IN VITRO INDUCTION OF T-LYMPHOCYTE-MEDIATED CYTOTOXICITY BY INFECTIOUS MURINE TYPE C ONCORNAVIRUSES

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Cell-mediated immune responses have been shown to play an important role in the rejection of tumors and in tumor immunity (see reference 1 for review). Among the immune responses to a tumor graft, cytolytic T lymphocytes capable of destroying target cells carrying the relevant tumor-associated antigens have been demonstrated in experimental animals. In the Moloney murine sarcoma virus (M-MSV)[1] system that has been extensively studied, M-MSV-induced tumors have been shown to have common antigens related to those induced by some murine leukemia viruses (MuLV) (2–5). Although cell-mediated immune responses induced by M-MSV have been extensively studied by many different techniques, such as the microcytotoxicity test, ^{51}Cr-release cytotoxicity assay and migration inhibitory factor (MIF) assay, there have been no conclusive indications about the antigens recognized by immune T lymphocytes (see reference 6 for review).

Recently, some studies with lymphocytes from M-MSV-immune mice have suggested that the recognition of type C virion proteins appears to account for at least a part of the immune reactivity. Enjuanes et al. (7) demonstrated that the type-specific envelope antigen, gp70, of Moloney murine leukemia virus (M-MuLV) could stimulate lymphoproliferative responses by M-MSV-immune lymphocytes and could specifically inhibit cell-mediated cytotoxicity. A study in our laboratory has indicated that gp70, p12, and p30 of M-MuLV could also stimulate M-MSV-immune lymphocytes to produce MIF.[2]

Initially, Plata et al. (8) and subsequently we (9, 10) reported that a secondary cytotoxic response could be induced in vitro by incubating spleen cells from M-MSV immune animals with intact tumor cells. More recently, we also demonstrated that macrophages functioned as accessory cells for the generation of a secondary cytotoxic response even when intact tumor cells were used as an antigen (10). This has led to a modified procedure for generating a secondary cytotoxic response, which facilitates

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[1] *Abbreviations used in this paper:* DMEM, Dulbecco's modified Eagle's minimal essential medium; FCS, fetal calf serum; FEF, feline embryo fibroblast; FeLV, feline leukemia virus; FIU, focus-inducing units; F-MuLV, Friend murine leukemia virus; ISC, immune spleen cells; MEF, mouse embryo fibroblast; MIF, migration inhibitory factor; M-MSV, Moloney strain of murine sarcoma virus; M-MuLV, Moloney murine leukemia virus; MuLV, murine leukemia virus(s); NSC, normal spleen cells; R-MuLV, Rauscher murine leukemia virus.

[2] Ng, A.-K., R. S. Ames, Jr., R. K. McIntire, and R. B. Herberman. 1979. In vitro studies of cell-mediated immunity to Moloney leukemia virus and Moloney leukemia-associated antigens. *Cancer Res.* In press.

TABLE I

Characteristics of the Tumors and Cell Lines Used in ^{51}Chromium-release Assay

Cells	Strain of origin	H-2 haplotype	Inducing agent	Condition	Reference
MBL-2	B6	b	Moloney-MuLV(M-MuLV)-induced lymphoma	TC*	3
RBL-5	B6	b	Rauscher-MuLV(R-MuLV)-induced lymphoma	TC	3
EL-4(G−)	B6	b	Benzo[a]pyrene-induced lymphoma GCSA−	Ascites	3
EL-4(G+)	B6	b	Benzo[a]pyrene-induced lymphoma GCSA+	Ascites	3
EδG2	B6	b	Gross-MuLV-induced lymphoma GCSA+	Spleen	3
B6-MEF	B6	b	Uninfected normal mouse embryo fibroblast	TC	7
B6-MEF-M-MuLV	B6	b	M-MuLV-infected B6-MEF	TC	7
B6-MEF-F-MuLV	B6	b	Friend-MuLV(F-MuLV)-infected B6-MEF	TC	7
B6-MEF-M-MSV	B6	b	M-MSV-infected B6-MEF	TC	7
B6-MEF-B-tropic	B6	b	B-tropic MuLV-infected B6-MEF	TC	7
LSTRA	BALB/c	d	M-MuLV-induced lymphoma	TC	3
RLδ1	BALB/c	d	Radiation-induced leukemia	TC	3
YAC-1	A	a	M-MuLV-induced lymphoma	TC	3
RDM-4	AKR	k	Spontaneous lymphoma	TC	11

* Tissue culture.

the examination of the specificity of M-MSV-immune cytolytic T lymphocytes. In the present report, we describe our finding that infectious type C virus can induce a secondary cytotoxic response, and explore the possibility that this technique can further be used to elucidate the specificity of cytotoxic T lymphocytes.

Materials and Methods

Mice. C57BL/6N (B6) mice were obtained from the Rodent and Rabbit Production Section, Division of Research Services, National Institutes of Health, Bethesda, Md.

Tumors. Primary tumors were induced by the M-MSV (>10^4 focus-forming units) in B6 mice as described previously (2). Usually, the tumors arose 6–7 after inoculation, reached maximum size on days 11–14, and regressed by days 22–25. For studies of the secondary cytotoxic response, spleen cells were obtained from mice 30–50 d after inoculation with M-MSV.

Target Cells. The characteristics and origins of the cell lines employed in this study are given in Table I (3, 7, 11). They were grown as stationary suspension or monolayer cultures in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N.Y.) containing 10% fetal calf serum (FCS), 100 μg/ml streptomycin, 100 U/ml penicillin, and 50 μg/ml gentamicin (hereafter referred to as RPMI-1640-10% FCS) (12). The in vivo passaged tumor lines were maintained by serial passage in normal histocompatible recipients. The respective cell lines were assayed for the expression of viral antigens by competition radioimmunoassay (7).

