In Vitro Lymphocyte Stimulation and the Generation of Cytotoxic Lymphocytes with Drug-induced Antigenic Lymphomas

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ABSTRACT

New antigenic specificities, not detectable on parental cells and transmissible after the withdrawal of the drug treatment, have been induced in mouse lymphomas.

Studies were conducted of proliferative stimulation of syngeneic lymphocytes and the generation of cytotoxic lymphocytes (CL’s) in a mixed lymphocyte-tumor cell culture system by 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC)-Induced antigens in L1210 and EL4 leukemia sublines. The DTIC-induced antigens were observed to stimulate [%H]thymidine uptake by normal and primed syngeneic lymphocytes. The specificity of the in vitro immune reactivity was demonstrated. Characteristics of lymphocyte triggering, including the optimal ratio of stimulating cells to responding cells, the kinetics of CL activation, and the quantitation of CL activity, were also evaluated.

DTIC antigens on leukemic cells can activate syngeneic lymphocytes and can act as target antigens in cell-mediated immunity.

The experimental data support the transplantation antigen-like nature of DTIC-induced antigens.

INTRODUCTION

Following in vivo treatment with certain drugs, the antigenicity of mouse leukemic cells increases markedly (2, 12, 13, 19). The new antigenic specificities, not detectable on parental cells, continue to be transmissible after withdrawal of the drug treatment (18).

Drug-mediated immunologically altered experimental lymphomas elicit immune responses in syngeneic hosts, as evaluated by classical rejection experiments and by transfer of immune lymphocytes (16). Specific immune reactions to drug-induced antigens by syngeneic animals detected by in vitro assays (14, 22) confirm the in vivo findings. As part of a program to elucidate the nature of new drug-induced antigens, studies of the reactivity of syngeneic lymphocytes cultured in vitro in the presence of antigenic tumor cells were undertaken. Cells from L1210 leukemia and EL4 lymphoma with altered immunological properties following treatment with DTIC, hereafter called L1210/DTIC and EL4/DTIC, respectively, were used in MLTC and for the in vitro generation of CL’s.

MATERIALS AND METHODS

Animals and tumors. Inbred DBA/2 Cr(H-2b) and C57BL/6J(H-26) mice and hybrid BALB/c x DBA/2 F1 (hereafter called CD2F) and DBA/2 x C57BL/6 F1 (hereafter called D2B6F,) mice, 8 to 10 weeks old, were obtained from Charles River Breeding Laboratory, Wilmington, Mass.

Stock L1210 (H-2b) and EL4 (H-2d) lymphomas were maintained by weekly i.p. injections of ascites cells into histocompatible animals. L1210/DTIC (16) and EL4/DTIC (17) sublines were developed as previously described. Briefly, CD2F1 mice given i.p. injections of L1210 leukemia or D2B6F1, mice challenged i.p. with EL4 lymphomas were treated for 4 and 12 transplant generations, respectively, with DTIC (100 mg/kg/day). DTIC tumor sublines were maintained in animals compatible with the original tumors, immunosuppressed by administration of cyclophosphamide (200 mg/kg i.p.) 24 hr before tumor implantation.

In Vitro Assays. The experiments were conducted with tumor cells and spleen lymphocytes explanted directly from animals. Cell viability was checked by trypan blue dye exclusion. The culture medium was Roswell Park Memorial Institute Tissue Culture Medium 1640 (Eurobio, Paris, France) supplemented with L-glutamine, Pen-Strep (100 units/ml, 100 µg/ml), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 2-mercaptoethanol (10⁻⁴ M), and 20% heat-inactivated fetal calf serum (Eurobio). Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with L-glutamine and 5% fetal calf serum was used in the ¹¹Cr release assay. Balanced salt solution was used for cell washing.

MLTC. Spleen cells (responding cells) (10⁶) with a varying number of tumor cells (stimulating cells) in 12- X 75-mm round-bottom glass tubes at a final volume of 2 ml were maintained in a moist atmosphere of 95% air and 5% CO₂ for 96 hr. Stimulating cells had been previously incubated for 30 min at 37° with mitomycin C (35 µg/10⁷ cells/ml) (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan).

Allogeneic and syngeneic spleen lymphocytes were the positive and negative stimulating cell controls for the assay. After 48 hr of incubation, in most experiments, the medium was carefully replaced with fresh medium. Sixteen hr before the end of the experiments, [%H]thymidine (2 µCi; specific activity, 28 Ci/mmmole; Sorin, Saluggia, Italy) was added to each tube.

Cultures were harvested onto glass-fiber filters by a multiple automatic cell harvester (Skatron, Lierbyen, Norway).

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Radioactivity was determined in a Packard liquid scintillation counter, and results were expressed as mean cpm of triplicate cultures ± S.E.

