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Augmentation of Human Cytotoxic T Lymphocytes against Autologous Tumor by a Factor Released from Human Monocytic Leukemia Cell Line

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A human acute monocytic leukemia cell line, THP-1, releases a factor which activates human cytotoxic (killer) T lymphocytes (CTL) against autologous tumor *in vitro*. The factor, named cytotoxic (killer) T cell activating factor (KAF), is an acidic protein of 70,000 to 100,000 dalton molecular size. Peripheral blood leukocytes from two patients, bearing epithelioid sarcoma or malignant schwannoma, were cultured for 7 days with individual autologous tumor to induce CTL directed to the corresponding tumor. Monocyte-depleted peripheral leukocytes generated lesser CTL activity than the monocyte-containing leukocyte population. However, the KAF was able to replace the monocyte function. The KAF acted at the CTL generation phase as well as the effector phase. The KAF-activated killer cells possessed $CD4^-8^+$ surface phenotype. The CTL killed autologous tumor or other unrelated tumor cell lines only when they shared some of the HLA class I antigens. It was also demonstrated that the KAF does not activate killer cells without proper antigenic stimuli, because the KAF-augmented CTL possess specificity against autologous tumor or other HLA-A or -B matched tumor cell lines. The therapeutic applicability of human KAF for anti-tumor CTL therapy against autologous tumor is discussed.

Key words: Cytotoxic T lymphocytes — Tumor immunity — Tumor immunotherapy — Soluble mediator — Cytokine

Cytotoxic T lymphocytes (CTL) specific for syngeneic tumor are one of the effector cell populations for both establishing and maintaining anti-tumor immunity in the animal. During the last decade, extensive investigations have revealed that the generation of cell-mediated immune responses is regulated by both helper and suppressor T lymphocytes specific for the given antigens.[1] However, the precise mechanisms in regulating cellular immunity especially against autologous tumors, are not yet fully understood.[2,3]

In our previous studies we have observed that the murine CTL directed to syngeneic sarcoma S1509a were not induced after depletion of the monocyte population from the immune splenocyte population at the *in vitro* CTL generation phase.[4] The activity was recovered by the addition of radioresistant splenic or peritoneal adherent cells from normal mice. Interestingly, the adherent cell function was completely replaced by culture supernatant of either peritoneal exudate cells (PEC) or of murine macrophage tumor cell line P388D1. The soluble factor, termed cytotoxic (killer) T cell activating factor (KAF), released from P388D1 is a protein with a molecular size of 60,000 to 70,000 daltons. The KAF has no known cytokine activity such as interleukin-1 or interferon.[5]

In the present report we describe the human cytokine homologous to murine KAF which was obtained and partially purified from human monocytic leukemia cell line THP-1. In order to elucidate the biological implications of the activity, the human KAF was added at the CTL generation phase against autologous tumor, with lymphocytes obtained from the tumor-bearing patient. The human KAF derived from THP-1 augmented the CTL generation against the tumor.

Abbreviations used in this paper: CTL, cytotoxic T lymphocyte(s); DMEM, Dulbecco's modified Eagle's medium; KAF, killer activating factor; KAF^m, murine KAF; KAF^h, human KAF, PBL, peripheral blood leukocyte; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells.

MATERIALS AND METHODS

Animals Specific pathogen-free female A/J mice and Sprague-Dawley rats, aged 5 to 6 weeks and 12 weeks, respectively, were purchased from Shizuoka Agricultural Cooperative Association, Shizuoka.

Human materials Case 1: A skin tumor biopsy specimen of 1 cm diameter from a 29-year-old male patient, who was pathologically diagnosed as having epithelioid sarcoma, was taken aseptically. The tumor mass was minced in Ca^{2+},Mg^{2+}-free phosphate-buffered (10 m*M*, pH7.2) saline (PBS) and was digested for 4 h with 0.2% (w/v) collagenase (Seikagaku Kogyo Co., Ltd., Tokyo) and 0.1% (w/v) deoxyribonuclease (Sigma Chemicals Co., Ltd., St. Louis, USA) at ambient temperature. The single cell suspension was collected and washed twice with PBS.

The tumor cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4% pooled human cord serum and antibiotics. The culture was maintained in 25 cm^2 plastic flasks (Nunc, Roskilde, Denmark) at 37°C in a humidified incubator (5% CO_2 and 95% air). The human epithelioid sarcoma line (ES020488) was propagated after a one-month culture period. Case 2: A biopsy specimen of a locally recurrent tumor was taken from a 22-year-old female patient. The tumor was pathologically diagnosed as a malignant schwannoma by the Department of Pathology of our Medical School. The tumor cell line (MS122387) was established according to the methods used for case 1. Other human tumor cell lines, i.e. two ovarian cancers (OC092987, OC070988), neuroblastoma (NB102287), malignant melanoma (MM042888), breast cancer (BC-101287), lung cancer (LC071488), gastric cancer (GC-022588) and osteosarcoma (OS062488) were also established in our laboratory. Case 1 and 2 patients' peripheral blood leukocytes (PBL) were purified from heparinized blood by regular Ficoll-Conray gradient centrifugation. The case 1 patient had multiple subcutaneous tumor nodules systemically at the time when PBL were collected. The case 2 patient had a metastatic tumor nodule in lung when PBL were collected. The HLA typing of the various tumor donor patients was performed by standard methods using typing trays of National Sakura Hospital, Central Kidney Transplantation Center, Sakura, Chiba.

