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Growth and Antigenic Properties of a Spontaneously Regressing Subline of Leukemia L1210*

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Abstract—*A subline (1210/MR) of the lethal parental L1210 leukemia cell line has been demonstrated to undergo dose-dependent spontaneous regression in both syngeneic and hybrid mice, although the regressing and non-regressing cells differed only slightly in growth rate. The regressed mice, like those actively immunized, were specifically protected from the subsequent challenge with L1210 cells. In addition, L1210/MR cells when coinjected could protect CD2F$_1$ mice from low but lethal doses of L1210 cells. These two cell lines shared at least one cross-reacting cell surface antigen as determined by an isologous anti-L1210/MR immune serum. Such immune serum after being absorbed by L1210 cells still retained its* in vitro *complement-dependent cytotoxicity against L1210/MR cells. We conclude that the cell lines are antigenically related but L1210/MR cells may possess additional novel antigens, and that spontaneous regression of L1210 may be an L1210-specific immunological phenomenon.*

INTRODUCTION

THE PHENOMENON of spontaneous regression of cancer was first documented in detail by Everson and Cole [1]. Amongst a number of causative factors which may be involved, stimulation of the immune process is considered as the major cause in most of the regressions [2–6]. This has raised the hope that such cancer models may provide the opportunity for identification of specific immune mechanisms for the regressions. To date, only a few transplantable and heritable spontaneously regressing cancer models are available. That includes a murine erythroleukemia induced by the regressing strain of Friend virus which is more highly immunogenic than the conventional strains of Friend virus [6, 7], and a series of murine lymphoma lines (L1210, L5178Y and LSTRA) which became highly immunogenic and heritable after many transplant generations in drug-treated syngeneic recipients [8–11] or *in vitro* [12]. Recently this laboratory has characterized the immunological factors associated with a spontaneously regressing subline of L1210 leukemia [13, 14]. In this report the growth and antigenic properties of the regressing and the non-regressing L1210 cell lines have been compared.

MATERIALS AND METHODS

Animals and cell lines

Eight to twelve-week-old female DBA/2J and C3HeB/FeJ mice were purchased from the Jackson Laboratory, Bar Harbor, ME. CD2F$_1$ hybrids (BALB/c × DBA/2, 20–25 g) were obtained from Laboratory Supply Co., Indianapolis, IN. CD2F$_1$ mice accepted skin grafts from DBA/2J females.

The sources of the parental L1210 (or P), the spontaneously regressing subline, L1210/MR (or MR), and the P815-X2 mastocytoma have been described in detail elsewhere [14]. The cell lines were routinely passaged in irradiated (650 R) DBA/2J mice in ascites form.

Ascitic fluids and tumor cell-free extracts

Each of the irradiated (650 R) mice were inoculated i.p. with MR or P cells (1×10^6). Seven to ten days later the cells and ascitic fluid were harvested from the peritoneal cavity with Hank's

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balanced salt solution (HBSS, Ca^{2+}- and Mg^{2+}-free, Grand Island Biological Co., Grand Island, NY). The viability of cells was usually better than 90% as estimated by the trypan blue exclusion method. The cell suspension was centrifuged at 5000 *g* for 20 min and the clear supernatants thus obtained were respectively designated as MR-AF and P-AF. The tumor cell pellets obtained above were washed with HBSS at 5°C, resuspended in small volumes of HBSS and then lysed by freeze–thaw procedures. The clear supernatants (designated as MR-CE and P-CE) were obtained by centrifugation (10,000 *g* for 20 min) to remove cell debris.

Isologous immune serum

$CD2F_1$ mice were inoculated subcutaneously in the back with irradiated (7000 rad) MR or spleen cells (1×10^7 cells per 0.2 ml RPMI-1640 medium) emulsified with complete Freund's adjuvant at a volume ratio of 1:1. Each mouse was boosted twice i.p. with 1×10^7 irradiated MR or spleen cells in HBSS at 3-week intervals. The animals were bled 7 days later and the sera pooled.

Complement-dependent cytotoxicity assay

P or MR cells were radiolabeled by incubating 0.5 ml of cells ($\sim 0.5 \times 10^7$ cells/ml) with 0.5 ml sodium [^{51}Cr]-chromate for 60 min at 37°C in a 5% CO_2 incubator. Cells were washed in RPMI-1640 and counted for radioactivity incorporated (as total release).

