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Tumor-specific T Cell Lines: Capacity to Proliferate and Produce Interleukin 2 in Response to Various Forms of Tumor Antigens

Jun Shimizu, Takashi Suda, Tatsuo Katagiri, Hiromi Fujiwara[1] and Toshiyuki Hamaoka
Biomedical Research Center, Osaka University Medical School, 2-2 Yamadaoka, Suita-shi, Osaka 565

Anti-tumor proliferative T cell lines were established from cultures of lymph node cells from BALB/c mice immunized to syngeneic CSA1M fibrosarcoma with the CSA1M tumor cell membrane. The cultures were maintained throughout in the absence of exogenous interleukin 2 (IL2). Cell surface phenotypes of all T cell lines established were Thy-1^+, Ig^-, $L3T4^+$ and Lyt-2^-. Their proliferation was induced in a tumor antigen dose-dependent fashion and a tumor antigen-specific way. Such proliferative responses were inhibited by the addition to cultures of anti-class II H-2^d (anti-I-A^d) or anti-L3T4 but not of anti-class I H-2^d or anti-Lyt-2 monoclonal antibody. None of the T cell lines exhibited any cytotoxic T lymphocyte activity but they all produced IL2 upon stimulation with CSA1M tumor antigens, indicating that they represent helper-type T cell (Th) lines. The activation of these tumor-specific Th lines was induced with either CSA1M tumor cells themselves, or their membrane or detergent-solubilized fraction depending on the presence of antigen-presenting cells (APC). Most importantly, activation was also inducible by membranous tumor antigen-pulsed APC, which were capable of producing potent anti-tumor protective immunity when administered *in vivo* into syngeneic BALB/c mice. These results indicate that the tumor-specific Th lines established here can be activated with various forms of tumor antigens for their expression of helper function. Since Th lines of this type have not been described previously, our Th lines provide an intriguing tool for investigating the cellular and molecular mechanisms by which tumor-specific Th recognize tumor antigens.

Key words: Helper T cells — Tumor antigen — Interleukin-2 production

An efferent phase of anti-tumor immune responses is mediated by various types of effector cells including tumor-specific cytotoxic T lymphocytes (CTL[2])[1,2] as well as non-specific tumoricidal effectors such as macrophages and lymphokine-activated killer cells.[3–7] It has been shown that these tumor-attacking cells require lymphokines produced by other lymphoid cells, i.e., helper T cells (Th) for their induction and/or activation.[3–9] Therefore, the recognition of tumor antigens by helper (non-cytolytic, lymphokine-producing)-type T cells, which is an initial event in a series of anti-tumor immune responses, would represent a key process for facilitating the effective induction of tumor immunity.

In general, helper-type T cells are stimulated with antigen associated with class II major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APC),[10–12] and therefore the role of APC in activating this type of T cells has been well established.[13–15] While such studies have been done in Th against nominal soluble protein antigens, APC requirement for the recognition of cell-bound antigens such as tumor antigens by Th is poorly understood. Thus, it remains obscure how tumor-specific Th recognize cell-bound tumor antigens through cooperation with APC. This situation appears to be mainly due to the fact that no Th line or clone against tumor antigens has been thus far established, in contrast to the availability of Th clones against various soluble antigens or CTL clones against tumor cells. Therefore, establishing tumor-specific Th lines or clones could contribute to investigating the cellular and molecular mechanisms underlying the recognition of tumor antigens on tumor cell surfaces by tumor-specific Th in the presence of APC.

In the present study, we have tried to establish tumor-specific Th lines and to initiate the investigation of tumor antigen-recognition by such Th lines. The results demonstrate that continued *in vitro* culturing of lymph node cells from CSA1M tumor-hyperimmune mice together with CSA1M membrane fraction resulted in the generation of T cell lines reactive to CSA1M tumor antigens. It was found that all of the T cell lines were of helper type and were effectively stimulated with various forms of tumor antigens including tumor cells themselves in the presence of APC or with tumor antigen-pulsed APC. Thus, this represents the first report of the establishment of tumor-specific Th lines. Its potential applicability to investigating the mechanism underlying the APC-

[1] To whom all correspondence should be addressed.

[2] Abbreviations used in this paper: CTL, cytotoxic T lymphocytes; APC, antigen-presenting cells; MHC, major histocompatibility complex; RSV, Rous sarcoma virus; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; SN, supernatant; MTD, mean tumor diameter.

dependent recognition of cell surface-bound tumor antigens by tumor-specific Th is discussed.

