

CLONAL ANALYSIS OF CYTOTOXIC AND REGULATORY T CELL RESPONSES AGAINST HUMAN MELANOMA

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Studies of T cell-mediated immune response against spontaneously arising autologous human tumors have been markedly facilitated by functional analyses of T cells at the clonal level (1-7). This particular approach has now provided a strong support for the long-held belief by some, although viewed skeptically by others, that T cell-mediated host responses do indeed exist in different types of human cancers. The evidence for such T cell antitumor response has been particularly impressive in malignant melanoma (1-4, 6, 7). In this system, the existence of CTL response (1-4, 6, 7), proliferative T cell response (8, 9), suggestion for regulatory T cell response (10, 11), and delayed-type hypersensitivity response (12) have all been demonstrated. While this impressive body of evidence certainly represents a serious beginning in investigation on T cell responses against autologous cancer, a critical analysis of T cell-immune responses against a large number of autologous melanoma at clonal levels is necessary for a more comprehensive understanding of T cell-immune response in host defense against human cancers or, for that matter, against malignant melanoma.

We have undertaken clonal analyses of T cell-immune response in a larger group of patients with melanoma. In this work, we present our observation of clonal analyses of CTL and regulatory responses in 31 autologous case studies. Here we show that T cell responses (CTL as well as regulatory T cell responses), taken together, are demonstrable in approximately half of this cohort of subjects. Results of our studies clearly document the involvement of the entire T cell repertoire in response to autologous melanoma. The melanoma-specific (CTL) responses show appropriate MHC class restriction, and the cytotoxic response in the PBL is subject to regulation by the helper and suppressor arms of the T cell network. The amplification of cytotoxic response by Th cell clones is mediated by the elaboration of IL-2 and IFN- γ , and the T cell-mediated downregulatory responses can be specific as well as nonspecific.

Materials and Methods

Patients. All 31 patients had recurrent and/or advanced metastatic disease. Specimens (tissue or blood) were obtained with informed consent.

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Tumor Cells. We performed all experiments with melanoma cell lines established in our laboratory (from 20 of these 31 cases) or with freshly isolated tumor cells from melanoma explants (11:31 cases). The procedure of isolating fresh cells from tissue explants has been described earlier (13). Cells were cryopreserved (-180°C) in filtered FCS with 10% DMSO in multiple aliquots. Quickly thawed cells were 90% viable by trypan blue dye exclusion test. By cytologic and morphologic criteria, the single cells were 95% monomorphic, consistent with the cytology of melanoma cells, and expressed one or more of the three antigens characteristically expressed by melanoma cells (D/DR antigen and ganglioside antigens GD3 and GM2) when tested by immunofluorescence tests with mAb anti-D/DR framework I2 (Coulter Electronics Inc., Hialeah, FL), mAb anti-GD3 R24 (a gift of Alan Houghton, Memorial Sloan-Kettering Cancer Center, New York, NY), and mAb anti-GM2 (a gift of Philip Livingston, Memorial Sloan-Kettering Cancer Center).

Lymphocytes. PBL were isolated on a Ficoll-Hypaque gradient fresh for each experiment. Further, autologous lymph node-derived lymphocytes (LNL)¹ were also isolated by a technique described earlier (10) on a Ficoll-Hypaque gradient from a single cell preparation of lymph nodes that were partially infiltrated with melanoma cells. All cultures and every experiment were initially performed in Ham's F-10 medium and, subsequently, in Iscoves medium (Gibco Laboratories, Grand Island, NY) containing 10% FCS.

In Vitro Coculture (IVC). The basic IVC technique has been described previously (10). Briefly, effector cells ($10^6/\text{ml}$) were cocultured with irradiated (3,000 rad) autologous or allogeneic targets ($10^4/\text{ml}$) in medium supplemented with 10% FCS. Depending on experimental protocol, 64 U of lectin-free and purified IL-2 (Electronucleonics, Silver Spring, MD) was added to the cultures on day 3 of the cultures or cocultures. Unless mentioned otherwise, the standard in vitro activation coculture protocol for activation of cytotoxicity consisted of a coculture of effector cells and target cells in medium containing IL-2 (64 U/ml) from day 3 followed by cytotoxicity assay on day 7.

Lymphocyte Cloning. Lymphocytes were cloned by the limiting dilution microculture technique as described earlier (1), with minor modifications. To generate clones, irradiated (3,000 rad), autologous PBL (alone or with allogeneic PBL from one or two healthy donors) were used as feeder cells at concentrations of 10^4 cells/well. 10–20 96-well U-bottomed cluster plates (Costar, Cambridge, MA) were seeded with the sensitized B lymphocytes or LNL at concentrations such that one of three wells, or each well, would have received a single cell. Microwells were fed with a drop of 64 U/ml of IL-2-containing medium every other day. Visible colonies were removed to another 96-well plate and were longitudinally expanded by dividing the contents of one well into two wells. The contents of six to eight wells were subsequently pooled into 48-well and, then, into 12-well cluster plates. Bulk cultures ($0.5\text{--}1.0 \times 10^6$ cloned cells) were restimulated with autologous melanoma cells (100 Ly-1 melanoma cells) with or without autologous lymphoid cells (cloned cell(s)/accessory lymphoid cell = 2:1). The stimulatory cells were irradiated (3,000 rad).

Phenotypic Analysis. Procedures for phenotypic analysis in FACS has been previously described (1). mAb Tac was a gift of Thomas Waldman (National Institutes of Health, Bethesda, MD). mAb W632 was a gift of Soldano Ferrone (New York Medical College, Valhalla, NY). Anti-CD2, -CD3, -CD4, and -CD8 reagents were purchased from Ortho Diagnostic Systems Inc., Westwood, MA. Anti-D/DR mAb I2 was purchased from Coulter Electronics Inc.

