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CELL-MEDIATED AMPLIFICATION AND DOWN REGULATION OF CYTOTOXIC IMMUNE RESPONSE AGAINST AUTOLOGOUS HUMAN CANCER[1]

BIJAY MUKHERJI,[2] AMALA GUHA, REGINA LOOMIS, AND M. T. ERGIN

From the Departments of Medicine and Surgery, University of Connecticut School of Medicine, Farmington, CT

The cytotoxic host immune response toward autologous human cancer may be regulated by the immunoregulatory network. Here we show that helper T cells, cloned from peripheral blood lymphocytes that were sensitized in vitro against an autologous human malignant paraganglioma, proliferated against and made interleukin 2 when cocultured with the tumor-associated antigen in the presence of autologous accessory cells. Furthermore, the helper cell clones amplified cytotoxic immune response by peripheral blood lymphocytes against the paraganglioma cells in coculture with the blood lymphocytes and the paraganglioma cells. An autologous T cell line bearing suppressor phenotype, established from a lymph node that had been infiltrated with the paraganglioma tumor cells, in contrast to the helper cells, selectively suppressed the cytotoxic immune response by the blood lymphocytes against the paraganglioma cells in identical coculture. These results, therefore, demonstrate the existence of cell-mediated immunologic regulations of the cytotoxic immune response (concurrent amplification and suppression in the same host) against an autologous human tumor.

It has become increasingly clear that tumor-bearing hosts are capable of mounting cell-mediated immune responses against their tumors through a number of different pathways (1–3). It has also become quite evident that the host immune response toward cancer can be subject to regulation, particularly down regulation (4). For cooperative and regulatory purposes, different subpopulations of immunocompetent cells interact with each other within the immune system and influence the immune response positively (up regulation) or negatively (down regulation). Functional analyses of the immunoregulatory network (5) potentially capable of regulating host immune responses against autologous human cancers, therefore, are of considerable interest. We have recently presented evidence of an operational existence of a T cell-mediated regulatory circuitry that is capable of selectively down regulating the generation of autologous cytotoxic effector response in vitro in a human melanoma system (6, 7). Despite reports of suppressor cell activities in tumor immunity in various model systems (4), the evidence for both up and down regulation of the immune response in the host against spontaneously arising autologous human tumors has not been demonstrated. To investigate the potential existence of regulatory interactions in tumor immunity further, we studied the regulatory network that could modulate the cytotoxic immune response against another autologous human tumor system. We demonstrate here that cell-mediated cytotoxic immune response toward a spontaneously arising autologous human tumor can be amplified as well as suppressed by the host's own regulatory apparatus.

MATERIALS AND METHODS

Patient. The patient was a 57-yr-old female who developed a primary laryngeal paraganglioma (a tumor of neuroectodermal origin) 5 yr ago. At the time of our study, she had developed multiple sites of metastasis, predominantly involving numerous subcutaneous nodules. Histologically, the tumor consisted of relatively small cells arranged in cords and nests. Ultrastructurally, secretory granules were present. Several subcutaneous nodules and a tumor-involved lymph node were obtained for studies.

Tumor cells. We performed all experiments in short-term culture in fetal bovine serum-free culture medium and employed freshly isolated tumor cells from an explant of a human malignant paraganglioma in order to eliminate potential culture-induced artifacts in in vitro experiments requiring tumor cells derived from cell lines. Accordingly, single tumor cells were obtained by first mechanically preparing a fine homogenate of a subcutaneous explant of the paraganglioma tissue. Approximately 0.5 ml of the finely minced tissue suspended in 5 ml phosphate buffer saline and 100 μg collagenase type II (Sigma Chemical Co., St. Louis, MO) was vigorously shaken in a 37°C shaking water bath. Fifteen milliliters of buffered saline was added, and the suspension was allowed to settle. The supernatant containing mostly single cells was collected and washed thoroughly. Cells were cryopreserved (−180°C) in filtered fetal calf serum with 10% dimethylsulfoxide 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 and consistent with the cytology of tumor cells. Henceforth, the paraganglioma cells will be referred to as VG-T. The VG-T cells were negative for D/DR antigen but expressed ganglioside GD3 when tested by immunofluorescence tests with monoclonal anti-D/DR framework antibody I2 (Coulter, Hialeah, FL) and monoclonal anti-GD3 antibody R24 (a gift of Alan Houghton, Memorial-Sloan Kettering Cancer Center, New York, NY).

