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Research Article

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Relationship of Cell-Cycle Expression of Ia-like Antigenic Determinants on Normal and Leukemia Human Granulocyte-Macrophage Progenitor Cells to Regulation In Vitro by Acidic Isoferritins

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ABSTRACT An association has been established between human Ia-like (Ia) antigenic determinants, expression during DNA synthesis on granulocyte-macrophage colony forming cells (CFU-GM) and the regulatory action of acidic isoferritins in vitro. Treatment of human bone marrow cells with monoclonal-anti-Ia-like (Ia) plus complement inhibited colony and cluster formation by ~50% but did not affect pre-CFU-GM. Reduction of colonies and clusters was similar whether bone marrow cells were exposed to anti-Ia plus complement, high specific activity tritiated thymidine (^{3}HTdr) or acidic isoferritins. No further decrease was apparent with ^{3}HTdr or acidic isoferritins after Ia-antigen$^+$ CFU-GM were removed, or with anti-Ia plus complement or acidic isoferritins after DNA synthetic phase (S-phase) CFU-GM were removed. Anti-Ia, without complement, did not reduce colony or cluster formation but did block the inhibitory action of acidic isoferritins. A relationship existed between Ia antigens and the activity of acidic isoferritins in the following ways: (*a*) The apparent loss of Ia-antigens from CFU-GM by 5 h in culture at 37°C, but not at 27° or 4°C, was associated with nonresponsiveness to inhibition with acidic isoferritins, (*b*) Ia-antigen$^-$, noncycling pre-CFU-GM that were insensitive to acidic isoferritins could generate a population of Ia-antigen$^+$ cycling CFU-GM in vitro that were responsive to inhibition by acidic isoferritins, and (*c*) nondetectability of Ia-antigens on CFU-GM from patients with leukemia was associated with nonresponsiveness to inhibition by acidic isoferritins. These results implicate Ia-antigen$^+$ progenitor cells in the regulation of myelopoiesis in vitro and demonstrate that absence of Ia-antigens on patient CFU-GM is associated with imbalances in normal regulatory interactions in vitro. These findings may be of relevance to normal regulation and to the progression of leukemia.

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INTRODUCTION

Progenitor cells (CFU-GM)[1] for the granulocyte-macrophage lineage can be assessed by their capacity to form colonies and clusters of granulocytes and/or macrophages in semisolid medium in vitro after stimulation by granulocyte-macrophage colony stimulatory factors (GM-CSF) (1, 2). Human CFU-GM contain Ia-like antigenic determinants on their surface (3–8) and these progenitor cells appear to be generated from pluripotential stem cells, which lack Ia-like antigens (6). For simplicity, the term Ia will be used in this paper to denote Ia-like. Myelopoiesis is a dynamic process regulated, at least in vitro, by stimulatory and inhibitory feedback systems (1, 2) and it has been suggested that a function of the Ia-antigenic system may relate to the control of proliferative events in the marrow (3, 6) in addition to its role in immune responsiveness (7). Evidence for such a relationship has been presented in that lactoferrin, a metal binding glycoprotein shuts off GM-CSF production and/or release from a subpopulation of Ia-like antigen positive monocytes and macrophages (9).

Aberrations in regulatory interactions in vitro have been detected with cells from patients with leukemia, lymphoma, myeloproliferative, and myelodysplastic

[1] *Abbreviations used in this paper*: AML, acute myeloid leukemia; CFU-GM, granulocyte-macrophage colony or cluster forming cells; CML, chronic myeloid leukemia; GM-CSF, granulocyte-macrophage colony stimulatory factors; PGE, prostaglandin E; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; S-phase, DNA synthetic phase; ^{3}HTdr, high specific activity tritiated thymidine.

disorders (1, 2). An example of this is a leukemia-associated inhibitory activity (10–12), which has recently been identified as acidic isoferritins (13). Acidic-isoferritin inhibitory activity can be found in normal bone marrow and blood cells but is present in much greater concentrations in such cells from patients with acute and chronic myeloid and lymphoid leukemia (10, 11, 13). This activity has also been detected in mice infected with Abelson and Friend virus (14, 15). In contrast to its inhibitory action on normal CFU-GM, which is restricted to the DNA synthetic phase of the cell cycle (S-phase), acidic isoferritin inhibitory activity is not effective in suppressing the growth of CFU-GM from many patients with leukemia and from mice infected with Abelson and Friend virus and blast progenitor cells even though the CFU-GM and blast progenitor cells are in cycle (10–16). The mechanisms involved in the lack of responsiveness of "leukemia" CFU-GM to inhibition by acidic isoferritins have not been elucidated but would be of importance for a better understanding of the progression of leukemia.

Evidence is now presented that certain monoclonal antibodies to human Ia-like antigens recognize normal CFU-GM during S-phase, acidic isoferritins act on CFU-GM expressing Ia-like antigens and that loss of these antigens on normal CFU-GM in culture and the absence of these antigens on CFU-GM from patients with leukemia is associated with nonresponsiveness to inhibition by acidic isoferritins.

METHODS

Cells. Bone marrow cells were obtained from normal healthy volunteers and from patients with acute and chronic myeloid leukemia from Memorial Hospital and from New York Hospital, New York. Cells were obtained from donors who had given informed consent and patient cells were obtained during routine hematologic assessment of clinical status. Nucleated bone marrow cells were separated by a density "cut" procedure with bovine serum albumin (1.070 g/cm^3, 270 mosmol) (1). Low density cells (<1.070 g/cm^3) were sometimes separated further by adherence to plastic tissue culture dishes (Falcon 3001 Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) for 1.5 h at 37°C (1) to obtain nonadherent low density cells depleted of cells producing GM-CSF. These fractions contained >98% of the colony and cluster forming cells present in the original unseparated nucleated cell fraction.

