Serotherapy of L1210 murine leukaemia—reasons for ineffectiveness of *in vivo* treatment by L.1 monoclonal antibody

C. Testorelli, G. Canti, P. Franco, A. Goldin¹ & A. Nicolin²

Institute of Pharmacology, School of Medicine, Milan, Italy, ¹N.C.I., N.I.H., Bethesda, Maryland, USA & ²Institute for Cancer Research and Treatment, Genoa, Italy

Summary A monoclonal antibody (L.1), reacting *in vitro* specifically with L1210 leukaemia cells in a complement-dependent cytotoxicity assay (CDC), has been exploited for serotherapy studies. Different regimens of L.1 treatment of CD2F₁ mice bearing the semi-syngeneic L1210 leukaemia did not prolong the life span of tumor-bearing animals. Moreover, the administration of L.1 did not enhance the antitumour effects of cyclophosphamide. Studies of *in vivo* localization showed that L.1 was able to bind specifically to L1210 leukaemic cells, although 30–40% of the cells remained negative. The presence of L.1 in mouse blood was demonstrated up to 15 days after the inoculation. On the other hand, *in vivo* administration of L.1 was probably accompanied by loss of the cytotoxic activity, perhaps through a mechanism of complement inactivation, since the presence of undiluted normal mouse serum in a CDC assay inhibited the cytotoxic activity of L.1. Moreover, serum from L.1-treated mice did not display any cytotoxic activity, although the presence of the antibody could be demonstrated by indirect immunofluorescence.

Shedding of the antigen defined by L.1 was probably not responsible for the failure of the serotherapy, since the L.1 neutralizing antigen could be found in body fluids only long after the start of therapy.

The treatment of malignant diseases by passive administration of antibodies (Wright & Bernstein 1980) has been pursued as a promising therapy, although the life span after antiserum administration has not so far been improved. The failure of conventional serotherapy has been mainly attributed to the low specificity of the antisera and the poor immunogenicity of tumour-associated antigens (TAA). The new era in serology since hybridoma technology (Köhler & Milstein 1975) was devised might be exploited for new modalities of cancer immunotherapy.

Tumour-specific antigens and specific antibodies are the basic necessities for successful passive immunotherapy of cancer. Although tumour-specific antigens in human tumours (Metzgar *et al.*, 1981; Iwaki *et al.*, 1982; Ball *et al.*, 1982) have not yet been definitely demonstrated, monoclonal antibodies (MAb) highly specific to TAA (Gunn *et al.*, 1981) or to tissue and differentiation antigens (Kirch & Hammerling, 1981) might provide optimal tools to study the anti-tumour efficacy of passive serotherapy (Kirch & Hammerling, 1981; Bernstein

et al., 1980; 1980; Scheinberg & Strand 1982; Ritz *et al.*, 1981; Trowbridge & Lopez, 1982; Sears *et al.*, 1982).

In a previous report from this laboratory (Testorelli *et al.*, 1982), the production and the characterization of a MAb reacting specifically with the cells of the L1210 murine leukaemia (L.1) was described. The L.1 MAb displayed *in vitro* very high and specific complement-dependent cytotoxicity against L1210 cells. On the other hand, it did not react with normal adult and foetal murine tissues, with cells from several independent haplotypes or with a number of chemical- and virus-induced experimental tumours.

In the present report, the therapeutic effects of the administration of L.1 alone or combined with chemotherapeutic agents on the survival rates of leukaemic mice are presented. Possible reasons for the ineffectiveness of serotherapy with L.1 were also investigated and are discussed.

Materials and methods

Animals and tumours

Inbred DBA/2, Balb/c and hybrid (Balb/c × DBA/2) CD2F₁ male mice, 10–15 weeks old, were obtained from Charles River (Calco, Italy). L1210/Cr leukaemia was maintained by weekly passages (i.p.) into compatible CD2F₁ mice. Balb/c or CD2F₁

Correspondence: C. Testorelli; Institute of Pharmacology, School of Medicine, Via Vantivelli 32, 20129 Milano, Italy.

