

THE DETECTION OF A SPLEEN FOCUS-FORMING VIRUS NEOANTIGEN BY LYMPHOCYTE-MEDIATED CYTOLYSIS*

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Murine erythroleukemia cells induced by Friend (FLV)¹ and Rauscher (RLV) leukemia viruses have been shown to express tumor-associated cell surface antigens (TASA) (1-3). The genes coding for these antigens have remained ill-defined, although present evidence indicates that the helper virus (lymphatic leukemia virus, LLV) genome may be responsible (4-6).

Until recently, it was not possible to examine putative cell surface antigens coded for by the replication-defective portion (spleen focus-forming virus, SFFV) of the viral genome due to an excess of helper LLV present in viral isolates. Troxler et al. (7) as well as Bernstein et al. (8) have recently obtained the SFFV genome free of LLV in both murine and rat cell lines through virus and cell cloning procedures. These developments have permitted the investigation of possible cell surface antigens contributed by genetic information contained in SFFV.

We have applied the techniques of secondary mixed tumor-lymphocyte culture (MTLC) stimulation followed by lymphocyte-mediated cytotoxicity (LMC) assays (tests used to demonstrate immunity in the murine sarcoma virus, MSV, system [9, 10]) to this SFFV nonproducer model system to investigate whether or not the SFFV genome alone codes for a cell surface antigen. We now report that BALB/c 3T3 clones containing the SFFV genome free of infectious LLV, express a cell surface antigen which is not a virus structural protein, yet which is present on the surfaces of syngeneic, productively infected RLV and FLV-induced erythroleukemia cells.

Materials and Methods

Mice. Age-matched BALB/c female, 8-10-wk-old mice were used in immunization experiments and as sources for normal thymocytes in LMC assays. All mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

* Supported in part by a research grant (CA 17643) and a research contract (N01-CB-74141) from the National Cancer Institute.

¹ *Abbreviations used in this paper:* C, core; E, envelope; FCS, fetal calf serum; FeLV, feline leukemia virus; FLV, Friend leukemia virus; FOCMA, feline oncornavirus-associated cell membrane antigen; GCSA, Gross cell surface antigen; GLV, Gross leukemia virus; gp, glycoprotein; KiSV, Kirsten sarcoma virus; LLV, lymphatic leukemia virus; LMC, lymphocyte-mediated cytotoxicity; LU, lytic unit; MCSA, Moloney cell surface antigen; Mo-MuLV, Moloney leukemia virus; MSV, murine sarcoma virus; MTLC, mixed tumor-lymphocyte culture; MuLV, murine leukemia virus; p, protein; PFU, plaque-forming units; RIA, radioimmunoassay; RLV, Rauscher leukemia virus; RT, reverse transcriptase; SFFV, spleen focus-forming virus; TASA, tumor-associated cell surface antigen.

Target Cells. Two nontransformed, nonproducer BALB/c 3T3 fibroblast cell lines containing the SFFV genome and free of replicating LLV (1902 B Cl-4 and 1902 B Cl-6) were obtained from Dr. David H. Troxler, Laboratory of Tumor Virus Genetics, National Cancer Institute, Bethesda, Md. (7).

Dr. Troxler also provided the parent BALB/c 3T3 cell line from which the SFFV nonproducers were cloned; a BALB/c 3T3 cell line productively infected with a cloned Friend LLV (BALB/c 3T3 F-murine leukemia virus [MuLV]); the 1902 B Cl-1 cell line (a normal BALB/c 3T3 cell line cloned in the same experiment which successfully isolated the SFFV nonproducer clones); the SFFV-FRE cell line (11); a Fischer rat epithelial cell which also contains the SFFV genome free of replicating LLV; and the FRE cell line, a Fischer rat epithelial cell which lacks SFFV genetic information. Other target cells syngeneic to the BALB/c mouse included the RLV-induced, MCDV-12 leukemia cell (obtained from Dr. Ronald Herberman, National Cancer Institute) and the FLV-induced, FLD-3 erythroleukemia cell (obtained from Dr. W. Ostertag, Max Planck Institute, Göttingen, W. Germany).

The 1902 B Cl-4 cell line was productively infected with two LLV preparations resulting in the generation of two additional target cells. 1902 B Cl-4-R-MuLV was produced by the co-cultivation of the 1902 B Cl-4 cell with TNF leukemia cells. The TNF cell line was initiated by Dr. T. N. Fredrickson et al. (12) from a leukemic mouse injected with an end-point diluted stock of RLV. The TNF cell line was adapted to tissue culture in our laboratory and produces RNA tumor virus as measured by XC (2,002 plaque-forming units [PFU]/ml) and reverse transcriptase (RT) assays. However, TNF tissue culture fluid lacks *in vivo* SFFV activity (20,000 XC PFU failed to produce spleen foci, data per Dr. Richard Steeves, Albert Einstein College of Medicine, Bronx, N. Y.). 1902 B Cl-4-Moloney leukemia virus (Mo-MuLV) was produced by the co-cultivation of 1902 B Cl-4 cells with MBL-2 leukemia cells. The MBL-2 is an Mo-MuLV-transformed tumor cell syngeneic to the C57Bl/6 mouse which produces large amounts of MuLV as detected in XC (5,200 PFU/ml) and RT assays.

