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Specific immune recognition of autologous tumor by lymphocytes infiltrating colon carcinomas: analysis by cytokine secretion

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Summary. Tumor-infiltrating lymphocytes (TIL) were grown in the presence of interleukin-2 from 19 colon carcinoma specimens, including 1 primary lesion and 18 metastatic lesions. These cultures showed a median proliferation of 606-fold (range 13-fold to 28000-fold) over 49 culture days (range 26–76 days). By phenotype, mature cultures were 69%–99% CD3+ (mean 93%) and contained mixed populations of CD4+ and CD8+ cells (CD4>CD8 in 10 of 19 cultures). Fresh cryopreserved colon tumors were not lysed by autologous TIL in short-term ^{51}Cr-release assays, and were poorly lysed by lymphokine-activated killer cells. Ten TIL cultures were assayed for cytokine secretion in response to autologous and allogeneic tumors during a 6- to 24-h coincubation. Culture supernatants were tested by ELISA for the presence of granulocyte/macrophage-colony-stimulating factor, interferon γ, and tumor necrosis factor α. Of 10 TIL, 4 secreted at least two of these cytokines specifically in response to autologous and/or HLA-matched fresh allogeneic colon carcinomas, but not to melanomas or HLA-unmatched colon carcinomas. Cytokine secretion was mediated by both CD4+ and CD8+ TIL, and could be inhibited by mAb directed against the appropriate class of MHC antigen. These data provide evidence for specific, MHC-restricted immune recognition of human colon carcinomas by T lymphocytes.

Key words: Colon carcinoma – Tumor-infiltrating lymphocytes – Cytokines

Introduction

Colon cancer is one of the commonest cancers occurring in the United States today. Despite standard treatment modalities, approximately 40% of patients with colon carcinoma will die of their disease. Immunotherapy represents an alternative approach to treating patients with colon carcinoma. Reports that lymphoid infiltrates in colon carcinoma may correlate with a favorable prognosis have suggested that patients can mount an immune response against this tumor [19]. Tumor-infiltrating lymphocytes (TIL) grown in the presence of interleukin-2 (IL-2) have induced tumor regressions in some patients with metastatic melanoma [13]. However, clinical experience using TIL in the treatment of patients with metastatic colon carcinoma has so far been anecdotal. Administration of systemic IL-2 without TIL in those patients has shown limited effectiveness [12].

The mechanism by which adoptively transferred TIL exert clinical antitumor effects is currently unknown and is the subject of ongoing studies. One possibility is that transferred TIL may mediate direct tumor lysis, as suggested in a recent report correlating in vitro cytolysis of autologous tumor with clinical response in melanoma patients treated with TIL [1]. Several in vitro studies have shown that TIL from colon carcinoma are often only weakly cytolytic for autologous tumor [7, 17, 22, 23]. This might suggest that TIL would be relatively ineffective in treating colon carcinoma.

However, it has recently been reported that in murine transplanted tumor models, specific secretion of interferon γ (IFNγ) by TIL in response to autologous tumor in vitro is more likely to predict their in vivo antitumor efficacy than is cytolysis [2]. TIL derived from human melanomas and breast carcinomas have been shown to secrete granulocyte/macrophage-colony-stimulating factor (GM-CSF), IFNγ, and tumor necrosis factor α (TNFα) specifically in response to autologous and HLA-matched allogeneic tumors, demonstrating MHC-restricted T-cell/tumor interactions in these systems [6 a, 14]. The clinical relevance of these findings is currently under investigation.

This study was undertaken to characterize a series of colon carcinoma TIL by phenotype, cytolytic activity, and cytokine secretion. We show that, while colon TIL are nonlytic for fresh colon tumors, select TIL cultures are capable of MHC-restricted recognition of autologous and

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HLA-matched allogeneic colon carcinomas, as manifested by cytokine secretion.

Materials and methods

Tumor-infiltrating lymphocyte cultures. TIL cultures were established as described previously [20] from tumor specimens resected from 19 patients with colon carcinoma. In 14 cases, the tissue source was hepatic metastases. The 5 remaining specimens included one each from an adrenal metastasis, a pulmonary metastasis, a subcutaneous metastasis, ascites, and a primary colon lesion. Briefly, the resected tumor specimen was minced into 3- to 5-mm pieces and enzymatically digested overnight. The resulting tumor digest was then washed and placed over a Ficoll density gradient to remove nonviable and red blood cells. The gradient interface containing mononuclear and tumor cells was then harvested and either frozen in aliquots for use as fresh tumor targets/stimulators in later studies, or cultured in TIL medium. Medium consisted of RPMI-1640+10% heat-inactivated human AB serum with 50 IU/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin, 250 ng/ml amphotericin, 10 mM HEPES buffer, and 2 mM L-glutamine. Conditioned medium from 4-day allogeneic lymphokine-activated killer (LAK) cell cultures was added at a final concentration of 20% (v/v). Recombinant IL-2 (kindly provided by the Cetus Corporation, Emeryville, Calif.) was added at a final concentration of 1000 Cetus U/ml (6000 IU/ml). Cells were maintained at concentrations less than 2×10^6/ml by passaging weekly. All TIL cultures except one (TIL 917) were passaged without tumor restimulation and were used in assays after 22–62 days in culture. Aliquots of TIL were cryopreserved for subsequent studies.