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mice were used to produce antibody-containing ascites.

Hybrid cell lines and production of ascitic fluid

The production of the hybridoma cell line secreting the monoclonal antibody L.1 (IgM) to a surface antigen of L1210 cells has been described previously (Testorelli *et al.*, 1982). The hybridoma was maintained in culture in RPMI 1640 medium (Eurobio, Paris), supplemented with 10% heat-inactivated foetal calf serum (FCS, Seromed GmbH, München, Germany) and $100 \mu\text{l ml}^{-1}$ gentamycin (Seromed). The supernatant from the cultures was stored at -70°C . For production of ascitic fluid, Balb/c or CD2F₁ mice, preprimed with pristane (0.5 ml, i.p.), were injected i.p. with 10^6 hybrid cells. Ascitic fluid was collected after 15–20 days, centrifuged to remove the cells and frozen at -70°C until use. The cytotoxic titers of both supernatant and ascitic fluid were assessed by a complement-dependent cytotoxicity assay (see below) using ^{51}Cr -labelled L1210 cells as target. Titres are expressed as the highest dilution that killed 50% of leukaemic cells. When not otherwise specified, the cytotoxic titre was 1:64 for the supernatant from the hybridoma culture and 10^{-6} for ascites.

Complement-dependent cytotoxicity assay (CDC)

^{51}Cr -release assay was performed as described elsewhere (Testorelli *et al.*, 1982). Briefly, $25 \mu\text{l}$ of target cells labelled with [^{51}Cr] sodium chromate were incubated with $25 \mu\text{l}$ of the L.1 (P3X63/Ag8 supernatant was used as control) for 45 min at 37°C . Rabbit complement diluted 1:5 was then added ($25 \mu\text{l}$) and the cells were incubated for 30 min at 37°C in a humidified 5% CO_2 atmosphere. The percentage of ^{51}Cr release into the supernatants of triplicate samples was calculated according to Ozato *et al.* (Ozato *et al.*, 1980).

Indirect membrane immunofluorescence (IMF)

The L1210 cells were tested after extensive washing for their viability (cells $<95\%$ viable were excluded) and incubated with $20 \mu\text{l}$ of supernatant from the hybridoma culture for 40 min at 4°C . The cells were washed twice and subsequently incubated with 0.15 of a 1:10 dilution of fluorescein isothiocyanate-conjugated (FITC) rabbit anti-mouse IgG (Cappel Lab., Cochranville, PA) for 30 min at 4°C . The cells were washed $3 \times$ and analyzed under a microscope with epifluorescence optics and in the fluorescence-activated cell sorter (FACS II, Becton Dickinson, Mountain View, Calif.).

Serotherapy of L1210-bearing mice with L.1

CD2F₁ were challenged i.p. with different numbers of L1210 cells (day 0). Experimental animals were given injections of L.1 (ascitic fluid containing $30 \text{ mg MAb ml}^{-1}$) alone or combined with rabbit complement, starting 24 h after challenge. Different schedules of treatment were adopted. In the combined therapy, L.1 was given i.p. with cyclophosphamide 120 mg kg^{-1} s.c. Tumour growth was evaluated by recording the median survival time (MST) and the number of dead animals per group (D/T) of control and treated animals.

In vitro evaluation of serotherapy

In vivo binding of L.1 to L1210 At different intervals after the i.v. infusion of L.1 into L1210-bearing mice, tumour cells were collected from peritoneal cavity and divided into 2 pools. The first pool was analysed by IMF for the presence of L.1 on the cells surface. The cells from the second pool were labelled with ^{51}Cr and assayed for their susceptibility to lysis by the CDC system.

Detection of L.1 in the serum of normal mice Blood samples from CD2F₁ mice infused i.v. with L.1 were collected at different times after the injection and the serum tested for the presence of L.1 by both the IMF and CDC systems.

Detection of circulating antigen in L1210 mouse fluids The presence of the antigen defined by L.1 in the serum and in the ascites of L1210-bearing mice was detected by incubating at 4°C for 45 min an appropriate dilution of L.1 (supernatant from hybridoma culture) with an equal volume of serum or ascites (undiluted, 1:2, 1:4 etc.) from mice at different days after tumour challenge. The resulting L.1 cytotoxic activity was assayed against L1210 target cells.

