CHARACTERIZATION OF A MONOCLONAL ANTIBODY TO L1210 LEUKAEMIA

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Received 30 September 1981  Accepted 10 November 1981

Summary.—A mouse monoclonal cell line (L1) was produced by fusing the mouse myeloma P3X63/Ag8 with CD2F1 spleen cells immunized with a highly immunogenic subline of L1210 leukaemia (L1210/DTIC). A very few positive clones (1%) were isolated and one of these was chosen for detailed study. The monoclonal antibody L1 is an IgM immunoglobulin strongly reacting in a complement-dependent cytotoxicity assay against L1210/Cr leukaemia and its more or less immunogenic sublines. The specificity of the L1 antibody against L1210 leukaemia was studied by extensive screening with normal adult and foetal tissues, lymphoid tissues from several independent strains and a panel of the most common experimental tumours, to all of which it was unreactive. Attempts at immunotherapy were carried out in DBA/2 mice challenged with L1210 leukaemia and treated with L1 (ascites) and complement. Although the in vitro cytotoxic titre of ascites fluid from mice bearing hybridoma was very high (10⁻⁷), no therapeutic effect was obtained in vivo.

TUMOUR CELLS have been shown to express surface antigens in both animal and human experimental systems. These include histocompatibility antigens and tumour-associated antigens (TAA). Since they were identified (Prehn & Main, 1957; Klein et al., 1960; Old & Boyse, 1964), TAA have been regarded as a natural target for cancer immunotherapy. Yet immunotherapy through humoral or cell-mediated manipulations has been so far ineffective (Rosenberg et al., 1977; Hawrylko, 1978; Motta, 1971). The failure of passive immunotherapy has been ascribed to the low immunogenicity (Hewitt et al., 1976) of TAA, fast shedding of antigen (Black, 1980), neutralizing antigens circulating in the blood (Nadler et al., 1980a) and antigenic modulation elicited by serum itself (Boyse et al., 1967; Stackpole et al., 1980). Although TAA have been found in a great number of tumour cells, they have not yet been well characterized molecularly. Conventional anti-tumour sera have not been completely reliable for the analysis of TAAs, owing to their low affinity and low titre. Since monoclonal antibodies overcome the above limitations (Gunn et al., 1980; Simrell & Klein, 1979; Nadler et al., 1980b), they should provide, in principle, an ideal means of study and treatment of tumours immunologically. In this paper the preparation and the characterization of a monoclonal antibody specific to a L1210 leukaemia-associated antigen is reported.

The monoclonal antibody, secreted by a mouse–mouse hybridoma, has been obtained by fusing the mouse myeloma P3X63/Ag8 cells and spleen cells from mice immune to a syngeneic highly immunogenic subline of the L1210 leukaemia.

MATERIALS AND METHODS

Mice and tumours.—Inbred DBA/2, C3H, C57BL/6J, BALB/c and hybrid CD2F₁ male mice, 10–15 weeks old, were obtained from Charles River (Calco, Italy). AKR,
B10.S male mice were kindly provided by Dr G. Parmiani, N.C.I., Milan, Italy. Low-immunogenic L1210/Cr leukaemia was maintained by weekly i.p. passage in compatible CD2F1 mice. The immunogenic L1210/DTIC sublines, obtained as previously described (Mihich & Kitano, 1971; Bonmassar et al., 1970; Nicolin et al., 1974), were grown in CD2F1 mice immunosuppressed by 200 mg/kg cyclophosphamide i.p. 24 h before challenge. The properties of the tumour cells used to assess the specificity of monoclonal antibodies are indicated in Table V and the tumours were maintained by in vivo or in vitro passages under standard methods.

