Brief Definitive Report

HETEROGENEITY OF CLONES FROM A HUMAN METASTATIC MELANOMA DETECTED BY AUTOLOGOUS CYTOTOXIC T LYMPHOCYTE CLONES

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The analysis of cell surface antigenic determinants associated with malignant transformation of cells in the melanocyte lineage has been carried out in recent years (1–4) with autologous sera as well as with mAb to HLA and melanoma-associated antigens (MAA).

This approach has revealed the existence of a high degree of phenotypic heterogeneity not only among tumors derived from different patients (4), but also between autologous primary and metastatic lesions (5), among different metastases removed from the same patient (6) and even among clones isolated from the same cell line (7, 8).

The differential expression of tumor-associated antigens (TAA) on different melanomas and in different cells of the same tumor has profound implications for the design of both diagnostic and therapeutic approaches with antibodies to MAA, since neoplastic clones not expressing the relevant antigen will not be detected or affected by these methods. The outcome of alternative therapeutic strategies, now under study in many laboratories, which are based on the infusion of autologous activated cytotoxic lymphocytes as antitumor reagents (9), might be equally influenced by the differential expression of the target structure recognized by the cytolytic effectors in a single melanoma. At the present time, however, no information is available concerning the heterogeneity of a single human tumor as determined by susceptibility to autologous CTL.

To address this issue, we used cloned CTL, previously shown (10) to react against the autologous metastatic melanoma (Me28) to screen 31 clones derived from Me28. We looked for significant differences in the cytotoxic activity of the CTL tested against the clones of the tumor and the uncloned melanoma line.

Materials and Methods

T Cell Clones. CTL clones B11 and A4 were derived by limiting dilution after mixed lymphocyte–tumor culture and/or IL-2 activation of PBL from a patient with metastatic melanoma (Me28) as previously described (10). Both clones have been previously shown (10) to lyse Me28 and to manifest different specificities as assessed in tests on a panel of allogeneic melanomas. Six clones obtained by micromanipulation (10) from B11 and A4 were used in this study.

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Melanoma Cell Cloning. Melanoma Me28 was cloned by a two-layer soft agarose technique at the fifth in vitro passage. Briefly, 5×10^2 Me28 cells in 2.5 ml of 15% FCS-RPMI 1640 containing 0.35% agarose (Seaplaque Agarose 50101; FMC Corp., Rockland, ME) were seeded in tissue culture dishes (3506; Costar, Cambridge, MA) containing a first layer of 2 ml of 0.5% agarose/15% FCS RPMI 1640 and incubated at 37°C in 5% CO_2. 2 wk later, individual colonies growing at the interface between the two agarose layers were harvested by micromanipulation and transferred to flat-bottom plates (3596; Costar) in 0.2 ml of 15% FCS/RPMI 1640. 54 clones were eventually expanded to 25-cm^2 tissue culture flasks (25100; Corning Glass Works, Corning, NY).

mAb and FACS Analysis. w6/32 (Seralab, Crawley Down, United Kingdom) recognizes a monomorphic determinant on class I HLA molecules; D1-12 is directed to HLA-DR antigens (11), L-3 to HLA-DQ antigens (12), and FA to HLA-DP antigens (13). mAb 763-74T, 376-96, and 606 are anti-MAA (14). mAb 6B, 7B, 8B, and 2A were provided by Dr. J. De Vries, Schering Corp., Lyon, France. The specificities are as follows: 6B recognizes a 225–450 kD proteoglycan expressed on melanomas, nevus cells, and melanocytes; 7B reacts with melanomas, and weakly with nevi, and recognizes a 95–120 kD protein; 8B reacts with melanomas but also with some leukemias; 2A is not melanoma-specific and was used as a positive control in the FACS analysis. Indirect immunofluorescence was performed on Me28 cells and the tumor clones as previously described (10). The cells were then analyzed by a FACS IV (Becton Dickinson, Sunnyvale, CA).

Cytotoxic Assay. Me28 cells and the tumor clones were removed mechanically from tissue culture flasks and labelled for 90 min at 37°C with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham Corp., Amersham, United Kingdom). The cytotoxic assay was then performed as described in detail elsewhere (10).

Results were calculated as follows: Percent cytotoxicity = ([experimental release] − [spontaneous release])/([total release] − [spontaneous release]) × 100. All ^{51}Cr release was measured in cpm. Spontaneous release of all targets ranged between 15 and 25% of the total release.

