Changes of the Immunogenic Properties of a Radiation-induced Mouse Lymphoma following Treatment with Antitumor Drugs

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SUMMARY

Eight sublines of the radiation-induced lymphoma S-1033 of C57BL/10 (hereafter called B10) origin were established by exposing the cells in vivo to eight antineoplastic agents for a number of transplant generations. The parental and drug-treated sublines were tested for immunogenic properties, i.e., the ability to elicit allograft reactions in the host of origin and in congenic-resistant mice differing for the S-D or K-I-S regions of the H-2 complex. Lymphoma S-1033 and all drug-treated sublines except one were found to be essentially nonimmunogenic for B10 mice. The S-DIC subline, when exposed for 8 to 12 transplant generations to dimethyltriazenoimidazolecarboxamide, became immunogenic for syngeneic B10 mice, as judged from prolongation of survival time.

Large i.v. inocula (10⁷ cells) of S-1033 and of the drug-treated sublines, with the possible exception of the cyclophosphamide-treated and dimethyltriazenoimidazolecarboxamide-treated lymphomas, were more effectively rejected by K-I-S- than by S-D-incompatible mice. Dilution escape (i.e., tumor rejection after challenge with large inocula, and lethal tumor growth after injection of small inocula of lymphoma cells in allogeneic recipients) occurred in K-I-S-incompatible mice that were inoculated with S-1033 and three drug-treated (5-fluorouracil, cyclophosphamide, and pyrazolocarboxamide) sublines. No dilution escape occurred with dimethyltriazenoimidazolecarboxamide or bischloroethylnitrosourea sublines.

These data favor the hypothesis that various types of immunogenic changes of neoplastic cells may occur in tumor-bearing hosts following treatment with antineoplastic agents in vivo.

INTRODUCTION

Changes in the immunogenic properties of tumor cells following treatment with antitumor drugs in vivo have been repeatedly observed in our laboratory (2, 3, 9, 25) and elsewhere (17, 21, 22, 26). One of the most active compounds was DIC. Highly immunogenic tumor sublines were obtained following DIC treatment of L1210 or L5178Y leukemia cells in vivo (2, 3, 23) so that large inocula were rejected by nonimmunodepressed mice for which the original (parental) lines were histocompatible. Cytotoxic lymphocytes were found in recipient mice after tumor rejection (23, 24), and the kinetics of peritoneal growth and rejection of DIC sublines were similar to those shown by H-2-incompatible lymphomas (14). Almost all studies with DIC or other antineoplastic agents were performed with leukemias homozygous for the H-2ᵇ haplotype. In order to determine whether the drug-dependent changes in immunogenicity would occur also under different conditions, we extended our investigation to tumor cells originated in an H-2ᵇ mouse. The effect of drug treatment in vivo on a radiation-induced lymphoma of C57BL/10 ScSn origin was investigated. The immunogenic properties of the parental and drug-treated sublines were compared in the host of origin and in congenic-resistant recipients differing for the K-I-S or S-D regions of the H-2 complex (18). The results of the present investigation indicate that cells of lymphoma S-1033 were essentially compatible with mice of the strain of origin and remained so after in vivo treatment with a number of drugs except for DIC, which rendered the cells immunogenic after 8 transplant generations. Moreover, the immunogenicity of some of the drug-treated tumor lines changed also for allogeneic hosts.

MATERIALS AND METHODS

Mice. Animals of the congenic-resistant lines C57BL/10ScSn [abbreviated B10 (H-2ᵇ)], B10.A(5R) (H-2ᵇ), and B10.A(2R) (H-2ᵇ) were obtained from the Mammalian Genetics and Animal Production Section of the National Cancer Institute, Bethesda, Md. Mice of these lines share most of the B10 genome, but the B10.A(5R) strain differs from B10 at the S-D regions of H-2 and

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the B10.A(2R) strain at the K-I-S regions (18, 29). Mice of both sexes, 4 to 6 months old were used.