Inhibition of complement activity by normal mouse serum Normal sera from different strains of mice were mixed for 45 min at 4°C with ^{51}Cr -labelled L1210 cells (10^6) and L.1 culture supernatant. The mixture ($50 \mu\text{l}$) was seeded in a 96-well microplate, complement was added ($25 \mu\text{l}$) and the cytotoxic activity of L.1 was evaluated as reported above (CDC).

Results

Effects of L.1 administration on survival of leukaemic mice

The results of passive serotherapy of leukaemic mice with L.1 and rabbit serum, as source of

Table I Serotherapy of L1210-leukaemic CD2F₁ mice with L.1 and complement

<i>L1210</i> Challenge, i.p.	<i>L.1</i> ^a Day + 1 ml/mouse, i.p.	<i>Rabbit</i> <i>complement</i> ^b Day + 1 ml/mouse, i.p.	<i>MST</i> ^c (range)	<i>D/T</i> ^d
10 ³ *	—	—	12(11–14)	8/8
10 ³ *	1.0	—	13(11–14)	8/8
10 ³ *	—	0.4	13(12–14)	8/8
10 ³ *	0.01	0.4	13(12–14)	8/8
10 ³ *	0.1	0.4	12(12–13)	8/8
10 ³ *	1.0	0.4	12(12–14)	8/8

^aThe source of L.1 was an ascitic fluid containing 30 mg antibody ml⁻¹ with a cytotoxic titre of 10⁻⁶.

^bComplement activity was checked *in vitro*.

^cMST: Median Survival Time (days).

^dD/T: Dead Mice/Treated Mice.

*The same treatment to mice challenged with 10⁵ L1210 cells was ineffective.

complement, are reported in Tables I and II. The life spans of CD2F₁ mice, challenged with as few as 10³ L1210 cells, was not improved by treatment with different amounts of L.1 plus 0.4 ml of complement. No increase in survival time of leukaemic mice was obtained with the treatment schedules in Table II, either. In order to see

whether the MAb acted synergistically with a chemotherapeutic agent, leukaemic mice were treated with an effective dose of cyclophosphamide plus L.1. As shown in Table III serotherapy did not significantly increase the survival time over cyclophosphamide alone.

L.1 binding to L1210 cells in vivo

In order to study the reasons for the failure of L.1 therapy, experiments were designed to determine whether the L.1 could bind specifically to tumour cells *in vivo*. Figure 1 shows that, although 24 h after L.1 administration the majority of L1210 cells were stained, a remarkable proportion of unstained cells was still detectable. Moreover, the fluorescence intensity of stained cells was never >20% of that of L1210 cells treated *in vitro* with L.1. The % of +ve cells and fluorescence intensity were completely restored when L1210 cells interacting with L.1 *in vivo* were further exposed to the MAb *in vitro*.

Results of time-course experiments are also illustrated in Figure 1. The % of L.1 +ve cells analysed by automatic flow fluorocytometry peaked

Table II Serotherapy of L1210-leukaemic CD2F₁ mice with L.1 and complement

<i>L1210</i> Challenge i.p.	<i>L.1</i> ^a Days and route of treatment 0.5 ml/mouse	<i>MST</i> (Range)	<i>D/T</i>
10 ³ *	—	13(12–13)	8/8
10 ³ *	1, 3, 5 i.p.	14(11–15)	8/8
10 ³ *	1, 3, 5 i.v.	12(11–13)	8/8
10 ³ *	1→7 i.p.	14(13–15)	8/8
10 ³ *	1→7 i.v.	14(12–15)	8/8

^aSee Table I. Rabbit serum as source of complement (0.4 ml/mouse, i.p.) was given after every L.1 inoculation.

*The same treatment in mice challenged with 10⁵ L1210 cells was ineffective.

