Proliferation of Human Malignant Hematopoietic Cells in Immunodeficient Mice: Suppression by Antibody to Pluripotent K-562 Leukemia Cells Involves Direct Cytolysis and Effector Cells

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Six human hematopoietic cell lines were successfully heterotransplanted into athymic (nude) and asplenic-athymic (lasat) neonatal mice. The tumors arising from leukemia and lymphoma cells could then be serially transplanted into adult nude mice. Seven days after the fourth serial mouse passage, each mouse was treated with goat immune gamma globulin against K-562 cells. One control group was treated similarly, but with nonimmune (normal) gamma globulin, while another control group was not treated. The goat gamma globulin was not toxic for nude and lasat mice, and the immune, but not the normal, gamma globulin suppressed local subcutaneous growth of myelosarcomas, lymphosarcomas, and Burkitt lymphoma cells. On the other hand, the growth of lung, breast, and prostatic carcinomas and a melanoma of human origin were not altered by the immune gamma globulin. Since suppression of cell growth occurred equally well in decomplemented mice, a complement-mediated cytotoxicity apparently cannot be considered as responsible for the abrogation. The Fab fragment of the immunoglobulin did not suppress the growth of the myelosarcomas. We conclude that antibody suppression of the in vivo proliferation was specific for malignant hematopoietic cells and that the Fe portion of IgG is necessary for in vivo cytosis of leukemia cells. The most probable mechanisms are direct antibody cytosis and antibody-dependent macrophage-mediated cytotoxicity.

MATERIALS AND METHODS

Mice

Human malignant hematopoietic cells were transplanted into 706 BALB/c lasat and nude mice. We have recently reviewed the characteristics of our colonies of these mutant strains of mice.

Heterotransplantation

Cells were maintained in long-term culture by standard techniques. An aliquot of 50 μl containing 10³ viable cells was routinely transplanted s.c. in the dorsal region of newborn lasat and nude mice. Subcutaneous tumors arising from leukemia and lymphoma cells were excised and serially transplanted into adult mice. Samples of a bronchogenic carcinoma, an adenocarcinoma of the prostate, a pigmented melanoma, and a carcinoma of the breast were obtained soon after surgical excision and transplanted into adult nude or lasat mice. Cylindrical pieces, 4 mm in diameter by 6 mm in length, were cut from the non-necrotic part of each tumor and
transplanted s.c. into the anterior part of the body of the mice. Tumor growth was limited to the site of implantation. All serially transplanted tumors, maintained in adult mice only, were used on the fourth serial mouse-to-mouse passage, which we considered to be the passage when predictable growth was definitively established. (Reproducible rates of growth occur in nude and lasat mice after the third serial passage.) The K-562 myelosarcomas, the prostatic adenocarcinoma, and the melanoma were used in the immunotherapeutic protocol after 24, 32, and 42 mo of serial transplantation, which corresponded to 36, 37, and 42 mouse-to-mouse passages, respectively.

To study the immune mechanisms, K-562 cells were cultured as previously described.13 Viable cells (5 x 10⁵), enmeshed in a fibrin clot, were implanted s.c. in the dorsal-anterior region of the body of mice, while other mice received a portion (4 x 6 mm) from serially transplanted K-562 myelosarcoma.

The growth of s.c. tumors was monitored by measuring the major and minor diameters to calculate tumor volume.23,25 The cumulative incidence of localized growths was confirmed by macroscopic and microscopic examinations.19

Histopathologic Examinations
A careful autopsy of each neonatally injected mouse was performed at the time of death. A gross examination of all organs was made under 4 x magnification. Light microscopic examinations were made19 to confirm the effectiveness of the treatment. Groups of at least 3 adult mice bearing human tumors were killed at intervals of 5 days after transplantation during exponential growth of the tumors as well as at the end of each experiment. When tumors were not macroscopically visible after a treatment, a round or square piece was removed from the site of implantation, keeping intact the relationship between the skin, s.c. tissue, and underlying connective and muscular layers. The entire block was fixed in 10% formaldehyde, pH 7.0, and processed for light microscopy for the determination of microproliferations.

