Immunoprotective Capability of Somatic Hybrid Cells in Comparison with Parental Tumor Cells Maintained In Vitro

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MIZUSHIMA, Y. and COHEN, E.P. *Immunoprotective Capability of Somatic Hybrid Cells in Comparison with Parental Tumor Cells Maintained In Vitro.* Tohoku J. exp. Med., 1985, **145** (4), 447-453 — The immunogenicity of X-irradiated hybrid cells derived from fusion of ASL-1 leukemia (A origin) and LM (TK⁻) fibroblasts (C3H origin) was compared to X-irradiated parental ASL-1 leukemia cells maintained in vivo (V-ASL-1) and to X-irradiated ASL-1 leukemia cells maintained in vitro (C-ASL-1). Immunization with hybrid cells induced transplantation resistance against tumor rechallenge with V-ASL-1 more effectively than did immunization with V-ASL-1 tumor cells. Immunization with X-irradiated C-ASL-1 cells produced the same, or slightly stronger level of transplantation resistance than that with X-irradiated hybrid cells. These findings were observed both in A/J and in (C3H/HeJ × A/J) F₁ mice. These results raise a question about whether the apparent increased immunogenicity of hybrid cells is due to a result of cell fusion or a result of their growth in vitro. ——— immunogenicity; somatic hybrid cells; in vitro cultivation

The immunogenicity of some tumors is very weak or unrecognizable. In these cases it has been difficult to prove that the host can elicit an effective antitumor immune response to the tumor. If antigens on tumor cells could be amplified or exposed, it might be possible to boost the host defenses against cancer. Several different approaches have been made to increase the immunogenicity of tumor cells. These include, chemical modifications of tumor cells, viral xenogenization, or cell hybridization (Kobayashi et al. 1970; Prager et al. 1974; Kataoka et al. 1977; Klein and Klein 1979; Klein 1979; Takatsu et al. 1980). The ultimate purpose behind all of these studies has been to increase the immunogenicity of tumor cells so that an effective antitumor immune response can be stimulated in the tumor-bearing host.

Previous studies from our laboratory (Liang and Cohen 1976, 1977a and b)

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have shown that specific transplantation resistance to parental ASL-1 leukemia (A origin) could be induced in (C3H/HeJ $\times$ A/J) F₁ mice by immunization with viable somatic hybrid of ASL-1 and LM (TK⁻) (C3H origin) cells. Significant resistance could not be induced by immunization with mitomycin C-treated in vivo passaged ASL-1 tumor cells (V-ASL-1) in A/J mice. The studies reported in this article were undertaken to further determine if the immunogenicity of hybrid cells is qualitatively greater than that of parental tumor cells. In an attempt to obtain an objective comparison of the immunogenicity of the different cell types the immunoprotective capability of hybrid and parental tumor cells, was evaluated using an X-irradiated tumor cell inoculum to immunize the animals.

MATERIAL AND METHODS

Animals. A/J and (C3H/HeJ $\times$ A/J) F₁ mice, 6-8 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, Maine, U.S.A.).

Cells. ASL-1 is a spontaneously occurring leukemia of A strain. In vivo ASL-1 cells (V-ASL-1) are maintained in A/J mice by weekly i.p. passage of 5×10^6 tumor cells which are obtained from the spleens of terminally ill mice. Greater than 90% of the spleen cells were neoplastic by morphologic criteria at the time of cell transfer. A long term culture

TABLE 1. *Some properties of V-ASL-1, C-ASL-1 and hybrid cells*

Characteristic	Cells		
	V-ASL-1	C-ASL-1	LA
1) <i>H-2 antigens</i>			
H-2 ^a antigens	+++ ^a	+++	+++
H-2 ^k antigens	—	—	+++
TL antigens	++	+++	++
2) <i>Tumorigenicity</i>			
5×10^3 ip in A/J	4/4 (12.8) ^b	4/4 (15.0)	Non-tumorigenic in A/J and F ₁
5×10^6 ip in A/J	4/4 (6.5)	4/4 (10.0)	Non-tumorigenic in A/J and F ₁
3) <i>Type of growth in vitro</i>		Suspension	Adherent
4) <i>NK sensitivity</i> ^c	Insensitive	Insensitive	NT ^e
5) <i>Antigenic Modulation</i> ^d			
with $\bar{a}$ -TL serum	NT	Yes	Yes
with $\bar{a}$ -H-2 ^a serum	NT	Yes	Yes

a. Positive cells (%) by indirect immunofluorescence.

