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Augmentation of interleukin-2-induced activation of human melanoma tumor-infiltrating lymphocytes by heteroconjugate antibody*

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Summary. Heteroconjugate (HC) antibody (anti-CD3 mAb × anti-p97 melanoma mAb) or monomeric anti-CD3 mAb by itself did not induce proliferation of uncultured melanoma tumor-infiltrating lymphocytes (TILs). They also failed to induce IL-2 production in uncultured TILs, although anti-CD3 mAb, but not HC antibody, stimulated IL-2 production in peripheral blood mononuclear cells (PBMCs). Sequential treatment of uncultured TILs from p97-antigen-positive (p97+) melanomas with HC antibody, followed by washing and incubation with interleukin-2 (IL-2), induced significantly higher proliferation than incubation with IL-2 alone. HC antibody pretreatment led to significantly greater results than with anti-CD3 mAb at a 1 ng/ml level in IL-2-induced proliferation of TILs from p97+ melanomas, similar to those with anti-CD3 mAb at a level of 100 ng/ml. HC antibody (1 ng/ml) pretretment did not enhance IL-2-induced proliferation of either TILs from p97− melanomas or PBMCs, while anti-CD3 mAb enhanced the proliferation of TILs from some p97− melanomas and PBMCs. Regardless of the pretreatment of uncultured TILs with HC antibody or anti-CD3 mAb, IL-2-activated TILs were cytotoxic primarily only to autologous tumor cells, and their phenotypes remained the same. Thus, HC antibody can augment IL-2-induced activation of TILs only from p97+ melanomas, without altering their pattern of cytotoxicity or phenotype. The findings were consistent with observations at the clonal level. In contrast to anti-CD3 mAb, HC pretreatment of uncultured TILs from only p97+ melanoma prior to limiting-dilution analysis increased the number of proliferating TIL clones, including autologous tumor-specific cytotoxic T lymphocyte clones. These results suggest that use of HC antibody in vivo would be more advantageous than anti-CD3 mAb, with regard to augmentation of IL-2-induced TIL activation.

Key words: Heteroconjugate antibody – Interleukin-2 – T cell activation – Human melanoma tumor-infiltrating lymphocytes

Introduction

Most human cancers contain a tumor-infiltrating lymphocyte (TIL) population composed predominantly of T cells. One type of current immunotherapy research is directed toward identifying a method by which tumors can be treated using TILs in combination with interleukin-2 (IL-2). In vitro studies of some human cancers, including melanoma, have shown that T cells within the TIL population are specifically cytotoxic to autologous tumor cells after activation with IL-2 [2, 9, 10, 15, 19, 23, 27, 34, 35]. Animal studies have shown that adoptive transfer of IL-2-activated TILs was 50–100 times more efficient than that of lymphokine-activated killer (LAK) cells in eradicating disseminated cancers [28]. In a clinical trial of a small number of melanoma patients, IL-2/TIL therapy resulted in a clinical response of 55% [29].

One obstacle to developing an effective regimen, however, is that the majority of freshly isolated TILs do not bind to autologous tumor cells, nor are they cytotxic to tumor cells [2, 9, 10, 15, 18, 19, 23, 27–29, 34, 35, 42]. Furthermore, less than 10% of TILs express the IL-2 receptor recognized by the anti-CD25 mAb (IL2Rα-p55) [4, 6, 36, 42]. These results suggest that the majority of TILs are inactive in vivo.

The crux of research in this area therefore rests in identifying a way to activate T cells in TILs in vivo. Monomeric anti-CD3 monoclonal antibody (mAb), acting by antigenic mimicry, can activate resting T cells [1, 8, 38–40] in vitro and thus has great potential to activate

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TILs in vivo. One drawback, however, is that the effects of anti-CD3 mAb are nonselective. A recent murine study showed that activation of T cells induced by in vivo administration of anti-CD3 mAb was followed by severe immunosuppression [7]. Hence, use of a monomeric anti-CD3 mAb may prove impractical as a therapy for humans.

Heteroconjugate (HC) antibody is composed of two covalently linked mAbs, each with a different specificity. HC antibody (anti-CD3 × anti-tumor mAb or anti-T-cell-receptor × anti-tumor mAb) can mimic antigenic specificity and induce direct lysis by in-vitro-activated lymphocytes against tumor cells at the effector phase [3, 5, 12–14, 16, 20–22, 25, 26, 30, 31, 33, 37, 43]. In vivo administration of LAK cells precoated with HC antibody (anti-CD3 × anti-glioma mAb) has resulted in tumor regression in human cancers [24]. HC antibody has localized at the tumor site with high efficiency in human tumors xenografted to nude mice [17]. Collectively, these results suggest that HC antibody has great potential in vivo both to induce tumor cell lysis by activated TILs and to activate resting TILs.

