Jpn. J. Cancer Res. (Gann), 79, 99–108 ; January, 1988

Mechanisms for Recognition of Tumor Antigens and Mediation of Anti-tumor Effect by Noncytolytic Lyt-2$^+$ T Cell Subset*1

Kohichi SAKAMOTO, Takayuki YOSHIOKA, Jun SHIMIZU, Soichiro SATO, Hiroto NAKAJIMA, Hiromi FUJIWARA*2 and Toshiyuki HAMAOKA

Department of Oncogenesis, Institute for Cancer Research, Osaka University Medical School, 1-1-50, Fukushima, Fukushima-ku, Osaka 553

The mode of anti-tumor function *in vivo* of noncytolytic Lyt-2$^+$ T cells from C3H/He mice hyperimmune to syngeneic MH134 hepatoma was investigated in a double diffusion chamber system which was recently established in our laboratory. C3H/He mice were implanted intraperitoneally with the double diffusion chamber unit in which each chamber contained either L3T4$^+$ T cell-depleted MH134-hyperimmune spleen cells plus mitomycin C-treated MH134 tumor cells or other syngeneic X5563 viable tumor cells plus normal spleen cells as a source of macrophages. Inclusion of anti-MH134 Lyt-2$^+$ T cells together with MH134 tumor cells in one chamber resulted in comparable growth inhibition of viable X5563 tumor cells in the other chamber to that obtained by unfractionated MH134-hyperimmune spleen cells. The induction in the Lyt-2$^+$ T cell-containing chamber of anti-tumor effect to be delivered into the other chamber was dependent on the co-existence of Ia-positive adherent cells along with Lyt-2$^+$ T cells. Although adherent cell-depleted Lyt-2$^+$ T cells regained the inducibility of anti-tumor immunity when supplemented with splenic adherent cells, the addition of adherent cells pretreated with chloroquine failed to restore the ability of Lyt-2$^+$ T cells to induce their anti-tumor effect. In addition, paraformaldehyde-treated MH134 tumor cells instead of untreated tumor cells were not capable of activating Lyt-2$^+$ T cells. These results indicate that a portion of Lyt-2$^+$ T cells exerts their anti-tumor effect by a mechanism distinct from direct tumor cell lysis and that their activation for mediation of this type of tumor immunity requires the recognition of tumor antigens processed and presented by Ia-positive adherent cells.

Key words: Lyt-2$^+$ T cell subset — Lymphokine — Anti-tumor effect

Despite ample evidence for the quintessential role of T lymphocytes in rejecting tumor cells as well as allografts,[1,2] the effector mechanisms responsible for the graft rejection have been poorly understood. Since the discovery of a subclass of T lymphocytes capable of specifically lysing target cells expressing tumor or histocompatibility antigens, it has been naturally assumed that such cells would be the effector cells in rejection responses.[3] Although a great deal of evidence has been presented to implicate cytotoxic T lymphocytes (CTL)*3 in the phenomenon of tumor or allograft rejection, it should be stressed that all of the evidence is circumstantial, and it has become increasingly apparent that there probably exists more than one mechanism to mediate the rejection responses.

Recent studies from several laboratories have revealed that tumor immunity *in vivo* can be transferred with T cells bearing the markers which have been considered to represent T cell subset for helper/delayed-type hypersensitivity (DTH) but not for the direct cytolytic function.[4–7] Such findings in tumor rejection parallel the results of similar experiments in histoincompatible allograft rejection systems.[8–11] Although the publication of these studies has placed strong emphasis on the role of the helper/DTH T cell subset in rejection responses, further experimentation has led to a reevaluation of the relative importance of

*1 This constitutes Part II of a series entitled "Role of tumor-specific Lyt-2$^+$ T cells in tumor growth inhibition *in vivo*."

*2 To whom correspondence should be addressed.