In Vitro Microcytotoxicity (Cell-mediated Cytotoxicity) Assay. The ^{51}Cr release microcytotoxicity assay has been described earlier (1). Cultured target cells, freshly prepared targets, and the cryopreserved targets were labeled with ^{51}Cr with good efficiency, the mean spontaneous release of ^{51}Cr from 20 separate experiments was 11.5% (range, 4–19%). For the purpose of quantitative comparison, the cytotoxic ability of a given activated PBL population was expressed as LU (^{51}Cr 1,000)/ 10^6 BMN. 1 LU equals the number of lymphocytes required to lyse 1,000 target cells (i.e., 50% lysis of 2,000 target cells/well). The standard cold target competition assay for analysis of specificity of cytotoxicity has been described earlier (14).

¹ Abbreviations used in this paper: AMLR, autologous mixed leukocyte reaction; ICAM, intercellular adhesion molecule; IVC, in vitro coculture; LFA, lymphocyte function-associated antigen; LNL, lymph node-derived lymphocytes.

Assay for Regulation of Cytotoxicity. The basic in vitro assay to examine cell-mediated regulation of cytotoxic response in in vitro coculture has been published earlier (10, 11, 13). Briefly, to assay regulation of generation of cytotoxicity in the PBL (at induction phase), standard in vitro activation cocultures between PBL and irradiated targets (3,000 rad) were set up in the presence or in the absence of irradiated (3,000 rad) cloned lymphocytes as potential regulatory cells at different PBL to regulatory cell ratios. Cytotoxicity was assayed on day 7 at different E/T ratios. Percent enhancement and percent suppression were calculated with the following formulas: percent suppression = $100 \times [1 - (\text{percent specific lysis with regulatory cells})/(\text{percent specific lysis without regulatory cells})]$; percent enhancement = $100 \times [1 - (\text{percent specific lysis without regulatory cells})/(\text{percent specific lysis with regulatory cells})]$. Mean cpm of three replicate samples from wells containing the E/T ratio of 20:1 was used to calculate percent changes. To assay potential regulation of cytotoxicity at the effector phase, cytotoxicity of the CTL clones was assayed in the presence or absence of autologous sera or of PBL as potential regulatory cells.

Results

Cytotoxic Response Against Autologous Target Cells. Fig. 1 shows representative examples of cytolytic responses by the freshly derived and in vitro activated autologous PBL or LNL in three autologous melanoma systems. In none of these three systems were the fresh PBL or LNL cytotoxic against the respective autologous targets. In two cases, the activated effectors, however, exhibited significant levels of cytotoxicity against the autologous targets. In a composite analysis of 31 autologous systems, the freshly derived PBL or LNL were marginally cytotoxic against the autologous targets in only two cases. In 19 of 31 cases, the activated effector cells (PBL, LNL, or both) exhibited significant cytotoxicity ($\geq 10^{LU} 1,000/10^6$ effector cells) (combined data not shown). In continuous culture the cytotoxic responses by the effector cells usually reached their peak levels by 2-3 wk and declined to the base line levels by the 5th to 7th week (data not shown). Restimulation with autologous melanoma cells as antigen and PBL or spleen cells (whenever available) as accessory cells helped the cytotoxic activity to rebound somewhat, but seldom to their earlier peak levels (data not shown). Although differences in the cytotoxic response (ranging from minor to marked) between autologous PBL and LNL was observed in several cases, cytotoxic unresponsiveness did not reside exclusively, however, with the PBL or with the LNL (composite data not shown).

The cytotoxic responses by the activated PBL or LNL were almost always seen

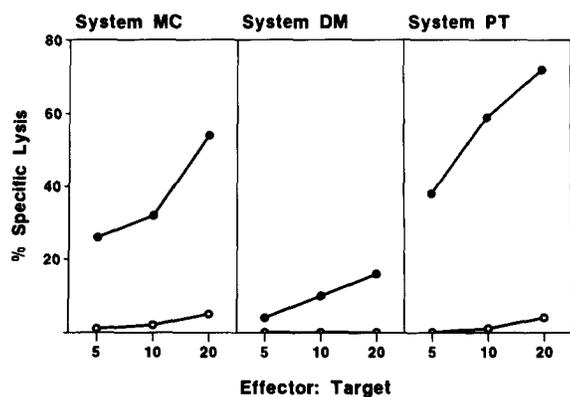


FIGURE 1. Cytotoxicity by the fresh PBL (O) and by the activated PBL (●) against the corresponding autologous melanoma cells. Cytotoxicities of the activated PBL were tested at 2 wk after the initiation of IVC.

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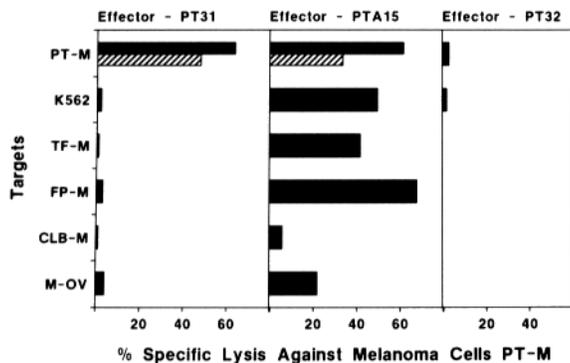


FIGURE 2. Cytotoxic profiles of three clones against the autologous melanoma cells PT-M. Percent specific lysis represented by the solid bars was at E/T ratio = 20:1 and was E/T ratio = 10:1 as represented by the broken bars. PT-M, TF-M, FP-M, and CLB-M, melanoma cells; K-562 erythroleukemia cell line; and M-OV, ovarian cancer cells.

