INHIBITION OF HUMAN ERYTHROPOIETIC COLONY FORMATION IN CULTURE BY TREATMENT WITH Ia ANTISERA*

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The membrane glycoproteins that characterize human B lymphocytes and serve to distinguish these cells from T cells have been the subject of considerable recent interest. The dominant complex of 28,000 and 37,000 daltons is a product of genes mapping in the major histocompatibility complex and was initially recognized through alloantibodies contained in pregnancy sera (1). More recently they have been studied through the use of heteroantisera raised against these molecules isolated from B-cell line membranes (2, 3). Because of the biologic and chemical homologies to the murine I region antigen system (Ia) the 28,000–37,000 dalton complex in man has been termed "Ia-like" or for simplicity "Ia".

From the earliest studies relating the presence of the Ia alloantigens to circulating B cells, it was evident that certain nonlymphoid cells of hematopoietic origin expressed these determinants (1). The recognition of the Ia determinants on monocytes (1, 2) was followed by their demonstration on malignant (2, 4, 5) and normal myeloblasts (6, 7), as well as on the stem cells that were the committed progenitors of granulocyte and macrophage (CFU-C) colonies (6, 8). In the granulocyte lineage, the Ia antigens became undetectable after the promyelocyte stage (6, 8).

In a previous study it was demonstrated that Ia antigens were expressed on the surface membrane of some cells with the appearance of pronormoblasts or basophilic normoblasts (6), but were not present on members of the erythroid series that were more mature than basophilic normoblasts.

The present study sought to document the presence of Ia determinants on the erythropoietin-sensitive progenitor cells committed to differentiation into these erythroid cells. Assays were performed on two cells in this lineage distinguishable by the kinetics and properties of the colonies they gave rise to: the colony and burst-forming cells (CFU-E and BFU-E, respectively) (9).

Materials and Methods

Bone Marrow Preparation. Bone marrow was obtained from normal volunteers who had given informed consent. Washed bone marrow buffy coat cells were mixed with bovine serum albumin (1.070 g/cm³ density, 270 mosM) over which bovine serum albumin at a density of 1.050 g/cm³ was

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layered. The gradient was centrifuged at 3,600 g for 10 min and the supernatant cells present in the lower density fraction were harvested and washed as described previously (10). A nonadherent population of the low density cell fraction was prepared by sequential adherence on plastic tissue culture Petri dishes as described (10, 11). 10–20% of the cells in the low density fraction were removed by this procedure. >98% of the erythroid and myeloid progenitors present in the whole marrow sample were recovered in the low density nonadherent preparation.

Lymphoid Lines. Lymphoid lines were maintained in culture in medium RPMI-1640 supplemented with 10% fetal calf serum (Reheis Chemical Co., St. Louis, Mo.). They were harvested and washed four times with Dulbecco's saline. The B-cell pool was formed from Epstein-Barr virus transformed lymphocytes obtained from six normal subjects. The T-cell pool consisted of CEM-T, MOLT-4, and 1301.

Ia Specific Antiserum. Ia specific antiserum was prepared by immunizing rabbits with a 65,000 dalton bimolecular glycoprotein complex isolated from the membranes of B35M, a B-cell lymphoblastoid line. The 65,000 dalton component contained the Ia alloantigenic activity and dissociated to 28,000 and 37,000 dalton polypeptides upon denaturation at 100°C. The resulting antiserum had been previously documented to react with the Ia bimolecular complex (2, 6, 12). 1 ml of the antiserum was diluted to 1:20 and absorbed with 1 ml of washed, packed pooled T-cell lymphoid lines. In the case of the B-cell-absorbed control sample, 2 ml of washed packed B cells were added to the packed T cells and a single absorption was performed. The cells and diluted serum were incubated at 4°C with frequent resuspension. The cells were removed by centrifugation (10 min, 8,000 g) and the diluted serum passed through a 0.22 μm Millipore filter (Millipore Corp., Bedford, Mass.).

Complement-Dependent Cytotoxicity. Dilutions of antiserum or control serum were prepared in McCoy's serum supplemented with 10% fetal calf serum. Cells (2 × 10⁶) in 0.4 ml were incubated with 0.4 ml of diluted antiserum at 4°C for 30 min. They were washed once by layering on a 2-ml cushion of fetal calf serum and incubated at 37°C for 45 min with 0.4 ml of rabbit complement diluted 1:10 that was previously selected for the absence of heterospecific antibodies. The cells were washed twice through fetal calf serum cushions and divided for subsequent assays.