Acidic isoferritins. Acidic isoferritins were isolated from the spleen of a patient with chronic myeloid leukemia by a sequence of procedures including dialysis, ultracentrifugation, Sephadex G-200 gel filtration, carboxymethylcellulose ion exchange chromatography, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and analytical isoelectric focusing. Isolation procedures are detailed elsewhere (12, 13). Most studies were done with the ferritin isolated to the stage of carboxymethylcellulose that contained at least 94% ferritin as determined by radioimmunoassay. At least one study for each experimental group was done with the more purified acidic isoferritins isolated at a pI of 4.7 after isoelectric focusing. This material migrated as a single band in the apparent molecular weight region of 550,000 after SDS-PAGE and as two bands with apparent molecular weights of 19,000 and 21,000 after treating with 10% 2-mercaptoethanol at 100°C for 10 min, running on SDS-PAGE and staining with Coomassie Blue (13). These studies have been substantiated more recently using ^{125}I-labeled acidic isoferritins and autoradiography.[2] The material contained at least 95% acidic isoferritins as determined by radioimmunoassay using ^{125}I-labeled goat antinormal heart ferritin absorbed with basic heart ferritin (pH 5.8) in a three-step procedure as described (13). The specificity of the goat antinormal heart ferritin absorbed with basic heart ferritin has been described in Fig. 5 of reference 13. Please note that in the figure legend to Fig. 5 of reference 13, F2 should be the basic heart ferritin, pH 5.8, and F1 the Hodgkin's spleen ferritin. Acidic isoferritins depleted of iron are as active as those samples containing iron within the molecules (13).

Assays. (*a*) Colony (>50 cells per aggregate) and cluster (3–50 cells per aggregate) formation of bone marrow cells was stimulated by exogenously supplied colony stimulatory factors. 1×10^5 nonadherent low density or 7.5×10^4 low density human bone marrow cells were suspended in 1 ml of 0.3% agar culture medium (Difco Laboratories, Detroit, Mich.) that included McCoy's 5A medium supplemented with additional essential and nonessential amino acids, glutamine, serine, asparagine, and sodium pyruvate (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) and contained 10% heat-inactivated (56°C for 30 min) fetal calf serum (Microbiological Associates, Walkersville, Md.). No background colonies and only rare clusters (up to 20) were observed under these conditions when no exogenous stimulatory factors were supplied. CSF were present in medium conditioned by the GCT cell line (Gibco Laboratories) (17). Acidic isoferritins (10% volume) were either used to pulse bone marrow cells for 30 min before washing and plating the cells or were mixed with the GCT-conditioned medium (10%) and the suspensions of bone marrow cells before gelling of the agar culture medium. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air and scored for colonies and/or clusters after 7 and 14 d of incubation. GCT-conditioned medium was used as a source of GM-CSF as it increased sensitivity of the assay to detect the percentage of CFU-GM in DNA-synthesis and inhibition by acidic isoferritins compared with that apparent when human placental cell- and human monocyte-conditioned media are used to stimulate colony and cluster formation. It also allows detection of inhibition of colony and/or cluster formation after 14 as well as after 7 d of incubation (unpublished observations).

The approximate percentages of colony and cluster types stimulated by GCT-CM after 7 d of incubation are 40% neutrophil, 10% eosinophil, 30% macrophage, and 20% neutrophil-macrophage. These results are in relatively good agreement with those published by others (17).

(*b*) Pre-CFU-GM are cells that generate CFU-GM in suspension culture and that can be separated from CFU-GM by their lower velocity sedimentation rate (18). In the present studies low density marrow cells (10^6) were incubated in 1 ml of supplemented McCoy's medium with 10% fetal calf serum and 10% GCT-conditioned medium. After 7 d in suspension culture, the marrow cells were washed twice and plated as described above.

[2] Bognacki, J., and H. Broxmeyer. Unpublished observation.

Measurement of CFU-GM and pre-CFU-GM in S-phase. To measure the proportion of cells in DNA synthesis (S-phase) marrow cells were treated with McCoy's medium, thymidine (500 μg), 50 μCi/ml tritiated thymidine (^{3}HTdr, New England Nuclear, Boston, Mass., sp act 20 Ci/mmol) or 50 μCi ^{3}HTdr plus 500 μg thymidine. After a 20-min incubation at 37°C the cells were washed once in ice-cold thymidine (2,000 μg), twice more in McCoy's medium, and then plated in the appropriate assays. The reduction in the number of colonies and/or clusters after exposure to ^{3}HTdr was expressed as the percentage of the control compared with McCoy's medium or thymidine-treated cells. The latter incubation of ^{3}HTdr plus excess thymidine was used to demonstrate the counteraction of the ^{3}HTdr kill and thus the S-phase specificity of the ^{3}HTdr.

Ia-like antigen-antibodies. Monoclonal anti-human Ia-like antigen (m-anti-Ia) (NE1-011) was purchased from New England Nuclear. It had been isolated from hybrid 7.2 (19), is an IgG_{2b} antibody that had been purified by affinity chromatography with protein A and is >95% homogeneous. It recognizes two polypeptides of 28,000 and 33,000 daltons and is found on >99% B cells, on <1% T cells, and on monocytes and activated T cells from peripheral blood as well as on B cell lines, B cell leukemias, and certain myeloid and "null" cell leukemias. It fixes rabbit complement and binds with high avidity to protein A of *Staphylococcus aureus* (New England Nuclear Communication).

M-anti Ia (BD) was purchased from Becton, Dickinson & Co., Sunnyvale, Calif. It had been isolated from clone L243 (20), is an IgG_{2a} antibody that had been purified by ion exchange chromatography. It recognizes an HLA-DR nonpolymorphic antigen of 28,000 and 34,000 daltons. It is found on human B lymphocytes, monocytes/macrophages, thymic epithelium, B cell-dependent areas of spleen and lymph node, B cell lymphomas, and activated T cells and is a complement-fixing antibody (Becton, Dickinson Communication).