+++ , >80% ; ++ , 80%-60% ; + , 60%-40% ; — , <10%.

b. Lethal growth (MSD) in normal adult mice.

c. 4 hour—⁵¹Cr release assay was performed with normal spleen cells from A/J, BALB/c, and C3H/HeJ.

d. Cells were incubated with antiserum at 37°C for 30-120 min., and then washed and stained with fluorescein-labeled rabbit anti-mouse Ig (Dakopatts, Denmark).

e. NT = Not tested.

subline of the ASL-1 was established (C-ASL-1). Cultures which were 3-6 months old were used in this study. LM (TK⁻) is a thymidine kinase-deficient subline of LM cells (C3H origin). Hybrid cells were obtained by the fusion of LM (TK⁻) and V-ASL-1 cells using polyethylene glycol (PEG 1000), and selection was done with HAT medium (1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine). LM (TK⁻) $\times$ ASL-1 hybrid cells (LA) used in this study were prepared approximately 36 months prior to the initiation of these studies. The clonal isolate of the hybrid cells was stable and it possessed 74 ± 8 chromosomes including marker chromosomes of each parental source. C-ASL-1, LM (TK⁻) and LA cells were maintained in RPMI-1640 supplemented with heat-inactivated fetal bovine serum (10%) (Hyclone, Utah, U.S.A.), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml), fungizon (0.25 μ g/ml), 2-mercaptoethanol (5×10^{-5} M), and Hepes buffer (20 mM). As a wash medium, Eagle's minimum essential medium (MEM) (Gibco, NY, U.S.A.) supplemented only with antibiotics and Hepes buffer solution was used. Both LM (TK⁻) and the hybrid cells have adherent properties and therefore it was necessary to incubate cultural dishes with 0.6 mM EDTA in PBS to remove the cells from plastic flasks. Some of the properties of V-ASL-1, C-ASL-1, and the hybrid cells are summarized in Table 1.

Irradiation of tumor cells. Cells suspended in MEM were irradiated 7,000 rads at 90 rads/min. with a Cobalt-60 apparatus standardized before use.

Statistical analysis. The significant differences in mean survival time, and in lethal rate were tested by the Student's *t*-test and Fisher's exact probability test, respectively.

RESULTS

Immunoprotective capability of hybrid cells in A/J and (C3H/HeJ $\times$ A/J) F₁ mice

Immunoprotective capability of X-irradiated hybrid cells (LA) was first compared to X-irradiated ASL-1 cells maintained in vivo (V-ASL-1). Both cells were irradiated and inoculated i.p. into A/J mice, and then all animals were

TABLE 2. *Comparison of immunoprotective capability between hybrid cells and V-ASL-1 leukemia cells in A/J mice*

Expt	Immunization ^a with	No. of cells challenged	Lethal growth	MSD $\pm$ s.d. ^b
I	Irrad. LA 1×10^7 i.p. once	4×10^4 sc	12/13	16.8 ± 7.7^c
	Irrad. V-ASL-1 1×10^7 i.p. once	4×10^4 sc	12/12	12.6 ± 2.1
	None	4×10^4 sc	12/12	11.6 ± 0.6
II	Irrad. LA 5×10^6 i.p. twice	1×10^4 i.p.	10/10	$16.1 \pm 4.2^{c,d}$
	Irrad. V-ASL-1 5×10^6 i.p. twice	1×10^4 i.p.	10/10	11.8 ± 0.7
	None	1×10^4 i.p.	10/10	11.5 ± 1.2

a. In Exp. I, A/J mice were immunized i.p. once with x-irradiated (7,000 rads) cells, and 10 days later V-ASL-1 cells (4×10^4) were challenged sc. In Exp. II, A/J mice were immunized i.p. twice with one week interval between injections. Two week after the 2nd immunization V-ASL-1 cells (1×10^4) were challenged i.p.