Lymphokine-activated-killer (LAK) cell cultures. Cultures were established as previously described [20] by placing normal donor buffy coats over a Ficoll density gradient to retrieve mononuclear cells. These cells were cultured at 1×10^6 cells/ml in RPMI-1640+2% heat-inactivated human AB serum, antibiotics, HEPES buffer, L-glutamine, and recombinant IL-2 at 1000 U/ml. A 4 day LAK cell culture supernatant was utilized as described above in TIL culture medium, and 3- to 8-day LAK cells were used as effectors in cytotoxicity assays.

Stimulator/target cells. Aliquots of fresh tumor digest, from which TIL cultures were initiated, were cryopreserved until used as stimulators in cytokine assays, or as targets in cytotoxicity assays. After the tumors had been and washed to remove dimethylsulfoxide, cell viability was assessed. If viability was less than 50%, tumor preparations were placed over a Ficoll density gradient to remove nonviable cells before use in assays.

Six cultured colon carcinoma lines (American Type Culture Collection, Rockville, Md.) were maintained as monolayers in RPMI-1640 medium+10% fetal calf serum. These included: HT29, LoVo, SK-CO-1, SW48, SW480, and WiDr. Fresh cryopreserved melanomas and sarcomas and several cultured melanoma lines were also used as stimulators/targets in some assays. The Daudi lymphoma line, which is lysed by LAK cells but not by natural killer cells, was used as a standard target in cytotoxicity assays.

Cytokine assays. Secretion of cytokines by cultured TIL was measured as described previously [14]. Briefly, TIL used in cytokine assays were washed at least twice to remove IL-2 and conditioned medium and then cultured at 1×10^6 TIL/ml with $(2-5) \times 10^5$ irradiated tumor stimulators/ml in RPMI-1640 medium+10% heat-inactivated human AB serum and 20 U/ml IL-2. Fresh cryopreserved tumor stimulators were thawed rapidly and washed to remove dimethylsulfoxide before use in assays. Cultured tumor stimulators were harvested with brief exposure to 0.05% trypsin plus 0.02% EDTA (trypsin/Versene, Biofluids, Rockville, Md.) and washed. A ^{137}Cs source was used to deliver 15 Gy to fresh tumors and 30 Gy to cultured tumors before their use as stimulators. TIL were cocultured with fresh autologous tumor or allogeneic fresh or cultured tumors in 1-ml volumes in 24-well tissue-culture plates at 37° C. As a positive control in each assay, TIL were cultured in 24-well plates containing immobilized anti-CD3 mAb (OKT3, Ortho Pharmaceuticals, Raritan, N.J.). Cultures of TIL alone and tumor alone were also included as controls in each assay. Culture medium was collected after incubation for 6–24 h, centrifuged to remove cells, and stored at –70° C until assayed for the presence of cytokines. Optimization of this assay system using melanoma TIL has been previously described [14].

Culture supernatants were tested for the presence of various cytokines using commercially available enzyme-linked immunosorbent assay (ELISA) kits for GM-CSF (Factor-Test human GM-CSF ELISA test kit, Genzyme Corporation, Boston, Mass.; minimum detectable concentration = 4 pg/ml), IFNγ (human gamma interferon ELISA test kit, Amgen Biologicals, Thousand Oaks, Calif., minimum detectable concentration = 0.3 U/ml, or Intertest human interferon gamma ELISA, Genzyme Corporation, minimum detectable concentration = 100 pg/ml), and TNFα (Quantikine human TNFα immunoassay, R&D Systems, Minneapolis, Minn., minimum detectable concentration = 4.8 pg/ml, or Factor-Test human TNFα ELISA test kit, Genzyme Corporation, minimum detectable concentration = 12 pg/ml). Concentrations below the minimum detectable values for each kit are reported in this paper as zero. Determinations of cytokine concentrations were performed on 24-h supernatants for GM-CSF and IFNγ, and on 6- to 24-h supernatants for TNFα.

Cytokine secretion in response to tumor stimulation was considered significant if it exceeded twice the secretion by unstimulated TIL and exceeded twice the minimum detectable concentration of the ELISA.