M-anti Ia (OKlal) was purchased from Ortho Pharmaceutical Corp., Raritan, N. J. as a lyophilized ascitic fluid. It reacts with the HLA-DR framework (21). It recognizes B lymphocytes, monocytes, some null cells, and activated T lymphocytes, and is a complement-fixing antibody (Ortho Communication).

M-anti Ia (BRL) was purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. It had been isolated from clone 59.5, is an IgG antibody that had been purified by DEAE ion exchange chromatography. It recognizes the common membrane determinant of the DR antigen system and is found on B lymphocytes and monocytes collected from peripheral blood as well as mitogen-activated T lymphoblasts and DR^+ human lymphoblastoid cells lines. It is not bound by Staphyloccal protein A and does not bind complement (Bethesda Research Laboratory Communication).

M-anti Ia-like antigen (Q5/13) was a generous gift from Dr. John Fitchen, University of California at Los Angeles and Dr. S. Ferrone, Scripps Clinic and Research Institute, La Jolla, Calif., and has been described previously (22). Rabbit anti-human Ia-like antiserum and the B cell absorbed Ia antiserum were generous gifts from Dr. Robert Winchester, Hospital for Joint Diseases, New York, and have been described previously (3).

The purified antibodies were stored frozen as 100-μl aliquots containing 0.1 mg reagent and the hybridoma supernatants and antisera were stored frozen in undiluted form. All were thawed and diluted in McCoy's medium as needed.

Complement-dependent cytotoxicity and complement-independent studies. These procedures have been described previously (3, 9). Rabbit complement was preselected for absence of heterospecific antibodies or purchased (Low-Tox-H-Rabbit complement, Accurate Chemicals Co., Westbury, N. Y.) and used at a 1:8 dilution. Results were similar using both sources of complement.

In all experimental protocols, cells were washed two times after each individual treatment procedure.

Statistical analysis. Five plates were scored for each sample and the probability of significant differences between samples was determined by use of Student's *t* test.

RESULTS

Certain monoclonal antibodies to human Ia-like antigens detect determinants on a subpopulation of CFU-GM. The average results of three separate experiments in which m-anti-Ia (NE1-011) was titrated in a complement cytotoxicity assay for inhibition of colony and cluster formation of pre-CFU-GM, day 14 CFU-GM and day 7 CFU-GM is shown in Fig. 1. It has been shown previously that pre CFU-GM represent an immature progenitor cell compartment that appears to give rise to day 14 CFU-GM, which in turn, appear to give rise to the more mature day 7 CFU-GM (17). No inhibition of the pre-CFU-GM compartment was detected but plateau curves of inhibition in the range of 40 to 50% were detected against days 14 and 7 CFU-GM. The antibody titered out furthest against the day 7 CFU-GM.

Two other complement-fixing monoclonal HLA-DR antibodies: OKlal and BD were assessed at dilutions of 1:100, 1:200, 1:400, and 1:800 for their capacity to inhibit colony and cluster formation of day 7 CFU-

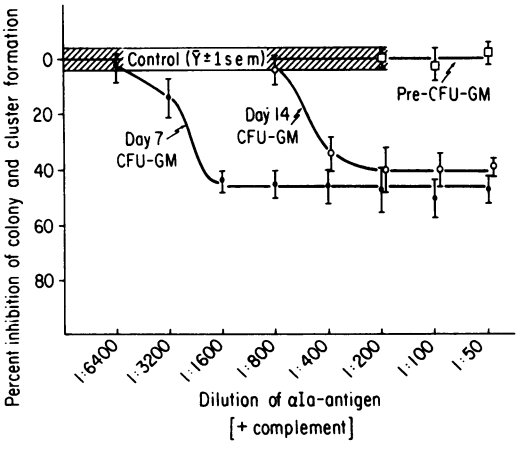

FIGURE 1 Inhibition of myeloid colony and cluster formation at day 7 and day 14 and of pre-CFU-GM following preincubation of bone marrow cells with m-anti-Ia (NE1-011) plus complement. Data are based on the mean±SE of three separate experiments. Percent inhibition is relative to complement control values that were equal to McCoy's medium control values. The number of control colonies and clusters ranged from 150 to 485 in individual experiments.

GM in a complement-dependent cytotoxicity assay and compared with the effects of NE1-011 at these same dilutions in two separate experiments. The three antibodies behaved similarly and significantly decreased colony and cluster formation by 40 to 46% ($P < 0.005$) at each dilution of antibody. These results contrast with those previously reported by us using a hetero-antiserum against human Ia-like antigens (3, 6). The rabbit anti-human Ia in a complement cytotoxicity assay inhibited the three progenitor cell compartments to >90% at high antiserum concentrations with the titers being greatest against the pre-CFU-GM and least against day 7 CFU-GM (6). The differences between m-anti-Ia (NE1-011, OKlal, BD) and the rabbit anti-human Ia did not appear to be due to the source of complement. Using the same preparation of complement at the same dilutions as for the data expressed in Fig. 1 it was possible to duplicate exactly the >90% inhibition of pre-CFU-GM, day 14 CFU-GM and day 7 CFU-GM using the rabbit hetero-antiserum (1:100 dilution). (Control values for the three populations were 198±8, 130±6, and 120±4.) Another m-anti-Ia ($Q5/_{13}$) has been reported to inhibit colony formation of CFU-GM by >90%.[3] Using the same sources and dilutions of complement as for the data in Fig. 1 and for the antibodies described above (NE1-011, OKlal and BD) the m-anti-Ia ($Q5/_{13}$) at a dilution of 1:50 reduced colony and/or cluster formation of pre-CFU-GM and days 14 and 7 CFU-GM by >94%. These results suggest that differences in the extent of lysis of CFU populations obtained with particular polyvalent and monoclonal antibodies is probably not related to the source or dilution of complement used. Furthermore, m-anti-Ia NE1-011, OKlal, and BD appear unique to those antibodies tested in eliminating a subpopulation of CFU-GM.

