

SECONDARY INDUCTION OF CYTOTOXIC
T LYMPHOCYTES WITH SOLUBILIZED SYNGENEIC
TUMOR CELL PLASMA MEMBRANES

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It is well established that tumor cells express unique antigens not present on nontransformed cells. Although this fact has been amply documented in a variety of *in vivo* and *in vitro* assay systems (1-3), there has yet been no clue as to the biochemical identity of the putative tumor-specific transplantation antigen (TSTA). Our laboratory has been interested for some time in the biochemical elucidation of the unique antigens on tumor cells. Attempts have been made to purify and solubilize the plasma membrane as a first step to isolate the TSTA (4). Having established that the TSTA copurifies with plasma membrane and can be solubilized in an active form by detergent, we began to investigate if it was possible to induce tumor-specific cytotoxic lymphocytes in a syngeneic secondary *in vitro* stimulation. Success with this technique should enable us to study the relationship between the antigens recognized in *in vitro* cytotoxicity assays and those inducing tumor rejection activity *in vivo*. Recently, serologically active detergent solubilization of the major histocompatibility (H-2) antigens was achieved (5-8) and also shown to be capable of eliciting *in vitro* allogeneic responses (9, 10). In this report, we demonstrate the feasibility of inducing cytotoxic effector lymphocytes in a syngeneic tumor system with purified, detergent-solubilized plasma membrane.

Materials and Methods

Mice. Adult female [(BALB/c × C57BL/6)]F₁ (CBF₁) mice were purchased from Charles River Breeding Laboratories, Wilmington, Mass.

Tumor-Cell Lines. RBL-5 is a lymphoma induced by Rauscher murine leukemia virus; it has strong cross-reacting antigens with other Friend-Moloney-Rauscher (FMR)-induced lymphomas. It is maintained either in ascitic form in female C57BL/6 (B6) mice or *in vitro* as a continuous suspension culture. E δ G2 is a Gross-murine leukemia virus-induced lymphoma and MBL-2, a Moloney leukemia virus-induced lymphoma; both are also maintained in B6 mice. mKSA is an undifferentiated sarcoma induced by SV40 in BALB/c mice and EL-4 is a chemically-induced lymphoma, syngeneic to B6 mice. mKSA, E δ G2, and EL-4 do not cross-react with RBL-5.

Plasma Membrane Purification. The purification scheme for RBL-5 plasma membrane was essentially as described by Snary et al. (11) and as adapted by Rogers et al. (4). Plasma membrane yield was based on histocompatibility antigen (H-2^b) which has been shown to be expressed almost exclusively on the plasma membrane (PM). Solubilization was done in 0.14 M NaCl, 0.01 M Na phosphate, pH 8.0 (PBS) containing 2% sodium deoxycholate (DOC). Insoluble material was removed by centrifugation at 100,000 *g* for 45 min; the solubilized plasma membrane (SPM) was filter sterilized and stored at -20°C until used. The mKSA plasma membrane preparation was that described by Rogers et al. (4) and had good tumor specific rejection activity.

Secondary In Vitro Sensitization of Spleen Cells. Responding spleen cells were obtained from mice that had previously rejected a subcutaneous challenge of 5×10^6 RBL-5 tumor cells after prior immunization with two doses of 50 $\mu\text{g}/\text{ml}$ solubilized plasma membrane antigens. The immune spleen cells were cultured alone, as control, or with (a) purified RBL-5 vesicular plasma membrane, (b) purified plasma membrane solubilized with 2% DOC or (c) mitomycin C-inactivated RBL-5 tissue culture cells. Mitomycin C (60 $\mu\text{g}/\text{ml}$) inactivation was performed at 37°C for 20 min and the cells were then washed three times in Eagles' minimal essential medium before culture. After each wash, cells were allowed to incubate for 10 min at 37°C. Cultures were established in 30 ml RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) consisting of 40×10^6 responding cells and 4×10^5 stimulating cells, or PM, particulate or solubilized, at concentrations indicated in the text. RPMI-1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, 10% fetal calf serum (FCS) (heat inactivated), 1% fresh L-glutamine, and 1% penicillin-streptomycin, was used. Cultures were established in tissue culture flasks and incubated at 37°C in a humidified chamber with air containing 5% CO_2 for 5 days.