Inhibition of cytokine secretion with monoclonal antibodies. We attempted to inhibit cytokine secretion from three TIL cultures by pretreatment of tumor stimulators with either the irrelevant anti-Thy1.2 mAb (against murine T cells, 250 μg/ml; Becton Dickinson, Mountain View, Calif.), W6/32 (anti-HLA-A,B,C, 500 μg/ml; Sera Lab, Accurate Chemical & Scientific Corporation, Westbury, N.Y.), IVA12 (anti-HLA-DR, DP, DQ, purified hybridoma supernatant, 500 μg/ml) or the combination of W6/32 and IVA12. Tumor was incubated with a 1 : 5 dilution of mAb

Table 1. Proliferation of tumor-infiltrating lymphocytes (TIL) derived from colon adenocarcinomas

Tumor	Tissue source	Lymphocytes[a] (%)	Expansion index	Time in culture (days)
556[c]	Subcutaneous metastasis	27	152	52
565[c]	Hepatic metastasis	37	782	49
709[c]	Hepatic metastasis	53	2.2×10^4	63
802[c]	Hepatic metastasis	42	19	26[b]
810[c]	Primary colon carcinoma	53	1.5×10^3	76
824[c]	Hepatic metastasis	66	419	34
863[c]	Hepatic metastasis	52	606	34
867[c]	Pulmonary metastasis	63	2.1×10^3	42
881	Hepatic metastasis	33	359	42
908	Hepatic metastasis	26	1.5×10^4	49
917	Adrenal metastasis	70	13	57[b]
920	Hepatic metastasis	27	2.1×10^3	58[b]
926	Hepatic metastasis	39	4.2×10^3	59
934[d]	Hepatic metastasis	36	155	53
936[d]	Hepatic metastasis	36	506	48
946[c]	Ascites	93	64	54
1085[c]	Hepatic metastasis	32	102	41
1099	Hepatic metastasis	73	2.8×10^4	43
1156	Hepatic metastasis	64	2.9×10^3	40
Median		42	606	49

[a] Estimated microscopically from single-cell tumor digests
[b] TIL cultured beyond day indicated. Maximum expansion index noted
[c] TIL cultured from cryopreserved tumor
[d] TIL previously cryopreserved, thawed and further expanded

in 0.5-ml volumes in 24-well plates for 30 min at room temperature before TIL were added and cocultured as described above.

Cytotoxicity assays. The cytolytic activity of TIL against autologous and allogeneic tumor targets was measured in standard 4-h ^{51}Cr-release assays as described previously [20]. Target lysis exceeding 10% at an effector-to-target (E : T) ratio of 40 : 1 was considered significant. Lysability of all tumor targets was assessed using LAK cells as effectors.

Phenotypic analyses. Flow-cytometric analyses of TIL cultures were performed as previously described [20], using the mAb anti-Leu4 (anti-CD3), anti-Leu3 (anti-CD4), anti-Leu2 (anti-CD8), anti-Leu19 (anti-CD56), anti-HLA-DR (all from Becton Dickinson, Mountain View, Calif.), and W6/32 (anti-HLA-A,B,C) (Sera-Lab, Accurate Chemical & Scientific Corporation, Westbury, N.Y.).

Cultured colon tumor lines were phenotyped to assess MHC and adhesion molecule expression using anti-HLA-DR, W6/32, and 84H10 (anti-ICAM-1, intercellular adhesion molecule, kidnly provided by Dr. S. Shaw, NIH, Bethesda, Md.). Anti-Thy1.2 (specific for murine T lymphocytes, Becton Dickinson) was used as a negativestaining control. Counterstaining, when necessary, was performed with fluorescein-conjugated goat anti-mouse IgG (Becton Dickinson).

HLA typing. HLA typing of peripheral blood lymphocytes from donors of TIL and fresh tumors was performed by the HLA Laboratory of the Clinical Center, National Institutes of Health (Bethesda, Md.) [6]. HLA phenotypes of cultured colon carcinoma lines were obtained from the American Type Culture Collection (Rockville, Md.).

Results

Colon tumor characteristics and TIL proliferation

TIL were successfully cultured from 19 consecutive colon carcinoma specimens, as shown in Table 1. Of these TIL cultures, 10 were initiated from cryopreserved tumor specimens, and 7 from fresh tumor digests. Two additional cultures (TIL 934 and 936) were initiated from fresh tumor digests, established in culture and cryopreserved; these TIL were thawed and further expanded before use in assays.

Single-cell tumor digests, used to initiate TIL cultures, contained a median of 42% lymphocytes (range 26%–93%). All TIL cultures proliferated without tumor restimulation except TIL 917, derived from an adrenal metastasis, which proliferated only with biweekly tumor restimulation.

The TIL expansion index was defined as the number of TIL grown in culture divided by the starting number of lymphocytes. Expansion indices ranging from 13 to over 28000 (median 606) were attained at 26–76 days of culture (median 49 days). All cultures were still expanding when electively terminated at the completion of studies except TIL 802, 917 and 920, which were maintained until their demise at days 41, 100 and 80 respectively.

