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Effects of interferon-gamma and tumour necrosis factor-alpha on the development of cytotoxic T lymphocytes in autologous mixed lymphocyte tumour cultures with human melanoma

A. D. ROTH, F. J. HORNICEK, C. G. GERSTNER & J. M. KIRKWOOD *Division of Medical Oncology, Department of Medicine, and Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA*

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SUMMARY

We have studied the influence of tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) on the development of cytotoxic T lymphocytes (CTL) against melanoma in mixed lymphocyte tumour cultures (MLTC). In these MLTC, TNF-α at 10^4 U/ml increased the expansion of the CTL up to 10^4-fold over recombinant IL-2 (rIL-2) alone. IFN-γ at 10^4 U/ml and combinations of TNF-α plus IFN-γ at 10^2–10^3 U/ml promoted the proliferation more variably. MLTC generated with rIL-2 showed a predominance of $CD8^+$ cells, while 2 weeks of culture in the presence of IFN-γ at 10^4 U/ml, or with IFN-γ and TNFα at 1×10^2–10^3 U/ml, favoured the emergence of $CD4^+$ cell populations. The cytotoxic activity of the lymphocytes generated in these MLTC showed a consistent decline of K562 cytotoxic activity following exposure to the combination of IFN-γ and TNF-α. Despite the altered T cell subset distribution with different combinations of cytokines, no consistent alteration in the specific anti-tumour cytotoxicity against melanoma was detected. These results suggest that TNF-α and IFN-γ influence the activation, phenotypic, and functional outcome of MLTC-generated CTL, and may account for the phenotypic variations observed in T cell populations generated *in vitro*.

Keywords melanoma interferon-gamma tumour necrosis factor-alpha lymphocytes cytotoxicity

INTRODUCTION

Advances in biotechnology have allowed for the production of large amounts of recombinant IL-2 (rIL-2) which has been used extensively in the development, expansion and study of cytotoxic T lymphocytes (CTL) (Morgan, Ruscetti & Gallo, 1976; Gillis *et al.*, 1978; Kern *et al.*, 1981; Erard *et al.*, 1985; Cheever *et al.*, 1986). However, several laboratories have shown that additional factors in the conditioned medium from activated T cells, originally designated T cell growth factor (TCGF), are important in the development of CTL (Garman & Fan, 1983; Mills *et al.*, 1986; Takai *et al.*, 1986, 1988; Wong *et al.*, 1988). Among these factors, interferon-gamma (IFN-γ) has been studied extensively for its immunomodulatory properties and its ability to enhance the expression of the MHC antigens by normal and neoplastic cells (Bonnem & Oldham, 1987). The increase of MHC class II antigen expression by tumour cells and T lymphocytes induced by IFN-γ may constitute a positive feedback loop in the development of CTL against autologous tumour (Guerry *et al.*, 1987). A co-factor in the production of IFN-γ by lymphocytes appears to be tumour necrosis factor-alpha (TNF-α) (Guerry *et al.*, 1987). TNF-α has been shown to have pleiotropic effects upon neoplastic as well as normal cells (Phillip & Epstein, 1986; Zucali *et al.*, 1987; Ranges *et al.*, 1987; Iigo *et al.*, 1988; Talmadge *et al.*, 1988). The wide range of activities of IFN-γ and TNF-α reported in studies of the development of CTL in different models suggests that these cytokines may play a significant role, in addition to IL-2, in the development and expression of cytotoxicity against autologous tumour *in vitro*. Enhancement of the recognition of melanoma-associated antigens by CTL may be possible by using IFN-γ and/or TNF-α with rIL-2.

We have been able to obtain CTL active against the autologous tumour by incubating peripheral blood mononuclear cells (PBMC) from melanoma patients with autologous tumour, in the presence of low doses of a crude preparation of TCGF and rIL-2 (Roth & Kirkwood, 1988). Here we have

A.D.R. present address: Division of Hemato-Oncology, Hospital Cantonal Geneva, Switzerland.

Correspondence: John M. Kirkwood, MD, Associate Director, Pittsburgh Cancer Institute, Professor and Chief, Division of Medical Oncology, Department of Medicine, University of Pittsburgh, Montefiore University Hospital, Main Level North 3459 Fifth Avenue, Pittsburgh, PA 15213, USA.

investigated the influence of IFN-γ, TNF-α, and the combination of these cytokines upon the development and persistence of CTL in IL-2 containing autologous mixed lymphocyte tumour culture (MLTC).

MATERIALS AND METHODS

Materials

All cell lines were maintained in either complete Ham's F-10 medium or complete RPMI 1640 medium at 37°C in a 5% CO_2 humidified atmosphere. Supplements to each medium were 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from GIBCO, Grand Island, NY). For MLTC cultures heat-inactivated human AB serum was used instead of FCS.

Monoclonal antibody (MoAb) W6/32 was purchased from Seralab (Crawley Down, UK) and MoAbs CD45RA and CD29 were obtained from Coulter Immunology (Hialeah, FL). All other MoAbs, as well as the FITC goat anti-mouse antibody and the PE and FITC anti-mouse isotypes, were purchased from Becton Dickinson (Mountain View, CA).