Statistical Analysis. The F-test value was determined for each clone at one effector/target (E/T) (50:1) ratio using the percent cytotoxicity from each of the triplicate wells against each of the targets. The significance of the differences between the lysis of any two targets in the panel by the same clone was then evaluated by the protected least significant difference method (PSD) (15). The PSD test was carried out only if the F test was significant at least at the level $p = 0.001$.

Results

Fig. 1 shows the phenotype of Me28 and of 15 representative tumor clones performed by FACS analysis with mAb to MAA and HLA antigens. In agreement with other reports (7, 8), a considerable degree of heterogeneity was found for the majority of the antigens considered, as shown by the differences among the clones, and between them and Me28 in terms of percentage of positive cells and of mean channel intensity (which correlates with antigenic density). However, all clones were positive for most of the anti-MAA mAb.

To screen the tumor clones for possible heterogeneity in their susceptibility to lysis by autologous CTL, six effectors (B11.1, B11.8, B11.11, A4.7, A4.8, A4.10) derived by micromanipulation from clones B11 and A4 and having the same phenotype as B11 and A4 ($T3^+$, $T4^-$, $T8^+$, HNK-1^-, B73.1^-) were tested for cytotoxicity against Me28 and against 31 tumor clones randomly selected among the 54 sublines obtained from the autologous tumor.

Results of this analysis are shown in Fig. 2, and revealed that: (*a*) B11 subclones expressed very similar lytic patterns against the same target; (*b*) large differences in the lysis of many targets by B11 subclones could be observed, with specific

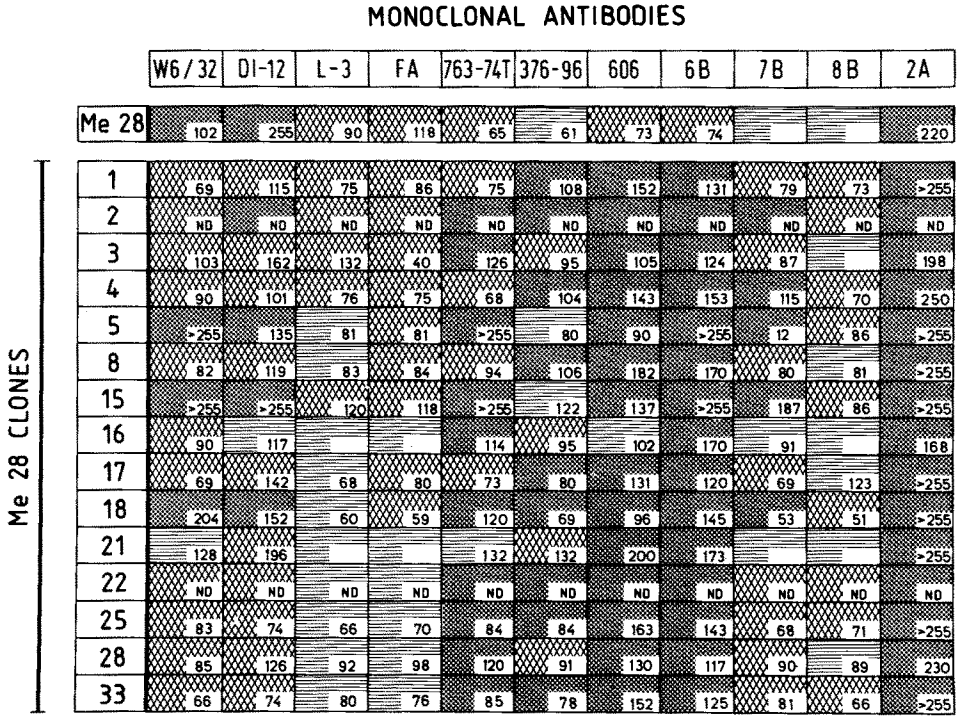

FIGURE 1. FACS analysis of Me28 cells and Me28 clones with mAb to HLA antigens (w6/32, D1.12, L-3, FA) and MAA (763-74T, 376-96, 606, 6B, 7B, 8B). 2A is not melanoma-specific and was included as positive control. Percentage positive cells with each mAb is reported as follows: horizontal bars, ≤10% positive cells; crosshatched, 11–50% positive cells; stippled, >50% positive cells. Numbers refer to the mean channel intensity (MCI) in arbitrary units. MCI was not evaluated when the percentage positive cells was <1%. ND, not done.

lysis ranging from <20% (targets 5 and 15) to >80% (target 25) at the highest E/T ratio; (*c*) A4 subclones expressed more heterogeneous lytic patterns than did B11 subclones tested against the same target, but all tumor clones could be efficiently lysed by at least one of the three A4 effectors, proving that all targets in the panel were susceptible to lysis by some effectors.