Acidic isoferritins act on the subpopulation of CFU-GM recognized by m-anti-Ia NE1-011, OKlal, and BD. One of three representative experiments is shown in Fig. 2, which demonstrates the effect of acidic isoferritins on day 7 CFU-GM pretreated with m-anti-Ia (NE1-011) plus complement (Fig. 2A) or m-anti-Ia (NE1-011) without complement (Fig. 2B). As shown in Fig. 2A, acidic isoferritins (1 nM) reduced colony formation by 64% and colony plus cluster formation by 53%. M-anti-Ia (NE1-011) reduced colony and/or cluster formation by an amount equal to the acidic isoferritins and the acidic isoferritins had no effect on the colonies and clusters surviving pretreatment with m-anti-Ia plus complement. Acidic isoferritins were tested at concentrations in the range of 10 nM to 10 fM and the results were similar (data not shown).

[3] Fitchen, J. Personal communication.

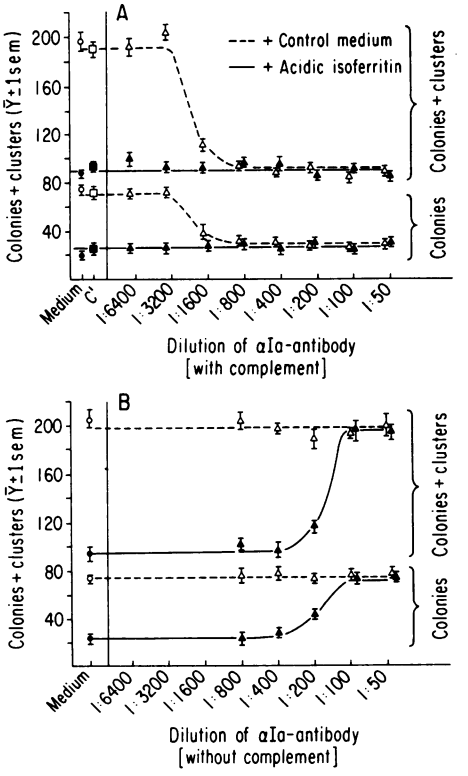

FIGURE 2 Effect of various dilutions of m-anti-Ia (NEl-0ll) on colony and/or cluster formation by day 7 CFU-GM and on inhibitory activity of acidic isoferritins. (A) Cells were pretreated with McCoy's medium, complement (C') or various dilutions of m-anti-Ia plus C'. (B) Cells were treated with McCoy's medium or m-anti-Ia. The treated cells were plated with or without 1 nM acidic isoferritin and assessed for colony formation. One of three representative experiments is shown.

As shown in Table IA, m-anti-Ia OKlal and BD (1:100 dilution) in the presence of complement also reduced colony and cluster formation by an amount equal to the acidic isoferritins and the acidic isoferritins had no effect on the colonies and clusters surviving pretreatment with these antibodies. M-anti-Ia BRL is not a complement-fixing antibody and was not assessed for an effect in this assay.

M-anti-Ia (NE1-011) in the absence of complement had no influence on colony and cluster formation but 1:50 to 1:100 dilutions of the m-anti-Ia blocked the inhibitory activity of 1 nM acidic isoferritins (Fig. 2B). Table IB demonstrates the results of one of two similar experiments in which five monoclonal Ia antibodies (NEl-0ll, $Q5/_{13}$, BD, OKlal, and BRL) and one hetero-Ia antiserum were assessed in the absence of complement for their capacity to block the inhibitory activity of 1 nM acidic isoferritins. Antibodies were tested at dilutions of 1:50, 1:100, 1:200, 1:400, 1:800, and

TABLE I

Comparative Effects of Various Human Ia-like Antibodies

Treatment	Colonies and clusters (mean Y ±1 SE)*			
	+ McCoy's medium	%Δ‡	+ 1 nM acidic isoferritins	%Δ
A With complement				
Exp. 1				
Control medium	452±10	—	282±6	−38‖
m-NEI-011 (1:100)§	290±5	−36‖	264±13	−42‖
m-OKlal (1:100)	274±18	−39‖	270±2	−40‖
Exp. 2				
Control medium	193±5	—	109±2	−44‖
m-NEI-011 (1:100)	103±4	−47‖	106±3	−45‖
m-BD (1:100)	107±1	−45‖	107±4	−45‖
B Without complement				
Control medium	121±8	—	57±4	−53‖
m-NEI-011 (1:100)	119±9	−2	117±9	−3
m-Q5/13 (1:10)	119±5	−2	118±9	−2
m-BD (1:200)	120±4	−1	117±6	−3
m-OKlal (1:100)	113±5	−7	118±4	−2
m-BRL (1:200)	118±6	−2	114±4	−6
Rabbit anti-human Ia (1:800)	123±6	+2	115±5	−5
B-cell absorbed rabbit anti-human Ia (1:20)	119±5	−2	49±6	−60‖

* Low density human bone marrow cells were scored after 7 d of incubation.

‡ Percent change from control medium.

§ Dilution of antibody.

‖ Significant change $P < 0.001$.

1:1,600 (plus 1:10 for $Q5/_{13}$ and 1:20 for B cell absorbed hetero-anti-Ia) and the results shown are for the highest dilution, which completely blocked the effect of the acidic isoferritins. None of the antibodies influenced colony and cluster formation but the monoclonal Ia-antibodies and the hetero-Ia-antiserum blocked the inhibitory action of acidic isoferritins. The B-cell absorbed hetero-Ia-antiserum did not block the inhibitory activity.

