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T-cell-mediated cytotoxicity against autologous malignant melanoma: Analysis with interleukin 2-dependent T-cell cultures

(cancer immunology/cellular immunity)

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ABSTRACT **The cytotoxic reactivity of lymphocytes for autologous melanoma cells was studied in a group of 13 melanoma patients. No cytotoxicity was observed with lymphocytes freshly isolated from peripheral blood or with lymphocytes cocultured for 7 days with autologous melanoma cells. Growth of lymphocytes previously sensitized with autologous melanoma *in vitro* in interleukin 2 (IL-2)-containing medium, however, resulted in cytotoxic reactivity for autologous melanoma in 7/13 patients. The reactivity of IL-2-dependent lymphocytes for autologous melanoma was particularly striking in one patient (A.V.) who has had an unexpectedly favorable clinical course and, because of their consistently high reactivity, AV lymphocytes were selected for detailed specificity analysis. After 2–3 weeks in culture in IL-2-containing medium, AV lymphocytes were cytolytic for autologous melanoma cells but not autologous Epstein–Barr virus-transformed B cells, autologous fibroblasts, or allogeneic tumor targets. Specificity of autologous melanoma reactivity was confirmed by competitive inhibition assays. The IL-2-dependent AV lymphocytes formed rosettes with sheep erythrocytes and expressed OKT 3 and Ia antigens. After longer periods of culture, AV lymphocytes were found to react with a wider range of target cells, and repeated attempts to isolate cultures with restricted reactivity to autologous melanoma by resensitization with autologous melanoma and limiting-dilution techniques were unsuccessful. The restricted reactivity of early cultures could be preserved, however, in frozen storage, but shifted again toward broader reactivity after several weeks in culture. The recognition of cytotoxic T cells with initial restricted reactivity for autologous melanoma suggests reinvestigation of the question of specific cellular immunity to human cancer.**

Few topics in cancer research have received as much attention as the issue of tumor-specific antigens. In the case of tumors in experimental animals, the individually distinct antigens of chemically induced tumors, demonstrable by their ability to induce transplantation resistance in syngeneic hosts, represent the most critical and still most compelling evidence for antigens that appear to be restricted to cancer cells (1). In the case of human cancer, the search for cancer-specific antigens has taken two general approaches. One involves the analysis of heteroantibody to human cancer cells, and this approach has identified a number of quantitative antigenic differences between normal and malignant cells. None of the antigens identified to date by heteroantibody have been tumor specific. However, the advent of hybridoma technology has given new impetus and promise to the use of heteroantibodies as probes for tumor antigens. The other approach has focused on immune reactions of cancer patients to human cancer cells by use of *in vitro* techniques to measure humoral or cellular immunity. Although there is no doubt that a variety of immunological reactions against cancer can be detected, the nature, specificity, and significance of these reactions remain open to question (2). To eliminate problems of investigating reactions obtained when serum or lymphocytes from one individual are tested with tumor cells from another individual (allogeneic combinations), attention has now shifted to the analysis of reactions observed when serum, lymphocytes, and tumor cells are from the same individual (autologous combinations). Application of this approach to the analysis of humoral immunity to several types of human cancer has resulted in the definition of three classes of cell surface antigens demonstrable with autologous antibody (3–7). Although defining specificity is relatively straightforward using serological methods, analyzing specificity of T-cell reactions to autologous tumor cells is far more difficult. The most extensive study to date is that of Livingston *et al.* (8), who examined the cytotoxicity of peripheral blood lymphocytes for autologous cultured melanoma cells in 32 patients. Eighteen patients showed autologous reactivity that appeared to correlate with more limited disease and better clinical prognosis. In these studies, a major problem in defining the specificity of cell-mediated cytotoxic responses was the limited supply of effector cells. With the recognition of T-cell growth factor (interleukin 2, IL-2) (9, 10), it should be possible to overcome this difficulty, as cultures of functional T cells can be cloned and propagated for extended periods.

In the present study, we have examined the generation and specificity of IL-2-dependent cytotoxic T cells sensitized *in vitro* with autologous melanoma cells.