against a broad range of target cells, including the NK-sensitive target, K-562. The activated PBL or LNL usually contained both CD4⁺ and CD8⁺ populations, although in some cases there was a significant preponderance of one subset or another. For example, in the DM system (Fig. 1), the activated PBL were predominantly CD4⁺ (80%); whereas, in the PT system, they were predominantly CD8⁺ (90%). Fig. 2 shows the cytotoxic profiles of three CD8⁺ clones in the PT system. In this system, clone A15 demonstrated a broad range of cytotoxic activity; while clone PT 32 was noncytotoxic. Clone PT 31, on the other hand, exhibited cytotoxicity that was restricted against the autologous target PT-M. All three clones were phenotypically identical (CD3⁺, CD8⁺, CD4⁻, Ia⁺), and the two cytotoxic clones (PT 31 and A15) required periodic (every 1–2 wk) stimulation with the autologous melanoma cells PT-M with or without autologous PBL or splenocytes (irradiated to 3,000 rad) for maintenance of their cytotoxic functions. Unstimulated cultures rapidly lost cytotoxic function.

Fig. 3 shows the cytotoxic profiles of four CTL clones that were derived from activated PBL or LNL from four separate cases. Clone PT 31 and RN C8 were derived from activated PBL; whereas, the other two clones were derived from activated LNL from melanoma-involved lymph nodes. All four clones expressed identical pheno-

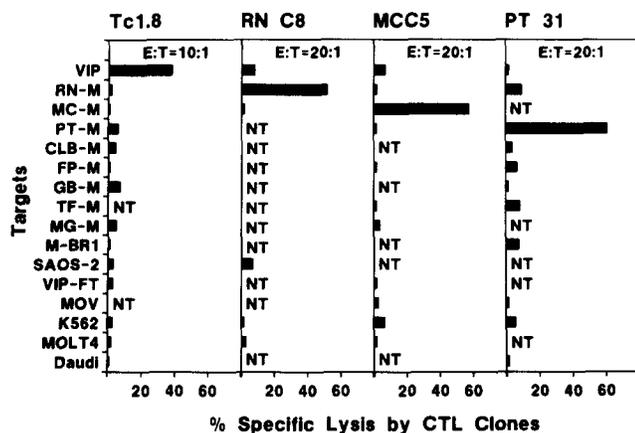


FIGURE 3. Cytotoxicity by CTL clones Tc1.8, RN C8, MCC5, and PT 31 against the respective autologous melanoma cells (VIP, RN-M, MC-M, and PT-M) and against a panel of allogeneic targets. VIP, RN-M, MC-M, PT-M, CLB-M, FP-M, GB-M, TF-M, and MG-M, melanoma lines; M-BR-1, breast cancer line; SAOS-2, osteosarcoma line; VIP-FT, transformed fibroblast line; M-OV, ovarian cancer cells; K-562 and MOLT4, NK sensitive targets; Daudi, Burkitt's lymphoma line.

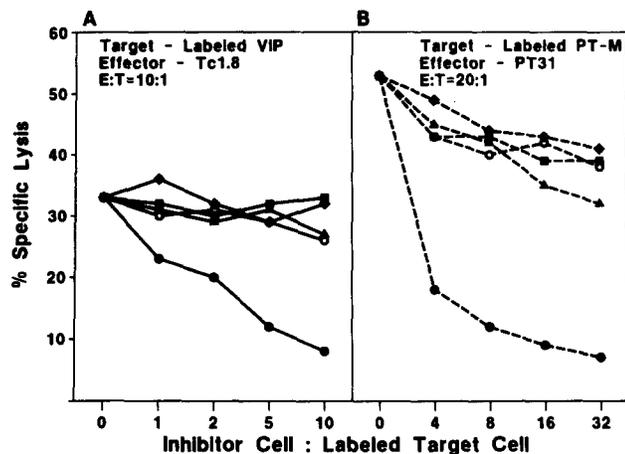


FIGURE 4. Cold target inhibition assays with CTL clones Tc1.8 (in the VIP system) and PT 31 (in the PT system). (A) Clone Tc1.8 was tested against labeled autologous melanoma cells VIP (at effector/labeled VIP target of 10:1) in the presence of unlabeled VIP (●), of unlabeled K-562 (○), of unlabeled Daudi cells (▲), of unlabeled PJ-M cells (■), and of unlabeled MOLT 4 cells (◆). (B) Clone PT 31 was tested against labeled autologous melanoma cells PT-M (at effector/labeled PT-M target of 20:1) in the presence of unlabeled PT-M cells (●), of unlabeled FP-M cells (◆), of unlabeled K-562 cells (○), of unlabeled CLB-M cells (■), and of unlabeled GB-M cells (▲).

type (CD3⁺, CD8⁺, CD4⁻, WT31⁺, and HNK1⁻), and all lysed the respective autologous melanoma cells in a restricted fashion. The CTL clone RN C8 could not be maintained in continuous culture long enough to perform more detailed analyses. The other three clones grew for a long enough time to allow more detailed analyses. The restricted specificities for the respective autologous melanoma cells were confirmed in cold target competition assays in two systems. In both systems, only the autologous melanoma cells avidly competed with the corresponding labeled autologous melanoma cells for the corresponding CTL receptors (Fig. 4). Three CTL clones (clone Tc1.8 in the VIP system, clone PT 31 in the PT system, and clone MCC5 in the MC system) demonstrated MHC class I-restricted killing, while clone RN C8 in the RN system showed no MHC restriction (Table I).