Ia-antigens recognized by m-anti-Ia (NE1-011) are expressed on CFU-GM during S-phase. Acidic isoferritins act on CFU-GM during S-phase (10) and the results in Figs. 1 and 2A of ~50% inhibition were consistent with a cycle related expression of Ia-antigens. Experimental evidence for an expression during S-phase is presented in Fig. 3, which shows one of three similar experiments. The decrease in colony and/or cluster formation (day 7 CFU-GM) was exactly equal whether the marrow cells were pretreated with m-anti-Ia plus complement or by ^{3}HTdr. The kill by ^{3}HTdr was blocked by coincubation of ^{3}HTdr with an excess of cold thymidine substantiating the S-phase

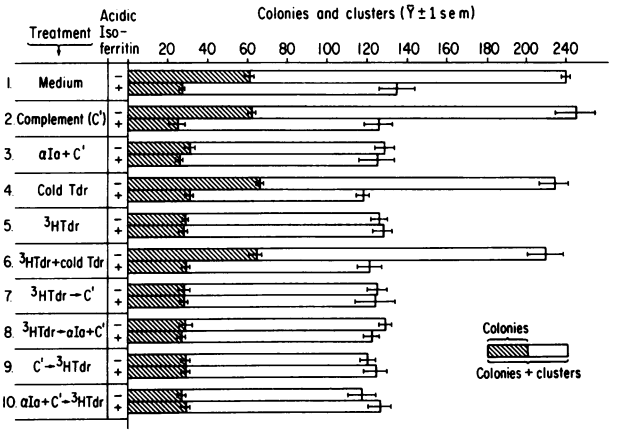

FIGURE 3 Comparative effect of m-anti-Ia (NE1-011) + C' and treatment with ^{3}HTdr on colony and/or cluster formation by day 7 CFU-GM and on inhibitory activity of acidic isoferritins. Cells were pretreated as shown above and as explained in the text and were plated with or without 1 nM acidic isoferritin and assessed for colony and cluster formation. One of three representative experiments is shown.

specificity of the ^{3}HTdr results. Moreover, no further decrease in colony and/or cluster formation was detected with m-anti-Ia plus complement after the S-phase cells had been killed by ^{3}HTdr. No further kill by ^{3}HTdr was noted for those cells surviving treatment with m-anti-Ia plus complement. Acidic isoferritins (1 nM) decreased colony and/or cluster formation of cells treated with medium, complement or ^{3}HTdr in the presence of excess cold thymidine but the acidic isoferritins had no effect on CFU-GM surviving treatment with m-anti-Ia plus complement, ^{3}HTdr, or sequential treatments with m-anti-Ia plus complement and ^{3}HTdr.

Loss or absence of Ia-antigens, on normal progenitor cells, which are detected by m-anti-Ia (NE1-011) results in insensitivity to the inhibitory activity of acidic isoferritins. Only a proportion of CFU-GM are in S-phase at the time bone marrow cells are removed from donors but all CFU-GM must move through S-phase in order to form colonies and clusters in culture. Acidic isoferritins act on CFU-GM during S-phase, yet when left in the culture plates for the full 7–14 d of incubation still result in the same 40–60% plateau curve of inhibition over a >6 log dilution of acidic isoferritins (10, 13). The inhibition is similar if marrow cells are pulsed with acidic isoferritins for only 10–30 min before washing and plating the cells (10). This suggested either that the acidic isoferritins were being inactivated within a few hours within the cultures or that with time in culture the CFU-GM became insensitive to the actions of acidic isoferritins even though they were preceding through S-phase. In the absence of certain exogenously added agents the biosynthesis and surface expression of Ia-antigens on mouse peritoneal macrophages is terminated during the 1st d in culture (23–25). This work suggested a testable hypothesis that CFU-GM were losing their Ia antigens with time in culture and were therefore insensitive to the actions of acidic isoferritins. As shown in Fig. 4 this turned out to be the case. Bone marrow cells were incubated at 37, 27, and 4°C for varying time periods and assessed for their sensitivity to kill by m-anti-Ia plus complement and to inhibition by acidic isoferritins. Day 7 CFU-GM from marrow cells left at 27 or 4°C for up to 24 h were still sensitive to both, whereas CFU-GM from the same marrow began losing sensitivity by 3 h when left at 37°C and completely lost sensitivity to both treatments by 6 h in culture. Similar results were apparent for day 14 CFU-GM and another experiment demonstrated that sensitivity to both treatments was lost by 5 h in culture (data not shown). Results shown in Table II demonstrate that loss of susceptibility to killing with m-anti-Ia plus complement and inhibition by acidic isoferritins was not due to loss of S-phase CFU-GM by 6 h in culture. Furthermore,

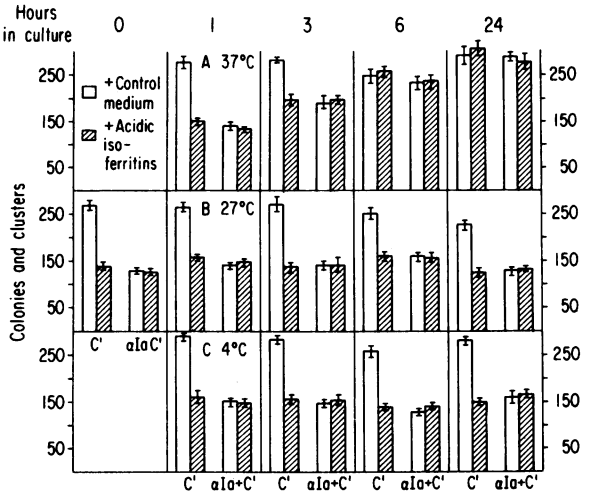

FIGURE 4 Effect of temperature on inhibition of colony and cluster formation of day 7 CFU-GM by treatment of m-anti-Ia (NE1-011) and acidic isoferritins. Bone marrow cells were placed at 37, 27, or 4°C for 1, 3, 6, and 24 h, washed two times and treated with C' or m-anti-Ia antigen + C', washed two times and plated with or without 1 nM acidic isoferritins and assessed for colony and cluster formation.

the loss of sensitivity occurred whether the bone marrow cells were placed at 37°C for 6 h in the absence or presence of an exogenously added source of GM-CSF.