MATERIALS AND METHODS

Mice Male BALB/c mice were obtained from Shizuoka Experimental Animal Laboratory, Shizuoka, and used at 6–9 weeks of age.[16]

Tumors Rous sarcoma virus (RSV)-induced CSA1M fibrosarcoma was kindly provided by Dr. T. Yoshida, Hamamatsu University School of Medicine, Hamamatsu. This tumor cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere with 5% CO_2. 3-Methylcholanthrene-induced Meth A sarcoma was maintained in an ascitic form. Both tumors were of BALB/c origin.

Monoclonal antibodies Anti-L3T4 (GK 1.5)[10] and anti-Lyt-2 (3.155) (ATCC, Rockville, MD) monoclonal antibodies were obtained from culture supernatants of hybridomas producing the relevant antibody. Monoclonal anti-I-A^d, anti-I-$E^{d\ or\ k}$, anti-K^dD^d and anti-D^d antibodies were obtained as culture supernatants of the respective hybridoma cell lines, 34-5-3S,[17] 14-4-4S,[18] 34-1-2S[17] and 34-5-8S.[17] Fluorescein isothiocyanate (FITC)-conjugated anti-Lyt-2, anti-Thy-1.2 and rat anti-mouse IgG were the products of Becton Dickinson Immunocytometry Systems, Mountain View, CA, of Bio Yeda Ltd., Rehovot, Israel, and of Jackson Immunoresearch Laboratories, Inc., West Grove, PA, respectively. FITC-conjugated anti-L3T4 was prepared with the use of anti-L3T4 (GK 1.5) monoclonal antibody as previously described.[19]

Culture media Complete culture medium was RPMI 1640 supplemented with 10% fetal calf serum (FCS), 5×10^{-5} *M* 2-mercaptoethanol (2-ME), 12 m*M* Hepes, 100 U/ml penicillin and 100 μg/ml streptomycin, and 2×10^{-3} *M* L-glutamine. This was used for maintaining T cell lines. For initial cultures of fresh immune lymph node cells and tumor antigens to establish T cell lines, medium supplemented with 10% horse serum instead of FCS was used. The horse serum-supplemented culture medium was further supplemented with non-essential amino acids and sodium pyruvate for use in *in vitro* proliferation assays and for lymphokine production and assays.

Preparation of plasma membrane fraction of tumor cells and its solubilization The procedures used were described previously.[20] Plasma membrane fractions were prepared according to the method of Tsushima and Friesen[21] with some modifications. Briefly, solid tumors were divided into small pieces and homogenized in 5 volumes of 0.3 *M* sucrose solution. This homogenate (first homogenate) was filtered through 2 layers of gauze, and the filtrate was further homogenized with a Polytron PT-10 for 1 min. The second homogenate was centrifuged at 1,500*g* for 25 min, and the resulting supernatants were centrifuged sequentially at 15,000*g* for 25 min, and 100,000*g* for 90 min. The 100,000*g* pellet was suspended in 25 m*M* Tris-HCl buffer (pH 7.6) containing 10 m*M* $CaCl_2$ and 1% aprotinin (Sigma) and homogenized with a Teflon-glass homogenizer. This homogenate was diluted with RPMI 1640, sonicated for 1 min on ice, filtered through a 0.45 μm filter and used as the plasma membrane fraction.

The plasma membrane fraction was extracted with 4 volumes of 0.25% sodium dodecyl sulfate (SDS) in Tris-borate-EDTA (TBE, 10.75 g:5.04 g:0.39 g/liter, pH 8.35) buffer containing 2 m*M* (*p*-amidinophenyl)methanesulfonyl fluoride (APMSF; Wako Pure Chemical Industries) at 37°C for 30 min, and the insoluble matrix was removed by centrifugation at 10,000*g* for 30 min. Saturated ammonium sulfate (SAS) solution was slowly added to the SDS-solubilized membrane fraction (approximately 10 mg protein/ml) to achieve 20% saturation, then the solution was stirred for 1 h at room temperature, and centrifuged at 10,000*g* for 20 min. The resulting pellet was dissolved in 0.2% SDS-TBE containing 2% 2-ME and 2 m*M* APMSF, extensively dialyzed against distilled water and PBS sequentially, and used as a source of solubilized tumor antigens.

Preparation of APC Splenic accessory cells were enriched on a bovine serum albumin (BSA) gradient according to the method described by Glimcher *et al.*[22] Briefly, the BSA gradient was prepared by layering 5 ml of 11% BSA over 5 ml of 35% BSA containing 5×10^8 normal spleen cells and centrifuging at 4°C for 30 min. Cells banding between 35% and 11% were collected. They comprised roughly 3–5% of the starting population and were found to be approximately 40% Mac-1-positive cells by means of a flow microfluorometric (FMF) study. These fractionated cells were used as an APC-enriched population (APC population).