HC-antibody-directed cytotoxicity by in-vitro-activated lymphocytes, including IL-2-activated TILs, has been well characterized [3, 5, 12–14, 16, 20–22, 25, 26, 30, 31, 33, 37, 43]. In contrast, HC-antibody-directed activation of TILs has not been fully characterized. Since the majority of in vivo TILs are inactive, administration of HC antibody by itself may not result in the same type of efficient tumor cell lysis that was observed in in vitro experiments [3, 5, 12–14, 16, 20–22, 25, 26, 30, 31, 33, 37, 43]. Instead, in vivo it may potentially activate TILs by itself or augment IL-2-induced activation of TILs. In this study, we have investigated activation of melanoma TILs by HC antibody in comparison with monomeric anti-CD3 mAb.

Materials and methods

Preparation and purification of heteroconjugate. OKT3 mAb (anti-CD3 mAb, IgG2a) and 96.5 mAb (anti-p97 mAb, IgG2a), which is specific for a 97-kDa melanoma-associated antigen [41], were kindly provided by Ortho Pharmaceutical (Raritan, N. J.) and Hybritech (La Jolla, Calif.), respectively. These mAbs were coupled using the bifunctional crosslinking reagents *N*-succinimidyl-3-(2-pyridyldiothio)propionate (SPDP) (Pharmacia, Piscataway, N. J.) and 2-imminothiolane (Sigma, St. Louis, Mo.). In brief, anti-p97 mAb was incubated for 1 h at 25° C with a 5 M excess of SPDP. Uncoupled SPDP was removed by gel filtration on a Sephadex G-25 column in a sodium phosphate buffer, pH 7.0. Anti-CD3 mAb was incubated with 1 mM 2-imminothiolane in tetraethylammonium/HCl, pH 8.0, for 90 min at 4° C under N_2. Unreacted 2-imminothiolane reagent was removed by gel filtration using a Sephadex G-25 column in a NaCl buffer, pH 5.8. The modified antibodies were then allowed to react for 18 h at 4° C under N_2. The reaction was stopped by adding iodoacetamide to a final concentration of 2 mM. The reaction mixture was concentrated and dialyzed against phosphate-buffered saline (PBS). Dimeric and trimeric conjugates were separated from uncoupled parental antibodies and polymers using fast protein liquid chromatography on a Superose 6 column under isocratic conditions with a buffer containing 0.1 M Na_2HPO_4 and 0.1 M NaCl, pH 6.8. The purity of HC antibody (anti-CD3 mAb/p97 mAb) was confirmed by 5% sodium dedecyl sulfate/polyacrylamide gel electrophoretic analysis and was >97% as determined by densitometry. As a control, a homoconjugate of anti-p97 mAb and anti-p97 mAb was prepared and purified using the same method described above.

Preparation of cells. Single-cell suspensions of TILs and tumor cells were prepared from surgical specimens of non-lymph-node (subcutaneous or visceral) melanoma metastases from five different patients and lymph node metastases that had severe infiltration of melanomas in three patients. Tumor samples were confirmed histologically and subjected to mechanical dissociation. Necrotic areas or tissues surrounding the tumor were carefully removed before the preparation of cells. The tumors were then digested enzymatically with 0.4 μg/ml DNase and 2.0 μg/ml collagenase (both from Sigma Chemical; St. Louis, Mo.) for 2 h. Lymphocytes and tumor cells were separated from debris and non-mononuclear cells by Ficoll density cushion. Peripheral blood mononuclear cells (PBMCs) from melanoma patients (n = 3) or healthy donors (n = 3) were also isolated by a Ficoll/Isopaque cushion. None of the patients had prior chemotherapy, radiotherapy or biotherapy. Cell viability exceeded 80%. Lymphoid cells were discriminated from tumor cells by cell size, morphology, and cytoplasmic pigmentation for melanoma cells [9]. MAbs specific for human lymphocytes (anti-CD3, -CD4, CD8, and CD16) or melanoma cells (anti-p97 mAb and anti-GD2 mAb) were used to confirm the identity of these cells in certain experiments. Cell suspensions were used directly for experiments or were cryopreserved at –170° C in 90% fetal calf serum (Flow Labs, McLean, Va.) and 10% dimethylsulfoxide until use.

Treatment of cells, cell culture, and proliferation assay. Dissociated cells from tumors or PBMCs were incubated at 37° C in the presence of various antibodies at various concentrations (0.1 ng–10 μ/ml). Following incubation, the cells and medium were aspirated and transferred to centrifuge tubes and spun at 1200 rpm for 6 min. The supernatants were collected and stored at 4° C for later evaluation of IL-2 activity. RPMI-1640 medium (Gibco, Grand Island, N. Y.) supplemented with 10% fetal calf serum was used as the medium. The cell pellet was washed three times, and then resuspended in the medium containing 100 U/ml human recombinant IL-2 (rIL-2) kindly provided by Hoffman LaRoche (Nutley, N. J.). The cells were expanded and fed, as needed, with medium containing IL-2 every 3–5 days. After 14–21 days in culture, viable lymphocytes and tumor cells were counted using a trypan blue dye exclusion test. For the proliferation assay the -fold increase in numbers of lymphocytes was calculated from the numbers of originally plated lymphocytes.