Table III Chemotherapy plus serotherapy of L1210-leukaemic CD2F₁ mice

<i>L1210</i> Day 0 Challenge i.p.	<i>Cy</i> ^a Day + 1 120 mg Kg ⁻¹ s.c.	<i>L.1</i> ^b Day + 2 0.5 ml/mouse i.v.	<i>MST</i> (Range)	<i>D/T</i>
10 ⁵	—	—	9(8–10)	8/8
10 ⁵	+	—	12(11–14)	8/8
10 ⁵	—	+	10(9–10)	8/8
10 ⁵	+	+	11(10–13)	8/8

^aCyclophosphamide.

^bL.1 and rabbit complement; see footnote Table II.

Figure 1 Binding of L.1 to L1210 cells *in vivo*. L1210 leukaemic cells, obtained from tumour-bearing animals at different intervals from an i.v. administration of L.1 (0.2 ml of ascites diluted 1:4) were analyzed for the presence of the MAb on their surface by IMF. The fluorescence staining (—) and the fluorescence intensity (----) of L1210 leukaemic cells treated *in vivo* with L.1 (●) and further exposed to L.1 *in vitro* (○) are reported. Tumour cells from untreated animals were used as -ve controls.

Figure 2 *In vitro* lysis of L1210 cells treated *in vivo* with L.1. The *in vivo* treatment was performed as reported in Figure 1. L1210 tumour cells collected from L.1 treated mice were labelled with ^{51}Cr and assayed in the CDC assay in the presence of rabbit complement alone (●) and after further exposure to L.1 *in vitro* (○) (supernatant from culture). Controls were performed using L1210 cells from untreated mice incubated *in vitro* with rabbit complement alone (×) or with L.1 + complement (*).

24 h after the L.1 inoculation and decreased gradually, although 15% of +ve cells were still detected 6 days after the treatment. As shown in Table IV, the amount of L.1 bound to L1210 cells after *in vivo* treatment was dependent upon the dose of the antibody injected. Findings in keeping with those reported in Figure 1 were obtained in the cytotoxic assay. (Figure 2). A % of tumour cells derived from L1210-bearing mice previously treated with L.1 underwent lysis upon *in vitro*

incubation with rabbit complement. However, full susceptibility to complement-mediated lysis was obtained upon cell re-exposure to L.1 *in vitro*.

Studies of L.1 properties after in vivo administration

The time course of blood L.1 levels in CD2F₁ mice, as evaluated by IMF (dashed line) or by the ^{51}Cr -release assay (full line), was studied (Figure 3).

Table IV Binding of L.1 to L1210 cells *in vivo*; dose-response relationship

L.1 ^b antibody dilution	Fluorescence % stained cells ^a	Dye Test %dead cells ^a
—	3±0.1	9±0.3
10 ⁰	64±6.9	78±0.8
10 ⁻¹	67±6.2	75±2.5
10 ⁻²	34±7.6	33±1.3
10 ⁻³	18±4.1	24±0.9
10 ⁻⁴	6±0.3	19±0.1

^aMean ± s.e..

^bTumour-bearing mice were infused i.v. with L.1 (ascites, 0.5 ml/mouse) and, 24 h later, L1210 leukaemic cells were collected and assayed for the presence of L.1 by IMF and by the Dye exclusion test of Gorer & O'Gorman (1956).

Figure 3 Blood kinetics of L.1 in CD2F₁ mice. Serum samples from CD2F₁ mice i.v. infused with 1 ml of L.1 (0.2 ml of ascites diluted 1:4) were analyzed for the presence of the MAb in the IMF (●----●) and CDC (●—●) assays at different times after inoculation.

L1210 cells incubated with serum from L.1 treated mice were stained positively in the IMF assay. After 4 days, the binding activity dropped progressively, although it was still detectable 15 days after L.1 inoculation. In sharp contrast, no cytotoxic activity was present in serum from L.1-treated mice. The possibility that complement inactivation by mouse serum was responsible for the *in vivo* loss of L.1 cytotoxic activity, was investigated. Serum samples from CD2F₁ mice inoculated i.v. with rabbit complement did not show any complement activity in the CDC (data not shown). Moreover, the presence of undiluted mouse serum in the CDC assay inhibited the cytotoxic reaction, whatever the source of complement (Table V). However, a normal lytic reaction was observed when the mouse serum was removed before the addition of complement.