Preparation of tumor antigen-pulsed APC Pulsing of APC with tumor antigens was performed as previously described.[23] Briefly, an APC-enriched population (1×10^7 cells) was cultured *in vitro* with 200 μg of tumor cell membrane fraction in one ml of RPMI 1640 medium. After culturing at 37°C for 2 h, cells were washed 3 times and used as tumor antigen-pulsed APC.

Immunization with tumor antigens and *in vitro* cultures for establishing anti-CSA1M T cell lines BALB/c mice were inoculated s.c. with 10^6 tumor antigen-pulsed APC 3 times.[23] Seven days after the last inoculation, the mice were challenged i.d. in the right flank with 2×10^5 viable CSA1M tumor cells. Mice which resisted the tumor cell challenge received the booster immunization with 100 μg of tumor membrane fraction emulsified in complete

Freund's adjuvant (CFA) s.c. at the tail base. Seven days later, inguinal lymph nodes were removed and used as donor cells for establishing anti-tumor T cell lines.

Cell cultures for the establishment of T cell lines were performed according to the procedure described by Kimoto and Fathman.[24] A single cell suspension of lymph node cells from the above immunized mice was cultured in 24-well culture plates (Corning No. 25820, Corning Glass Works, Corning, NY) with 100 μg/ml tumor membrane fraction at a cell number of 5×10^6 cells/well in 2 ml of complete medium supplemented with horse serum. Four days later, the cells were harvested, washed twice, and recultured with 1×10^7 irradiated (3000 R) syngeneic spleen cells (filler cells) at a concentration of 1×10^5 cells/well in 2 ml of complete medium supplemented with FCS (RPMI 1640-FCS). After 14 days, the cells were harvested and viable recovered cells were counted. Viable recovered cells (1×10^5) were restimulated with 100 μg/ml tumor antigen in the presence of 1×10^7 syngeneic irradiated filler cells in 2 ml of RPMI 1640-FCS. After 14 days, the cells were harvested, and recovered cells were recultured with syngeneic irradiated filler cells in the absence of antigen at a concentration of 1×10^5 cells and 1×10^7 filler cells/well in 2 ml of RPMI 1640-FCS for an additional 14 days. Tumor antigen-reactive T cells were maintained by this 14-day antigen restimulation followed by 14-day resting culture. Tumor antigen-reactive T cells after culture for 4 months were regarded as T cell lines.

Assay for proliferative responses Tumor antigen-reactive T cell line (1.5×10^4 cells) were stimulated with various forms of tumor antigens in the presence of 2×10^4 APC (2000 R irradiated) in 0.2 ml of complete culture medium in 96-well flat-bottomed microplates (Corning No. 25860, Corning Glass Works) for 48 h at 37°C in a humidified incubator (5% CO_2). Twenty kBq/well of [^{3}H]thymidine (^{3}H-TdR) was added 8 h before harvest. Cells were harvested on a filter paper and radioactivity was counted using a liquid scintillation spectrometer. Results were expressed as the mean±SE of triplicate cultures.

Production of lymphokine and its assay Culture conditions were the same as those used for proliferative responses. After incubation at 37°C in a humidified incubator (5% CO_2) for 48 h, culture supernatants (SN) were harvested by centrifugation.

SN were assayed for IL2 activity in terms of their ability to support the proliferation of the IL2-dependent T cell line, CTLL-2.[25] CTLL-2 (10^4/well) were cultured with the SN in a volume of 0.2 ml in 96-well flat-bottomed microplates (Corning 25860) for 24 h at 37°C. Proliferation was assessed in terms of the uptake of ^{3}H-TdR during 4-h pulsing with 20 kBq of ^{3}H-TdR/well.

Immunofluorescence staining and flow microfluorometry (FMF) Tumor antigen-reactive cell lines were incubated at 4°C for 30 min with FITC-conjugated antibodies, washed twice, resuspended and analyzed for fluorescence. These procedures were performed in Hanks' balanced salt solution (without phenol red) containing 0.1% BSA and 0.1% sodium azide. FMF analysis was performed by using a FACStar (Becton Dickinson, Immunocytometry Systems). All data were collected by using log amplification, and dead cells were rejected from analysis on the basis of forward light scatter.