Xenogeneic Gamma (γ) Globulin
The production of goat antiserum to K-562 blasts, absorptions with normal human peripheral blood leukocytes and normal human bone marrow, and the separation of gamma globulin have been described.15,35 Currently, all preparations of IγG are standardized in terms of the concentration of immune IgG and are then lyophilized. The cytoxic titer of the antiserum and IγG does not change when absorbed with human normal marrow cells and/or murine lymphohematopoietic tissues and maintains the therapeutic efficacy on heterotransplanted neoplastic blood cells.5,7 Preparations have remained stable for nearly 3 yr. For these experiments, IγG was absorbed with pooled cells from nude mouse lymphoid and hematopoietic tissues.7 The Fab fragment was prepared from normal γ-globulin (γG) and IγG by papain digestion followed by separation on carboxymethylcellulose.15,30 Varying doses of γG or IγG were mixed with cells and fibrinogen before clot formation and implantation into mice. In other experiments, 6 mg/mouse (between 250 and 300 mg/kg) were given intraperitoneally (i.p.) every 3 days for 19 days. The treatment was started 7 days after implantation of K-562 cells, because at that time, cells proliferate rapidly within a developing vascular network. No further treatment was given.

Treatment of Mice With Cobra Venom Factor (CoF)
Mice were decomplemented41 with neurotoxin-free CoF (Cordis Laboratories, Miami, FL), in which 0.5 U/g of body weight was injected i.p. every 6 hr for 24 hr before transplantation of K-562 cells. Finally, a 0.5-U was given intravenously (i.v.) at 24 hr after the last i.p. injection and every 24 hr thereafter, as indicated in Figs. 6 and 7. One unit of CoF is the amount of protein that will inhibit 50% of the hemolytic activity of 0.5 ml of a 1:20 dilution of normal human serum using 0.4 ml of 2% antibody-sensitized sheep erythrocytes as target cells.21 Decomplementation was determined by the failure of serum to cause hemolysis in the hemolytic plaque assay27 and by lack of cytosis of K-562 target cells using treated mouse serum as the source of complement in ADCMC assays.44 Serum from nondecomplemented mice (positive controls) were included in each assay.

Antigenicity of Cell Proliferating In Vivo
Cells obtained from s.c. tumors were grown in suspension cultures for 4 days. The presence of leukemia antigen related to that on K-562 cells was determined by the ADCMC assay.44 In addition, sections of myelosarcomas, lymphomas, and other neoplastic tissues used in these experiments were fixed in 10% formaldehyde in methanol containing 0.3% hydrogen peroxide to inhibit endogenous mouse cell peroxidase activity. The fixed sections were washed with 0.9 M Tris buffer (pH 7.6), incubated with goat IγG for 1 hr, and then with rabbit anti-goat IgG fraction conjugated with peroxidase. The procedure was essentially the same as reported previously.18

Cytogenetic Studies
Chromosome analyses were made prior to transplantation and in cells recovered from the s.c. tumors by standard techniques.25

RESULTS
Cells obtained from myelosarcomas, lymphosarcomas, and the Burkitt lymphoma reacted with anti-K-562 γ-globulin in the ADCMC assay. Also, tissue sections processed by the immunoperoxidase technique gave a strong brown-orange reaction when stained with benzidine, indicating that leukemia and lymphoma cells growing within the tumors had retained the antigen. The other carcinomas (lung, prostate, breast) and a melanoma did not react with the IγG to K-562 cells either in an ADCMC reaction or as detected in tissue sections processed for the immunoperoxidase method.

Cells that gave rise to tumors in nude and lasat mice maintained the human karyotype.

