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Tumor-specific T Cells which Form Clusters with Dendritic Cells and Tumor Cells and Deliver Macrophage-activating Factors

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T cells prepared from tumor (Meth A)-bearing mice were cocultured with homologous tumor cells and splenic dendritic cells to enrich tumor-specific T cells by the separation of clusters. T blasts generated from clusters were capable of inhibiting the *in vivo* tumor cell growth. The culture supernatant of clustering cells (CLSN) was effective in activating macrophages (Mϕ) to be cytostatic and cytocidal against tumor cells. Moreover, it was found that CLSN contains at least 3 distinct factors; one was identified as interferon-γ (IFN-γ), and the others are so far unidentified, but one acts synergistically with IFN-γ, possibly as the second signal, and the other cooperates with lipopolysaccharide but not with IFN-γ. We propose that the tumor-specific T cells secrete soluble mediators which cooperate with each other in Mϕ activation against tumor cells.

Key words: Macrophage-activating factors — Tumor-specific T cells — Dendritic cells — Clustering cells

Cellular immunity mediated by T cells has been postulated to be the basis for the transplantation resistance of animals immunized with tumor cells. One of the well known instances is the case of Meth A, which is a highly immunogenic sarcoma cell line induced by methylcholanthrene in BALB/c mice.[1)] On the other hand, Macrophage (Mϕ)[6] are known to play an important role in the host defense against tumors.[2)] It has been indicated that tumor-specific T cells are required for the Mϕ-mediated response against tumors.[3-5)] In the previous report,[6)] we have shown that culture supernatant of spleen cells obtained from Meth A-bearing mice with homologous tumor cells contains the humoral factors that activate Mϕ to inhibit the *in vitro* tumor cell growth. This seems to indicate that T cells specific for Meth A are activated in spleen of mice bearing progressive tumors.

In order to elucidate the contribution of tumor-specific T cells in rejection of tumor cells by Mϕ-mediated response, we have attemped to enrich them. In the present study, we employed lymphoid dendritic cells (DC), since DC are known to be the most potent accessory cells (A cells) in T cell responses[7)] and to form clusters with antigen-specific T cells in the presence of homologous antigen.[8,9)] It is shown here that the isolation of clustering cells from a culture of Meth A-primed T cell population with DC and homologous tumor cells is an efficient procedure to obtain the tumor-specific T cells. In addition, we demonstrate that culture supernatant of such clusters (cluster supernatant; CLSN) exhibits a strong Mϕ-activating capacity which is mediated by at least 3 distinct factors; one is IFN-γ, which provides the priming signal. The others are so far unidentified, but one of them serves as the secondary triggering signal, like lipopolysaccharide (LPS) of gram-negative bacteria, and the other cooperates with LPS but probably not with IFN-γ.

MATERIALS AND METHODS

Animals BALB/cCrSlc, BALB/c *nu/nu*, and C3H/HeSlc mice of both sexes, purchased from the Shizuoka Laboratory Animal Center (Shizuoka), were used at 7–9 weeks of age.

Reagents Recombinant murine IFN-γ (rIFN-γ, 10^7 IU/mg protein) was a gift from Shionogi Research Laboratories (Osaka). The immunopurified rat monoclonal antibody against murine IFN-γ (7FD9) and rabbit anti-murine IFN-γ antiserum were provided by Drs. Y. Watanabe and Y. Kawade (The Institute for Virus Research, Kyoto Univ.). Proteose peptone (PP), Brewer's thioglycollate medium (TGC), and LPS from *E. coli*

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[6] The abbreviations used are: A cells, accessory cells; CLSN, supernatant of clustering cells; DC, dendritic cells; HBSS, Hanks' balanced salt solution; LPS, lipopolysaccharide; MAF, macrophage activating factor(s); Mϕ, macrophage(s); PB, polymyxin B; PEC, peritoneal exudate cells; PP, proteose peptone; rIFN-γ, recombinant interferon-γ; TGC, thioglycollate medium.

0111:B4 were purchased from Difco Lab., Detroit, MI. Polymyxin B sulfate (PB, Sigma Chemical Co., St. Louis, MO) was employed to inactivate the biological activity of LPS. Anti-Ia and anti-Lyt-2 antibodies were the culture supernatants of B21-2 (anti-Ia^d) and HO-2.2 (anti-Lyt-2.2), respectively, from the American Type Culture Collection (Rockville, MA).

The tissue culture medium was RPMI-1640 (Nissui Seiyaku Co., Tokyo) supplemented with 10% fetal bovine serum (Sterile Systems Inc., Logan, UT), 2 m*M* L-glutamine, 100 mg/ml streptomycin, and 100 U/ml penicillin. The tissue culture medium and serum were screened for endotoxin contamination by means of the test with *Limulus* amebocyte lysate (Wako Pure Chemical Industries, Ltd., Tokyo) or by chromogenic endotoxin-specific assay (Endspecy; Seikagaku Kogyo Co., Ltd., Tokyo). [Methyl-^{3}H]thymidine (60 Ci/mmol, ICN Radiochemicals, Irvine, CA) was obtained through the Japan Isotope Association, Tokyo.