Lymphocytes. Peripheral blood lymphocytes (PBL) were isolated on a Ficoll-Hypaque gradient fresh for each experiment. Further, autologous lymph node resident lymphocytes (LNL)[3] were also isolated by a technique described earlier (6) on a Ficoll-Hypaque gradient from a single cell preparation of a lymph node that was partially infiltrated with the paraganglioma cells. All cultures and experiments were performed in Ham's F-10 medium (GIBCO, Grand Island, NY) containing no bovine serum but supplemented with 20% NU serum (Collaborative Research, Waltham, MA).

In vitro coculture (IVC) and lymphocyte panning. IVC technique has been described (6). Briefly, effector cells (1×10^6/ml) were

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[2] To whom correspondence should be addressed: Department of Medicine, The University of Connecticut School of Medicine, Farmington, CT 06032.

[3] Abbreviations used in this paper: IVC, in vitro coculture; CMC, cell-mediated cytotoxicity; LNL, lymph node-derived lymphocyte; APC, antigen-presenting cell.

cocultured with irradiated (4500 rad) autologous or allogeneic targets (1×10^4/ml) in Ham's F-10 medium supplemented with 20% NU serum. Depending on experimental protocol, 10% lectin-free and purified interleukin 2 (IL 2; Electronucleonics, Silver Spring, MD) was added to the cultures either at the initiation (day 1) or on day 7 of the cultures or cocultures. Unless mentioned otherwise, the standard coculture protocol for generation of cytotoxicity consisted of a coculture in F-10 medium containing 10% IL 2 from day one.

Lymphocyte cloning. Lymphocytes were cloned in limiting dilution microculture technique as described earlier (7, 8), with some modifications.

To generate clones, autologous PBL, irradiated with 2000 rad, were used as feeder cells at concentrations of 10^4 cells/well. Twenty 96-well U-bottom cluster plates (Costar, Cambridge, MA) were seeded with the sensitized PBL or LNL at concentrations such that one of three wells would have received a single cell. Microwells were fed with a drop of 20% lectin-free purified IL 2 (Electronucleonics) 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. Contents of six to eight wells were subsequently pooled into 48-well, and then into 12-well, cluster plates.

Phenotypic analysis and enrichment of lymphocyte in panning. Procedures for phenotypic analysis in a fluorescence-activated cell sorter (FACS) has been described (8). Lymphocytes bearing the desired phenotype were isolated by positive selection in panning. Details have been described (6, 9).

In vitro lymphocyte proliferation assay. Because thymidine incorporation could not be consistently blocked even after 6000 rad to the VG-T cells, a crude VG-T cell lysate was used as an antigen preparation for proliferation assays. The cell lysate stimulated lymphocyte proliferation consistently. Approximately 1×10^3 thoroughly washed cloned lymphocytes were seeded in 0.1 ml F-10 medium supplemented with 20% NU serum in triplicate samples in the microwells of a U-bottomed 96-well microtiter plate (Costar). Approximately 1×10^6 appropriate tumor cells were lysed in 1 ml of medium by alternately freezing (−180°C) and thawing (37°C) three times. Lysate (0.1 ml) was added to each well as stimulator antigen preparation. Approximately 1×10^3 irradiated (3000 rad) autologous unfractionated PBL were used in each well as accessory cells wherever indicated. The total volume in each microwell was 0.2 ml. After 48 hr of incubation at 37°C, the cultures were pulsed with 1 Ci of [methyl-^{3}H]thymidine (^{3}H-TdR; 6.7 Ci/mmol, New England Nuclear, Boston, MA). Seven hours later, the cultures were harvested with the aid of a semi-automatic harvester (TiterTek; Microbiological Associates, Walkersville, MD), and the incorporated radioactivities were counted in a liquid scintillation counter (Beckman Instruments, Palo Alto, CA).