**Tumors.** Transplantable radiation-induced lymphoma S-1033 of B10 origin was obtained through the courtesy of G. D. Snell in 1968, 2 years after the tumor was induced by X-rays and serially transplanted in B10 mice (27), and was then kept frozen.

Treatment schedules and dosages used to obtain drug-treated sublines are reported in Table 1. The original S-1033 (parental) line and all drug-treated sublines grew as generalized lymphomas after i.v. or i.p. injection of dispersed cells.

**Tumor Transplantation and Evaluation of Growth.** Grossly enlarged spleens were removed aseptically from leukemic mice, and the cells were dispersed by flushing the organs with 5 ml of chilled Medium 199 from a syringe and by gently pressing with blunt forceps. The cell suspension, kept in an ice bath, was then filtered through a stainless steel screen and counts of unstained nucleated cells were made in a hemocytometer in the presence of trypan blue. The desired number of viable cells were injected i.v. in 0.5 ml and i.p. in 0.2 ml. Only suspensions with more than 70% viable lymphoma cells were acceptable. Mortality of mice was recorded for 60 days after tumor inoculation and deaths that were attributable to generalized lymphoma were confirmed by gross autopsy.

**Drugs.** All drugs were obtained from Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Md. 5-FU, BCNU, and CY were dissolved in 0.9% NaCl solution, MTX and DCM were dissolved in 2% sodium bicarbonate, and 6-TG was dissolved in distilled water that was alkalinized with a few drops of 1 N NaOH (the pH was brought to 9.5 to 10 with 0.1 N HCl). DIC and PCA were suspended in a chilled solution of 2% carboxymethylcellulose. The solutions of MTX and DCM were stored in dark glass bottles at 4° for periods not exceeding 4 days. All other drugs were prepared immediately before use.

**RESULTS**

Establishment of Drug-treated Sublines of Lymphoma S-1033. Lymphoma cells were transplanted i.p. into compatible B10 male mice (transplant generation 0). The recipient animals were divided into groups receiving no further treatment or to be inoculated with one of the drugs (5-FU, 6-TG, MTX, DCM, CY, BCNU, PCA, or DIC) using the doses and schedules shown in Table 1. The administration of all drugs except PCA and 6-TG prolonged survival times as compared with those of untreated mice, but in most instances the animals died within 60 days with generalized lymphoma (Charts 1 and 2). Tumor growth was monitored by spleen palpation and cells were collected from mice of each treatment group, for retransplantation (generation 1) into syngeneic B10 mice. The recipients were divided again into drug-treated and untreated groups. Mice of the drug-treated groups were always used for serial transplantation up to 12 to 17 passages. The efficacy of 5-FU, MTX, CY, and BCNU in prolonging survival times decreased in the subsequent transplant generations 6 and 12, indicating that these drug-treated sublines acquired resistance to the antitumor effect of the compounds. PCA and 6-TG did not prolong survival times at every transplant generation tested.

The survival patterns of mice given injections of cells of the S-DIC lymphoma subline differed markedly from those described above (compare Chart 1 with Chart 2). Starting with the 8th transplant generation, B10 mice given injections of S-DIC cells but not treated with the drug lived longer than B10 mice treated with DIC. At the 12th generation the prolongation of survival time of untreated recipients of S-DIC cells was even greater. However, a single i.p. injection of CY (200 mg/kg) given before tumor transplantation reduced the median survival time of these animals from 28.5 to 13 days, down to the survival time of the DIC-treated group (Chart 2). This observation suggests that S-DIC cells (of H-2b origin) became capable of eliciting transplantation reactions in B10 mice, the susceptible strain of origin of the parental tumor line.