Anti- θ Antibody. AKR anti-Thy 1.2 C3H antibody was provided by Dr. H. Holden, National Cancer Institute. The preparation and activity of this antiserum has been reported elsewhere in detail (12).

Target Cell Labeling. All target cells were labeled with ^{51}Cr by incubating $3\text{--}6 \times 10^6$ cells with 200 μCi of [^{51}Cr]NaCrO₄ (New England Nuclear, Boston, Mass.) for 1 h in PBS.

Cell-Mediated Cytotoxicity Assay. The standard ^{51}Cr -release assay involved the incubation of 1×10^4 ^{51}Cr -labeled target cells with 3×10^5 , 1×10^5 , and 1×10^4 effector cells in 0.2 ml RPMI-1640 containing 10% FCS for 4 h at 37°C. Percentage of cytotoxicity was defined as:

$$\frac{\text{Se-So}}{\text{Sm-So}} \times 100$$

where Se = isotope release from wells containing effector cells plus targets, So = spontaneous release of isotope from wells containing target cells plus medium, Sm = maximum isotope-release after 3 cycles of freezing and thawing of target cells. Percentage of specific cytotoxicity = percent cytotoxicity of immune cells cultured with stimulating antigen-percent cytotoxicity of immune cells cultured alone.

Results

Induction of Cytotoxic Lymphocytes by Purified Plasma Membrane. Although we have established that as low as 2 μg of purified RBL-5 plasma membrane, both in the particulate and solubilized forms, was capable of inducing immunity against a subcutaneous challenge of viable tumor cells, it was not known whether this same antigen(s) could induce cytotoxic lymphocytes in vitro. To test if this was possible, RBL-5 immune lymphocytes were incubated with inactivated RBL-5 leukemia cells, 1 $\mu\text{g}/\text{ml}$ PM or 1 $\mu\text{g}/\text{ml}$ SPM. The final concentration of DOC in the cultures was 1.2 $\mu\text{g}/\text{ml}$. After 5 days stimulation cytotoxicity was determined and the results (Fig. 1) clearly indicated that syngeneic tumor cell PM, particulate or solubilized with a modest amount of detergent, was fully capable of inducing a secondary generation of cytotoxic lymphocytes. There appeared to be little or no difference in the efficiency of the tumor antigens before or after detergent solubilization. In at least four different experiments (Fig. 1, Table I), the induction of cytolytic lymphocytes by 1 $\mu\text{g}/\text{ml}$ of tumor PM or SPM was nearly as efficient as an equivalent number of intact tumor cells.

Specificity of the Induction Phase by PM and SPM. All cultures tested, including the controls, contained the same amount of DOC. However, to be certain that nothing in our membrane preparation was causing nonspecific stimulation, normal unimmunized spleen cells were incubated with RBL-5 cells, PM, or SPM. In all cases, cytotoxic cells could not be generated (Fig. 1). Likewise, incubation of RBL-5 immune cells with

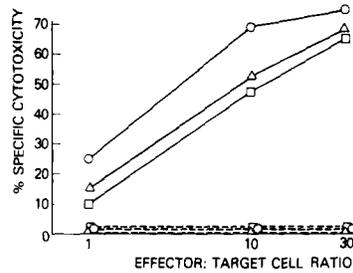


FIG. 1. Cytotoxic activity of immune spleen cells (—) or normal spleen cells (---) restimulated in vitro with mitomycin C-inactivated RBL-5 tumor cells (○), 1 µg/ml plasma membrane (□), and 1 µg/ml DOC solubilized plasma membrane (Δ).

TABLE I
Induction of Cytotoxic Effector Cells by Whole Cells, PM, and Solubilized PM

Stimulating antigen	RBL-5 cells		% Specificity cytotoxicity			
			Plasma membrane (1 µg/ml)		Solubilized plasma membrane (1 µg/ml)	
E/T*	30	10	30	10	30	10
Exp. 1	58	21	49	16	53	19
Exp. 2	71	38	66	30	70	33
Exp. 3	62	40	60	34	60	36

* E/T, effector cell:target cell ratio.

purified PM or SPM of the mKSA tumor induced no cytotoxic effector cells capable of lysing RBL-5 targets (data not shown).