MATERIALS AND METHODS

Patients. All patients studied had recurrent or metastatic malignant melanoma. The patient selected for detailed study (A.V.) is a 31-year-old male who underwent resections of extensive metastatic melanoma of axillary, supraclavicular, and cervical lymph nodes in 1976 and 1978 and has remained free of detectable melanoma since that time. From 1976 to 1978, the patient's lymphocytes were found to be strongly cytotoxic for autologous cultured melanoma cells on several occasions (8). At the time of the present study, only minimal lymphocyte cytotoxicity for autologous melanoma cells was detectable. Antibodies reactive with cell surface antigens of autologous melanoma cells were not detectable in the patient's serum at any time.

Target Cells. See refs. 3–7.

Effector Cells. Peripheral blood lymphocytes (PBL) were separated from heparinized blood by using Ficoll-Hypaque. The medium used was Eagle's minimal essential medium

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Abbreviations: IL-2, interleukin 2; IL-2 CM, IL-2-conditioned medium; EBV, Epstein–Barr virus; E:T, effector cell/target cell ratio; PBL, peripheral blood lymphocytes.

(ME medium) supplemented with 2 mM glutamine, 1% nonessential amino acids, penicillin at 100 international units/ml, streptomycin at 100 μg/ml, and 10% prescreened fetal bovine serum (complete medium, CM). Sensitization with melanoma cells or fibroblasts: PBL, 1–2 × 10^6/ml, were added to melanoma cells or fibroblasts growing as 80% confluent monolayers (Falcon 3013 flasks, Falcon Plastics, Oxnard, CA) in a total vol of 4 ml. Sensitization with Epstein–Barr virus (EBV)-transformed B cells: PBL, 1–2 × 10^6/ml, were mixed with irradiated (5000 rads; 1 rad = 0.01 gray) EBV-transformed B lymphocytes at a ratio of 1:1 or 1:2 in a total vol of 4 ml. After 7 or 9 days of coculture, the responder lymphocytes were transferred to CM containing IL-2 (IL-2 CM) and passaged in T30 flasks or subcultured by limiting dilution in 96-well flat-bottomed microtest plates (3040, Falcon Plastics) with or without irradiated (2500 rads) allogeneic PBL (5 × 10^4 per well) as feeder cells. For subculturing, 32 central wells in 10 plates were seeded, 8 replicate wells each, at a starting concentration of five cells per well and at three doubling dilutions and maintained for 2–3 weeks. In a representative experiment, 13/80 growing cultures were recovered from wells seeded at five cells per well, 8/80 at the next serial dilution (2.5 cells per well), 3/80 (1.25 cells per well), and 2/80 (0.625 cells per well) at subsequent dilutions. Subcultures obtained in this way were again subjected to limiting dilution, generating second- and third-generation subcultures (with expansion in 24-well Linbro plates between each subculturing). The results of limiting dilution subculturing presented in this report were derived from an analysis of nine initial AV lymphocyte cultures previously sensitized with autologous AV melanoma, 55 subcultures after the first series of limiting dilutions, 28 after the second series of limiting dilutions, and 39 after the third series of limiting dilutions.

IL-2 CM was prepared by a modification of the procedure of Morgan *et al.* (9).

Cell-Mediated Cytotoxicity Assay. Target cells growing as monolayers were cultured in tissue culture flasks (3013, Falcon Plastics) to 80% confluence, washed in complete ME medium and incubated with 200 μCi of $Na_2{}^{51}CrO_4$ (1 Ci = 37 GBq; New England Nuclear, Boston, MA) in 1 ml of ME medium for 2 hr at 37°C in 5% CO_2/95% air. Target cells growing in suspension culture were labeled in a similar fashion at a cell concentration of 2 × 10^6/ml in 0.5 ml of RPMI 1640 medium. After labeling, target cells growing as adherent monolayers were detached by treatment with 0.05% EDTA in phosphate-buffered saline. The labeled target cells were washed twice and seeded into 96-well round-bottomed microtest plates (163320, Nunc, Roskilde, Denmark), 1000 cells per well for nonlymphoid target cells and 2000 cells per well for lymphoid target cells. Target cells were incubated with effector cells at specified effector cell:target cell (E:T) ratios for 6 hr in 5% CO_2/95% air at 37°C. The supernatants were harvested using the Titertek harvesting system (Flow Laboratories) and assayed for radioactivity in a gamma counter. Cytotoxicity is expressed as follows: % specific lysis = $[(a - b)/(c - b)] \times 100$, where a = cpm of the supernatant of target cells incubated with effector cells, b = cpm of the supernatant of target cells without effector cells, and c = cpm of the supernatant of target cells lysed with Nonidet P-40 (Sigma).

