DTIC xenogenized lines obtained from an L1210 clone: Clonal analysis of cytotoxic T lymphocyte reactivity

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Summary Antineoplastic compounds can induce on tumour cells new antigens that indetectable on parental cells and which are transmissible as a genetic character. In this study mouse leukaemia L1210 was cloned in vitro by limiting dilution and one cloned line was recloned in vivo. Four subcloned tumour cell lines (A, D, R, S) were xenogenized in vivo by DTIC treatment (A/DTIC, D/DTIC, R/DTIC, S/DTIC) following a schedule previously described. Up to 10⁷ cells of these xenogenized subclones, injected i.p., were rejected by syngeneic hosts, although they grew in immunosuppressed hosts.

The DTIC treated subclones were lysed by in vivo-primed, in vitro-restimulated (with the relevant subclone) lymphocytes.

The cytotoxic lymphocyte activity was not strictly specific since parental, DTIC-untreated cells were also lysed, although less efficiently.

CTL directed against the D/DTIC subclone were cloned by limiting dilution. Ninety-four CTL clones were assayed against L1210 subcloned cells, DTIC-treated and untreated, and against different murine tumours (syngeneic or allogeneic). Three specific antigens could be identified in the ³¹Cr release assay. The DTIC subclones expressed one antigen that was specifically recognized by a set of CTL clones. A number of CTL clones were able to lyse the L1210 subcloned cell exclusively, targeting a tumour-associated antigen that did not appear to be modified in the DTIC-treated subclones. A third antigen was demonstrated in the parental and DTIC treated D subclone.

On the basis of these results it was postulated that there was at least one common DTIC-inducible antigen specific and reproducible within an identical cell population. Moreover, DTIC treatment did not modify histocompatibility existing in L1210 antigens or TAA pre-existing in L1210 antigens.

The findings discussed here provide new information about permanent xenogenization of tumour cells, which might be exploited for experimental chemo-immunotherapy of cancer.

Materials and methods

Animals

Hybrid (BALB/c x C57BL/6) F₁ mice (hereafter called CD2F₁) mice of both sexes, 6-8 weeks old were obtained from Charles River Breeding Laboratories (Calco, Italy).

Tumours

L1210 Cr (H-2b) (Law et al., 1949; Dunham et al., 1953) obtained from the National Cancer Institute (Bethesda, USA) and maintained in the laboratory by weekly i.p. injection into CD2F₁ mice, was cloned in vivo by limiting dilution. Twenty cells from one clone (20 cells/4ml) were injected (0.2ml/mouse) into 20 syngeneic mice. Four of 8 subcloned tumour lines (referred to as A, D, R, S) were transformed in vivo with DTIC, as previously described (Bonomassar et al., 1970). Briefly, 10⁶ tumour cells were injected i.p. into CD2F₁ mice treated for 7 consecutive days with DTIC (100 mg/kg/d, i.p.) starting one day after tumour challenge. When the ascitic tumours developed in the DTIC-treated mice, they were collected and 10⁶ cells were transplanted into untreated mice. The same procedure was followed for 4-5 transplant generations.

Immunogenic subclones A/DTIC, D/DTIC, R/DTIC, S/
DTIC were maintained in compatible, total body X-irradiated (3.5 Gy; Securix Compact CGD), mice.

- YAC-1(H-2b) tissue culture cell line originating from YAC (Klein & Klein, 1964) was maintained in RPMI 1640 medium (Flow Lab.) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Flow Lab.), 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin.
- P388 (H-2b) tumour cells (Dave & Potter, 1957) were maintained by weekly i.p. injections into syngeneic DBA/2 mice.

Preparation of T-cell growth factor (TCGF) from rat spleen cells

Rat spleen cells (5–10 x 10⁶ cells ml⁻¹) were cultured for 24 h in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 5 x 10⁻⁴ M 2-mercaptoethanol, 100 μg ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 2-5 μg ml⁻¹ Concanavalin A (Flow Lab.).

The supernatant that contained TCGF was collected by centrifugation, filtered and stored at -20°C.

Cytotoxic T lymphocytes (CTL) and ⁵¹Cr release assay

Spleen cells from CD2F1, mice injected 3–5 weeks before with 10⁷ DTIC treated subclones were stimulated secondarily in vitro.