To analyze the broad target range of the cytotoxic behavior of clone A15 in the PT system, we carried out its specificity analysis in cold target competition assay. Interestingly, in cytotoxic interaction between clone A15 and the autologous PT-M

TABLE I
Effect of Anti-MHC Antibody on Cytotoxicity by CTL Clones

In the presence of:	Percent specific lysis of:			
	Tc1.8 vs. VIP	MC C5 vs. MC-M	PT 31 vs. PT-M	RN C8 vs. RN-M
Medium	55	49	66	40
Control mouse Ig	61	51	68	36
I-2 (anti-D/DR)	49	47	65	33
W6-32 (anti-MHC-I)	16*	26*	37*	37

Percent specific lysis at E/T ratio is 20:1.

* When compared with percent specific lysis by the corresponding clones in the presence of control mouse Ig, the difference is significant at $p < 0.001$ by student's *t* test.

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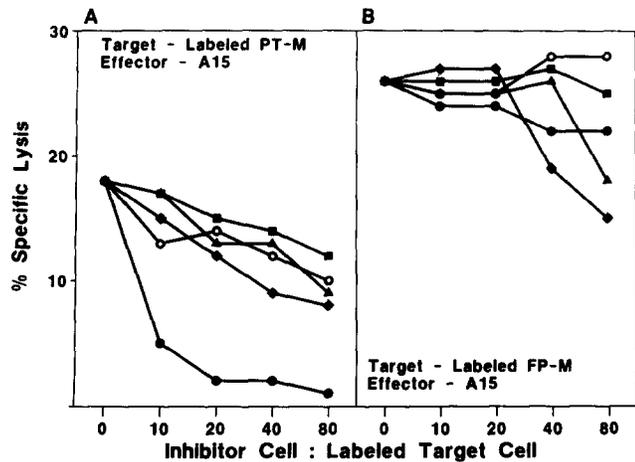


FIGURE 5. Cold target inhibition assays with CTL clone A15 (in the PT system) tested against the labeled autologous melanoma cells PT-M (A) and against the labeled allogeneic melanoma cells FP-M cells (B) in the presence of unlabeled PT-M cells (●), of unlabeled K-562 cells (○), of unlabeled GB-M cells (■), of unlabeled CLB-M cells (▲), and of unlabeled FP-M cells (◆).

melanoma cells, only the unlabeled autologous melanoma cells showed positive competition (Fig. 5). Conversely, against an allogeneic target (FP-M), which was lysed quite well by A15, no competition between the relevant autologous melanoma cells and the allogeneic targets, or between the irrelevant allogeneic targets, was noted (Fig. 5). Since these results could be explained on the basis of possible contamination of other "nonspecific" populations of cytotoxic cells, clone A15 was recloned. Unfortunately, our initial attempt to reclone A15 was unsuccessful.

Modulation of Cytotoxic Activities of the CTL Clones. The cytotoxic activities of all three clones against the autologous targets could not be modulated at the effector phase by autologous sera and autologous unactivated PBL (Table II). The cytotoxic activities of the CTL clones against the corresponding autologous targets in the MC and PT systems were blocked by anti-CD3 mAb (data not shown). In contrast, the cytotoxicity of the Tc1.8 (VIP system) was enhanced significantly by the anti-CD3 treatment. A more detailed analysis of anti-CD3-mediated enhancement of the cytotoxic function of CTL clone Tc1.8 has been communicated earlier (15). Further, with reference to the restricted and broad target range of the CTL clones PT31 and A15, the roles of lymphocyte function-associated (LFA) antigen 1 and intercellular adhesion molecule (ICAM)-1 were examined. Table III shows that while the cytotoxic activities by A15 against the allogeneic targets were abrogated by anti-LFA-1 and anti-ICAM-1 antibodies, the same antibodies failed to block the cytotoxicity of A15

TABLE II
Effect of Autologous Sera and Autologous Fresh PBL on Cytotoxicity

In the presence of:	Percent specific lysis of:		
	Tc1,8 vs. VIP	MC C5 vs. MC-M	PT 31 vs. PT-M
Medium	36	46	52
AB ⁺ normal serum	38	52	57
Autologous serum	33	43	44
Autologous PBL	34	47	54

Percent specific lysis at E/T ratio is 20:1.

TABLE III
Effect of Anti-LFA-1 and Anti-ICAM-1 on Restricted and
"Promiscuous" Cytotoxicity by CTL Clones

In the presence of:	Percent specific lysis of targets		
	PT-M	FP-m	TF-M
Medium	23 (42)	27	17
Anti-LFA-1 α	13 (37)	24	8
Anti-LFA-1 β	23 (41)	9	3
Anti-ICAM-1	27 (48)	11	4
Control mouse Ig	26 (43)	37	18

Percent specific lysis (E/T ratio is 20:1) is by CTL A15. Percent specific lysis values shown in parentheses were by the CTL clone PT 31.

and PT31 against the autologous melanoma cells PT-M (mAbs anti-LFA-1 and anti-ICAM-1 are gifts of Timothy Springer, Harvard Medical School, Boston, MA).

Antigen Dependence, Life Span, and Stability of the CTL Clones. The CTL clones were restimulated every 1–3 wk with irradiated autologous melanoma cells (CTL/melanoma = 100:1) and, whenever available, with irradiated autologous PBL or with spleen cells in the PT system. This patient underwent splenectomy, which allowed us to freeze multiple vials of freshly isolated splenocytes. Despite repeated restimulation, the life span of all three CTL clones in continuous culture varied from 2 to 6 mo. At the end of their life span, the clones entered into "crises" leading to almost abrupt death. Restimulation at the crisis stage or the addition of higher dosages of IL-2, rIL-2, or rIL-4 seldom resurrected them.