Tumor cells Meth A (methylcholanthrene-induced fibrosarcoma of BALB/c mice), P815 (mastocytoma of DBA/2 mice) and L1210 (B lymphoma of DBA/2 mice) were maintained by *in vitro* culture. All of the cell lines were free of mycoplasma.

Cell preparations Mϕ: Peritoneal exudate cells (PEC) were collected by peritoneal lavage of mice which had received an intraperitoneal injection of 2 ml of 10% PP 3 days previously or TGC 4 days previously. PEC were plated onto 24- or 96-well flat-bottomed tissue culture plates (A/S Nunc, Kamstrup, Roskilde) and incubated for 2 h at 37°C. Then, nonadherent cells were removed by washing with Hanks' balanced salt solution (HBSS; Nissui) to prepare the Mϕ monolayer.

DC: DC were prepared from low-density adherent spleen cells according to the methods of Nussenzweig and Steinman.[10]

T cells: Spleens and popliteal lymph nodes were obtained from BALB/c mice which had been inoculated with $5–10\times10^5$ Meth A cells into the footpads 6 days before, or spleens were obtained from mice which survived for more than 30 days after the intraperioneal transplantation of 10^6 Meth A cells and therapeutic treatment with human rIFN-α A/D.[6] T cells were prepared by passing cell suspensions of spleens and/or lymph nodes through a nylon wool column and depleting Ia-bearing cells by treatment with anti-Ia antibody and rabbit complement (Cederlane Lab. Ltd., Hornby, Ontario). In some experiments, T cells were also depleted of Lyt-2^+ cells.

Isolation of clusters and T blasts, and preparation of CLSN Five million T cells thus prepared were cultured with 5×10^4 DC and 10^5 Meth A, inactivated by ultraviolet irradiation with a germicidal lamp, in 1 ml of medium in a 24-well culture plate. After 2 days, cell clusters were isolated by Percoll (Pharmacia Fine Chemicals, Uppsala) gradient centrifugation as described.[8] T blasts were separated from residual clusters by the same procedure 2 days after the beginning of culture of the clusters, the supernatant of which served as CLSN. CLSN was filtered and stored at −80°C until use. Blasts in the cluster were also obtained as described.[7] In order to prepare Lyt-2^- T blasts, the isolated T blasts were treated with anti-Lyt-2.2 antibody and complement.

Winn assay Inhibition of tumor growth under *in vivo* conditions was assessed by a Winn test[11] in the right hind footpads of BALB/c mice. Meth A (5×10^5) mixed with or without various types of cells (described in "Results") were inoculated into mice in a volume of 25 μl. The thickness of the footpad was measured every day or every 2 days with a dial caliper.

Tumor growth inhibition *in vitro* L1210 (2×10^4), P815 (2×10^4), or Meth A (3×10^4) cells were cultured on an Mϕ monolayer in 24-well plates in the presence or absence of CLSN for 48 h, and the number of viable tumor cells was determined by means of the trypan blue dye exclusion test. LPS and/or rIFN-γ were also used as controls for Mϕ activation. In Mϕ-preactivation experiments, Mϕ were precultured with CLSN or these activators for 8 h, washed, and cultured with tumor cells for an additional 48 h. In some experiments, T blasts prepared as described above were pretreated with mitomycin C (Kyowa Hakko, Co., Ltd., Tokyo) to block their proliferation and added to the culture of Mϕ and tumor cells.

The percentage growth of tumor cells was calculated as; (number of tumor cells cultured with Mϕ)/(number of tumor cells without Mϕ)$\times$100.

Cytolytic activity of Mϕ against tumor cells *in vitro* Mϕ monolayers in 96-well flat-bottomed plates (10^5 Mϕ/well) were pretreated for 8 h with graded concentrations of CLSN, 10 ng/ml LPS or 10 IU/ml rIFN-γ, or their combinations, and washed twice with HBSS prior to the addition of target tumor cells. In the two-step activation procedure, Mϕ were treated with the first and the second agents for 4 h each. The target cells were P815, 10^6 of which had been cultured for 10 h in the medium containing 0.2 μCi/ml [^{3}H]thymidine at 37°C. After washing, 2×10^4 labeled P815 cells were added to a preactivated Mϕ monolayer, and cultured for 36 h in 0.2 ml of medium. The radioactivity of released [^{3}H]thymidine in the supernatant was measured with a liquid scintillation counter after centrifugation at 125*g* for 5 min.

Tumor cytolysis was calculated as specific [^{3}H]-thymidine release according to the following formula:
% Specific [^{3}H]thymidine release=[(experimental cpm −spontaneous cpm)/(maximum cpm − spontaneous cpm)]$\times$100. Experimental cpm was cpm released in wells containing target cells and Mϕ; spontaneous cpm

was cpm released from target cells in the absence of Mϕ; maximum cpm was radioactivity of the lysate of target cells treated with 1% v/v sodium dodecyl sulfate. Spontaneous cpm was less than 15% of the maximum cpm. Results are given as the mean of triplicate assays.