*3 Abbreviation used in this paper: CTL, cytotoxic T lymphocyte(s); C, complement; AC, accessory cells; MHC, major histocompatibility complex; TAA, tumor-associated antigens; APC, antigen-presenting cells; id, intradermal or intradermally; MMC, mitomycin C; Con A, concanavalin A.

this T cell subset and a reexamination of the role of T cells bearing the classical CTL phenotype (Lyt-2 in the mouse) in the graft rejection.[13] In fact, we have also found in the preceding study that Lyt-2$^+$ as well as Lyt-2$^-$ (L3T4$^+$) T cell subsets functioned as *in vivo* tumor-neutralizing T cells.[14] However, the efficacy of the Lyt-2$^+$ T cell subset has been demonstrated in the tumor model in which tumor cells are not lysed by T cells from tumor-immunized hosts. Thus, these findings permitted us to investigate the mechanism(s) by which this Lyt-2$^+$ T cell subset exerts its *in vivo* anti-tumor effect.

In the present study, we have addressed ourselves to the above issue. The results demonstrate that Lyt-2$^+$ T cell subset from C3H/He mice immunized to syngeneic MH134 hepatoma exhibited a potent *in vivo* tumor growth-inhibiting effect. With the use of the double diffusion chamber system which was recently developed,[15] it was found that this T cell subset is capable of exerting its effect through a mechanism similar to that described for Lyt-2$^-$ (L3T4$^+$) T cell-mediated tumor growth inhibition; namely, the production of lymphokine(s) able to activate non-T lymphoid cell populations which function as the ultimate tumoricidal effectors. More importantly, such activation of Lyt-2$^+$ T cells depended on the recognition of tumor antigens processed and presented by antigen-presenting cells (APC). The results are discussed in the context of the heterogeneity of Lyt-2$^+$ T cells in the mode of the recognition of antigen as well as implementation of its function.

Materials and Methods

Mice and Tumors Female C3H/HeN mice, obtained from Charles River Laboratory, were used at 6–9 weeks of age. MH134 hepatoma and X5563 plasmacytoma, both derived from C3H/He strain and maintained by serial intraperitoneal transplantation, were utilized.

Immunization to MH134 Hepatoma Cells C3H/HeN mice (7–8 weeks old) were inoculated intradermally (id) with 10^6 viable MH134 tumor cells, followed by the surgical resection of the tumor 7 days thereafter ("primed" mice). Seven days after the tumor resection, mice were challenged id with 10^5 viable MH134 tumor cells. The mice which did not develop a tumor mass 3 weeks after the tumor challenge received two additional challenges with 10^5 and 10^6 viable MH134 tumor cells and the resistant mice were used as "hyperimmune" mice.

Monoclonal Antibodies Monoclonal antibodies to L3T4 and Lyt-2 antigens were used. The GK1.5 monoclonal antibody-producing hybridoma line,[16,17] specific for the L3T4 molecule, was a gift from Dr. F. Fitch, University of Chicago, IL, and the 3.155 monoclonal antibody-producing hybridoma line, specific for the Lyt-2 molecule, was obtained from ATCC, Rockville, MD.

Treatment of Immune Spleen Cells with Antibody plus Complement Spleen cells (10^8) from immunized mice were incubated at room temperature for 30 min with ascitic form of anti-L3T4 or -Lyt-2 monoclonal antibody at a dilution of 1:50.[14] Cells were washed and incubated at 37° for 45 min with rabbit complement (C) preabsorbed with syngeneic mouse spleen cells at a final dilution of 1/20.

Tumor-neutralization Test (Winn Assay) Spleen cells (10^7) from normal or tumor-immunized mice were admixed with 10^5 viable tumor cells. The mixture was inoculated id in a volume of 0.1 ml into syngeneic C3H/HeN recipient mice, and tumor growth was expressed as the mean tumor diameter ± SE of five mice/group.

***In vitro* Sensitization and Cytotoxicity Test** *In vivo* sensitization and cytotoxicity assay were essentially the same as previously described.[18] Briefly, 5×10^6 spleen cells from tumor-immunized mice were cultured with 10^5 mitomycin C (MMC)-treated tumor cells at 37° in a humidified incubator (5% CO_2). Effector cells generated were harvested after 5 days of culture and tested on tumor target cells in a 4-hr ^{51}Cr release assay.[18]

Treatment of Spleen Cells with a Nylon Wool Column The procedure used was essentially the same as that described by Julius *et al.*[19] Briefly, nylon wool in LP-1 Leuko-Pak leukocyte filters (Fenwal Laboratories, Morton Grove, IL) was soaked, dried and packed into a 10 ml syringe. Spleen cells (10^8 in 1 ml of medium) were loaded onto the nylon wool column.