BALB/c 3T3 cells were also productively infected with R-MuLV and Mo-MuLV by co-cultivation with TNF and MBL-2 leukemia cells, respectively. These co-cultivations resulted in the generation of two nontransformed, LLV-producing fibroblast cell lines; BALB/c 3T3-R-MuLV and BALB/c 3T3-Mo-MuLV. The Kirsten sarcoma virus (KiSV)-transformed, replication-defective BALB/c 3T3 cell line (K-BALB; 13) was obtained from Dr. Robert Bassin, National Cancer Institute. The sarcoma virus genome of this transformed cell was also rescued by co-cultivation with TNF and MBL-2 leukemia cells resulting in the production of two KiSV-rescued cell lines; K-BALB-R-MuLV and K-BALB-Mo-MuLV.

Fibroblast cell lines were maintained in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% heat-inactivated fetal calf serum (FCS; Grand Island Biological Co.), 50 U/ml penicillin, 50 μ g/ml gentamicin, and 300 μ g/ml fresh L-glutamine (Grand Island Biological Co.). MBL-2, TNF, FLD-3, and MCDV-12 leukemia cells were maintained in suspension culture in RPMI-1640 (Grand Island Biological Co.) supplemented with 10% FCS, 2.5×10^{-5} M beta-mercaptoethanol/ml, 50 μ g/ml gentamicin, and 300 μ g/ml fresh L-glutamine. All cells were cultured in a humidified atmosphere of 5% CO₂ in air at 37°C.

Immunization. Mice were immunized subcutaneously in the right flank with 1×10^6 live 1902 B Cl-4 SFFV nonproducer cells biweekly for a period of 6 wk. Spleen and inguinal lymph node cells were used as responding lymphocytes in *in vitro* activation with 1902 B Cl-4 cells, 2-12 wk after completion of the immunization schedule.

Viral Characterization of Target Cells. Tests for RT activity (14) present in target cell tissue culture medium, and for production of mature MuLV (XC assay; 15) were conducted as previously described. As a more sensitive indicator of MuLV production in XC assays, 1×10^4 target cells were often co-cultivated with SC-1 cells for 4 days before the addition of the XC rat indicator cell. Target cells used in LMC assays were tested for the presence of cell surface viral proteins in antibody-dependent, complement-mediated cytotoxicity assays. Heat-inactivated, absorbed, monospecific goat antisera raised against purified murine ecotropic and xenotropic viral proteins were tested (obtained from Dr. Jack Gruber, Office of Program Resources and Logistics, Viral Oncology, National Cancer Institute). The antigenic specificity of all antisera used in these tests as defined by Dr. Gruber is detailed in Table I. Methods involved in antibody-dependent complement-mediated cytotoxicity assays have been previously described (10).

7-day assays for MSV production on SC-1 mouse fibroblasts, using target tissue culture supernates were conducted as previously described (16). Target cell tissue culture fluid was also

TABLE I
Specificity of Antisera Used in Antibody-Dependent Complement-Mediated Cytolysis Assays for Cell Surface Expression of Viral Structural Proteins

Goat antisera anti -	Preparation of immunizing antigen	Radioimmunoassay specificity data labeled antigen (50% binding titer)*
AKR MuLV p10	Ion exchange chromatography & gel filtration	AKR MuLV p10(150), RLV p30(<50), AKR MuLV p12(<50)
AKR MuLV p30	Ion exchange chromatography & gel filtration	AKR MuLV p30(800), RLV p30(24,000), AKR MuLV p10(<50), AKR MuLV p12 (<50)
BALB virus 2 p10	Guanidine HCl + agarose chromatography	BALB virus 2 p10(2720), BALB virus 2 p30(<50)
BALB virus 3 p30	Guanidine HCl + agarose chromatography	BALB virus 2 p30 (1000)
BALB virus 2 gp69/71	Ion exchange chromatography	BALB virus 2 gp69/71(9600), BALB virus 2 p30(<50)
GLV p12	Ion exchange chromatography	GLV p12(4800), GLV p30(<50)
GLV p30	Ion exchange chromatography	GLV p30(9800)
RLV p10	Guanidine HCl + agarose chromatography	RLV p10(9600), RLV p30(<50)
RLV p12	Guanidine HCl + agarose chromatography	RLV p12(18,000), RLV gp69/71(<50), RLV p30(75)
RLV p15	Guanidine HCl + agarose chromatography	RLV p15(2000), RLV p30(50)
RLV p30	Guanidine HCl + agarose chromatography	RLV p30(38,000)
RLV p60 precursor	Guanidine HCl + agarose chromatography	ND‡
RLV gp69/71	Ion exchange chromatography	RLV gp69/71(3200), RLV p30(<50)

* Expressed as reciprocal of serum dilution corresponding to 50% binding titer of labeled antigen.