Established tumor cell lines Murine macrophage tumor cell line P388D1[6] (DBA/2 origin) was supplied by the Japanese Cancer Research Resources Bank, Tokyo. Human acute monocytic leukemia cell line THP-1[7] was the gift of Dr. K. Kumagai, Tohoku University, School of Dentistry, Sendai. Spontaneous lymphoma L1117 (A/J mouse origin) and methylcholanthrene-induced sarcoma S1509a (A/J mouse origin) were the gifts of Dr. A. H. Sehon, University of Manitoba, Faculty of Medicine, Winnipeg, Canada. All tumor cell lines were maintained stationarily in RPMI1640 medium containing 4% fetal bovine serum and antibiotics.

Killer T cell activating factor Cytotoxic (killer) T lymphocyte activating factors (KAF) from either mouse or human were collected from P388D1 or THP-1 culture supernatant, respectively. Both P388D1 or THP-1 cell lines maintained confluently in fetal bovine serum-containing medium were harvested and washed twice with Hanks' balanced salt solution and resuspended in serum-free DMEM. The cells were cultured sub-confluently for 72 h in a CO_2-incubator and the culture supernatants were collected. The supernatants were concentrated about one hundred times by using a Pellicon Labo Cassette system (Nihon Millipore Ltd., Tokyo) equipped with a PT filter (Nihon Millipore Ltd.) having a molecular size cut-off of 10,000 daltons. The factors were then partially purified by a gel block electrophoresis (agar was used instead of starch)[8] and a high-performance liquid chromatography system equipped with gel filtration column (TSK G3000SW, Toyo Soda Manufacturing Co. Ltd., Tokyo). Blue Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden) affinity adsorbent was used to separate protein having an albumin nature. Protein determination was made by Bradford's dye-binding assay[9] using bovine serum albumin as a standard protein.

Murine CTL line Concanavalin A-stimulated conditioned medium was prepared using rat splenocytes by the method of Schreier and Tees.[10] DMEM supplemented with 10% (v/v) fetal bovine serum, 5×10^{-5} *M* 2-mercaptoethanol, 100 μg/ml kanamycin, 2 m*M* L-glutamine and 20 m*M* HEPES, was used as a basal medium. The conditioned medium consisting of 20% (v/v) concanavalin A-stimulated culture supernatant in the basal medium was used for the establishment and maintenance of murine CTL lines. T cell growth factor-dependent murine CTL line was propagated and maintained according to the methods described by Haas and von Boehmer[11] with slight modifications. In brief, S1509a tumor cell-specific CTL were induced in A/J mice by two intraperitoneal immunizations, with a two-week interval, of 1×10^6 mitomycin C-treated S1509a cells as described.[4] The spleen cells were stimulated twice *in vitro* with mitomycin C-treated S1509a cells at a 5-day interval. They were then cultured in the conditioned medium (2×10^5/well/2 ml) with 3300 R X-irradiated syngeneic feeder splenocytes at 37°C in a CO_2 incubator and fed the conditioned medium every 5 days, and growing cells were transferred to fresh feeder cells every 30 days. Four lines exhibiting cytotoxic activity (higher than 10% at 5:1 effector-to-target ratio) against S1509a were selected and maintained in the conditioned medium and feeder cells, and they were cloned by a single cell manipulation method at 90 days of culture. The four cloned lines (named TM-series) were finally established. The characteristics of these CTL lines were described in detail elsewhere[5] (Takata *et al.*, manuscript in preparation). The TM-series CTL clone-S1509a system was used for the assay system of human KAF activity contained in THP-1 culture supernatant.

Induction of human CTL Tumor-bearing patients' PBL were cultured with mitomycin C-treated autologous tumor cells at a density of 3×10^7 PBL and 1×10^5 tumor cells in 10 ml of 4% human cord serum-supplemented DMEM for 7 days in a CO_2 incubator. Monocyte population in the patients' PBL was depleted by passing the PBL through a nylon wool column[12] and then through a Sephadex G-10 column.[13] The *in vitro*-activated CTL were assayed for their cytotoxic activity by 16 h ^{51}Cr release assay as described.[14] Either CD4- or CD8-

positive T lymphocytes were depleted by the standard methods using monoclonal antibodies and rabbit complement. Monoclonal anti-human CD4 and CD8 monoclonal antibodies, OKT4 and OKT8, respectively, were the products of Ortho Diagnostics Systems Inc., Raritan, USA. Anti-CD4 and CD8 antibodies were used at the final concentration of 50 μg/ml. The low toxicity rabbit complement was prepared in our laboratory by the absorption of normal rabbit serum with mouse splenocytes.