30 x 10⁶ splenocytes (responders) were cultured in 25 cm² flasks (Sterilin) with 3 x 10⁶ mitomycin C (Kiowa, Tokyo)-treated tumour cells (stimulators) at a final volume of 20 ml RPMI 1640 medium, supplemented as previously described. After 5 days incubation, the effector CTL were cultured and their lytic activity against different target cells was evaluated in a ⁵¹Cr release microassay (Ferrini et al., 1987). Briefly, ⁵¹Cr-labelled target cells, 10⁴ in 100 μl, were seeded in 96 well plates (Sterilin) with 100 μl effector cells at different concentrations and incubated for 4 h at 37°C in a moist atmosphere of 95% air and 5% CO₂. The plates were then centrifuged and 100 μl supernatant counted in a gamma counter. Percent specific lysis was calculated as:

\[
\% \text{ lysis} = \frac{\text{cpm exp. release} - \text{cpm spontaneous release}}{\text{cpm max. release} - \text{cpm spontaneous release}} \times 100
\]

Maximum release was obtained by incubating 10⁴ ⁵¹Cr labelled target cells in 200 μl Triton-X100 (1% in distilled water) for 4 h. Spontaneous release was obtained by incubating 10⁴ target cells with different concentrations of non-secondarily activated syngeneic splenocytes. The spontaneous ⁵¹Cr release for the target cells used was in the range, 5-15%.

CTL cloning

Ten CTLs, obtained as previously described, were placed in flat-bottom 96 well plates (Linbro Chemical Co.) under limiting dilution conditions (1, 10 and 100 cells per well) with 5 x 10⁴ previously irradiated (60 Gy; ⁶⁰Co) stimulator cells, 10⁴ irradiated (40 Gy; ⁶⁰Co) syngeneic cells per well as feeders, and 10% TCGF.

The three plates seeded with 1 cell/well did not develop a colony, the 3 plates seeded with 10 cells/well developed a colony in 33% of the wells and when 100 cells were seeded colonies grew in almost all the wells. CTL clones used in this work derive from the plates seeded with 10 cells/well.

Virus detection

Cell culture supernatants were filtered (0.2 μm FlowPore D) and assayed for ecotropic murine retroviruses in mouse SC-1 cells by the XC plaque assay (Varmier & Levy, 1979; Rowe et al., 1970).

- No infectious virus (≤4 Plaque Forming Unit ml⁻¹) was detected in the samples.

The electron microscopic localization of MMTV antigens was performed by the pre-embedding protein A-gold technique.

Results

Cloned cells from the mouse L1210 leukaemia, obtained by ‘limiting dilution’ in vitro, were inoculated (20 cells in 4 ml), i.p. into 20 syngeneic CD2F1, mice (0.2 ml/mouse). Four of the 8 tumour subclones (A, D, R, S) that grew in the mice, were xenogenized in vivo by DTIC treatment, as previously described (Bonmassar et al., 1970). Following 4-5 transplant generations the A/DTIC, D/DTIC, R/DTIC, S/DTIC sublines were fully xenogenized, since 10⁷ cells of each subclone were completely rejected by CD2F1, mice. The experimental results in Table I show that xenogenized DTIC subclones retained the growth characteristics of the untreated L1210 parental subclones, since their tumorigenicity in immunosuppressed mice was fully retained.

The antigenic properties of the L1210/DTIC subclones and their reciprocal immunological relationships were studied with CTL. Spleen cells from CD2F1, mice that had rejected 10⁷ xenogenized cells were secondarily restimulated in vitro with the relevant DTIC subclones. The results for the immunological cross-reactivities among L1210 DTIC subclones are listed in Table II.

In the 4 h ⁵¹Cr-release assay, anti-D/DTIC CTL had high lytic activity against D/DTIC target cells as well as against the other DTIC subclone lines. The parental, DTIC-unxetured D subclone line was also lysed by anti-D/DTIC CTL, although the percentage of ⁵¹Cr release was lower than that released by D/DTIC. The other untreated cell lines (A, R, S) were recognized and lysed to a lesser degree than D. There was detectable activity against YAC and P388 lymphomas. Similar patterns of cross-reactivity were displayed by anti-A/DTIC, anti-R/DTIC and anti-S/DTIC CTL (data not shown). Indeed the CTL to each xenogenized subclone were fully cross-reactive with the other DTIC treated subclones.