IL 2 assay. IL 2 assay was performed in proliferation assay by using the IL 2-dependent CTLL cells (a gift of Kendal Smith). Briefly, the cloned cells to be tested for IL 2 synthesis capacity were washed four times. The washed cells (1×10^4/ml) were cultured or cocultured with appropriate autologous accessory cells (PBL) (cloned effector cells:PBL = 10:1) and/or autologous tumor cells (cloned effector cells:tumor cells, 100:1) in IL 2-free medium. Accessory cells and tumor cells were irradiated (3000 rad and 4500 rad, respectively). Forty-eight hours later, the supernatants were harvested and were tested for the presence of IL 2 in a 24-hr proliferation assay against the CTLL cells. Approximately 1×10^4 thoroughly washed CTLL cells were added to each well in 0.1 ml volume. Supernatants were added in 0.1 ml. After 24 hr of incubation at 37°C, the cultures were pulsed with [^{3}H]thymidine and processed as described above in reference to lymphocyte proliferation assay. Assays were done in three replicates. For confirmatory tests, approximately 1 ml of the relevant supernatant was absorbed (24 hr) with 2×10^6 washed CTLL cells. The absorbed and the unabsorbed supernatants were then tested for IL 2 activities in the same assay.

In vitro microcytotoxicity (CMC)[3] assay. The ^{51}Cr-release microcytotoxicity assay has been described earlier (8). The freshly prepared and the cryopreserved VG-T targets were labeled with ^{51}Cr with good efficiency, and the mean spontaneous release of ^{51}Cr from 10 separate experiments was 7.5% (range 4 to 11%).

Assay for regulation of cytotoxicity. To assay regulation of generation of cytotoxicity in the PBL (induction phase), standard cocultures between PBL and irradiated VG-T (4500 rad) were set up in the presence or in the absence of irradiated (2000 rad) cloned lymphocytes or the LNL cells as potential regulatory cells at different PBL to regulatory cell ratios. Cytotoxicity was assayed on day 7 at different effector to target ratios. Percentage of enhancement and percentage of suppression were calculated with the following formulae.

$$\% \text{ Suppression} = 1 - \frac{\% \text{ specific lysis with regulatory cells}}{\% \text{ specific lysis without regulatory cells}} \times 100$$

$$\% \text{ Enhancement} = 1 - \frac{\% \text{ specific lysis without regulatory cells}}{\% \text{ specific lysis with regulatory cells}} \times 100$$

Mean cpm of three replicate samples from wells containing effector to target ratio of 20:1 was used to calculate percentage of changes. To assay potential regulation of cytotoxicity at the effector phase, cytotoxic PBL were generated in standard IVC, and cytotoxicity of the PBL was assayed in the presence or absence of irradiated LNL as potential regulatory cells.

RESULTS

When the cytotoxic capacities of the autologous PBL and LNL were tested in a CMC assay against the VG-T target, significant levels of cytotoxic reactivities were observed with the PBL activated in culture in IL 2 or activated in IVC with the VG-T targets in the presence of IL 2. Under identical coculture, the LNL expressed no cytotoxic activity against the autologous target (Table I, Expt. 1). The autologous PBL and the LNL activated in IVC against the VG-T cells (cocultured for 7 days) and expanded thereafter in IL 2 were cytotoxic against the VG-T targets; the LNL, however, remained unreactive (Table I, Expt. 2). PBL, activated in IVC with IL 2 added on day 1 or day 7, consisted of mixed populations of helper ($T4^+$) and suppressor/cytotoxic ($T8^+$) lymphocytes as determined by phenotypic analyses with monoclonal antibody in a FACS IV (Becton Dickinson, Sunnyvale, CA).

Both PBL and LNL sensitized against the VG-T cells for 7 days followed by expansion of the responding lymphocytes in IL 2 for 7 days were cloned in limiting dilution technique with the use of irradiated autologous PBL as feeder cells. Seven colonies of sensitized PBL were obtained and three of the seven colonies were sufficiently expanded for phenotypic and functional analyses. All three colonies exhibited the phenotype of activated helper cells ($T3^+$, $T4^+$, $T8^-$, Ia^+, Tac^+) (monoclonal antibodies OKT3, OKT4, OKT8, and I2 were purchased from Ortho Pharmaceutical, Raritan, NJ; monoclonal antibody Tac was a generous gift from Thomas Waldman). The sensitized LNL could not be cloned after three separate experiments. However, the LNL cells were maintained in continuous culture containing IL 2, and a $T8^+$ LNL line exhibiting the phenotype of activated suppressor/cytotoxic T cells ($T3^+$, $T4^-$, $T8^+$, Ia^+, Tac^+) was established from the IL 2 expanded resensitized LNL cells by positive selection in panning.