RESULTS

Effectiveness of clustering cells in inhibiting Meth A growth *in vivo* We attempted to enrich Meth A-specific T cells by the isolation of clustering T cells with DC and Meth A. T cells were prepared from spleens of mice cured of cancer by treatment with human rIFN-α A/D. The culture of T cells, DC and Meth A cells proceeded for 2 days, and cell clusters were cutured for another 2 days, and dispersed to form a cell suspension. These cells (10^6) and Meth A cells (5×10^5) were mixed and injected into right hind footpads. Nonclustering cells or unfractionated spleen cells were used as controls in place of the clustering cells. As shown in Fig. 1A, clustering cells were very effective in inhibiting the growth of tumor cells in the living tissue. In contrast, unfractionated spleen cells were not efficacious, and nonclustering cells were rather slightly augmentative for the tumor growth. T blast cells and their subset in the cell cluster responsible for tumor growth inhibition were tested. Lyt-2$^-$ T blasts were prepared from clustering cells, and 10^6 Lyt-2$^-$ blasts or clustering cells with 5×10^5 Meth A were inoculated into footpads (Fig. 1B). The recovery of Lyt-2$^-$ blasts was 46% of total clustering cells. In comparison with unfractionated clustering cells, Lyt-2$^-$ blasts exerted a prominent inhibitory effect on the Meth A growth.

T cells prepared from spleens and popliteal lymph nodes of Meth A-bearing mice in an early stage (6 days after footpad inoculation) were also examined (Fig. 2). T blasts were obtained from the clusters formed in the culture of Lyt-2$^-$ T cells with DC and Meth A cells. Meth A cells (10^6) alone or with 10^6 T blasts or with nonclustering cells (2×10^6) were inoculated into footpads. As shown in Fig. 2A, the Lyt-2$^-$ T blasts significantly inhibited the tumor growth, although they were not as effective as those from mice cured of cancer by treatment with human rIFN-α A/D (Fig. 1). Similar results were also obtained in athymic nude mice, indicating that the collaboration of host T cells with adoptively inoculated cells was not necessary (Fig. 2B).

These results indicate that the isolation of clustering cells is an efficient procedure for enrichment of Meth A-specific T cells and that T cells of Lyt-2$^-$ phenotype are primarily responsible for tumor cell growth inhibition. It was also shown that tumor-specific T cells could be obtained from not only tumor-surviving mice but also tumor-bearing mice. So we usually used T cells from tumor-bearing mice in the experiments below.