Construction and Implantation of a Double Diffusion Chamber The procedure was essentially the same as that described for constructing a single diffusion chamber.[20] The Millipore double diffusion chamber was prepared from two Plexiglass rings covered at the outer ends and separated from each other by Millipore filter discs of 0.22 μm porosity (total of 3 discs). Filters were cemented to the rings before filling the chamber. The dimensions of each chamber were: outer diameter, 14 mm; inner diameter, 10 mm; thickness, 2 mm. The complete chamber was sterilized by exposure to ethylene oxide gas before use. Suspensions of 10^7 spleen cells and various numbers of tumor cells were injected in a volume of 0.1 ml into each

chamber through the filling holes with a syringe and fine-guage needle. The filling holes were sealed with nontoxic cement (Millipore Corp.). For implanting the chambers, mice were anesthetized with ether alone. An abdominal incision was made and the chamber was gently inserted into the peritoneal cavity.

Measurement of Tumor-growth Inhibition by Immune Spleen Cells in Diffusion Chambers After culturing of the diffusion chambers *in vivo*, the host animals were killed and the chambers were removed. The harvest of chambers was performed 3 days after the implantation. After detaching the filter at each end of a double chamber, each fluid of the double chamber was obtained by washing with Hanks' balanced salt solution (HBSS) containing 0.5% pronase to lyze the fibrin clot embedding the cultured cells. Viable tumor cells remaining in the chamber were enumerated as previously described.[20] Briefly, total cell number was counted by the trypan blue dye exclusion method (more than 99% of cultured tumor and spleen cells were viable). To discriminate tumor cells from spleen cells or peritoneal exudate cells (PEC), a cytocentrifuge preparation was made of total cells from each chamber. These cells were stained with Wrigth-Giemsa and observed by light microscopy. Tumor cells were discriminated on a morphological basis as previously described.[20] Tumor cell number per chamber was obtained by multiplying total viable cell number by the tumor cell: total cell ratio in the Wright-Giemsa preparations. Mean tumor cell number was obtained from 4 chambers/group. Tumor growth inhibition (%) was calculated according to the following formula:

$$\text{Tumor growth inhibition (\%)} = 100 \times [1 - (\text{viable tumor cell No. in experimental chamber} / \text{viable tumor cell No. in control chamber})].$$

Collection of Splenic Adherent Cells Splenic macrophages were enriched on a bovine serum albumin (BSA) gradient according to the method described by Glimcher *et al.*[21] 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° 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 70% Thy-1.2-negative or surface Ig-negative cells by the immunofluorescent antibody technique. These fractionated cells were used as a splenic macrophage-enriched population.

Paraformaldehyde (P-CH_2O) Fixation of Tumor Cells Tumor cells (MMC-treated) were fixed with 1% P-CH_2O (Merck Schuchandt, Munich, West Germany) at 2.5×10^6/ml in Hanks' balanced salt solution (HBSS) for 30 min on ice. Fixation was stopped by the addition of cold HBSS containing 5% FCS and the cells were washed extensively before addition to the culture.[22]

Chloroquine Treatment of AC Chloroquine (7-chloro-4-[4-diethylamine-1-methyl-butylamino]-quinoline diphosphate salt) was obtained from Sigma Chemical Co., St. Louis, MO. AC (5×10^6/ml) were incubated in 5% FCS-RPMI1640 containing 1m*M* chloroquine for 30 min at 37° with shaking and were washed extensively before being placed in culture.[22]

Results

We have confirmed the fact[6] that MH134 tumor cells can not be lysed by spleen cells from syngeneic C3H/He mice immunized to MH134 cells. As shown in Table I, X5563-primed spleen cells generated potent tumor-specific CTL activity after 5-day *in vitro* culture with the relevant tumor cells, whereas MH134-hyperimmune as well as MH134-primed mice failed to induce any significant CTL response.