For ELISA assays, peroxidase-conjugated goat anti-mouse antibodies and 2,2′-azino-bis(3 ethylbenzthiazoline sulphonic acid) were obtained from Chemicon International (El Segundo, CA) and Sigma Chemical Co. (St Louis, MO), respectively.

Cell lines

The melanoma cell lines used have been established in our laboratory from fresh surgical specimens of melanoma metastatic to lymph nodes, skin, or visceral organs. The cell lines were maintained in complete Ham's F-10 medium and were characterized as melanoma cells. All cell lines but one (PCI-MEL 108) were used in early passages (between passages 4–11) and routinely documented free of mycoplasma contamination. The erythroleukaemia cell line K562 (ATCC Cell Bank, Bethesda, MD) and the Burkitt lymphoma cell line Daudi (ATCC) were maintained in complete RPMI 1640 medium, and were generous gifts of Dr T. Whiteside.

Isolation and cryopreservation of PBMC

Peripheral blood was obtained from patients with metastatic melanoma. PBMC were isolated by Ficoll–Hypaque density gradient centrifugation (Pharmacia, Piscataway, NY). Washed PBMC were cryopreserved at $2–5 \times 10^6$ cells/vial, in RPMI 1640 supplemented with 40% (v/v) FCS and 10% (v/v) DMSO, in a controlled-rate liquid nitrogen freezer (Cryomed, Mt Clemens, MI). The percentages of monocytes and lymphocytes in Ficoll–Hypaque preparations were 6·5±2·1% and 85·4±2·1%, respectively. The remaining fraction of the cells were generally granulocytes as assessed by flow cytometry.

Establishment of MLTC

The MLTC were performed in 24-well plates (Costar, Cambridge, MA), in complete RPMI 1640 medium. In each well, $0·5 \times 10^6$ PBMC were incubated with 25000 irradiated autologous tumour cells (100 Gy) in the presence of 10 BRMP U/ml of rIL-2 (Cetus, Emeryville, CA). TNF-α (Knoll Pharmaceutical, Whippany, NJ) and/or IFN-γ (Biogen Research Corp., Cambridge, MA) were added to the culture at predetermined concentrations for the first 2 weeks of the culture. After 5 days of incubation, the rIL-2 concentration was raised to 50 BRMP U/ml in the cultures, and maintained at this level up to the

Table 1. Proliferation index of the mixed lymphocyte tumour cultures

Patient no./ cell line designation	Cytokines (U/ml)			Cumulative proliferation index (weeks in culture)				
	rIL-2	IFN-γ	TNF-α	2	4	6	8	10
1/PCI-MEL 136	10	—	—	2	3	10	D	—
	10	—	10^4	<1	46	646	2454	D
	10	10^2	10^3	<1	28	5346	81×10^3	220×10^6
2/PCI-MEL 107	10	—	—	3	27	43	D	—
	10	—	10^4	2	16	35	D	—
	10	10^2	10^3	15	23	D	—	—
	10	10^4	—	17	27	D	—	—
3/PCI-MEL 208	10	—	—	2	16	68	D	—
	10	—	10^4	6	156	7056	409×10^3	$1·3 \times 10^6$
	10	10^3	—	<1	2	20	D	—
	10	10^2	10^3	1	40	1558	31×10^3	179×10^3
	10	10^3	10^3	<1	10	706	26×10^3	D
	10	10^4	—	<1	3	117	3525	27×10^3
4/PCI-MEL 103	10	—	—	5	114	DC	—	—
	10	—	10^4	7	444	DC	—	—
	10	10^2	10^3	2	187	DC	—	—
	10	10^3	10^3	2	217	DC	—	—
	10	10^4	—	1	122	DC	—	—

D, dead; DC, discontinued.

termination of the cell cultures. The MLTC were refed every 3 days. After 1 week, 25 000 fresh irradiated autologous tumour cells were added to each MLTC. At the beginning of the second week of culture each MLTC was counted and replated at $0{\cdot}5 \times 10^6$ lymphocytes/well with 25 000 freshly irradiated autologous tumour cells on a weekly basis. When the cultures were split and refed, cell counts were performed and viability determined by Trypan blue dye exclusion. The proliferation index (PI) was defined as total viable cell no. at a particular time/viable cell no. at initiation of culture.

Cytotoxicity test

Cell-mediated anti-tumour cytotoxicity was measured *in vitro*, by using a ^{51}Cr release micro-assay as described elsewhere (Yron & Shohat, 1986). Briefly, monolayer melanoma cells were detached after 5–10 min of incubation in 0·02% (w/v) EDTA in PBS. Target cells ($1–2 \times 10^5$) were suspended in 50 μl of 0·3 M sucrose and were labelled with 100 μCi sodium^{51} chromate (New England Nuclear, Boston, MA) for 90 min at 37°C. The labelled target cells were plated after washing twice in 96-well V-bottomed microtitre plates (Costar) at 500, 1000, or 2000 target cells per microwell, and combined in triplicate, with RPMI 1640 containing 10% (v/v) FCS, and antibiotics with varying numbers of effector cells such that effector-to-target cell (E/T) ratios of 25:1, 12·5:1, 6·25:1 and 3·12:1 were achieved. Spontaneous release and total releasable isotope were determined in sextuplet in medium alone and medium with 0·5% (v/v) Triton X-100 (Sigma), respectively. The plates were centrifuged at 200 *g* for 3 min, and incubated at 37°C in a 5% CO_2