Equal volumes of chromated target cells (1×10^5/100 μl) and serially diluted isologous anti-MR serum (heat-inactivated, absorbed by DBA/2 spleen cells and with or without absorption by $2.5–5 \times 10^7$ P cells/ml serum) were incubated in the presence of 20 μl complement for 1 hr at 37°C. Guinea pig serum was used as the complement source previously absorbed with DBA/2J spleen cells. After incubation the mixtures were centrifuged to collect 100 μl of supernatants and counted for radioactivity released (specific release). To obtain spontaneous release HBSS was used instead of anti-MR serum. The percentage of specific lysis was calculated as follows:

$$100 \times \frac{\text{(specific release)–(spontaneous release)}}{\text{(total release)–(spontaneous release)}}.$$

Other methods

Papain digestion of washed MR cells were carried out according to Eady *et al.* [15]. After digestion about 90% of the treated cells were still viable and the soluble products collected by centrifugation.

Gel immunodiffusion and immunoelectrophoresis were carried out in a 1% agarose gel plate as previously described [16].

RESULTS

Comparative growth properties of regressing and non-regressing L1210 cells in syngeneic and hybrid mice

The results in Table 1 demonstrate that (i) all the P-mice invariably died with ascites tumor between 7 and 13 days, whereas mice transplanted either i.p. or i.m. with MR cells underwent dose-dependent spontaneous tumor regression; and (ii) $CD2F_1$ mice are more resistant to MR tumor than DBA/2 mice. DBA/2 mice which received higher doses (1×10^6, experiment No. 1) of MR cells had 100% mortality but died at a much slower rate; some survived for as long as 31 days after tumor transplantation.

MR cells taken from normal or irradiated DBA/2 mice retain the same growth characteristics in both normal DBA/2 and $CD2F_1$ when transplanted again (data not shown). This rules out the possibility that MR cells could only undergo a limited number of mitotic divisions *in vivo*.

The phenomenon of MR regression appears to be an immunologically mediated phenomenon, since all the irradiated (650 rad) $CD2F_1$ or DBA/2 mice injected with MR cells (1×10^5–1×10^6) invariably died of the tumor within 7 to 14 days (data not shown).

Immunoprophylactic property of MR tumor

$CD2F_1$ mice which survived for more than 4 months after a single i.p. inoculation of 1×10^4–1×10^7 MR were again challenged i.p. with 1×10^6 P or P815 cells per animal. As shown in Table 2, mice which survived higher doses of MR cells were better protected against P cell challenge. All the untreated controls similarly challenged died of P tumor within 12 days after inoculation. The regressed mice and those which were actively immunized by i.p. inoculation of irradiated (10,000 rad) P815-X2 mastocytoma cells (1×10^7) were not protected from P815-X2 and P cells respectively. These results suggest that MR may share common cell surface tumor-associated antigens with P cells and may provide specific anti-L1210 tumor immunity in these animals.

Occurrence of distinct tumor-associated antigens in the regressing and non-regressing L1210 cells and the induced cell-free ascitic fluids

As judged by the gel immunodiffusion method, an isologous immune serum raised against MR cells did not cross-react with normal mouse serum, ascitic fluid induced by complete Freund's

Table 1. Survival of DBA/2 and CD2F$_1$ mice after the inoculation of L1210/MR and L1210 cells

Experiment No.*	No. of cells inoculated	CD2F$_1$ Average survival, days†	CD2F$_1$ Percentage of 90-day survivors	DBA/2 Average survival, days†	DBA/2 Percentage of 90-day survivors
1	1×10^5 MR	>90.0	100	78.0	80
	1×10^5 P	11.7	0	9.6	0
	1×10^6 MR	>112.0	100	19.7	0
	1×10^6 P	11.0	0	8.8	0
2	5×10^6 MR	23.3	40		
	5×10^6 P	8.8	0		
3	6×10^4 MR	>90.0	100		
	6×10^5 MR	>90.0	100		
	6×10^6 MR	67.0	80		
	6×10^7 MR	8.3	40		

*In experiments Nos 1 and 3 mice were inoculated i.p. and in experiment No. 2 mice were injected i.m. with the number of cells indicated. There were 10 mice per group.

†Average survival values include only mice that died within 90 days after inoculation of the leukemia cells.

Table 2. Survival of CD2F$_1$ mice previously exposed to different doses of MR cells and then rechallenged with L1210 or P815 mastocytoma cells

Mice groups*	Average survival, days†	90-day survivors
1: 10^7 MR survivors	>90	9/9
2: 10^6 MR survivors	>90	10/10
3: 10^5 MR survivors	47	10/12
4: 10^4 survivors	29.5	8/10
5: 10^6 MR survivors rechallenged with 1×10^6 P815	13.7	0/9
6: 10^7 irradiated P815 immunized mice rechallenged with 1×10^6 P	11.7	0/10
7: 10^6 P	11	0/10

*Mice in groups 1–4 were rechallenged with 1×10^6 P cells. There were 9–12 mice per group.

