Immunologic Characterization of a Granulocytic Leukemia of Inbred Strain 13 Guinea Pigs: Presence of Ia-Positive Myeloblasts

By Loran T. Clement, Cheng-po Hu, Ethan M. Shevach, and Ira Green

Membrane markers expressed by a transplantable granulocytic leukemia induced in inbred strain 13 guinea pigs by N-nitroso-N-butylurea have been investigated by serologic and immunohemochemical methods. Although the leukemic myeloblasts have no detectable surface immunoglobulin or Fc receptors, they have been found to synthesize and express membrane antigens encoded for by the I-region of the major histocompatibility complex. Despite the presence of Ia antigens, these cells do not stimulate allogeneic T lymphocytes in the mixed lymphocyte reaction nor are they able to present soluble antigen to immune T cells. The presence of Ia antigens on immature myeloid cells suggests that these proteins may be important in cellular functions beyond those identified in the cellular immune system.

Recent studies have described the induction of a granulocytic leukemia in a single female inbred strain 13 guinea pig given N-nitroso-N-butylurea. Because this leukemia is transplantable and is derived from a genetically inbred strain, it offers a potentially valuable tool for the study of granulocytic leukemia. In this article, we wish to report studies demonstrating that neoplastic cells of this transplantable granulocytic leukemia express and synthesize Ia antigens. We shall also report studies investigating the functional significance of these antigens on myelogenous cells.

MATERIALS AND METHODS

Animals

Inbred strain 2 and strain 13 guinea pigs were obtained from the Division of Research Services, NIH, Bethesda, Md.

Granulocytic Leukemia Transplantation

Granulocytic leukemia cells, kindly provided by Dr. M. Mage, NCI, NIH, Bethesda, Md., were maintained by serial passage in strain 13 guinea pigs. Transplantation was achieved by inoculating 10 x 10⁶ leukemia cells intraperitoneally into strain 13 guinea pigs. Peripheral WBC counts were performed weekly, and WBC differential smears evaluated periodically. Early in the leukemic process, the elevation in the WBC count was mainly due to neutrophils. However, 4-5 wk after transplantation, a significant increase in the peripheral myeloblast population occurred and typically exceeded 60% of the peripheral WBC when the animals became moribund.

When the total WBC count reached approximately 70,000/cu mm, the leukemic animals were bled from the heart, and the WBC in the plasma were harvested. After washing, cells were counted and the percentage of leukemic blasts determined by microscopic examination of Giemsa-stained smears. Cells thus obtained typically consisted of 60%-95% leukemic myeloblasts. These cells were then used either for transplantation into new animals for in vivo propagation or were used without further separation for the studies described here.

Antisera

Anti-Ia sera were prepared as previously described by the cross-immunization of inbred strain 2 and strain 13 guinea pigs, whose MHC differ only in the I-region. Anti-B1 sera, which represent the guinea pig equivalent of alloantisera to the human HLA-A and B serologic determinants, were prepared as previously described.
goat and a rabbit antiserum to guinea pig Ig was produced as described by Schwartz et al.13

In Vitro Cellular Methods

Cytotoxicity testing of various antisera against the leukemic cells was determined by a complement-mediated 51Cr release cytotoxicity assay.13 Cells were labeled with Na251CrO4, then incubated for 30 min at 37°C with various dilutions of the antiserum as well as guinea pig complement at a final dilution of 1:6. Percent lysis was then determined by comparing the 51Cr released into the supernatant after 30 min in the test samples with that found using complement alone (spontaneous release) and that found following three freeze-thaw cycles (100% release) according to the formula:

\[
\text{Percent } 51\text{Cr release} = \frac{51\text{Cr cpm (test sample)} - 51\text{Cr cpm (spontaneous)}}{51\text{Cr cpm (100% release)} - 51\text{Cr cpm (spontaneous)}} \times 100
\]

Indirect Immunofluorescence and Double Staining for Morphological Correlation

A fluorescein-labeled rabbit anti-guinea pig Ig was used to stain living leukemic cells directly in testing for the presence of sfg by standard techniques. To determine which cell types (neutrophils, bands, mononuclear cells, or myeloblasts) had surface Ia antigens, an indirect technique was employed. First, 5 x 105 cells were treated with anti-Ia sera at a 1:5 or 1:10 dilution, washed, then treated with fluorescein-labeled rabbit anti-guinea pig Ig. After washing, the cell pellet was suspended in 50% rabbit serum and smeared on gridded slides (Brysen Coating Laboratories, Safety Harbor, Fla.), each sector of the grid having a number. The slides were fixed for 10 min, stored in 95% ethanol, mounted in buffered glycerol, and the position of stained (Ia-positive) and unstained (Ia-negative) cells was determined by fluorescent microscopy and recorded on paper. Coverslips were then gently removed, and the slides were stained with Giemsa stain. The same cells were then located by their position on the grids and were evaluated by light microscopy; the morphology of cells was correlated with the presence or absence of fluorescence.