Despite the failure to detect CTL response in the MH134 tumor system, C3H/He mice are capable of generating potent *in vivo* tumor immunity to MH134 hepatoma, and this has been demonstrated in various *in vivo* tumor growth inhibition assays.[6, 14, 15] The preceding paper further illustrated the efficacy of both L3T4$^+$ and Lyt-2$^+$ T cell subsets in mediating and/or initiating *in vivo* anti-tumor immunity.[14] This is also confirmed in Table II. Spleen cells from MH134-hyperimmune C3H/

Table I. Failure to Detect Anti-MH134 CTL Activity in Spleen Cells from MH134-primed or MH134-hyperimmune Mice

Responding cells from mice[a]	% Specific lysis on targets[b]			
	X5563		MH134	
	40:1	5:1	40:1	5:1
Primed to X5563	59.3	44.2	0.0	0.9
Primed to MH134	8.4	3.7	0.0	1.1
Hyperimmune to MH134	2.0	0.0	1.3	0.0

a) Spleen cells (5×10^6) from X5563- or MH134-immunized mice were stimulated *in vitro* with MMC-treated tumor cells (10^4) homologous to those used for *in vivo* immunization.

b) Effector cells were assayed on target cells at the effector-to-target ratios indicated. Percent specific lysis of effector cells resulting from unimmunized spleen cells was less than 4.0 irrespective of the stimulation with either tumor cell type.

Table II. Mediation of Tumor Growth Inhibition by Lyt-2$^+$ as Well as L3T4$^+$ T Cell Subsets

Effector cells[a] (treatment)	Tumor diameter (mm) in Winn assay[b]	Tumor growth inhibition (%) in single diffusion chamber[c]
Normal (−)	12.7±0.7	0.0
MH134-hyperimmune (C)	<3.0	68.1
MH134-hyperimmune (anti-Lyt-2+C)	<3.0	56.8
MH134-hyperimmune (anti-L3T4+C)	<3.0	75.1
MH134-hyperimmune (anti-Lyt-2 & -L3T4+C)	12.3±0.5	18.1

a) Spleen cells from MH134-hyperimmune mice were treated with various antibodies and complement (C) before examination (Winn assay and single diffusion chamber system).
b) Fractionated cells (10^7) were mixed with 10^5 viable MH134 tumor cells and the mixture was inoculated id into syngeneic recipient mice. Tumor growth on day 12 was expressed as the mean diameter±SE of 5 mice/group.
c) Fractionated cells (10^7) were mixed with 5×10^3 viable MH134 tumor cells in the single diffusion chamber which was placed in the peritoneal cavity of C3H/He mice for 3 days. Percent tumor growth inhibition was expressed according to the formula described in "Materials and Methods."

Table III. Ability of Anti-MH134 Lyt-2$^+$ T Cell Subset to Exert Its Anti-tumor Effect in Double Diffusion Chamber System

Culture in double diffusion chamber				Tumor growth inhibition (%) in chamber 2
Chamber 1		Chamber 2		
Tumor cells	Spleen cells[a] (treatment)	Tumor cells	Spleen cells[b]	
MH134	Normal (−)	X5563	Normal	0.0
MH134	MH134-hyperimmune (C)	X5563	Normal	61.6
MH134	MH134-hyperimmune (anti-L3T4+C)	X5563	Normal	64.5
MH134	MH134-hyperimmune (anti-L3T4 & -Lyt-2+C)	X5563	Normal	8.0

a) MH134-hyperimmune spleen cells (1×10^7) were treated with the indicated antibody+C before injection into chamber 1 together with mitomycin C (MMC)-treated MH134 tumor cells (5×10^5).
b) Normal spleen cells (10^7) as an adherent cell source and 2×10^4 viable X5563 tumor cells were injected into chamber 2.

He mice were fractionated into L3T4$^+$ or Lyt-2$^+$ T cell subset by treatment with anti-Lyt-2 or anti-L3T4 antibody plus complement (C). The resultant fractions of T cells were tested for ability to neutralize the growth of MH134 tumor cells in Winn assays. As shown in Table II, either fraction of T cells produced complete protection against the MH134 tumor.