TIL culture phenotypes

Cell-surface phenotypes of 19 TIL cultures were determined within 10 days of cytotoxicity and cytokine assays (exception: TIL 946, 17 days), and are shown in Table 2. The majority of TIL were CD3+ (92.5 ± 1.9%, mean ± SEM). There was a predominance of CD4+ cells

Table 2. Phenotypes of colon TIL cultures

TIL	Culture day	Positive cells (%)					
		CD3	CD4	CD8	CD56	W6/32	HLA-DR
556	45	69	25	58	33	ND[a]	78
565	38	96	33	65	1	ND	74
709	41	96	59	33	16	98	63
802	44	95	86	9	9	ND	34
810	22	93	77	13	4	ND	68
824	34	92	23	68	13	ND	68
863	35	97	23	74	2	ND	75
867	34	98	45	69	1	ND	66
881	35	87	69	25	11	ND	62
908	41	99	39	63	2	99	45
917[b]	42	75	96	0	1	ND	17
920	55	98	96	0	1	96	87
926	56	98	46	59	1	ND	ND
934	56	90	64	30	4	95	36
936	43	87	7	47	51	96	31
946	30	95	54	33	1	96	77
1085	57	97	58	37	3	99	64
1099	40	98	44	59	2	100	80
1156	31	98	80	19	2	100	70
Mean ± SEM	41 ± 2.2	92.5 ± 1.9	53.9 ± 5.9	40.1 ± 5.6	8.3 ± 3.0	97.7 ± 0.6	60.8 ± 4.6

[a] Not done
[b] TIL culture stimulated with irradiated autologous tumor on culture days 16, 31, 45, 76

Table 3. Cytolytic activity of colon TIL

TIL	Culture day	Target lysis[a] (%)					
		Autologous tumor	Fresh allogeneic colon tumor	Cultured allogeneic colon tumor	Cultured non colon tumor	Daudi	LAK[b] cell lysis of autologous tumor
556	44	1	–4	ND[c]	11	31	5
565	37	–6	1	ND	6	–3	–5
709	43	1	–1	ND	0	–22	0
802	41	5	6	ND	8	8	18
810	22	9	8	ND	3	12	18
824	34	–15	2	ND	11	41	–3
863	34	–2	–1	ND	–1	14	1
867	34	1	2	ND	–3	0	18
881	34	–2	–2	ND	2	18	11
908	36	4	7	ND	2	1	–1
917[d]	43	–1	2	ND	–5	–1	11
920	52	–1	2	10	1	–2	15
926	51	3	3	45	ND	46	27
934	62	–1	4	36	11	3	16
936	41	4	17	59	38	73	6
946	47	1	6	7	–1	8	12
1085	49	–5	2	22	1	–3	0
1099	37	2	1	5	ND	10	9
1156	ND						

[a] E : T = 40; results of 4 h ^{51}Cr-release assays
[b] Lymphokine-activated killer cells
[c] Not done
[d] TIL culture stimulated with autologous tumor at culture days 16, 31, 45, 76

Table 4. HLA phenotypes of colon carcinoma patients whose TIL were tested for cytokine secretion[a]

TIL	HLA-A	HLA-B	HLA-Cw	HLA-DR	HLA-DRw	HLA-DQw
556	2,26	13,49	–,–[b]	1,7	53,–	2,5
709	2,23	18,44	4,5	3,7	53,–	2,–
908	1,11	8,35	4,7	1,15	–,–	5,6
920	1,2	8,51	6,7	1,3	52,–	1,2
926	1,3	8,51	7,–	4,17	52,–	2,8
934	2,3	7,44	–,–	13,–	52,–	6,–
936	3,23	44,–	4,–	–,–	–,–	–,–
946	1,28	8,45	7,–	6,11	52,–	7,–
1085	3,11	39,44	7,–	15,–	52,–	6,–
1099	1,–	8,57	6,7	7,17	52,53	2,9

[a] HLA typing was performed on peripheral blood lymphocytes or cultured TIL
[b] Could not be determined

Table 5. Cytokine secretion by TIL 709[a]

Incubation	Stimulator	HLA match	Cytokine secretion[b]		
			GM-CSF (pg/ml)	IFNγ (pg/ml)	TNFα[c] (pg/ml)
TIL alone			27	109	0
+anti-CD3			**>512**	**3454**	**>800**
+autologous tumor	709[d]	Autologous	54	**269**	**50**
	908[d]	Cw4	28	0	0
	920[d]	A2,DR3,DQw2	24	0	0
	HT-29	Cw5	10	0	0
	SK-CO-1	φ	17	0	0
	SW48	φ	**76**	0	0
	SW480	A2	19	0	0
	WiDr	B18	8	0	0
	397-mel	Cw4,DRw53	**140**	0	14
	624-mel	A2,DR7, DRw53,DQw2	22	0	0

[a] TIL culture day 51
[b] Cytokine secretion by tumor alone subtracted from these values: granulocyte/macrophage-colony-stimulating factor (GM-CSF), 0–26 pg/ml; interferon γ (IFNγ), undetectable; tumor necrosis factor α (TNFα), 0–130 pg/ml. Values in bold type are considered significant (see Materials and methods)
[c] 14-h incubation for TNFα; 24-h incubation for other cytokines
[d] Fresh colon carcinoma

over CD8+ cells, with mean values of 53.9% and 40.1% respectively. Two cultures were pure CD4+ populations (TIL 917, 920), and 5 were predominantly CD4+ (CD4/CD8 >2; TIL 802, 810, 881, 934, and 1156). Four cultures were predominantly CD8+ (CD4/CD8 <0.5; TIL 556, 824, 863 and 936). The remaining 8 TIL cultures were mixed CD4/CD8 populations. Among 9 cultures stained with W6/32, 95% of cells expressed MHC class I molecules. The mean proportion of cells expressing HLA-DR, a marker for T cell activation, was 60.8%. Only 5 of 19 TIL cultures contained more than 10% CD56+ cells.