RESULTS

Establishment of T cell lines reactive to CSA1M tumor antigens BALB/c mice were consecutively immunized with various forms of CSA1M tumor antigens, including tumor antigen-pulsed APC,[23] viable tumor cells themselves and their membrane fraction emulsified in CFA. CSA1M-reactive cell lines were established from long-term cultures of lymph node cells from these CSA1M-

Table I. Establishment of CSA1M-reactive Cell Lines from Lymph Node Cells of CSA1M-hyperimmune BALB/c Mice

Anti-CSA1M cell line[a]	CSA1M tumor antigen	APC	^{3}H-TdR uptake[b] (cpm) of cell lines		
			A	B	C
−	−	+	401±25	378±11	330±15
+	−	−	467±5	369±3	347±5
+	−	+	1680±756	1372±197	1138±195
+	+	−	1057±70	ND[c]	368±23
+	+	+	10745±1246	11251±607	22206±510

a) Anti-CSA1M cell lines were established from three separate cultures. Each cell line (1.5×10^4 cells) was unstimulated or stimulated with 100 μg of CSA1M membrane fraction in the absence or presence of irradiated syngeneic APC (2×10^4 cells) in 0.2 ml of culture medium in 96-well microplates for 48 h.
b) ^{3}H-TdR uptake of each cell line was expressed as the mean±SE of triplicate cultures.
c) Not done.

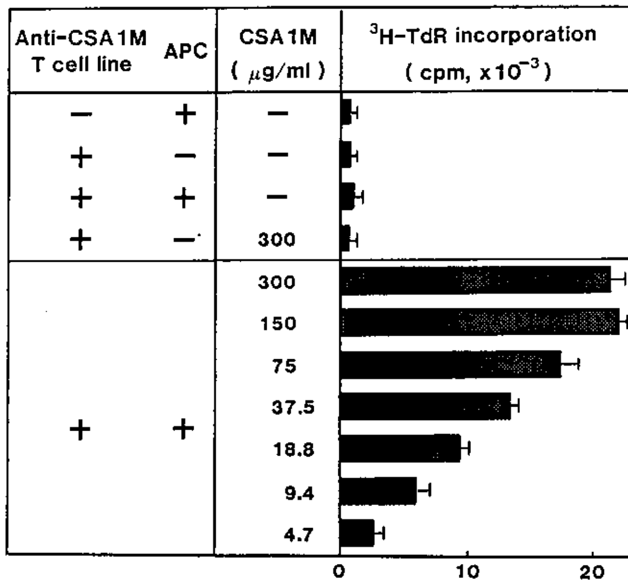

Fig. 1. Antigen dose-dependent proliferation of an anti-CSA1M cell line. An anti-CSA1M cell line (cell line C in Table I) (1.5×10^4 cells) was unstimulated or stimulated with various amounts of CSA1M membrane protein in the absence or presence of 2×10^4 APC for 48 h. ³H-TdR uptake of the cell line was expressed as the mean±SE of triplicate cultures.

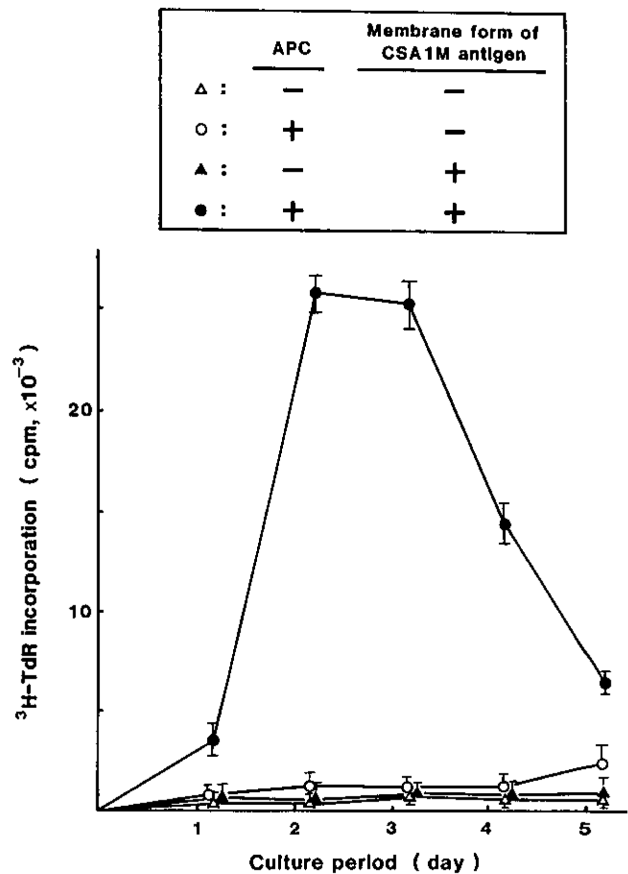

Fig. 2. Time course of proliferative responses of an anti-CSA1M cell line. An anti-CSA1M cell line (cell line C in Table I) (1.5×10^4 cells) was unstimulated or stimulated with 100 μg of CSA1M membrane fraction in the absence or presence of APC (2×10^4 cells) for various times.