Viruses. M-MSV was prepared from a stock originally obtained from Dr. J. B. Moloney, National Cancer Institute (NCI), Bethesda, Md., and maintained in our laboratory (2). Moloney MuLV clone IC (M-MuLV clone IC) from NIH/3T3 cells, Rauscher MuLV (R-MuLV) from JLS-V9 cells, Gross-MuLV and BALB virus-1 from NIH/3T3 cells, AKR-MuLV from AKR mouse embryo fibroblasts (originally obtained from Dr. W. P. Rowe, National Institute of Allergy and Infectious Diseases [NIAID], Bethesda, Md.), BALB virus-2 from human A673 cells, feline leukemia virus (FeLV), and baboon type C virus were obtained from the Viral Oncology Program of the National Cancer Institute through the courtesy of Dr. J. Gruber. These viruses, except for M-MSV, were purified by double sucrose density gradient ultracentrifugation and stored at −70°C until use. Ecotropic M-MuLV clone H, originally isolated by Dr. Janet Hartley, NIAID, Bethesda, Md. (13), and a variant (dualtropic) of M-MuLV clone 83 (13), both propagated on 3T3FL cells in McCoy 5A medium containing 10% FCS, were obtained from Dr. Alan Rein, NCI, Bethesda, Md. A nondefective dualtropic virus (HIX virus) from M-MuLV clone IC was propagated on feline embryo fibroblast (FEF) (HIX-FEF) or human RD cells (HIX-RD) and was obtained from P. J. Fischinger, NCI, Bethesda,

TABLE II

Characterization of Viruses Used in the Present Study

Virus	Cell line	Tropism	Virus particles/ml*	FIU/ml‡	Condition used
M-MuLV clone IC	NIH/3T3§	NB	1.5×10^{11}	1×10^{8}	Purified‖
M-MuLV clone H	3T3FL¶	NB	N.D.	2×10^{6}	Supernate**
M-MuLV clone 83	3T3FL¶	Dualtropic	N.D.	4×10^{5}	Supernate
HIX-FEF	FEF‡‡	Dualtropic	N.D.	5×10^{5}	Supernate
HIX-RD	RD human cells‡‡	Dualtropic	N.D.	5×10^{5}	Supernate
R-MuLV	JLS-V9§	NB	1×10^{12}	3×10^{7}	Purified
Gross-MuLV passage A	NIH/3T3§	N	2.5×10^{11}	1×10^{8}	Purified
AKR-MuLV	AKR-MEF§	N	2.2×10^{11}	5×10^{7}	Purified
BALB virus-1 BC194	NIH/3T3§	N	1.2×10^{11}	4×10^{7}	Purified
BALB B-tropic WN1802B	SC-1§§	B	ND	3×10^{6}	Supernate
BALB virus-2 BC177	A673 human cells§	Xenotropic	1.3×10^{11}	7×10^{6}	Purified
Feline LV Theilen	FL-74§		3.5×10^{11}	4×10^{6}	Purified
Baboon type C M7	A207 human cells§		2.0×10^{11}	3×10^{6}	Purified

* Determined by electron microscopy.

‡ Focus-inducing units per milliliter in S+L− FG-10 assay, S+L− mink cell or S+L− cat 81 cell assay.

§ Obtained from Dr. J. Gruber, National Cancer Institute, Bethesda.

‖ Purified by double-density gradient ultracentrifugation.

¶ Obtained from Dr. A. Rein, National Cancer Institute, Bethesda.

** 24-hour supernate from the relevant confluent culture.

‡‡ Obtained from Dr. P. Fischinger, National Cancer Institute, Bethesda.

§§ Obtained from Dr. R. Bassin, National Cancer Institute, Bethesda.

Md. (14). BALB B-tropic virus (WN 1802B), originally isolated by Dr. Janet Hartley, was propagated on SC-1 cells in McCoy's 5A medium containing 10% FCS and was obtained from Dr. Robert H. Bassin, NCI, Bethesda, Md. (15). The characteristics of the virus used in the present study are given in Table II.

Irradiation of Virus with Ultraviolet (UV)-light. Irradiation of infectious virus with UV-light (General Electric Co., Lamp Parts & Equipment Sales Operation, Cleveland, Ohio) was performed as described previously (16). In brief, virus was diluted in 1 ml of phosphate-buffered saline, placed onto a watch glass, and then irradiated with UV-light at 60 ergs/mm^2 per s. Treatment for 40 s (total 2,400 ergs/mm^2) was shown to inactivate 1 log of infectious virus, as determined by XC syncytial plaque assay (16) and the S+L− focus assay on FG-10 (17).

Virus Infectivity Assay. Ecotropic and dualtropic M-MuLVs were assayed according to the method of Bassin et al. (17), using the S + L − focus assay on FG-10 cells (kindly provided by Dr. R. Bassin, NCI) that had been pretreated with 20 μg/ml DEAE-dextran. Assays for xenotropic virus, FeLV, and baboon type C virus were done by the focus assay in the S + L − mink cells (18) (kindly given by Dr. J. Hartley, NIAID) or the S + L − cat cell 81 (19) (obtained from Dr. P. Fischinger, NCI) in the presence of polybrene (2 μg/ml). The results of the S + L − assay are given as focus-inducing units (FIU) per milliliter. M-MSV was titrated on 3T3FL (given by Dr. A. Rein, NCI) in the presence of an optimal concentration of helper virus, by the method of Bassin et al. (20), with the titer being expressed as focus-forming units per milliliter.

In Vitro Generation of a Secondary Cytotoxic Response. Spleens were removed aseptically from M-MSV-immune or normal animals and minced. The resulting single cell suspension was passed through sterile gauze and suspended in Dulbecco's modified minimal essential medium (DMEM) containing 10% FCS (lot RG1808, Reheis Chemical Co., Chicago, Ill.) 50 μM 2-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 34 μg/ml L-asparagine, 116 μg/ml arginine-HCl, and 10 μg/ml folic acid (hereafter referred to as complete DMEM). Secondary cell-mediated cytotoxicity was generated in vitro using a modification of the method described by Plata et al. (8). Briefly, unseparated immune spleen cells (ISC), suspended in complete DMEM, were adjusted to a final concentration of 5×10^6 cells/ml. Tumor cells to be used as stimulators were treated with 100 μg/ml mitomycin C/10^7 cells (Sigma Chemical Co., St. Louis, Mo.) for 60 min at 37°C and washed three times with RPMI-1640-10% FCS. For culture, 5×10^6 immune spleen cells were mixed with different

concentrations of purified virus, tissue culture supernates containing infectious virus or 2×10^5 mitomycin C-treated tumor cells in 2.0 ml of complete DMEM and dispensed in multiwell dishes (FB-16-24-TC, Linbro Chemicals, Hamden, Conn.). In a preliminary experiment, we found that with intact tumor cells as in the stimulus, the optimal responding cell to stimulator cell ratios were 25:1 and 20:1. Each group consisted of at least four cultures. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. After 5 d in culture, lymphocytes were harvested and used as effector cells in a 4-h ^{51}Cr-release assay.