Further evidence for the association of m-anti-Ia (NE1-011) with S-phase progenitor cells and with sensitivity to inhibition with acidic isoferritins is given in Table III, which presents one of three representative experiments. Pre-CFU-GM, assessed upon immediate removal of marrow cells from a donor, were not affected by treatment with m-anti-Ia plus complement, ^{3}HTdr or acidic isoferritins, but day 7 CFU-GM from the same marrow inoculum were decreased. Whereas pre-CFU-GM were not in cycle, a finding consistent with previous reports (1, 2, 18), and lacked detectable Ia-antigens recognized by m-anti-Ia (NE1-011), the day 7 CFU-GM generated in culture from the pre-CFR-GM compartment were in cycle, did contain Ia antigenic determinants and were susceptible to inhibition by acidic isoferritins.

Absence of Ia-antigens recognized by m-anti-Ia (NE1-011) is associated with insensitivity of CFU-GM from patients with leukemia to inhibition by acidic isoferritins. Acidic isoferritins are inactive against CFU-GM from nonremission patients with acute leukemia and from some patients with chronic leukemia and acute leukemia in remission (11–13, 26). Inactivity was not related to cycle status of patient CFU-GM as CFU-GM upon removal from patients were in cycle (11). The relationship between Ia-antigens and susceptibility to the action of acidic isofer-

TABLE II

Effect of Temperature on Inhibition of Colony and Cluster Formation of Day 7 CFU-GM by Monoclonal Anti-Ia, ^{3}HTdr, and Acidic Isoferritins

Preincubation			Treatment after preincubation (colonies mean ±1 SE)					
Hours of incubation	Temperature	GM-CSF*	C′	αIa + C′	C′ plus acidic isoferritins	Medium	^{3}HTdr	Cold Tdr + ^{3}HTdr
	°C							
0	–	–	84±4	33±3 (−61)‡§	36±3 (−57)§	83±3	34±3 (−59)§	87±3 (+5)
6	37	–	65±3	68±4 (+5)	59±2 (−9)	64±4	32±1 (−50)§	68±3 (+6)
6	37	+	84±6	86±5 (+2)	79±3 (−6)	78±6	25±2 (−68)§	72±3 (−8)
6	4	–	92±6	46±3 (−50)§	42±3 (−54)§	81±5	33±4 (−59)§	87±5 (+7)
6	4	+	77±3	33±2 (−57)§	30±4 (−61)§	83±2	29±3 (−65)§	78±3 (−6)

Bone marrow cells were treated with C′, α Ia + C′, or ^{3}HTdr, washed two times and plated or placed at 37°C or 4°C for 6 h in the absence or presence of GCT-CM, washed two times, and treated with C′, α Ia + C′, or ^{3}HTdr prior to washing two times and plating. Cells were washed prior to plating in the absence or presence of 1 nM acidic isoferritins.

* Presence or absence of GCT-conditioned medium during the preincubation phase.

‡ Percent change from control (C′ or medium).

§ Significant change $P < 0.001$.

ritins as described above suggested that insensitivity to acidic isoferritins might be related to a lack or paucity of m-anti-Ia (NE1-011)-recognizable-Ia antigens on patient CFU-GM. The results of such a study are shown in Fig. 5. Day 7 CFU-GM from 10 normal donors showed comparable decreases in colony and clus-

TABLE III

Effect of Monoclonal Anti-Ia, ^{3}HTdr and Acidic Isoferritins on Pre-CFU-GM and Day 7 CFU-GM

Progenitor cells assayed*	Complement (C′)	αIa + C′	C′ plus acidic isoferritin	Medium	^{3}HTdr
A					
Pre-CFU-GM	100±4	103±6 (+3)‡	110±5 (+10)	107±6	98±4 (−8)
B					
Day 7 CFU-GM	116±11	38±3 (−67)§	42±2 (−64)§	116±7	41±5 (−65)§
C					
Day 7 CFU-GM from pre-CFU-GM	110±4	50±4 (−55)§	46±2 (−58)§	107±5	45±3 (−58)§

* Bone marrow cells were treated with C′, αIa + C′, or ^{3}HTdr and either (A) placed in suspension culture for 7 d in the absence or presence of 1 nM acidic isoferritins prior to washing and plating in agar with control medium for the pre-CFU-GM assay, or (B) placed in agar directly with or without acidic isoferritins for the day 7 CFU-GM assay. Untreated bone marrow cells were (C) placed in suspension culture for 7 d, washed, and treated with C′, αIa + C′, or ^{3}HTdr and then plated in agar in the absence or presence of acidic isoferritins for the day 7 CFU-GM from pre-CFU-GM assay. In all prephase studies, cells were incubated with 10% GCT-conditioned medium.

‡ Percent change from control (C′ or medium).

§ Significant change, $P < 0.001$.