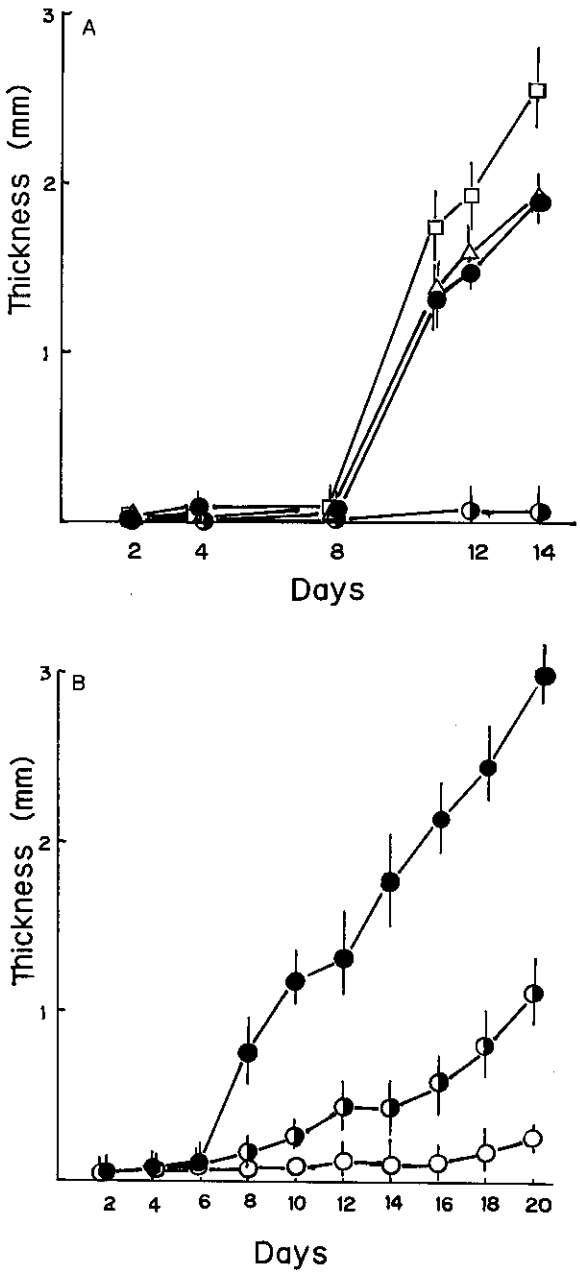

Fig. 1. Inhibition of tumor growth by immune T cells in the living tissue. The effects of clustering cells (A, B) and Lyt-2$^-$ blasts (B) are shown. Fresh Meth A cells (5×10^5) were inoculated into footpads of BALB/c mice with different preparations of lymphoid cells obtained from BALB/c mice cured of cancer (Meth A) by treatment with human rIFN-α A/D: ●, nothing; □, 10^6 nonclustering cells; △, 10^6 unfractionated spleen cells; ◑, 10^6 clustering cells; ○, 10^6 Lyt-2$^-$ blasts prepared from clustering cells. Thickness means the difference from footpad thickness before tumor inoculation. Each symbol and vertical bar represent the mean of 5 to 10 mice and the standard errors, respectively.

Collaboration of Lyt-2$^-$ blasts with Mϕ to inhibit *in vitro* tumor cell growth The effect of Lyt-2$^-$ T blast cells on *in vitro* tumor cell (Meth A and P815) growth was examined in the culture with or without either syngeneic or allogeneic Mϕ (Table I). T blast cells did not exert a suppressive effect on Meth A proliferation by themselves, but caused the inhibition of tumor cell division in the coculture with syngeneic, but not allogeneic, Mϕ. In contrast, the proliferation of P815 cells was not affected by any combination of Lyt-2$^-$ T blast cells and Mϕ.

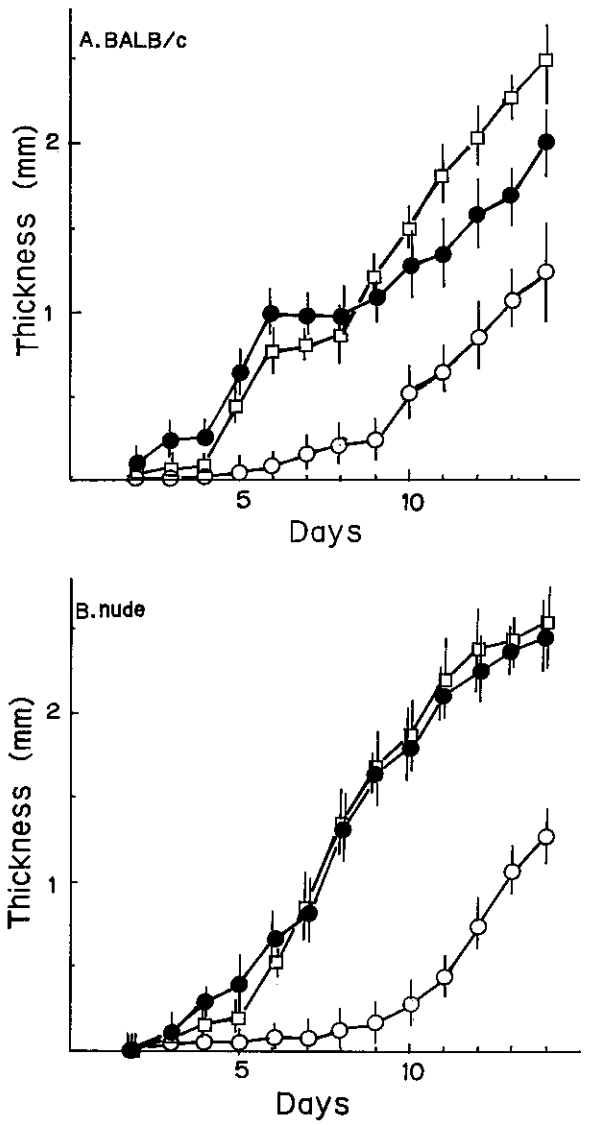

Fig. 2. Inhibition of tumor growth by immune T cells in the living tissue. Fresh Meth A cells (10^6) were inoculated into footpads of BALB/c (A) or BALB/c *nu/nu* (B) mice with different preparations of lymphoid cells, obtained from BALB/c mice inoculated with Meth A cells 6 days previously: ●, nothing; ○, 10^6 Lyt-2$^-$ blasts prepared from clustering cells; □, 2×10^6 nonclustering cells. Thickness means the difference from footpad thickness before tumor inoculation. Each symbol and vertical bar represent the mean of 5 to 10 mice and the standard errors, respectively.

These results reveal that the effect of Lyt-2$^-$ blast cells is antigen-specific and their collaboration with Mϕ is genetically restricted. It also seems likely that the actual effector is Mϕ, which are activated by the interaction with antigen-specific T blast cells. The results in Fig. 2B support this interpretation.