^{51}Cr-release Assay. The procedure was described previously (9, 10). Varying numbers of effector cells were mixed with 5×10^3 ^{51}Cr-labeled target cells in a final 0.2-ml vol in microtiter plates with U-shaped wells (Linbro Scientific Co.). The plates were then centrifuged at 65 *g* for 2 min and were incubated at 37°C for 4 h. Supernates were then harvested with the Titertek Supernatant Collecting System (Flow Laboratories, Inc., Rockville, Md.), and the percent cytotoxicity was determined as follows:

$$\% \text{ cytotoxicity} = \frac{\text{cpm of test supernate} - \text{cpm of autologous control}}{\text{total cpm incorporated into cells}} \times 100.$$

% specific cytotoxicity = % cytotoxicity of immune spleen cells stimulated with an antigen or a supernatant-containing infectious virus − % cytotoxicity of immune spleen cells cultured alone or with supernate from uninfected cells.

The percent cytotoxicity of immune spleen cells cultured alone (unstimulated) was 5.0 ± 4.4 (mean ± standard deviation) for 20 experiments. An autologous control, with unlabeled target cells in place of lymphocytes, was used to measure the spontaneous release. Each experimental group was tested in quadruplicate.

Elimination of T Lymphocytes. AKR anti-C3H, anti-Thy1.2, was prepared as previously described (21). To eliminate T lymphocytes, $10–20 \times 10^6$ effector cells were incubated with undiluted AKR anti-C3H Thy1.2 serum (0.3 ml) for 30 min at room temperature. Next, the cells were washed once with RPMI-1640 medium, resuspended in a 1:2 dilution of normal rabbit serum (Grand Island Biological Co.) (preselected for lack of toxicity against mouse lymphocytes) as a source of complement, and incubated for 45 min at 37°C. After being washed three times, the cells were then counted and used in the test.

Results

Induction of a Cytotoxic Response: Tumor Cells vs. Virus. As has been reported previously (8–10), M-MSV-immune spleen cells could be restimulated in vitro with the relevant intact tumor cells to produce a potent secondary cytotoxic response 5 d after the initiation of culture (Table III). In addition to the routine use of MBL-2 tissue culture cells as the stimulus, we decided to investigate whether supernatant fluids from cultures of MBL-2 could substitute for the intact tumor cells. As shown in Table III, the supernate from MBL-2 cultures was as good as or better than intact tumor cells for inducing a secondary response. Normal spleen cells were not stimulated to produce a cytotoxic response with either MBL-2 or culture supernates from MBL-2 cells. Furthermore, the stimulated immune cells did not react appreciably or consistently against RLδ1, a target that does not contain cross-reacting antigens.

These results prompted us to investigate the possibility that a virus released from MBL-2 was responsible for the induction of this response, because the supernate from MBL-2 tissue cultures was found to contain $>10^5$ FIU/ml of infectious ecotropic MuLV. M-MuLV clone IC was tested for its ability to induce a secondary cytotoxic response. Addition of 3×10^9 virus particles per culture stimulated high levels of cytotoxicity in spleen cells from animals 30 d after MSV inoculation. As with the supernate from MBL-2, no cytotoxic reactivity was seen when normal spleen cells were used as the responders.

TABLE III

Induction of Secondary Cytotoxic Responses by the Culture Supernate of MBL-2 or by M-MuLV Clone IC

Exp.	Responding* cells	Antigen	Dose	Percent specific cytotoxicity (± SE)		
				MBL-2		RLδ1
				50:1‡	10:1	50:1
1	ISC	MBL-2	2×10^5§	34.0 (1.8)	17.2 (0.5)	2.8 (0.8)
	ISC	Supernate-MBL-2	1 ml	46.8 (1.1)	37.9 (1.1)	9.3 (0.8)
	NSC	MBL-2	2×10^5	0 (0.6)	0.1 (0.5)	−1.0 (0.4)
	NSC	Supernate-MBL-2	1 ml	1.1 (0.5)	0.3 (0.4)	0.1 (1.0)
2	ISC	MBL-2	2×10^5	20.3 (0.3)	10.7 (0.2)	—¶
	ISC	M-MuLV clone IC	3×10^9‖	36.5 (0.8)	29.7 (1.2)	—
	NSC	MBL-2	2×10^5	0.1 (0.2)	−0.3 (0.6)	—
	NSC	M-MuLV clone IC	3×10^9	0.1 (0.3)	0.2 (0.7)	—

* 5×10^6 M MSV-immune spleen cells (ISC) or normal spleen cells (NSC) from B6 mice were cultured for 5 d in 24-well tissue culture plates. After the incubation, viable lymphocytes were harvested and tested for cytolytic activity in a 4-h ^{51}Cr release assay.

‡ Effector to target cell ratio.

§ Number of cells per culture.

‖ Virus particles per culture.

¶ Not done.

Kinetics of the Generation of Cytolytic T Lymphocytes by M-MuLV. To investigate the time course of the secondary cytotoxic response induced by M-MuLV clone IC, multiple cultures of M-MSV immune spleen cells were stimulated with either M-MuLV clone IC or MBL-2. At different times after initiation of culture, the responding cells were harvested and tested for their cytolytic activity against MBL-2. As shown in Table IV, significant cytotoxicity was induced by M-MuLV clone IC by day 4, and by day 5 had reached a peak. Thereafter, the cytotoxicity induced by M-MuLV clone IC decreased. These kinetics were comparable to those observed in cultures containing mitomycin C-treated MBL-2 as the stimulus.

Nature of the Effector Cells. Although it has been established that T lymphocytes are the effector cells generated when intact tumor cells are used as the stimulus (8–10), it was important to determine whether the effector cells generated in a secondary cytotoxic response by M-MuLV clone IC were also T lymphocytes. To test this, M-MSV-immune spleen cells obtained after 5 d of culture with M-MuLV clone IC were treated with anti-Thyl.2 plus complement before assaying their cytolytic activity on MBL-2. As shown in Table V, treatment with anti-Thyl.2 plus complement, but not complement alone, completely abolished the cytolytic activity, indicating that the effector cells generated in the secondary cytotoxic response by M-MuLV were T lymphocytes. Similar results were obtained when tissue culture supernate from MBL-2 was used as the stimulus (data not shown).