Mixed Lymphocyte Reaction (MLR) and Antigen Presentation Assays

These assays have been described in detail elsewhere.13,14 Briefly, the MLR was assessed by determining the triitated-thymidine (3H-TdR) uptake of allogeneic or syngeneic lymph node lymphocytes in response to mitomycin-treated leukemia cells, while antigen presentation assays utilized a T-cell proliferation assay in which the 3H-TdR uptake by peritoneal exudate lymphocytes in the presence of antigen-pulsed, mitomycin-treated leukemic cells was determined. In both assays, peritoneal exudate cells known to act as stimulator cells in the MLR and as antigen-presenting cells for T-cell proliferative responses were tested in parallel.

Biosynthetic Radiolabeling and Immunoprecipitation Experiments

The methods used for internally radiolabeling cells and for the immunoprecipitation and analysis of solubilized antigens have been previously described in detail.12 Briefly, leukemic cells (or peripheral WBC from nonleukemic animals) were internally labeled with 3H-leucine (New England Nuclear, Boston, Mass.) in short-term culture. Cells were then solubilized in 0.5% Non-Idet P-40 (Particle Data Laboratories, Elmhurst, Ill.) and nuclei and cellular debris removed by ultracentrifugation. A glycoprotein-enriched fraction known to contain the majority of the Ia antigens was then isolated by affinity chromatography using a lentil lectin-Sepharose 4B column. Aliquots of these fractions were treated with the various antisera being tested, and immune complexes were precipitated using heat-formalin fixed protein A-bearing Staphylococcus aureus, Cowan I strain. Precipitated antigens were then solubilized and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli.13 Ten percent polyacrylamide gels were utilized in these studies, and gels were run under nonreducing conditions, except those analyzing anti-Ig precipitates, which were run using reducing conditions.

RESULTS

Membrane Antigens Expressed by Granulocytic Leukemia Cells

Initial studies of the strain 13 granulocytic leukemia were directed at determining the membrane antigens borne by these cells. Cells obtained from leukemic animals (having >50% abnormal myeloblasts) or peripheral WBC prepared in identical fashion from nonleukemic control animals were first tested in a complement-mediated cytotoxicity assay using antisera against either strain-13-specific Ia antigens, strain-2-specific Ia antigens, or the GPLA B.1 antigen—the strain 13 guinea pig analog of the murine H-2K/D and human HLA-A and B antigens. Both the leukemic cells and normal peripheral WBC were equally susceptible to lysis by the anti-B.1 serum, and neither was lysed by the control antiserum recognizing strain-2-specific alloantigens (data not shown). In contrast, there was a significant difference in the susceptibility of these two cell populations to complement-mediated cytotoxicity effected by the antiserum against strain 13 Ia antigens. In Fig. 1, it can be seen

![Fig. 1. Complement-mediated cytotoxicity curves of antistrain-13 Ia antiserum tested against strain 13 granulocytic leukemia cells (■—■) or normal peripheral WBC from nonleukemic strain 13 animals (□—□). Greater than 50% of the cells from the leukemic animals are susceptible to lysis by anti-Ia serum and complement, whereas only 5% of the peripheral WBC from healthy control animals are lysed. The background release was less than 5% for both of the cell populations.](www.bloodjournal.org)
that at low dilutions, the anti-Ia serum lysed over 50% of the peripheral WBC obtained from the leukemic animal while lysing only 5% of the cells from the control nonleukemic animal. Since the differential count from the leukemic animal showed that very few of the cells (<5%) were normal lymphocytes or monocytes, these few Ia-positive cells could not account for the high percentage of cytotoxicity observed. Therefore, these findings strongly suggested that, in addition to bearing the B.1 GPLA alloantigen, the granulocytic leukemia blast cells also possessed significant amounts of membrane-bound strain 13 Ia antigens.