The effect of IγG on tumors serially transplanted into adult nude mice is illustrated in Figs. 1–3. The IγG abrogated the growth of myelosarcomas (K-562, KG-1, and HL6O), lymphosarcomas (JM and MOLT-4), and Burkitt’s lymphoma (RAJI). None of the mice had recurrent proliferation of malignant cells 9 wk after treatment. In contrast, the proliferation of lung, prostatic, and breast carcinomas, as well as a melanoma (Figs. 2 A and B) could not be arrested by antibody to K-562 cells.

To prove beyond doubt the specificity of the IγG to K-562 cells for leukemia and lymphoma cells, adult nude mice were implanted (Fig. 3) with a K-562 myelosarcoma in one flank, while either a prostatic carcinoma or a melanoma was implanted in the contralateral side. As in all treatments reported here, the
Fig. 1. (A–D) Suppression of the proliferation of myelogenous (KG-1; HL-60), T-lymphoblastic (JM; MOLT-4) leukemia, and B lymphoma (RAJI) cells by the IgG to K-562 cells. Cells (10^7) were injected s.c. in newborn mice and the s.c. myelosarcomas (K-562; KG-1; HL-60), lymphosarcomas (JM; MOLT-4), and a lymphoma (RAJI), arisen from a single cell inoculum, were then serially transplanted into adult nudes.
administration of IgG was started 7 days after transplantation, when each tumor was well vascularized. The IgG selectively abrogated the proliferation of the K-562 myelosarcoma cells, whereas the prostatic and melanoma cells grew to form characteristic tumors. Thus, the suppression of cell proliferation by the IgG to K-562 leukemia cells was restricted to malignant hematopoietic cells.

The histopathology of serially transplanted tumors was very similar from passage to passage, with minor variations, after several mouse-to-mouse passages in untreated mice or those injected with NγG (Fig. 4A). In contrast, tumors obtained from mice that had been given 1 or 2 injections of IgG showed early changes characterized by hydropic degeneration of a large portion of the tumors, as well as karyolysis and/or karyorrhexis randomly distributed throughout the tumors. After 4 or more injections, myelosarcomas, lymphosarcomas, and the lymphoma were totally necrotic, and the cell debris appeared as being rapidly absorbed. For example, the structural pattern of tumors treated with IgG was replaced by extensive areas (Fig. 4B) of coagulative necrosis in which the cells appeared as acidophilic homogeneous masses without nuclei. Cytoplasmic outlines, still preserved in some cells, appeared as "ghost" bodies. Neither residual proliferations nor recurrent growths could be demonstrated 9 wk after the myelosarcomas, lymphosarco-
ANTIBODY SUPPRESSES K-562 GROWTH IN MICE

Fig. 4. (A and B) Structural pattern of K-562 myelosarcomas growing in nude mice. The morphology was preserved after treatment with NγG (A). Panoramic view of a massive eosinophilic necrosis observed after 4 injections of IγG (B). Hydropic changes and nuclear picnosis of K-562 cells are also shown (arrow). The necrosis of other myelosarcomas, T-cell lymphosarcomas, and Burkitt's lymphoma are essentially similar. Silver impregnation (A) x400 and hematoxylin-eosin (B) x250.

mas, and lymphomas had been eradicated by specific immunotherapy. As expected, the IγG produced no changes in the structural pattern of carcinomas or melanoma used to demonstrate in vivo antibody specificity.

The NγG and IγG preparations were not toxic for immunodeficient mice. In fact, the IγG improved the general health of the mice, which gained more body weight than did the control mice. Also, several nu/nu females gave birth to homozygotes during the observation period following immunotherapy.

To study the mechanism(s) of action of the IγG, different concentrations of NγG or IγG were added to K-562 cells enmeshed in fibrin clots, and the growth of developing myelosarcomas was evaluated. The results are presented in Fig. 5. Increasing amounts of NγG produced a transient stimulation of tumor growth, whereas IγG diminished (at 0.5–1 mg/5 x 10⁶ cell/clot) or consistently prevented (at 1.5 mg/clot) the proliferation of K-562 cells.