Neither the clones expressing the helper cell phenotype nor the unfractionated LNL and the $T8^+$ LNL line exhibited any cytotoxic activity against the VG-T targets (individual data not shown). The unfractionated LNL and the $T8^+$ LNL, however, significantly reduced the generation of cytotoxicity against VG-T when added to the PBL in standard IVC. The $T4^+$ LNL had no suppressive effect. Interestingly, the generation of alloreactivity in the PBL was not affected by the LNL or the $T8^+$ LNL (Fig. 1). The $T8^+$ LNL line suppressed the generation of cytotoxicity in the PBL in a dose-dependent manner (Fig. 2). The $T8^+$ LNL line down regulated the cytotoxicity in the PBL in a

TABLE I

Cytotoxicity by the autologous PBL and LNL against the VG-T target[a]

Expt.		% Specific Lysis Against VG-T at E:T[b]				
		3	6	12	25	50
1	PBL in medium	0	0	0	1	2
	PBL in 10% IL 2	0	0	2	7*	14*
	PBL in IVC with VG-T	0	0	0	0	0
	PBL in IVC with VG-T in IL 2	4	5	13*	26*	38*
	LNL in IVC with VG-T	0	0	2	3	3
	LNL in IVC with VG-T in IL 2	0	1	4	4	6
2	PBL in medium → IL 2	0	2	4	12*	16*
	PBL in IVC with VG-T → IL 2	3	7*	16*	28*	40*
	LNL in IVC with VG-T → IL 2	0	0	2	1	3

[a] In experiment 1, IL 2 (10%) was added in the appropriate IVC from the beginning of the culture. In experiment 2, the culture or the cocultures were carried out for 7 days in medium, after which 10% IL 2 was added to the washed cultures for seven additional days before the cytotoxicity assay.

[b] When compared with percent specific lysis by PBL in medium, the percent specific lysis at identical E:T (indicated by asterisk) was significant at $p < 0.01$ or better by Student's *t*-test. E:T = effector to target cell ratio.

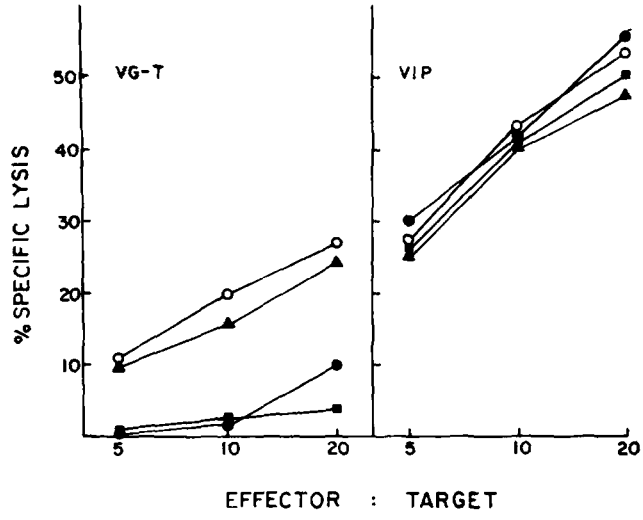

Figure 1. Effect of LNL and the T4$^+$ and T8$^+$ fractions on the cytotoxic response by the autologous PBL in IVC against VG-T and against the allogeneic target VIP. Cytotoxicity by the PBL in standard IVC (O——O), in the presence of the unfractionated LNL (●——●), in the presence of the T4$^+$ LNL (▲——▲), and in the presence of the T8$^+$ LNL (■——■). Percent reduction of cytotoxicity against VG-T induced by the LNL and the T8$^+$ LNL at all points were significant at $p < 0.01$ or better by Student's *t*-test. The reductions caused by the T4$^+$ LNL were not significant.