Specificity of Cytolytic T Lymphocytes Generated by Intact MBL-2 or M-MuLV Clone IC. To determine the specificity of the cytolytic T lymphocytes, a wide panel of target cells was employed in a 4-h ^{51}Cr-release assay. As shown in Table VI, cytolytic T lymphocytes generated by M-MuLV clone IC were capable of lysing MBL-2, RBL-

TABLE IV

Kinetics of the Secondary Cytotoxic Response Induced by M-MuLV or by Intact MBL-2 Cells

Day of culture	E/T ratio	Percent specific cytotoxicity (± SE)*	
		M-MuLV clone IC (3×10^9 VP/well)‡	MBL-2 (2×10^5 cells/well)
4	100:1	28.5 (0.5)	24.9 (0.6)
	30:1	24.6 (0.7)	22.0 (0.5)
	10:1	19.1 (0.7)	16.6 (0.8)
5	100:1	39.5 (0.6)	34.2 (0.7)
	30:1	32.4 (0.5)	30.6 (0.8)
	10:1	30.3 (0.5)	26.0 (0.6)
6	100:1	22.9 (0.8)	21.1 (0.7)
	30:1	20.5 (0.6)	18.7 (0.6)
	10:1	16.9 (0.5)	17.3 (0.8)

* MBL-2 cells were used as the target.

‡ Virus particles per culture.

5, and EL-4(G+) targets very efficiently. In contrast, the same effector cells failed to lyse significantly RDM-4, E♂G2, RL♂1, LSTRA, EL-4(G-), or YAC target cells. Similar patterns were obtained when MBL-2 culture supernate or MBL-2 tumor cells were employed as the stimulus. Hence, these results indicate that the antigen(s) being recognized by cytolytic T lymphocytes is associated with M-MuLV, but unrelated to Gross cell surface antigen (GCSA), because E♂G2, a syngeneic Gross-MuLV-induced lymphoma, was not lysed, and RBL-5 contains Moloney antigens (gp70 and p12) on their cell surface as determined by competition radioimmunoassay (J. N. Ihle. Personal communication.). Furthermore, the above results were confirmed by using B6-MEF infected with a variety of murine type C RNA viruses, which were regularly checked for viral contamination or activation and expression of endogenous type C viruses. The results shown in Table VI clearly indicate that no cytotoxicity was detected in the uninfected or F-MuLV- or B-tropic MuLV-infected B6-MEFs by cytolytic T lymphocytes generated by M-MuLV clone IC or intact MBL-2 cells. The same effector cells, on the other hand, lysed efficiently M-MSV- or M-MuLV-infected B6-MEF, thus indicating that antigenic determinants which are recognized by cytolytic T lymphocytes, are immunologically specific for the serotype of M-MuLV.

Consistent with previous reports (8, 9, 22, 23), the cytotoxicity observed in the present study was H-2 restricted, because YAC and LSTRA, M-MuLV-induced lymphomas of A and BALB/c mice were not lysed in a 4 h ^{51}Cr-release assay (Table VI). Moreover, the fact that good targets (YAC and RL♂1) for NK activity (24) were also not killed significantly by cytolytic T lymphocytes shows that the effector cells were immune T lymphocytes but not NK cells. This result was further confirmed by a cold target inhibition study in which experiments were performed using labeled MBL-2 target cells and unlabeled competitive inhibitor cells including MBL-2 and YAC. Only MBL-2 inhibitor cells abrogated the cytolysis of MBL-2 in a dose-dependent manner whereas YAC did not inhibit (data not shown).

The Role of the Infectivity of M-MuLV in Induction of a Secondary Cytotoxic Response. In the above experiments, infectious M-MuLV clone IC was shown to induce a secondary cytotoxic response. It was important to determine if infectivity was required for induction of the response, because previous reports with other nononcogenic viruses

TABLE V

Effect of Anti-Thy1.2 Plus Complement on Effector Cells Induced by Culture of M-MSV-immune Spleen Cells with M-MuLV

Treatment	Percent recovery of viable cells	Percent specific cytotoxicity (± SE)*
Medium	100	36.3 (1.5)
C	90	32.9 (1.6)
Anti-Thy1.2 + C	35	0 (0.7)

* Effector to target cell ratio of 60:1, against MBL-2 target cells.

TABLE VI

Specificity of Cytolytic Activity of T Lymphocytes Generated in Response to M-MuLV

Target cells	H-2 haplotype	Percent specific cytotoxicity*		
		M-MuLV clone IC‡	Supernatant-MBL-2‡	Intact MBL-2‡
MBL-2	b	37.5–60.9§	47.4	21.3
RBL-5	b	41.3§	22.1	18.5
EL-4(G+)	b	34.2§	—‖	—
EL-4(G−)	b	1.2–6.1§	—	1.4–8.8
EδG2	b	−1.2–1.0§	—	—
YAC	a	1.7§	—	—
RLδ1	d	2.9–3.9§	1.9	2.4–6.4
LSTRA	d	0.8§	1.5	4.2
RDM-4	k	2.6§	—	3.3
B6-MEF	b	0.2–2.1¶	—	0.1–3.5
B6-MEF-M-MuLV	b	20.5–25.8¶	—	14.2–16.8
B6-MEF-M-MSV	b	20.8–28.9¶	—	21.6
B6-MEF-F-MuLV	b	2.9–4.2¶	—	2.9
B6-MEF-B-tropic	b	−0.2–3.7¶	—	0.2–0.6

* Two values indicate the range of cytotoxicity from multiple tests; one value is percent specific cytotoxicity from one experiment.
‡ Antigen used as stimuli in secondary responses.
§ E/T ratios of 30:1–50:1.
‖ Not done.
¶ E/T ratios of 50:1–150:1.