MAF activity of CLSN in tumor cytostasis Inaba and Steinman showed that antigen-specific Lyt-2$^-$ T blasts secrete various kinds of soluble factors as a result of antigen recognition in an MHC-restricted manner.[7] This and the preceding results in Table I prompted us to consider that Mϕ function as effectors against tumor cells may be induced by factors secreted from T blasts. In order to confirm this, we investigated the Mϕ-activating activity of culture CLSN in an *in vitro* tumor cell system. Tumor cells were cultured in the medium containing various concentrations of CLSN in the presence or absence of Mϕ. Only a high concentration (10%) of CLSN was observed to exhibit a slight inhibitory effect in the absence of Mϕ. In the culture with Mϕ, the growth of three different kinds of tumor cells was suppressed in a CLSN-dose-dependent manner, and Mϕ either syngeneic or allogeneic to the T cells producing CLSN showed similar suppressive activity toward the tumors (Table II). These results suggest that CLSN activate Mϕ without genetic restriction and that the action of activated Mϕ is effective on various types of tumors.

Induction of tumoricidal activity of Mϕ by CLSN Mϕ were pretreated with graded concentrations of CLSN with or without 10 U/ml rIFN-γ or 10 ng/ml LPS for 8 h and cultured with [^{3}H]thymidine-labeled P815 target cells for 36 h. A high concentration of CLSN activated Mϕ without the aid of other agents, whereas a lower concentration (1%) of CLSN by itself was incapable of activating Mϕ, but it collaborated with IFN-γ and LPS to render Mϕ tumoricidal (Fig. 3). Although neither IFN-γ nor LPS was effective at the concentaration used in this experiment, they cooperated with each other in Mϕ activation. These results show that CLSN contains one or more factors that synergize with IFN-γ and with LPS for Mϕ activation.

IFN-γ is critical for MAF activity of CLSN IFN-γ is known to be a product of activated T cells and is one of the MAFs.[12] Therefore, we investigated the contribution of IFN-γ to MAF activity of CLSN. Eventually, 75 U/ml of IFN-γ was detected in CLSN employed in the experiment of Fig. 4A by anti-viral assay and only IFN-γ was detected by neutralization with different anti-IFN antibodies (not shown).

Table I. Inhibition of Meth A Growth by Mϕ and Tumor-specific T Blasts

Cells added to culture			Growth or tumor cells[d] (%)				
			Number or T cells				
Tumor[a]	T cells[b]	Mϕ[c]	10^6	3×10^5	10^5	3×10^4	none
Meth A	Lyt-2⁻ blasts[e]	—	nt	94.0	109.0	nt	100.0
		BALB/c	nt	39.0	47.1	63.1	101.2
		C3H/He	nt	91.0	99.1	108.1	96.1
	Noncluster[f]	—	96.0	106.0	nt	nt	nt
		BALB/c	89.0	110.1	104.2	nt	nt
		C3H/He	96.0	104.2	108.1	nt	nt
P815	Lyt-2⁻ blasts	—	95.1	100.0	nt	nt	100.0
		BALB/c	90.9	95.8	109.8	nt	103.7
		C3H/He	97.5	101.5	104.9	nt	102.5
	Noncluster	—	96.0	106.0	nt	nt	nt
		BALB/c	101.5	110.6	108.8	nt	nt
		C3H/He	97.5	104.9	107.4	nt	nt

a) 3×10^4 Meth A and 2×10^4 P815.
b) T cells were prepared from BALB/c mice.
c) 3×10^5 TGC-Mϕ in 16-mm-diameter well in a volume of 1 ml of medium.
d) The number of viable tumor cells was counted after 48 h. Numbers represent the arithmetic mean of triplicate assays. nt, not tested.
e) Obtained from clustering cells of Lyt-2⁻ T cells, DC and Meth A.
f) Nonclustering cells left in culture another 2 days after separation of clusters of Lyt-2⁻ T cells, DC and Meth A at day 2.

Table II. Inhibition of Tumor Cell Growth by Mϕ Activated with CLSN[a]

Exp.	Cells in culture		Growth of tumor cells (%)				
			Dose of CLSN[d] (%)				
	Tumor[b]	Mϕ[c]	10	3	1	0.3	none
A	P815	none	89.1	98.4	101.5	100.0	100.0
		BALB/c	10.7	15.5	25.5	48.1	105.7
		C3H/He	11.1	14.3	25.6	53.2	103.5
	L1210	none	89.6	101.2	99.7	103.0	100.0
		BALB/c	9.6	13.2	19.8	46.5	98.5
		C3H/He	10.6	10.9	20.5	50.3	97.7
	Meth A	none					100.0
		BALB/c	24.1	29.1	49.1	90.3	101.2
		C3H/He	21.0	29.1	47.8	91.6	96.1
B	P815	none					100.0
		BALB/c	22.1	23.8	41.9	80.0	103.7
		C3H/He	19.6	22.1	48.7	85.6	102.5
	Meth A	none					100.0
		BALB/c	24.9	39.1	69.1	98.3	101.2
		C3H/He	21.0	39.1	67.8	101.6	101.0

a) T cells used to prepare CLSN were from BALB/c mice.
b) 2×10^4 P815 and L1210, and 3×10^4 Meth A.
c) 3×10^5 TGC-Mϕ.
d) Tumor cells were cultured on Mϕ monolayer in the presence of graded doses of CLSN for 48 h and viable tumor cells were counted by trypan blue dye exclusion. Numbers represent the arithmetic mean of triplicate assays.