immunized mice together with CSA1M membrane fraction. The results of Table I indicate that cell lines established in three sepatate cultures exhibit potent proliferative responses when stimulated with CSA1M membrane fraction as a source of CSA1M tumor antigens in the presence of APC. Figures 1 and 2 show the tumor antigen dose-dependence and time course of such proliferative responses. The results indicate that an anti-CSA1M cell line (line C in Table I) exhibits peak response upon stimulation with as much as about 150 μg/ml of CSA1M membrane protein (Fig. 1) and around 2–3 days after the stimulation with CSA1M tumor antigens in the presence of APC (Fig. 2).

We have also determined the phenotypes of the anti-CSA1M cell lines. Representative results obtained with the use of one of the three lines (Line C) are shown in Fig. 3. All three lines including line C expressed Thy-1 and L3T4 antigens but neither surface Ig nor Lyt-2 antigen was detected. Thus, these results indicate the generation of $L3T4^+$ T cell lines capable of proliferating in response to tumor antigens.

Self-restriction element in antigen recognition of anti-CSA1M T cell lines In order to analyze self-restriction element which is involved in the recognition of CSA1M tumor antigens by anti-CSA1M T cell lines, various monoclonal antibodies were included in cultures of anti-CSA1M T cell lines stimulated with CSA1M tumor antigens (Fig. 4). The addition of the cultures of anti-class I $H\text{-}2^d$, anti-I-E^d or anti-Lyt-2 antibody did not affect the proliferative responses, whereas these responses were inhibited by addition of anti-I-A^d or anti-L3T4 antibody. Thus, these results illustrate that CSA1M-reactive $L3T4^+$ T cell lines recognize target tumor antigens in the context of self class II MHC antigens on APC.

Analysis of the function of anti-CSA1M T cell lines Which type(s) of function is mediated by CSA1M-reactive $L3T4^+$ T cell lines was examined. First, the T cell line from various stages of CSA1M-stimulated culture were tested for cytotoxic activity as determined by short-term (4 h) ^{51}Cr release assay. CSA1M-reactive T cell lines obtained from either early (2 day) or late (13 day) stage of stimulated cultures failed to exhibit any sig-

nificant cytotoxic activity towards CSA1M target cells (data not shown).

Second, the ability of CSA1M-reactive T cell lines to produce lymphokines in response to stimulation with CSA1M antigens was investigated. The T cell lines were stimulated with CSA1M membrane, and culture SNs were tested for IL2 activity (Table II). The results again demonstrate that potent proliferation of the CSA1M-reactive T cell lines was induced in cultures containing CSA1M membrane antigens and APC. Such cultures were found to produce high levels of IL2 activity. Thus, anti-CSA1M T cell lines represent a non-cytolytic, helper proliferative and lymphokine (IL2)-producing type of T cells.

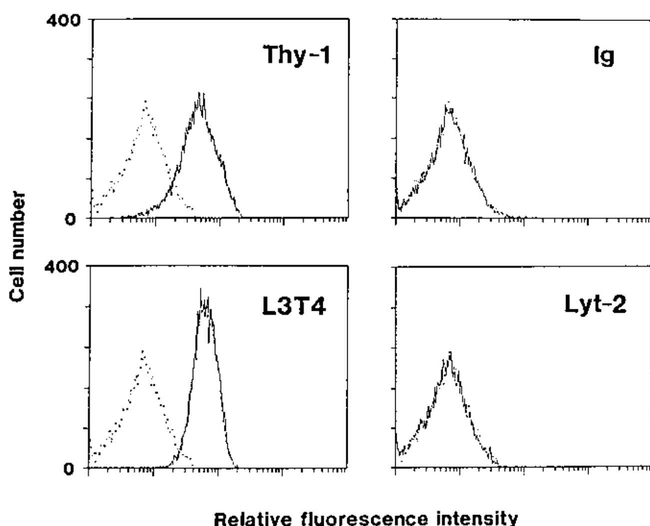

Fig. 3. Surface markers of anti-CSA1M cell line. An anti-CSA1M cell line (line C in Table I) was stained with FITC-conjugated antibodies (indicated).

Stimulation of anti-CSA1M T cell line with various forms of CSA1M tumor antigens We next investigated the stimulation of CSA1M-reactive T cell lines with various forms of CSA1M tumor antigens. The results (Table III) demonstrate that the proliferation of the T cell lines is inducible by stimulation not only with tumor cell membrane but also with intact tumor cells themselves or solubilized form of tumor cell membrane. It should also be noted that the presence of APC was an absolute requirement for inducing the proliferation of T cell lines with any form of tumor antigens.