Portions of the same fractions of cells were admixed with viable MH134 tumor cells and the mixture was injected into a single diffusion chamber which was subsequently implanted in the C3H/He mouse peritoneal cavity for 3-day *in vivo* culture. The results of Table II also demonstrate that Lyt-2$^+$ T cells exhibited comparable tumor growth inhibition to that obtained with either unfractionated or L3T4$^+$ splenic T cells.

The above results suggested the existence of a Lyt-2$^+$ T cell-mediated anti-tumor pathway which is distinct from CTL function. To investigate the mechanism(s) of such a pathway,

Table IV. Requirement of Ia$^+$ Adherent Cells for Activating Lyt-2$^+$ T Cells to Deliver Anti-tumor Potential into Chamber 2

Group	Lymphoid cells injected into chamber 1		Tumor growth inhibiton (%) in chamber 2
	Spleen cells (treatment)[a]	AC added back[b]	
A	Normal (−)	−	0.0
B	MH134-hyperimmune (C)	−	57.2
C	MH134-hyperimmune (anti-I-A^k+C)	−	16.6
D	MH134-hyperimmune (anti-I-A^k and -L3T4+C)	−	20.6
E	MH134-hyperimmune (anti-I-A^k and -L3T4+C)	+	52.7

a) MH134-hyperimmune spleen cells were treated with the indicated antibody +C. Anti-I-A^k antibody was obtained from 10-2.16 hybridoma cells, and the treatment was done according to the procedure described.[22]

b) Splenic adherent cells (10^6) were co-injected together with antibody-treated MH134-hyperimmune cells into chamber 1.

we have utilized a double diffusion culture system.[15] Each chamber of a double diffusion chamber unit was injected with either a combination of mitomycin C (MMC)-treated MH134 tumor cells plus MH134-hyperimmune spleen cells (normal spleen cells were used as a control) or viable X5563 tumor cells plus normal spleen cells as a source of adherent cells (Table III). The injection of MH134-hyperimmune spleen cells into chamber 1 together with stimulating MH134 tumor cells resulted in the generation of potent anti-tumor potential which is subsequently delivered to chamber 2 and leads to strong growth inhibition of X5563 tumor cells. The results of Table III further demonstrate that a comparably potent anti-tumor effect can also be initiated by cells obtained after the treatment of MH134-hyperimmune spleen cells with anti-L3T4 plus C. Thus, these results indicate that the Lyt-2$^+$ T cell subset is capable of initiating such an anti-tumor effect in the chamber in which this subset is contained.

Additional experiments were performed to investigate the mechanism of antigen recognition by the Lyt-2$^+$ T cell subset. When I-A positive cells (Table IV) or nylon-adherent cells (Table V) were eliminated from MH134-hyperimmune spleen cells in chamber 1 by treatment with anti-I-A^k antibody plus C[22] or a nylon wool column, the generation of potential to initiate tumor growth inhibition was inhibited. This anti-tumor potential was restored by adding splenic adherent cells back to chamber 1 (group E in Table IV and group D in Table V). Thus, these results indicate the requirement of Ia-positive adherent cells for the activation of Lyt-2$^+$ T cell subset, as was demonstrated for Lyt-2$^-$ (L3T4$^+$) T cell subset in the previous study.[15]

The results of Table V also demonstrate that pretreatment of the adherent cells which were added back with chloroquine resulted in almost complete inhibition of the restoring capability (group E). Moreover, pretreatment of stimulating MH 134 tumor cells with paraformaldehyde instead of adherent cell pretreatment also failed to initiate anti-tumor immunity in the immune T cell-containing chamber (Table VI). It has been established that the lysosomotropic agent, chloroquine, inhibits the ability of antigen-presenting cells to internally process antigen by alkalinizing lysosomes[23, 24] and that paraformaldehyde blocks the shedding off of cell surface antigens.[25] Our recent study has also established conditions in which adherent cells as antigen-presenting cells and stimulating tumor cells can be treated with chloroquine and paraformaldehyde, respectively, without inducing nonspecific toxicity to adherent cells or tumor cells. Since this study utilizes similar conditions to those established,[22] the results of Tables V and VI indicate that Lyt-2$^+$ as

Table V. Inhibitory Effect of Chloroquine Treatment of Adherent Cells on the Activation of Tumor-specific T Cells