**Search for Transplantation Resistance of B10 Mice to Drug-treated Lymphoma Sublines.** During the establishment of drug-treated lymphoma sublines, it became apparent that

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Drug treatment of lymphomas and subline designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor sublines</td>
<td>Drug</td>
</tr>
<tr>
<td>S-1033</td>
<td>None</td>
</tr>
<tr>
<td>S-FU</td>
<td>5-FU</td>
</tr>
<tr>
<td>S-TG</td>
<td>6-TG</td>
</tr>
<tr>
<td>S-MTX</td>
<td>MTX</td>
</tr>
<tr>
<td>S-DCM</td>
<td>DCM</td>
</tr>
<tr>
<td>S-CY</td>
<td>CY</td>
</tr>
<tr>
<td>S-BCNU</td>
<td>BCNU</td>
</tr>
<tr>
<td>S-DIC</td>
<td>DIC</td>
</tr>
<tr>
<td>S-PCA</td>
<td>PCA</td>
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</table>

* 10⁶ cells injected i.p. into B10 male mice.
only the S-DIC lymphoma was strongly immunogenic for B10 mice. A sensitive test exploiting synergism between chemotherapy and transplantation resistance (5) was applied to establish whether the other tumor sublines were at least weakly immunogenic. Cells of each drug-treated subline were injected into B10 female mice that were assigned to 1 of the following groups (Table 2): untreated (Group 1); immunodepressed with CY before transplantation (Group 2); treated with BCNU after transplantation (Group 3); pretreated with CY and treated with BCNU after transplantation (Group 4). Longer survival time of Group 1 relative to Group 2, or of Group 3 relative to Group 4, would be indicative of tumor-host incompatibility since mice of Groups 2 and 4 were immunodepressed. If tumor cells were weakly immunogenic, the therapeutic effect of BCNU should synergize with the antitumor-immune response of mice of Group 3 (5, 21, 25) and the median survival time should be longer than that of immunodepressed mice of Group 4, which are presumably incapable of mounting an effective anti-graft immune reaction. The results indicated that there was no detectable anti-graft reaction in B10 recipients of the drug-treated lymphoma sublines listed in Table 2.

Transplantation Resistance of Allogeneic Mice to Drug-treated Lymphoma Sublines. The immunogenic properties of drug-treated lymphoma sublines were compared with the parental line by transplanting the cells into mice of the congenic-resistant strains B10.A(5R) and B10.A(2R) differing from B10 at the S-D and K-I-S regions of the H-2 complex, respectively. Inocula of $10^3$, $10^4$, or $10^5$ cells were injected i.v. into recipient mice in order to determine: (a) differential allograft reactivity against transplantation antigens specified by the S-D and K-I-S regions (6, 7); and (b) escape of small numbers of lymphoma cells from immune surveillance (dilution escape), i.e., the increased incidence of tumor takes in K-I-S-incompatible recipients obtained by reducing the number of transplanted cells (6, 8).

When $10^7$ tumor cells were transplanted, the allograft reaction elicited by cells of lymphoma S-1033 and S-FU, S-MTX, S-DCM, and S-PCA sublines appeared to be stronger in K-I-S- than in S-D-incompatible mice, as judged by mortality data (Chart 3). The differential allo-