Titration of Induction by PM and SPM. The dose response for the secondary generation of cytotoxic effector cells by PM and SPM was determined by incubating graded doses of the antigen with RBL-5 immune lymphocytes. A concentration-dependent effect was observed in both cases (Fig. 2). Induction of cytotoxic cells was possible with a concentration as low as 0.5 µg/ml of PM or SPM.

Specificity of the Cytotoxic Reaction. In initial experiments, we have observed that effector cells generated in the presence of inactivated tumor cells maintained a strict specificity for RBL-5 and for the Moloney virus-transformed lymphoma, MBL-2. Similar results were also obtained with PM and SPM (Table II). The effector cells generated by PM and SPM significantly lysed RBL-5 and MBL-2 tumor cells (FMR cross-reactivity) although E δ G2, a syngeneic Gross virus-induced lymphoma was not lysed. EL-4 lysis was only marginal.

The cytotoxic effector cells generated were found, in all cases, to be sensitive to treatment with anti- θ antiserum plus complement, but not to complement alone, suggesting that the effector cells are T lymphocytes.

Discussion

The purpose of the present study was to investigate the possibility of generating syngeneic tumor-specific cytotoxic effector cells with particulate and solubilized PM. Although studies on in vitro stimulation of allogeneic effector cells with histoincompatible plasma membranes have been previously reported (5-10), we are aware of no attempt to stimulate specifically syngeneic effector cells with PM or SPM. In this

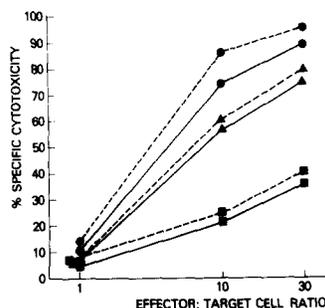


FIG. 2. Dose dependence of CTL induction. Graded amounts of plasma membrane (—) or DOC solubilized plasma membrane (---) were added to cultures of RBL-5 immune spleen cells at 5 $\mu\text{g}/\text{ml}$ (●), 1 $\mu\text{g}/\text{ml}$ (▲), and 0.5 $\mu\text{g}/\text{ml}$ (■). Final concentration of DOC in the cultures ranged from 6.0 to 0.6 $\mu\text{g}/\text{ml}$, respectively.

TABLE II
Specificity of CTL Generation by RBL-5 Cells, Plasma Membrane, and Solubilized Plasma Membrane

Stimulating antigen		% Specific cytotoxicity			
		Target cells			
		RBL-5	MBL-2	E δ G2	EL-4
Exp. I	RBL-5 cells	41	36	0	3
	RBL-5 PM	35	37	1	5
	RBL-5 solubilized PM	38	34	-1	3
Exp. II	RBL-5 cells	55	32	-2	ND*
	RBL-5 PM	45	30	-1	ND
	RBL-5 solubilized PM	51	30	-1	ND

* ND, not done.

report we present data indicating that the secondary generation of syngeneic cytotoxic effector cells with PM and SPM has been achieved. The specificity of the induction phase by PM and SPM, and of the effector phase by the cytotoxic cells generated was presented. We observed no difference in the specificity of these cells; all lysed syngeneic RBL-5 target cells and a cross-reacting tumor cell, MBL-2. Immunization with either RBL-5 or MBL-2 tumor cells will prevent the growth of a subsequent challenge of either or both lymphomas. The antigens shared by the two tumors, which protected one against the other, have not been fully elucidated. It was suggested that the FMR group-specific antigens may be the common antigens recognized on these cells (13). E δ G2, a syngeneic Gross virus-induced lymphoma was not lysed and the lysis of EL-4, a chemically induced tumor was, at best, marginal. EL-4 has been shown to be slightly contaminated with FMR group murine leukemia virus (14).

The ability to generate syngeneic cytotoxic lymphocytes with SPM should be expected to make possible the study of the antigens involved in *in vitro* induction of cytotoxic cells and those responsible for *in vivo* tumor rejection. We have already obtained data which show that upon solubilization and chromatographic fractionation of purified PM, the fractions that induced cytotoxic effector cells *in vitro* also were able to protect against an *in vivo* tumor challenge.¹ We believe that with this

¹ O. Alaba and L. W. Law. Manuscript in preparation.

approach, the elucidation of the molecular entity of TSTA antigen should be ultimately feasible.