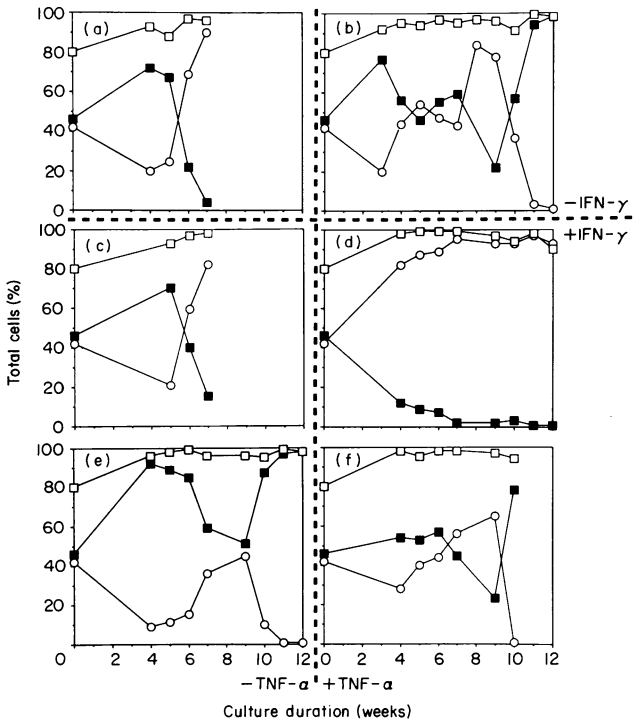

Fig. 1. Expression over time of CD4 (■), CD8 (○), and CD3 (□), by the lymphocytes in different MLTC during the same experiment (patient 3 (see Table 1), experiment 9. This is a representative graph of three experiments in which the phenotypic evolution of T cell populations were determined for patient 3. The same trends were evident in experiment 3 (at 2–6 weeks) and 5 (at 4–8 weeks). The MLTC were set up as described in Materials and Methods. All cultures contained rIL-2 10 U/ml.

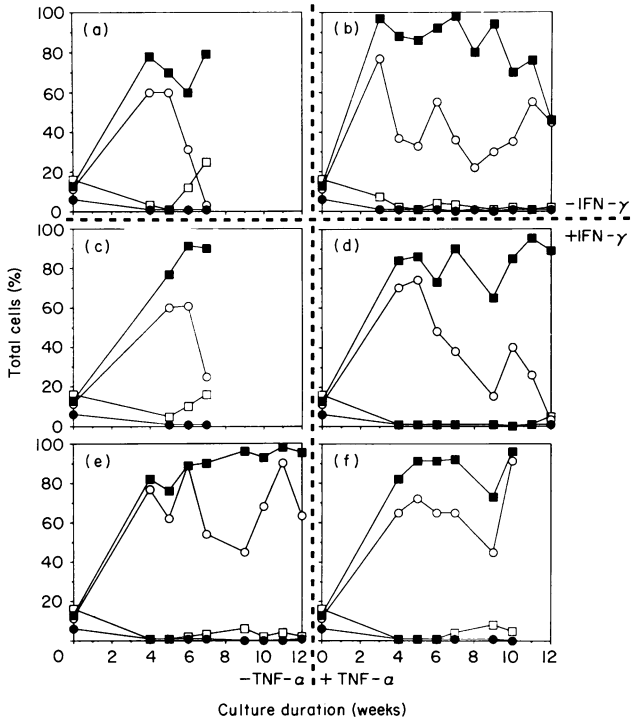

Fig. 2. Expression over time of CD56 (□), TAC (CD25) (○), CD16 (●), and HLA-DR (■), by lymphocytes in MLTC using different combinations of TNF-α and IFN-γ during the experiment for patient 3 (see Table 1), experiment 9. This is a representative graph of three experiments in which the phenotypic evolution of T cell populations was determined for patient 3. The same trends were evident in experiments at 2–6 weeks and at 4–8 weeks. The MLTC were set-up as described in Materials and Methods. All cultures contained rIL-2 at a concentration of 10 U/ml.

Panel	TNF-α (U/ml)	IFN-γ (U/ml)
A	0	0
B	10^4	0
C	0	10^3
D	10^2	10^3
E	0	10^4
F	10^3	10^3

Panel	TNF-α (U/ml)	IFN-γ (U/ml)
A	0	0
B	10^4	0
C	0	10^3
D	10^2	10^3
E	0	10^4
F	10^3	10^3

humidified atmosphere for 4 h. The assay supernatants were then harvested using an SCS Skatron harvester press (Skatron, Lier, Norway) and counted in a beta counter (LKB, 1209 Rackbeta, Gaitherburg, MI) following addition of aqueous scintillation cocktail (Beckman ready-Protein (+); Beckman Instruments, Fullerton, CA). The killing index (KI) was calculated as follows:

$$KI\ (\%) = \frac{\text{Experimental} - \text{Spontaneous release}}{\text{Total} - \text{Spontaneous release}} \times 100\%$$

Phenotyping

Lymphocytes. The lymphocytes were suspended in 400 μl of cold PBS 0·1% (w/v) sodium azide with 5 μl of FITC- or PE-labelled MoAb and incubated for 15 min at 4 C. Cells were resuspended in 200 μl of 2% (w/v) paraformaldehyde after three washes and were passed through a FACstar or a FACstar Plus flow cytometer (Becton Dickinson). FITC- and PE-labelled anti-mouse IgG isotypes were used as control.