Cytotoxicity and IL-2 assay. A 4-h ^{51}Cr-release assay was used to detect cytotoxic activity as described previously [1]. Cryopreserved, uncultured melanoma tumor cells or cells from primary cultures of melanoma or renal cell carcinoma cells were used as target cells after radiolabelling with $Na_2{}^{51}CrO_4$. The spontaneous release did not exceed 30% of the maximum release. The percentage specific lysis was calculated as described previously [10]. IL-2 activity in the culture supernatants was measured by a bioassay using a murine IL-2-dependent natural killer (NK) cell clone (NK8) as described previously [32]. Briefly, NK clones (10^4 cells/well) were incubated for 24 h with medium alone, IL-2, or serial dilutions of test samples, and proliferation of NK clones was measured by a regular [^{3}H]thymidine assay with a 6-h pulse of 0.5 μCi/well [^{3}H]thymidine (New England Nuclear, Boston, Mass.).

Immunofluorescence. Fluorescein-isothiocynate(FITC)-conjugated anti-CD8 and -CD16 mAb and phycoerythrin-conjugated anti-CD3, -CD4, and -CD25 (anti-Tac mAb) were purchased from Becton Dickinson Co. (Mountain View, Calif.). Cells were incubated with mAb at 4° C for 30 min, and then antigen expression on lymphocytes was analyzed using flow cytometry (EPICS C, Coulter, Hialeah, Flo.) by two-color analysis, as reported previously [11]. To detect p97 antigen expression on tumor cells, cells were incubated with 10 μg/ml anti-p97 mAb or HC antibody for 30 min at 4° C, followed by staining with FITC-conjugated $F(ab')_2$ fragments of goat anti-(mouse IgG) (Cooper Laboratories, Cochranville, Pa.). Lymphocytes were gated on the basis of their smaller size and less granularity, while tumor cells were gated on the basis of their larger size and greater granularity [11]. Normal mouse Ig or irrelevant mouse IgG2a was used as the first antibody for control staining during the indirect immunofluorescence assay. The term "p97$^+$ or p97$^-$ tumor" was used to denote ⩾10% or <10% of tumor cells among the total tumor cell population that were stained with anti-p97 mAb.

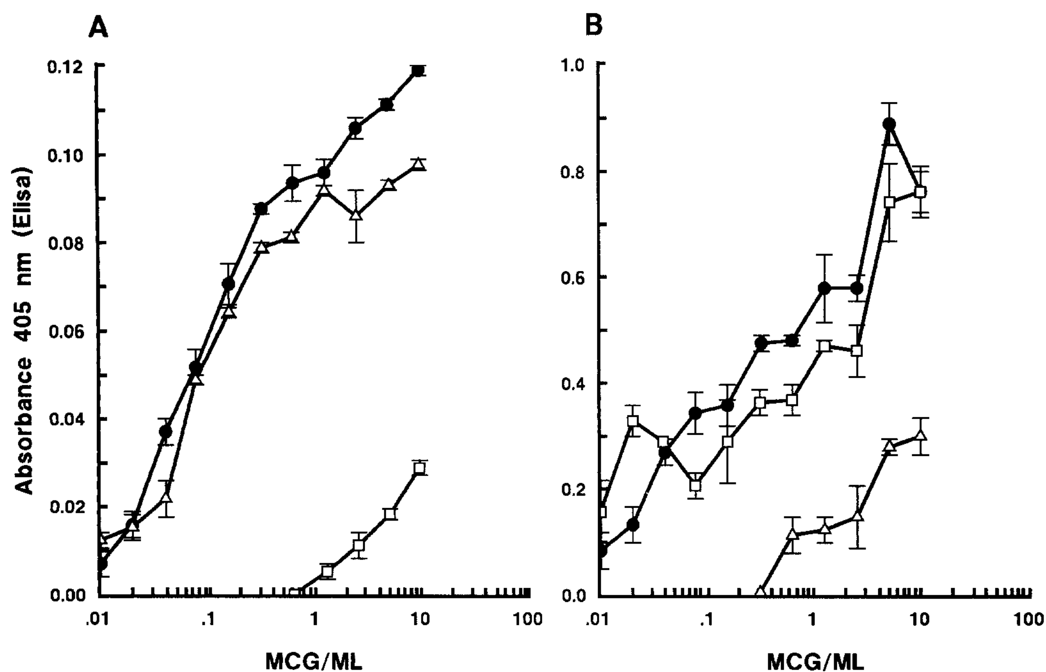

Fig. 1 A, B. Binding of heteroconjugate (HC) antibody to both melanoma cells and T cells. HC antibody (●), monomeric anti-CD3 mAb (□), and monomeric anti-p97 mAb (△) were tested for binding specificity in enzyme-linked immunosorbent assay *Elisa*. Each mAb served as both a relevant and irrelevant control for the other, since both were IgG2a. **A** Binding to glutaraldehyde-fixed M110 melanoma cells expressing a high level of p97 antigen as determined by immunofluorescent analysis and flow cytometry; **B** Binding to the dry-fixed Jurkat cell line, which has a high expression of the CD3 antigen. *MCG:* microgram