Colony Assays. Myeloid colony assays were carried out in 0.3% agar in supplemented McCoy's medium over a feeder layer of human peripheral leukocytes in 0.5% agar as previously reported (10). Colonies and clusters were scored with the inverted microscope at 7 days. Erythroid colony assays were carried out according to the method of Iscove et al. (11, 12). 1 × 10⁵ light density cells or 2 × 10⁵ nonadherent light density cells were incorporated in each plate. Cells were plated in alpha medium containing a final concentration of 0.8% methylcellulose, 30% fetal calf serum, 1% bovine serum albumin, and 1 U/ml of human urinary erythropoietin. The erythropoietin, which was kindly supplied by Norman Iscove, had been prepared from the urine of patients with aplastic anemia by chromatographic separation, including absorption by concanavalin A-sepharose to remove colony stimulating activity. The final activity of the erythropoietin was 110 U/mg protein. Erythroid colony-forming units (CFU-E) and burst-forming units (BFU-E) were scored by using the inverted microscope at 7 and 14 days, respectively. The data were based on counting of 10 replicates for each datum point and are expressed as mean ± standard error. Individual colonies were plucked and placed on glass slides. Staining with benzidine and counter staining with Wright-Giemsa confirmed the erythroid nature of the colonies.

Results

Table I contains the results of a representative experiment from a single donor demonstrating the complement-dependent inhibition of erythropoietic and granulocyte-macrophage colony formation by the Ia antiserum. The cloning efficiency of erythropoietin-dependent colonies, arising from CFU-E, and bursts, arising from BFU-E, were 0.22 and 0.56%, respectively, in this light density nonadherent fraction of normal bone marrow. Incubation with complement alone caused slight inhibition of erythroid colony formation. Substantial inhibition of CFU-E and BFU-E assays resulted after incubation with the Ia antiserum which was prepared with T-cell lymphoid line absorption, followed by complement. Significant inhibition was evident at a dilution of the Ia

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TABLE I

Complement Dependent Inhibition by Antiserum to Ia Determinants of Erythropoietic and Granulocytic Colony Formation by Bone Marrow Cells

Treatment of bone marrow cells	Colonies per 2 × 10⁵/plate		
	CFU-E (7 days)	BFU-E (13 days)	CFU-C (7 days)
Medium	444 ± 14	113 ± 2	387 ± 9
Complement	400 ± 15	102 ± 10	388 ± 17
Anti-Ia serum 1:40 + complement	41 ± 5	5 ± 2	13 ± 2
Anti-Ia serum 1:160 + complement	49 ± 5	12 ± 2	41 ± 4
Anti-Ia serum 1:640 + complement	69 ± 8	20 ± 3	141 ± 5
Anti-Ia serum 1:2500 + complement	71 ± 7	33 ± 4	238 ± 4
Anti-Ia serum 1:10,000 + complement	105 ± 6	47 ± 3	308 ± 5
Anti-Ia serum 1:40,000 + complement	213 ± 8	63 ± 1	390 ± 8
Anti-Ia serum 1:160,000 + complement	384 ± 12	92 ± 4	380 ± 10
Anti-Ia serum 1:40 absorbed with B cells + complement	367 ± 15	98 ± 12	375 ± 22

antiserum of 1:40,000 for the CFU-E and BFU-E assays. The inhibition of the Ia antiserum on colony formation was specifically abolished by absorption with pooled B-cell lymphoid lines, giving a cloning efficiency not significantly different from that obtained with complement alone. As a control for these experiments granulocyte-macrophage (CFU-C) colonies were also assayed on the same samples of bone marrow after treatment with Ia antiserum and complement. Specific inhibition of CFU-C colonies scored after 7 days of incubation was found up to a dilution of 1:10,000. Similar results were obtained in three other identical experiments by using different normal bone marrow donors with comparable titers of inhibition obtained in each experiment for each of the progenitor cell assays.

Experiments done using the light density fraction of normal bone marrow before removal of adherent cells gave a cloning efficiency for CFU-E and BFU-E of 0.16 and 0.029, respectively. These colonies were inhibited at titers of anti-Ia antiserum equivalent to those obtained with the nonadherent light density fraction.