Table 6. Cytokine secretion by TIL 920[a]

Incubation	Stimulator	HLA match	Cytokine secretion[b]		
			GM-CSF (pg/ml)	IFNγ (pg/ml)	TNFα[c] (pg/ml)
TIL alone			0	0	0
+anti-CD3			**>512**	**3117**	**>800**
+autologous tumor	920[d]	Autologous	**337**	**388**	**234**
	908[d]	A1,B8,Cw7, DR1	**9**	0	0
	926[d]	A1,B8,B51,Cw7, DRw52,DQw2	**>512**	**1682**	**671**
	HT-29	A1	0	0	0
	SK-CO-1	A1	4	0	0
	SW48	φ	5	0	0
	SW480	A2,B8	4	0	0
	WiDr	φ	0	0	0
	397-mel	A1,B8	0	0	0
	624-mel	A2,Cw7,DQw2	0	0	0

[a] TIL culture day 51
[b] Cytokine secretion by tumor alone subtracted from these values: GM-CSF, 0–26 pg/ml; IFNγ, undetectable; TNFα, 0–130 pg/ml. Values in bold type are considered significant (see Materials and methods)
[c] 14-h incubation for TNFα; 24-h incubation for other cytokines
[d] Fresh colon carcinoma

Cytolytic activity of colon carcinoma TIL

Cultures were tested for cytolytic activity during the period of active proliferation (days 22–62) against fresh autologous tumor, fresh and cultured allogeneic tumors, and Daudi cells. Lysis of tumor targets at an E:T ratio of 40:1 in standard 4-h ^{51}Cr-release assays was considered to be significant if it exceeded 10%. As shown in Table 3, cytolysis by 18 of the 19 colon TIL cultures was studied, and none of these TIL cultures exhibited significant lysis of autologous tumor regardless of their phenotypes. Only TIL 936 (51% CD56+) lysed any fresh colon tumor. Several TIL cultures lysed cultured colon tumors, cultured non-colon tumors, and/or Daudi cells. LAK cells lysed only 9 of the 18 fresh colon tumors from which TIL cultures were derived, confirming the relatively poor lysability of fresh colon adenocarcinomas [7, 22, 23].

Cytokine secretion by colon carcinoma TIL

Recent studies in our laboratory have suggested that specific (MHC-restricted) recognition of autologous tumor by CD8+ melanoma TIL or CD4+ breast carcinoma or lymphoma TIL can result in secretion of the cytokines GM-CSF, IFNγ, and TNFα [6a, 14–16]. Thus, we chose to explore this phenomenon with colon carcinoma TIL, analyzing 10 TIL cultures for which autologous tumor was still available.

In our previous studies of CD8+ melanoma TIL, cytokines could be secreted in response to autologous as well as HLA-matched allogeneic melanoma stimulators, indi-

Table 7. Cytokine secretion by TIL 926[a]

Incubation	Stimulator	HLA match	Cytokine secretion[b]		
			GM-CSF (pg/ml)	IFNγ (pg/ml)	TNFα[c] (pg/ml)
TIL alone			12	0	0
+anti-CD3			**>512**	**1396**	**591**
+autologous tumor	926[d]	Autologous	**467**	**142**	**156**
	709[d]	DQw2	**36**	0	0
	908[d]	A1,B8,Cw7	**42**	0	0
	934[d]	A3,DRw52	**40**	0	0
	HT-29	A1,3	4	0	0
	SK-CO-1	A1,3	6	0	0
	SW48	φ	9	0	0
	SW480	B8	11	0	0
	WiDr	φ	4	0	0
	397-mel	A1,B8,DR4	5	0	0
	624-mel	A3,Cw7,DQw2	4	0	0

[a] TIL culture day 50

[b] Cytokine secretion by tumor alone subtracted from these values: GM-CSF, 0–26 pg/ml; IFNγ, undetectable; TNFα, 0–125 pg/ml. Values in bold type are considered significant (see Materials and methods)

[c] 24-h incubation for all cytokines

[d] Fresh colon carcinoma

cating the presence of shared tumor antigens [6 a]. Thus prior to our studies of colon TIL cytokine secretion, HLA typing of the colon TIL or patient peripheral blood mononuclear cells corresponding to fresh tumor stimulators was performed (Table 4). HLA-A,B,C typing of the cultured colon tumor lines used as cytokine stimulators was obtained from the American Type Culture Collection and appears in Tables 5–7. Cell-surface phenotyping of cultured lines with flow cytometry showed that none expressed MHC class II antigens (no staining with mAb IVA12). In addition, the line SW48 did not express significant numbers of MHC class I molecules, as shown by negative staining using mAb W6/32. However, all colon lines stained positively for the adhesion molecule ICAM-1 (data not shown).