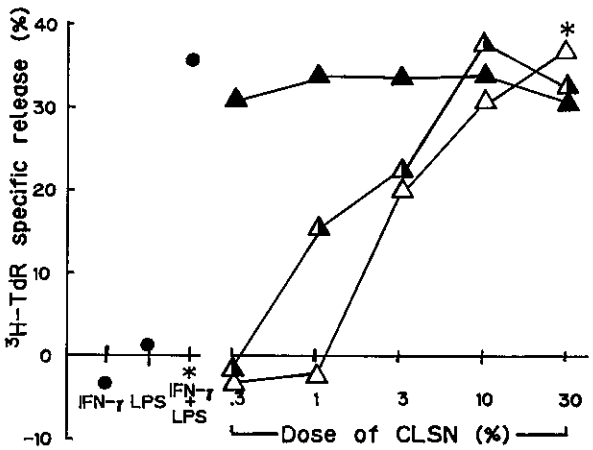

Fig. 3. Induction of tumoricidal activity of Mϕ by CLSN. PP-induced Mϕ (10^5) of C3H/He mice were pretreated with graded concentrations of CLSN alone (△), CLSN plus 10 U/ml rIFN-γ (▲), or CLSN plus 10 ng/ml LPS (▲) for 8 h, washed, and tested for cytotoxicity to [^{3}H]thymidine-labeled P815 cells. As controls (●), the results of pretreatment with 10 U/ml rIFN-γ or 10 ng/ml LPS alone, or their combinations are indicated. The effects of pretreatment of LPS or CLSN with PB (40 mg/ml) are also indicated (*). Each symbol represents the arithmetic mean of triplicate cultures.

Reduction of IFN-γ content from CLSN to less than 8 U/ml by passage through an anti-IFN-γ affinity column resulted in a marked decrease of MAF activity of CLSN. The addition of rIFN-γ back to the effluent, however, restored the activity (Fig. 4A). Likewise, the preincubation of CLSN with anti-IFN-γ antibody, which neutralized 20 U/ml IFN-γ, resulted in the abolition of MAF activity especially at low concentrations of CLSN (Fig. 4B). These results reveal that IFN-γ is critical for MAF activity in CLSN.

It is possible that the MAF activity of our CLSN is ascribable to the cooperation of IFN-γ with LPS which might contaminate CLSN and/or the culture medium. To check this possibility, CLSN was incubated with PB to neutralize LPS prior to use for Mϕ activation. The dose of PB (40 mg/ml) was not toxic and was sufficient for neutralizing 10 ng/ml LPS. In addition, our CLSN contains less than 0.1 ng/ml of LPS as determined by the test with *Limulus* amebocyte lysate. MAF activity in CLSN was not counteracted by PB, which blocked the cooperative activity of rIFN-γ and LPS (Fig. 3), indicating that MAF activity of CLSN cannot be attributed to contamination by LPS.

Cooperation of IFN-γ with additional factor(s) in CLSN is involved in Mϕ activation In the Mϕ activation with IFN-γ and LPS, IFN-γ acts as a primary signal and LPS as a secondary signal, while the same treatment in the