Tumour MHC antigen expression

Tumour cell lines were tested by flow cytometry and ELISA.

Flow cytometry analysis. Tumour cells were incubated in 25-cm^2 culture flasks (Costar) with or without TNF-α and/or IFN-γ for 5 days. The cells were then detached with 0·02% (w/v) EDTA in PBS, washed and resuspended in cold PBS containing 0·1% (w/v) sodium azide. The incubations with unlabelled W6/32, anti-DR, anti-DP, and anti-DQ antibodies were performed as described above for the lymphocytes. Labelling was performed with a FITC-conjugated goat anti-mouse antibody at 4 C for 15 min and antibody cells were washed, fixed, and analysed as already described.

ELISA. Confluent cultures of tumour cells plated in 96-well flat-bottomed microtitre plates (Costar), were irradiated (100 Gy) and then incubated in the presence of various concentrations of cytokines at 37 C, 5% CO_2, for 5 days. After removal of the supernatants the plates were air dried, fixed with 200 μl of methanol/well for 10 min, and then 100 μl of PBS with 10% agamma FCS were added to each well. Plates were analysed immediately or frozen at −70 C until used. The ELISA for class

Table 2. Phenotype of the lymphocytes generated in mixed lymphocyte tumour culture

Patient no./cell line designation	Cytokines (U/ml)			Antibody	Cells positive (%) Weeks in culture						
	rIL-2	IFN-γ	TNF-α		2	3	4	5	6	8	10
1/PCI-MEL 136	10	—	—	CD8	49	D					
				CD4	3						
	10	—	10^4	CD8	29		82		82	88	D
				CD4	6		15		16	1	
	10	10^2	10^3	CD8	*		28		4	1	0
				CD4			74		96	43	99
2/PCI-MEL 107	10	—	—	CD8	48	31	52	52	D		
				CD4	29	24	17	27			
	10	—	10^4	CD8	19	31	52	36	D		
				CD4	51	18	16	38			
	10	10^2	10^3	CD8	*	19	29	31	D		
				CD4		75	45	26			
3/PCI-MEL 208	10	—	—	CD8	*	*	63	D			
				CD4			18				
	10	—	10^4	CD8	*	*	79	74	86	89	
				CD4			16	27	11	7	
	10	—	10^5	CD8	*	*	80	73	67	D	
				CD4			2	19	23		
	10	10^2	10^3	CD8	*	*	*	20	27	13	
				CD4				56	57	83	
4/PCI-MEL 103	10	—	—	CD8	20	45	65		DC		
				CD4	72	55	35				
	10	—	10^4	CD8	16	21	23		DC		
				CD4	84	84	79				
	10	10^2	10^3	CD8	*	27	35		DC		
				CD4		73	65				
	10	10^4	—	CD8	*	12	23		DC		
				CD4		89	76				

* Not enough cells for testing.
D, dead; DC, discontinued.

I and II MHC antigens was performed using a peroxidase-conjugated goat anti-mouse antibody and the absorbance at 630 nm (ref. wavelength 490 nm) determined using a Dynatech MR-700 microplate autoreader (Dynatech Instruments, Santa Monica, CA).

RESULTS

Proliferation of the MLTC

Table 1 shows the proliferative capacity of MLTC established from four different melanoma patients. In all cultures, TNF-α and IFN-γ were present for the initial 2 weeks. All cultures but one contained 10 U IL-2/ml for 5 days and 50 U IL-2/ml thereafter. In one (PCI-MEL 208-2) the concentration of rIL-2 was increased to 50 U/ml at 2 weeks. In comparison to IL-2 alone, all experiments except for one (PCI-MEL 107) showed an increased proliferative index (up to 10^4) for the MLTC incubated with TNF-α and/or IFN-γ. The addition of TNF-α at 10^4 U/ml gave the most consistent results, whereas IFN-γ alone and the combination of TNF-α and IFN-γ influenced the outcome of the MLTC more variably.

Phenotypic evolution

Figures 1 and 2 show the phenotypic evolution of lymphocyte populations derived from MLTC during one experiment (against autologous PCI-MEL 208-1). The MLTC incubated from the outset with rIL-2 alone (Fig. 1a), or in conjunction with IFN-γ 10^3 U/ml (Fig. 1c), or with IFN-γ 10^2 U/ml and TNF-α 10^3 U/ml (Fig. 1d), show very similar evolution patterns. The $CD4^+$ population decreased proportionately with a concomitant increase in the population bearing CD8. A dramatic fall in the percentage of cells expressing CD25 precedes the decline in proliferation and termination of the MLTC (see Fig. 2 a,c,d). The MLTC which were started in the presence of TNF-α 10^4 U/ml (Fig. 1b), or TNF-α 10^3 U/ml and IFN-γ 10^3 U/ml and IFN-γ 10^3 U/ml (Fig. 1f), or IFN-γ 10^4 U/ml (Fig. 1e), show the persistence of the $CD4^+$ population and of CD25 expression (Fig. 2b,e,f) with variable proportions of $CD8^+$ cells. No significant changes in the evolution of CD56, CD3, CD16, and DR expression were observed among the different MLTC (Fig. 2).