†Average survival values include only mice that died within 90 days after inoculation of P or P815 cells.

adjuvant, normal spleen cell-free extracts or cell-free ascitic fluids of mice bearing the P815 mastocytoma, the EL4 thymoma and a mouse ovarian tumor. However, this immune serum recognized a common cross-reacting component in both MR-CE and MR-AF and an extra component in MR-AF. The common antigen can also be obtained by limited proteolysis of the MR cell surface with papain. A control supernatant similarly prepared in the absence of papain did not contain any cross-reacting materials detectable by this anti-MR serum. Therefore one of the MR tumor-associated antigens appears to be a cell surface antigen which is shedded into ascitic fluid (data not shown).

The anti-MR serum also cross-reacted with two components in P-CE, and at least one cross-reacting material is in complete identity with that of MR-CE and MR-AF. All the tumor-associated antigens detected by this anti-MR serum apparently have similar β_2 or γ electrophoretic mobilities at pH 8.5 (data not shown).

Differential susceptibility of MR and P cells to cytotoxicity by an isologous anti-MR serum

To further demonstrate whether MR and P cells share similar or different tumor-associated antigens a sensitive chromium release assay was carried out. Data presented in Table 3 demonstrate that unabsorbed anti-MR serum was cytotoxic to both MR and P cells, although it was clearly more effective against MR cells. The P cell-absorbed serum lost about 44–59% of its anti-MR activity, and further absorption of this serum by P cells was unable to completely remove its anti-MR cytolytic activity. The above experiments suggest that (i) MR and P cells contain cross-reactive tumor-associated antigens; (ii) MR cells are more susceptible to complement-dependent cytolysis; and (iii) MR may contain novel antigens not expressed in P cells.

Table 3. Differential susceptibility of MR and P cells to complement-dependent cytolysis

Antiserum	Dilutions	Percentage specific release MR as target cells	Percentage specific release P as target cells
Anti-MR (unabsorbed)	1:4	46	17
	1:16	33	4
	1:64	28	3
	1:256	18	4
	1:1024	1	-1
Anti-MR† (absorbed)	1:4	19	8
	1:16	26	1
	1:64	14	1
	1:126	0	-3
	1:1024	0	0

*For MR cells: total release = 21,675 counts/min, spontaneous release = 9724 counts/min; for P cells: total release = 7944 counts/min, spontaneous release = 2356 counts/min.

†Anti-MR serum absorbed with P cells.

Comparative growth rates of the regressing and non-regressing L1210 cells

To compare the growth rates of MR and P cells *in vivo*, irradiated (650 rad) mice in groups of 3 were injected i.p. with 2×10^5 P or MR cells at day 0. On days 3, 5 and 7, all the cells in the peritoneal cavity were harvested and counted in trypan blue solution. The results in Table 4 indicate that P cells grow out at a slightly (1.6- to 2.6-fold) faster rate than MR cells. As determined by a [^{3}H]-thymidine incorporation assay [16], MR and P cells initially did not differ significantly in their growth rates *in vitro*. After about 7 hr in culture the growth of P cells reached a plateau, whereas MR cells continued their growth for another 18 hr (data not shown).

Table 4. Comparative growth rates of L1210 and L1210/MR cells in vivo

	Average number of cells harvested ± S.D. ($\times 10^6$)		
	Day 3	Day 5	Day 7
MR	0.99 ± 0.06 ($P < 0.005$)*	23.5 ± 9.51 ($P < 0.05$)	193 ± 25.4 ($P < 0.025$)
P	1.76 ± 0.12	61.9 ± 22.9	317 ± 62.2

*The P-values are calculated according to Student's t-test.

Effect of coinjection of the regressing and non-regressing L1210 cells on leukemia development

Figure 1 demonstrates that mice inoculated with 1×10^3 or 1×10^5 P cells, or coinjected with 1×10^5 P and 1×10^6 MR cells, had 100% mortality by day 14. However, 70% of the mice which were coinjected with a smaller dose of P cells (1×10^3) and the same dose of MR cells (1×10^6) survived for at least 90 days after tumor inoculation. The survival curve of this experimental group is similar to that of the MR control group. The above results indicate that MR cells when coinjected can protect $CD2F_1$ mice from low but lethal doses of P cells.