Among the 10 TIL cultures studied for cytokine secretion in response to autologous tumor stimulation, 1 (TIL 709) secreted significant amounts of IFNγ and TNFα, and 2 cultures (TIL 920 and 926) secreted significant amounts of GM-CSF, IFNγ, and TNFα specifically when stimulated with autologous tumor. In addition, 2 cultures (TIL 908 and 920) secreted significant amounts of all three cytokines when stimulated with HLA-matched allogeneic colon tumors.

Cytokine secretion by TIL 709, which contained a mixture of CD4+ and CD8+ cells, is shown in Table 5. Unstimulated TIL produced 27 pg/ml GM-CSF, and stimulation with autologous tumor increased release to 54 pg/ml, which did not quite reach our criterion for significant secretion (greater than 2-fold increase over secretion by unstimulated TIL); however, an HLA-unmatched cultured colon tumor (SW48) and a cultured melanoma nonspecifically stimulated significant secretion of GM-CSF. Unstimulated TIL 709 secreted 109 pg/ml IFNγ and stimulation with autologous tumor resulted in 269 pg/ml IFNγ; stimulation by 9 allogeneic tumors (2 fresh colon tumors, 5 cultured colon carcinoma lines, and 2 cultured melanomas), 7 of which were matched for various HLA molecules expressed by these TIL, did not result in secretion of IFNγ. Likewise, stimulation with autologous 709 tumor, but not any allogeneic tumor, resulted in significant TNFα secretion. Autologous tumor therefore stimulated TIL 709 to secrete significant amounts of IFNγ and TNFα, but not GM-CSF. Large quantities of all three cytokines were secreted by TIL stimulated with anti-CD3 mAb. This assay was repeated once with similar results.

As shown in Table 6, unstimulated TIL 920 (exclusively CD4+) secreted none of the three cytokines but, on stimulation with autologous tumor, 337 pg/ml GM-CSF, 388 pg/ml IFNγ, and 234 pg/ml TNFα were secreted by these TIL. In addition, stimulation with tumor 926, an allogeneic fresh colon tumor sharing four HLA class I and two HLA class II antigens with TIL 920, resulted in secretion of large amounts of all three cytokines (>512 pg/ml GM-CSF, 1682 pg/ml IFNγ, and 671 pg/ml TNFα). One additional HLA-matched colon tumor, 908, stimulated significant secretion of GM-CSF only, although the amount secreted (9 pg/ml) was much lower than after stimulation with tumors 920 or 926. Thus, stimulation of TIL 920 with autologous tumor or the HLA-matched allogeneic colon carcinoma 926 resulted in secretion of significant amounts of all three cytokines, which was not seen on stimulation with six other allogeneic colon carcinoma or two melanomas. Similar results were obtained when this assay was repeated twice.

TIL 926 (Table 7), a mixed CD4+/CD8+ culture, secreted a baseline amount of 12 pg/ml GM-CSF, but no IFNγ or TNFα. Autologous tumor stimulated secretion of GM-CSF (467 pg/ml), IFNγ (142 pg/ml), and TNFα (156 pg/ml). While IFNγ and TNFα secretion was restricted to autologous tumor stimulation, low but significant levels of GM-CSF (36–42 pg/ml) were also secreted in response to three HLA-matched allogeneic fresh colon tumors.

Note that the experiments summarized in Tables 5–7 were performed simultaneously. Thus, while tumor 709 elicited secretion of IFNγ and TNFα by autologous TIL, it failed to stimulate secretion by TIL 926, further supporting the immune specificity of the autologous TIL/tumor interaction. Likewise, tumor 920 stimulated cytokine secretion by autologous TIL, but not by TIL 709. In contrast, tumor 926 stimulated cytokine secretion from both autologous TIL and HLA-matched TIL 920. The immune specificity of these interactions was further explored with mAb inhibition studies (see below).

In addition to the 3 TIL cultures described above, TIL 908 (mixed CD4+/CD8+) secreted significant amounts of all three cytokines upon stimulation with the allogeneic fresh colon carcinoma 920, which was matched for HLA-A1, -B8, -Cw7, and -DR1. However, stimulation by autologous tumor or other allogeneic tumors did not result in cytokine secretion (Table 8, discussed below).

Among the 6 remaining TIL assayed for cytokine secretion (TIL 556, 934, 936, 946, 1085, and 1099), none secreted significant amounts of any cytokine in response to

Table 8. Antibody blocking of cytokine secretion by colon TIL

TIL (culture day)	Stimulator	Cytokine secretion[a] (% inhibition)									
		GM-CSF (pg/ml)					TNFα (pg/ml)				
		No mAb	Anti-Thy1.2	W6/32	IVA12	W6/32+ IVA12	No mAb	Anti-Thy1.2	W6/32	IVA12	W6/32+ IVA12
709 (day 31)	None	104					54				
	Anti-CD3	>512					>800				
	709 tumor	261	281 (–8)	229 (12)	140 (46)	103 (61)	260	263 (–1)	94 (64)	88 (66)	51 (80)
908 (day 43)	None	0					0				
	Anti-CD3	>512					420				
	908 tumor	0					0				
	920 tumor	36	39 (–8)	59(–64)	0 (100)	4 (89)	78	84 (–8)	82 (–5)	0(100)	0(100)
920 (day 42)	None	18					0				
	anti-CD3	>2048					>800				
	920 tumor	532	676(–27)	512 (4)	157 (70)	156 (71)	248	271 (–9)	177 (29)	15 (94)	40 (84)