An earlier study from our laboratory investigated the role of APC in inducing tumor-specific *in vivo* protective immunity and demonstrated that tumor antigen-pulsed APC prepared by culturing APC with solubilized or membrane tumor antigens were capable of producing potent *in vivo* tumor immunity.[23] This is confirmed by the data in a part of Table IV. Administration of CSA1M membrane-pulsed APC into BALB/c mice produced protection against CSA1M. These results prompted us to examine whether anti-CSA1M T cell line can also be stimulated with an APC-pulsed form of tumor antigens

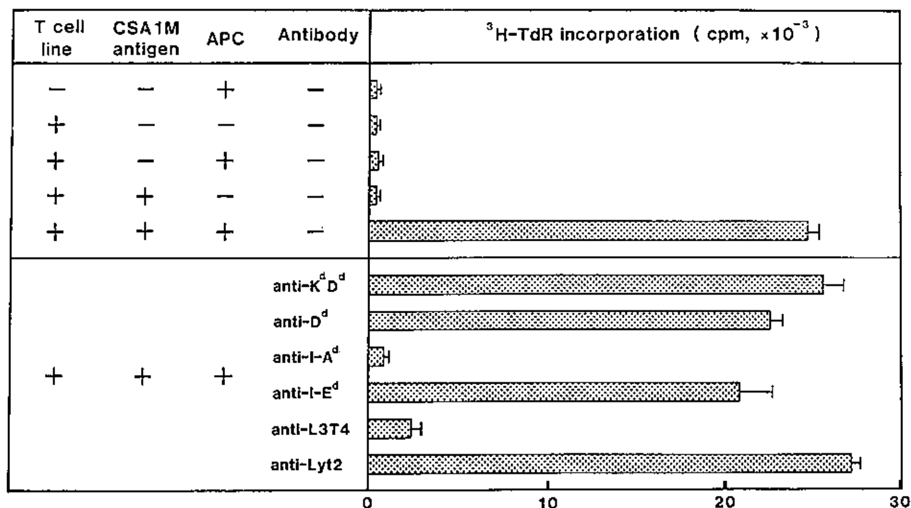

Fig. 4. Inhibition of proliferative responses of anti-CSA1M T cell line by anti-I-A^d or anti-L3T4 antibody. CSA1M-reactive T cell line (line C in Table I) (1.5×10^4 cells) was stimulated with CSA1M membrane fraction plus APC in the absence or presence of various monoclonal antibodies (shown). Culture SN containing each monoclonal antibody was added to the cultures at the final concentration of 10%.

Table II. Ability of Anti-CSA1M T Cell Lines to Produce IL2

Exp.	T cell line[a]	CSA1M antigen	APC	Proliferation of T cell line (cpm)	Production of IL2[b] (cpm)
1	−	−	+	399±13	1454±174
	+	−	−	414±10	1551±46
	+	−	+	1354±201	4654±651
	+	+	−	442±30	630±25
	+	+	+	25959±1168	35322±3532
2	−	−	+	65±2	1252±150
	+	−	−	94±9	1199±299
	+	−	+	83±3	1397±97
	+	+	−	84±8	2508±1354
	+	+	+	10896±642	13397±1741

a) CSA1M-reactive T cell lines (line C in Exp. 1 and line B in Exp. 2) was unstimulated or stimulated with CSA1M membrane antigens in the absence or presence of APC in 96-well microplastes. Some microplates were used to evaluate the proliferative responses of the T cell lines. Culture SNs from other microplates were used to assess the production of IL2.
b) IL2 activity in culture SNs was assessed in terms of the ability to support the growth of IL2-dependent CTLL-2 cell line.

Table III. Stimulation of Anti-CSA1M T Cell Lines with Various Forms of CSA1M Tumor Antigens

Form of CSA1M tumor antigens[a]		APC	^{3}H-TdR uptake (cpm) Exp. 1	Exp. 2
Membrane	−	+	1111±137	745±108
	+	−	167±33	ND
	+	+	19194±1266	15454±432
Solubilized	−	+	1187±212	1402±112
	+	−	500±38	ND
	+	+	7833±1308	10130±2390
Cellular	−	+	518±18	1607±908
	+	−	3495±104	2108±92
	+	+	12629±959	7156±300

a) Anti-CSA1M T cell line (line C in Table I) was stimulated with 100 μg of membrane or solubilized form of CSA1M tumor antigens or 1×10^4 irradiated (10,000 R) CSA1M tumor cells in the absence or presence of APC.

instead of tumor antigens plus APC. Thus, the anti-CSA1M T cell lines were cultured with APC which had been incubated with membrane form of CSA1M tumor antigens. The results (Table IV) also illustrate that CSA1M-pulsed APC induce potent proliferation of anti-CSA1M T cell lines. These results indicate that anti-tumor L3T4$^+$ Th line established here recognize APC-pulsed form of tumor antigens, and this form is capable of effectively inducing *in vivo* protective immunity.