b. Mean survival day. Only dead mice are included. Animals were observed for 60 days after tumor cell challenge.

c. $p < 0.05$ compared with the nonimmunized group.

d. $p < 0.05$ compared with the group having immunization with V-ASL-1 cells.

TABLE 3. *Comparison of immunoprotective capability among hybrid, C-ASL-1 and V-ASL-1 cells in A/J mice*

Immunization ^a with	Lethal growth (%)	MSD $\pm$ s.d.
Viable LA	18/24 (75)	15.6 $\pm$ 4.4 ^b
Irrad. LA	23/24 (96)	17.3 $\pm$ 5.1 ^b
Irrad. C-ASL-1	16/24 (67) ^b	17.3 $\pm$ 4.7 ^b
Irrad. V-ASL-1	19/21 (90)	14.9 $\pm$ 7.5
None	23/24 (96)	12.3 $\pm$ 1.3

- a. A/J mice were immunized with tumor cells (5×10^6) i. p. twice with one week interval between injections. Two weeks after the 2nd immunization V-ASL-1 cells (5×10^3) were challenged i.p.
- b. $p < 0.05$ compared with the nonimmunized group.

TABLE 4. *Comparison of immunoprotective capability among hybrid, C-ASL-1 and V-ASL-1 cells in (C3H/HeJ $\times$ A/J) F₁ mice*

Immunization ^a with	2 $\times$ 10 ³ i.p.		1 $\times$ 10 ⁴ i.p.	
	Lethal growth (%)	MSD $\pm$ s.d.	Lethal growth (%)	MSD $\pm$ s.d.
Viable LA	0/7 (0) ^b	—	3/8 (38)	25.7 $\pm$ 5.2 ^b
Irrad. LA	0/7 (0) ^b	—	4/8 (50)	26.5 $\pm$ 7.4 ^b
Irrad. C-ASL-1	0/7 (0) ^b	—	1/8 (13) ^b	21.0
Irrad. V-ASL-1	6/7 (86)	16.3 $\pm$ 2.9	5/8 (63)	12.3 $\pm$ 2.2
None	6/7 (86)	16.0 $\pm$ 1.9	6/8 (75)	12.5 $\pm$ 2.6

- a. F₁ mice were immunized with tumor cells (5×10^6) i.p. twice with one week interval between injections. Two weeks after the 2nd immunization, V-ASL-1 cells (2×10^3 or 1×10^4) were challenged i.p. Animals were observed for 45 days after tumor cell challenge.
- b. $p < 0.05$ compared with the nonimmunized group.

challenged sc or i.p. with V-ASL-1 tumor cells according to the schedule described in the footnote of Table 2. No transplantation resistance was induced by immunization with V-ASL-1 cells whereas immunization with LA cells caused a significant prolongation of the MSD (Table 2). Experiments were undertaken to compare the immunoprotective capability of X-irradiated hybrid cells to that of X-irradiated cultured ASL-1 tumor cells (C-ASL-1). As shown in Table 3, significant transplantation resistance was induced by immunization with either the hybrid cells or the C-ASL-1 cells, but immunization with X-irradiated V-ASL-1 did not cause any significant immunoprotection. The number of animals which died in irradiated-C-ASL-1-immunized group was significantly lower than that in irradiated-LA-immunized group (67% vs 96%).