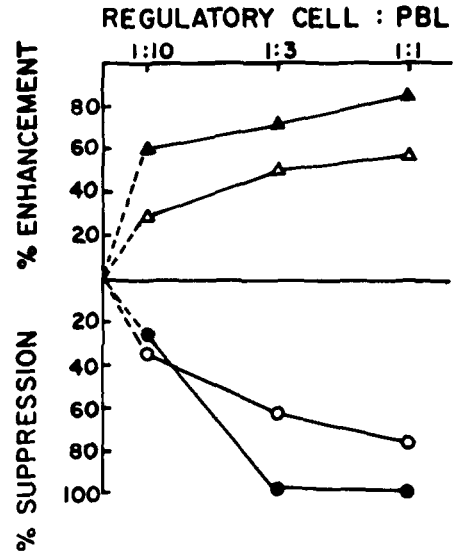

Figure 2. Effect of the helper cell clones and resident LNL lines on the generation of cytotoxicity in the PBL against the autologous tumor cells VG-T. ▲——▲, Clone I.3-6; △——△, Clone I.3.5; ●——●, LNL; O——O, T8$^+$ LNL. Percent enhancement or suppression at all points shown when compared with % specific lysis obtained by the activated PB in the absence of any regulatory cells (At E:T of 20:1, the % specific lysis was 29%) was significant at $p < 0.01$ or better by Student's *t*-test.

dose-dependent manner (Fig. 2). Although the LNL and the T8$^+$ LNL line down regulated the cytotoxic response, the helper clones also amplified the cytotoxic response in a dose-dependent manner (Fig. 2) (the amplification data of I-3-10 not shown). The down regulation of the cytotoxic response was observed at the induction phase of the immune response, as the presence of the T8$^+$ LNL cells had no effect on the cytotoxic PBL at the effector phase (Fig. 3).

Because the reduction of cytotoxicity in the coculture could have resulted from a depletion of IL 2 in the culture caused by the T8$^+$ LNL, experiments were performed with cocultures being fed with excess IL 2 (half of the culture medium being replaced daily with 50% IL 2). Table II shows the results of such an experiment. The reductions of cytotoxicities in the cocultures (standard IVC and IVC with excess IL 2) were essentially comparable.

Figure 4 shows the results of a proliferation assay with the three helper clones. As can be seen, clone I.3-6 showed proliferative activity against the VG-T lysate (prepared by freeze-thawing 1×10^6 VG-T cells in 1 ml phosphate buffered saline) in the presence of irradiated autologous PBL-containing monocytes/macrophages as

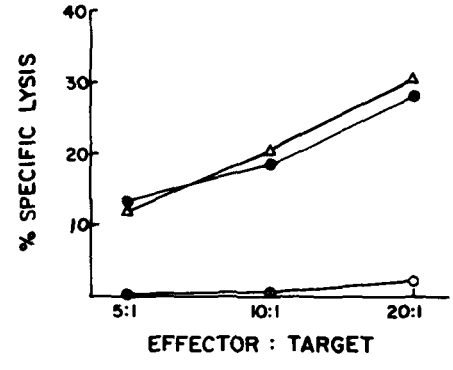

Figure 3. The effect of the T8$^+$ LNL on cytotoxicity by the autologous PBL sensitized in IVC against the VG-T cells at effector phase. Cytotoxicity by the sensitized PBL (●——●), by the sensitized PBL in the presence of the T8$^+$ LNL (△——△), and by the T8$^+$ LNL (O——O).

antigen-presenting cells (APC). Among the helper clones, clone I.3-6 grew better than the others. Hence, this line was used for further functional characterization. Clone I.3-6 synthesized IL 2 when exposed to autologous irradiated APC and tumor cells (Fig. 5). More detailed analyses of specificity of proliferation and IL 2 synthesis could

TABLE II

Effect of feeding coculture with excess IL 2 on reduction of cytotoxic response

	% Specific Lysis against VG-T by PBL in:					
	Standard IVC[a]			Excess IL 2[b]		
	5[c]	10	20	5	10	20
Without $T8^+$ LNL	10	16	21	13	18	24
With $T8^+$ LNL[d]	3	2	5	3	4	8

[a] Autologous PBL were cocultured with VG-T cells (PBL:VG-T = 100:1) in medium containing 10% IL 2 from day 1. Culture was fed every other day with fresh medium + IL 2.