Antigen-binding specificity of HC antibody by ELISA. Bioactivity of HC antibody was confirmed by enzyme-linked immunosorbent assay (ELISA). To detect CD3 antigen-binding activity, 5×10^4 Jurkat cells (a T cell line) were placed in each well of a 96-well plate. The cells were dried and fixed to the wells at 37°C overnight. M110 melanoma cells from an established cell line from a patient with metastatic melanoma were cultured overnight to allow adherence, and were used to detect p97 antigens bound by HC antibody. The wells were then washed with PBS containing 0.05% polysorbate and incubated for 90 min at 37°C with PBS containing 3% bovine serum albumin. The wells were washed again, and triplicate serial dilutions of antibodies were added and incubated for 1 h at 37°C. An antibody test kit (Boehringer Mannheim, Indianapolis, Ind.) was then used for the assay. HC antibody bound to both Jurkat and M110 melanoma cells (Fig. 1). Monomeric anti-CD3 or p97 mAb bound to Jurkat or M110 melanoma cells, respectively (Fig. 1).

Limiting-dilution analysis. Limiting-dilution analysis was carried out according to the methods previously described [10], with a slight modification. Freshly isolated cell suspensions of lymphocytes and tumor cells were pretreated for 2 h with 10 ng/ml anti-p97 mAb alone (as control), anti-CD3 mAb alone, or HC antibody. After being washed, these cells were then provided for limiting-dilution analysis. Different numbers of these TILs (8, 4, 2, 1, 0.5 cells/well) were incubated in 96-well U-bottomed microplates containing RPMI medium/fetal calf serum, 100 U/ml rIL-2 and irradiated PBMCs (90 Gy) from two different healthy donors (2×10^5 PBMCs/well). Two microplates per each plated TIL sample (a total of ten plates) were used for each of three groups. Half of the medium was replaced 7 and 14 days later with the same medium. The numbers of proliferating wells were scored from all ten plates in each group. Proliferating cells were transferred at 3–4 weeks from 96-well microculture plates to 24-well culture plates in the presence of irradiated autologous tumor cells (1×10^5 cells/well), and investigated for their surface antigens and cytotoxic activity.

Results

Failure to induce TIL activation with HC antibody alone

At first we incubated uncultured TILs with different doses (1–1000 ng/ml) of HC antibody or monomeric anti-CD3 mAb for 1 or 2 days and followed this by washing and incubation without antibody for up to 7–10 days. TILs were also cultured in the presence of HC antibody or anti-CD3 mAb for 7 days. However, HC antibody or anti-CD3 mAb by itself failed to induce TIL proliferation, and therefore exogenous IL-2 was necessary for this (data not shown). To understand the mechanisms of the failure, we investigated IL-2 production by uncultured TILs or PBMCs treated with HC antibody or the other mAbs (Table 1). HC antibody failed to induce IL-2 production in either uncultured TILs from any of five different tumors or in PBMCs from any of three donors. Anti-CD3 mAb treatment also failed to induce IL-2 in uncultured TILs, but stimulated PBMCs to produce IL-2 in all three donors (Table 1). Any of the other antibodies (anti-CD8, anti-p97, or homoconjugate of anti-p97 mAbs) failed to induce IL-2 production in either TILs or PBMCs.

HC-antibody-mediated selective augmentation of IL-2-induced TIL activation

Uncultured cells from p97+ (n = 6) and p97− tumors (n = 2) and PBMCs (n = 3) were pretreated for 2 h at 37°C with HC antibody or anti-CD3 mAb at various concentrations followed by washing and incubation with 100 U/ml rIL-2

Table 1. Failure to induce interleukin-2 (IL-2) production by uncultured tumor-infiltrating lymphocytes (TIL) with heteroconjugate antibody[a]

Cell sources	IL-2 activity in supernatant from cells treated with							
	Medium	100 U/ml IL-2	None	Anti-CD3	Anti-CD8	Anti-p97	HC	Hm
TIL ($n = 5$)	0.8	8.8	0.8	0.8	0.8	0.8	0.8	0.9
PBMC ($n = 3$)	1.1	8.4*	2.5	6.0*	2.5	2.4	2.2	2.6

[a] Supernatants of freshly isolated TILs ($n = 5$) or PBMCs ($n = 3$) cultured with the antibodies shown above (10 ng/ml) for 18–24 h were assayed for IL-2 activity using a bioassay. Medium alone served as negative control, and medium with 100 U/ml IL-2 as positive controls. Values represent means of 10^{-3}[^{3}H]thymidine uptakes (cpm) of IL-2-dependent NK8 clones from different donors. HC, heteroconjugate (anti-CD3 × anti-p97 mAb). Hm, homoconjugate (anti-p97 × anti-p97 mAb)
* $P < 0.05$ as compared to that from cells treated with HC antibody or control culture (Student's two-tailed t-test).