In order to provide more direct proof that the leukemic cells were indeed the Ia-positive cell, leukemic cells were examined by a double-staining technique. Cells were first treated with anti-Ia serum and fluorescent-labeled rabbit anti-guinea pig Ig to identify cells bearing Ia antigens, using a grid method to assess the position of fluorescent-stained cells. Slides were then stained with Giemsa stain to allow a subsequent morphological assessment of the Ia-positive cells. The results from one representative experiment are summarized in Table 1. It is clear that the cells with the highest percentage of staining have the morphology of leukemic blast cells. These blasts are not directly stained by the rabbit anti-guinea pig Ig reagent, nor are they stained when a guinea pig antiserum against strain-2-specific Ia antigens is used for the first antibody layer of the indirect immunofluorescent technique. Consequently, when viewed in conjunction with the cytotoxicity data previously discussed, it is clear that the granulocytic leukemia blasts do indeed have membrane-associated Ia antigens.

Although peripheral myeloblasts were clearly found to be Ia-positive, it is of interest that a small percentage of band forms and neutrophils were also weakly stained by the method employed. In studies of human myelocytic maturation, bands and mature neutrophils have been found to be Ia-negative cells.16,17 Since whole antisera (rather than F(ab)2 fragments of IgG) were used in these studies, it is possible that the occasional weakly positive fluorescence of bands and neutrophils is attributable to nonspecific binding of guinea pig IgG to the Fc receptor of these cells. It is very unlikely that this mechanism accounted for the staining of the blast cells, however, as these cells did not express Fc receptors when tested by EA-rosetting assays (data not shown) nor did they demonstrate even weak fluorescence when guinea pig antisera to the irrelevant strain-2-specific Ia antigens were tested.

**Demonstration of Biosynthetically Radiolabeled Ia Antigens by Granulocytic Leukemia Cells**

The previous data have indicated that the strain 13 granulocytic leukemia blasts have membrane-associated Ia antigens. However, to establish that these cells were, in fact, responsible for the synthesis of these molecules and to characterize biochemically the Ia antigens produced by these cells, biosynthetic radiolabeling experiments were performed. Equivalent numbers of peripheral white cells from either leukemic strain 13 animals (>80% blast cells on differential) or normal strain 13 animals were internally labeled with 3H-leucine, solubilized in Non-Idet P-40, and a glycoprotein-enriched fraction from each cell lysate was isolated by lentil lectin affinity chromatography. Aliquots of each were then treated with either normal guinea pig serum (NGPS), anti-Ia serum, anti-B.1 serum, or a goat anti-guinea pig Ig serum, and the immune complexes were precipitated and analyzed by SDS-PAGE. The results from these studies are shown in Fig. 2. In the normal strain 13 peripheral WBC population (shown in the lower panel B), it can be seen that, while no proteins are precipitated by the NGPS from the cell lysate, the anti-B.1 serum precipitates a single protein of 40,000 daltons, analogous structurally to the HLA-A and B antigens in man, and electrophoretically identical to that previously reported for guinea pig lymphocytes.12 (The β2-microglobulin molecule, which is associated with the 40,000 dalton molecule on the cell surface, migrates in the low molecular weight running front to the right of the 10% polyacrylamide gel.) In contrast, the anti-Ia antisem precipitates virtually no Ia antigens from the normal peripheral WBC, and only trace amounts of slg molecules are seen from the anti-Ig precipitate. Since B lymphocytes represent only 5%-7% of the peripheral WBC in the guinea pig (and only 5% of the normal peripheral WBC were lysed by anti-Ia serum and complement in Fig. 1), the absence of detectable radiolabeled Ia antigens from the peripheral WBC population is not surprising. These same reagents have also been used to successfully precipitate large amounts of immunoglobulin molecules from lysates of both guinea pig L2C cells (a B-cell leukemia) as well
Fig. 2. SDS-PAGE analysis of radiolabeled antigens synthesized by either normal or leukemic peripheral WBC. (A) The lysate from leukemia cells contains the B.1 GPLA alloantigen as well as substantial amounts of strain 13 Ia antigens (see text for discussion), but does not contain detectable amounts of slg molecules. (B) In contrast, the lysate from normal strain 13 cells contains equivalent amounts of the B.1 antigen yet has only trace amounts of Ia antigens or slg demonstrable. These findings indicate that strain 13 myeloblasts, and not peripheral B lymphocytes or monocytes, are responsible for the synthesis of the Ia antigens found in the leukemic cell population. The marker arrows denote the migration distances for the mu chain (left arrow) and the light chain (right arrow) of radiolabeled guinea pig surface IgM molecule.

as normal guinea pig lymph node cells (unpublished observations).