Similar phenotypic alterations following incubation with TNF-α and/or IFN-γ were observed in other experiments. Table 2 shows the evolution of the CD8- and CD4-bearing cell populations in cultures from a second PCI-MEL 208 and three other patients. An increase in the $CD4^+$ cell population was observed when IFN-γ was used at 10^2 or 10^3 U/ml in combination with TNF. In contrast, incubation with TNF-α alone resulted in a variable outcome of a predominance of $CD8^+$ or $CD4^+$ cells. No systematic changes were noted in the expression of antigens detected by CD45RA and CD29 using the different culture conditions tested (results not shown).

Cytotoxic activity of the MLTC

As shown in Tables 3 and 4, and Fig. 3, natural killer (NK) cell cytotoxicity (K562) was depressed by the combination of IFN-γ and TNF-α. IFN-γ and TNF-α failed to generate consistent alterations of antitumour specific cytotoxicity of T cells from MLTC. The non-specific anti-tumour cytotoxicity of the MLTC was influenced variably by each cytokine from experiment to experiment. In general, we did not detect superiority of one

Table 3. Cytotoxic activity of effector cells* generated in mixed lymphocyte tumour culture (four experiments)

Patient no./cell line designation	Cytokines (U/ml)			Target cells: Killing index (%)							
							Allogeneic melanoma				
	rIL-2	IFN-γ	TNF-α	K562	Daudi	Autologous	Patient 2	Patient 5†	Patient 1	Patient 6‡	Patient 3
1/PCI-MEL 136	10	—	—	100	21	64	18	—	—	—	—
	10	—	10^4	41	5	15	18	5	—	12	10
	10	10^2	10^3	17	0	0	0	5	—	0	5
2/PCI-MEL 107	10	—	—	95	25	31	—	4	2	0	5
	10	—	10^4	97	75	65	—	5	21	18	28
	10	10^2	10^3	64	9	18	—	0	0	5	0
	10	10^4	—	61	5	29	—	0	0	9	3
3/PCI-MEL 208	10	—	—	66	14	62	21	5	57	53	—
	10	—	10^4	61	17	66	33	5	56	67	—
	10	—	10^5	61	23	60	26	0	56	38	—
	10	10^2	10^3	15	10	37	5	0	20	20	—
4/PCI-MEL 103	10	—	—	66	16	28	—	—	5	—	—
	10	—	10^4	56	12	28	—	—	3	—	—
	10	10^2	10^3	59	58	46	—	—	8	—	—
	10	10^3	10^3	36	40	43	—	—	4	—	—
	10	10^4	—	49	28	27	—	—	4	—	—

* Tested at 3 weeks (patients 2 and 4), 4 weeks (patients 1 and 3).
† Cell line PCI-MEL 102.
‡ Cell line PCI-MEL 108.

culture condition over the others for generating cytotoxic activity against the autologous melanoma. However, the MLTC of PCI-MEL 136 performed in the presence of IFN-γ 10^2 U/ml and TNF-α 10^3 U/ml led to the development of a pure CD4-bearing cell line (Table 2) without any detectable anti-tumour cytotoxic activity. This culture population exhibited a very high proliferative response in the presence of the autologous tumour, while no proliferation could be detected against a range of other tumours (results not shown). The lymphocytes grown in MLTC were tested extensively against several allogeneic melanoma lines and no consistency related to the culture conditions could be observed. In Table 4, the evolution of the anti-tumour cytotoxic activity of lymphocytes generated in MLTC containing TNF-α or high concentrations of IFN-γ over time is presented. A decline in the non-specific cytotoxic activity observed over time confirms observations reported previously (Slingluff *et al.*, 1988). The cytotoxic activity against the autologous tumour, once established, persisted throughout the culture period without significant change.

MHC antigen expression by tumour cell lines exposed to IFN-γ and TNF-α

In order to assess whether a major change in MHC antigen expression by the tumour could be correlated with the proliferative and phenotypic alterations observed among the MLTC incubated with or without IFN-γ and/or TNF-α, we performed a phenotypic analysis of the tumour cell lines involved in the MLTC. This analysis was performed by ELISA and flow cytometry. For the ELISA, the same dose of irradiation (100 Gy) applied to the tumour cells used in the MLTC was given before incubation with the different cytokines. By inhibiting tumour growth, irradiation also resolved the potential problem of differential anti-proliferative activities of IFN-γ and TNF-α, and allowed the use of comparable cell concentrations per well in each condition. However, a significant decrease of viability was noted, particularly when the irradiated tumour was subsequently incubated with IFN-γ and TNF-α ($\leq 65\%$). Therefore, this analysis was repeated by flow cytometry using non-irradiated cells.