Hybridization.—The hybridization technique described by Nowinski et al. (1979) has been followed, with minor modifications. Briefly, CD2F1 mice were challenged with 10⁷ viable L1210/DTIC cells i.p. on Days 0 and 7, and i.v. on Day 17. Three days later spleen cells from immune mice were mixed with BALB/c myeloma P3X63Ag8 cells in a ratio of 4:1 in serum-free RPMI 1640 (Eurobio, Paris, France). The mixture was centrifuged in 50ml plastic tubes (Falcon, Cockeysville, Md., U.S.A.) and the pellet resuspended in 50% polyethylene glycol, mol. wt 1500 (PEG 1500, Merek-Schuchardt, Hohenbrunn, W. Germany) at 37°C. The concentration of PEG was gradually decreased by the slow addition of serum-free RPMI 1640 with gentle stirring. The cell suspension was washed and resuspended in 40 ml of complete RPMI 1640 (20% foetal calf serum, Seromed, München, Germany) and distributed on 4 96-well, flat-bottomed microtitre plates (Falcon). On Days 1, 2, 4, 7 and 11 the culture medium was replaced with conditioned hypoxanthine–aminopterin–thymidine (HAT) medium (Littlefield, 1964). Two to three weeks after fusion, 100 µl of culture supernatant from wells with growing hybrids, as seen under the microscope, were collected for preliminary screening. Cytotoxic antibodies in hybridoma cultures were detected by the ⁵¹Cr-release assay, using ⁵¹Cr-labelled L1210/DTIC cells as targets (Cerottini et al., 1974). Briefly, 25 µl containing 10⁶ target cells/ml were exposed to 25 µl culture supernatant for 30 min at 37°C. An appropriate dilution of rabbit complement was added (25 µl) and cells were incubated for 30 min at 37°C. The mean cytotoxicity of triplicate samples was calculated as:

\[
\text{\textit{51}Cr released by hybridoma supernatant - \textit{51}Cr released by control} \\
\text{\textit{51}Cr released into detergent} \\
\text{\textit{51}Cr released by control} \times 100
\]

Cultures showing >15% cytotoxicity were scored as positive (Ozato et al., 1980).

Cloning and establishment of hybridoma cell lines.—Antibody-producing hybridoma cultures were cloned in a 96-well microplate by limiting dilution on a feeder layer of macrophages (0.3 to 1 cell/100 µl/well). Positive clones were grown on a larger scale and divided into 3 pools. The first pool was stored at -80°C for future use, the second injected i.p. (10⁶ cell/mouse) into BALB/c mice primed with tetramethylpentadecane (Pris- tane, Schillings, Steinheim, W. Germany) 0.5 ml/mouse i.p., to obtain large amounts of antibodies, and the last pool was maintained in vitro.

Ig class analysis.—The inhibition of cytotoxic activity after incubation with goat antisera to mouse IgM, IgG1, IgG2, IgA immunoglobulins (Meloy Laboratories, Springfield, Virginia) was used to identify the heavy-chain class of the monoclonal antibody.

Fluorescence.—Normal cells or cells of L1210/Cr leukaemia (10⁶ cells in 0.1 ml) were incubated with 20 µl of monoclonal antibody (supernatant of Clone E10 diluted 1:64) for 40 min in an ice bath. After washing, fluorescein isothiocyanate-conjugated (FITC) rabbit anti-mouse IgG (Cappel Laboratories, Cochranville, PA) was added at a 1:10 dilution and the tubes incubated for 30 min in an ice bath. The cells were washed and the fluorescence was evaluated under the 40× lens of an Olympus microscope with epifluorescence optics.

RESULTS

Cell fusion and production of the monoclonal antibody

Two weeks after fusion, hybrid cells were detected in 95% of the wells. Although after further growth in 24-well plates most of them lost their activity (Nowinski et al., 1979; Ozato et al., 1980), in the early screening about 5% of positive supernatants were obtained (specific ⁵¹Cr-release 15–60%). One of 3 definitely positive hybrids, immediately minicloned,
**Table I.—** *L1* reactivity to different sublines of *L1210* leukaemia

| Target cells       | Immunogenicity* | % 51Cr release (±s.e.) | Absorbing† capacity |
|--------------------|-----------------|------------------------|---------------------|
| *L1210/Cr*         | ±               | 80 ± 1.8               | 5 x 10⁶             |
| *L1210/Ha*         | ++              | 75 ± 1.6               | NT                  |
| *L1210/DTIC*       | +++             | 78 ± 1.2               | 5 x 10⁶             |
| *(in vitro)*       | ±               | 12 ± 0.2               | NT                  |
| *L1210/DTIC*       | +++             | 20 ± 0.2               | NT                  |
| *(in vitro)*       | ±               | 76 ± 1.9               | 10⁷                 |
| *(Clone 2)*        | +++             | 77 ± 1.3               | 10⁷                 |
| *L1210/Cr*         | ±               | 68 ± 0.9               | NT                  |
| *(Clone 4)*        | ++              | 84 ± 1.5               | 5 x 10⁶             |
| *L1210/DTIC*       | +++             | 69 ± 1.3               | 10⁷                 |
| *(Clone 5)*        | ±               | 73 ± 0.9               | 10⁷                 |