[b] Coculture between PBL and VG-T at identical rates was started in and fed daily with the same medium with 50% IL 2.

[c] E:T.

[d] PBL:$T8^+$ LNL = 1:1.

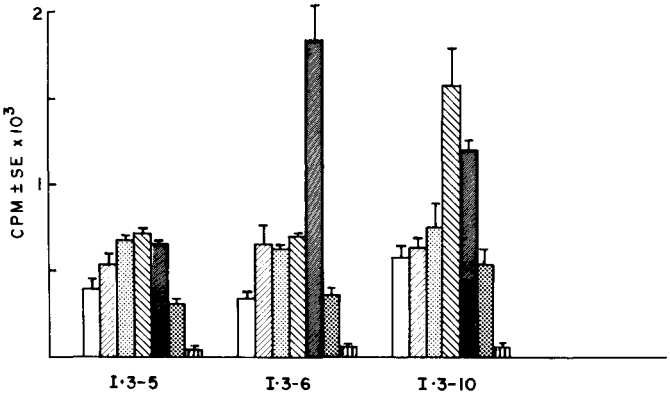

Figure 4. Result of a proliferation assay with three helper T cell clones against lysates of the VG-T and an allogeneic melanoma tumor cell line, VIP. □, Cloned responder cells in medium; ▨, against melanoma cell lysate; □, against the same melanoma lysate in the presence of 1×10^3 irradiated (2000 rad) autologous PBL as APC; ▧, against the autologous VG-T lysate; ■, against the autologous VG-T lysates in the presence of APC; ▩, against the autologous APC; ▥, APC in medium.

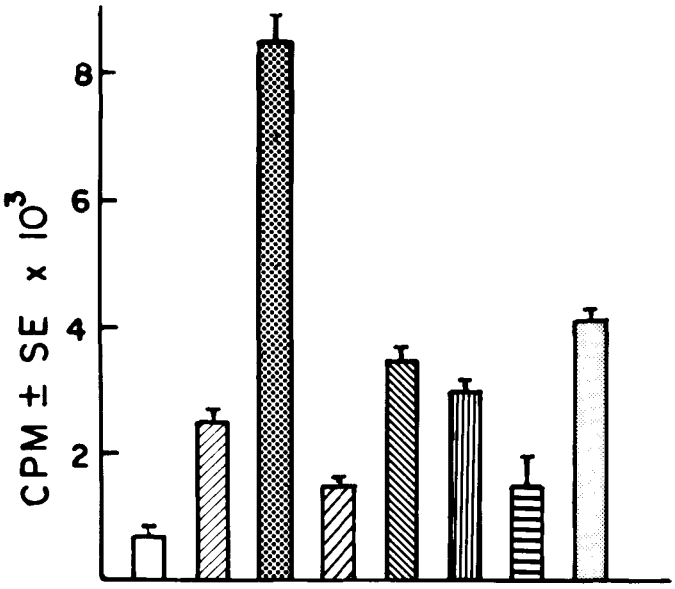

Figure 5. IL 2 assay against the CTLL cells. □, in medium alone; ▨, supernatant from unstimulated clone I.3-6; ▩, unabsorbed supernatant for I.3-6 stimulated with VG-T lysate + APC; ▨, same supernatant absorbed with CTLL cells; ▧, supernatant from I.3-6 stimulated with APC; ▥, supernatant from I.3-6 stimulated with VG-T lysate alone; ▤, supernatant from APC alone; □, in 10% IL 2.

not be performed, although analyses of specificity of the regulations of cytotoxic response were carried out. Table III shows the results. IVC were set up with the PBL against the autologous target VG-T and two other allogeneic target lines in the presence or absence of the regulatory cells

TABLE III

Specificity of suppression or amplification of cytotoxicity in the PBL[a]

By:	% Suppression (−) or Enhancement (+) of Cytotoxicity in the PBL against:			
	VG-T	VIP[b]	PJ-M[c]	K-562[d]
LNL $T8^+$	−85	−2	0	NT
I.3-6	+61	+47	NT[e]	+31

[a] Percent suppression or enhancement were calculated from cpm values obtained in the microcytotoxicity assay with an effector to target ratio of 20:1. Percent suppression against VG-T and percent amplification against VG-T, VIP, and K562 were significant at $p < 0.001$ by Student's *t* test.