Table 4. Cytotoxic activity of effector cells generated in mixed lymphocyte tumour culture over time (E:T ratio 25:1) (patient no. 3; cell line PCI-MEL 208)

Cytokines (U/ml)			Week	Target cells: Killing index (%)						
				K562	Daudi	Autologous	Allogeneic melanoma			
rIL-2	IFN-γ	TNF-α	no.			(Pt 3)	Pt 2	Pt 5	Pt 1	Pt 6
10	—	—	4	67	33	32	27	5	34	32
			5	79	47	24	—	—	—	—
			6	86	74	26	48	25	40	35
			7	87	63	33	—	—	—	—
10	—	10^4	4	56	12	57	7	6	57	40
			5	29	5	85	—	—	—	—
			6	3	0	65	0	2	99	62
			7	0	0	60	—	—	—	—
			8	0	0	58	0	0	60	39
			9	3	3	32	2	0	40	46
10	10^3	—	5	72	44	59	—	—	—	—
			6	77	23	49	15	7	64	76
10	10^2	10^3	4	24	0	12	14	0	15	14
			5	0	0	15	—	—	—	—
			6	0	0	42	0	0	45	22
			7	2	0	39	—	—	—	—
			8	0	0	58	0	0	60	39
			9	3	0	30	2	1	48	30
10	10^3	10^3	4	14	0	39	1	0	49	34
			5	10	0	51	—	—	—	—
			6	0	0	41	0	0	89	49
			7	0	6	47	—	—	—	—
10	10^4	—	5	9	3	57	—	—	—	—
			6	9	3	71	0	0	99	44
			7	6	2	53	—	—	—	—
			8	0	12	47	0	0	76	54
			9	0	5	29	0	0	42	48

Cell line designations: Pt 2, PCI-MEL 107; Pt 5, PCI-MEL 102; Pt 1, PCI-MEL 136; Pt 6, PCI-MEL 108; Pt 4, PCI-MEL 103; Pt 3, PCI-MEL 208.

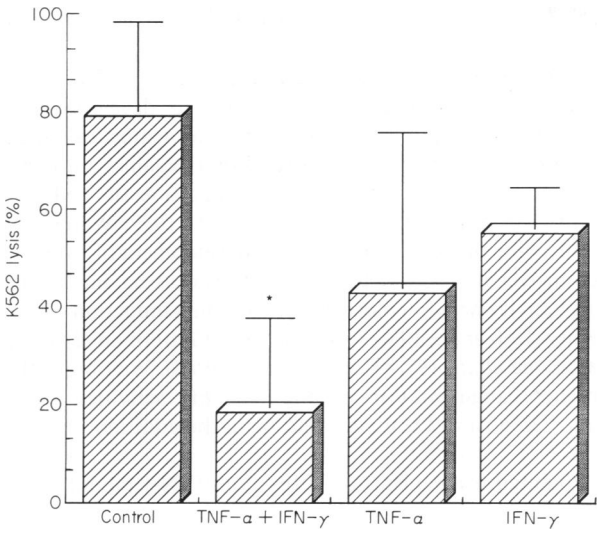

Fig. 3. Depression of K562 activity in cultures of lymphocytes established from MLTC containing different combinations of cytokines. K562 cells were suspended in 0·3 M sucrose and labelled with ^{51}Cr. Target cells were placed into 96-well plates and effector cells were then added so that E:T ratios of 25:1, 12·5:1, 6·25:1, and 3·12:1 were obtained. Following centrifugation, plates were incubated for 4 h, supernatants were then collected and radioactivity determined. A killing index (KI) was determined as

$$\text{KI (\%)} = \frac{\text{Experimental} - \text{Spontaneous release}}{\text{Total} - \text{Spontaneous release}} \times 100\%.$$

The mean of 13 determinations with s.d. are given. $^*P < 0{\cdot}001$.

The viability after 5 days of incubation without irradiation was ≥85% as assessed by Trypan blue dye exclusion, and phenotypic results were the same as those determined by ELISA. In PCI-MEL 208, PCI-MEL 136, and PCI-MEL 103 melanoma cells lines, the incubation in the presence of different doses and combinations of TNF-α and IFN-γ led to an increase in MHC class I, DR, and DP antigen expression but led to less significant augmentation of DQ expression (Table 5). By contrast, the tumour cell line designated PCI-MEL 107 enhanced class I MHC expression but failed to express DR, DP or DQ altogether in two experiments, and showed a marginal induction of DR expression in one experiment, using a relatively late passage. This latter result implies a lack of correlation between the change in MHC class II by the melanoma and the proliferative and phenotypic alterations described above.

DISCUSSION

In vitro culture of human effector cells provides a model system in which one may examine the role of cytokines alone and in combination; the use of adoptive cellular therapy has made this explanation both analytically and therapeutically relevant. For example, it has not been possible to predict outgrowth of T cell subsets from individuals for tumour-infiltrating lymphocyte (TIL) therapy (Rosenberg *et al.*, 1988; Belldegrun, Muul & Rosenberg, 1988).