To further understand the antigenic properties of DTIC subclones, D/DTIC CTL were cloned in microtitre plates by limiting dilution and cultured in the presence of irradiated D/DTIC cells, syngeneic feeder spleen cells and rat IL-2. Three weeks later, it was possible to detect growing colonies in 94/288 microwells. Since the percentage of negative wells was ~67%, a high probability of cloning was ensured. The cytotoxic activity (lytic activity) of individual clones was assayed against the xenogenized cells, the untreated parental subclones and unrelated tumours. Twenty-one of the 94 CTL clones obtained had cytotoxic activity and 73 had cytolytic activity. Among the 73 effective CTL clones, 36 had high cytotoxic activity against the 4 xenogenized subclone cells only (Table III), in contrast to the 12 CTL clones that had lytic activity against the L1210 subclone cells also (Table IV), whether xenogenized or not. None of the CTL clone groups had any effect on the unrelated tumour cells. Two CTL clones were weakly but highly specifically lytic to the

| Tumour cell* | MST* | D/T | MST | D/T |
|--------------|------|-----|-----|-----|
| D            | 6.5  | 10/10 | 6   | 10/10 |
| D/DTIC       | -    | 0/10  | 6.5 | 10/10 |
| A            | 7    | 0/10  | 6.5 | 10/10 |
| A/DTIC       | -    | 0/10  | 7   | 10/10 |
| R            | 6.5  | 10/10 | 6   | 10/10 |
| R/DTIC       | -    | 0/10  | 7   | 10/10 |
| S            | 7    | 10/10 | 7   | 10/10 |
| S/DTIC       | -    | 0/10  | 7.5 | 10/10 |

*10⁷ viable cells/mouse, i.p.; *MST = mean survival time; *D/T = dead mice/treated mice; *3.5 Gy/mouse (day-1).
Table II  Anti-D/DTIC CTL activity: percentage $^{51}$Cr release (±s.d.)

| Target cells | 20:1 | 10:1 | 5:1 | 25:1 | 1:1 |
|--------------|------|------|-----|------|-----|
| D/DTIC       | 94.4±7.8 | 81.4±7.2 | 71.4±5.2 | 56.9±4.2 | 35.9±2.1 |
| D            | 51.9±4.3 | 31.9±1.8 | 17.1±9.9 | 11.1±0.3 | 2.5±0.1 |
| A/DTIC       | 43.9±3.1 | 34.6±1.9 | 28.5±1.1 | 23.7±1.2 | 17.2±0.7 |
| A            | 15.0±1.2 | 9.6±0.5  | 7.7±0.4  | 2.7±0.1  | 0.2±0.1 |
| R/DTIC       | 79.3±6.1 | 66.2±4.0 | 58.9±4.1 | 52.4±3.9 | 25.9±1.5 |
| R            | 39.9±2.9 | 26.2±1.1 | 21.8±1.2 | 12.9±0.9 | 5.5±0.4 |
| S/DTIC       | 84.3±7.1 | 73.4±5.1 | 64.4±4.2 | 48.9±3.1 | 25.9±1.9 |
| S            | 24.9±1.9 | 19.0±0.1 | 17.0±1.2 | 5.6±0.3  | 2.1±0.1 |
| YAC          | 14.2±0.9 | 9.9±0.5  | 2.6±0.1  | 1.6±0.2  | 0.4±0.1 |
| P388         | 19.4±1.2 | 9.6±0.8  | 9.2±0.4  | 6±0.4    | 3.4±0.2 |

*Effector: target cell ratio.

Table III  Anti-D/DTIC CTL clones: Specific lysis of DTIC subclones

| Target cells | Clone no. |
|--------------|-----------|
| D            | 11        |
| D/DTIC       | 14        |
| A            | 33        |
| A/DTIC       | 69        |
| R            | 85        |
| S/DTIC       | 62        |
| YAC          | 78        |
| P388         | 85        |
| EL4          | 11        |
| EL4          | 14        |
| EL4          | 33        |
| EL4          | 69        |
| EL4          | 85        |

Similar activities were displayed by 31 CTL clones not reported here; *% $^{51}$Cr release ± s.d.; Effector: target cell 5:1.