The SI was calculated by the following formula:

\[
SI = \frac{cpm \text{ in experimental cultures}}{cpm \text{ in control cultures}}
\]

A SI of 2 or more was considered positive in accordance with previous reports (5).

**Generation of CL's and \(^{51}\text{Cr} \) Release Assay.** The basic procedure described for MLTC was used. In brief, \(6 \times 10^6\) lymphocytes with a varying number of mitomycin C-treated stimulating cells (2.5 ml, final volume) were seeded in wells of tissue culture plates (Costar tissue culture cluster, 24 wells, 16-mm diameter; Costar, Cambridge, Mass.) and incubated under the usual conditions.

The CL's were harvested with Pasteur pipets, and the lytic activity was evaluated by the \(^{51}\text{Cr} \) release microassay, as described by Goldstein and Blomgren (6). Conditions were: \(^{51}\text{Cr} \) (Na\(^{251}\text{CrO}_4\); Sorin) and V-bottom microplates (Sterilin, Teddington, Middlesex, England), and results are expressed as:

\[
\% \text{ cytotoxicity} = \frac{cpm \text{ in experimental samples} - cpm \text{ in controls}}{cpm \text{ in detergent samples} - cpm \text{ in controls}} \times 100
\]

**RESULTS**

The experimental results show the capacity of the altered DTIC cells to stimulate DNA synthesis and to generate CL's from virgin or primed syngeneic lymphocytes.

CD2F, lymphocyte reactivity to L1210/DTIC cells, as measured by \(^{3}\text{H} \)thymidine incorporation, is reported in Table 1. Uptake of radioactive label and the SI showed the specific stimulation of lymphocytes by L1210/DTIC cells and the failure of parental L1210 cells to induce any proliferative response. Unprimed lymphocytes were less reactive than were cells sensitized in vivo. However, the SI of 3.4 for the unprimed lymphocytes was notable when compared with the SI induced by allogeneic lymphocytes.

The optimal ratio of responding cells to stimulating tumor cells is shown in Chart 1. For the maximal SI, following a 96-hr incubation, a lower number of stimulating cells was needed in experiments with primed lymphocytes than was needed in experiments with unprimed cells. When the stimulating cell number was increased further, inhibition of \(^{3}\text{H} \)thymidine uptake was observed.

The specific *in vitro* activation of *in vivo* primed syngeneic lymphocytes by L1210/DTIC cells (Table 2) or by EL4/DTIC cells (Table 3) was demonstrated by the \(^{51}\text{Cr} \) release assay. Spleen cells from syngeneic animals immune to DTIC-altered cells after incubation with mitomycin C-treated DTIC cells showed high, specific, cytotoxic activity when assayed against \(^{51}\text{Cr} \)-labeled DTIC cells. The parental cells (namely, L1210 cells or EL4 cells) did not activate syngeneic lymphocytes, nor were they lysed by CL's. Thus, DTIC-induced antigen(s) on leukemic cells could fully generate CL's in

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**Table 1**

<table>
<thead>
<tr>
<th>Source of responding cells(^a)</th>
<th>Source of stimulating cells</th>
<th>(^{3}\text{H} )Thymidine uptake (cpm)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2F, (10^6)</td>
<td>CD2F, (10^6)</td>
<td>12,448 ± 3,668</td>
<td>3.6</td>
</tr>
<tr>
<td>CD2F, (10^6)</td>
<td>L1210 (2.5 \times 10^8)</td>
<td>11,566 ± 1,404</td>
<td>0.9</td>
</tr>
<tr>
<td>CD2F, (10^6)</td>
<td>L1210/DTIC (2.5 \times 10^8)</td>
<td>44,877 ± 4,963</td>
<td>3.4</td>
</tr>
<tr>
<td>CD2F, (10^6)</td>
<td>C57BL/6J (10^6)</td>
<td>56,478 ± 1,833</td>
<td>4.6</td>
</tr>
<tr>
<td>p-CD2F, (10^6)</td>
<td>p-CD2F, (10^6)</td>
<td>6,307 ± 1,297</td>
<td>1.2</td>
</tr>
<tr>
<td>p-CD2F, (10^6)</td>
<td>L1210 (6.25 \times 10^4)</td>
<td>8,072 ± 741</td>
<td>1.2</td>
</tr>
<tr>
<td>p-CD2F, (10^6)</td>
<td>L1210/DTIC (6.25 \times 10^4)</td>
<td>32,609 ± 3,058</td>
<td>5.1</td>
</tr>
<tr>
<td>p-CD2F, (10^6)</td>
<td>C57BL/6J (10^6)</td>
<td>21,023 ± 1,478</td>
<td>3.4</td>
</tr>
</tbody>
</table>

\(a\) Spleen cells (10^6) from normal CD2F, mice or CD2F, mice immunized (primed; p-CD2F,) to L1210-DTIC tumor cells by i.p. challenge of 10^7 viable cells 30 to 40 days before MLTC. Time of culture, 96 hr.