In Fig. 2A, the results for the granulocytic leukemia cells (prepared in identical fashion to the normal peripheral WBC) are shown. Once again, the anti-B.1 serum precipitates a 40,000-dalton molecule from the cell lysate, which is quantitatively comparable to that found in the normal WBC population. However, when the anti-Ia serum is tested with the leukemic cell lysate, 3 major proteins having molecular weights of 58,000, 33,000, and 25,000 daltons (left to right) are seen. This pattern is quite characteristic of that previously reported for strain 13 guinea pig Ia antigens.\textsuperscript{12,18} The 58,000-dalton protein, which bears the guinea pig Ia.7 determinant, is comprised of a 33,000-dalton chain and a 25,000-dalton chain covalently associated by sulfhydryl linkage. The 33,000-dalton and 25,000-dalton proteins seen on the gel are associated by noncovalent forces (which are disrupted by solubilization in SDS prior to electrophoresis) and bear the Ia.3 and Ia.5 determinants in strain 13 animals. The presence of significant quantities of Ia antigens in the granulocytic leukemia lysate is in marked contrast to the virtual absence of these proteins in the normal peripheral WBC population. Furthermore, since the granulocytic leukemia cell lysate has no detectable slg molecules (Fig. 2A), it is very unlikely that B lymphocytes present among the leukemic myeloblasts are responsible for the Ia antigens found in this lysate; indeed, the percentage of contaminating B lymphocytes in the leukemia cell preparation was less than 2% as detected by direct immunofluorescence determination of Ig-bearing cells (results not shown). Consequently, these data indicate that the Ia antigens found in the leukemia cell population are actively synthesized by the myeloblasts.

Functional Significance of Ia Antigens on Granulocytic Leukemia Cells

Although Ia antigens on mature lymphoid cells and macrophages have been shown to be important in a number of immune phenomena, their presence on early myeloid cells is of unknown significance. Consequently, we studied these Ia-positive myeloblasts by two in vitro immunologic assays in which Ia-positive cells are known to perform vital functions. The first of these, stimulation of an allogeneic MLR, involves T-cell recognition of allogeneic Ia antigens; and, in the guinea pig, the stimulator cell is an Ia-antigen-bearing macrophage.\textsuperscript{19} The second assay, the proliferation of immune T lymphocytes to soluble antigen, also requires Ia-antigen-positive macrophages for presentation of the immunogenic material.\textsuperscript{20}

The results of these experiments are summarized in Tables 2 and 3. It is evident that in comparison with

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<th>Table 2. Failure of Ia-Positive Granulocytic Leukemia Cells to Stimulate a Mixed Lymphocyte Reaction*</th>
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<td>Responder Cells</td>
<td>Stimulator Cell</td>
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<tr>
<td>2 lymph node lymphocytes</td>
<td>Strain 2 macrophages</td>
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<td>Strain 13 macrophages</td>
<td>Strain 13 macrophages</td>
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*Column-purified strain 2 lymph node cells (2 x 10^\textsuperscript{2}) were cultured with strain 2 macrophages, strain 13 macrophages, or the strain 13 granulocytic leukemia cells (10^\textsuperscript{5}) in the presence of 5% normal guinea pig serum for 6 days. Eighteen hours before harvesting, 1.0 \( \mu \)Ci of tritiated thymidine was added to each well.

†cpm/well; each value is the mean of three determinations.
strains 13 macrophages, the Ia-positive strain 13 leukemia cells do not stimulate an MLR in allogeneic, strain 2 lymph node responder lymphocytes. This inability of the leukemic cells to stimulate an MLR is not due to macrophage depletion from the responder T-lymphocyte population, as the addition of strain 2 macrophages (syngeneic to the responder T cells) to the culture does not result in positive MLR stimulation by the allogeneic leukemic cells (data not shown). Similarly, as shown in Table 3, the antigen-pulsed granulocytic leukemia cells could stimulate only a small proliferative response when added to the immune T lymphocytes. It is conceivable that the low degree of proliferation seen with the leukemia cell preparation is secondary to contaminating normal monocytes. Consequently, although strain 13 leukemia myeloblasts synthesize and bear Ia antigens, these cells cannot perform these two functions ascribed to Ia-positive macrophages and monocytes.