Since the amount of IγG necessary to prevent the development of K-562 myelosarcomas in 100% of the nude mice with normal levels of complements was known, we repeated the experiment in decomplemented nude mice. As shown in Fig. 6, the IγG incorporated in the clot inhibited the proliferation of K-562 cells even in decomplemented mice. Normal γ-globulin and CoF have no effect on the proliferation of K-562 cells when incorporated in the fibrin clots or injected parenterally (Figs. 6 and 7). Identical results were obtained when portions of serially transplanted myelosarcomas (eighth passage) were transplanted into normal and decomplemented mice (Fig. 7). The IgG abrogated the growth of the tumors in both groups.
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Fig. 5. Growth of myelosarcomas arising from $5 \times 10^6$ K-562 cells enmeshed in a fibrin clot implanted s.c. in adult lasat mice. NγG or IγG globulin was mixed into the developing clot before implantation. Each point is the mean ±1 SEM of 10-14 mice. Again, CoF and NγG had no effect on tumor growth. No complement activity was demonstrated in the serum of mice treated with CoF as evidenced by the lack of hemolysis of sheep erythrocytes sensitized with rabbit anti-mouse erythrocyte serum. Similarly, K-562 target cells were not lysed by the IγG when the pooled serum from nude mice injected with CoF was used as a source of complement in ADCMC assays.

The results described above demonstrate that the mechanism of abrogation of heterotransplanted K-562 cells by the antibody was not complement-mediated. Further, abrogation occurred whether the cells were proliferating in a matrix of fibrin or within the fibrovascular stroma of the mouse, as was the case in serially transplanted tumors.

In other experiments, 0.8 mg of Fab (equivalent to 1.5 mg of IgG) fraction either from NγG or IγG was mixed with K-562 cells in the clot prior to implantation. At comparable doses or greater (3–6 mg), Fab from IγG had no effect on tumor growth (Fig. 8). We conclude that the Fc portion of the IgG is necessary for the immune clearance of K-562 cells.

DISCUSSION

We have shown that a heterologous antibody to K-562 cells suppresses the growth of various neoplastic human hematopoietic cells transplanted in nude and lasat mice. The transplanted malignant hematopoietic cells maintained the leukemia-specific antigen (LSA) that is expressed in the K-562 leukemia stem cells and early committed bone marrow progenitor cells, such as the cell lines used in these experiments. It is known that IγG does cross-react with T lymphoblasts (JM and MOLT-4), promyelocytes (HL60), Burkitt's lymphoma cells (RAJI), and myeloblasts (KG-1), but not with normal bone marrow cells from healthy individuals, since the antiserum was extensively absorbed on normal bone marrow cells before IγG was isolated.
The fact that leukemia cells grew as s.c. tumors is not surprising, since leukemia is a hyperplastic process that usually spreads to the bloodstream, infiltrates all organs of the body, and gives rise to tumor masses in humans. In an adult xenogeneic host, leukemia cells grow as solid tumors, whereas tumors and infiltrations are produced in neonates of the same species, perhaps owing to the sparsity of natural killer cells. The preservations of the human karyotype, structural pattern, and antigenic markers confirm the human nature of the neoplastic cells. It is precisely the human nature of the hematopoietic cells transplanted and the specificity of the antibody used that make the results of these experiments important as a model for immunotherapy.

The models described here are useful for the analysis of specific immunotherapeutic procedures, such as pertains to the dissection of the mechanism whereby antibody is cytotoxic in vivo and the establishment of requirements for effective therapy. Thus, a phase of the study was undertaken to determine if the abrogation of growth of K-562 myelosarcomas by IgG was complement-mediated or if an analogous in vitro cytolysis of K-562 cells by antibody alone could be occurring. To this end, we used two in vivo approaches to determine the requirements for antibody-dependent cytolysis. First, by incorporating the IgG in the fibrin clot, or giving the IgG parenterally, we could elucidate the need for complement by determining whether in vivo lysis of K-562 cells occurs in decomplemented mice as compared to that in mice with normal levels of complement. The results indicated that abrogation of cell proliferation is effective in decomplemented mice, that is, CoF-treated mice, and therefore ADCMC is apparently not the in vivo mechanism. In the second approach, we compared intact IgG with Fab fragment and found that only intact globulin, not Fab, restricts tumor growth. We conclude that for antibody cytolysis to occur in vivo, the whole IgG molecule is needed, which is in contrast to our in vitro findings.