Competitive Inhibition Tests. See Fig. 1.

RESULTS

Reactivity of PBL with Autologous Melanoma Cells. PBL from 13 melanoma patients were tested for cytotoxic reactions with autologous melanoma cells. No or minimal cytolytic activity was found at E:T ratios ≥40:1. The percentage specific lysis never exceeded 20% with freshly separated PBL even at E:T ratios of 200:1.

Generation and Reactivity of IL-2-Dependent Lymphocytes. No cytotoxicity against autologous melanoma cells was detected with PBL co-cultured for 7 or 9 days with autologous tumor in the absence of exogenous IL-2. However, after outgrowth of lymphocytes in IL-2 CM, cytotoxic reactivity of 20–60% specific lysis of autologous melanoma cells at E:T ratios of 40:1 was observed in 7/13 patients. High levels of cytotoxicity could be generated most regularly with cultured lymphocytes from patient A.V. For this reason, the reactivity of AV lymphocytes was selected for detailed analysis.

Growth Characteristics of AV Lymphocytes in IL-2 CM. After co-culture with autologous melanoma cells for 7 days, AV lymphocytes placed in IL-2 CM showed initial proliferation, followed by a lag phase of 3–5 weeks, followed by renewed proliferation. In contrast, AV lymphocytes co-cultured with AV EBV-transformed B cells or AV skin fibroblasts ceased to grow after approximately 1 month of culture in IL-2 CM. When irradiated Daudi cells were added to AV lymphocytes co-cultured with autologous melanoma cells, subsequent lymphocyte proliferation in IL-2 CM was often sustained without an initial intervening lag phase. Lympho-

Table 1. Cytotoxicity of AV lymphocytes for autologous AV melanoma cells before and after *in vitro* sensitization with AV melanoma and subsequent culture in IL-2 CM

Effector cells*	*In vitro* sensitization,† days	Growth in IL-2, days	E:T ratio	Cytotoxicity at doubling dilutions of E:T ratio, % specific lysis			
				Undiluted	1:2	1:4	1:8
AV-PBL	—	—	40:1	12	8	2	3
AV-PBL	—	—	80:1	−3	−1	−7	−4
AV-PBL	—	—	200:1	5	6	3	1
AV1	7	—	20:1	7	0	−1	−1
AV4	7	—	40:1	25	0	1	2
AV7	9	—	40:1	10	12	7	2
AV1	7	14	35:1	100	82	60	31
AV4	7	8	18:1	56	53	22	10
AV7	9	7	40:1	81	69	59	43

Spontaneous ^{51}Cr release was 11–25%.
*AV lymphocytes prepared by Ficoll separation from heparinized blood. AV1, AV4, and AV7 are designations for cultures of AV lymphocytes obtained from patient A.V. on separate occasions.
†*In vitro* sensitization with autologous AV melanoma.

cyte cultures generated in this way showed a median doubling time of 2–4 days. Occasional doubling times of 24 hr or even less were observed.

Cytotoxic Reactions of AV Lymphocytes with AV Melanoma. The results of cytotoxic tests with AV lymphocytes are given in Table 1. No or minimal cytotoxicity was observed with AV PBL obtained on three separate occasions or with AV lymphocytes sensitized for 7 or 9 days with autologous melanoma. In contrast, substantial cytotoxicity was seen with the presensitized AV effector cells cultured in IL-2 CM. AV lymphocytes grown in IL-2 CM had the surface phenotype of activated T cells: >90–95% of cells formed rosettes with sheep erythrocytes and expressed T3 and Ia antigens. For control purposes, the cytotoxicity of IL-2-dependent cultures of AV lymphocytes presensitized in the following ways was determined: stimulation with phytohemagglutinin or co-culture with Daudi cells or with autologous EBV-transformed B cells or autologous skin fibroblasts. (In contrast to the IL-2-dependent growth of AV lymphocytes following melanoma sensitization, lymphocytes without prior melanoma sensitization grew poorly in IL-2 CM and could be cultured for only a short period.) Tests with these cultures showed little or no cytotoxic activity for autologous melanoma cells or only broad nonspecific reactivity.