\(b\) Numbers in parentheses, number of cultures.

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**Table 2**

<table>
<thead>
<tr>
<th>Activating cells</th>
<th>Target cells</th>
<th>(^{51}\text{Cr} ) release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210</td>
<td>L1210</td>
<td>2</td>
</tr>
<tr>
<td>L1210</td>
<td>L1210/DTIC</td>
<td>–1</td>
</tr>
<tr>
<td>L1210</td>
<td>EL4</td>
<td>–2</td>
</tr>
<tr>
<td>L1210/DTIC</td>
<td>C3H-GL(^a)</td>
<td>1</td>
</tr>
</tbody>
</table>

L1210/DTIC\(^a\)

| L1210            | L1210        | 2                              |
| L1210            | L1210/DTIC   | 53                             |
| L1210            | EL4          | –1                             |
| L1210/DTIC      | C3H-GL       | 3                              |

\(a\) A transplant leukemia originally induced in C3H mice by Gross leukemia virus.

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**Chart 1.** MLTC with spleen cells from CD2F, mice and mitomycin C-treated L1210/DTIC cells at various spleen cell to tumor cell ratios. A responding cells (10^6) were spleen cells from normal CD2F, mice; B responding cells were spleen cells from CD2F, mice primed by a challenge of 10^7 L1210/DTIC viable cells i.p. 30 to 40 days before MLTC.
MLTC, as in the already described experiments with allogeneic lymphoid cells (3) or with tumor-associated antigens, such as the Moloney sarcoma in BALB/c mice (21) or the Gross leukemia in W/Fu rats (1).

The specificity of the cytotoxic reactions was evaluated further by the inhibition assay (20). L1210 cells (10^5) added to ^51Cr-labeled L1210/DTIC cells (10^4) partially inhibited ^51Cr release by L1210/DTIC, possibly by an overcrowding mechanism (Chart 2). No competition was exerted by a lower number of L1210 parental cells, in contrast with inhibition observed with the addition of cold L1210/DTIC cells. EL-4/DTIC ^51Cr release was not reduced by EL-4 parental cells, whereas marked inhibition was exerted by EL-4/DTIC cells. These experimental results provide further evidence for the special nature of the DTIC-induced antigens.

Following incubation in the presence of DTIC leukemic cells, CL's were generated from unprimed lymphocytes. Chart 3 shows the kinetics of activation. D2B6F1 CL's showed lytic activity against EL-4/DTIC cells from Day 4 to Day 6 of incubation. CD2F1 CL's were cytolytic for L1210/DTIC from Day 4 to Day 7. In assays not reported here, the specificity of the reaction was checked, as shown, for secondary activation (Tables 2 and 3; Chart 2). In most experiments primary generation of anti-L1210/DTIC CD2F1 CL's did not elicit ^51Cr release more than 10% above that of the controls, whereas anti-EL-4/DTIC CL's caused a stronger response (Chart 3). Secondary MLTC elicited greater CL activity over a more extensive incubation period (Chart 4), as compared with the primary MLTC.

The optimal activating cell number in the generation of CL's in secondary culture is shown in Chart 5. Twenty primed CD2F1 spleen cells to each activating L1210/DTIC cell brought about the maximal cytotoxic activity. A smaller number of activating EL-4/DTIC cells was needed to obtain the highest ^51Cr release.

The quantitative relationship of the degree of cytotoxic activity to effector cell frequency in the lymphoid cell population is reported in Chart 6. Specific cytotoxic activity varied linearly with the logarithm of the number of effector cells, in agreement with experiments (8) originally designed for this type of study.

**DISCUSSION**

The specificity of the *in vitro* immune reactions to DTIC-leukemic cells supports the hypothesis that DTIC treatment induced the appearance of additional novel antigen(s) in the tumor cells rather than producing an increase in preexistent determinants. In the latter instance normal or immune

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**Table 3**

Secondary in vitro generation of CL's by EL-4/DTIC cells

Experimental conditions were the same as those described in Table 2. Spleen cells were obtained from D2B6F1 mice primed with 10^7 EL-4/DTIC viable cells.