Group	Culture in chamber 1		Tumor growth inhibition (%) in chamber 2
	Spleen cells (fraction)	AC (treatment)[b]	
A	Normal	−	0.0
B	MH134-hyperimmune	−	44.1
C	MH134-hyperimmune (nylon-nonadherent)[a]	−	0.0
D	MH134-hyperimmune (nylon-nonadherent)	Splenic AC	56.3
E	MH134-hyperimmune (nylon-nonadherent)	Splenic AC (chloroquine)	7.9

a) MH134-hyperimmune spleen cells were passed through a nylon wool column, and nylon wool column-effluent (nylon-nonadherent) cells were used.
b) Splenic adherent cells were treated with chloroquine before being added back into chamber 1 containing adherent cell-depleted MH134-hyperimmune cells.

Table VI. Inhibitory Effect of the Fixation of Tumor Cells with Paraformaldehyde on the Activation of Tumor-specific T Cells

Culture in chamber 1		Tumor growth inhibition (%) in chamber 2
Spleen cells	Tumor cells (treatment)[a]	
Normal	MH134 (−)	0.0
MH134-hyperimmune	MH134 (−)	80.3
MH134-hyperimmune	MH134 (paraformaldehyde)	8.9

a) MMC-pretreated MH134 tumor cells were treated with paraformaldehyde before co-injection into chamber 1.

well as L3T4$^+$ T cells can be activated with tumor antigens which are shed off from tumor cell surfaces and presented by adherent cells (antigen-presenting cells) after internal processing.

Discussion

In order to determine the nature of the T cell subsets responsible for tumor cell eradication *in vivo*, several animal models have been developed in which the adoptive transfer of tumor-specific immune T cells results in the rejection of the subsequent challenge with tumor cells or the eradication of growing tumor cells preinoculated. These models have provided insights into how appropriately selected and amplified T cells from tumor-immune hosts might be utilized to promote tumor eradication, although studies along this line have yielded somewhat conflicting results.

The administration of purified noncytolytic Lyt-1$^+$2$^-$ T cells in the mouse, or their analog in the rat, has been shown to be effective in specific therapy or prevention of chemically induced and virally induced sarcoma,[4] a spontaneous plasmacytoma,[5] chemically induced hepatoma,[6] and virally induced leukemia,[7] and recent studies have demonstrated that these noncytolytic T cells mediate an anti-tumor effect without the participation of CTL.[5,26] However, studies of other tumor models of sarcomas and leukemias have suggested that CTL are critically important for therapy.[27,28] Moreover, recent studies along this line have demonstrated that infusion of cloned CTL can mediate a therapeutic effect.[29–32]

Understanding the reasons for these apparently disparate results is important for the prospective development of effective therapeutic regimens. However, it could be that

different types of tumor antigens stimulate predominantly either type of anti-tumor T cell subset. It is also possible that a certain tumor antigen may induce both Lyt-2$^+$ and Lyt-2$^-$ T cell subpopulations which are capable of initiating or mediating tumor cell eradication *in vivo*. In addition, it has been reported that Lyt-2$^+$ T cell subset does not necessarily represent the CTL population, but at least a part of Lyt-2$^+$ T cells is responsible for mediating helper T cell activity against cell surface antigens such as major histocompatibility complex (MHC) class I antigens.[33] Thus, the most important issue is not to try to reconcile the above conflicting observations concerning anti-tumor effector T cell phenotype, but to elucidate the cellular and molecular mechanisms by which either Lyt-2$^-$(L3T4$^+$) or Lyt-2$^+$ T cell subset exerts its anti-tumor effect *in vivo*.

The preceding study[14] and Table II of this study have demonstrated the efficacy of Lyt-2$^+$ T cell subset from MH134-hyperimmune mice to neutralize MH134 tumor cells *in vivo*, in addition to the effectiveness of Lyt-2$^-$ (L3T4$^+$) T cells as has been reported.[5, 6] Concerning the mechanism by which the Lyt-2$^+$ T cells function as *in vivo* anti-tumor effector T cells, this paper also confirms that tumor-specific CTL responses as observed in a short-term cytotoxicity test were not generated even from mice which had acquired potent immunity in the MH134 tumor model and implies the existence of a Lyt-2$^+$ T cell-initiated anti-tumor pathway which is distinct from CTL responses.