were conflicting. Ertl et al. (25) reported a requirement for infectious vaccinia virus to induce virus-specific cytotoxic T lymphocytes in vivo. However, inactivated influenza virus (26) and Sendai virus (27, 28) were able to induce cytotoxic responses in vitro. Experiments were therefore performed to investigate whether inactivated M-MuLV was able to induce a secondary cytotoxic response against MBL-2. The results shown in Fig. 1 A indicated that when the infectious virus was exposed to UV-light for a period resulting in a decrease to 1/1,000 of the original infectivity, 1,000-fold more virus particles were required to induce the same degree of the cytotoxicity. We considered the possibility that this treatment somehow rendered the infectious virus immunologically inactive (e.g., by conformational change of the antigens). Therefore, various doses of UV-irradiation were tested and the results are shown in Fig. 1 B. Treatment of the infectious M-MuLV for 40 s (2,400 ergs/mm^2) or 1 min 20 s (4,800 ergs/mm^2), which decreased infectivity by 1 or 2 logs, respectively, decreased the

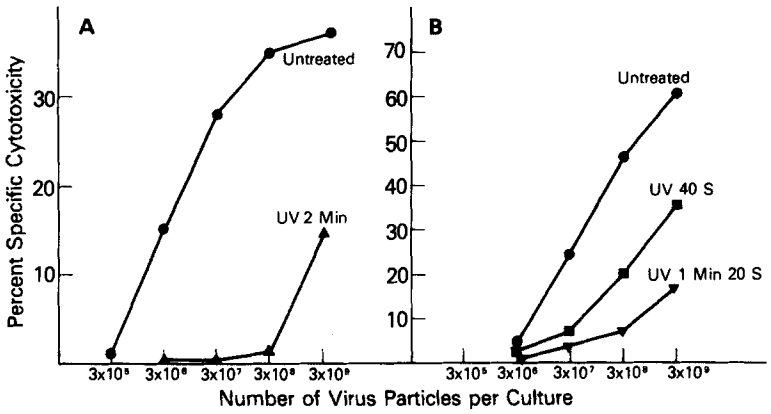

FIG. 1. Effect of UV irradiation on the ability of M-MuLV clone IC to induce a secondary cytotoxic response. The infectious M-MuLV was irradiated with UV-light at 7,200 ergs/mm^2 (2 min, ▲) (Fig. 1 A), which has been shown to decrease infectivity by 3 logs, or at 2,400 (40 s, ■) or 4,800 (1 min 20 s, ▼) ergs/mm^2 (Fig. 1 B), which have been shown to decrease infectivity to 1 or 2 logs, respectively. The ability of UV-treated M-MuLV to induce a secondary cytotoxic response was compared to that of untreated virus (●). MBL-2 cells were used as the target, with an effector to target cell ratio of 100:1. Note that the ability of M-MuLV to induce a secondary cytotoxic response decreased in parallel with the infectivity of M-MuLV.

ability of the virus to induce a secondary cytotoxic response by ~12- to 50-fold and 100- to 300-fold, as compared to the untreated virus control. Taken together, these results suggest that there is a close correlation between the infectivity of M-MuLV and the ability to induce a secondary cytotoxic response in this system.

Specificity of a Secondary Cytotoxic Response in the Induction Phase. Having examined the specificity of the cytolytic T lymphocytes induced by M-MuLV clone IC, it was of interest to determine whether other murine leukemia viruses, such as ecotropic and xenotropic virus, and nonmurine type C viruses, such as FeLV and baboon type C viruses, could also induce a secondary cytotoxic response of M-MSV-immune spleen cells. The results of experiments with an exogenous ecotropic type C virus (R-MuLV) and three endogenous N-tropic and one B-tropic type C viruses are shown in Tables VII and VIII. AKR-MuLV, Gross-MuLV, BALB virus-1, and B-tropic virus were unable to induce a secondary cytotoxic response by M-MSV-immune spleen cells, even when the number of virus particles per culture was varied from 10^5 up to 10^9. M-MuLV clone IC, on the other hand, was consistently capable of inducing high levels of reactivity. Furthermore, no cytotoxic response was detectable against E♂G2, a Gross-MuLV-induced lymphoma, in the cultures of M-MSV-immune spleen cells and Gross-MuLV or M-MuLV (data not shown). However, when M-MSV-immune spleen cells were cultured with R-MuLV (Table VII), low but significant levels of cytotoxicity were detectable.

Recently, some variants of M-MuLV, which were shown to be recombinants in the *env* gene region between ecotropic M-MuLV and a xenotropic virus, have been isolated (13, 14). It was of interest to determine whether these variants of M-MuLV (M-MuLV clone 83, HIX-FEF, or HIX-RD) as well as ecotropic M-MuLVs clone IC and clone H could induce a secondary cytotoxic response by M-MSV-immune spleen cells. The results shown in Table IX indicate that two ecotropic M-MuLVs consistently showed an ability to induce a secondary cytotoxic response. However, M-MuLV

TABLE VII

Ability of Ecotropic Viruses To Induce a Secondary Cytotoxic Response by M-MSV-immune Spleen Cells

Exp.	Virus‡		Percent specific cytotoxicity (± SE)*					
			Number of virus particles					
			10^5	10^6	10^7	10^8	10^9	10^{10}
Exp. 1	M-MuLV clone IC	(3 ×)§	2.4 (0.4)	6.4 (0.5)	4.7 (0.8)	18.3 (0.5)	36.8 (0.5)	—‖
	R-MuLV	(2 ×)	—	2.2 (0.8)	2.6 (0.7)	3.8 (0.5)	6.9 (0.4)	5.0 (0.5)
	AKR-MuLV	(4 ×)	0.1 (0.5)	0 (0.7)	0.3 (0.7)	1.0 (0.4)	0 (0.7)	—
Exp. 2	M-MuLV clone IC	(3 ×)	0.1 (0.9)	0 (0.7)	1.7 (1.0)	23.1 (1.0)	55.8 (3.0)	—
	Gross-MuLV	(3 ×)	0.8 (1.0)	1.9 (0.7)	4.3 (0.8)	3.3 (0.9)	0 (0.7)	—
Exp. 3	M-MuLV clone IC	(1.5 ×)	—	2.0 (0.5)	6.3 (0.6)	16.8 (0.6)	28.4 (0.6)	—
	BALB-virus-1	(1.5 ×)	—	0.1 (0.7)	1.0 (0.5)	0 (0.5)	0.2 (0.5)	—

* MBL-2 cells were used as a target. E/T ratio was 80:1 in Exp. 1, 100:1 in Exp. 2 and 80:1 in Exp. 3.