Regulation of Cytotoxic Effector Response by T Cell Clones and Lines. The evidence for autologous tumor-specific downregulation of induction of cytotoxic response in autologous melanoma (10, 11) and in malignant paraganglioma (a tumor of neuroectodermal origin [13]) has been published earlier. These studies were continued in order to further explore T cell-mediated regulation of cytotoxic responses in other systems. Fig. 6 shows three experiments in three separate systems in which the cytotoxic responses by the autologous PBL were regulated by three respective autologous CD4⁺ T cell clones exhibiting CD3⁺, CD4⁺, and CD8⁻ phenotypes. In the

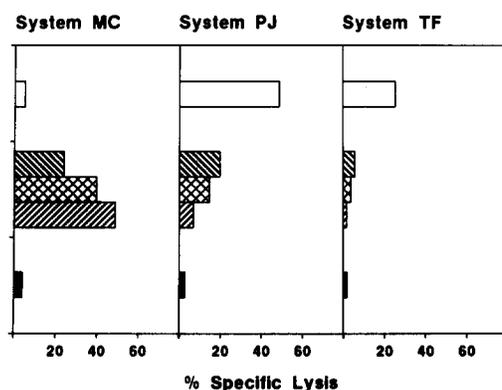


FIGURE 6. Effect of regulatory T cell clones on cytotoxic responses in three separate autologous systems. In the MC system the effect of clone I5 is shown in coculture with MC PBL and with MC-M melanoma cells in the absence of exogenous IL-2. In the PJ and TF systems, the effects of clones I-10 and TF14 are shown in cocultures with the corresponding autologous PBL and melanoma cells in the standard in vitro activation coculture containing exogenous IL-2. Percent specific lysis at effector/target of 20:1 in the absence of any regulatory cells (\square) and in the presence of regulatory cells at PBL/regulatory cells of 1:1 (hatched), of 3:1 (cross-hatched), and of 10:1 (solid black). (■) Percent specific lysis by the respective regulatory cells themselves.

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MC system, the CD4⁺ autologous clone, I5, upregulated the cytotoxic response. I5 was not cytotoxic against the autologous melanoma cells MC-M. In the other two systems (PJ and TF), the cytotoxic responses, regardless of the magnitudes of cytotoxicity, were markedly downregulated by the respective CD4⁺ T cell clones. In all three systems, the regulation by the T cell clones was observed in a dose-dependent manner.

The amplification of cytotoxic response in the PBL induced by CD4⁺ T cell clones was observed in two other systems (systems DM and JC). In all three systems (MC, DM, and JC) the T cell clones exhibited the phenotype of "helper-inducer" cells (CD3⁺, CD4⁺, CD8⁻, 4B4⁺, and 2H4⁻), and they amplified cytotoxicity in the PBL in IVC with the autologous melanoma cells in the absence of any exogenous IL-2 (Fig. 6 and Table IV). Table IV also shows that the amplification of cytotoxicity by the helper cell clones in the DM and JC systems could be blocked partially by anti-IL-2 antibody or by anti-IFN- γ antibody.

Table V shows examples of downregulation of cytotoxic responses in three separate systems and illustrates three specificity patterns of downregulation. In the MM system, the cytotoxic response by the PBL was significantly suppressed by a CD4⁺ clone, MM T-4. The suppression, however, was unrestricted as the cytotoxic responses against both the autologous and allogeneic targets (D/DR antigen-positive as well as D/DR antigen-negative) were suppressed. A similar pattern of nonrestricted downregulation of cytotoxic response was observed in the CLB system in which a CD4⁺ T cell line, CLB Ts4 (expanded from a well seeded with 100 cells that grew vigorously in continuous culture for >2 mo), completely suppressed the induction of cytotoxic response by the autologous PBL against the autologous melanoma cells CLB-M and another allogeneic melanoma cell line. CLB Ts4 did not suppress cytotoxic response against a third melanoma cell line, PJ-M. It is noteworthy that the melanoma cells CLB-M and TF-M expressed abundant D/DR II antigen, while the melanoma cell line PJ-M did not express any D/DR antigen. Interestingly, CLB Ts4 upregulated its Tac receptor when stimulated with the autologous and allogeneic targets expressing D/DR antigens and did not do so when stimulated with the D/DR antigen-negative target PJ-M (Fig. 7). In both systems, the cytotoxic responses in the in vitro activa-

TABLE IV
*Effect of Anti-IL-2 and Anti-IFN- γ on Th Cell-induced
Amplification of Cytotoxic Response*

Auto IVC* with:	Percent specific lysis (E/T = 20:1) in systems		
	DM	JC	MC
Medium alone	4	4	2
+ Helper cells [†]	32	21	31
+ Helper cells + anti-IL-2	9	3	16
+ Helper cells + anti-IFN- γ	8	8	NT
+ Helper cells + control Ig	28	16	27

* IVC between PBL and respective autologous melanoma cells.

[†] CD4⁺ T cell clone DM5 in DM system, A5 in JC system, and I5 in MC system. Control Ig was either mouse Ig in MC system or mouse and rabbit Ig in DM and JC system.

tion cultures were quite modest. Even the modest cytotoxic responses were almost totally suppressed by the regulatory cells. CLB Ts4 were not cytotoxic for the autologous melanoma cells nor for several other melanoma targets (data not shown).

In another melanoma system (GB), the CD4⁺ clone, GB 1.7, in contrast to the nonrestricted downregulation shown by the clones discussed earlier, downregulated the induction of cytotoxic response in a more restricted manner. The induction of cytotoxic response against allogeneic targets was minimally suppressed against one target and was not suppressed against the other (Table V). In addition, coculture between GB PBL and GB 1.7 in the presence of the allogeneic target PT-M (D/DR antigen-positive) or against PJ-M (D/DR antigen-negative) led to no suppression, or only marginal suppression, of cytotoxic response against the respective allogeneic targets (Table V).