RESULTS

Cloned murine CTL line Details of the cloned murine CTL lines which exhibit cytotoxic activity upon KAF addition were described elsewhere[5] (Takata *et al.*, manuscript submitted). In brief, the outstanding characteristics of the T cell growth factor-dependent murine CTL line were that the two lines, named TM2008 and TM4001, showed no significant cytotoxicity when they were cultured without feeder cells, but turned out to be cytotoxic upon the addition of X-irradiated splenocytes or peritoneal exudate cells as feeder cells. This result is crucial supporting evidence for our previous finding that the S1509a tumor immune splenocytes did not generate CTL without a monocytic cell population.[4]

The effect of PEC addition on the cloned murine CTL activity is shown in Table I. CTL clones, TM2008 or TM4001 showed no cytotoxicity against S1509a tumor cells in the absence of accessory cells (lines 1 and 4). However, the activity was recovered by the addition of a small number of PEC (lines 2 and 5). In order to investigate the role of the molecule released from monocytes, the culture supernatant of murine macrophage tumor cell line P388D1 was added. The addition of P388D1 culture supernatant was able to replace the PEC function which resulted in a significant cytotoxic activity in both TM-2008 and TM4001 (lines 3 and 6). The KAF activity was enriched from P388D1 culture supernatant in a 60,000 to 70,000 dalton protein fraction having a pI of 3.9 to 4.3. The KAF fraction showed neither antiviral (interferons) nor interleukin 1 (IL-1) activity. The KAF obtained from P388D1 is termed KAF^m (murine KAF) hereafter.

Table I. Monocyte- or KAF-dependent Killing by Cloned CTL Line against S1509a Tumor

CTL line[a]	Additional cells or factor[b]	% Specific lysis against S1509a (E:T=20:1)
TM2008	None	0.1±0.3
	Mouse PEC (2.5×10^6)	48.3±3.0
	P388D1 sup.	42.5±2.2
TM4001	None	2.1±0.5
	Mouse PEC (2.5×10^6)	54.7±4.1
	P388D1 sup.	59.6±2.3
TM4001	None	0.5±0.2
	THP-1 sup.	39.3±1.5

a) 0.2×10^6 CTL clones and 10^4 ^{51}Cr-labeled target S1509a tumor cells were mixed and % ^{51}Cr release was measured 16 h later. Surface phenotypes of the clones were: TM2008, Thy-1$^+$, Lyt-2^{low}; TM4001, Thy-1$^+$, Lyt-2^{high}.
b) Mouse PEC were collected from normal A/J mice by flushing their peritoneal cavities with Hanks' balanced salt solution. Culture supernatant of either P388D1 or THP-1 was concentrated about 100 times by ultrafiltration. Final concentration of both supernatants was 10 times concentrated (about 200 μg protein/ml) at the CTL assay.

We extended the experimentation to investigate the KAF activity from human materials by utilizing the TM4001 and S1509a combination as an assay system. We sought for activity in the culture supernatant of human monocytic leukemia cell line THP-1 analogous to the murine P388D1 derived KAF^m. The addition of THP-1 supernatant to the TM4001-S1509a system enhanced killer cell activity significantly (Table I, line 8). The results indicate that the THP-1 culture supernatant contains a human homologue to the KAF^m. The KAF activity found in THP-1 supernatant is termed KAF^h hereafter.

Biochemical and biological characteristics of KAF^h In order to enrich and purify the KAF^h, the activity was monitored by using the TM4001-S1509a system. The serum-free THP-1 supernatant was concentrated through ultrafiltration, since the KAF^m was enriched by the same procedures. The fraction was then subjected to agar gel block electrophoresis. The KAF^h activity was found in the range between albumin and α_1-globulin fraction. The fraction was then analyzed by using high-performance liquid chromatography equipped with a gel filtration column. Figure 1 represents the elution pattern of KAF^h from the gel filtration column. The results indicate that the KAF^h is an acidic protein of 70,000 to 100,000 daltons in molecular size. The gel-filtered KAF^h fraction was then applied to a blue Sepharose CL-6B column and both passed and potassium thiocyanate-eluted fractions were dialyzed and assayed for their KAF^h activity. More than 98% of the KAF^h activity was found in the blue Sepharose CL-6B passed fraction but the potassium thiocyanate-eluted fraction contained little activity (data not shown). The blue-Sepharose passed fraction was then subjected to anion-exchange chromatography (high-performance liquid chromatography system equipped with a SynChropak AX-300 column, SynChrom, Inc., Linden, USA). KAF active fraction was concentrated and analyzed by SDS-PAGE (7.5% acrylamide) in an unreduced condition. Though the data are not shown here, the purified fraction still exhibited 3-4 bands rang-