L1210 antigen in mouse fluids

The detectability of antigen recognized by L.1 shed by tumour cells into the blood or the ascites of

Table V Inhibition of complement activity by normal mouse serum

Serum from mouse strain	Source of complement*	cpm $\pm$ s.e.	% ^{51}Cr release
—	rabbit	2495 $\pm$ 207	80.3
CD2F ₁	rabbit	430 $\pm$ 38	0.2
CD2F ₁	rabbit	426 $\pm$ 41	0
CD2F ₁	rabbit	448 $\pm$ 39	0
CD2F ₁	guinea pig	428 $\pm$ 46	0
CD2F ₁	guinea pig	431 $\pm$ 37	0.2
CD2F ₁	human	445 $\pm$ 48	0.8
DBA/2	rabbit	412 $\pm$ 40	0
Balb/c	rabbit	429 $\pm$ 45	0
C57B1/6	rabbit	410 $\pm$ 28	0
C3H/f	rabbit	425 $\pm$ 33	0
CD2F _{1(w)}	rabbit	2360 $\pm$ 250	75.5
CD2F _{1(w)}	guinea pig	2405 $\pm$ 198	77.2
CD2F _{1(w)}	human	2381 $\pm$ 279	76.3

The cytotoxic activity of L.1 was evaluated in a CDC with L1210 target cells, in the presence of normal serum from different strains of mice as described in **Materials and Methods**. When indicated (w), L.1 and mouse serum were washed off before adding the complement. L.1 (supernatant from hybridoma culture) was present in all samples. Replacement of L.1 with rabbit anti mouse serum caused similar results.

*Sources of rabbit complement were selected from non-cytotoxic blood samples from our breeding colony. Guinea pig sources were purchased (Bio Mérieux, France). Human complement was from healthy donors. All samples were assayed *in vitro* for complement activity before use, and for each complement the appropriate dilution was selected. This Table uses rabbit complement as a +ve control.

L1210-bearing mice was examined. Inhibition of cytotoxic activity of L.1 by serum and ascites fluid from leukaemic mice obtained at varying intervals after the challenge is reported in Figure 4. The inhibitory capacity of mouse fluids, not detectable until 4 days after the tumour inoculation, increased rapidly thereafter and was more impressive for the ascites fluid than for the serum. Titration of soluble antigen (Figure 5) showed that a relatively large amount of free antigen was detectable in the ascites fluid on day 7, whereas a lesser degree of inhibitory activity was demonstrable on day 5.

Figure 4 Inhibition of the cytotoxic activity of L.1 by ascites and serum from L1210-bearing mice. The supernatant from the hybridoma culture was incubated at 4°C for 45 min with the serum (●—●) and the ascites (●---●) from L1210-bearing mice on different days after the challenge i.p. with 10⁴ leukaemic cells. The mixture was added to L1210-labelled cells and incubated at 37°C (45 min). After washing an appropriate dilution of complement was added as described for the CDC assay. The results are expressed as % inhibition of L.1 cytotoxicity.

Discussion

In principle, specific and cytotoxic MAb should provide selective bullets for the *in vivo* eradication of cancerous cells leaving normal cells undamaged. These theoretical conditions, hypothesized since the Ehrlich era, were obtained in our laboratory by the production of L.1. In fact, L.1 is absolutely specific to L1210 leukaemic cells and as an IgM immunoglobulin displays great efficiency in fixing complement (Testorelli *et al.*, 1982). Furthermore, since the CD2F₁ mice used in these studies were of

Figure 5 Inhibition of the cytotoxic activity of L.1 by cell-free ascites from L1210-bearing mice. Varying dilutions of cell-free ascites from L1210-bearing mice challenged i.p. with 10^4 leukaemic cells were incubated at 4°C for 45 min with the supernatant from the hybridoma culture. The residual cytotoxic activity of L.1 was determined in a CDC assay as reported in Figure 4. The inhibitory activity of the ascites obtained on Day 5 (●—●) and on Day 7 (●---●) after the L1210 challenge is illustrated.