The results of the experiments described above using the model of autologous MLTC can be summarized as follows: (i) the proliferative potential of MLTC-generated lymphocytes cultured in the presence of TNF-α and/or IFN-γ was greater than that which was obtained with rIL-2 alone; (ii) high concentrations of IFN-γ or the combination of TNF-α and IFN-γ significantly alter the phenotypic profile of cells generated in the MLTC; (iii) the combination of TNF-α and IFN-γ did not alter specific or nonspecific cytotoxic activity consistently, except for the inhibition of NK cell activity; and (iv) these observations are not correlated with the induction of MHC class II antigen expression by tumour cells, and may be direct effects of these cytokines upon effector lymphocytes and possibly monocytes.

The mechanisms of TNF-α and IFN-γ action *in vitro* may involve direct or indirect effects of these cytokines upon lymphocytes and/or monocytes. Initial cultures contained small percentages of granulocytes and other effector cells which were irrelevant at the time points assessed in this study. Our results showing increased proliferative capacity of lymphocytes exposed to TNF-α and/or IFN-γ, above rIL-2 alone, are consistent with other studies that have reported activity of these cytokines as single agents in the development of CTL (Scheurich *et al.*, 1987; Siegel, 1988; Talmadge *et al.*, 1988). Proliferative rates of lymphocytes sensitized to tumour in the presence of IFN-γ and TNF-α varied between individuals. One account for this degree of variability may be that the established melanoma lines were derived from patients whose metastatic disease varied in site and volume. The ability of lymphocytes to proliferate in response to melanoma cells from early and advanced disease has been reported to vary according to disease state (Guerry *et al.*, 1984). Moreover, the antigenicity of tumour lines relate to cell membrane expression of tumour-associated antigens (Knuth *et al.*, 1989). Hence, the capacity of these tumour cells to stimulate host T cells may be expected to differ. In addition, a further source of variability may be the different susceptibility of host T cells to modulation by IFN-γ and TNF-α. High doses of IFN-γ (10^4 U/ml) were expected to decrease proliferation. Effects of TNF-α, increasing proliferative responses, were perhaps the most predictable of all that we observed in these studies.

In the MLTC generated with TNF-α and/or IFN-γ, the proportion of cells expressing the CD25 antigen remained high throughout cultures. This increased expression of high-affinity IL-2 receptors induced by both cytokines may account for the enhanced proliferative capacity as reported elsewhere (Scheurich *et al.*, 1987; Siegel, 1988). TNF-α alone also enhances the production of IFN-γ by T lymphocytes, which results in the amplification of the T cell proliferative process (Hackett, Davis & Lipsky, 1988).

The phenotypic evolution of various lymphoid populations exposed to IFN-γ alone, or to TNF-α plus IFN-γ, differed in the present studies. The combination of IFN-γ and TNF-α concentrations as low as 10^2–10^3 U/ml alone induced an inversion of the CD4/CD8 ratio. Without TNF-α, as much as 10^4 U/ml of IFN-γ were required in order to obtain the same effect. TNF-α alone at 10^4 U/ml generated only moderate increases of CD4 or CD8 populations. These observations suggest some synergistic activity between TNF-α and IFN-γ. Such synergism between IFN-γ and TNF-α in the induction of MHC class II antigen expression in normal and neoplastic cells, and in the differentiation of several malignant cell lines, has been reported (Dinarello *et al.*, 1986; Malek, Danis & Codias, 1989). The induction of TNF-α receptor synthesis by IFN-γ may account in part for this

phenomenon (Ruggerio *et al.*, 1986). TNF-α may act synergistically with IFN-γ upon monocytes/macrophages, thymocytes, and T cells, but the mechanism of this synergy is still incompletely understood (Nakano *et al.*, 1989; Wang *et al.*, 1989). The phenotypic changes associated with use of these cytokines in our study endured long after the withdrawal of IFN-γ and/or TNF-α, implying that a durable regulatory process or selection occurred early during the sensitization phase. Each cytokine may exhibit selective properties and may inhibit or enhance the proliferative activity of only subsets of T cell clones (Gajewski, Joyce & Fitch, 1989). These findings may lend insight into the dilemma of variable outgrowth of CD4 or CD8 effector populations observed in clinical studies of adoptive therapy (Rosenberg *et al.*, 1988).

Inhibition of NK cytotoxic activity of MLTC-generated lymphocytes cultured in the presence of TNF-α and IFN-γ could be an indirect or direct effect. Inhibition could have been mediated through monocytes affected by these cytokines; otherwise, consistent changes in the levels of specific cytotoxic activity were absent. With rare exceptions, a level of >25% autologous tumour-specific cytolytic activity developed in the MLTC, with variable effector function against allogeneic tumour cell lines. Specific cytolytic activity against autologous melanoma was not enhanced by the addition of TNF-α, as observed in other systems (Nakano *et al.*, 1989; Wang *et al.*, 1989). While the role of IFN-γ in the development of LAK cells is controversial, TNF-α has been reported as having a synergistic activity with low-dose IL-2, promoting induction of LAK activity (Itoh *et al.*, 1985; Owen-Schaub, Gutterman & Grimm, 1988; Sone *et al.*, 1988; Chouaib *et al.*, 1988). We observed no consistent augmentation of NK or LAK activities in tests against K562 and Daudi cell lines with the addition of TNF-α alone.