‡ Purified by double sucrose density gradient.

§ Number of virus particles per culture is the number in parentheses times the heading of each column.

‖ Not done.

clone 83, HIX-FEF, and HIX-RD were found to be inactive, suggesting that the *env* gene product, gp70, was important in this response.

Aoki et al. (29) had reported that SIRC cells infected with some xenotropic viruses inhibited the cytolysis by anti-M-MSV cytolytic T lymphocytes. Therefore, an experiment employing xenotropic BALB virus-2 was performed. As shown in Table X, BALB virus-2 was inactive, whereas M-MuLV clone IC was able to induce a secondary cytotoxic response in the same experiment. We also evaluated whether type C viruses derived from other species could generate a secondary cytotoxic response by M-MSV-immune spleen cells. The results shown in Table XI clearly indicate that FeLV and baboon type C viruses were inactive. Taken together, these results strongly suggest that the induction of a secondary cytotoxic response by M-MSV-immune spleen cells was immunologically type specific for M-MuLV.

Discussion

Results obtained in the present study indicate that M-MuLV can induce a strong secondary cytotoxic response by M-MSV-immune spleen cells. The specificity of the response induced by M-MuLV was the same as that induced by the primary response and the effector cells were shown to be T lymphocytes. Although the supernate of tumor cells (MBL-2 in these experiments) stimulated a cytotoxic response as strong as that induced by the tumor cells themselves, it is not clear what proportion of the response induced by the intact cells can be accounted for by the production of virus. First, the concentration of MBL-2 tumor cells that we used to generate the supernate was 5- to 10-fold higher than the concentration employed in the secondary response culture and, therefore, the increase in cell concentration could have yielded higher titers of virus in the supernate than are normally produced in the secondary response cultures. In addition, we have recently found that infectious virus is most effective at inducing a secondary response when added during the first 24 h of culture (T. Taniyama. Unpublished observations.). Therefore, it would be important to have high concentrations of virus during the early phase of the in vitro secondary response and further testing would have to be performed to determine whether the rate of virus production by the tumor cells was sufficient to meet these requirements. Second, although LSTRA induces a potent secondary cytotoxic response in syngeneic BALB/

TABLE VIII

Inability of B-Tropic Endogenous Type C Virus To Induce a Secondary Cytotoxic Response by M-MSV-immune Spleen Cells

Virus	Dose (FIU/well)	Percent specific cytotoxicity (± SE) vs. MBL-2		
		100:1*	30:1	10:1
M-MuLV clone IC	4×10^6	45.4 (2.1)‡	40.8 (1.6)	39.4 (1.1)
M-MuLV clone IC	4×10^5	38.5 (1.3)	36.3 (1.4)	33.5 (1.8)
M-MuLV clone IC	4×10^4	25.0 (1.3)	17.2 (0.6)	10.6 (1.2)
B-tropic virus	4×10^6	1.0 (0.7)§	0.6 (0.3)	−1.2 (0.4)
B-tropic virus	2×10^6	−1.2 (0.3)	−1.2 (0.8)	0.3 (1.2)
B-tropic virus	1×10^6	−1.4 (0.4)	0.8 (1.2)	−1.9 (0.7)
Supernatant-MBL-2	8×10^5	32.6 (1.5)‡	16.7 (0.2)	12.4 (0.3)
Supernatant-MBL-2	4×10^5	27.3 (1.2)	14.4 (0.7)	9.6 (0.9)

* Effector to target cell ratio.

‡ Specific cytotoxicity was calculated by subtracting percent cytotoxicity of immune cells cultured alone from percent cytotoxicity of immune cells cultured with virus or supernate.

§ Specific cytotoxicity was calculated by subtracting percent cytotoxicity of immune cells cultured with control-uninfected supernate from the relevant cell line from percent cytotoxicity of immune cells cultured with the supernate containing the infectious virus.

c M-MSV-immune spleen cells, it does not as readily stimulate development of cytotoxicity to tumor-associated antigens in B6 M-MSV-immune spleen cells. If the virus produced by the tumor cells were the only stimulus, then one might expect the same cells to be an equally capable inducer for both responding populations. However, MHC products on the envelope of the released viruses might contribute to differences in responses by syngeneic vs. allogeneic responding cells, perhaps by influencing the rate at which the virus was adsorbed to the cells. Alternatively, the situation may be the same as mentioned in the first point, namely that there is not enough virus produced by the tumor cells (LSTRA in this case) during the early phase of the culture, to act as an efficient stimulus. Regardless of the explanation, it appears that the tumor cells are more restricted than infectious virus in their ability to induce a secondary response and also that the sequence of events after stimulation by tumor cells may be different from that when infectious virus is the inducing agent.

The results in the present study also suggest that for virus to be able to induce a secondary cytotoxic response, it must be infectious. When M-MuLV was irradiated with UV-light at doses which caused a decrease in infectivity of either 1, 2, or 3 logs as determined by the XC-plaque assay, the ability of irradiated M-MuLV to induce a secondary cytotoxic response decreased in parallel to the infectivity. Although this result suggests that there was an association between infectivity of the virus and its ability to induce a secondary cytotoxic response, at least two other possible mechanisms for this can be considered. The first is that the UV irradiation rendered the virus immunologically inactive. Although this is certainly possible, it is not likely because it has been shown that UV irradiation mainly affected RNA species and not proteins (30). The second alternative is that infection with, and replication of, M-MuLV, and not just adsorption of virus, was required for the formation of immunologically active antigens on the cell surface. Although low doses of infectious M-MuLV could induce detectable cytolytic T lymphocytes, little cytotoxicity was generated in the presence of 10^3- to 10^4-fold higher concentrations of virus that had

TABLE IX

Ability of Ecotropic M-MuLVs and Variants (Dualtropic) of M-MuLV Clone IC To Induce a Secondary Cytotoxic Response