Specificity Analysis of AV Lymphocytes: Direct Cytotoxicity Tests and Competitive Inhibition Assays. The results of a series of experiments with AV lymphocytes grown in IL-2 CM for 11, 16, or 22 days following a 7-day (AV1, AV5) or 9-day (AV7) sensitization with AV melanoma are summarized in Table 2. At an E:T ratio of 40:1, strong cytotoxicity for AV melanoma was observed, whereas there was little or no reactivity with two other melanoma target cell lines (BD, AH) or a renal cancer line (AG). In addition, AV lymphocytes with autologous melanoma reactivity were not cytotoxic for autologous AV EBV-transformed B cells or AV fibroblasts. K562 cells, the standard test cell for natural killer cell activity, and Daudi cells used as the "third party" stimulator cell during *in vitro* co-culture, were not lysed by AV lymphocytes sensitized with autologous AV melanoma.

Table 2. Specificity of AV lymphocyte cytotoxicity for autologous AV melanoma cells: Analysis by direct tests

Effector cells*	Target cells†	Cytotoxicity at doubling dilutions of E:T ratio, % specific lysis			
		Undiluted	1:2	1:4	1:8
AV1	AV melanoma cells	100	82	60	31
	AV B cells (EBV)	20	17	6	−1
	AV fibroblasts	28	1	−7	−6
	BD melanoma cells	16	9	2	2
	AG renal cancer cells	17	10	4	3
AV5	AV melanoma cells	97	61	43	28
	BD melanoma cells	4	1	0	−1
	AH melanoma cells	6	5	6	1
	AG renal cancer cells	3	5	4	1
AV7	AV melanoma cells	102	103	98	72
	BD melanoma cells	3	−2	−1	4
	AH melanoma cells	30	13	7	7
	AG renal cancer cells	50	20	7	5

*AV effector cells were co-cultured with autologous melanoma cells for 7 days (AV1, AV5) or 9 days (AV7) and then in IL-2 CM for 16 days (AV1), 22 days (AV5), or 11 days (AV7). The selective cytotoxicity of AV lymphocytes for autologous melanoma could be recovered from cryopreserved AV1, AV5, or AV7 cultures.

†At 1000 target cells per well; spontaneous ^{51}Cr release was 10–16%, except for B cells (26%) and fibroblasts (37%). E:T ratio, 40:1. AV, BD, and AH melanoma cells and AG renal cancer cells show comparable sensitivity to allogeneic cytotoxic reactions.

The specificity of AV lymphocytes for autologous melanoma cells was repeatedly confirmed in competitive inhibition assays. As shown in Fig. 1, AV lymphocyte cytotoxicity for autologous AV melanoma could be inhibited by AV melanoma but not by autologous EBV-transformed B cells or by allogeneic melanoma cells, renal cancer cells, EBV-transformed B cells, or Daudi cells.

Change in the Cytotoxic Reactivity of Melanoma-Sensitized AV Lymphocytes During Long-Term Culture. The reactions of two independently derived cultures of AV effector cells (AV5 and AV7) grown in IL-2-containing medium for various periods are shown in Fig. 2. AV5 lymphocytes cultured for 22 days showed a restricted pattern of cytotoxicity—i.e., cytotoxicity for autologous AV melanoma cells but not for the allogeneic target cells. After 27–34 days in culture, AV5 lymphocytes began to show cytotoxicity for AG renal cancer and BD melanoma at a high E:T ratio, and by 49 days cytotoxicity for AG renal cancer was as strong as it was for autologous melanoma. With longer culture, autologous melanoma reactivity of AV5 disappeared whereas cytotoxicity for AG renal cancer persisted at high levels. In the case of lymphocytes from AV7 culture, a similar pattern of shift in cytotoxic reactivity against autologous and allogeneic target cells was observed. The initial restricted cytotoxicity against AV melanoma gradually ceased and reactivity against AG renal cancer cells increased. After prolonged culture (>150 days), reactivity of AV lymphocytes against allogeneic target cells decreased or disappeared. Efforts to preserve the restricted pattern of cytotoxicity by repeated restimulation of AV lymphocytes with autologous melanoma cells were unsuccessful.