### Table 2

<table>
<thead>
<tr>
<th>Lymphoma line</th>
<th>No. of cells (i.p.)</th>
<th>MST*</th>
<th>Dead/total</th>
<th>MST</th>
<th>Dead/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-1033</td>
<td>$10^3$</td>
<td>10.5</td>
<td>8/8</td>
<td>10.5</td>
<td>8/8</td>
</tr>
<tr>
<td>S-FU (14)</td>
<td>$10^4$</td>
<td>11.5</td>
<td>8/8</td>
<td>11.5</td>
<td>8/8</td>
</tr>
<tr>
<td>S-MTX (12)</td>
<td>$10^4$</td>
<td>10.5</td>
<td>8/8</td>
<td>10</td>
<td>8/8</td>
</tr>
<tr>
<td>S-DCM (11)</td>
<td>$10^4$</td>
<td>10</td>
<td>8/8</td>
<td>10.5</td>
<td>8/8</td>
</tr>
<tr>
<td>S-CY (15)</td>
<td>$10^4$</td>
<td>15</td>
<td>6/6</td>
<td>15</td>
<td>6/6</td>
</tr>
<tr>
<td>S-BCNU (9)</td>
<td>$10^4$</td>
<td>15</td>
<td>6/6</td>
<td>17</td>
<td>6/6</td>
</tr>
<tr>
<td>S-PCA (6)</td>
<td>$10^4$</td>
<td>12.5</td>
<td>6/6</td>
<td>12</td>
<td>6/6</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, transplant generations of drug treatment. Data for S-TG subline are omitted because a number of mice died without evidence of tumor growth.
* After tumor transplantation.
* $10$ mg/kg i.p. on Day 5 after tumor transplantation. Mice grafted with cells of the S-BCNU subline (BCNU-resistant) were given instead 5-FU (70 mg/kg i.p.) on Day 3 after challenge.
* $130$ to $150$ mg/kg i.p., 1 day before tumor transplantation.
* MST, median survival time.

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**Chart 2.** Median survival time of B10 male mice (5 to 8 animals/point) given injections i.p. of $10^3$ cells of S-DIC lymphoma subline, at transplant generations 0, 4, 8, and 12. ●, recipient mice not treated with the drug after transplantation; O, DIC-treated recipients; ▲, mice immunodepressed with CY (200 mg/kg i.p. 6 hr before challenge), but not treated with DIC.
graft reactivity was minimal, however, with the 5-CY and completely rejected by both the Bl0.A(5R) and Bl0.A(2R) lymphoma target cells.

Specified by different subregions of H-2 is questionable with ble recipients and for 3 of 6 K-I-S-incompatible Bl0.A(2R) transplanted i.p. into the congenic mice and the results exploration of this point, 2 additional experiments were done.

(Chart 3). The incidence of tumor takes and mortality was higher in the K-I-S-incompatible animals that were transplanted i.v. with graded numbers of cells of lymphoma S-1033 or drug-treated sublines. ●, Bl0.A(2R) mice (K-I-S-incompatible hosts); ○, Bl0.A(5R) mice (S-D-incompatible hosts); △, Bl0.A(2R) mice pretreated with CY (108 mg/kg i.p. 1 day before challenge with S-DIC); Δ, Bl0.A(5R) mice pretreated with CY (108 mg/kg i.p. 1 day before challenge with S-DIC). Numbers in parentheses, transplant generations of each tumor line. The numbers associated with circles or triangle are the median survival times (in days). S-1033 and drug-treated sublines were not rejected by Bl0 mice. Survival times (in days) of Bl0 recipients of i.v. transplants of 10⁶ or 10⁷ cells ranged as follows: S-1033, 6 to 12.5; S-FU, 8 to 17; S-PCA, 6 to 14; S-CY, 8.5 to 15; S-BCNU 10 to 17.5; and S-DIC, 10 to 20. Data for S-MTX, S-DCM, and S-TG were incomplete and not reported here (10⁷ cells i.v. of S-MTX and S-DCM were rejected by K-I-S-incompatible recipients and accepted by S-D-incompatible mice, like cells of the parental line S-1033).

graft reactivity was minimal, however, with the S-CY and not detectable with the S-DIC subline. For further exploration of this point, 2 additional experiments were done. First, a large number of S-CY cells (18 x 10⁶/mouse) were transplanted i.p. into the congenic mice and the results confirmed that differential allograft reactivity against cells of this subline was minimal; 9 of 10 Bl0.A(2R) mice died with a median survival time of 19.5 days compared with 100% mortality (10 of 10) in Bl0.A(5R) mice with median survival time of 16 days. Second, mice to be grafted with S-DIC lymphoma were immunodepressed with CY as indicated in the legend to Chart 3. The 10⁷ number of S-DIC lymphoma cells were lethal for 7 of 8 S-D-incompatible recipients and for 3 of 6 K-I-S-incompatible Bl0.A(2R) mice. It follows that differential reactivity to alloantigens specified by different subregions of H-2 is questionable with S-DIC lymphoma target cells.