**DISCUSSION**

Characterization of the membrane constituents of tumor cells has become an important tool in investigating the origin and differentiation pathway of hematopoietic neoplasms. In the studies reported herein, surface markers present on a carcinogen-induced transplantable strain 13 guinea pig granulocytic leukemia have been investigated. These leukemic cells have been found to express the B1 GPLA alloantigen found on normal strain 13 cells. The blasts do not express slg nor do they express Fc receptors or C3 receptors (data not shown). However, the leukemic cells do synthesize and express surface Ia antigens but are unable to present soluble antigen to immune T cells and do not stimulate an allogeneic MLR. Taken together, these findings indicate that these Ia-positive leukemic cells, which have been classified by morphological criteria as being myelogenous, are also immunochemically and functionally distinct from both B lymphocytes and monocytes.

Although Ia antigens have been clearly demonstrated on B-cell-transformed lymphoblastoid cell lines and on leukemic cells bearing other B-lymphocyte markers, it is only recently that these MHC gene products have been identified on neoplastic cells not having an obvious B-lymphocyte origin. For instance, acute lymphoblastic leukemia cells having neither T-cell markers nor additional B-cell markers have now been shown, in the vast majority of cases, to express human Ia-like antigens. In addition, membrane Ia-like antigens have also been found in a significant number of cases of human acute myelogenous leukemia as well as on peripheral WBC present in chronic myelogenous leukemia (CML), particularly when the cells tested were immature cells seen in CML blast crisis or transformed myeloid cell lines derived from such immature blasts. Our finding that guinea pig leukemia myeloblasts also bear Ia antigens is in accord with these observations and indicates that the detection of Ia-positive cells in the peripheral blood may provide an additional tool for gauging the maturity, if not lineage, of peripheral neoplastic cells.

The expression of Ia antigens during cellular ontogeny has been studied most extensively for human and murine B-lymphocyte differentiation, and it appears that most, if not all, B cells express Ia antigens early in differentiation and continue to express these molecules until their terminal differentiation into plasma cells. The presence of Ia antigens on cells differentiating along the myeloid pathway has raised several intriguing questions. Studies of the effects of anti-Ia sera and complement on murine bone marrow cells have concluded that both the pluripotential stem cell and the committed granulocyte–monocyte progenitor cell do not express Ia antigens. In contrast, similar studies of human bone marrow cells have reported that the granulocyte–monocyte committed progenitors as well as erythropoietin-sensitive progenitor cells in erythroid differentiation are susceptible to inhibition by anti-Ia and complement lysis. Furthermore, human cells differentiating along the myeloid pathway have been shown to express Ia antigens that are rapidly lost with increasing cellular maturation and are no longer detectable on metamyelocytes or more mature granulocytic elements.

Ia antigens have clearly been shown to be intimately involved in a number of important immune regulatory and recognition functions. However, it should be noted that Ia antigens are a necessary but not sufficient
The presence of Ia antigens on these malignant cells as well as on spermatozoa and cultured malignant melanoma cell lines, suggest, if only by inference, that these MHC-linked gene products may be important in the regulation of multiple cellular interactions and differentiation pathways in the bone marrow and, perhaps, elsewhere. Documentation of this possibility will require specific inquiry addressing this question.

One final point merits discussion. The presence of Ia antigens on the membrane of leukemic cells does not establish that it is, in fact, the leukemic cells that synthesize Ia antigens. Several laboratories have reported that Ia antigens are normally present in the serum of other species, and serum Ia-like antigens have also been reported in leukemic patients in blast crisis. Consequently, it is conceivable that the expression of Ia antigens on the leukemic cells might result from the passive absorption of such serum Ia antigens to the blasts by some unknown mechanism, which would result in artifactual immunofluorescent staining. Similarly, biochemical characterization of the antigens on these cells by radiolabeling techniques would also fail to rule out passive absorption. In the studies reported herein, radiolabeling experiments were performed in order to document the active synthesis of Ia antigens by the guinea pig granulocytic leukemic cells, and within the constraints of the methods employed, it appears that the Ia antigens synthesized and expressed by the leukemia cells are biochemically and serologically identical to those produced by normal guinea pig B cells, macrophages, and Langerhans cells.

The transplantable strain 13 guinea pig granulocytic leukemia characterized in this report offers a new, potentially valuable tool for the study of myelogenous leukemia and, with further study, may provide important information on the significance of Ia histocompatibility antigens for the diagnosis, classification, and treatment of hematopoietic neoplasia.

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Immunologic characterization of a granulocytic leukemia of inbred strain 13 guinea pigs: presence of la-positive myeloblasts

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