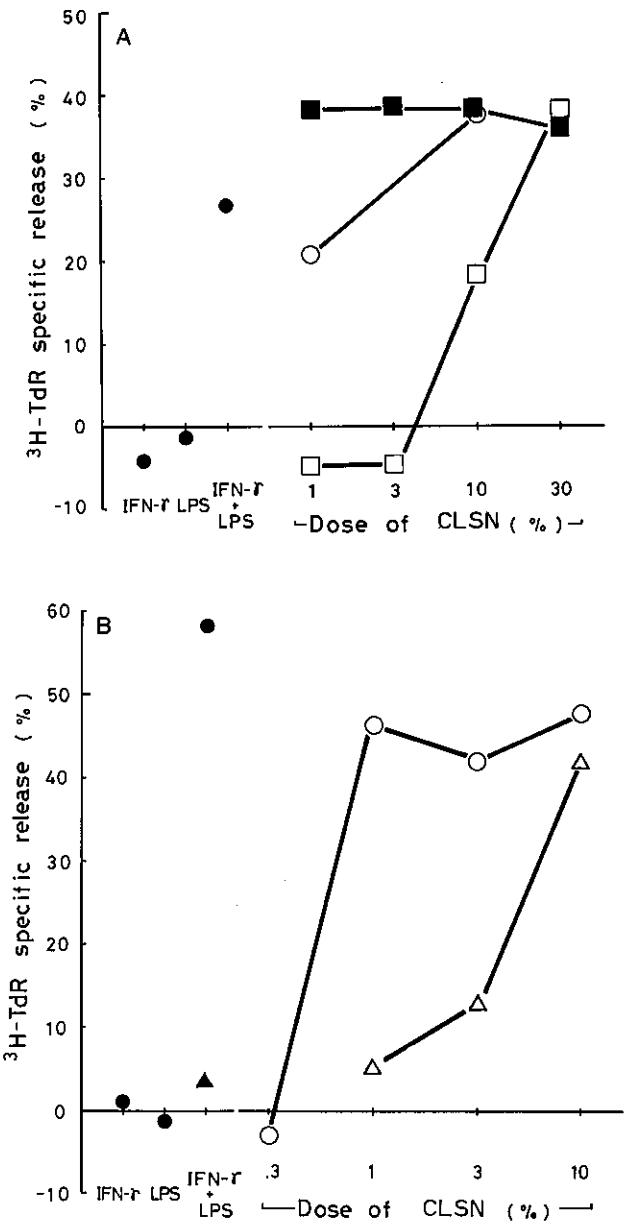

Fig. 4. The effects of reduction of IFN-γ content from CLSN by passage through an anti-IFN-γ affinity column (A) or by pretreatment with anti-IFN-γ antibody (B) on MAF activity. PP-induced Mϕ (10^5) of C3H/He mice were pretreated with graded doses of CLSN (○) or with affinity column effluent of CLSN (□) or with 10 U/ml rIFN-γ plus effluent (■) or with anti-IFN-γ antibody pretreated CLSN (△) or IFN-γ plus LPS (▲) for 8 h, washed, and tested for cytotoxicity to [^{3}H]-thymidine-labeled P815 cells. As controls (●), the results of pretreatment with 10 U/ml rIFN-γ or 10 ng/ml LPS alone, or their combination are indicated. Each symbol represents the arithmetic mean of triplicate cultures.

Table III. MAF Activity of CLSN Is Manifested by the Cooperation of IFN-γ with an Additional Factor Analogous to LPS

Mϕ preincubated with[a]		Cytotoxicity (%)[b] specific [^{3}H]thymidine release
first step	second step	
CLSN	CLSN	41.6
CLSN	medium	−10.6
CLSN	LPS	67.7
CLSN (anti-IFN-γ)	LPS	−11.1
medium	CLSN	−9.5
rIFN-γ	CLSN	54.2
rIFN-γ	CLSN (anti-IFN-γ)	43.3
rIFN-γ	LPS	59.5
CLSN		55.1
CLSN (anti-IFN-γ)		−4.8

a) 10^5 PP-Mϕ in a 96-well plate were preincubated with the first agent and the second agent for 4 h each. CLSN, LPS, and rIFN-γ were used at 10%, 10 ng/ml, and 10 U/ml, respectively. Neutralization of IFN-γ in CLSN was performed by preincubation with rabbit anti-murine IFN-γ antiserum prior to the experiment.
b) After culture of ^{3}H-labeled targets on a Mϕ monolayer for 36 h, isotope released into the culture medium was measured. Numbers represent the mean of triplicate assays.

Table IV. Cooperative Activity of a Factor Distinct from IFN-γ with LPS in Mϕ Activation

Dose (%)	Mϕ pretreated with neutralization of IFN-γ in CLSN[a]	LPS (10 ng/ml)	Cytotoxicity (%)[b] specific [^{3}H]thymidine release
10	−	−	38.9
	−	+	74.3
	+	−	−3.9
	+	+	18.7
30	−	−	72.5
	−	+	67.5
	+	−	48.2
	+	+	69.8

a) Rabbit anti-murine IFN-γ would neutralize 100 U/ml rIFN-γ.
b) Isotope released from ^{3}H-labeled targets into the culture medium was measured. Numbers represent the mean of triplicate assays.

reversed sequence is ineffective.[13] We attempted to clarify the presence of LPS-analogous factor(s) in CLSN by treating Mϕ with agents in two separate steps for 4 h each. CLSN used in this experiment was supplemented with PB to neutralize LPS, if present. The concentration of CLSN was chosen to be minimum but sufficient for Mϕ activation and for the abrogation of MAF activity by anti-IFN-γ in a single-step activation procedure. CLSN was incapable of activating Mϕ by preincubation for 4 h in either the first or the second step, but could provide both primary and secondary signals in the combined treatment with LPS as a secondary signal and IFN-γ as a primary signal, respectively (Table III). When CLSN was pretreated with anti-IFN-γ antiserum, its activity as a primary signal was ablated, but that as a secondary signal was not.

Factor distinct from IFN-γ in CLSN induces Mϕ activation and augments it in the combination with LPS The MAF activity of a high dose of CLSN was tested again after neutralizing IFN-γ with specific antibody (Table IV). The amount of anti-IFN-γ antibody used in this experiment was sufficient to block the MAF activity of 100 U/ml IFN-γ plus 10 ng/ml LPS. MAF activity of 10% CLSN was abrogated by anti-IFN-γ antibody, whereas that of 30% CLSN was reduced but was still significant. Furthermore, abolished or diminished MAF activity of CLSN was restored by the supplementation with LPS. These results confirm that a factor distinct from IFN-γ in CLSN activates Mϕ without the aid of IFN-γ and also suggest that it cooperates with LPS for activation of Mϕ.