* The degree of immunogenicity was established from the *in vivo* rejection by DBA/2Cr mice (Nicolin et al., 1980).
† The number of tumour cells needed to absorb 50% of the cytotoxicity of *L1* assayed against *L1210/Cr* target cells. The source of *L1* was the supernatant from Clone E10 diluted 1:128.
‡ *L1210/Cr* clones were obtained by cloning the *in vitro* line by limiting dilution. Clones were then injected into CD2F₁ mice and maintained *in vivo* by weekly passages i.p.
§ *L1210/DTIC* clones were obtained by *in vivo* treatment of *L1210/Cr* clones with DTIC, as described by Nicolin et al. (1974).

Characterization of *L1* reactivity

As shown in Table I, *L1* is cytotoxic against both *L1210/Cr* leukaemia and its immunogenic sublines, and the less related *L1210/Ha* leukaemia. The pattern of reactivity is independent of the immunogenicity of the tumour lines, as established by rejection experiments in syngeneic mice (Kitano et al., 1972; Nicolin et al., 1980). The reactivity of *L1* against *L1210* leukaemia was confirmed by absorption at 5 cells/well (Nowinski et al., 1979), has yielded 95% positive hybrids. Cell culture were allowed to grow on a larger scale and supernatants were collected and frozen at −80°C.

Cells from 3 positive miniclones were further cloned by limiting dilution, with cloning efficiency of 10–20%, to yield 20 highly cytotoxic supernatants. Since all the supernatants showed the same pattern of reactivity and contained a monoclonal antibody of IgM subclass, a single designation, namely *L1*, has been adopted.

**Fig. 1.—** Quantitative absorption of *L1* activity by *L1210* cells. *L1* was absorbed with increasing numbers of *L1210* cells at 4°C for 1 h. Residual cytotoxicity was evaluated by a 51Cr-release assay using *L1210/Cr* cells as targets. The absorbing capacity was expressed as % inhibition of *L1* cytotoxicity. Three different dilutions of culture supernatant from Clone E10 were absorbed: ○ 1:64, △ 1:128, □ 1:256. Absorption with P815 (DBA/2) tumour cells was included as a negative control (×).
The cytotoxicity of L1 was completely absorbed by as few as $10^7$ tumour cells (Fig. 1).

The titration of L1 activity is shown in Fig. 2. The cytotoxicity was much higher in the ascitic fluid, (dilution $10^{-6} - 10^{-7}$) than in the supernatants (dilution $10^{-3}$), of in vitro hybridoma cultures. Hybrids established for more than 6 months or thawed after storage at $-80^\circ$C retained their capacity to secrete L1.

The cytotoxicity of L1 was completely removed by preincubation with goat anti-\(\alpha\) mouse Ig7S (\(\alpha\) light chains) and with goat anti-\(\alpha\) mouse (\(\mu\)-heavy chain) (Fig. 3). Since a single band has been obtained by standard immunoelectrophoresis (not reported here) and the inhibitory effects of other immunoglobulin subclasses were significatively less effective, L1 is considered to belong to the IgM subclass.

The specificity of L1 activity for L1210 leukaemia has been established by extensive screening on syngeneic and allogeneic lymphoid cells, on normal adult and foetal cells, and on a number of experimental mouse tumours.

Table II shows that spleen cells from several independent strains, both syngeneic and allogeneic, failed to remove L1 activity. The reactivity of L1 to normal and foetal tissues was studied by the indirect fluorescence method. As shown in
TABLE II.—Absorption of L1* activity by 5 × 10⁷ spleen cells from independent strains

| Strain          | Haplotype | Absorption† |
|-----------------|-----------|-------------|
| DBA/2 (L1210/Cr cells) | H-2d     | +           |
| DBA/2 (spleen cells) | H-2d     | —           |
| BALB/c          | H-2d     | —           |
| CD2F1           | H-2d/a   | —           |
| AKR             | H-2k     | —           |
| C3H             | H-2k     | —           |
| C57BL/6         | H-2b     | —           |
| B10. S          | H-2s     | —           |

* The supernatant from clone E10 diluted 1:64, assayed for cytotoxicity against ⁵¹Cr-labelled L1210/Cr target cells, was the source of L1.
† Ability to completely absorb the L1 activity, assayed as in Fig. 1.