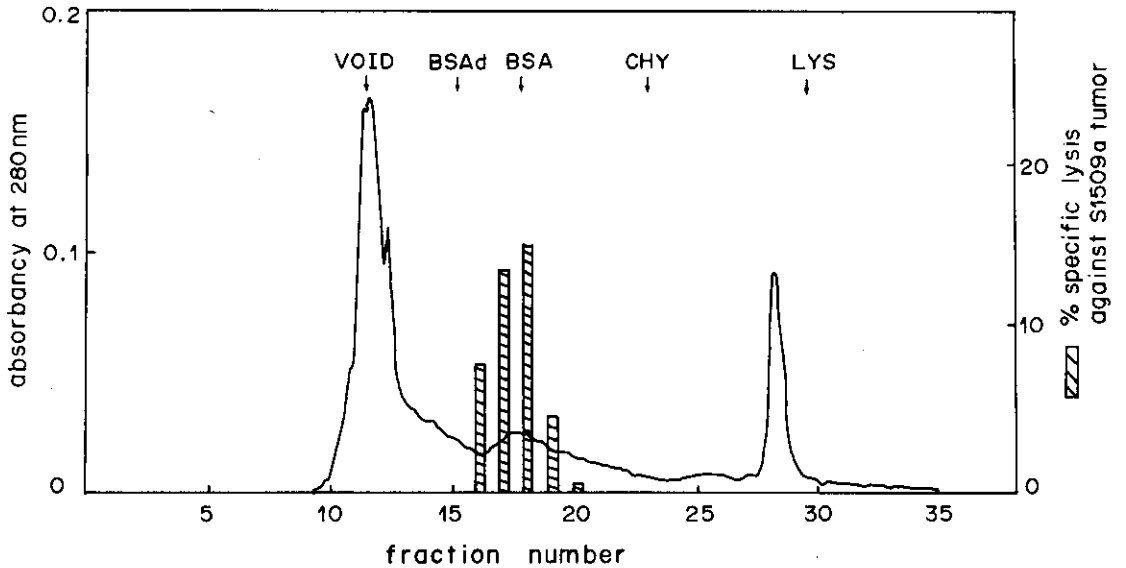

Fig. 1. Gel filtration pattern of KAF^h. Serum-free culture supernatant of THP-1 was concentrated about 100 times by ultrafiltration and then separated by agar gel block electrophoresis. The active fraction was then applied to a high-performance liquid chromatograph equipped with a gel filtration column equilibrated with PBS. The elution profile was monitored by measuring the absorbancy at 280 nm. The KAF^h activity was determined by using the TM-4001-S1509a system. Molecular size markers are arrowed: VOID (blue dextran 2000); >2,000,000, BSAd (dimeric form of bovine serum albumin); 138,000, BSA; 69,000, CHY (chymotrypsinogen); 25,700, LYS (lysozyme); 14,300.

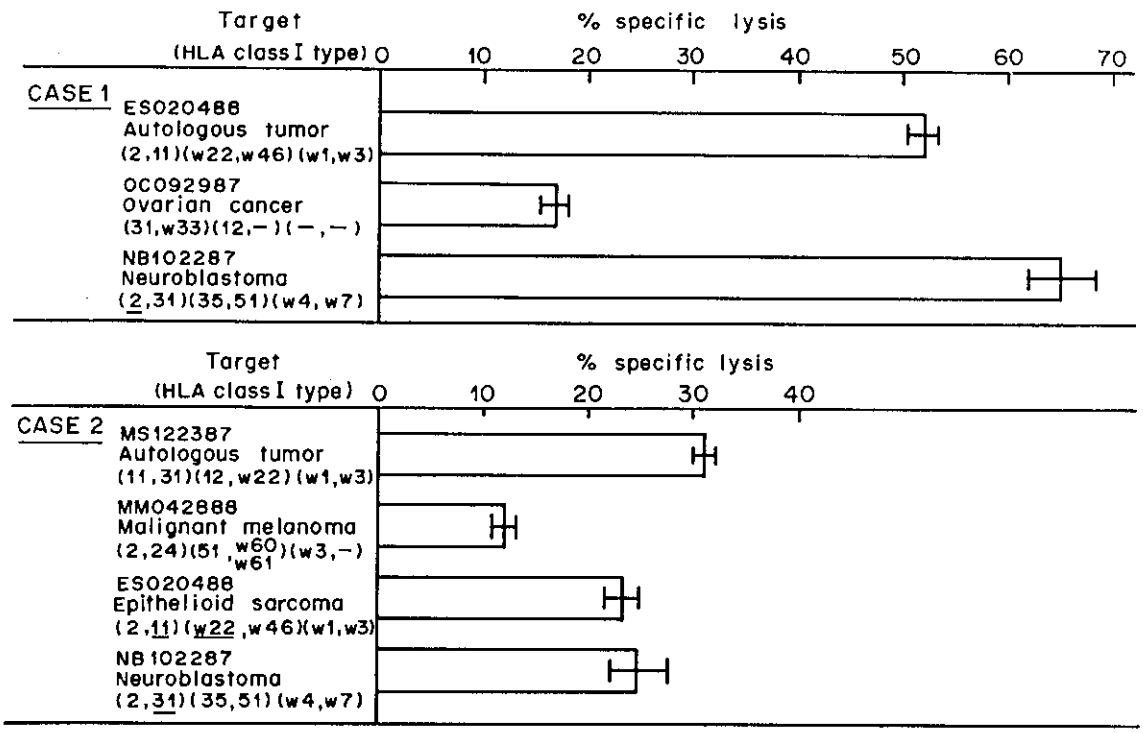

Fig. 2. Selective killing activity of autologous tumor-stimulated CTL. Either case 1 or case 2 patient's PBL were cultured for 7 days with each autologous tumor cell line at a responder: stimulator ratio of 300:1. The cytotoxic activity was measured by 16-h ^{51}Cr release assay at an effector:target ratio of 20:1.

ing from 50,000 to 100,000 daltons in molecular size. Because the KAF^h activity could not be extracted from the SDS-PAGE gel, the actual molecular size of the KAF^h is not yet determined.