Table 2. HC-antibody-mediated augmentation of IL-2-induced proliferation of TILs from p97-positive tumors[a]

Lymphocyte sources	p97 antigen on tumor cells (% positive)	Antibody pretreatment	Lymphocyte proliferation with antibody pretreatment (ng/ml) (-fold increase)				
			0	0.1	1	10	100
p97+ tumor 1	+ (>70)	HC	10	15	67	101	100
		Anti-CD3 mAb	10	13	27	42	97
p97+ tumor 2	+ (>70)	HC	0.5	8	10	10	12
		Anti-CD3 mAb	0.5	1	4	8	8
p97+ tumor 3	+ (60)	HC	12	38	50	ND	85
		Anti-CD3 mAb	12	18	35	ND	62
p97− tumor 1	− (0)	HC	3	3	4	20	ND
		Anti-CD3 mAb	3	8	20	35	ND
p97− tumor 2	− (6)	HC	2	3	5	5	6
		Anti-CD3 mAb	2	5	4	4	6
PBMC ($n = 3$)	No tumor	HC	2±2	1±1	2±2	55±5	ND
	(0)	Anti-CD3 mAb	2±2	25±9	80±15	65±10	ND

[a] Uncultured cells were pretreated for 2 h at 37°C with 0–100 ng/ml HC antibody or monomeric anti-CD3 mAb. After pretreatment, cells were washed three times and incubated for 14–18 days with the medium and 100 U/ml rIL-2 alone. Viable numbers of lymphocytes were enumerated, and the magnitude of proliferation was expressed as the -fold increase, calculated from the numbers of originally plated lymphocytes. The values from PBMCs represent the means ± SD from three different patients. P97 antigen expression on tumor cells was investigated by immunofluorescence and flow cytometry. ND, not determined

alone for 14–18 days. Proliferation of TILs was measured by counting lymphocyte numbers. Representative results are shown in Table 2. HC antibody pretreatment of cells augmented IL-2-induced proliferation of lymphocytes from p97+ tumors. In these tumors, HC-antibody-mediated enhancement was greater than that by monomeric anti-CD3 mAb at lower concentrations (0.1–10 ng/ml), but was nearly the same at a concentration of 100 ng/ml. Lymphocyte numbers from these six p97+ tumors, which were pretreated with 1 ng/ml HC antibody or anti-CD3 mAb followed by IL-2 culture (14 days), were respectively 12.5±7.4 or 5.9±2.4 times greater than those pretreated with normal mouse Ig followed by IL-2 culture. Thus, the magnitude of proliferation after HC antibody followed by IL-2 was significantly ($P < 0.05$ by Student's two-tailed t-test) higher than that after anti-CD3 mAb followd by IL-2, which in turn was significantly ($P < 0.01$) higher than that following IL-2 alone. Treatment for 1 h with HC antibody was sufficient to enhance IL-2-induced proliferation, and prolonged pretreatment (24–48 h) or coculture with IL-2 was often inhibitory for IL-2-induced proliferation (data not shown).

For lymphocytes from either p97− tumors or PBMCs, HC antibody pretreatment at 0.1–1 ng/ml did not augment IL-2-induced proliferation. anti-CD3 mAb pretreatment increased IL-2-induced proliferation in TILs from one p97− tumor, but not the other (Table 2). Pretreatment of cells with p97 mAb alone or homoconjugate antibody (anti-p97× anti-p97 mAb) did not augment IL-2-induced lymphocyte proliferation (data not shown).

Cytotoxicity

Uncultured cells were pretreated with 1 ng/ml normal mouse Ig, anti-CD3 mAb, or HC antibody. Cells were washed and incubated with IL-2 alone for 14–17 days, and these lymphocytes were investigated for both proliferation and cytotoxicity against autologous and allogeneic tumor cells (Table 3). Proliferation of TILs pretreated with HC antibody was higher than that of TILs treated with monomeric anti-CD3 mAb, which in turn was higher than that after treatment with normal mouse Ig. Regardless of antibodies used for pretreatment, IL-2-activated TILs from all four melanomas displayed significant cytotoxicity against autologous tumor cells, but no or low cytotoxicity against any of the three allogeneic melanoma cells. Thus, different pretreatments did not alter the pattern of TIL-mediated cytotoxicity. Furthermore, the length of time for the pretreatment did not alter the pattern of cytotoxicity (Table 4).