Discussion

The present studies demonstrated that erythropoietic bursts and colonies, arising from the more primitive BFU-E, as well as the relatively more mature CFU-E, were both essentially eliminated by antisera specific for Ia determinants. The marked sensitivity to the anti-Ia antiserum of both of these distinct erythroid progenitors was comparable and furthermore was similar to that of the cell that is the progenitor of granulocyte-macrophage colonies (CFU-C). The specificity of these reactions was demonstrated by abolishing the inhibition through absorption with B but not T-cell lymphoid lines. In view of these results and the large numbers of previous studies demonstrating the specificity of this antiserum (2, 6, 13), it appears likely that the inhibition of the erythroid precursor colonies is mediated through elimination of Ia bearing cells.

One point regarding the interpretation of these findings is whether the Ia antiserum is reacting directly with Ia determinants expressed on the erythroid progenitors or whether colony formation depends on an essential accessory cell bearing Ia determinants that would have been eliminated by the antiserum treatment. Support for the interpretation that Ia molecules are present on the

erythropoietic progenitors comes from the evidence that cells with the appearance of the subsequent stages in the erythroid lineage—proerythroblasts and basophilic nomoblasts—express Ia determinants on their membranes (6). Furthermore there has thus far been no evidence for the participation of an accessory cell likely to bear Ia antigens in erythropoietic colony formation. Recent evidence by Nathan et al. (14) has implicated a soluble component produced by a cell in the T-cell fraction as an enhancing factor in generation of BFU-E from progenitors circulating in peripheral blood. However, since the vast majority of cells isolated by sheep erythrocyte rosette formation do not react with anti-Ia sera it is very unlikely that the inhibition was mediated through T cells. Indeed, the antiserum is employed only after T-cell lymphoid line absorption. Therefore the pattern in the erythroid lineage of Ia antigen expression on progenitor cells and the first cytologically distinct stages of maturation, followed by the disappearance of these antigens on the more mature stages appears to be precisely analogous to the finding in the granulocyte lineage (2, 4, 8).

Particularly with respect to the granulocytic lineage, it appears that there are some differences in Ia antigen expression between the findings in man and in the murine studies. Basch et al. (15) concluded that Ia determinants were not expressed on the pleuripotential stem cell, CFU-S. Furthermore, Kincade et al. reported that CFU-C are not eliminated by an anti-Ia (murine) serum (16). It is unlikely that the relatively minor differences in reagent preparation account for this divergence. The anti-Ia (human) heteroantiserum used in these studies was prepared by immunization with a 65,000 dalton complex that contains the alloantigenic activity obtained from B-cell lymphoid lines. The murine Ia reagents have usually been prepared by intraspecies immunization and recognize a very similar bimolecular complex (17) with many points of genetic and biologic homology with the human system.

Furthermore, the studies on the presence of Ia antigens on progenitors and early members of the normal human granulocyte lineage using various anti-Ia heterosera were strengthened considerably by the use of cells from patients with granulocytic leukemias. These leukemias permitted more extensive documentation of the chemical and allo-antigenic characteristics of the Ia system present on the myeloblast stage than would be possible on the more limited material available from normal bone marrow. Thus the demonstration that the anti-Ia heteroantisera identified the bimolecular complex of 28,000 and 37,000 daltons on radiolabeled myeloblasts (6), as well as the selective reactivity of these cells with a panel of selected Ia specific alloantisera (2, 6) provide strong evidence for the Ia nature of these determinants on the myeloblasts and support the interpretation that the inhibition of the granulocyte-macrophage progenitor cells is due to the Ia specificity of the hetero anti-Ia reagents.

Thus it appears that in man the Ia system is more broadly expressed on various progenitor cells and very early members of the major marrow lineages than has been reported in the mouse. In this context the findings of the present, as well as previous studies, raise the question of the function served by the occurrence of the Ia molecule on cells derived from the bone marrow that do not serve a primary immune function. One possibility is the conjecture that Ia determinants have a function relating to the control of proliferation within the marrow that is distinct from their role in the immune response.

Summary

Incubation with Ia antiserum, followed by complement, markedly inhibited erythroid colonies arising from hematopoietic cells present in the nonadherent low density fractions of normal bone marrow. Both erythropoietin-dependent colonies and bursts were eliminated at dilutions of antiserum equivalent to, or greater than the dilutions required to abolish the granulocyte-macrophage colony formation. The inhibitory effect of the Ia antiserum was abolished by absorption with B but not T cells from lymphoid lines. Available evidence suggested that Ia determinants are expressed on the erythropoietin-sensitive progenitors of the erythroid series in precise analogy to their sequence of expression on the granulocyte lineage. In both lineages, as shown previously, the Ia determinants become undetectable during subsequent stages of differentiation.

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