The KAF^h has no known monokine activities. The IL-1 activity, which was measured by murine thymocyte proliferating activity, was separated from KAF activity into a lower molecular mass fraction (from 10,000 to 20,000 daltons) by gel filtration chromatography. The KAF^h has no antiviral (interferon) activity (kindly analyzed by Dr. B. Shimizu, Department of Microbiology, School of Medicine, Chiba University, Chiba). The monoclonal anti TNFα antibody (supplied by Asahi Chemical Industry Co. Ltd., Fuji) did not inhibit KAF^h activity, at a dose which inhibited TNFα activity.

KAF^h activity on human CTL directed to autologous tumor The utilization of CTL which have specificity to autologous tumor is a potential therapeutic strategy against human tumors. If the CTL activity is not able to be induced strongly enough *in vitro,* various manipulations to augment the activity will be required. To examine the immuno-therapeutic applicability of KAF^h, human CTL were induced *in vitro* and the effect of KAF^h on the generation of the CTL activity was investigated. Selective cytotoxic killing activities of stimulated CTL against autologous and other tumors are shown in Fig. 2. In case 1, the PBL obtained from the patient bearing epithelioid sarcoma were restimulated *in vitro* with autologous tumor (ES020488) for 7 days and the CTL activity was measured using three tumor cell lines as targets. Case 1 CTL killed autologous tumor as well as HLA-A2 matched allogeneic neuroblastoma (NB102287) but killed an unrelated tumor (ovarian cancer cell line, OC092987) less effectively. In case 2, the CTL induced *in vitro* by the autologous malignant schwannoma (MS122387) killed the tumor. CTL crossreactivity was observed against either HLA-A11 or HLA-A31 matched tumor (epithelioid sarcoma, ES020488 or NB102287, respectively) but it was lower in the mismatched tumor (malignant melanoma, MM042888). One may speculate as to whether or not there exists a killer cell-sensitivity difference among various tumor cell lines. However, since the CTL obtained from the patient bearing ovarian cancer specifically killed the autologous tumor cell line (OC092987) (data not shown) which was relatively resistant to case 1 CTL, this possibility can be excluded. Thus, these results suggest that the killing activity is closely related to HLA-A or -B product of the patients but not to the origin of tumor cells. In order to examine the effect of KAF^h on *in vitro* CTL induction, the monocytic adherent cells contained in case 1 patient's PBL were depleted by consecutively passing them through nylon wool and Sephadex G-10 columns and then restimulated *in vitro* with the autologous tumor in the presence or absence of KAF^h (gel-filtered fraction was used throughout the human CTL studies). As shown in Table II, the killing activity was significantly reduced in the absence of monocytic adherent cell population but the activity was recovered by the addition of either nylon wool adherent monocytic cell population (line 3) or KAF^h (line 4). A basically similar pattern of KAF^h effect was observed in the case of patient 2 (lines 5–7). It is worth noting that the PBL showed low but significant killing activity without *in vitro* restimulation with the tumor cells or without

Table II. Augmentation of Autologous Tumor Killing by KAF^h

Patient[a]	Autologous tumor stimulation[b]	Added	% Specific lysis against autologous tumor (E:T=20:1)
Case 1	−	−	13.8±1.0
	+	−	9.1±1.0
	+	Monocytes[c]	23.0±0.9
	+	KAF^h [d]	22.0±1.3
Case 2	−	−	29.3±1.3
	+	−	31.6±2.6
	+	KAF^h	43.8±2.9

a) PBL were obtained from: case 1, epithelioid sarcoma-bearing patient; case 2, malignant schwannoma-bearing patient. Adherent monocytic cells were depleted by consecutively passing PBL through nylon wool and Sephadex G-10 columns.
b) The ratio of PBL:autologous tumor was 300:1.
c) Nylon wool-adherent cells were used as a monocyte source. They were collected by gently teasing the nylon wool and added to the culture at a density of one-tenth of the responder cells.
d) Partially purified (gel-filtered) KAF^h preparation was used at a final concentration of 12 μg protein/ml.

monocytes. These primary killer cells might come from circulating CTL, because these two patients had received several *in vitro*-restimulated autologous PBL injections in the course of CTL therapy. The surface phenotype of the cells possessing killing activity from the patient in case 2 was analyzed by using monoclonal anti-CD4 (OKT4) or anti-CD8 (OKT8) antibody and low toxicity rabbit complement. The killing activity was meaningfully removed by treatment with anti-CD8 and complement (Table III). It is not clear whether the residual cytotoxicity is mediated by $CD8^{low}$ or $CD8^-$ cell population. On the other hand, anti-CD4 and complement treatment did not reduce the activity. Taking these results collectively, it is suggested that the killing activity is mediated by HLA-A or -B restricted CTL. It is important to note that the KAF^h do not potentiate killer cell activity without antigenic stimuli. The CTL or precursor cells might be activated by the KAF^h only when they are stimulated with appropriate tumor cells.