TABLE III.—Distribution pattern of L1 antigen on DBA/2 cell surfaces

| Cell type | Normal mouse serum (± s.e.) | L1 |
|-----------|-----------------------------|----|
| L1210/Cr  | 0.6 ± 0.0-1                 | 100 ± 0-1 |
| L1210/Ha  | 0.3 ± 1-0                   | 100 ± 0-2 |
| Spleen    | 32 ± 6-9                    | 30 ± 5-4 |
| Marrow    | 11.3 ± 7-2                  | 16.4 ± 3-4 |
| Thymus    | 1.5 ± 0-2                   | 1.3 ± 0-1 |
| Foetal liver | 7.3 ± 1-0               | 8.0 ± 0-1 |
| Foetal thymus | 2.3 ± 0-8             | 4.0 ± 0-0 |

% ⁵¹Cr release by rabbit anti-α-mouse serum

| L1                       | 78.0 ± 0-9 |
|--------------------------|------------|

B-enriched population*  

* B-enriched population was obtained by treating DBA/2 spleen cells with anti-Thy-1-2 antisera and rabbit complement. Viable cells were harvested after separation on an Isopaque/Ficoll gradient. B-enriched spleen cells were labelled with ⁵¹Cr and used as targets in a complement-dependent cytotoxicity assay.

Table III, both normal and foetal tissues, including marrow, thymus, liver and spleen cells, were not stained. Moreover, since L1210 leukaemia could have originated from a B-cell lineage (Shevach et al., 1972; Cooper et al., 1977), it was relevant to study its reaction with B cells. A B-enriched population, obtained by pre-treatment of spleen cells from DBA/2 mouse with anti-Thy-1-2 serum and rabbit complement, was not lysed by L1 (Table III). In addition, L1 did not modify the activity of natural killer (NK) cells (Table IV).

Finally, the reactivity of L1 was matched (Table V) in a complement-dependent cytotoxicity test against several lines of mouse tumours, most of them of lymphoid origin, induced by either chemical agents or oncogenic viruses. The panel of tumour cells expressed T or B cell-surface antigens: namely Thy, TL, Ly antigens, immunoglobulins, Fc and C3 receptors. Moreover, the tumour cells carried the most common structural viral antigens or non-virionic specificities that accompany oncogenic virus infection. None of the tumour lines reacted with L1. Lack of reactivity against P388 and P815 tumours rules out the possibility that the specificity recognized by L1 is a surface antigen of macrophages or mast-cells. Furthermore, a B-lymphocyte tumour line obtained by infection with Abelson virus (Abelson & Rabstein, 1970) was also completely unreactive.

Because of its high specificity and its potent cytotoxicity in vitro, L1 was used for immunotherapeutic studies in L1210-bearing animals. L1 and rabbit complement were inoculated, following various schedules of treatment, into leukaemic mice challenged with L1210 cells i.p. on Day 0, but failed to improve their life
Table V.—Characteristics of tumour cells tested for reactivity to L1

| Target cells | Strain of origin | Haplotypes | Type of tumour | % 51Cr release |
|--------------|------------------|------------|----------------|----------------|
| L1210/Cr     | DBA/2            | H-2k       | Carcinogen     | 80             |
| L5178Y       | DBA/2            | H-2b       | Carcinogen     | 0              |
| EL4 (G +)    | C57BL/6          | H-2b       | Carcinogen     | 3              |
| EL4 (G -)    | C57BL/6          | H-2b       | Carcinogen     | 3              |
| P388         | BALB/c           | H-2k       | Carcinogen     | 1              |
| P815         | DBA/2            | H-2k       | Carcinogen     | 0              |
| BALB/urethan 2 | BALB/c       | H-2k      | X-rays         | 2              |
| RL d 1       | BALB/c           | H-2k       | Gross virus    | 2              |
| E 5 G2       | C57BL/6          | H-2k       | Spontaneous    | 0              |
| K36          | AKR              | H-2k       | Moloney virus  | 1              |
| LSTRA        | BALB/c           | H-2k       | Moloney virus  | 1              |
| GLV          | C3H              | H-2k       | Moloney virus  | 3              |
| Abelson      | BALB/c           | H-2k       | Prednisolone   | 1              |