[a] Results of 24-h coincubation of 1×10^6 TIL plus 4×10^5 tumor stimulators in 1-ml volumes. mAb added as indicated

autologous tumor stimulation. In addition, none secreted IFNγ in response to any allogeneic tumor. Four cultures (TIL 556, 934, 936, 1085) secreted significant amounts of GM-CSF and/or TNFα nonspecifically when stimulated with allogeneic tumors including HLA-unmatched colon carcinomas or melanomas. All TIL secreted cytokines in response to anti-CD3 mAb (GM-CSF 171 to more than 512 pg/ml, TNFα 396 to more than 800 pg/ml, IFNγ 252 to more than 6400 pg/ml), proving that they were capable of this function.

Inhibition of cytokine secretion by anti-MHC monoclonal antibodies

We attempted to block cytokine secretion by TIL 709 and 920 when stimulated with autologous tumor or, in the case of TIL 908, when stimulated with the allogeneic tumor 920, using mAb directed against MHC class I (W6/32) and class II (IVA12) molecules (Table 8). Unfortunately, tumor 926 was not available for further studies.

TIL 709 were a mixed CD4+/CD8+ culture (Table 2). As shown in Table 8, both GM-CSF and TNFα were secreted in response to autologous tumor, and secretion was not inhibited by the irrelevant mAb anti-Thy1.2. While W6/32 inhibited GM-CSF secretion by 12% and IVA12 inhibited GM-CSF secretion by 46%, the combination of both mAb showed augmented inhibition over that of either mAb alone (61%), suggesting that both CD8+ and CD4+TIL were involved in cytokine secretion. A similar pattern was seen for inhibition of TNFα secretion by TIL 709; however, W6/32 was somewhat more effective in blocking TNFα secretion (64% inhibition) than GM-CSF secretion by these TIL. Low levels of IFNγ secreted by these TIL were effectively blocked by both W6/32 and IVA12 (data not shown).

For TIL 908, a mixed CD4+/CD8+ population, autologous tumor failed to stimulate cytokine secretion but the HLA-matched allogeneic colon carcinoma 920 did stimulate, as also noted in a second experiment (data not shown). Neither mAb anti-Thy1.2 nor W6/32 inhibited secretion of GM-CSF or TNFα by TIL 908 in response to tumor 920, whereas mAb IVA12 completely inhibited secretion of both cytokines. Detectable levels of IFNγ were not secreted by TIL 908 in this experiment. Thus, although TIL 908 contain a population of CD8+ cells, it seems that cytokine secretion is mediated by CD4+ cells operating in an MHC class II context.

TIL 920 was shown to be a pure CD4+ cell population by phenotyping at culture day 55 (Table 2), which was approximately the age of the culture when cytotoxicity and cytokine assays were performed (Tables 3, 6). However, antibody-blocking studies were performed on culture day 42. Phenotyping of these TIL on day 38 showed a population that was 87% CD3+, 71% CD4+, and 16% CD8+. Anti-Thy1.2 did not inhibit secretion of either GM-CSF or TNFα in response to autologous tumor. TNFα but not GM-CSF secretion was partially inhibited by W6/32, but both cytokines were effectively blocked by IVA12. It is possible that, although most cytokine is secreted by the phenotypically dominant CD4+ TIL population, some cytokine is secreted by the small CD8+ population as well, which is inhibited by the mAb W6/32.

In summary, these data indicate that cytokine secretion by colon-carcinoma-derived TIL in response to autologous or HLA-matched allogeneic colon tumors is an MHC-restricted event that can be mediated by both CD4+ and CD8+ cells.

Discussion

The successful treatment of some patients with metastatic melanoma using adoptively transferred TIL [13] has prompted us to evaluate TIL for the treatment of more common cancers including breast carcinoma [14, 15] and colon carcinoma. In this study, TIL harvested from 18 metastatic colon tumors as well as 1 primary tumor could be expanded in the presence of IL-2 without tumor restimulation in 18 of 19 cases; one culture required autolo-

gous tumor restimulation for proliferation. The 606-fold median expansion in 49 culture days suggests that many of these cultures could have reached cell numbers suitable for TIL therapy, especially since 16 cultures were proliferating actively at the time they were electively terminated.