Specificity of anti-CSA1M T cell lines We finally determined the tumor specificity of anti-CSA1M T cell lines. Membrane fractions from BALB/c-derived tumors (RSV-induced CSA1M and RSV-unrelated Meth A) were obtained, and the existence of tumor antigens in these membrane fractions was examined in *in vivo* tumor protection assays before testing the ability to stimulate the anti-CSA1M T cell lines. Administration of CSA1M tumor membranes emulsified in CFA into BALB/c mice produced protection against CSA1M but not against Meth A tumors; likewise, inoculation of Meth A tumor antigens induced Meth A-specific protection, demonstrating that CSA1M and Meth A tumor membrane preparations contain the respective tumor-specific antigens (Table V).

When CSA1M-reactive T cell lines were stimulated *in vitro* with the above tumor antigens in the presence of APC, these T cell lines exhibited potent proliferation only in response to CSA1M but not to Meth A tumor antigens (Table V). It was also confirmed that CSA1M membrane plus APC did not stimulate some T cell lines against various nominal antigens thus far established (data not shown). These results indicate that anti-CSA1M T cell lines respond specifically to tumor antigens expressed on CSA1M tumor cells.

DISCUSSION

Unlike B cells, which directly interact with an antigen, T cells possess an antigen-specific receptor that recognizes a bimolecular ligand composed of foreign antigen and self major histocompatibility complex (MHC) antigens.[13, 26, 27] In particular, helper (proliferative and lymphokine-producing)-type T cells require APC and its

Table IV. Ability of CSA1M-pulsed APC to Produce *in vivo* Protective Immunity and to Stimulate Anti-CSA1M T Cell Line

Exp.	APC preparation[a)]	Tumor protection[b)] tumor diameter in mm	Stimulation of T cell line[c)] ^{3}H-TdR uptake (cpm)
1	unpulsed	12.7±2.1	2165±627
	CSA1M-pulsed	<3.0	13713±713
2	unpulsed	11.9±0.7	4586±132
	CSA1M-pulsed	<3.0	13453±538

a) An APC-enriched population (1×10^7 cells) was cultured *in vitro* with 200 μg of CSA1M membrane fraction for 2 h.
b) BALB/c mice were given three rounds of inoculation with CSA1M-pulsed APC (10^6). One wk after the final inoculation, the mice were challenged with 2×10^5 CSA1M cells. Tumor diameter was measured 40 days after the tumor cell challenge and expressed as the mean±SE of 7 mice/group.
c) Anti-CSA1M T cell line (line C in Table I) (1.5×10^4) was stimulated with a normal (unpulsed) or CSA1M-pulsed population (2×10^4).

Table V. Tumor Antigen Specificity in the Generation of *in vivo* Protective Immunity and Stimulation *in vitro* of Anti-CSA1M T Cell Lines

Tumor antigen used	Tumor protection[a)] to:		Stimulation of anti-CSA1M[b)]	
	CSA1M	Meth A	Line B	Line C
—	13.5±1.7	22.3±2.9	1402±112	4628±545
CSA1M	<3.0	21.3±3.2	12223±1307	27806±1696
Meth A	12.6±3.1	<3.0	3303±293	2226±422

a) BALB/c mice were given a single administration of tumor membrane (200 μg) emulsified in CFA. Two wk later, the mice challenged with CSA1M (2×10^5) or Meth A (1.5×10^5) tumor cells. Tumor diameter was measured 30 days after the tumor cell challenge and expressed as the mean±SE of 5 mice/group.
b) Anti-CSA1M T cell lines (1.5×10^4) were stimulated with tumor membrane (100 μg) plus APC (2×10^4).

surface MHC antigens for recognizing antigen. Analyses concerned with the mechanism of antigen presentation by APC have demonstrated that APC pick up an antigen, convert it from a native to a non-native form (processing) and present the processed antigen in association with self Ia molecules.[13–15] This series of processes is applied for a variety of nominal soluble protein antigens, and recent studies have suggested that this may also hold true for cell surface antigens such as allo-class I MHC antigens[28, 29] or other antigens.[30–33] However, these studies investigated the mechanisms of the activation of helper T cells against cell surface antigens by monitoring the generation of CTL responses requiring the assistance of such helper T cells. Thus, there have been few studies designed to analyze directly the mechanism by which helper-type T cells recognize cell-bound antigens, and most of the foregoing results can be regarded as being indirect observations.