Although the Lyt-2 marker was originally described as representing the CTL phenotype, the ability of Lyt-2$^+$ T cells to function as non-CTL T cells such as helper T cells in immune responses has recently been documented.[33, 34] Evidence has also emerged that portions of tumor-specific Lyt-2$^+$ T cells are capable of producing lymphokine(s).[35] Thus, stimulation of FBL tumor-immune T cells with FBL leukemia cells results in production of γ-interferon (γ-IFN) which renders macrophages capable of lysing FBL. Analysis of γ-IFN production by T cell subsets revealed that Lyt-2$^-$(L3T4$^+$) subset secreted γ-IFN following stimulation by FBL. Moreover, the Lyt-2$^+$ subset produced large amounts of γ-IFN particularly depending on the presence of interleukin 2 (IL2). Thus, the anti-tumor activity of T cells bearing classical CTL marker (Lyt-2) may reflect activation of non-T cell effector mechanisms in addition to direct cytolysis.

In considering the ability of tumor-immune Lyt-2$^+$ T cells to exert their anti-tumor effect by producing chemical mediator(s) (lymphokine) capable of activating non-T cell populations, we have investigated the existence of such a mechanism in the immune response to MH134 tumor by utilizing the double diffusion chamber system. It was previously demonstrated that when one chamber of a double chamber unit contained tumor-immune Lyt-2$^-$ (L3T4$^+$) T cells, the growth of viable tumor cells admixed with adherent cells in the other chamber was inhibited.[15] The results also indicated that lymphokine(s) produced by tumor-immune Lyt-2$^-$ T cells passes through the cell-impermeable membrane into the other chamber where the growth of viable tumor cells is inhibited by adherent cells activated with lymphokine.[15] These observations are consistent with the notion that tumor-specific Lyt-2$^-$ T cells have the potential to reject antigenically irrelevant (bystander) tumor cells on activation with the specific target tumor cells.[36] Thus, the present model which utilized this double diffusion chamber system and tested the capability of Lyt-2$^+$ T cells from tumor-hyperimmune mice illustrated that at least a portion of Lyt-2$^+$ T cell subset mimics the anti-tumor pathway thus far obtained for Lyt-2$^-$ (L3T4$^+$) T cells.

It has been demonstrated that the activation of L3T4$^+$ T cell subset for the production of lymphokine requires the participation of adherent cells which are capable of internally processing tumor antigens shed off from tumor cell surfaces.[22, 37] In the present double chamber system, the elimination of Ia-positive adherent cells from tumor-hyperimmune spleen cells abolished the generation of anti-tumor potential. Moreover, pretreatment of adherent cells and stimulating tumor cells with chloroquine and paraformaldehyde, respectively, inhibited the activation of both Lyt-2$^+$ and L3T4$^+$ T cells. Therefore, it is conceivable that Ia-positive adherent cells are required for the antigen recognition by and activation of both T subsets through a similar mechanism. It should be noted that the recog-

nition of tumor antigens by such a part of Lyt-2^+ T cells contrasts with that which has been observed for Lyt-2 marker-bearing classical CTL subset, i.e., CTL can recognize tumor antigens directly on tumor cells even after the pretreatment with paraformaldehyde.[22]

Thus, it is increasingly evident that in addition to the direct cytolytic function of Lyt-2^+ T cells as CTL, at least a part of Lyt-2^+ T cells is responsible for an anti-tumor mechanism similar to that observed for Lyt-1^+2^- (L3T4^+) T cell subset. Our present observations, together with others,[33-35] indicate the functional heterogeneity of Lyt-2^+ T cells as well as distinct mechanisms underlying the recognition of tumor antigens. This reinforces the importance of understanding the mechanism by which each effector T cell subset exerts its anti-tumor effect, rather than mere identification of effector T cell phenotypes.

Acknowledgments

The authors are grateful to Miss Kazue Yanagi and Miss Yuko Osawa for their expert secretarial assistance.

(Received Aug. 24, 1987/Accepted Oct. 29, 1987)

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