Analysis of variance performed for the lytic patterns of B11 subclones tested against all targets at E/T ratios of 50:1 gave F values of 22.8 (B11.1), 19.3 (B11.8), and 33.1 (B11.11), which are far beyond the tabular value of F (2.52) for significance at the level of $p = 0.001$. This indicates the presence of highly significant differences in the lysis of some targets in the panel. By employing the PSD test, we found for example that the lysis of Me28 by clone B11.8 was significantly higher at the level $p = 0.001$ than the lysis of targets 5 and 15, while tumor clones 3, 4, 8, 10, 16, 18, 21, 25, 30, and 32 were significantly more lysed than Me28. Similar results were obtained with CTL clones B11.1 and B11.11. By constrast, the same analysis performed on one of A4 effectors (A4.8) gave only one significant difference, with target 2 being less lysed than Me28.

Finally, the comparison between the lytic patterns shown in Fig. 2 and the phenotype of the tumor targets did not reveal any consistent correlation between

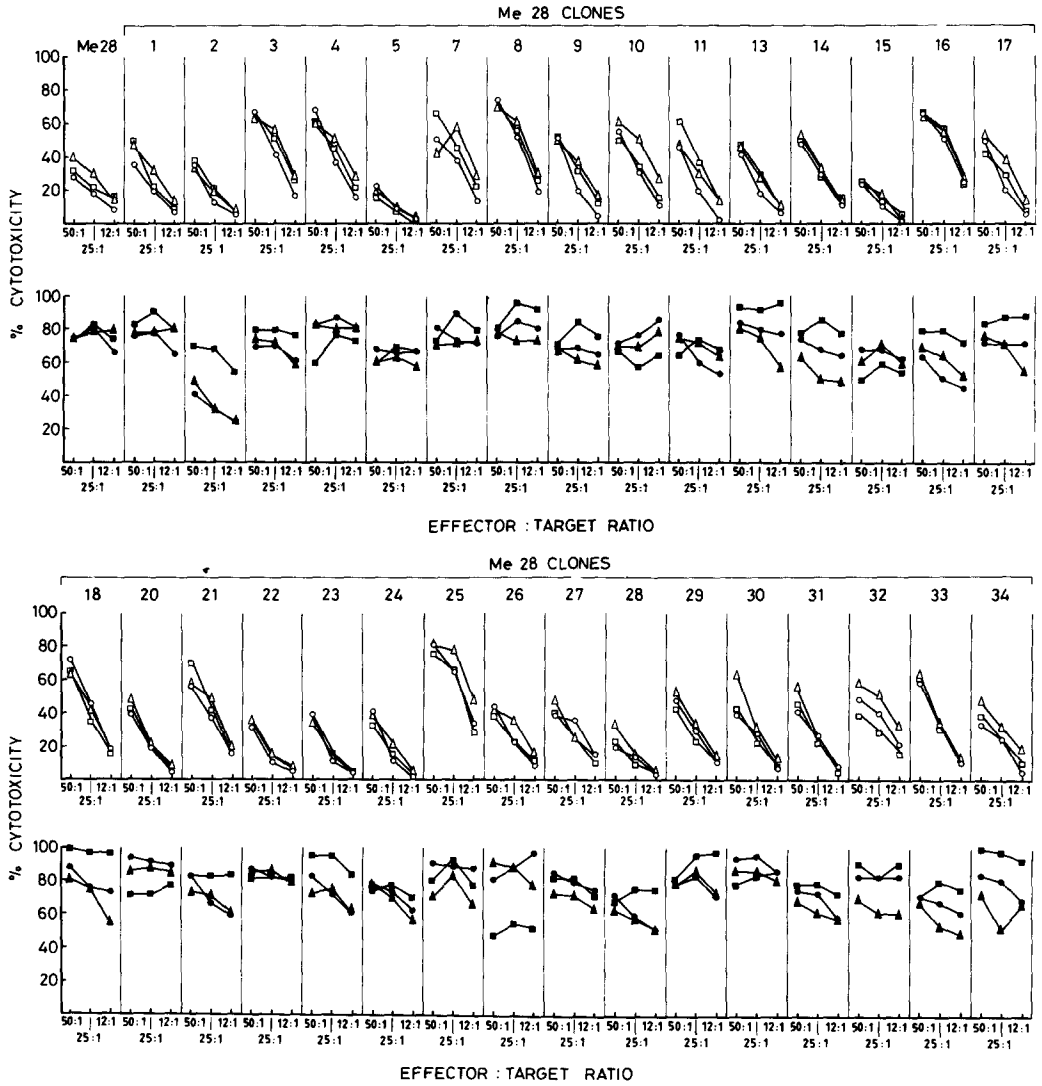

FIGURE 2. Lytic patterns of CTL clones against Me28 and Me28 clones. CTL clones B11.1 (○), B11.8 (△), B11.11 (□), A4.7 (●), A4.8 (▲), and A4.10 (■) were tested for cytotoxicity in an 18-h ^{51}Cr-release assay at E/T ratios of 50:1, 25:1, and 12:1 against Me28 and against 31 clones derived from Me28. Results are expressed as percent cytotoxicity.

the presence or absence of one type of MAA or HLA antigen and the level of lysis. This suggests that the target structures recognized by B11 and A4 subclones are independent from the antigens considered for the FACS analysis.

Discussion

The results of this study show that highly significant differences in the lysis by autologous CTL clones can be observed among tumor cell clones derived from a metastatic melanoma. Although B11 effectors discriminated among the tumor

clones more than A4 effectors, the strong cytotoxicity expressed by A4.7, A4.8, and A4.10 proves that all targets could be lysed and supports the hypothesis that the heterogeneity revealed by B11 subclones may depend upon differential expression of a target structure recognized by a particular CTL clone. Furthermore, no target was completely resistant to lysis, and a wide range of cytotoxic activity (20–80% at E/T ratios of 50:1) was expressed by B11 effectors against the tumor clones, suggesting that the differences in lysis among the targets may reflect quantitative changes in the level of expression of a target determinant rather than a simple lack or presence of such structures.