Reactivity of Subcultures of AV Lymphocytes. In view of the loss of specificity of AV lymphocytes for autologous melanoma cells during culture, we asked whether this change affected all effector cells in the culture or reflected a growth advantage of more broadly reactive clones over clones with restricted reactivity. The results of tests with a panel of AV lymphocyte subcultures derived by limiting di-

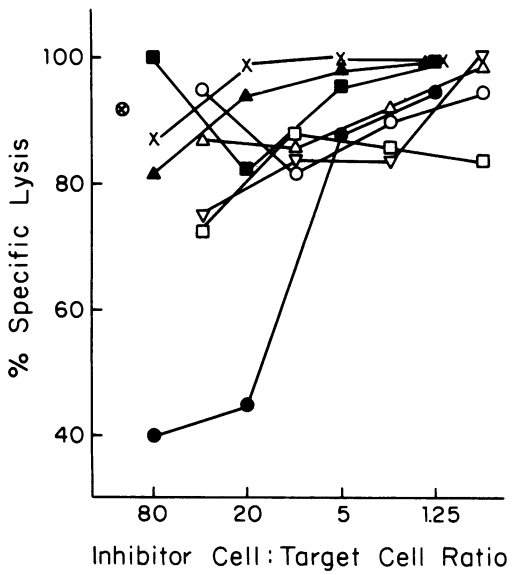

FIG. 1. Competitive inhibition assays with AV effector cells previously sensitized with autologous melanoma and cultured for 26 days in IL-2 CM. Effector cells were incubated with various numbers of inhibitor cells for 20 min at 37°C before addition of labeled AV melanoma target cells (1000 cells per well). E:T ratio, 20:1. Inhibitor cells: ⊗, none; ●, autologous AV melanoma; △, AH melanoma; □, BD melanoma; ▽, AG renal cancer. EBV-transformed B cells: ○, AV; ▲, AH; ■, BD; ×, Daudi cells.

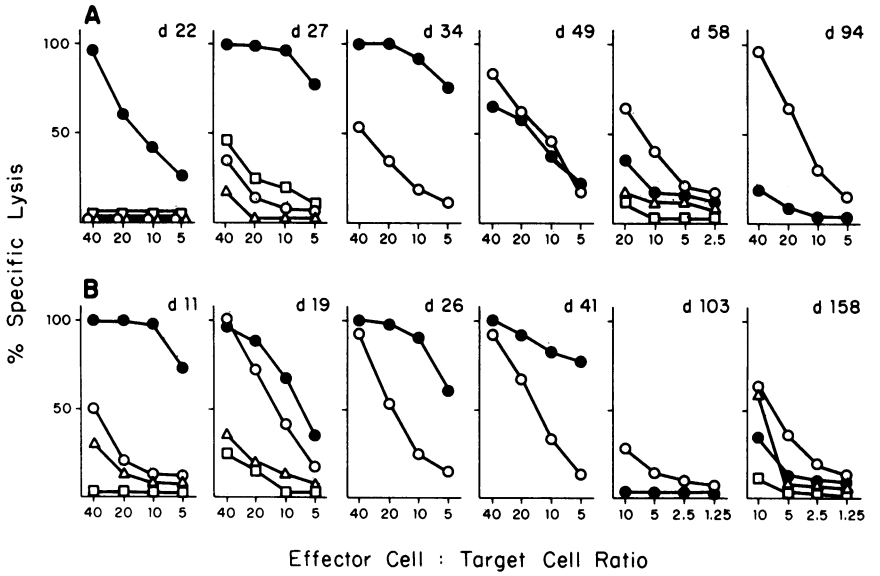

FIG. 2. Changes in reactivity of AV lymphocytes previously sensitized with autologous AV melanoma and cultured in IL-2 CM for various numbers of days (d). (*A*) Culture AV5. (*B*) culture AV7. Target cells: ●, autologous AV melanoma; △, AH melanoma; □, BD melanoma; ○, AG renal cancer.