identical haplotype to that of the L.1 hybridoma cells, strictly syngeneic conditions could be obtained. However, even under these optimal theoretical conditions, L.1 serotherapy was almost completely ineffective in increasing the survival time of L1210-bearing CD2F₁ mice. Variations of the schedule of route or treatment, of the amounts of antibody or of complement were consistently unsuccessful. One reasonable cause for failure, the low level of endogenous complement in mice, was counteracted by excessive inoculation of exogenous complement, but this manoeuvre was without improvement. Since the activation of an endogenous immune response (Kirch & Hammerling, 1981) is a possible effector mechanism of serotherapy, L.1 might synergize with an effective antitumour drug. Following this line, a number of treatments with cyclophosphamide or with other anticancer compounds (not reported here) were combined with L.1 serotherapy, without any additional increase in the life-span of the mouse.

To account for the failure of L.1 serotherapy several experiments were devised. The first set was conceived to see whether or not L.1 given i.v. to tumour-bearing mice bound to peritoneal L1210 cells. Although the results were positive (Figure 1) a lower percentage than expected of dead cells and of fluorescent cells was regularly observed. It is

noteworthy that the fluorescence intensity of *in vivo* L.1-treated cells was 80% below the intensity achieved in the *in vitro*-treated control cells. Decreased susceptibility to *in vitro* complement-mediated lysis by L1210 cells exposed *in vivo* to L.1 was confirmed by the ^{51}Cr -release assay. Finally, we found that the susceptibility to both complement-mediated lysis and to staining with a second antibody were completely restored after *in vitro* re-exposure to L.1.

The *in vivo* studies suggest the following conclusions: (a) a fraction of L1210 cells did not bind L.1 *in vivo*; (b) the amount of L.1 bound to the surface cells +ve by IMF was less than for *in vitro*-treated controls, as demonstrated by the lower fluorescence intensity; (c) the *in vivo* L.1 +ve cells, in spite of their low fluorescence intensity, were still susceptible to *in vitro* lysis in presence of an appropriate source of complement; (d) the antigenic specificity defined by L.1 did not undergo modulation, since fluorescence intensity and susceptibility to lysis were fully recovered after *in vitro* re-exposure to L.1; (e) cells -ve by IMF and those resistant to complement-mediated lysis might belong to the same subpopulation.

Although blood clearance of L.1 in the mouse had the half life already described by others for the same antibody class (Bernstein *et al.*, 1980; Kirch & Hammerling 1981), blood serum samples containing L.1 and complement did not lyse L1210 cells *in vitro*. Furthermore, complement from different sources mixed with mouse serum *in vitro* was completely inactivated.

Antigen shedding cannot be responsible for the failure of L.1 serotherapy since, at the start of serotherapy, soluble antigen was not found in body fluids. L.1 inhibition by mouse serum and even more by ascites fluid from tumour-bearing mice became evident 4 days after L1210 challenge. Therefore, circulating antigen might negatively influence late treatments only.

The experimental system used in these studies although adhering to optimal theoretical conditions, did not bring about increased survival time for leukaemic mice. Incomplete antibody binding to target cells and blood inactivation of complement might be major factors in these disappointing results.

A number of claims (Kirch & Hammerling, 1981; Bernstein *et al.*, 1980; Scheinberg & Strand 1982; Miller & Levy, 1981; Ritz *et al.*, 1980; Trowbridge & Lopez 1982; Herlyn & Koprowski, 1981), of therapeutic benefit from the use of MAb in cancer therapy have been reported recently in the literature. Partial successes in tumour systems other than that used here could be attributed to different Ig isotypes, acting through an antibody-dependent

cellular cytotoxicity (ADCC) rather than complement-mediated lysis. The limited achievements of this approach might result in greater efforts (Pimm *et al.*, 1982; Raso *et al.*, 1982)

to exploit monoclonal technology to carry toxic compounds specifically to their target cells.

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