DISCUSSION

We report in the present study the growth and antigenic properties of one of the rare and heritable spontaneously regressing tumor cell lines available. Our present data demonstrate that MR is a spontaneously regressing tumor in both syngeneic and hybrid mice. However, the animal's ability to reject MR cells appears to depend upon the tumor load and the immune status of the animals.

The possibility that the MR cell may be a much less virulent cell line by virtue of its slower growth rate than the P cell has been examined. P cells appear to have less than 3-fold faster growth rate than MR cells (Table 4). However, P cells at doses as low as 1×10^3 have been consistently fatal to $CD2F_1$ mice within 12 days of transplantation, (Fig. 1), whereas MR cells at doses as high as 6×10^7 could not kill all the experimental mice for as long as 3 months (Table 1). Therefore, we conclude that the growth rate of P and MR cells alone cannot account for their differential survival in both $CD2F_1$ and DBA/2 mice, although the slower proliferation rate of MR may be one contributing factor.

The finding that MR cells do not regress in DBA/2 mice as readily as they do in $CD2F_1$ mice suggests that DBA/2 and $CD2F_1$ mice may differ in their immunological competence [17, 18]. In addition, the hybrid resistance [19] could be one etiological factor involved in spontaneous tumor regression in hybrid animals.

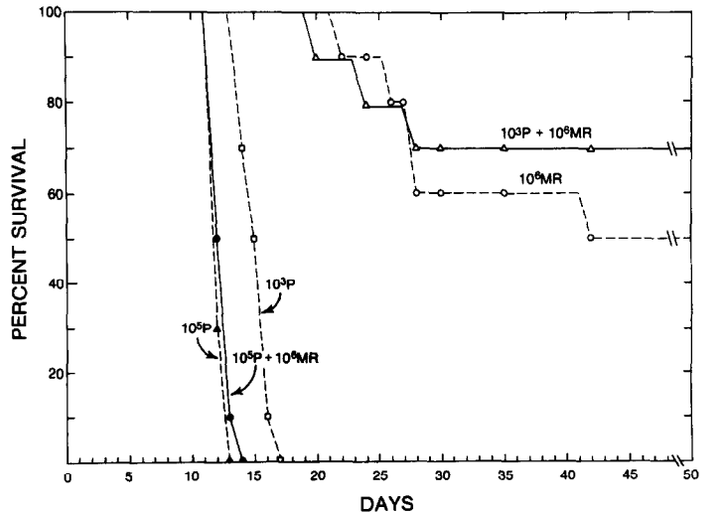

Fig. 1. The survival of $CD2F_1$ mice injected i.p. on day 0 with MR (1×10^6) or P (1×10^3 or 1×10^5) cells alone, or coinjected with MR (1×10^6) and P (1×10^3 or 1×10^5) cells. There were 10 mice in each group.

Allogeneic tumors have been commonly demonstrated to grow out initially *in vivo* and then spontaneously regress. Several lines of evidence collected here demonstrate that MR and P cells are antigenically related albeit distinct: (i) MR and P cells share at least one tumor-associated cell surface antigen of β_2 or γ-electrophoretic mobility; (ii) anti-MR serum absorbed with P cells lost most of its cytolytic activity against both P and MR cells (Table 3); and (iii) $CD2F_1$ mice recovered from various doses of MR cells are specifically immune against P cells but not an unrelated syngeneic tumor.

Mice which survived larger doses of MR cells were better protected against P cells (Table 2). In addition, MR cells when coinjected (Fig. 1) could indeed have a positive effect on the animal's ability to reject P cells. $CD2F_1$ mice immunologically crippled by sublethal X-irradiation, on the other hand, were unable to undergo spontaneous tumor regression; these mice invariably died of low doses (e.g. 2×10^5) of MR tumor within 8 to 10 days. This finding demonstrates that MR cells may modulate appropriate host responses (e.g., the immune responses) required for the spontaneous regression of MR or P cells. The immunomodulatory agents involved have not been completely elucidated.

Our recent data [20] show that MR can produce or induce *in vivo* production of a potent and regressing tumor-specific immunostimulatory factor which can greatly enhance the secondary IgM antibody response [14]. The significance of this immunostimulatory factor in the spontaneous tumor regression is currently being investigated. Although spontaneous regression of L1210 leukemia may be a multifactorial event, we suggest that the immunogenicity of L1210/MR cells and the specific immune response elicited by this tumor may be the major factors involved.

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