The cell killing that results when IgG is incorporated in fibrin clots is dose dependent, and the effective doses are 50 times less than those required (30 mg) for parenteral therapy. However, the dose of IgG (1.5
mg/clot) required for total inhibition of cell proliferation is such as to suggest that direct antibody sensitization and cytolysis of target cells appears to be operative in vivo rather than the more sensitive ADCC, at least before a vascular network is established. The migration of killer cells and/or macrophages into the fibrin clot may be physically impeded. This restricted migration and the catabolic decay of the antibody may necessitate an initially high level of antibody if ADCC is the only mechanism. On the other hand, histologic examinations indicated that target K-562 cells throughout the clot are being lysed continually in the apparent absence of macrophages. This suggests direct antibody cytolysis.

We propose that the most probable mechanisms of cytolysis in vivo involving antibody are direct antibody cytolysis and secondary cytolysis mediated by effector cells (ADCC). The lysis of K-562 cells by nude mouse spleen cells occurred in vitro even at very high (1:1000) antibody dilutions.

It is known that the higher the number of macrophages within a tumor, the more probable is tumor regression. To kill target cells, macrophages or killer cells require a recognition mechanism and activation. In ADCC, antibody allows recognition of tumor-associated antigen with appropriate stimulus for macrophage activation. After stimulation, the killing potential of the macrophages appears related to their increased capability for generating superoxide anions (O_2^-) and hydrogen peroxide.

Since cell-mediated rather than humoral immunity, presumably complement-dependent, appears to be important in tumor resistance, ADCC should be the primary mechanism when the abrogation of a tumor is dependent on antibody injected parenterally. The rapid proliferation of K-562 cells in vivo during the first week after implantation suggests that soluble antigen may be released from the cells during that time. A part of the injected antibody, therefore, could form soluble antigen–antibody complexes that might inhibit the ADCC component of host defense.

Exogenous antibody can sensitize the tumor cells directly by reacting with cell surface antigens. It can also attach to effector cells first, either via the Fc receptor, which is a weak interaction and requires more antibody (up to 100-fold) than sensitization of tumor cells, or by binding directly to the cellular membrane of effector cells. After attachment to effector cells, the antibody then would selectively recognize and direct the lysis of target cells and not other adjacent cells.

Recent demonstrations have shown that monoclonal antibodies of IgG2a isotype specifically abrogate the growth of heterotransplanted human tumors in nude mice through ADCC specifically mediated by macrophages. As in our studies with a polyclonal antibody, complement (C3) depletion had no effect in the suppression by antibody of the proliferation of human cancer cells in nude mice. Further, other potential effector cells, such as T and killer cells or granulocytes, had no significant function as mediators of the tumoricidal activity of the monoclonal antibodies used. The data presented here confirm and support the previous findings.

It can be argued that the models and/or immunotherapeutic trials reported here may not be directly relevant to the treatment of patients with leukemia with a complex immune reaction to their own leukemia cells. However, IgG has produced similar beneficial effects in eight patients with terminal leukemia, including acute lymphoblastic (ALL), myeloblastic (AML), and myelomonocytic (AMML) leukemias, as well as CML. Thus, the results presented do suggest that studies for effective immunotherapy of human leukemia can be developed by use of heterotransplanted human leukemia cells in immunodeficient mice.

REFERENCES

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Proliferation of human malignant hematopoietic cells in immunodeficient mice: suppression by antibody to pluripotent K-562 leukemia cells involves direct cytolysis and effector cells

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