Table III. Surface Phenotype Analysis of Killer Cells against Autologous Tumor

Patient case 2[a]; effector cells treated with:	% Specific lysis against autologous tumor (MS122387) (E:T=20:1)
None	31.8±2.9
Complement (C) only	33.9±3.2
Anti-CD4[b]+C	31.0±2.9
Anti-CD8 +C	17.0±1.0

a) Case 2 patient's PBL were cultured for 7 days with autologous MS122387 tumor cells at a responder:stimulator ratio of 300:1. Effector cells were then treated with monoclonal antibodies and rabbit complement.
b) Both anti-CD4 and anti-CD8 antibodies were at a final concentration of 50 μg antibody protein/ml.

The tumor specificity of the KAF^h augmented CTL was then examined. Monocytic adherent cells of case 2 patient's PBL were depleted by consecutively passing through nylon wool and Sephadex G-10 columns and then stimulated *in vitro* with the autologous tumor cell line (MS122387). As shown in Fig. 3, the KAF^h-augmented CTL population from the case 2 patient killed autologous tumor (MS122387) and other tumor cell lines when they shared HLA-A31 (NB102287) or HLA-Bw22 (osteosarcoma, OS062488) (Bw22 is split into Bw54 and Bw55), and killed unrelated tumor cells less effectively. The results indicate that the KAF^h-augmented CTL also kill the target tumor cells in an HLA-restricted manner. A cold target inhibition test in case 2 patient's CTL was done to confirm the HLA-restricted pattern of lysis. The results summarized in Fig. 4 clearly show that the KAF^h-augmented CTL activity induced by the autologous tumor (MS122387) was inhibited by the HLA-A or -B matched tumor cells as well as the

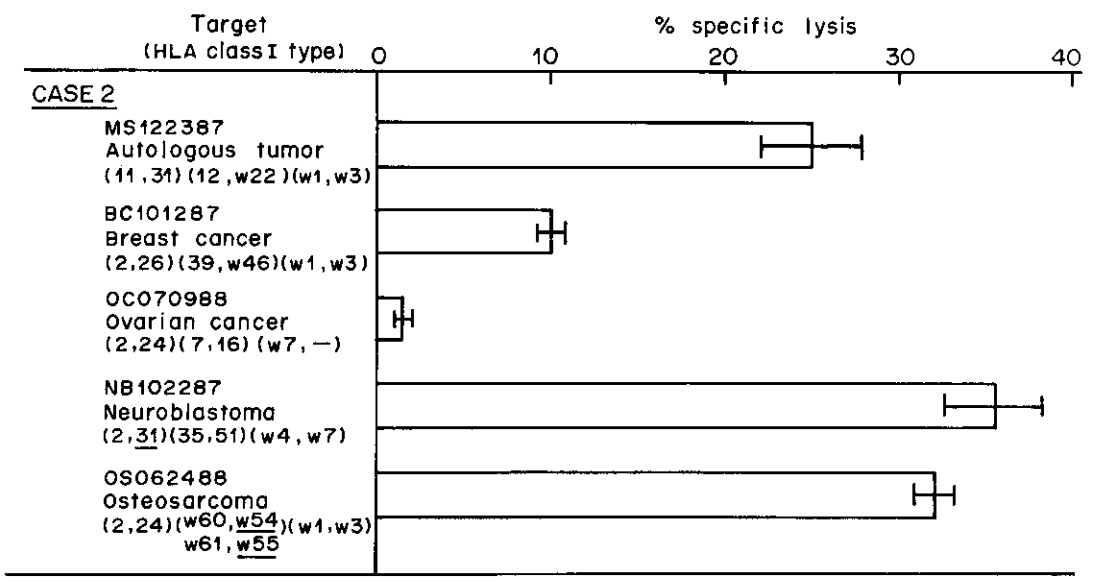

Fig. 3. Specificity of the KAF^h-augmented CTL. Case 2 patient's PBL were passed consecutively through nylon wool and Sephadex G-10 columns to deplete adherent monocytic cells. They were then cultured for 7 days with autologous tumor cell line (MS122387) in the presence of KAF^h (final KAF^h protein concentration was 12 μg/ml) at a responder: stimulator ratio of 300:1. The cytotoxic activity was measured by 16-h ^{51}Cr release assay at an effector: target ratio of 20:1.