<table>
<thead>
<tr>
<th>Activating cells</th>
<th>Target cells</th>
<th>^51Cr release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL-4</td>
<td>L1210</td>
<td>2</td>
</tr>
<tr>
<td>EL-4</td>
<td>EL-4</td>
<td>4</td>
</tr>
<tr>
<td>EL-4/DTIC</td>
<td>L1210</td>
<td>2</td>
</tr>
<tr>
<td>C3H-GL</td>
<td>EL-4</td>
<td>4</td>
</tr>
<tr>
<td>EL-4/DTIC</td>
<td>L1210</td>
<td>5</td>
</tr>
<tr>
<td>C3H-GL</td>
<td>EL-4</td>
<td>4</td>
</tr>
<tr>
<td>EL-4/DTIC</td>
<td>C3H-GL</td>
<td>61</td>
</tr>
<tr>
<td>EL-4/DTIC</td>
<td>C3H-GL</td>
<td>3</td>
</tr>
</tbody>
</table>

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**Chart 3**

Kinetics of generation of CL's in primary MLTC. Unprimed CD2F1 spleen cells (6 x 10^6) were incubated with 2 x 10^6 mitomycin C-treated L1210/DTIC cells and assayed against ^51Cr-labeled L1210/DTIC (●). Anti-EL-4/DTIC D2B6F1, CL's were obtained with the same procedure (○). CL's were ineffective for lysis of ^51Cr-labeled parental cells.

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**Chart 2**

Inhibition assay of CL's by unlabeled target cells. CD2F1 CL's and D2B6F1 CL's active against L1210/DTIC cells or EL-4/DTIC cells, respectively, were obtained as indicated in Tables 2 and 3. The ^51Cr release assay was modified from that described in Table 2 by the addition of a number of unlabeled target cells to the effector cell-labeled target cell suspension. A, anti-L1210/DTIC CD2F1, CL's tested against ^51Cr-labeled L1210/DTIC cells; B, anti-EL-4/DTIC D2B6F1, CL's tested against ^51Cr-labeled EL-4/DTIC cells.

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**Chart 4**

Kinetics of generation of CL's in secondary MLTC. Conditions were as described in Chart 3. lymphocytes were from animals sensitized as reported in Table 2. ▲, anti-L1210/DTIC CL's assayed against ^51Cr-labeled L1210/DTIC cells; ○, anti-EL-4/DTIC CL's assayed against ^51Cr-labeled EL-4/DTIC cells.
lymphocytes would have been stimulated or activated by parental drug-untreated cells. Furthermore, the parental L1210 and EL4 lymphoma cells would have been lysed by CL's generated in vitro to DTIC-altered cells. The current experimental data provide evidence for the special nature of the DTIC-induced antigen(s), both as inducers of lymphocyte reactivity and as CL targets. The observations are in agreement with recent studies (8) of the in vitro stimulation of lymphocytes and in vitro generation of CL's by syngeneic tumor systems. Although the nature of drug-induced antigen(s) remains unknown and the relationship between proliferative and cytotoxic activity is still obscure, the current experimental data do demonstrate the similarity of DTIC-induced antigens to transplantation antigens.

A possible relationship could exist between DTIC-induced antigen(s) and Ia-antigens, the latter coded for by the I region of the MHC (9) and considered to be of primary relevance in stimulating lymphocyte proliferation.

However, it seems more probable that there is a relationship of DTIC-induced antigens to serologically defined antigens coded for by the K and D end of the MHC, since serologically defined antigens are possibly the main targets for CL's on cellular membranes. Indeed, 1 of the tumor sublines studied here (namely, the EL4/DTIC lymphoma) reacted with an antiserum to private specificity 31 normally expressed on the H2^b strain (A. Nicolin, unpublished data). More detailed studies with antisera monospecific for the products of the MHC might reveal new aspects of the nature of DTIC-induced antigenicity.

Viral infection, which frequently accompanies transplantable tumors and which may be prevalent in our experimental system because of the tumor passages in immunosuppressed mice, did not appear to influence the observations in the current studies. In unpublished studies (A. Nicolin), CD2F1 spleen cells after incubation with mitomycin C-treated L1210/DTIC cells and D2B6F1 spleen cells after incubation with mitomycin C-treated EL4/DTIC cells did not show cytotoxic activity when assayed against ^51Cr-labeled EL4/DTIC cells and ^51Cr-labeled L1210/DTIC cells, respectively. Therefore, these sublines treated with the same drug, DTIC, did not show immunological cross-reactivity, as would be expected if there were modification of surface membrane identity by infecting viruses (10, 23). In only 1 instance did a tumor subline obtained from the same parental tumor treated with DTIC under the standard conditions already described show immunological cross-reactivity (15).

Drug-induced derepression of normally silent foreign haplotypes, in accordance with both recent reports for other tumor systems (4, 7, 11) and preliminary data from this laboratory, could also be of interest in relation to the alteration of tumor cell immunogenicity.

In conclusion, 2 experimental tumors altered by in vivo treatment with DTIC induced lymphocyte proliferation and generated CL's in a syngeneic system in vitro. The nature of the drug-induced antigens remains unknown. However, the present data and unpublished results from our laboratory illustrate the histocompatibility antigen-like activity of DTIC-induced antigen(s).

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