lution from an 11-day IL-2 CM culture of AV lymphocytes (after a 9-day *in vitro* sensitization period with autologous AV melanoma) with restricted cytotoxicity for autologous melanoma are shown in Fig. 3. At the time of the tests with lymphocyte subcultures shown in Fig. 3, the parental culture AV7 showed a strong preferential cytotoxicity for autologous AV melanoma targets and weaker cross-reactivity with allogeneic AG renal cancer cell targets (as shown in Fig. 2*B*, days 26–41). The subcultures were tested at 28 days (SC 15), 33 days (SC 3, 8, 19, and 21), 35 days (SC 2, 4, 11, 14, 16, and 17) and 56 days (SC 23) after initiating IL-2-dependent growth. Four patterns of reactivity were observed with AV subcultures: (*i*) reactivity restricted to autologous melanoma (SC 17); (*ii*) reactivity for both autologous AV melanoma and AG renal cancer, with some subcultures showing higher reactivity with AV melanoma (e.g., SC 2) and others, higher reactivity with AG renal cancer (e.g., SC 16); (*iii*) reactivity restricted to AG renal cancer (SC 15) or (*iv*) no reactivity (SC 19 and SC 23). In our experience, AV subcultures with reactivity restricted to autologous melanoma cells could not be expanded for further analysis whereas AV subcultures with nonrestricted reactivity or no reactivity generally grew well. No cultures with reactivity restricted to autologous AV melanoma were obtained by further subculturing.

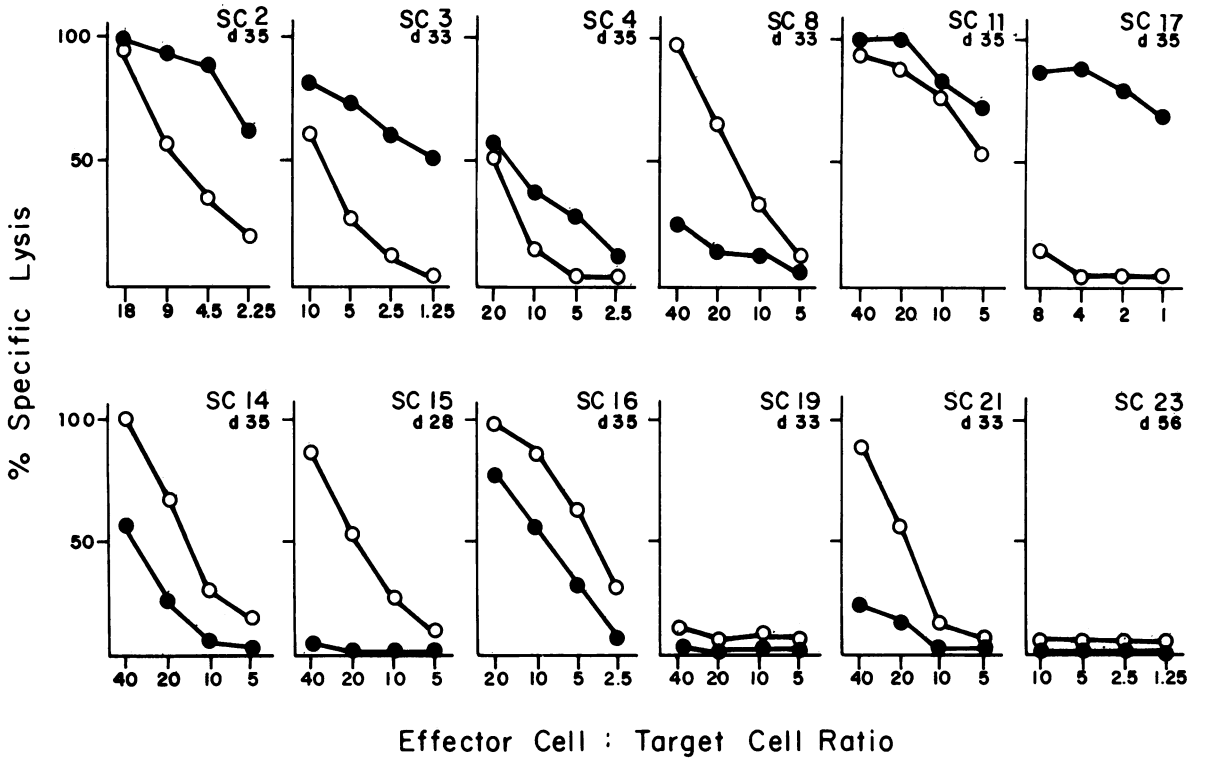

FIG. 3. Reactivity of subcultures (SC) of AV7 lymphocytes derived by limiting dilutions and cultured for various numbers of days (d). Target cells: ●, autologous AV melanoma; ○, AG renal cancer.