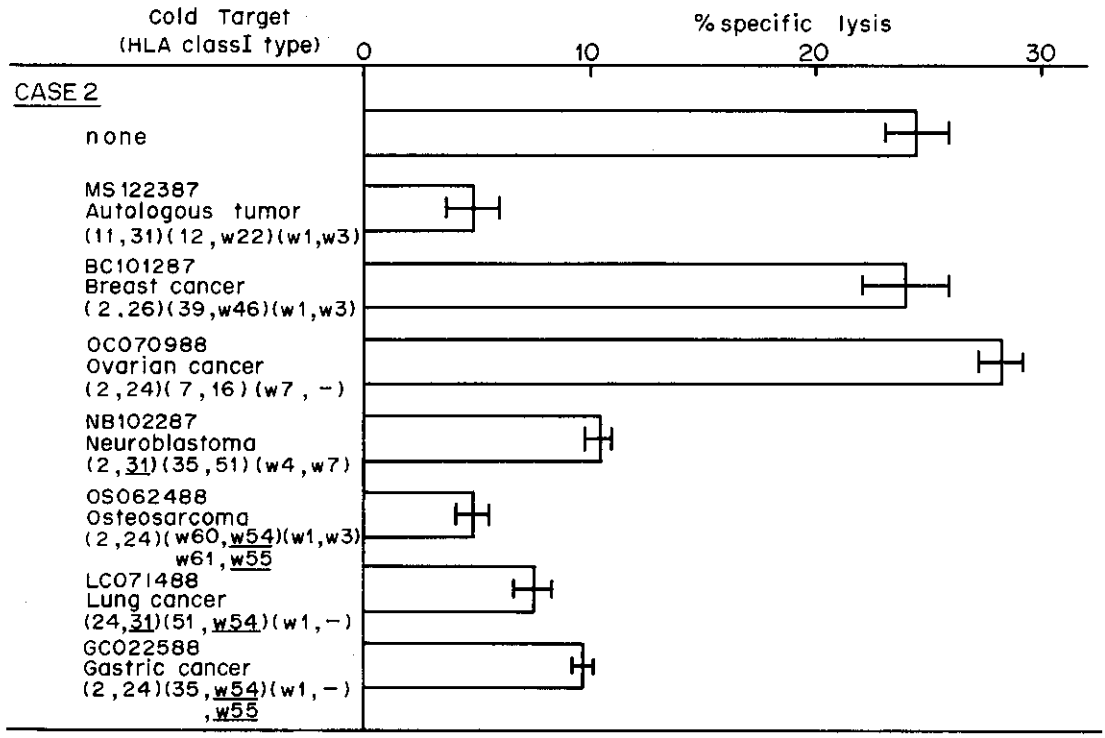

Fig. 4. Cold target inhibition test of KAF^h-activated CTL. KAF^h-activated CTL from case 2 patient's PBL were prepared (see Fig. 2, legend). CTL activity against autologous tumor (MS122387) was measured at an effector: target ratio of 5:1. Unlabeled competitor cell lines including autologous tumor were added to the 16-h ^{51}Cr release assay at an effector: labeled target:cold target ratio of 5:1:10. A similar inhibitory pattern at 5:1:5 was also observed (data not shown).

Table IV. KAF^h-augmented CTL Activity at the Effector Phase

Patient case 2; CTL population treatment[a)]	Added	% Specific lysis against autologous tumor (MS122387) (E:T=20:1)
None	None	13.5±2.5
Monocyte depleted	None	5.0±2.5
Monocyte depleted	KAF^h [b)]	12.6±1.7

a) Case 2 patient's PBL were cultured for 7 days with autologous MS122387 tomor cells at a responder:stimulator ratio of 300:1. Effector cells were then consecutively passed through nylon wool and Sephadex G-10 columns.
b) The final KAF^h concentration was 12 μg protein/ml.

autologous tumor. Case 2 CTL-sensitive target cells, such as both NB102287 and OS062488, inhibited the CTL against autologous tumor, but neither BC101287 nor OC070988 was inhibitory. Additionally to the cell lines used in the Fig. 3 experiment, both LC071488 and GC022588, which shared HLA-A or -B, exhibited the inhibition. Thus, HLA-restricted tumor cell lysis was confirmed.

The KAF^h activity at the effector phase was investigated in the case of patient 2. The CTL population restimulated *in vitro* with autologous tumor for 7 days was harvested and was consecutively passed through nylon wool and Sephadex G-10 columns. The depletion of monocytic adherent cells resulted in a reduction of CTL activity and the KAF^h successfully replaced the monocytic cell function (Table IV). It was concluded that the KAF^h can augment CTL activity in both induction and effector phases.

DISCUSSION

Since the discovery of methods for establishing long-term-cultured T lymphocyte lines using so-called T cell growth factor,[15] the T lymphocyte activation and growth mechanisms have been intensively investigated. On the other hand, the mechanisms of tumor cell lysis by CTL are still largely unresolved. The unique property of the CTL-tumor target system employed by us for the detec-

tion of murine KAF activity is that it requires 12 to 16 h for S1509a tumor cell lysis by the specific CTL, and an longer incubation time than 16 h increases spontaneous tumor cell lysis. The 4-h ^{51}Cr release assay system which has been commonly used detects no meaningful S1509a cell lysis. This may be due to the relative resistance of the S1509a sarcoma cell line to CTL, since the alloantigen-activated CTL, for example H-2^b anti-H-2^a CTL, mounts only 1/10 to 1/5 specific ^{51}Cr release compared with H-2^a-positive L1117 lymphoma cells (unpublished observation). These results suggest that there exist different types of CTL-mediated target cell killing mechanisms from regular 4-h CTL killing. As is evident, we have succeeded in establishing several CTL clones which require additional monocytic cells or the soluble factor derived from monocytic cells for optimal S1509a tumor cell lysis. It is also possible to speculate that there exist two CTL subpopulations, KAF-independent and KAF-dependent CTL for the exertion of their lytic activity.

