Clin. Invest.* 65:1182–1191.
7. Larrick, J. W., and P. Creswell. 1979. Modulation of cell surface iron transferrin receptors by cellular density and state of activation. *J. Supramol. Struct.* 11:579–586.
8. Hamilton, T. A., H. G. Wada, and H. H. Sussman. 1979. Identification of transferrin receptors on the surface of human cultured cells. *Proc. Natl. Acad. Sci. USA.* 76:6406–6410.
9. Ward, J. H., J. P. Kushner, and J. Kaplan. 1982. Regulation of HeLa cell transferrin receptors. *J. Biol. Chem.* 257:10317–10323.
10. Galbraith, R. M., and G. M. P. Galbraith. 1981. Expression of transferrin receptors on mitogen-stimulated human peripheral blood lymphocytes: relation to cellular activation and related metabolic events. *Immunology.* 44:703–710.
11. Hamilton, T. A. 1982. Regulation of transferrin receptor expression in concanavalin A-stimulated and Gross virus-transformed rat lymphoblasts. *J. Cell. Physiol.* 113:40–46.
12. Frazier, J. L., J. H. Caskey, M. Yoffe, and P. A. Seligman. 1982. Studies of the transferrin receptor on both reticulocytes and nucleated human cells in culture. Comparison of factors regulating receptor density. *J. Clin. Invest.* 69:853–865.
13. Enns, C. A., J. E. Shindelman, S. E. Tonik, and H. H. Sussman. 1981. Radioimmunochemical measurement of the transferrin receptors in human trophoblast and reticulocyte membranes with a specific anti-receptor antibody. *Proc. Natl. Acad. Sci. USA.* 178:4222–4225.
14. Tormey, D. C., and G. C. Meuller. 1972. Biologic effects of transferrin on human lymphocytes *in vitro*. *Exp. Cell Res.* 74:220–226.
15. Hayashi, I., and G. H. Sato. 1976. Replacement of serum by hormones permits growth of cells in defined media. *Nature (Lond.).* 259:132–134.
16. Breitman, T. R., S. J. Collins, and B. R. Keene. 1980. Replacement of serum by insulin and transferrin supports growth and differentiation of the human promyelocytic cell line HL-60. *Exp. Cell Res.* 126:494–498.
17. Trowbridge, I. A., and F. Lopez. 1982. Monoclonal antibody to transferrin receptor blocks transferrin binding and inhibits human tumor growth *in vitro*. *Proc. Natl. Acad. Sci. USA.* 79:1175–1179.
18. Scott, R. E., D. L. Florine, J. J. Wille, Jr., and K. Yun. 1982. Coupling of growth arrest and differentiation at a distinct state in the G_1 phase of the cell cycle: G_D. *Proc. Natl. Acad. Sci. USA.* 79:845–849.
19. Collins, S. J., R. C. Gallo, and R. E. Gallagher. 1977. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature (Lond.).* 270:347–349.
20. Collins, S. J., F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo. 1978. Terminal differentiation of human promyelocytic leukemia cells induced by dimethylsulfoxide and other polar compounds. *Proc. Natl. Acad. Sci. USA.* 75:2458–2462.
21. Yeh, C. G., M. Papamichael, and W. P. Faulk. 1982. Loss of transferrin receptors following induced differentiation of HL-60 promyelocytic leukemia cells. *Exp. Cell Res.* 138:429–433.
22. Bates, G. W., and M. R. Schlambach. 1973. The reaction of ferric salts with transferrin. *J. Biol. Chem.* 248:3228–3232.
23. Harris, D. C., and P. Aisen. 1975. Magnetic resonance studies of the spatial relation of the metal and anion in human transferrin. *In* Proteins of Iron Storage and Transport in Biochemistry and Medicine. R. R. Crighton, editor. North Holland/American Elsevier, New York. 59.
24. Tarella, C., D. Ferrero, E. Gallo, G. L. Pagliardi, and F. W. Ruscetti. 1982. Induction of differentiation of HL-60 cells by dimethylsulfoxide: evidence for a stochastic model not linked to the cell division cycle. *Cancer Res.* 42:445–449.
25. Karin, M., and B. Mintz. 1981. Receptor-mediated endocytosis of transferrin in developmental totipotent mouse teratocarcinoma stem cells. *J. Biol. Chem.* 256:3245–3252.