Table 3. Cytotoxicity by IL-2-activated melanoma TILs pretrated with HC antibody[a]; target cell spectrum

TILs from	Antibody pretreatment	Increase (-fold)	Cytotoxicity (%) vs											
			M25			M46			M48			M73		
			40	20	10	40	20	10	40	20	10	40	20	10
M25	NMG	5	30	23	14	0	0	0	5	0	0	4	1	0
	HC	60	30	11	5	0	0	0	6	2	0	10	3	0
	Anti-CD3	27	27	19	7	0	0	0	3	0	0	5	2	0
M46	NMG	5	0	0	0	12	11	7	0	0	0	0	0	0
	HC	33	0	0	0	24	31	22	0	0	0	0	0	0
	Anti-CD3	21	0	0	0	22	18	22	0	0	0	0	0	0
M48	NMG	2		NA			NA			NA			NA	
	HC	19	13	8	6	0	0	0	25	14	9	0	0	0
	Anti-CD3	11	0	0	0	0	0	0	19	17	15	0	0	0
M73	NMG	ND	15	2	0	10	5	2	1	0	0	68	39	16
	HC	ND	10	0	0	8	3	1	3	0	0	68	42	11
	Anti-CD3	ND	13	6	0	9	4	1	2	0	0	72	44	15

[a] Uncultured cells from four different p97+ melanomas were pretreated for 2 h with 1 ng/ml normal mouse Ig (NMG), HC antibody or monomeric anti-CD3 mAb. Cells were washed three times and incubated with medium and 100 U/ml rIL-2 for 17 days. Viable numbers of lymphocytes were enumerated, and the magnitude of proliferation was expressed as -fold increase, calculated from the numbers of originally plated lymphocytes. Their cytotoxicity against four different uncultured melanoma cells was measured by a 4-h ^{51}Cr-release assay at E:T ratios of 40, 20, and 10. Values represent means for the percentage cytotoxicity in the triplicate assays. NA, not available; ND, not determined

Table 4. Cytotoxicity by IL-2 activated lymphocytes pretreated with HC antibody[a]: kinetic study

Pretreatment with	Pretreatment time (h)	Mean lysis (%) of	
		Auto tumors	Allo tumors
HC antibody	1	68.1	7.0
	4	65.3	3.3
	24	64.5	3.9
Anti-CD3 mAb	1	72.9	4.5
	4	64.5	3.6
	24	61.1	2.9

[a] Freshly isolated TILs from p97+ tumors (n = 2) were pretreated with 10 ng/ml HC antibody or anti-CD3 mAb for various times (1, 2, 4, 8, 24, and 48 h) followed by washing and incubation with rIL-2 (100 U/ml) alone for 21 days. Values represent the mean percentage lysis by IL-2-activated lymphocytes from two different tumors against autologous or allogeneic tumor cells at an E:T ratio of 20:1. Cytotoxicity by lymphocytes pretreated for 1, 4, and 24 h is shown here. Similar results were obtained from the other periods of preincubation (data not shown).

Table 5. Surface antigens of proliferating TILs[a]

Pretreatment	Mean percentage of positive cells			
	CD3+CD16− T cells	CD4+ T cells	CD8+ T cells	CD3−CD16+ NK cells
None	84.5	36.0	50.5	3.5
HC antibody	94.6	23.0	57.2	0.8
Anti-CD3 mAb	96.0	30.0	68.6	0.8

[a] Uncultured cells from p97+ tumors (n = 4) were pretreated for 4 h with medium alone, 1 ng/ml monomeric anti-CD3 mAb, or the HC antibody. The cells were then washed and incubated in medium with 100 U/ml rIL-2 for 18–21 days. Two-color immunofluorescence analysis was carried out to analyze surface antigens of the IL-2-activated TILs using flow cytometry. The vast majority (>98%) of CD4+ T cells were CD8−, and CD8+ T cells were CD4−

IL-2-activated TILs lysed autologous tumor cells, but not allogeneic tumor cells, regardless of different pretreatment times (Table 4).

Surface phenotypes

Uncultured cells from p97+ tumors were pretreated with HC antibody or anti-CD3 mAb and then were incubated with IL-2 alone. Surface phenotypes of the IL-2-activated TILs were investigated (Table 5). CD3+CD16− T cells predominated in all four cases. Percentages of CD4+ T cells in TILs pretreated with HC antibody were slightly lower than those in the control group, although it was not significant. Percentages of CD3−CD16+ NK cells were very low (3.5%) in the control group, and <1% in pretreated groups. Thus, there were no fundamental differences in T-cell subsets of IL-2 activated TILs, regardless of different pretreatment schedules.