Table IV  Anti-D/DTIC CTL clones: Lysis of L1210 subclones

| Target cells | Clone no. |
|--------------|-----------|
| D            | 6         |
| D/DTIC       | 16        |
| A            | 48        |
| A/DTIC       | 52        |
| R            | 78        |
| S            | 78        |
| S/DTIC       | 78        |
| YAC          | 85        |
| P388         | 85        |
| EL4          | 85        |
| EL4          | 85        |

Similar activities were displayed by 7 CTL clones not reported here; *% $^{51}$Cr release ± s.d.; Effector: target cell 5:1

Table V  Anti-D/DTIC CTL clones: Specific lysis (% ± s.d.) of D subclones

| Target cells | Clone no. |
|--------------|-----------|
| D            | 32        |
| D/DTIC       | 32        |
| A            | 57        |
| A/DTIC       | 57        |
| R            | 57        |
| R/DTIC       | 57        |
| S            | 57        |
| S/DTIC       | 57        |
| YAC          | 57        |
| P388         | 57        |
| EL4          | 57        |

*% $^{51}$Cr release; Effector: Target cell 5:1.

Table VI  Recognition pattern of anti-D/DTIC CTL clones

| CTL clone groups of activity |
|------------------------------|
| Target cells | I | II | III |
|----------------|---|----|-----|
| D/DTIC         | + + +  | + + + | +  |
| D              | -   | + + + | +  |
| A/DTIC         | -   | + + + | +  |
| A              | -   | + + + | +  |
| R/DTIC         | + + + | + + + | +  |
| R              | -   | + + + | +  |
| S/DTIC         | + + + | + + + | +  |
| S              | -   | + + + | +  |
| YAC            | -   | -   | -   |
| P388           | -   | -   | -   |
| EL4            | -   | -   | -   |

Note: + + + 60--80% specific $^{51}$Cr release; + 30% specific $^{51}$Cr release.
D/DTIC subclone and its corresponding D parental subclone (Table V). The specific activity of CTL clones could therefore be differentiated into three groups, (Table VI). The last 23 CTL clones had no specific activities, since they showed different patterns of reactivity. All of them were able to lyse the L1210 lines, YAC cells and different non-related tumour cell lines (P388, L12175, EL4), but the degree of lysis of each target cell was different, clone by clone (data not shown). The pattern of activity of these clones is the pattern of reactivity described for activated NK (Henney et al., 1981; Suzuki et al., 1983) and/or LAK cells (Rosenstein et al., 1984; Merluzzi, 1984).

Discussion

This laboratory has been involved in attempts to elucidate the immunological alterations in L1210 leukaemia after treatment with the anticancer agent DTIC. Briefly, DTIC-treated L1210 cells are rejected by immunocompetent syngeneic animals, even after very heavy cell challenges in spite of the fact that these cells show the same kinetic properties as those of parental L1210 cells both in vitro and in immunodeficient hosts (Silvestrini et al., 1977). The modification at the molecular level is still unknown. Studies of DTIC-induced antigenicity and of similar immunological alterations produced in different tumour cells by other anticancer compounds (Mihich, 1969; Nicolin et al., 1972; Frost, 1984), or mutagenic agents (Boon, 1983; Altevogt et al., 1985), have been hampered by difficulties in raising effective polyclonal or monoclonal antibodies. Further characterization of DTIC antigens might be useful in shortening the time schedule required to xenograft the tumour cells, in order to achieve the tumour rejection in primary hosts. Furthermore the DTIC-induced antigens might be exploited for active vaccination or passive immunotherapy.

Previous studies with cell-mediated cytotoxic assays from this laboratory (Marelli et al., 1986) have excluded the likelihood that DTIC positively selects naturally occurring non-tumorigenic cells and indicate that there is a limited number of antigens in DTIC-treated L1210 cells. The number of antigens expressed by DTIC-treated L1210 cells is still not exactly defined because of the genetic heterogeneity of the tumour populations.

In this study, we found that four L1210 subclones, submitted separately to DTIC treatments, were not tumorigenic in syngeneic hosts although they were as tumorigenic as treated subclones in immunodepressed mice. The genetic homogeneity within the four subclones rendered it impossible for DTIC to select preexisting non-tumorigenic variant cells from the L1210 subclones. Therefore, we assume that the antigenic properties of treated L1210 subclones were directly altered by DTIC treatment.