The immunoprotective capability of the different X-irradiated cells was studied in histocompatible F_1 ($H-2^{a/k}$) mice (Table 4). Immunization with X-irradiated V-ASL-1 cells did not induce any transplantation resistance to rechallenge with V-ASL-1 tumor cells. Significant transplantation immunity was observed by immunization with either X-irradiated hybrid or C-ASL-1 tumor cells or with viable LA cells.

DISCUSSION

Many reports have been published concerning the possible increase in immunogenicity of hybrid cells (Watkins and Chen 1969; Parkman 1974; Liang and Cohen 1976, 1977a and b; Kim 1979; Klein and Klein 1979; Klein 1979; Yefenof et al. 1982; Toffaletti et al. 1983). In some studies the comparison of immunogenicity was performed between *viable* hybrid cells and *irradiated* parental tumor cells, while in other studies the comparison was performed between hybrid cells maintained in vitro and parental tumor cells maintained in vivo. Previous reports from our laboratory (Liang and Cohen 1976, 1977a and b) have shown that viable somatic hybrid cells could induce specific transplantation resistance although V-ASL-1 cells could not. In the present study, both hybrid cells (maintained in vitro) and parental tumor cells (maintained in vivo and in vitro) were lethally x-irradiated, and their immunogenicity was studied in parental A/J and in F_1 mice. The results showed that the immunogenicity of X-irradiated hybrid cells was higher than that of ASL-1 cells maintained in vivo (V-ASL-1), and that immunogenicity of X-irradiated hybrid cells was not significantly higher than that of X-irradiated ASL-1 cells maintained in vitro (C-ASL-1). These results were observed in both A/J ($H-2^a$) and F_1 ($H-2^{a/k}$) mice, and this indirectly implies that $H-2^k$ antigens which were present on the surface of hybrid cells are most likely not responsible for the apparent increase in immunogenicity. Viable hybrid cells were unexpectedly less immunogenic than irradiated C-ASL-1 cells. The loss of antitumor immunogenicity of somatic cell hybrid has been reported to occur with increasing subculture (Favre et al. 1975) and may reflect progressive chromosomal loss after prolonged in vitro growth. Better immunoprotection may have been obtained if younger cultures of the hybrid cells were employed. Anyway, our results raised a simple question about whether the increased immunogenicity of hybrid cells is due to a result of cell fusion or a result of their growth in vitro. Klein G and Klein E (1979) have reported that X-irradiated in vitro somatic cell hybrids of YAC lymphoma and mouse fibroblasts induced a stronger transplantation resistance than did X-irradiated parental in vivo YAC tumor cells. Klein's group (Devens et al. 1979; Galili et al. 1978) have shown that the YAC lymphoma changes aspects of its antigenic properties after in vitro cultivation. So our question above still remains to be solved. Further studies will be required to clarify this point.

Enhancement of tumor immunogenicity after in vitro cultivation has been

reported with several tumors, e.g., Moloney virus-induced YAC lymphoma, chemically-induced MSC-10 squamous-cell carcinoma, and chemically-induced line 10 hepatocarcinoma (Morgan et al. 1970; Jamaski and Nettesheim 1977; Galili et al. 1978; Devens et al. 1979; Klein et al. 1981; Correll et al. 1983). Klein et al (1981) speculated that suppressogenic determinants expressed on tumor cells might be inactivated by in vitro cultivation. Correll et al. (1983) speculated fetal bovine serum which (FBS) bound to tumor cells might activate T-cell helper functions in vivo. In their studies, it is of interest that cells cultured in medium with 10% calf bovine serum, or 10% normal guinea pig serum, or reduced concentration of fetal bovine serum were less effective in inducing antitumor resistance. Conceivably the contamination of in vitro cultures by virus or mycoplasma may affect the immunogenicity of tumor cells. We have not investigated as yet the mechanism about the enhanced immunogenicity of C-ASL-1 cells after in vitro cultivation. However, if this is a generally observed phenomenon applicable to other systems, it seems to be very important from the view point of immunotherapy of cancer.

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