Clonal analysis

Uncultured cells from a p97+ (M63) or a p97− (M2) tumor were pretreated with p97 mAb as a control antibody, HC antibody, or anti-CD3 mAb prior to limiting-dilution analysis (Table 6). HC antibody pretreatment of TILs from the p97+ tumor, but not from the p97− tumor, increased the number of proliferating clones as compared with the control group (experiment 1; 26 by HC antibody vs 15 clones

Table 6. The effects of pretreatment of uncultured TILs with HC antibody or anti-CD3 mAb on the development of cytottoxic T lymphocyte (CTL) clones[a]

Pretreatment	No. prolif. wells	No. clones studied	No. clones (surface antigens)			No. clones showing cytotoxicity		
			$CD3^+4^+8^-$	$CD3^+4^-8^+$	$CD3^+CD4^-8^-$	CTL activity[b]	LAK activity[c]	No cytotoxicity[d]
Expt. 1 ($p97^+$ tumor)								
Anti-p97 mAb	15	12	7	2	3	2	2	8
HC antibody	26	21	13	6	2	6	2	13
Anti-CD3 mAb	14	10	8	2	0	2	1	7
Expt. 2 ($p97^-$ tumor)								
Anti-p97 mAb	16	11	7	4	0	3	0	2
HC antibody	17	14	8	6	0	4	1	6
Anti-CD3 mAb	40	36	30	6	0	0	0	12

[a] Uncultured cells from a p97+ tumor (M63) or a p97− tumor (M2) were pretreated for 2 h with 10 ng/ml p97 mAb (as control), anti-CD3 mAb, or HC antibody. After incubation, the cells were washed and used as effector cells to establish T cell clones with limiting-dilution analysis. Cytotoxic patterns in T cell clones were defined as autologous restricted (CTL activity), MHC-nonrestricted cytotoxicity (LAK activity), or no cytotoxicity

[b] ⩾10% lysis against autologous tumor cells but not any of two different allogeneic melanoma cells (E:8T = 10)

[c] ⩾10% lysis against both autologous and allogeneic melanoma cells (E:T = 10)

[d] <10% lysis against either autologous or allogeneic melanoma cells (E:T = 10)

by anti-p97 mAb). In the control group of experiment 1, 2 of 12 clones displayed cytotoxicity against autologous tumor cells (⩾10% lysis) but did not lyse either of two allogeneic melanoma cell lines (cytotoxic T lymphocyte, CTL, activity). The other two clones showed major-histocompatibility-complex-nonrestricted cytotoxicity (LAK activity), and the remaining eight clones did not lyse either autologous or allogeneic melanoma cells (no cytotoxicity). In the HC antibody group of experiment 1, 6 of 21 T cell clones displayed CTL activity. Two clones had LAK activity and the remaining 13 clones had no cytotoxicity. CD4+ T cell clones were predominant among clones, and there were some CD8+ T cells and double-negative T cell clones either in the control or the HC antibody group (experiment 1). HC antibody pretreatment of TILs from the p97− tumor did not affect numbers of proliferating clones or their pattern of cytotoxicity (Table 6, experiment 2).

Anti-CD3 mAb pretreatment of TILs from p97+ tumor did not increase the number of proliferating clones or CTL clones, and had no double-negative T cell clones (experiment 1, Table 6). anti-CD3 mAb pretreatment of TILs from p97− tumor increased the number of proliferating clones as compared with the control group or HC antibody group (40 vs 16 or 17 respectively). However, none of the 12 clones tested from the anti-CD3 mAb group displayed CTL activity, while 3 of the 5 clones from the control group or 4 of the 11 clones from the HC antibody group exhibited CTL activity. Thus, monomeric anti-CD3 mAb pretreatment resulted in more noncytotoxic CD4+ T cell clones from the p97− tumor (experiment 2).

Discussion

This manuscript demonstrates that HC antibody pretreatment can augment IL-2-induced activation of only p97+ melanoma TILs without altering their phenotypes or cytotoxicity, which is mostly restricted to autologous tumor cells. Although HC-antibody-directed lysis of tumor cells by lymphocytes activated in vitro including IL-2-activated TILs, has been well described [3, 5, 12, 14, 16, 20–22, 25, 26, 30, 31, 33, 37, 43], this is the first report describing the ability of HC antibody to augment IL-2-induced activation of TILs without changing their functional activity. Anti-CD3 mAb is a potent T cell activator by antigenic mimicry [1, 8, 38–40]. However, in vivo administration of monomeric anti-CD3 mAb in mice induced T cell activation followed by severe immunosuppression [7]. IL-2 is pivotal for T cell activation, but the vast majority of TILs are resting and do not express CD25 antigen (IL2Rα) on the surface [4, 6, 36, 42]. Consequently, activation of lymphocytes only at the tumor site by HC antibody followed by IL-2 may be critical to developing more effective but less toxic IL-2-based immunotherapy. Our results described here may provide the scientific basis for sequential treatment of malignancy with HC antibody (anti-CD3 × anti-tumor mAb) followed by administration of IL-2.