Table VI shows an analysis of the characteristics of the 15 cases in which we were able to undertake a detailed functional analysis of such T cell responses. Interestingly, all the CTL clones in this study were CD8⁺; and all the regulatory clones exhibiting downregulation of CTL responses were CD4⁺. An analysis of the magnitude of cytotoxic response by the activated PBL at population level (at the height of cytotoxic response, i.e., at 2 wk after initiation of the IVCs) and of the nature of the clonal responses revealed an interesting correlation. While CTL responses at clonal levels correlated with substantial cytotoxic responses by the corresponding activated PBL, the emergence of downregulatory responses was associated with very low cytotoxic responses by the corresponding activated PBL populations (Table VI). In this analysis, the cytotoxicity values at 2 wk after the initiation of IVC were used for calculation of LU.

TABLE V
Specificity Pattern of Downregulation by Cloned T Cell Lines

Exp.	IVC condition	Percent suppression (-) or enhancement (+) of cytotoxicity in the PBL against*:		
		CLB-M (Ia ⁺) [†]	PT-M (Ia ⁺)	PJ-M (Ia ⁻)
1.	CLB PBL + Ts4 + CLB-M [‡]	-92	-70	-44
	CLB PBL + Ts4 + PT-M	-82	-62	-50
	CLB PBL + Ts4 + PJ-M	-11	-7	+10
		MM-T (Ia ⁻)	VIP (Ia ⁺)	PJ-M (Ia ⁻)
2.	MM PBL + T-4 + MM-T	-38	-49	-35
	MM PBL + T-4 + VIP	-30	-46	-17
	MM PBL + T-4 + PJ-M	-25	-27	-31
		GB-M (Ia ⁺)	PT-M (Ia ⁺)	PJ-M (Ia ⁻)
3.	GB PBL + GB 1.7 + GB-M	-45	-16	+11
	GB PBL + GB 1.7 + PT-M	0	-21	0
	GB PBL + GB 1.7 + PJ-M	0	-2.5	0

* Percent suppression or enhancement were calculated from percent specific lysis values at E/T = 20:1. Percent lysis by the auto-sensitized BL were 26% against CLB-M, 24% against MM-T, and 33% against GB-M.

[†] The Ia⁺ or Ia⁻, as shown in parentheses, represent MHC II antigen expression by the melanoma cells.

[‡] The BL/Regulatory Clone ratios in Exp. 1 and 2 were 3:1, while the ratio in Exp. 3 was 10:1.

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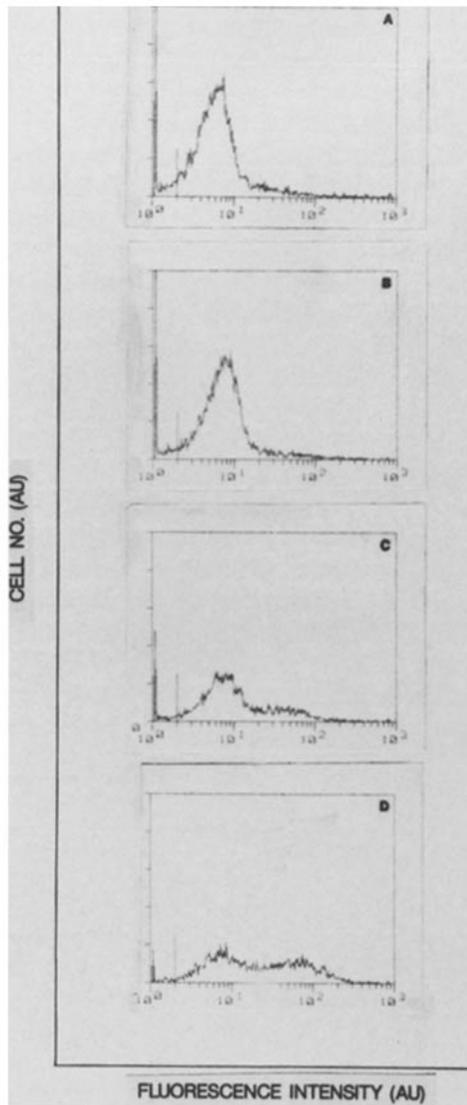


FIGURE 7. Tac antigen expression by CLB T4 line. Thoroughly washed CLB T4 cells were incubated in medium (A), with irradiated Ia⁻ Pj-M melanoma cells (B), with Ia⁺ allogeneic RN-M melanoma cells (C), and with Ia⁺ autologous melanoma cells CLB-M (D) for 24 h (T4/melanoma cells = 10:1). After incubation, the CLB T4 cells were phenotyped for Tac expression by immunofluorescence technique in the FACS.

Discussion

The results described here reaffirm earlier observations on CTL response against autologous melanoma from our laboratory (1) and other laboratories (2-4, 6, 7). In addition to providing considerable credibility for the notion that true T cell effector response can indeed be demonstrated in autologous human melanoma, this study also supports the concept that cytotoxic effector response against autologous melanoma cells is subject to regulation by T cells. Besides providing support for these concepts, several interesting points have emerged from this study.