‡ ND, not done.

tested for xenotropic virus production on mink lung fibroblast cells as measured by insipient RT activity (17). AT5-Dog and NZB-Dog cell tissue culture fluid was used as a positive control in all assays for xenotropic virus production (cells obtained from Dr. Sandra K. Ruscetti, Laboratory of Tumor Virus Genetics, National Cancer Institute). 24-h tissue culture fluid harvested from cultures of SFFV nonproducer and nonproducer-rescued cell lines were assayed for in vivo SFFV activity by inoculation into CDF₁ newborn mice. Assays for in vivo SFFV activity (18) were conducted by Dr. Richard Steeves.

In Vitro Activation of 1902 B Cl-4-Immune Lymphocytes. Spleen and inguinal lymph node cells from immunized mice were teased apart from the intact organ with forceps, minced, and passed through successively smaller gauge needles to insure single cell suspension. Erythrocytes were removed from the cell population by hypotonic shock, and the remaining cells were counted with a Coulter electronic counter (Coulter Electronics Inc., Hialeah, Fla.). After we determined cell viability via trypan blue exclusion, we diluted cells in Click's medium (Altick Associates, Hudson, Wis.), supplemented with 2% FCS, 300 µg/ml fresh L-glutamine, 50 U/ml penicillin, 50 µg/ml gentamicin, 16 µmol/ml NaHCO₃, and 25 µmol/ml HEPES buffer (Calbiochem, San Diego, Calif.). Spleen cell populations were adjusted to a final concentration of 2.5×10^6 cells/ml. In vitro activation of 1902 B Cl-4 immune lymphocytes was conducted by mixing 10 ml of spleen cells with 10 ml of UV-irradiated (6,000 electroretinograms/mm²) 1902 B Cl-4 fibroblasts which had been adjusted in supplemented Click's medium to a concentration of 1.25×10^5 cells/ml. In vitro

lymphocyte activations were conducted in 30-ml tissue culture flasks (3012; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) cultured upright in a humidified atmosphere of 5% CO₂ in air at 37°C. Activated lymphocytes were harvested after 5 days in culture, and viable cells were used as effector cells in LMC assays.

LMC Assays. 4-h ⁵¹Cr-release assays were conducted in 96-well V-bottom microplates (1S-MVC-96 TC; Linbro Chemical Company, New Haven, Conn.) using a methodology previously described (10). Percent specific lysis was determined by using the following equation:

$$\text{percent specific lysis} = 100 \times \frac{\text{experimental cpm} - \text{medium control cpm}}{\text{maximum release cpm} - \text{medium control cpm}}$$

One lytic unit (LU) was defined as the number of effector cells required to cause 30% specific lysis. Data is displayed both graphically and in tabular form as percent cytotoxicity observed at an effector/target cell ratio of 100/1, and as LU/25 × 10⁶ effector cells. In cases where 30% specific lysis was not achieved even at a relatively high effector/target cell ratio, LU were determined by graphical interpolation.

Results

Target Cell Viral Genome Expression. The virological characterization of all target cells used in these studies is displayed in Tables II and III. SFFV nonproducer fibroblast cell lines (1902 B Cl-4, 1902 B Cl-6, and SFFV-FRE) showed no virus production as measured by XC, RT, and in vivo SFFV activity assays. In addition, all three SFFV nonproducers lacked cell surface expression of viral structural antigens protein (p)10, p12, p15, p30, or glycoprotein (gp)71 as measured by antibody-dependent complement-mediated cytolysis. BALB/c 3T3, 1902 B Cl-1 and KiSV-transformed K-BALB cells were also void of viral genome expression. The SFFV genome contained in 1902 B Cl-4 cells was effectively rescued after co-cultivation with TNF and MBL-2 murine leukemia cells. Both 1902 B Cl-4-R-MuLV, and 1902 B Cl-4-Mo-MuLV were markedly positive in XC co-cultivation assays. 24-h tissue culture medium harvested from cultures of SFFV-rescued cell lines, contained high titers of RT and in vivo SFFV activity. 1902 B Cl-4-R-MuLV expressed p15, p12, gp71 at the cell surface, and 1902 B Cl-4-Mo-MuLV was positive for surface expression of gp71. BALB/c 3T3 cells infected with R-MuLV, F-MuLV, and Mo-MuLV tested positive for virus production as measured in XC and RT assays, and in cytotoxicity tests for cell surface expression of viral structural antigens. The replication-defective MSV genome contained in the K-BALB cell was rescued after co-cultivation with TNF and MBL-2 leukemia cells. Both K-BALB-Mo-MuLV and K-BALB-R-MuLV produced high titers of both MuLV and transforming virus (as measured by XC, RT, and in vitro focus-formation assays) and tested positive for cell surface expression of gp71.