Summary

Secondary induction of *in vitro* cytotoxic T lymphocytes in a syngeneic system has been achieved with plasma membrane, both in the particulate and solubilized forms. Both the induction and the lytic phases were shown to be immunologically specific. The effector cells generated were completely susceptible to treatment with anti- θ antibody and complement, suggesting that they are T lymphocytes.

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References

1. Law, L. W., E. Appella, O. Henriksen, and M. J. Rogers. 1976. Some biologic and biochemical properties of soluble tumor antigens. *Ann. N. Y. Acad. Sci.* **276**:11.
2. Alaba, O., and I. D. Bernstein. 1978. Tumor growth suppression *in vivo*: cooperation between immune lymphoid cells sensitized *in vitro* and immune bone marrow cells. *J. Immunol.* **120**:1941.
3. Ting, C. C., G. S. Bushar, D. Rodrigues, and R. B. Herberman. 1975. Cell-mediated immunity to Friend virus-induced leukemia. I. Modification of ^{125}I UdR release cytotoxicity assay for use with suspension target cells. *J. Immunol.* **115**:1351.
4. Rogers, M. J., L. W. Law, M. Prat, S. Oroszlan, and E. Appella. 1978. Separation of the tumor rejection antigen (TSTA) from the major viral structural proteins associated with the membrane of an R-MuLV-induced leukemia. *Int. J. Cancer* **21**:246.
5. Linna, T. J., H. D. Engers, J.-C. Cerottini, and K. T. Brunner. 1978. Inhibition of cytolytic T lymphocytes activity with subcellular alloantigen preparations and with unlabelled allogeneic target cells. *J. Immunol.* **120**:1544.
6. Hayry, P., and L.-C. Anderson. 1976. Generation of T memory cells in one-way mixed lymphocyte culture. IV. Primary and secondary responses to soluble and insoluble membrane preparations and to ultraviolet light inactivated simulator cells. *Scand. J. Immunol.* **5**:391.
7. Wagner, H., M. Hess, M. Feldman, and M. Rollinghoff. 1976. Secondary cytotoxic allograft response *in vitro*. III. The immunogenicity of allogeneic membrane fragments. *Transplantation (Baltimore)*. **21**:282.
8. Fast, L. D., and D. P. Fan. 1978. Dissociated and reconstituted subcellular alloantigen capable of stimulating mouse cytotoxic T lymphocytes *in vitro*. *J. Immunol.* **120**:1092.
9. Lemonnier, F., M. Mescher, L. Sherman, and S. Burakoff. 1978. The induction of cytolytic T lymphocytes with purified plasma membranes. *J. Immunol.* **120**:1114.
10. Mescher, M., L. Sherman, F. Lemonnier, and S. Burakoff. 1978. The induction of secondary cytolytic T lymphocytes by solubilized membrane proteins. *J. Exp. Med.* **147**:946.
11. Snary, D., B. Goodfellow, W. F. Bodmer, M. J. Hayman, and M. J. Crumpton. 1974. Subcellular separation and molecular nature of human histocompatibility antigens (HL-A). *Nature (Lond.)*. **247**:457.
12. Herberman, R. B., M. E. Nunn, D. H. Lavrin, R. Asofsky. 1973. Effect of antibody to antigen on cell-mediated immunity induced in syngeneic mice by murine sarcoma virus. *J. Natl. Cancer Inst.* **5**:1509.
13. Glynn, J. P., J. L. McCoy, and A. Fefer. 1968. Cross-resistance to the transplantation of syngeneic Friend, Moloney and Rauscher virus-induced tumors. *Cancer Res.* **28**:434.
14. Herberman, R. B., T. Aoki, M. Nunn, D. H. Lavrin, N. Soares, A. Gazdar, H. Holden, and K. S. S. Chang. 1974. Specificity of ^{51}Cr -release cytotoxicity of lymphocyte immune to murine sarcoma virus. *J. Natl. Cancer Inst.* **53**:1103.