Table VI.—Life span of L1210 leukaemic CD2F1 mice treated with L1 and complement

| L1210/Cr | L1* (0.2 ml/mouse) | Rabbit complement† (0.4 ml/mouse) | Mean survival in days |
|----------|--------------------|-----------------------------------|----------------------|
|          | Day 0             | Days and route of treatment       | Days and route of treatment |
| 10^3     | 1, 3, 5 i.p.      | —                                 | —                    |
| 10^5     | 1 i.p.            | 1, 2, 3, 4, 5 i.p.                | 1, 2, 3, 4, 5 i.p.   |
| 10^6     | 1, 3, 5 i.p.      | 1, 2, 3, 4, 5 i.p.                | 1, 2, 3, 4, 5 i.p.   |
|          | 1, 3, 5 i.v.      | 1, 2, 3, 4, 5 i.p.                | 1, 2, 3, 4, 5 i.p.   |

* The ascitic fluid of pristane-primed BALB/c mice challenged with producing clones was the source of L1. The in vitro cytotoxicity was at 10^-6 dilution.
† Checked in vitro for complement activity.

span over that of untreated animals (Table VI).

DISCUSSION

The L1210 lymphoblastoid cell line is a mouse lymphoma originally induced in DBA/2 mice by exposure to methylcholanthrene (Law et al., 1949). As a consequence of several generations of transplants carried out in different laboratories, L1210 sublines have been originated with different degrees of immunogenicity in syngeneic hosts. The line L1210/Cr used in this study is a tumour that is weakly immunogenic in eliciting both in vitro immunoreactivity and in vivo rejection. Although tumour-associated antigens in transplantable tumours have been the object of a number of studies, their intimate features are still quite undefined. The availability of monoclonal antibodies might facilitate the biochemical and functional characterization of TAA. In the present report we describe an IgM monoclonal antibody (L1) strictly specific for an antigen associated with L1210 leukaemia. The antibody (L1) obtained from ascitic fluid has shown high cytotoxicity at dilutions as high as 10^-6-10^-7. The L1 antigenic specificity was expressed by every cell in the L1210 population, all of which stained in the indirect fluorescence assay. The L1210 cells cultured in vitro showed decreased susceptibility to L1 cytotoxicity, probably because of increased shedding of the antigen into the supernatant (unpublished observations from this laboratory).
Extensive screening with normal and malignant cells ruled out any reactivity of L1 to histocompatibility antigens, to antigens defining different subsets of lymphoid cells and to surface determinants of macrophages, mast cells, or marrow cells. Moreover, L1 did not recognize foetal tissues, namely liver, thymus and spleen, with which L1210 leukaemia might share some differentiation antigens. Finally, a panel of laboratory tumour cells, transformed by different mechanisms and expressing on their surfaces a number of antigenic determinants and receptors, did not express the antigen recognized by L1. The specificity of L1 to L1210 leukaemia cells was established by its lack of reactivity with a variety of target cells and from the schedule of hybridization (syngeneic immunization and mouse–mouse fusion). In spite of the high in vitro cytotoxicity of L1 and lack of toxicity to the host tissues, attempts at immunotherapy of L1210 mice were unsuccessful. Other sche 'ules of treatment, not reported here, were also ineffective. Complement inactivation in vivo, neutralization of L1 by antigens circulating in the blood or present in the ascitic fluid are first among the hypotheses that should be experimentally tested in attempts to explain the negative in vivo findings. Antigenic modulation by L1 itself, or poor binding to target tumour cells, might also be worth attention. Whatever the reason for the failure of these and other (Houston et al., 1980; Ritz et al., 1980) preliminary attempts at serological therapy, the exploitation of the monoclonal antibody technology in experimental cancer might be furthered by studies with L1. Since L1210 leukaemia has been widely used over the last 30 years and there is good knowledge of its in vivo and in vitro biological properties, studies with this experimental model might easily and quickly provide some essential pieces of information. The high specificity of L1, and its lack of reactivity to normal tissues, might make it an optimal tool in carrying toxic agents to target cells or developing experimental methodologies for highly sensitive immunodiagnosis.

This research was supported in part by PFCCN from C.N.R., Rome, Italy.

We wish to thank Dr Giorgio Corte, Istituto di Biochimica, Genoa, Italy, for the generous gift of P3X63Ag8 myeloma cells.

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