DISCUSSION

Previous studies have shown that patient A.V. lymphocytes were cytotoxic for cultured autologous melanoma cells without prior *in vitro* sensitization (8). By the time the present study was initiated, autologous cytotoxicity of unsensitized lymphocytes could not be demonstrated. However, *in vitro* sensitization with autologous melanoma cells, especially in the presence of an allogeneic stimulator cell line (Daudi) and subsequent growth in IL-2-containing medium resulted in appearance of cytotoxic T cells with autologous reactivity.

Reactivity of AV T cells with the panel of autologous and allogeneic target cells showed four distinct patterns: (*i*) cytotoxicity restricted to autologous melanoma cells, (*ii*) nonrestricted cytotoxicity for both autologous and allogeneic targets, (*iii*) cytotoxicity restricted to the allogeneic target, and (*iv*) no cytotoxicity. Time in culture appeared to determine which reaction pattern predominated, with early cultures showing a restricted pattern, later cultures a nonrestricted pattern, and prolonged cultures no reactivity. To determine whether these altered reactivity patterns were due to the outgrowth of cells with nonrestricted or no reactivity (present but not detected in the original culture) or to changes in the specificity of individual cells occurring during propagation *in vitro*, early cultures of AV lymphocytes with a restricted pattern of cytotoxicity were subcultured at limiting dilutions. The recovery of nonrestricted T cells from restricted cultures argues for their preexistence in the initial cultures. The fact that T cells with restricted cytotoxicity subcultured poorly, whereas nonrestricted (and nonreactive) T cells were easily propagated, would account for the emergence of nonrestricted cells from cultures containing predominantly restricted cells. However, we cannot formally exclude the possibility that individual cytotoxic AV cells go through a sequential change in specificity and reactivity. Whatever explanation is correct, restimulation of cultures of restricted cells with autologous melanomas has not resulted in stabilizing the restricted phenotype nor has earlier subculturing or changing culture conditions. Thus, conditions for the prolonged culture of AV T cells with specificity for autologous melanoma cells need to be defined. One approach that is being explored involves immortalization of reactive clones with human T-cell leukemia virus (HTLV).

Despite the changes in reactivity of cultured AV T cells with time, sufficient numbers of T cells showing restricted reactivity could be repeatedly obtained from this patient, and direct tests and competitive inhibition analysis showed that these T cells had specificity for autologous melanoma cells. *In vitro* sensitization with autologous malignant melanoma was necessary to generate AV cells with restricted cytotoxicity; autologous B cells or autologous fibroblasts were not effective in this regard. Although these findings are consistent with the possibility that AV T cells recognize a melanoma-specific antigen, the possibility that an autoimmunogenic differentiation antigen expressed by melanoma cells and/or melanocytes may be involved cannot be excluded. The panel of autologous target cells is too limited to distinguish between these possibilities, and the unknown requirement for HLA-associated recognition of the AV melanoma antigen by AV cytotoxic T cells adds a complication to interpreting results with allogeneic melanomas. Nevertheless, the finding of such restricted activity provides an opportunity to characterize the antigen being recognized.

In this study of 13 patients, only A.V. had sufficiently high and reproducible reactivity against autologous melanoma cells for detailed analysis. This low incidence of patients having demonstrable T-cell cytotoxicity with specificity for autologous melanoma parallels the low incidence of patients showing specific humoral reactivity against autologous melanoma (2). These studies of cellular and humoral immune reactions in patients with melanoma have focused on patients with stage II or more advanced disease, because of the difficulty of obtaining sufficient tissue from patients with primary melanoma to establish tissue culture lines. A comparable study of a series of patients with primary melanoma will be instructive to determine whether the frequency of specific immune reactions will be higher in patients with more limited disease. As patient A.V. has had a remarkable clinical course, with prolonged survival in the face of advanced disease, it will be important to identify other patients with similar T-cell reactivity for autologous melanoma cells and determine whether a correlation with survival will be found. In addition, the nature of the target cell in detecting immune reactions in melanoma patients needs to be stressed, as emphasized in the studies of Albino *et al.* (11). Of melanoma cell lines derived from six separate metastases of a single patient, only one line expressed sufficient antigen to detect autologous antibody. From these and other considerations (see ref. 12), it is evident that an assessment of the frequency, specificity, and significance of humoral and cellular reactions against autologous melanoma has barely begun. In this context, it is encouraging that Mukherji and MacAlister (13) have recently reported another melanoma patient with autologous T-cell cytotoxicity showing restricted specificity.

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