First, the T cell clones Tc1.8, MCC5, and PT 31 share characteristics that are exhibited by most bonafide CTL. All of them expressed restricted specificity; all

TABLE VI
An Analysis of the 12 Cases from which CTL and
Regulatory Clones were Obtained

No.	System	^{LU} 1,000/10 ⁶ activated PBL	Clone derived from:	Phenotype of clone	Function	Specificity*
1	VIP	60	LNL	CD8	CTL	S
2	MC	48	LNL	CD8	CTL	S
3	PT	72	PBL	CD8	CTL	S
4	RN	50	PBL	CD8	CTL	S
5	JC	35	PBL	CD4	Helper	S
6	MC	48	LNL	CD4	Helper	ND
7	DM	18	PBL	CD4	Helper	S
8	PJ	61	LNL	CD4	Suppressor	S
9	RG	13	LNL	CD4	Suppressor	NS
10	JG	18	PBL	CD4	Suppressor	NS
11	MM	20	PBL	CD4	Suppressor	NS
12	TF	22	PBL	CD4	Suppressor	NS
13	CLB	20	PBL	CD4	Suppressor	Ia
14	GB	11	LNL	CD4	Suppressor	S
15	AS	32	PBL	CD4	Suppressor	ND

* S, specific; NS, nonspecific.

demonstrated MHC class I-restricted function; and they all used their TCR complexes one way or another. mAb to CD3 (a molecule associated with the TCR complex and quite possibly involved in signal transduction) blocked the cytotoxic function in two cases and enhanced cytotoxicity in the other. It is of interest to note that, although the cytotoxicity of Tc1.8 was enhanced by the treatment with CD3 mAb, Tc1.8 needed the full expression of the receptor complex for its function, since modulation of the complex completely abrogated such function (15).

Second, the burdensome demands both of effort and of time involved in carrying out a completely satisfactory and thorough functional analysis of hundreds of emerging clones in individual systems do not allow a credible estimate of the frequency of CTL response in patients with advanced disease. It should be noted that, although we isolated true CTL clones demonstrating specificity in limited cases only, we were able to generate cytotoxic responses at population levels against the autologous melanoma cells in IVC in the majority of these patients. Even if the cytotoxic responses induced in IVC in the other systems reflected lymphokine-activated killer cell-type responses and/or NK cell-type responses, it is quite clear that we were not always successful in selecting true CTL clones in other systems. In this context, it should be mentioned that in the PT system, the MHC class I-restricted autospesific CTL clone PT 31 was isolated in our second attempt. In another system (system AS), although we were able to demonstrate MHC class II-restricted melanoma-specific CD4⁺ CTL response at the population level (data not shown), we failed to isolate an MHC class II-restricted CD4⁺ CTL clone from a total of 60 expanded and well-studied CD4⁺ clones from two separate clonings of 2,000 cells. Thus, these types of technical uncertainties and difficulties in obtaining CTL clones might account for an observed low CTL frequency in advanced melanoma.

Third, our data clearly demonstrate that a CTL clone demonstrating a wide target

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range, or "promiscuous" cytotoxic behavior, may also exhibit antigenic specificity. Although we cannot rule out the possibility that the CTL clone PT A15 did not have another irrelevant population(s) of cytotoxic cells, the cold target competition experiments with A15 and the relevant autologous and irrelevant allogeneic targets (Fig. 5) clearly ruled out antigenic similarities between the autologous and allogeneic targets. Thus, in reference to cytotoxicity against the irrelevant allogeneic targets, clone A15 could have been activated through interactions of accessory molecules and their respective ligands on the target cells. Hence, the possibility exists that a bonafide CTL clone can be activated through alternate activation pathways (i.e., CD2-LFA-3 or through other homotypic and/or heterotypic adhesion molecules that may also serve in signal transduction). Indeed, the cytotoxicity against the irrelevant allogeneic targets by CTL clone A15 could be substantially blocked by mAbs against LFA-1 and ICAM-1, while the cytotoxicity of A15 against the autologous melanoma cells was not blocked. Similarly, cytotoxicity of PT 31 against the autologous PT-M melanoma cells also was not blocked by these reagents (Table III).

Fourth, frequent restimulation by the appropriate antigen and/or autologous accessory cells was not enough to maintain these clones indefinitely in continuous culture. A different strategy, namely, the insertion of a different growth factor gene or a growth factor receptor gene, such as has been accomplished recently by Pierce et al. (16), or the induction of immortality through infection or transfection by a suitable retroviral transforming gene, needs to be considered.

Last, it is clear that once the CTL are activated and amplified, their cytotoxic function cannot be blocked at the effector phase by autologous PBL or by autologous sera. Since the fresh PBL and LNL (from which the CTL clones were obtained) were not cytotoxic against their respective autologous melanoma cells, even though a CTL clone(s) could be isolated from its *in vitro* activated counterparts, one might argue that the cytotoxic unresponsiveness in the fresh effector population was not due to "clonal deletion." Alternatively, such cytotoxic unresponsiveness might have been a reflection of their numeric insufficiency or of the lack of "help" required for activation and amplification of T cell response. It might have resulted also from the influence of the regulatory apparatus.

In reference to regulation of antitumor CTL response, although the role of "suppressor cell activity" has received considerable attention (17, 18), the authenticity of the phenomenon has only been established in the animal tumor system (18-28). Earlier, we presented the evidence of T cell-mediated downregulation of cytotoxic response in two human systems (10, 11, 13). More recently, Cozzolini et al. (29) also have presented indirect evidence of suppressor cell activities in proliferative T cell response in tumors of the head and neck region. Further, Livingston et al. (30) have shown cyclophosphamide-sensitive suppressor cell activities in patients with melanoma. Clearly, then, the issue of cell-mediated suppression of immune response to human tumors has assumed more relevance.