LMC Detection of an SFFV-Specific Cell Surface Antigen. Spleen and inguinal lymph node cells from BALB/c mice immunized with the SFFV nonproducer 1902 B Cl-4, were stimulated in a 5-day in vitro activation with UV-irradiated, 1902 B Cl-4 cells. Viable effector cells harvested from activated cultures were then tested for cytotoxic reactivity against a battery of target cells. Results of a typical experiment are displayed in Fig. 1A. 1902 B Cl-4-immune lymphocytes were highly efficient in effecting the lysis of two SFFV nonproducer BALB/c 3T3 clones (1902 B Cl-4 and 1902 B Cl-6). BALB/c 3T3 cells and BALB/c 3T3 fibroblasts infected with a cloned F-MuLV remained uninfected. The data displayed in Fig. 1A implied the existence of an antigen on the

TABLE II
Virological Characterization of Target Cells

Target cell	XC Activity		RT activity*	In vivo SFFV activity‡	MSV activity§	Xenotropic virus production
	Tissue culture supernate	Target cells co-cultivated				
	PFU/ml	PFU/10 ⁴				
1902 B Cl-4	0	0	0.04	0	0	<0.01
1902 B Cl-4-R-MuLV	0	131	39.7	350	0	0.01
1902 B Cl-4-Mo-MuLV	1,390	TNC¶	60.5	6	0	<0.01
1902 B Cl-6	0	0	0.01	0	0	<0.01
SFFV-FRE	0	0	0.01	0	0	0.04
FRE	0	0	0.01	nd**	0	<0.01
MCDV-12	2,818	TNC	48.2	8	0	<0.01
FLD-3	1,773	TNC	37.0	<10	0	<0.01
BALB/c 3T3	0	0	0.67	nd	0	<0.01
BALB/c 3T3-F-MuLV	2,265	TNC	68.3	nd	0	<0.01
BALB/c 3T3-R-MuLV	70	TNC	29.2	nd	0	<0.01
BALB/c 3T3-Mo-MuLV	490	TNC	46.2	nd	0	<0.01
1902 B Cl-1	0	0	0.07	nd	0	<0.01
K-BALB	0	0	0.03	nd	0	0.04
K-BALB-R-MuLV	1,050	TNC	40.4	nd	2,170	<0.01
K-BALB-Mo-MuLV	5,550	TNC	41.4	nd	3,250	0.05
ATS-Dog	nd	nd	nd	nd	nd	12.5
NZB-Dog	nd	nd	nd	nd	nd	25.0

* Expressed as $\text{cpm} \times 10^{-4}$ of [³H]thymidine triphosphate ([³H]TTP) incorporated by oligo dT poly rA template aliquot minus cpm of [³H]TTP incorporated by oligo dT poly dA template aliquot.

‡ Expressed in terms of spleen focus-forming units (FFU)/ml of target cell tissue culture supernate. Only pertinent target cell fluids were assayed for in vivo SFFV activity.

§ Expressed in terms of in vitro FFU/ml of target cell tissue culture supernate.

|| Expressed in terms of RT activity of mink lung fibroblasts after 7-day culture in the presence of target cell tissue culture supernate.

¶ TNC, too numerous to count.

** nd, not done.

surface of SFFV nonproducer cells which was capable of provoking meaningful cell-mediated immune reactivity. The fact that BALB/c 3T3 F-MuLV fibroblasts were not lysed by 1902 B Cl-4 immune lymphocytes suggested that this cell surface antigen was not coded for by the helper virus genome. This suggestion was confirmed by results of subsequent experimentation displayed in Fig. 1 B. 1902 B Cl-4-Mo-MuLV and 1902 B Cl-4-R-MuLV SFFV-rescued targets were effectively lysed by SFFV nonproducer-immune activated lymphocytes. However, BALB/c 3T3 cells productively infected with either R-MuLV or Mo-MuLV remained unaffected. Therefore, the lysis of the SFFV-rescued cell lines was not due to cytotoxic reactivity directed against an LLV-coded cell surface antigen. The data displayed in Fig. 1 along with the observation that the SFFV nonproducer cells lacked cell surface expression of type-, group-, or interspecies-specific viral structural antigens, indicated that the antigen detected by LMC was coded for solely by the SFFV genome.