A possible interpretation of the phenomenon described in this communication may be a consequence of the fact that we used CTL clones derived by micromanipulation; these effectors are derived from a single CTL and should function as monospecific reagents recognizing the same determinant on Me28 and on the tumor clones. Therefore, the observed heterogeneity, at least for the targets lysed significantly more than Me28, should not result from processes following the cloning of Me28 and inducing the expression of new surface determinants absent in the uncloned tumor. Rather, the results presented here are consistent with the hypothesis that different neoplastic cells having high or low susceptibility to lysis exist in Me28 and can be separated from one another by cloning the tumor. The composite nature of the uncloned melanoma therefore might explain the intermediate cytotoxic activity of B11 subclones against Me28 as compared to targets 5 or 15 (low cytotoxicity) and target 25 (high cytotoxicity).

We cannot rule out the possibility that the observed heterogeneity was generated during the in vitro culture of Me28 before cloning. However, this seems unlikely since the tumor was cloned at an early (fifth) passage.

A further level of complexity is revealed by the finding that B11 effectors and A4 effectors expressed quite different lytic patterns. This suggests that a human tumor may express multiple determinants that can be recognized as target structures by different cytolytic clones. In other words, the experimental approach described herein might be useful to throw light on the heterogeneity of both the neoplastic cells and the cytotoxic effectors interacting with the tumor.

In conclusion, these results show that a new type of tumor heterogeneity can be demonstrated by autologous cloned cellular reagents, and suggest that this may be critical to the planning of therapeutic protocols involving autologous lymphocytes as antitumor reagents; multiple different clones may be required to optimize the probability of recognition and destruction of most neoplastic cells in vivo.

Summary

The possibility that a single human tumor may be composed of an heterogeneous population of cells with respect to susceptibility to lysis by autologous CTL clones was investigated by testing six cytolytic clones derived by micromanipulation against the autologous metastatic melanoma, Me28, and against 31 clones derived from Me28 by cloning in soft agar. Highly significant differences in the lysis of many tumor clones were observed by three of the CTL effectors in comparison with the cytotoxicity achieved on Me28. These results indicate that cloned cellular reagents can detect heterogeneity among cells isolated from the

same melanoma, and suggest that the target determinants recognized on the autologous tumor might be differentially expressed on different neoplastic cells.

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