This experiment used intact mAbs for HC antibody preparation. For each microgram of protein, HC antibody, as compared with the parental antibodies, has roughly one half the number of each specific binding site but twice the number of binding sites for the second antibody [anti-(mouse IgG)] per molecule. The binding curves detected by ELISA would be expected to overlap as observed in Fig. 1, although binding curves of HC antibody were primarily superior to those of monomeric antibody.

HC antibody pretreatment augmented IL-2-induced activation of TILs from p97+ tumors but not from p97− tumors or from PBMCs at a 0.1–1 ng/ml concentration. However, at a higher concentration (100 ng/ml), the effects of the HC antibody were similar to those of anti-CD3 mAb in IL-2-induced activation of TILs or PBMCs. This may be due to the Fc-mediated nonspecific binding of HC antibody to Fcγ-positive cells in uncultured cell fractions. Therefore, one cannot expect an obvious advantage in the use of HC antibody instead of anti-CD3 mAb with regard to in vitro activation of T cells or TILs at higher doses.

Of eight metastatic melanomas tested, three strongly expressed p97 antigens (>50% positive cells by flow cytometry), three modestly expressed p97 antigens (10%–

50%), and the remaining two had no, or low levels of p97 antigens on the surface (<10%). HC antibody pretreatment at 1 ng/ml did not enhance IL-2-induced proliferation of TILs from p97$^-$ tumors. anti-CD3 mAb enhance IL-2-induced proliferation of TILs from one p97$^-$ tumor but not the other tumor (M2) at the bulk culture level. This failure of anti-CD3 mAb to enhance TIL proliferation from an M2 tumor may partly be due to low levels of Fcγ receptor expression on the M2 tumor cells, since binding of normal mouse Ig to M2 tumor cells was much lower than that to the other melanoma cells, as judged by immunofluorescence and flow cytometry (data not shown).

Neither HC antibody nor monomeric anti-CD3mAb induced uncultured TILs (n = 5) to produce IL-2. In contrast, monomeric anti-CD3 mAb, but not HC antibody, induced IL-2 production by PBMCs. Failure to produce IL-2 in TILs may partly be due to fewer numbers of activated lymphocytes among the uncultured populations. This hypothesis is supported by the fact that the vast majority of uncultured TIL were inactive [2, 4, 6, 9, 10, 15, 18, 19, 23, 27–29, 34–36, 42]. Although one may expect an inability of T cells in melanoma TILs with regard to lymphokine production, uncultured TILs produced interferon γ in response to anti-CD3 mAb or IL-2 (data not shown). An alternative explanation is that tumor cells suppress anti-CD3-mAb-induced IL-2 production in TILs. This hypothesis is unlikely, since addition of tumor cells to the culture of PBMCs with anti-CD3 mAb did not suppress IL-2 production (data not shown).

HC antibody pretreatment consistently induced a greater than five- to tenfold increase in the number of proliferating TILs from p97$^+$ tumors as compared with the control IL-2 culture. Its effect at the clonal level was less dramatic than the effect at the bulk culture level. Furthermore, anti-CD3 mAb pretreatment did not increase the number of proliferating clones from p97$^+$ tumors. Anti-CD3 mAb pretreatment did not augment IL-2-induced proliferation of TILs from p97$^-$ (M2) tumor at the bulk culture level, but increased the number of noncytotoxic T cell clones in the same tumor. These discrepancies in the efficiency of HC antibody or anti-CD3 mAb pretreatment between the clonal and bulk culture levels may be partly due to the different culture conditions. Allogeneic feeder cells were required to establish T cell clones, while IL-2 alone was sufficient for bulk culture. in limiting-dilution analysis, the numbers of plated cells were very small, while numerous lymphocytes were plated at the bulk culture level.

HC antibody pretreatment (1 ng/ml) in vitro augmented IL-2-induced TIL activation from only p97$^+$ tumors without altering their phenotype or cytotoxic pattern both at the bulk culture and clonal levels. Monomeric anti-CD3 mAb augmented IL-2-induced TIL activation in both p97$^+$ and p97$^-$ melanomas and also increased IL-2-induced proliferation of PBMCs. Furthermore, alteration of the immunological properties of TIL by anti-CD3 mAb pretreatment was suggested at the clonal level (induction of noncytotoxic clones). In vivo administration of anti-CD3 mAb risks causing nonspecific T cell activation followed by severe immunosuppression [7]. Therefore, HC antibody (anti-CD3 mAb × anti-tumor mAb) may be superior to anti-CD3 mAb for clinical use to activate TILs. Use of HC antibody as a vehicle for transporting anti-CD3 mAb to tumor sites prior to IL-2 therapy for solid cancers may induce more efficient TIL activation in vivo, possibly resulting in higher efficacy of tumor regression and less toxicity than to IL-2 therapy alone.

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