It is interesting to note that both MCDV-12 (an RLV-induced leukemia cell)

TABLE III
Target Cell Surface Expression of Virus Structural Proteins*

Target cell	AKR MuLV p10	AKR MuLV p30	BALB virus 2 p10	BALB virus 2 p30	BALB virus 2 gp69/71	GLV p12	GLV p30	RLV p10	RLV p12	RLV p15	RLV p30	RLV p60 pre- cursor	RLV gp69/71
1902 B Cl-4	-‡	-	-	-	-	-	-	-	-	-	-	-	-
1902 B Cl-4-R-MuLV	-	-	-	-	-	-	-	-	1,750	50	-	-	750
1902 B Cl-4-Mo-MuLV	-	-	-	-	450	-	-	-	-	-	-	-	-
1902 B Cl-6	-	-	-	-	-	-	-	-	-	-	-	-	-
SFFV-FRE	-	-	-	-	-	-	-	-	-	-	-	-	-
FRE	-	-	-	-	-	-	-	-	-	-	-	-	-
MCDV-12	-	-	-	-	-	-	-	-	250	-	128	-	2,500
FLD-3	-	-	-	-	-	-	-	-	-	-	-	-	320
BALB/c 3T3	-	-	-	-	-	-	-	-	-	-	-	-	-
BALB/c 3T3-F-MuLV	-	-	-	-	450	-	-	-	-	-	-	-	-
BALB/c 3T3-R-MuLV	-	-	-	-	1,350	-	-	-	-	-	-	-	150
BALB/c 3T3-Mo-MuLV	-	-	-	-	450	-	-	-	-	-	-	-	-
1902 B Cl-1	-	-	-	-	-	-	-	-	-	-	-	-	-
K-BALB	-	-	-	-	-	-	-	-	-	-	-	-	-
K-BALB-R-MuLV	-	-	-	-	450	-	-	-	450	150	-	-	1,350
K-BALB-Mo-MuLV	-	-	-	-	2,500	-	-	-	-	-	-	-	-

* Expressed as the reciprocal of the serum dilution which caused 20% specific lysis in antibody-dependent complement-mediated cytotoxicity assays.

‡ -, No specific lysis observed.

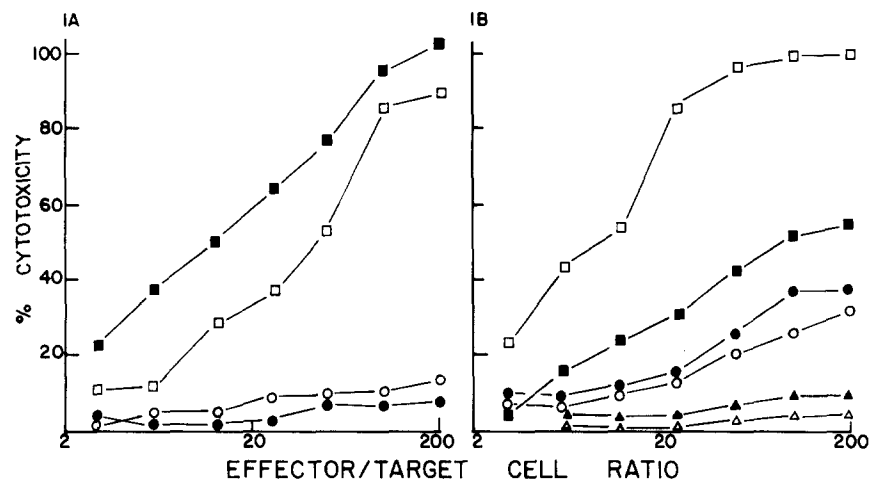


FIG. 1. (A), Cytotoxic reactivity of 1902 B Cl-4-immune, in vitro activated spleen cells, directed against 1902 B Cl-4 (■), 1902 B Cl-6 (□), BALB/c 3T3 (●), and BALB/c 3T3-F-MuLV (○) target cells. (B), Cytotoxic reactivity of 1902 B Cl-4-immune, in vitro activated spleen cells directed against 1902 B Cl-4-Mo-MuLV (□), 1902 B Cl-4-R-MuLV (■), MCDV-12 (●), FLD-3 (○), BALB/c 3T3-R-MuLV (▲), and BALB/c 3T3-Mo-MuLV (△) target cells.

and FLD-3 (an FLV-induced erythroleukemia cell) were effectively lysed by 1902 B Cl-4 immune lymphocytes. This suggested the cell surface expression of the SFFV-specific antigen by in vivo transformed leukemia cells as well.

The results of replicate experiments in which 1902 B Cl-4-immune, in vitro activated lymphocytes were used as LMC effector cells are shown in Table IV. The data is presented in terms of $LU/25 \times 10^6$ effector cells, and as the percent specific lysis observed at an effector/target cell ratio of 100/1. Experimentation