Virus	Dose (FIU/well)	Percent specific cytotoxicity (± SE) vs. MBL-2		
		100:1*	70:1	10:1
M-MuLV clone IC	4×10^6	52.1 (0.8)‡	50.0 (1.9)	34.5 (1.4)
M-MuLV clone IC	4×10^5	42.3 (1.4)	40.8 (1.0)	27.8 (0.6)
M-MuLV clone IC	4×10^4	13.2 (0.6)	11.8 (0.5)	8.8 (0.9)
M-MuLV clone H	2×10^5	30.1 (0.5)§	27.7 (0.6)	21.5 (0.6)
M-MuLV clone H	4×10^4	21.6 (0.3)	17.6 (0.4)	15.1 (0.4)
HIX-FEF	5×10^5	2.2 (0.3)§	2.9 (0.5)	2.4 (0.8)
HIX-FEF	1×10^5	2.5 (0.8)	1.5 (0.7)	1.8 (0.6)
HIX-RD	5×10^5	1.2 (0.4)§	1.8 (0.1)	0.9 (0.6)
HIX-RD	1×10^5	2.0 (0.6)	2.1 (0.9)	1.2 (1.0)
M-MuLV clone 83	4×10^5	1.5 (1.0)§	2.1 (0.8)	1.5 (0.6)
M-MuLV clone 83	8×10^4	−1.2 (0.8)	1.3 (0.6)	2.1 (0.8)

* As in Table VIII.
‡ As in Table VIII.
§ As in Table VIII.

been UV-irradiated to reduce the titer by 7 logs (data not shown). In recent experiments using purified M-MuLV gp70 (5 μg/ml), we have been unsuccessful in inducing a secondary cytotoxic response (unpublished data), although it may be possible to induce a response with much higher concentrations of M-MuLV gp70. Therefore, infectivity of virus appears to be necessary for the induction of a secondary cytotoxic response. Our results should be compared to those by Braciale and Yap (26) and others (27, 31). Inactivated Sendai virus or virion proteins were shown to induce virus-specific cytotoxic responses (27, 28), and to sensitize target cells for lysis by cytolytic T lymphocytes (27, 31). Braciale and Yap also found that influenza virus inactivated by UV light was able to induce a secondary cytotoxic response but failed to sensitize target cells for lysis (26). Nevertheless, it should be noted that Sendai virus possesses a fusion protein which allows for the efficient integration of virion antigens into cell surface cytoplasmic membranes (32). Thus, the requirement for infectivity with M-MuLV suggests that absorption of the inactivated virus is not sufficient for immunogenicity. It seems likely that integration of M-MuLV into the cell membranes of responding cells or accessory cells, via infection, is needed for the formation of stimulatory antigens. These results are compatible to those of Ertl et al. (25) and Braciale and Yap (26) who showed that infectivity of viruses was required for the induction of cytolytic T lymphocytes in vivo by using vaccinnia and influenza viruses. Currently, we are investigating which types of the cells are infected with M-MuLV.

In some other studies involving cytotoxic reactivity against type C viral antigens, it appears that noninfectious materials were capable of inducing an in vitro secondary cytotoxic response. Alaba and Law (33) have recently reported that spleen cells from mice immunized with antigen solubilized from the plasma membrane of a Rauscher virus-induced lymphoma could be restimulated in vitro with the same antigen to become cytotoxic. Bruce et al. (34) found that disrupted MuLV could stimulate a secondary response by spleen cells from rats immunized with a Gross virus-induced lymphoma. In contrast, we have previously tested several materials (other than

TABLE X

Failure of a Xenotropic Virus (BALB Virus-2) To Induce a Secondary Cytotoxic Response by M-MSV-immune Spleen Cells

Number of virus particles	E/T ratio	Percent specific cytotoxicity (± SE) vs. MBL-2	
		M-MuLV clone IC	BALB virus-2
3×10^6	100:1	0 (0.6)	2.0 (0.9)
	30:1	0 (0.7)	1.9 (0.7)
	10:1	0 (0.5)	0.6 (0.6)
3×10^7	100:1	13.8 (1.0)	1.9 (0.7)
	30:1	7.1 (0.9)	0.9 (0.7)
	10:1	3.9 (0.7)	1.9 (0.6)
3×10^8	100:1	34.7 (0.7)	2.2 (1.0)
	30:1	27.2 (1.5)	1.1 (0.9)
	10:1	17.3 (0.8)	2.9 (1.0)
3×10^9	100:1	41.0 (0.8)	0.5 (0.8)
	30:1	37.0 (1.0)	0 (0.5)
	10:1	25.5 (1.1)	2.1 (0.7)

infectious virus) derived from MBL-2 tumor cells (among these were subcellular extracts obtained by freezing and thawing, and fractions solubilized by NP-40, deoxycholate, and 3M KCl) for their ability to stimulate a secondary cytotoxic response by M-MSV-immune spleen cells and found them to be inactive (unpublished observations). This may have been a result of insufficient antigenicity of the solubilized material or, in light of the data discussed in this paper, to insufficient titers of infectious virus in these preparations. Alternatively, some of the different results that have been obtained in various studies may be related to the method of primary immunization; we used virus for immunization whereas Alaba and Law (33) used soluble antigen and Bruce et al. (34) employed viable tumor cells.

Although tumor immune responses in the MSV system have been extensively studied in many laboratories, there is no agreement on the nature of the antigenic specificities of the cellular response. Gomard et al. (23) examined the nature of the cell surface antigens, and found that Friend-Moloney-Rauscher-like antigens appeared to be major antigenic determinants. Herberman et al. (3) reported that the surface antigens recognized by anti-M-MSV cytolytic T lymphocytes were related to the expression of endogenous type C virus. Furthermore, Aoki et al. (29), using a cold target cell inhibition assay, reported that xenotropic virus-producing SIRC cells could inhibit the anti-M-MSV response. Gorczynski and Knight (35) found p30 to be one of the antigenic determinants. More recently, Enjuanes et al. (7) found that the primary anti-M-MSV cytotoxic response was specific for M-MuLV, and especially for its envelope gp70, as determined by ^{51}Cr release inhibition by purified virion components. In the present study, we examined the specificity of the cytotoxic response in both the induction and effector phases. Effector cells generated by M-MuLV clone IC could lyse efficiently MBL-2, EL-4(G+), B6-MEF-M-MSV, and B6-MEF-M-MuLV, but did not significantly affect EL-4(G−), YAC, RLδ1, RDM-4, B6-MEF, B6-MEF-B-tropic, B6-MEF-F-MuLV, and EδG2, thus indicating that the determinant(s) recognized by cytolytic T lymphocytes was closely associated with M-MuLV but not with a variety of other murine type C oncornaviruses. These results