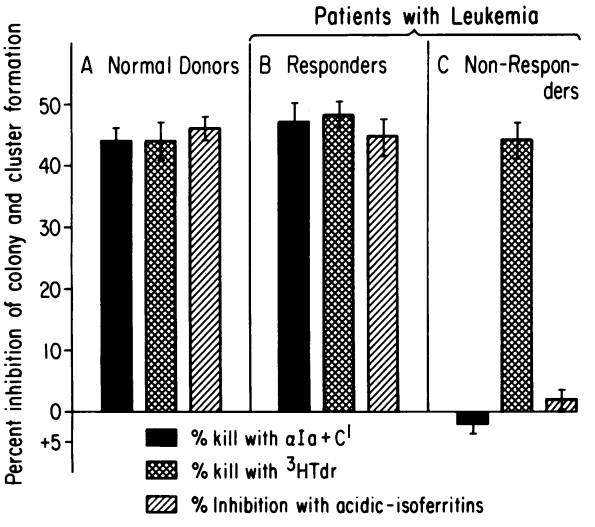

FIGURE 5 Comparative effect of m-anti-Ia (NE1-011) + C', ^{3}HTdr and 1 nM acidic isoferritins on colony and/or cluster formation of bone marrow cells from normal donors and patients with leukemia. Responders refer to those patients whose day 7 CFU-GM were inhibited by acidic isoferritins and nonresponders refer to those patients whose day 7 CFU-GM were insensitive to inhibition. The results are expressed as the mean percent inhibition±1 SE for (A) n = 10, (B) n = 17, (C) n = 13 donors or patients.

ter formation after exposure to m-anti-Ia, ^{3}HTdr or acidic isoferritins (Fig. 5A). 17 patients, including 10 with chronic myelogenous leukemia (CML) and 7 with acute leukemia in chemotherapy-or bone marrow transplantation-induced remission had day 7 CFU-GM, which responded to inhibition with 1 nM acidic isoferritins (Fig. 5B). This inhibition equalled the reduction observed after treatment of marrow cells with m-anti-Ia plus complement or ^{3}HTdr. While marrow cells from normal donors in this study contained 35–54% of day 7 CFU-GM in cycle, some of the patients in the responding groups (Fig. 5B) contained a lesser or greater percentage of day 7 CFU-GM in cycle. In all cases the percent decrease in colony and cluster numbers after treatment with m-anti-Ia plus complement, ^{3}HTdr, or acidic isoferritins were similar. The percent decreases were, respectively, for patient 1 (CML): 26, 30, 31%, patient 2 (CML): 30, 30, and 38%, patient 3 (acute myeloid leukemia [AML] in remission and on maintenance chemotherapy): 70, 65, and 68% and patient 4 (AML in remission, postmarrow transplantation): 76, 78, and 80%. 13 patients, including 7 with CML (1 of whom was untreated), 2 with untreated AML, 1 with AML in relapse and 3 with AML in remission, contained day 7 CFU-GM that were not responsive to inhibition with 1 nM acidic isoferritins (Fig. 5C). The CFU-GM from all the patients in this group were in cycle but none contained Ia antigens recognized by m-anti-Ia (NE1-0-1) plus complement. The results expressed in Fig. 5 are based on control colony and cluster numbers of at least 100 and the killing effect of ^{3}HTdr on CFU-GM from all patients was counteracted by coincubation with an excess of cold thymidine.

DISCUSSION

CFU-GM are considered to be a heterogeneous population of maturing cells. These are present in low concentrations in bone marrow (~1 to 4 per 10^3 low density nucleated cells) and in even lower concentrations in blood and spleen and are recognized by their focal proliferation in semisolid culture medium (1, 2). These cells appear to lack T cell antigens, Fc receptors, and complement receptors but several markers have been recognized on the human CFU-GM (reviewed in 1, 2). The best studied surface determinant is that recognized by antibodies to human Ia-like (HLA-DR) antigens (3–8). The apparent absence of Ia-antigenic determinants on probable human pluripotential stem cells, their presence on the progenitor cells that have gained commitment to the various hematopoietic cell lineages and their selective loss as these cells mature has suggested that human Ia-like antigens may play a role in the regulation of hematopoietic cellular proliferation (3, 6). However, there have thus far been no reports that have experimentally established a link between Ia-antigens on human CFU-GM and the regulation of this progenitor cell compartment. The present communication describes the recognition by certain monoclonal antibodies of Ia-like antigens associated with the S-phase cycle of CFU-GM, which may have significance in terms of regulation of cell proliferation. The recognition of such an antigenic determinant is not surprising as monoclonal antibodies have been reported that recognize subpopulations as well as epitopic regions of human Ia-like molecules (22, 27, 28). A low density appearance of such Ia-like antigens on portions of the CFU-GM cycle other than S-phase has not been ruled out. Moreover, it is possible that other monoclonal antibodies may recognize different Ia-like antigens that are displayed on portions of the cycle of CFU-GM other than or including S-phase and these may not be involved in the particular regulatory interactions described in this communication. Murine I-A and I-E/C region products have been shown to be independently regulated at various stages of the cell cycle of an established B lymphoma cell line (29). One of the monoclonal antibodies to Ia-antigens ($Q5/_{13}$) that was used as a control for the activity of complement reduced CFU-GM to <6% of control values suggesting that this antibody may be recognizing Ia-antigens expressed at high density through all phases of the cell cycle of CFU-GM.

The relevance of the present communication is that the Ia-like antigens associated with the S-phase of CFU-GM have been implicated in the regulation of these cells in vitro by purified acidic isoferritins, molecules that may play a regulatory role in vivo and that may be involved in the progression of acute leukemia (13). The acidic isoferritins did not act on Ia-antigen negative CFU-GM and the action of these molecules was blocked by pretreatment of the cells with various antibodies against Ia-antigens in the absence of complement. The blocking action suggests that the acidic isoferritin "receptors" may be in close proximity to these Ia-antigenic molecules, but the loss of sensitivity to acidic isoferritin-inhibitory activity with loss of Ia-antigens suggests the possibility, not yet proven, that Ia-antigens may be necessary for the suppressive effect of acidic isoferritins on these cells in vitro. The ~10-fold higher concentrations of monoclonal antibodies necessary to block acidic isoferritin action in the absence of complement as compared to the killing of CFU-GM in the presence of complement, may mean that only a portion of the total NE1-011 antibody-binding sites are needed for complement-mediated killing and mediation of the action of acidic isoferritins, but that all or most of the sites must be covered to insure that the acidic isoferritin effect is blocked.