The phenomenon of dilution escape occurred in the series of mice given injections i.v. with 10⁶ or 10⁷ lymphoma cells (Chart 3). The incidence of tumor takes and mortality was higher in the K-I-S-incompatible animals that were transplanted with the smaller number of cells than in those given 10⁶ cells of the S-1033, S-FU, S-PCA, and S-CY sublines. No evidence of dilution escape was obtained with the S-BCNU and S-DIC sublines, cells of which were almost completely rejected by both the Bl0.A(5R) and Bl0.A(2R) mice.

DISCUSSION

A number of drug-resistant (i.e., serially transplanted and drug-treated) sublines derived from L1210 leukemia have shown various degrees of immunogenicity for BALB/c x DBA/2 F₁ (hereafter called CD2F₁) mice which otherwise were histocompatible with the parental tumor (2, 3, 17, 21, 22, 25, 26). Attempts were now made to influence the immunogenicity of a radiation-induced lymphoma of Bl0 origin by treatment with antineoplastic agents of different classes and mechanisms of action. The tumor-host system used in these experiments was chosen because it enabled us (a) to extend the analysis of drug influences on tumor immunogenicity to a lymphoma that differs from L1210 leukemia for being of H-2 acces instead of H-2 acces genotype and radiation rather than chemically induced (19, 27); (b) to detect acquired antigenicity or increased immunogenicity of lymphoma cells for mice of the strain of origin since the parental S-1033 line does not elicit detectable primary or secondary responses in Bl0 recipients of both sexes (5); and (c) to evaluate transplantation resistance across histocompatibility barriers involving defined regions of the H-2 complex.

Only 1 of the 8 drug-treated lymphoma sublines studied, the S-DIC line, became immunogenic for syngeneic Bl0 mice in which the untreated lymphoma S-1033 failed to meet with, or induce, transplantation resistance. The evidence for immunogenicity of S-DIC cells rests on longer survival of tumor-bearing Bl0 mice not given immunodepressive or chemotherapeutic drugs as compared with the survival of Bl0 mice either pretreated with CY or given DIC after tumor transplantation (Chart 2). The enhancing effect of a single administration of CY on subsequent tumor progression was similar to the effect of multiple injections of DIC given after transplantation. This finding rules out the possibility that S-DIC cells were biochemically dependent on DIC for growth and suggests instead an immunodepressive mechanism. In fact, DIC was shown to inhibit allograft reactions and humoral immunity in mice (2, 3, 28).

The evidence for lack of immunogenicity of the other drug-treated lymphoma sublines rests on comparable survival times of immunocompetent and immunodepressed tumor-bearing Bl0 mice given BCNU chemotherapy after transplantation (Table 2). This experimental design is adequate to detect weak tumor-host incompatibilities, as indicated by the survival of H-I-incompatible congenic-resistant Bl0.129(5M) mice given injections of lymphoma S-1033 and given BCNU chemotherapy (5). This test system has also been used to measure the antilymphoma allograft response of mice incompatible for components of the H-2 complex (7) and to detect incompatibility between CD2F₁ mice and drug-treated L1210 sublines (25). Although incomplete results were obtained with cells of the S-TG line that were transplanted into CY-treated mice (see Table 2, Footnote a), no differences in survival times were observed in untreated tumor-bearing mice and in those given 6-TG after transplantation (Chart 1). Since 6-TG is a strong immunosuppressive agent (15), this finding suggests
that the S-TG line was relatively nonimmunogenic for B10 mice.