TABLE XI

Ability of M-MuLV Clone IC or Nonmurine Type C Viruses To Induce a Secondary Cytotoxic Response by M-MSV-immune Spleen Cells

Exp.	Virus	E/T ratio	Percent specific cytotoxicity (± SE) vs. MBL-2				
			Number of virus particles				
			3×10^6	3×10^7	3×10^8	3×10^9	3×10^{10}
1	M-MuLV clone	80:1	6.2 (0.7)	11.7 (1.0)	31.3 (1.2)	43.7 (1.6)	—*
	IC	30:1	4.2 (1.0)	8.6 (1.6)	26.3 (1.9)	42.3 (0.5)	—
	FeLV Theilen	80:1	0.7 (0.5)	1.8 (0.6)	1.3 (0.5)	1.6 (0.6)	—
		30:1	0.3 (0.7)	0 (0.5)	0.3 (0.5)	0.6 (0.7)	—
2	M-MuLV clone	50:1	0 (0.7)	1.5 (0.7)	19.4 (0.6)	32.5 (0.9)	41.3 (1.8)
	IC	30:1	0 (0.4)	0.9 (0.7)	0.9 (0.9)	18.3 (0.6)	25.6 (1.3)
	Baboon M7	50:1	0 (0.6)	0 (0.3)	0.1 (0.8)	1.6 (0.5)	2.1 (1.3)
	type C	30:1	0.1 (0.7)	0.7 (0.7)	0.1 (0.8)	1.3 (0.4)	1.4 (0.6)

* Not done.

support the findings of Cerny and Essex (36) and Cloyd et al. (37) showing that Moloney antigens are different from Friend and Rauscher antigens as determined by immunofluorescence microscopy.

The M-MuLV-stimulated effector cells also lysed RBL-5 (induced by R-MuLV) and EL-4(G+) targets, which would appear to be against an M-MuLV-restricted specificity. However, RBL-5 cells contained a significant amount of M-MuLV antigens (gp70 and p12), as determined by competition radioimmunoassay (J. Lee, personal communication) and therefore, it is not surprising that intact RBL-5 or supernate of RBL-5 were also capable of inducing a secondary cytotoxic response (unpublished data). The present results with EL-4(G+) are compatible with previous data from our laboratory (3) indicating that these cells are susceptible to lysis by primary anti-M-MSV immune cells and can inhibit the cytolysis of RBL-5 or MBL-2 in a cold target inhibition assay. Ng et al. (38) also have shown that anti-M-MSV serum lysed EL-4(G+) in the presence of complement. EL-4(G+) cells susceptible to the lysis by the effector cells were shown to contain NB-tropic and xenotropic endogenous type C viruses (39). Thus, although EL-4(G+) expresses serologically detectable GCSA, it seems possible that EL-4(G+) contains an endogenous MuLV closely associated to M-MuLV, instead of or in addition to Gross-MuLV.

In the induction phase, only ecotropic M-MuLVs clone IC and clone H were able to induce a secondary cytotoxic response by M-MSV-immune spleen cells. In contrast, AKR-MuLV, Gross-MuLV, BALB-B-tropic virus, and BALB virus-1 failed to induce such responses, indicating that this response was not a result of these characterized endogenous murine type C viruses. However, it is still possible that a particular endogenous type C virus was responsible for the induction of this response. When R-MuLV was used as the stimulus, low but significant levels of cytotoxicity could be induced, thus indicating some cross-reactivity, perhaps of a minor antigenic determinant(s), between M- and R-MuLVs, because type-, group- and interspecies-specific determinants of gp70 and p30 have been found by radioimmunoassay (40). Thus, it appears from the above results that the induction of in vitro secondary cytotoxic responses by virus is specific for M-MuLV and related viruses.

Because some variants (M-MuLV clone 83, HIX-FEF, or HIX-RD) of M-MuLV

clone IC have been isolated and characterized as recombinants in the *env* gene between an ecotropic M-MuLV and xenotropic virus (13, 14, 41), we tested for the ability of M-MuLV clone 83, HIX-FEF or HIX-RD to induce a secondary cytotoxic response by M-MSV-immune spleen cells. The two ecotropic M-MuLV clone IC and clone H were able to induce the response, whereas dualtropic M-MuLV clone 83, HIX-FEF, and HIX-RD failed to do so, indicating that type-specific determinants of M-MuLV gp70 are important in this system. It should be noted that when mouse cells were infected with M-MuLV clone 83 or HIX-FEF, infectious virus was released into the supernate, thus indicating that the lack of stimulation by M-MuLV clone 83 or HIX-FEF was not a result of an inability to infect mouse cells (data not shown).

Thus, by studying the ability of infectious viruses to induce a secondary response, we have found that both the induction and effector phases of the in vitro secondary cytotoxic response in the M-MSV system are strongly associated with the type-specific determinants of M-MuLV gp70. Furthermore, this experimental approach should facilitate the detailed analysis of the cell-mediated cytotoxic response to oncornaviruses.

Summary

We have developed a system to induce oncornavirus-specific secondary cytotoxic response in vitro. When Moloney strain of murine sarcoma virus-immune spleen cells were cultivated with purified infectious Moloney murine leukemia virus (M-MuLV) or with supernates of tissue culture cells containing infectious virus, a virus-specific secondary cytotoxic response directed against type-specific determinant(s) of M-MuLV was generated in vitro, as determined by a 4-h ^{51}Cr-release assay. The effector cells were susceptible to the treatment with anti-Thyl.2 plus complement, but were unrelated to natural killer cells (NK), because they could not lyse some target cells that are highly sensitive to NK activity. This response was primarily (or largely) type-specific for M-MuLV in both the induction phase and the interaction between effector cells and target cells. Furthermore, a product of the *env* gene of M-MuLV, perhaps gp70, appeared to be responsible for this response, because viruses with recombinations in the *env* gene between ecotropic M-MuLV and a xenotropic virus failed to induce a response. When infectious M-MuLV was exposed to UV-light at different doses, the ability of UV-treated M-MuLV to induce a secondary cytotoxic response decreased in parallel with infectivity, indicating that infectivity was necessary for the induction of this response.

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