In the present study we have found that the *in vitro* stimulation of patients' PBL with autologous tumor cells generated potent cytolytic lymphocytes. The depletion of monocytic cell population from PBL resulted in reduced cytolytic activity, and the KAF, which was obtained from the culture supernatant of human monocytic leukemia cell line, was able to replace the monocyte function. The cytolytic cells induced *in vitro* by stimulation with autologous tumor were revealed to be CTL from the following observations. First, though there remained some cytolytic activity after the treatments, anti-CD8 and complement largely removed the activity (Table III). It is not yet known whether the residual killer cell activity is mediated by $CD8^{low}$ or $CD8^-$ lymphocytes. Second, the cytolytic cell activity has crossreactivity against other tumor cell lines only when they share HLA-A or B antigens (Fig. 3). Lastly, the HLA-restricted cytolytic pattern was confirmed by cold target inhibition tests (Fig. 4). It is interesting to note that human CTL against autologous tumor are restricted only as regards HLA and there is no relationship to the tissues from which the tumor originated. Purification of tumor-specific antigens which stimulate host's immune systems has been extensively studied by many investigators.[16] However, at present there is no firm evidence whether tumor or tumor cell-type specific antigens exist even in highly immunogenic experimental tumor systems. It might be extremely difficult to identify the tumor specific antigens in spontaneous human tumors that induce host anti-tumor T cell-mediated immune responses.

The KAF^h activates CTL at the effector phase as well as at the induction phase. The requirement of non-lymphoid cells or their factors for the generation of CTL is an unusual observation, with a few exceptions.[17, 18] Finke *et al.*[17] reported that CTL induction against alloantigenic determinants depends on a helper factor derived from macrophages for their maturation and that the factor was distinct from the growth factor. Though we have to wait until the use of molecularly cloned KAF^h to exclude the possibility of combinatorial cytokine effects for CTL induction, the KAF seems to be an activating factor rather than a differentiation factor, because it generates CTL activity within only 16 h at the CTL effector phase. The KAF itself has no direct cytotoxic activity towards tumor cells but it acts on CTL. The 30 min pre-incubation of KAF^m with TM-series CTL clone in the presence of antigen tumor (S1509a) cells followed by a washing procedure turns the clone cytotoxic. The question arises, how does the KAF activate CTL? The CTL lines established by Blakely *et al.*[18] showed unique properties similar to ours. Some of their CTL lines exert lytic activity upon brief incubation with phorbol ester plus ionomycin. Though the intracellular mechanisms mediated by these chemical reagents have not yet been clarified, the investigation of KAF-induced biochemical changes in our CTL clones may unveil the activation mechanisms. Thus the KAF material, though it has not yet been purified to a single protein, is a monocyte-derived novel cytokine which activates immune CTL to final effector T lymphocytes. The KAF is able to be enriched in the culture supernatant of monocytic tumor cell lines from both mouse and human, and it is different from previously reported monokines such as IL-1, interferon or TNF. The KAF is an activating factor but not a differentiation factor, because it acts on cloned murine CTL line and induces cytolytic activity. Moreover, the unique feature that it also induces CTL activity at an effector phase strongly suggests that KAF is distinct from other cytokines.

The clinical therapeutic potentiality of CTL against autologous tumor has been highlighted during the last decade. The lymphokine-activated killer cells (LAK) developed by Rosenberg's group received much attention for clinical application.[19, 20] However, in regard to tumor cell destruction, CTL may be more effective than LAK, because CTL have specific lytic activity against the tumor. In quite clear contrast to LAK activation, which is simply induced by culturing the patient's PBL with IL-2, CTL therapy requires several critical steps for induction. First, because the CTL should be activated by autologous tumor cells in order to magnify the tumor-specific cytolytic activity, obtaining a large number of tumor cells from each individual patient is required. Secondly, consistent and effective *in vitro* methods for inducing potent CTL are needed. And lastly, *in vitro* expansion of autologous tumor-specific CTL is necessary for bringing about complete tumor regression. The first and the third steps could be circumvented by establishing individual tumor cell lines and the addition of growth

factors such as IL-2, respectively, but the second step, augmenting the CTL activity, is the most crucial step for the therapy. Although we have begun clinical CTL therapy and several tumor remission cases have been experienced (Hamasato *et al.*, Kitsukawa *et al.*, manuscripts in preparation), induction of much more potent CTL based on the above concept is required for the establishment of a general CTL therapy protocol. The purification and molecular cloning of KAF^h, and a deeper understanding of the precise cellular and molecular mechanisms remain to be achieved. As reported here, the KAF^h augments CTL activity towards autologous tumor *in vitro*. The KAF^h should offer a specific immuno-therapeutic potential against human cancer.

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