In order to initiate the investigation regarding the mode of recognition of cell-bound tumor antigens by anti-tumor Th, we tried to establish Th lines directed against an RSV-induced murine fibrosarcoma, CSA1M. Several anti-CSA1M T cell lines were isolated from long-term liquid cultures of lymph node cells from CSA1M-immunized BALB/c mice together with CSA1M membrane fraction. These T cell lines were shown to be of $L3T4^+$ $Lyt\text{-}2^-$ phenotypes, $I\text{-}A^d$ restricted, stimulated to proliferate with CSA1M (RSV-induced tumor) but not with RSV-unrelated tumor membrane fractions, and capable of producing IL2 upon CSA1M stimulation. More importantly, it was found that these Th lines can be stimulated with various forms of tumor antigens. These included (a) CSA1M tumor cells themselves, (b) subcellular fractions such as membrane and detergent-solubilized forms and (c) APC pulsed with subcellular CSA1M membrane fractions.

The present study permits us to consider the mechanisms by which tumor-specific Th can be stimulated with cell-bound tumor antigens. In contrast to CTL or CTL clones that can recognize tumor antigens directly on

tumor cell surfaces, anti-CSA1M Th were not stimulated with CSA1M tumor cells *per se*. The activation of these Th with tumor cells themselves was induced only when APC were added to the cultures. This is reminiscent of fact that anti-CSA1M Th lines were stimulated with subcellular tumor antigens depending on the presence of APC. Thus, it appears that tumor cells themselves fail to stimulate anti-tumor Th and instead, this type of T cells is activated with tumor antigens associated with APC. Previous studies have suggested that APC play a crucial role in picking up tumor antigens shed from tumor cells, processing these tumor antigens and presenting the processed tumor antigens to anti-tumor Th which function to activate tumor-specific CTL[30, 31] and non-specific tumoricidal effectors such as macrophages.[32] This notion is supported by the present results, which demonstrated the requirement of APC for the activation of tumor-specific Th with various forms of tumor antigens. Moreover, if the above scenario of anti-tumor Th activation is the case, it is conceivable that tumor-specific Th can be stimulated with APC which have already picked up and processed tumor antigens through preincubation with tumor antigens. This was, indeed, substantiated by the observation that anti-tumor Th lines can be efficiently activated with APC pulsed with subcellular tumor antigens. Thus, the precent results provide direct evidence that anti-tumor Th are activated by recognizing tumor antigens which are associated with APC through pulsing processes.

Another important aspect of the precent results is concerned with the immunobiologic nature of tumor antigens which are pulsed and presented by APC. Subcellular (membrane or solubilized form) CSA1M tumor antigens were shown to contain molecules capable of eliciting anti-CSA1M *in vivo* protective immunity (ref. 20 and Table V). Moreover, it has also been demonstrated that administration of APC pulsed with such subcellular tumor antigens resulted in highly efficient induction of tumor-specific protective immunity (ref. 23 and Table IV). It should be noted that the same preparations of tumor antigens or tumor antigen-pulsed APC population as used in those *in vivo* protection assays effectively stimulate CSA1M-specific T cell lines. Thus, the present study taken together with the above observations indicates that various forms of tumor antigens are, in parallel, capable of stimulating the Th lines established here and producing *in vivo* protective tumor immunity. It remains, however, to be analyzed whether the anti-CSA1M Th line-stimulating antigens are identical to the *in vivo* tumor immunity-inducing antigens. Studies are in progress to determine the molecular relationship between these two tumor antigen activities by purifying solubilized CSA1M tumor antigen preparations.

Irrespective of the relationship between the above two tumor antigen activities, it is clear that both are detected on tumor antigen-pulsed APC. In contrast to difficulties in analyzing the mechanisms of the activation *in vivo* of tumor-specific Th, it is possible to investigate the mechanisms underlying the generation of tumor antigen-pulsed APC by utilizing tumor-specific Th lines. Therefore, tumor-specific Th lines provide an *in vitro* model for analyzing the mechanism by which APC process tumor immunity-inducing antigens *in vivo*. Such an approach could also contribute to a better understanding of how helper-type T cells, in general, recognize tumor antigens expressed on tumor cell surfaces. Clearly the utilization of tumor-specific Th lines could be very fruitful.

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