TABLE IV
Lysis of Target Cells by 1902 B-CI-4 Immune Spleen Cells

Target cell	LU/25 × 10 ⁶ effec- tor cells	Cytotoxicity at effec- tor/target cell ratio of 100/1
		%
1902 B CI-4	3,472	78
1902 B CI-4-R-MuLV	313	88
1902 B CI-4-Mo-MuLV	2,477	68
1902 B CI-6	2,750	62
SFFV-FRE	2,500	72
FRE	0.5	12
MCDV-12	41.7	25
FLD-3	312.5	32
BALB/c 3T3	1.8	4
BALB/c 3T3-F-MuLV	6.3	8
BALB/c 3T3-R-MuLV	0.6	7
BALB/c 3T3-Mo-MuLV	6.7	9
1902 B CI-1	7.1	11
K-BALB	0.5	8
K-BALB-R-MuLV	5.0	6
K-BALB-Mo-MuLV	<0.01	4
BALB/c thymocytes	<0.01	6

displayed in Table IV emphasizes that the cytotoxic reactivity of 1902 B CI-4-immune lymphocytes appears to be directed only against target cells containing the SFFV genome. 1902 B CI-4 and 1902 B CI-6 cells, along with the SFFV rescued targets, were quite susceptible to lysis by 1902 B CI-4 activated effector cells. Once more, BALB/c 3T3 (both the parent BALB/c 3T3 population and the 1902 B CI-1 cloned 3T3 cells) and 3T3 MuLV-infected target cells were not effectively lysed by SFFV nonproducer immune lymphocytes. It is important to note that both K-BALB cells and the KiSV-rescued, K-BALB-Mo-MuLV and K-BALB-R-MuLV targets were not lysed by SFFV nonproducer-activated spleen cells. The lack of cytotoxic reactivity against these cells further demonstrated that the antigen present on the 1902 B CI-4 SFFV nonproducer was not coded for by a helper virus genome, nor was it shared by MSV genome-containing target cells. Data displayed in Table IV also demonstrated that 1902 B CI-4 immune effector cells were capable of killing the xenogeneic SFFV-FRE cell. This observation emphasizes the SFFV-specific nature of the antigen in question and furthermore, provides indirect evidence that the antigen in question is not coded for by endogenous murine genome(s) activated in the BALB/c 3T3 cells by the SFFV genome.

Discussion

The results of these studies indicate that the SFFV genome codes for a cell surface antigen which is capable of provoking an antigen-specific cell-mediated immune response. The antigen was detected on the surface of cloned BALB/c 3T3 and FRE fibroblasts which contained the SFFV genome, free of replicating LLV. The SFFV nonproducer fibroblasts were void of cell surface expression of viral structural proteins as determined in antibody-dependent complement-

mediated cytotoxicity assays using monospecific antisera raised against purified murine ecotropic and xenotropic structural polypeptides. Normal BALB/c 3T3 fibroblasts and BALB/c 3T3 cells infected with three types of helper LLV were not susceptible to lysis by SFFV nonproducer-immune lymphocytes. Based upon these observations, it is reasonable to assume that the antigen detected in these LMC experiments is not coded for by genetic information contained in the helper component of FLV and represents a true SFFV-specific cell surface antigen.

Several nonproducer-specific antigens have been investigated in experimental sarcoma and leukemia virus systems. However, most reported nonproducer TASAs have been confirmed serologically. The existence of such humorally defined TASAs remains a controversial subject, due to reports claiming that the antigens detected are simply viral structural proteins present at the cell surface.

Cats infected with feline leukemia virus (FeLV) contain in their sera antibodies specific for an antigen present on the surface of FeLV-transformed cat cells (19). Antisera harvested from FeLV-infected cats retains its specificity and affinity for the feline leukemia cell surface, even after absorption with FeLV structural proteins gp70 and p30 (20). Furthermore, humoral reactivity against this serologically detected feline-oncornavirus-associated cell membrane antigen (FOCMA) has been shown to positively correlate with the natural resistance of cats to FeLV-induced leukemia or sarcoma (21). Data intimating that FOCMA expression may be the result of sarcoma virus genetic sequences was reported in studies detailing FOCMA cell surface expression on replication-defective FeLV-transformed mink cells (22). Whether or not FOCMA truly represents a cell surface neoantigen distinct from virion structural proteins remains open to question. Recently, Ruscetti and Parks (23), using immune precipitation of radiolabeled cell membranes and polyacrylamide gel electrophoresis presented evidence indicating that anti-FOCMA immune reactivity may indeed be directed against a class of 85,000 mol wt proteins which contain determinants of the major FeLV glycoprotein.

A similar antigen present on the surface of cells transformed by Gross leukemia virus (GLV) has also been recently characterized. Antibody directed against the Gross cell surface antigen (GCSA) has been traditionally prepared by immunization of C57Bl/6 mice with the transplanted AKR spontaneous leukemia (K-36) (24). Anti-GCSA antibody has been found to be cytotoxic against cells infected with GLV and absorbed by the normal lymphoid tissues of mice with high incidences of spontaneous leukemia (24, 25). Investigators using immunoelectron microscopy have determined that anti-GCSA antisera does not bind to portions of GLV-transformed lymphoma cell membranes that are associated with budding virions (26). This observation prompted the suggestion that GCSA was not a structural component of GLV. However, two groups have simultaneously reported (in radioimmunoprecipitation studies) that the GCSA complex is actually composed of glycosylated precursors of MuLV core proteins which contain antigenic determinants specific for p30, p12, and p10 (27, 28).