DISCUSSION

Several categories of cells are known to participate in the host defense against tumors. In this study, we investigated the interaction between Mϕ and T cells in tumor-bearing hosts. For this purpose, we attempted to enrich tumor-specific activated T cells (T blasts). Based on the fact that DC are the most potent A cells in T cell-dependent immune responses and form discrete clusters with antigen-specific T cells in the presence of homologous antigen, we isolated clustering cells or T blasts released from clusters during *in vitro* culture. Clustering cells and Lyt-2$^-$ T blasts from tumor-surviving mice more effectively inhibited *in vivo* tumor growth than non-clustering cells and unfractionated spleen cells (Fig. 1A and B). We obtained essentially similar results in the experiments using cells from tumor-bearing mice (Fig. 2A), and this was also the case when nude mice were used as recipients (Fig. 2B). These results reveal that isolation of clustering cells from either tumor-surviving or tumor-bearing mice is an efficient procedure to enrich tumor-specific T cell and also indicate that tumor-specific activated T cells exert an antitumor effect *in vivo*.

Lyt-2$^-$ blasts specific for Meth A by themselves did not affect the growth of Meth A *in vitro* (Table I). Antitumor effect of the blasts was disclosed for only Meth

A, when cultured in the presence of BALB/c Mϕ but not C3H Mϕ (Table I). Inaba and Steinman have demonstrated that antigen-specific Lyt-2$^-$ blasts can be reactivated to proliferate and secrete lymphokines by syngeneic A cells, even if Mϕ, with homologous antigen.[7] Based on this, we interpret the results as showing that syngeneic Mϕ as A cells in the presence of Meth A restimulate the blasts, and such blasts activate Mϕ to effectors. The Mϕ activation is mediated by some factors derived from tumor-specific T cells with Lyt-2$^-$ phenotype (Table II), in agreement with the results of Fujiwara *et al.*[4,5] We have no evidence, but it is also feasible that antigen-presenting Mϕ are activated by cell-to-cell interaction with Lyt-2$^-$ T blasts as in the case of B cells.

It is known that Mϕ activated by T cell-derived natural lymphokines or the combination of rIFN-γ and LPS are able to exhibit cytocidal activity against tumors.[14] Our CLSN by itself was capable of activating Mϕ at high concentrations, but the MAF activity disappeared at low concentrations. Supplementation of the dilute CLSN with either rIFN-γ or LPS, at concentrations which were insufficient to serve as autonomous MAF, resulted in the recovery of MAF activity of CLSN (Fig. 3). This raises two possibilities, that CLSN contains a single factor that synergizes with both IFN-γ and LPS, or that CLSN possesses at least two different factors, of which one cooperates with IFN-γ and the other with LPS. The results shown in Fig. 4 clearly indicate that the latter possibility is the case. The activity of CLSN was agrogated by treatment with anti-IFN-γ (Fig. 4B) or by passage through an anti-IFN-γ affinity column (Fig. 4A), revealing that IFN-γ is critical for the MAF activity. IFN-γ in CLSN serves as the first or priming signal and synergizes with LPS, which serves as the second or triggering signal (Table III). However, there also seems to be another unidentified factor cooperating with LPS (Fig. 3 and Table IV).

So, what factor does synergize with IFN-γ? This factor remains in the CLSN treated with anti-IFN-γ, and triggers the Mϕ primed with IFN-γ (Table III). The possibility that LPS contaminating CLSN may contribute to the MAF activity seems implausible, since the LPS content of our CLSN was less than 0.1 ng/ml, which is insufficient for synergizing with IFN-γ, and since the pretreatment of CLSN with PB at a concentration which is effective for neutralizing 10 ng/ml of LPS exerted no effect on the MAF activity. Thus, we conclude that the MAF activity of CLSN is attributable to the synergism between IFN-γ and another unidentified factor(s).

Our present results coincide with those of several investigators who have documented the existence of a T-cell-derived factor which synergizes with IFN-γ, like LPS, to display MAF activity.[15,16] To date, many papers have presented results suggesting that other cytokines than IFN-γ, such as IL-1, IL-2, IL-4 (BSF-1), TNF-α, TNF-β, GM-CSF, or CSF-1, participate in the Mϕ activation.[17-22] Our CLSN actually contains considerable amounts of IL-2, IL-4, and GM-CSF in addition to IFN-γ (not shown). We are therefore convinced that T cell-derived lymphokines act on Mϕ independently or in cooperation with each other in natural host antitumor responses. Identification of the non-IFN-γ factor(s) in CLSN should contribute to a better understanding of the Mϕ activation mechanism in the immune response against tumors *in vivo*.

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