Allograft reactions directed against transplantation antigens associated with the K-I-S regions of H-2 are usually stronger than those elicited by S-D incompatibilities. This was repeatedly observed with transplants of normal tissues (13) and several radiation-induced lymphomas (6–8). Cells of lymphoma S-1033 and of most of our drug-treated sublines were not rejected by B10.A(5R) mice and, therefore, conformed to this pattern whenever 10^6 cells were transplanted. Possible exceptions were S-CY and S-DIC cells that were rejected by both S-D- and K-I-S-incompatible recipients. The differential immunogenicity of antigens associated with distinct regions of the H-2 complex is apparently a property common to cells of varied origin not readily altered by drug treatments in vivo.

The prevalence of the K-I-S over the S-D regions in causing allograft reactions was not detectable with lymphoma grafts of smaller size (10^4 or 10^5 cells). Paradoxically, the K-I-S-incompatible mice did not reject the smaller grafts owing to dilution escape (6, 8). This occurred with lymphoma S-1033, S-FU, S-PCA, and S-CY, but not with S-BCNU and S-DIC.

The absence of dilution escape in mice grafted with cells of the S-DIC and S-BCNU lines is difficult to interpret and only tentative hypotheses can be formulated. There is experimental evidence that an IDF of viral origin associated with lymphoma cells plays a key role in the dilution escape phenomenon (1, 8, 16). Small inocula of allogeneic lymphoma cells release this factor, which inhibits the allograft reaction of the host before the initially small tumor cell population reaches the size required to be immunogenic. Hence, lethal tumor growth occurs despite major histoincompatibility. On the other hand, large inocula of lymphoma cells promptly trigger antigen-sensitive lymphocytes before the immunodepressive factor can interfere with the host’s allograft reactivity. In this case the allogeneic tumor is rejected.

In the light of this interpretation, the absence of dilution escape in recipients of S-DIC and S-BCNU cells could be ascribed either to lack of IDF in these tumor lines or to increased immunogenicity of tumor cells for allogeneic recipients. The 1st possibility was ruled out for the S-DIC line as this tumor is IDF positive (1); it seemed unlikely for the S-BCNU line because the parental S-1033 lymphoma is also IDF-positive (1) and BCNU treatment does not inhibit IDF replication (unpublished observations). The 2nd explanation (increased immunogenicity) faces the difficulty that other radiation-induced lymphomas, highly immunogenic for congenic-resistant mice, do undergo dilution escape in K-I-S-incompatible recipients (6, 8). Also, while cells of the S-DIC line were more immunogenic than those of the parental line for B10 mice, this was not so for S-BCNU cells, which were similar to S-DIC cells with respect to dilution escape.

Radiation-induced lymphomas share with normal hemopoietic cells tissue-specific alloantigens determined by Hh (Hemopoietic-histocompatibility) genes (4, 10–12). Hh-incompatible cells are recognized as foreign and rejected by nonradiated as well as by heavily radiated mice. Macrophages seem to play an important role in the rejection process as effector cells (20). Resistance to lymphoma grafts is effective in inhibiting tumor growth when small but not large inocula are used for challenge (26), and IDF does not inhibit the anti-Hh reaction (M. C. Fioretti, C. Riccardi, E. Bonmassar, and G. Cudkowicz, manuscript in preparation).

It is therefore possible to speculate that increased immunogenicity of “Hh type” could be responsible for rejection of small number of S-DIC and S-BCNU lymphoma cells by B10.A(2R) and B10.A(5R) mice. If so, incompatible mice congenic with B10 would reject small inocula of cells of these lymphoma lines despite the presence of IDF. The hypothesis could be tested by transplanting the tumor cells into radiated mice and by selectively impairing the function of host macrophages (20).

In conclusion, heritable changes of the immunogenic properties of B10 lymphoma cells, detected as transplantation resistance in mice of the strain of origin and/or allogeneic recipients, occurred following treatment in vivo with antineoplastic agents. The mechanisms underlying such modifications are still obscure. Nevertheless, the possibility that antitumor drugs directly or indirectly affect tumor cell immunogenicity in vivo should be taken into consideration for its possible relevance to antitumor immunotherapy.

ACKNOWLEDGMENTS

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