Potential mechanisms of TNF-α and IFN-γ modulation of

Table 5. Relative MHC expression by tumour cell lines ELISA*

Patient no./ cell line	Cytokines (U/ml)			OD Index			
	rIL-2	IFN-γ	TNF-α	HLA A-B-C	DR	DP	DQ
1/PCI-MEL 136	10	—	—	1·0	1·5±0·4	0·7±0·1	0·5±0·2
	10	—	10^3	1·8±0·2	1·8±0·2	0·7±0·1	0·5±0·1
	10	—	10^4	2·0±0·6	2·0±0·5	0·8±0·2	0·6±0·2
	10	10^2	—	2·3±0·9	2·4±0·7	1·0±0·3	0·7±0·2
	10	10^3	—	3·0±1·9	3·0±1·5	1·8±0·2	1·1±0·3
	10	10^2	10^3	2·2±0·5	2·0±0·2	0·8±0·1	0·8±0·1
2/PCI-MEL 107	10	—	—	1·0			
	10	—	10^3	1·7±0·6	No HLA-DR, -DP, and -DQ detected		
	10	—	10^4	1·7±0·4			
	10	10^2	—	1·8±0·6			
	10	10^3	—	2·7±0·1			
	10	10^2	10^3	2·2±0·4			
3/PCI-MEL 208	10	—	—	1·0	1·1±0·2	0·7±0·3	ND
	10	—	10^3	1·3±0·4	1·2±0·2	0·9±0·2	0·6±0·2
	10	—	10^4	1·2±0·6	1·3±0·2	0·8±0·2	0·5±0·1
	10	10^2	—	2·2±0·5	2·5±0·8	1·5±0·3	0·8±0·1
	10	10^3	—	3·1±1·3	3·4±1·4	1·8±0·2	1·3±0·7
	10	10^2	10^3	3·1±1·5	2·9±1·1	1·3±0·8	1·1±0·4
4/PCI-MEL 103†	10	—	—	1·0	5·6	6·0	0·6
	10	—	10^3	0·7	3·9	5·0	ND
	10	—	10^4	0·8	5·6	5·9	ND
	10	10^2	—	2·2	6·0	6·9	ND
	10	10^3	—	1·5	9·7	8·7	0·6
	10	10^2	10^3	1·1	4·7	5·4	ND

OD index = (OD of condition tested) × (OD of control with second antibody only)$^{-1}$. Cultures assayed for class I MHC antigen with the absence of cytokines were designated 1·0 and the relative changes in expression were determined as follows:

$$\text{Relative OD index} = \frac{\text{OD index exp.}}{\text{OD index of class I MHC samples cytokines}}$$

* Tumour cells during passages 5–11 were preirradiated (100 Gy).

† One determination.

ND, none detected.

the emerging pattern of lymphocyte subsets include the induction of tumour-associated and MHC antigen expression by the tumour cells. Because of the importance of MHC class II molecules in the antigen recognition process by T cells ($CD4^+$), the expression of MHC class I and II antigens by the tumour cells was studied in the absence and in the presence of IFN-γ and/or TNF-α (Bottomly, 1988). The expression of MHC class I and II antigens by tumour cell lines detected in this study corresponded to the results reported by others (Taramelli *et al.*, 1986). One of the tumour cell lines studied in MLTC did not express detectable MHC class II antigens, indicating that MHC class II antigens of melanoma cells may not mediate the effects induced by IFN-γ and TNF-α here. However, increased expression of MHC class I antigens might influence the cytotoxic T cell response. An effect of IFN-γ and/or TNF-α upon tumour-restricted antigens in the modulation of the MLTC (Taramelli *et al.*, 1986; Holzmann *et al.*, 1987) cannot be excluded. Transforming growth factor-beta (TGF-β) has been shown to inhibit the generation of CTL (Jin *et al.*, 1989). Preliminary results showed no relationship between TGF-β production and tumour cytotoxicity, class I or II expression, and proliferation (data not shown).

IFN-γ and TNF-α may have directly influenced the generation of mononuclear effector cells in MLTC in many different ways. IFN-γ and TNF-α alone and in association with other lymphokines have been shown to affect accessory cell function and B and T cell development (Bonnem & Oldham, 1987; Beutler & Cerami, 1987; Talmadge *et al.*, 1988). The temporal sequence of *in vitro* exposure to cytokines is also a determinant of outcome, as it has been demonstrated *in vivo* in a murine system (Zimmerman *et al.*, 1989) and in a human system (Kirkwood & Ernstoff, 1990). IFN-γ has been shown to favour the expansion of T_H1 clones by inhibiting the proliferation of T_H2 clones (Gajewski *et al.*, 1989). It is possible that the addition of one or both cytokines at the beginning of an MLTC alters the sensitization process, leading to the selective outgrowth of different T cell populations. Several reports have shown variations in the phenotype of cytotoxic cells generated *in vitro* from TIL derived from different kinds of tumours, and $CD4^+$ cytotoxic clones have been shown to be generated (Taramelli *et al.*, 1986; Holzmann *et al.*, 1987; Bottomly, 1988; Owen-Schaub *et al.*, 1988). Both cytokines may account for the phenotypic variations reported in the studies cited above (Whiteside *et al.*, 1986; Itoh, Tilden & Balch, 1986; Muul *et al.*, 1987; Belldegrun *et al.*, 1988).

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