We have previously demonstrated that some CD8+ melanoma TIL can lyse autologous and HLA-matched allogeneic tumors in an MHC-class-I-restricted manner [6]. However, in this study we have shown that colon TIL were most commonly mixed CD4+/CD8+ populations and were not cytolytic for autologous tumor in any of the 18 cultures tested. The finding that colon tumors are resistant to TIL lysis is consistent with the experience of other investigators [7, 17, 22, 23]. Cytolysis could possibly have been optimized under different culture conditions, perhaps by using tumor restimulation of long-term TIL cultures. It is also possible that CD8+ TIL did not lyse colon tumors because of decreased or absent MHC class I molecule expression by these tumors. HLA typing of our colon carcinoma patients was performed on PBL or TIL from those patients and, because of limited amounts of tissue, their fresh tumors were not directly HLA typed. A recent report that expression of both MHC class I and II antigens can be reduced in colon carcinoma supports this possible explanation for failure of at least some tumors to be lysed [8]. Nevertheless, our fresh colon tumors proved relatively resistant to lysis by non-MHC-restricted LAK cells as well as TIL, suggesting that these tumors may be inherently resitant to lysis in short-term in vitro assays. The high percentage of lymphocytes in some of our fresh tumor preparations may have contributed to poor lysis.

Our analysis of cytokine secretion by colon TIL was prompted by the recent finding that some CD8+ melanoma TIL can secrete the cytokines GM-CSF, IFNγ, and TNFα specifically when stimulated by autologous tumor [14], and that some HLA-matched allogeneic melanomas can also stimulate significant cytokine secretion by these TIL [6 a]. Among a total of 42 melanoma TIL cultures tested, 17 manifested specific recognition of autologous tumor via cytokine secretion (some data unpublished). In addition, we have found that 3 of 11 breast carcinoma TIL, including 2 cultures that were at least 96% CD4+ and not lytic for autologous tumor, were nevertheless able to secrete cytokines specifically when stimulated with autologous tumor [14, 15], providing evidence for immune recognition of human breast carcinoma. Furthermore, a study of 10 TIL cultures derived from B cell lymphomas has revealed 1 culture that secreted cytokines specifically to autologous tumor, and this was mediated by CD4+ cells [16]. In the current study of cytokine secretion by 10 colon TIL cultures, 2 (TIL 709, 926) secreted significant amounts of IFNγ and TNFα only when stimulated with autologous tumor, and not when exposed to 9 or 10 different allogeneic tumors, respectively. A 3rd TIL culture (TIL 920) secreted substantial amounts of these two cytokines as well as GM-CSF specifically when stimulated with autologous tumor or 1 fresh HLA-matched allogeneic colon tumor. Finally, TIL 908 did not secrete cytokines in response to autologous tumor stimulation, but did secrete GM-CSF, IFNγ and TNFα when stimulated with the HLA-matched allogeneic 920 colon carcinoma. The ability of colon carcinoma TIL to secrete cytokines when stimulated with autologous or select HLA-matched allogeneic colon tumors suggests specific recognition of a tumor-associated antigen that can be shared among different patients. Again, since our fresh colon tumors were not directly HLA-typed, it is possible that the failure of some tumors to induce cytokine secretion may be explained by decreased or absent expression of MHC molecules. At this point, it is speculative whether TIL are capable of tumor-stimulated cytokine secretion in situ as well as in vitro.

The immune specificity of colon TIL cytokine secretion was shown with antibody-blocking studies suggesting that both CD4+ and CD8+ cells are capable of secreting GM-CSF and TNFα in an MHC-restricted manner. Some reports have suggested that IFNγ is secreted by both CD4+ and CD8+ human T cell clones, but that GM-CSF and TNFα are secreted only by CD4+ T cells [9, 10]. However, our data suggest that CD8+ colon TIL can secrete TNFα and GM-CSF when stimulated by autologous tumor, since the mAb W6/32 inhibited secretion by both TIL 709 and TIL 920 to some extent. Examination of purified CD4+ and CD8+ melanoma TIL subpopulations has shown definitively that CD8+ cells can secrete IFNγ, TNFα, and GM-CSF upon tumor stimulation [14]. We have not addressed the possibility that LAK cell subpopulations in TIL cultures might also contribute to cytokine secretion. In this regard, it should be noted that Chong et al. have documented the ability of human LAK cells to secrete IFNγ and TNFα on contact with a variety of lymphomas or leukemias [4].

This evidence for MHC-restricted cytokine secretion by colon TIL stimulated with autologous or select HLA-matched allogeneic tumors suggests that TIL therapy for patients with colon carcinoma may be worthy of investigation. Direct antitumor effects of IFNγ and TNFα [18, 21] and activation of leukocytes with antitumor functions by GM-CSF [5] have been reported, and are possible mechanisms by which colon TIL secreting cytokines might exert antitumor effects. Another reported function of both IFNγ and TNFα is to enhance expression of MHC molecules and tumor-specific antigens on neoplastic cells, possibly making these cells more susceptible to immune recognition [3, 11]. Recent studies by Barth et al. [2] have shown a strong correlation of specific cytokine secretion by murine TIL and their therapeutic effectiveness against established lung metastases in vivo.

Specific cytokine secretion by colon carcinoma TIL stimulated with autologous or some HLA-matched allogeneic colon tumors affords a reproducible and readily quantifiable assay for specific T cell recognition of these tumors, alternative to the proliferation and lysis assays that have been the mainstays of past studies. The demonstration of this phenomenon provides new impetus for the development of immunotherapeutic regimens for patients with colon cancer.

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