[b] VIP, an allogeneic melanoma line.

[c] PJ-M, an allogeneic melanoma line.

[d] K-562, erythroleukemia natural killer-sensitive target line.

[e] NT, not tested.

in standard coculture protocols. The down regulation of the cytotoxic immune response induced by the $T8^+$ LNL cell was selectively observed against the autologous target VG-T, as the generation of cytotoxic alloreactivity was unaffected by the $T8^+$ line. The amplification of the cytotoxicity by the helper clone I.3-6, on the other hand, showed no autospecificity.

DISCUSSION

Thus, the generation of cytotoxic immune response in the PBL against an autologous tumor could be enhanced by amplifier T cell clones (one of which was shown capable of synthesizing IL 2 and recognizing the autologous target in proliferation assay) and could be selectively down regulated as well by suppressor cells that were derived from a lymph node that was involved with the same tumor and that was devoid of autoreactive cytotoxic cells or their precursors (as evidenced by a lack of generation of autoreactive cytotoxic cells in assay, which could readily induce cytotoxicity in the PBL). The reduction of cytotoxic response by the PBL in the presence of the $T8^+$ LNL could not be explained by the depletion of IL 2 by the regulatory cells. First, the suppression caused by the $T8^+$ LNL was not mitigated by the addition of excess IL 2 in the culture (Table II). Second, the cytotoxic allo-responses were not reduced by the same regulatory $T8^+$ LNL (Fig. 1 and Table III). Finally, in the autologous coculture (in which the $T8^+$ LNL reduced the cytotoxic response) the $T4^+$ LNL (derived from the same parent LNL population and grown in IL 2 for the same length of time) had no down regulatory activity. The mechanism for amplification of cytotoxic response by the helper clones remains unclear. It is conceivable that the amplification could have been mediated by IL 2 synthesis or by a cytokine other than IL 2. Unfortunately, these questions could not be pursued further as fresh autologous PBL could not be obtained for an extended period of time. Regardless of the mechanisms of amplification of cytotoxic immune response, the experiments presented in this paper provide a clear example of the operational existence of both forms of regulatory interventions in immune response in the same host against a spontaneously arising tumor. To our knowledge, these types of modulations of cytotoxic response against autologous human tumor system have not been shown before. The helper cell clone I.3-6 synthesized IL 2, proliferated when exposed to the autologous tumor-associated antigen plus APC, and amplified the cytotoxic response in the PBL. Extensive analyses of specificities of these functions

could not be undertaken as fresh autologous PBL were not available at the end of the study period.

Although the cellular and molecular mechanism of amplification could not be further explored in this system, from a biological point of view and because the tumor grew and metastasized in this host despite the existence of a mechanism for amplification of cytotoxic immune response, one can speculate that the net result of host immune response toward this tumor may have been determined by the balance between or, more appropriately, by the dominance of one opposing arm of the network over the other (in this case, suppression over help). We have reason to believe that the phenomena of suppression of cell-mediated immune response against autologous tumor, such as in this case and as recently communicated (6, 7), are not exceptions.

We have also observed autoregulation of anti-tumor immune response in other human tumor systems (unpublished observation). These types of autospecific regulation of anti-tumor immune response may explain the cytotoxic unresponsive state in a large proportion of patients with cancer against their respective cancer cells (10–12) while cytotoxic responsiveness of such patients against allogeneic tumors was essentially intact (10). The results of this study and our interpretation are consistent with earlier observations of concomitant as well as sequential induction of immunity and immunosuppression in animals bearing different types of tumors (13–15). In addition to extending such earlier findings, we now believe that in a spontaneously arising autologous human tumor system, the selectivity of the down regulation of the cytotoxic immune response in this system and in the autologous melanoma system (6, 7) is of considerable interest, from the point of view of the network theory (5, 16) and from our present understanding of T cell receptors (17, 18). Importantly, since the regulatory cells exhibited selectivity toward effector response against the autologous tumor, further studies of receptors through which these regulatory cells exhibit specificity (i.e., via clonotypic-anticlonotypic determinants or any other receptor-mediated interactions) could provide unique opportunities of controlling the regulatory interference of effective immune response, which in turn might provide novel opportunities for control of neoplasia.

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