Serological studies carried out in collaboration with the Tissue Typing Research Laboratories of the London Hospital Medical College (Marelli & Kimura, unpublished observations) provide no evidence for qualitative or quantitative differences of MHC class I antigen expression in the parental and DTIC treated cells. All the subclones (DTIC treated and untreated), as well as the L1210 line from which they were derived, were negative for MHC class II antigen expression.

It was therefore of interest to establish the number of antigens expressed on each DTIC subclone as well as to study the immunological relationships among the subclones. Since all four CTL populations lysed all four DTIC subclones, the immunological cross-reactivity of the DTIC subclones was proven. However, parental subclones and NK sensitive YAC cells were also lysed although less efficiently. Therefore, we have not established the number of DTIC antigenic specificities on the cells, nor whether the DTIC subclones expressed new antigens or overexpressed the L1210 TAA putatively responsible for the partial CTL cross-reactivity. Moreover the possibility that the increased antigenicity was due to the induction of a product that behaves like a restriction element for the recognition of an antigen already expressed on untreated tumour cells, could not be ruled out. These questions were approached by testing the CTL clones obtained from the anti-D/DTIC CTL population against the battery of relevant target cells. As shown in Table VI, three types of specific antigens could be identified. A number of CTL clones were able to effectively lyse only the DTIC subclones.

These results concur with the hypothesis that a new antigen (or set of antigens) is induced by DTIC treatment. To date, every property related to the drug treatment and expressed on D/DTIC cells is shared by all the other DTIC-modified lines. However, the design of our studies does not exclude in principle that within A/DTIC, R/DTIC or S/DTIC cell populations there might be other products not shared with the D/DTIC cell population. Likewise, within D/DTIC there might be cells carrying determinants unrecognized by the CTL clones studied here and unique for D/DTIC lines. Investigations relating to virus infection have excluded differences among parental and DTIC subclones. All L1210 tumour lines and P388 used here were positive for mammatory tumour virus (Squattini & Marchetti, unpublished data), as determined in an immunocytochemical study with rabbit anti-MTV serum. Studies of DTIC-induced antigens might be useful in the development of vaccines against these viruses.

The biochemical structure of the DTIC induced antigen is unknown. This study shows that the TAA of L1210 is not functionally modified, such that a set of cytotoxic clones DTIC did not discriminate between parental and DTIC-treated cells. This antigen(s) cannot be responsible for host rejection, since challenge with as few as 10 cells of parental subclones was lethal. Although it is quite likely that this common determinant is comparable with a TAA expressed on L1210, since P388 and YAC cells are not recognized, we cannot rule out the possibility that these cross-reactivities might be due to the presence of viral products (Racevskis & Sarkar, 1982; Naejmark et al., 1978).

Two CTL clones showed weak but highly specific reactivity against the immunizing target (D/DTIC) cells and the respective untreated lines (D). Although the parental cell lines D, A, R, S were derived from one clone of L1210, a mutational event occurred before the DTIC treatment could be responsible for the induction of a determinant on the untreated D cell line that is retained on D/DTIC cells. The antigens recognized by the 23 clones that behave in a nonspecific way could belong to the category of molecules that are targets for LAK or activated NK.

We did exclude that they are classical NK targets, since L1210 cells (Fuji & Irie, 1986) were not NK sensitive. Moreover the parental and DTIC treated subclones involved in this study were not lysed in classical NK experiments (data not shown).

Little is known so far about the mechanism by which alkylating compounds, such as DTIC (Audette et al., 1973), have such a strong effect on the immunogenicity of tumour cells. Since DTIC treatment has to be protracted to obtain tumour lines rejected by a syngeneic host, it is quite likely that more than one mutational event occurs, but probably only a few of these lead to increased antigenicity. Furthermore, our results suggest that DTIC induces mutations, leading to the recurrent expression of new antigens at preferential sites.

Studies are in progress to elucidate the biochemical and genetics of DTIC induced-antigens.

Whatever the chemical structure of the DTIC-induced antigens, the data presented in this paper may prove useful for a chemo-immunological approach to tumour therapy.

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