In the Mo-MuLV system, a similar nonvirion TASA has been detected (Moloney cell-surface antigen; MCSA) using sera of syngeneic mice immunized with irradiated virus-producing lymphoma cells (29). Mouse anti-MCSA serum

has been found to contain virus-neutralizing antibodies which represent reactivity against gp71, the major envelope glycoprotein. However, virus-neutralizing activity could be removed by absorption either with cells which expressed surface concentrations of gp71 or with intact Mo-MuLV. This treatment had little effect on the antisera's cytotoxicity directed against lymphoma cells (30). Recently, MCSA activity (mol wt 110,000) has been physically separated from Mo-MuLV structural proteins by concanavalin A affinity chromatography, gel filtration, and velocity centrifugation (30). These results suggest that MCSA indeed represents a neoantigen separate from virion structural proteins. An approach employing virus-transformed nonproducer tumor cells, similar to that used in the present studies, would lend further evidence in support of this interpretation.

Greenberger et al. (31) have demonstrated (using immunoelectron microscopy) a nonvirion TASA expressed on the surface of nonproducer K-BALB cells which could not be detected on the parent BALB/c 3T3 cell line. Because administration of this K-BALB antigen-specific antisera to normal mice did not protect against subsequent tumor challenge, these investigators concluded that the neoantigen present on the K-BALB cell did not function as a tumor-specific transplantation antigen. Recently, we reported the detection of a similar nonvirion K-BALB-specific antigen using the techniques of secondary and tertiary MTLIC followed by LMC. Transfer of *in vitro* activated lymphocytes along with a lethal challenge of K-BALB cells to normal BALB/c mice mediated almost complete protection against subsequent K-BALB tumor development (32). These findings suggest that a nonvirion antigen present on K-BALB cells does function as a TSTA. Whether or not this antigen is coded for by the replication-defective portion of the KiSV genome has not been established. However, this TSTA is not present on parent BALB/c 3T3 cells as detected by LMC (K. A. Smith, M. M. Ferm, S. Gillis, and A. E. Gillis, unpublished observations).

A common cell surface antigen (FMR) has been described on leukemia cells induced *in vivo* by FLV, RLV, and Mo-MuLV. Initial studies reporting cross-reactivity between all three virally-induced leukemias have recently been questioned by results showing that cells induced by Mo-MuLV were antigenically distinct from those induced by FLV and RLV (33). These data imply that such a serologically detected antigen common only to Friend and Rauscher leukemias may have determinants coded for by the SFFV genome. Regardless of the cross-reactivity of antigenic determinants detected by anti-FMR antisera, the nonvirion nature of these determinants has not been thoroughly established. It has been shown that anti-FLV antisera remain cytotoxic against FLV-transformed spleen cells despite exhaustive absorption with purified FLV (34). However, the demonstration that the cytotoxic reactivity of anti-FMR antisera could be removed by absorption with disrupted virus suggests that FMR antibody reactivity may be directed against internal virion proteins (6). Despite these results, the observation that SFFV-immune lymphocytes mediated the lysis of FLV and RLV transformed leukemia cells suggests that some portion of classically defined FMR antigenic determinants may be coded for by SFFV gene sequences.

The SFFV genome as it exists in the fibroblast nonproducer target cells studied in these investigations has recently been analyzed by molecular hybridization. The genome appears to be a recombinant between a portion of the F-MuLV genome and RNA sequences that are highly related (hybridizable) to three separate strains of murine xenotropic virus (11). Further investigation has confirmed that the recombinational event between ecotropic and xenotropic virus sequences (responsible for the elaboration of SFFV genome) occurred somewhere in the envelope region of the xenotropic viral genome (35). It is plausible that the specific cell surface antigen described in this report might be coded for by the xenotropic viral information contained within the SFFV genome. It would appear unlikely that the ecotropic sequences of the SFFV genome were responsible for coding for the SFFV antigen, in that BALB/c 3T3 cells productively infected with three types of ecotropic helper virus were not susceptible to lysis by SFFV nonproducer-immune lymphocytes.

Recently, Bernstein et al. (8) in radioimmunoassay (RIA) studies of SFFV-containing murine and rat fibroblasts has determined that SFFV nonproducers contain high levels of cytosol p15, whereas the remaining gag-gene coded core proteins and the envelope glycoprotein gp71 were not present in detectable concentrations. Similar RIA studies of the SFFV nonproducer cells used in this report confirmed the cytosol presence of both p15 and p12 (D. H. Troxler, personal communication). These results intimate that the portion of ecotropic virus which remains complexed with the xenotropic sequences of SFFV RNA represent early gag-gene regions.