From the above perspective, the present study provides several noteworthy points. First, our data provide additional support for the view that cell-mediated effector response against autologous human cancer is subject to both up- and downregulation. The upregulation of cytotoxic response in coculture induced by the CD4⁺ helper or "helper-inducer" clones in the MC, DM, and JC systems (Fig. 6) is similar to one of our earlier observations (13). It appears that the amplification of cytotoxic

response in the PBL in IVC by the helper cell clones was mediated primarily by IL-2 and by IFN- γ (Table IV). Since these cytokines can influence the behavior of a diverse group of immunocompetent cells (e.g., CTL, LAK, NK, and effector macrophages), these Th cells, therefore, can exert a broad range of regulatory influences. It is noteworthy that, in the MC system, we were also able to document a CTL response (Fig. 3).

Second, the cells participating in downregulatory activities are predominantly CD4⁺ T cells. It is unclear, presently, whether these CD4⁺ regulatory T cells act as suppressor effector cells themselves or act as intermediaries (suppressor inducers). In an earlier communication (11) we demonstrated that CD4⁺ T cells can, indeed, induce a CD8⁺ suppressor effector population.

Third, the regulatory process includes autorestricted as well as nonrestricted interventions. The autorestricted downregulation by the CD4⁺ clone GB 1.7 of cytotoxic immune response in the GB systems is reminiscent of similar autorestricted downregulations shown earlier in two other autologous human tumor systems (10, 11, 13). Further, the higher level of downregulation induced by the T cell clone GB 1.7 against the autologous target was not due to differential expression of D/DR antigen between the melanoma cells GB-M and PT-M. In fact, the autologous melanoma cells GB-M expressed D/DR antigen quantitatively at a lower level than did the PT-M cells (data not shown). Additional evidence for autologous melanoma-associated specificity of the regulatory T cell clone GB 1.7 has been observed. For example, the GB 1.7 cells selectively upregulate their Tac receptors when they are stimulated by the autologous melanoma cells GB-M; and they proliferate when stimulated by the melanoma cells GB-M and exogenous IL-2 (N. G. Chakravorty, and B. Mukherji, manuscript submitted for publication).

Fourth, it is clear that the nonselective downregulation of cytotoxic immune response by CD4⁺ T cell clones in the melanoma system may represent regulation resulting from interaction with the D/DR antigen. The interactions between the PBL and the MHC class II-bearing melanoma cell CLB-M led to a CD4⁺ T cell response that profoundly suppressed cytotoxic responses against both the autologous and allogeneic melanoma targets (Table V). This is analogous to autologous mixed lymphocyte reaction (AMLR), which may lead to downregulation of immune response in different assay systems (31, 32). All nonrestricted downregulations seen in our study, however, did not represent similar situations as evidenced by the nonrestricted downregulation against D/DR antigen-negative allogeneic target shown in the MM system (Table V). The D/DR antigen-induced regulation, nevertheless, is quite intriguing, considering that a significant percentage of human melanomas constitutively express D/DR antigens (33). D/DR antigens have been shown to play a regulatory role in T cell proliferative responses against melanoma cells (34, 35). From an operational point of view, whatever molecular specificities the regulatory cells recognize ("tumor antigen," D/DR antigen, or some other specificity), when such recognition leads to suppression of CTL response, the phenomenon assumes considerable significance in the context of immune response to human melanoma. Interestingly, in this group of patients the emergence of a regulatory response in our in vitro cocultures was associated with only a modest cytotoxic response in the effector populations (Table VI).

Finally, it is of interest that, in our group of patients, identical in vitro activation

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techniques led to the CTL responses, in some, and regulatory responses (specific or nonspecific), in others, without any discernable correlation with GD3, GM2, or D/DR antigen expression by the respective melanoma cells (cumulative data not shown). Circumstances leading to these two opposite results (CTL vs. regulatory response) remain unclear. In this context, it should be noted that immune responses in the chemically induced tumor system in animals have been known to go through sequential expression of CTL responses followed by the emergence of regulatory responses (18, 26). Careful analysis of immune responses in patients with primary melanomas and sequential analysis of immune responses in a group of patients over a length of time will provide much insight into this issue.

Summary

T cell-mediated immune response against autologous melanoma cells was analyzed, at population and clonal levels, in 31 patients with recurrent and/or metastatic disease. Fresh PBL and lymph node lymphocytes (LNL) from melanoma-involved nodes were not cytotoxic against the respective melanoma cells. When activated in *in vitro* coculture (IVC) against the autologous melanoma cells in the presence of IL-2, a majority of the activated PBL and LNL became cytotoxic against the autologous targets. The activated effector cells were cloned in limiting dilution microcultures, and growing clones were phenotypically defined and were functionally characterized for cytotoxicity and for potential regulatory function.

Functional T cell clones were obtained from 15 of 31 cases. Of these, CTL responses exhibiting cytotoxicity restricted against the autologous melanoma were seen in four cases. All four CTL clones were CD3⁺, CD8⁺, and CD4⁻. Three of these four CTL clones were studied extensively. All three of these CTL clones expressed MHC class I-restricted cytotoxicity. mAb anti-CD3 blocked cytotoxicity in two and enhanced cytotoxicity in the other. Neither autologous sera nor autologous nonactivated fresh PBL modulated the cytotoxic functions of the CTL clones at the effector phase. T cell lines exhibiting regulatory function were obtained in 11 cases. The regulatory T cell lines were CD3⁺, CD4⁺, and CD8⁻. In three cases CD4⁺ clones amplified the cytotoxic response in the PBL in coculture, while in eight other cases the T cell lines downregulated the cytotoxic responses. Such T cell-mediated downregulations were either restricted to the autologous system, induced by D/DR antigens expressed by the autologous or allogeneic melanoma cells, or induced by stimulus other than D/DR antigens. Taken together, these findings clearly demonstrate the existence of T cell-mediated cytotoxic and regulatory responses against human melanoma.

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