The loss of detection of Ia-antigens from normal CFU-GM that occurs within 3 to 5 h in cultures at 37°C is simultaneous with the loss of responsiveness to inhibition with acidic isoferritins. Neither of the two reappeared within 24 h in culture under the conditions used in the present studies. Shedding of Ia-antigens from mouse macrophages is known to occur within a few hours when cells are placed at 37°C (23–25) but, until purified populations of human CFU-GM are obtained it will not be possible to examine the actual synthesis and shedding of Ia-antigens from these cells, and to confirm the indirect evidence obtained by the complement-dependent cytotoxicity tests. Recent evidence by L. M. Pelus[4] has implicated E type prostaglandins ($PGE_{1,2}$) in the "induction" of the responsiveness of a portion of human CFU-GM to killing by anti-Ia (mNE1-011, mBD, or mOKlal) plus complement and to inhibition by acidic isoferritins after 24, but not after 6 h, in culture at 37°C if PGE is present in the culture at time zero. It is clear from the present studies that Ia-antigen positive CFU-GM responsive to acidic isoferritins, can be generated in culture from an Ia-antigen negative population of pre-CFU-GM and this may relate to the presence of PGE and/or other endogenously released mediators that could induce and/or stabilize Ia-antigens on CFU-GM. A precedence for such mediator involvement in the expression of Ia-antigens exists for mouse macrophages (23–25).

With reference to the detection of inhibition by acidic isoferritins of the Ia-antigen-positive subpopulation of cycling CFU-GM derived in suspension culture from pre-CFU-GM of normal donors and of CFU-GM from some patients with leukemia, it has recently been found that acidic isoferritin-inhibitory activity is present in and released from normal bone marrow and blood cells only of the mononuclear phagocytic lineage (monocytes and macrophages)[5] as well as from non-T, non-B, lymphoid like cells or promonocytes from patients with leukemia (30). Since monocytes are present at the initiation of the culture, monocytes and macrophages are generated during the culture period, and acidic isoferritins act to suppress cycling CFU-GM it might seem that the release of acidic isoferritins in the culture should have suppressed the responsive CFU-GM, cycling CFU-GM should not have been detected and exogenously added acidic isoferritins should not have influenced colony formation. This was not the case. Monocytes from normal donors contain and release much less acidic isoferritin inhibitory activity than cells from patients with leukemia but the pattern of release is similar (10, unpublished observations). Medium conditioned by monocytes from normal donors or by cells from patients with leukemia first contains detectable acidic isoferritin-inhibitory activity by 12–24 h of culture, but after 4–5 d no inhibitory activity is detectable. This release is influenced by the presence or absence of subpopulations of lymphocytes, which can suppress or enhance the release of the inhibitory activity, monocytes that also can enhance the release, as well as the release of proteases that can inactivate the inhibitory activity already released (10, 26, unpublished observations). Peak generation of CFU-GM from pre-CFU-GM in suspension culture occurs after 7 d and it is understandable that CFU-GM generated in suspension culture would still be in cycle and sensitive to the action of exogenously added acidic isoferritins. Since a pulse treatment for <30 min in vitro with exogenously added acidic isoferritins results in maximum detectable inhibition of normal CFU-GM (10) and the patients's CFU-GM were in cycle, the endogenous production of acidic isoferritin-inhibitory activity in vitro should have had no influence on the capacity to detect an inhibition of colony formation if CFU-GM were responsive. This was the case for CFU-GM from some of the patients. Moreover, the inability to inhibit colony formation of CFU-

[4] Pelus, L. M. Personal communication.

[5] Broxmeyer, H. E., J. Bognacki, P. Ralph, M. H. Dorner, L. Lu, and H. Castro-Malaspina. Monocyte-macrophage derived acidic isoferritins. Normal feedback regulators of granulocyte-macrophage progenitor cells. Submitted for publication.

GM from other patients, an event associated with lack of detectable Ia-antigens on these CFU-GM, suggests that these CFU-GM may contain an intrinsic or recently induced defect in responsiveness. Acidic isoferritins suppressed colony and/or cluster formation of neutrophils, eosinphils, macrophages, and neutrophils plus macrophages in a similar manner (10, 30) and lack of responsiveness of CFU-GM from patients with acute leukemia in remission and chronic leukemia to inhibition by acidic isoferritins was not due to the types of colonies and clusters formed (11). It is not known if the nonresponsive CFU-GM from patients with acute leukemia in remission are part of the normal or leukemia cell population, but the nonresponsive CFU-GM from nonremission patients with acute myeloid leukemia and from patients with chronic myeloid leukemia probably represent the leukemia cell population (2).

The progression of leukemia may be due to, or exacerbated by, imbalances in normal regulatory interactions. The insensitivity of patient CFU-GM to inhibition by acidic isoferritins occurs simultaneously with or prior to the increase in acidic isoferritin inhibitory activity (11). This in vitro sequence of events has been substantiated in animal models of virally induced disease (14, 15) and has been suggested as a potential means for the proliferative advantage of leukemia cells that can proliferate while normal progenitor cells are suppressed (11, 14). The absence of Ia-antigens on patient CFU-GM may be an early event associated with the progression of leukemia. Current studies suggest that microgram quantities of acidic isoferritin-inhibitory activity can act in vivo to dampen rebound myelopoiesis in mice recovering from sublethal dosages of cyclophosphamide.[6] It would thus be of theoretical as well as clinical interest if Ia-antigen negative patient CFU-GM could be induced to express Ia-antigens in vitro and concomittantly gain responsiveness to the regulatory action of acidic isoferritins and these studies are in progress.

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[6] Gentile, P., and H. E. Broxmeyer. Unpublished observations.

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