It is important to note that the 15,000 mol wt protein detected in the above-mentioned RIA studies was not further characterized as the core (C) polypeptide p(15)C or the envelope (E) polypeptide p(15)E. The localization of a 15,000 mol wt protein [p(15)E] on the surface on intact C-type virus particles was originally described in neutralization experiments using anti-p(15)E antisera (36). Furthermore, p(15)E has been isolated from virus-infected cells in stable association with gp71 as a common polypeptide precursor (37). Although specific chemical differences between p(15)E and p(15)C have not been detailed, the antigenic characteristics of both molecules have been described. p(15)E appears to carry antigenic determinants with strong interspecies specificity as observed by the neutralization of FeLV and several murine xenotropic viruses by anti-FLV-p(15)E antisera (38). This observation is especially intriguing in light of the recently defined genetic composition of the SFFV genome as a recombination of ecotropic sequences into the envelope region of xenotropic MuLV.

Although one cannot rule out the possibility that the antigen described in these studies is a gag-gene-coded polypeptide, the observation that the p15 and p12 positive K-BALB R-MuLV target cell (Table III) was not lysed by 1902 B C1-4-immune spleen cells (Table IV) further supports the nonvirion SFFV-specific nature of the antigen detected. It is possible, however, that the antibody-dependent, complement-mediated cytolysis assay used in virological characterizations was not sensitive enough to detect extremely small cell surface concentrations of viral structural proteins. Perhaps an isotopic antiglobulin binding assay such as that described by Ruscetti and Parks (23) would be a more sensitive indicator of cell surface virion protein expression. Similarly, the

use of radiolabeled target cell membranes in radioimmunoprecipitation competitive RIA studies might provide the most accurate means of determining the cell surface viral antigen expression of SFFV nonproducer cells.

Several analogies have been consistently drawn between the MSV and SFFV genome. Both are replication-defective and require rescue by tropic LLV. Both genomes are accepted as being responsible for the malignant transformation event *in vivo*. Studies conducted in this report (Table IV) indicate that a cell surface antigen coded expressly by SFFV genetic information is not shared by MSV-transformed fibroblasts, regardless of their capacity to produce infectious transforming virus. The lack of cross-reactive antigenicity in MSV and SFFV nonproducer cells is an observation which is understandable in light of recent hybridization investigations, indicating that little if any genetic information is common to both SFFV and MSV genomes. However, the detection of cell surface antigens by LMC on KiSV and SFFV replication-defective nonproducer cells suggests that nonvirion cell surface antigens may be coded for by genetic information intimately associated with those genes responsible for malignant transformation.

Summary

The existence of a nonvirion tumor-associated cell surface antigen (TASA) on cells transformed with Friend (FLV) on Rauscher (RLV) leukemia virus has been difficult to demonstrate. Antisera raised against classically defined Friend-Moloney-Rauscher antigenic determinants have been shown to react with virus structural proteins coded for by genetic information contained in the lymphatic leukemia or helper (LLV) virus genome. The recent development of nontransformed fibroblast cell lines which contain the replication-defective spleen focus-forming virus (SFFV) genome, free of replicating LLV, has allowed investigation of an SFFV-specific antigen. We have applied the techniques of mixed tumor-lymphocyte culture stimulation followed by lymphocyte-mediated cytotoxicity assays to search for the cell surface expression of an antigen coded expressly by SFFV genetic information.

SFFV nonproducer-immune, *in vitro* activated spleen cells were capable of effecting the lysis of SFFV-containing BALB/c 3T3 and Fischer rat epithelial, cloned cell lines. Normal BALB/c 3T3 and BALB/c 3T3 cells infected with three types of ecotropic LLV were unaffected. Syngeneic FLV and RLV-induced murine leukemia cells were also killed by SFFV nonproducer-immune lymphocytes. In addition, Kirsten sarcoma virus-transformed, replication-defective and replication-rescued BALB/c 3T3 fibroblasts were not susceptible to SFFV antigen-directed cytotoxicity. Antibody-dependent complement-mediated cytotoxicity assays using monospecific goat antisera confirmed that SFFV nonproducers lacked cell surface expression of virion structural proteins.

These observations suggest that the antigen detected in LMC experiments was not coded for by genetic information contained in the helper component of FLV, and that it represents a true SFFV-specific cell surface antigen. Based upon the recent molecular evaluation of the SFFV genome as consisting of both xenotropic and ecotropic virus sequences, it appears reasonable that xenotropic genetic information may be responsible for expression of the SFFV-specific

antigen. Since the replication-defective SFFV genome is also responsible for the malignant transformation associated with FLV-induced erythroleukemia, one might postulate that gene sequences capable of programming transformation may also code for the TASA detected in these studies.

The authors gratefully acknowledge Doctors D. H. Troxler and E. M. Scolnick (Laboratory of Tumor Virus Genetics, National Cancer Institute, Bethesda, Md.) for providing many of the cell lines used in these studies, and Dr. Richard A. Steeves for conducting in vivo SFFV assays. We also wish to thank Ms. Susan Kennedy, Ms. Mary Ferm, and Ms. Rachel Stocking for their invaluable assistance in performing virological characterizations of all target cells tested. We also wish to thank Ms. Linda Waidlick for her expert editorial and secretarial assistance.

Received for publication 15 February 1978.

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