26. Renswoude, J. V., K. R. Bridges, J. B. Harford, and R. D. Klausner. 1982. Receptor-mediated endocytosis of transferrin and the uptake of Fe in K_{562} cells: identification of a nonlysosomal acidic compartment. *Proc. Natl. Acad. Sci. USA.* 79:6186–6190.
27. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* 51:660–672.
28. Segal, A. W. 1974. Nitroblue-tetrazolium tests. *Lancet.* II:1248–1252.
29. Seligman, P. A., R. H. Allen, S. J. Kirchenski, and P. J. Natale. 1983. Automated analysis of reticulocytes using fluorescence staining with both acridine orange and an immunofluorescent technique. *Am. J. Hematol.* 14:57–66.
30. Crissman, H. A., and J. A. Steinkamp. 1973. Rapid, simultaneous measurement of DNA, protein, and cell volume in single cells from large mammalian cell populations. *J. Cell Biol.* 59:766–771.
31. Thompoulos, P., J. Roth, E. Lovelace, and I. Pastan. 1976. Insulin receptors in normal and transformed fibroblasts: relationship to growth and transformation. *Cell.* 8:417–423.
32. Omary, M. B., and I. S. Trowbridge. 1981. Biosynthesis of the human transferrin receptor in cultured cells. *J. Biol. Chem.* 256:12888–12892.
33. Omary, M. B., I. S. Trowbridge, and J. Minowada. 1980. Human cell-surface glycoprotein with unusual properties. *Nature (Lond.).* 286:888–891.
34. Cram, L. S., and A. Bunsting. 1973. Fluorescence and light scattering measurements of hog cholera-infected PK-15 cells. *Exp. Cell Res.* 78:209–213.
35. Loken, M. R., and L. A. Herzenberg. 1975. Analysis of cell populations with a fluorescence-activated cell sorter. *Ann. NY Acad. Sci.* 254:163–171.
36. Sephton, R. G., and A. W. Harris. 1975. ^{67}Ga citrate uptake by cultured tumor cells, stimulated by serum transferrin. *J. Natl. Cancer Inst.* 54:1263–1266.
37. Rasey, J. S., N. J. Nelson, and S. M. Larson. 1981. Re-

lationship of iron metabolism to tumor cell toxicity of stable gallium salts. *Int. J. Nucl. Med. Biol.* 8:303–313.
38. Sutherland, R., D. Delia, C. Schneider, R. Newman, J. Kemhead, and M. Greaves. 1981. Ubiquitous cell-surface glycoprotein on tumor cells is proliferation associated receptor for transferrin. *Proc. Natl. Acad. Sci. USA.* 78:4515–4519.
39. Trowbridge, I. S., and M. B. Omary. 1981. Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. *Proc. Natl. Acad. Sci. USA.* 78:3039–3043.
40. Rudland, P. S., H. Durbin, D. Clingan, and L. J. deAsua. 1977. Iron salts and transferrin are specifically required for cell division of cultured 3T6 cells. *Biochem. Biophys. Res. Commun.* 75:556–562.
41. Johnston, G. S. 1981. Clinical applications of gallium in oncology. *Int. J. Nucl. Med. Biol.* 8:249–255.
42. Hart, M. M., and R. H. Adamson. 1971. Antitumor activity and toxicity of salts of inorganic group IIIa metals: aluminum, gallium, indium, and thallium. *Proc. Natl. Acad. Sci. USA.* 68:1623–1626.
43. Ferrero, D., T. C. Tarella, E. Gallo, F. W. Ruscetti, and T. R. Breitman. 1982. Terminal differentiation of the human promyelocytic leukemia cell line HL-60 in the absence of cell proliferation. *Cancer Res.* 42:4421–4426.
44. Pelicci, P. G., U. Testa, P. Thomopoulos, A. Tabilio, W. Vainchenker, M. Titeux, and J. Breton-Gorius. 1982. Regulation of the expression of the transferrin receptor of hematopoietic cell lines by phorbol esters. *Blood.* 60:90*a*. (Abstr.)
45. Warrell, R. P., C. J. Coonley, D. J. Strauss, M. J. Lacher, and C. W. Young. 1983. Clinical evaluation of gallium nitrate (NSC-15200) by 7 day continuous infusion in advanced malignant lymphoma. *Cancer.* 51:1982–1987.