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Failure of specific adoptive immunotherapy owing to survival and outgrowth of variant cells*

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Summary. Adoptive immunotherapy, the transfer of spleen cells from immunized mice to mice with a small tumor, was usually curative for mice with the P815 mastocytoma provided that steps were taken to prevent the generation of tumor-induced suppressor cells in the recipient animal. However, failure of adoptive immunotherapy of the P815 tumor, resulting in regrowth of either the primary intradermal or a metastatic tumor, was observed in 10 out of 112 animals receiving graded doses of 7.5×10^7 to 3.0×10^8 immune spleen cells. Examination of the ten tumors in mice that failed to respond to therapy revealed that seven of them were significantly less susceptible than the original P815 tumor to rejection in vivo by transferred anti-P815-specific effector cells. In addition, nine of the ten therapy-failure tumors were also less susceptible than the original P815 tumor to lysis in vitro by P815-specific, but not DBA/2-specific, cytotoxic T lymphocytes. Sensitivity to lysis by tumor-specific cytotoxic T cells was not, however, strongly correlated with sensitivity to rejection in vivo by P815-specific effector spleen cells. Neither in vivo sensitivity to rejection, nor sensitivity to cytotoxic T cells, was correlated with alterations in class I major histocompatibility complex antigen expression. These results suggest that the survival and outgrowth of variant tumor cells was frequently the cause of failure of specific adoptive immunotherapy of the P815 tumor, and that selection for cells with a reduced sensitivity to killing by cytotoxic T cells was only one mechanism that might lead to an immunotherapeutic failure.

Introduction

Studies of adoptive immunotherapy in a variety of animal tumor models have demonstrated that the transfer of specifically sensitized T lymphocytes can affect the regression of established tumors [1, 3, 12, 20, 26]. The findings from animal studies have also identified obstacles that must be considered and overcome, before adoptive immunotherapy is likely to be of human clinical benefit. These obstacles include the need to minimize in the recipient the inhibitory effect of tumor-induced immunosuppression on the expression of immunity by the transferred cells [1, 8], and the need for large numbers of syngeneic tumor-specific lymphocytes [5, 17]. Several methods may be clinically relevant for reducing tumor-induced immunosuppression including the use of cytotoxic drugs, such as cyclophosphamide [22], and irradiation [23]. Furthermore, recent advances in the development of in vitro methods for the generation and expansion of immune cells from hosts with progressively growing tumors may provide large numbers of syngeneic tumor-specific lymphocytes [4, 6]. However, another potential obstacle to adoptive immunotherapy, which has not been systematically investigated even in animal models, is the phenotypic diversity that may exist within neoplasms. Intratumor heterogeneity has serious implications for the treatment of neoplastic disease. For example, a major cause of failure of antitumor chemotherapy is the selection and outgrowth of stable, drug-resistant tumor cells [25]. In addition, it is known that cells within a tumor can differ in both expression of tumor-associated antigens [2, 19, 28, 29], and in sensitivity to immune attack [11, 27].

The purpose of this study was to investigate the significance of selection of phenotypically variant cell populations as a cause of failure of adoptive immunotherapy. The metastatic, weakly immunogenic P815 mastocytoma was used for these studies because escape of this tumor from rejection during progressive growth in immunocompetent hosts has been attributed to the selection of antigen-loss variants [29], thereby suggesting that the tumor may be phenotypically heterogeneous with regard to immunological properties. It was reasoned that if selection for immunologically variant cells within the P815 tumor was the cause of failure of adoptive immunotherapy, then tumor cells recovered from animals in which therapy had failed should have a reduced sensitivity to immune attack by P815-specific effector cells. It will be shown that a high percentage of the tumors re-emerging in mice in which adoptive immunotherapy of the P815 tumor failed were indeed less susceptible to rejection in vivo by transferred P815-specific immune effector cells than was the original tumor. It will also be shown that sensitivity to rejection in vivo was not correlated with sensitivity to killing by P815-specific cytotoxic T lymphocytes (CTL) in vitro.

Materials and methods

Mice. B6D2F1 (C57BL/6 × DBA/2) mice and AB6F1 (A × C57BL/6), 8–10 weeks old, were supplied by the Tru-

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deau Institute Animal Breeding Facility. These mice were free of known pathogens, as demonstrated by the result of routine testing for the presence of pathogenic bacteria, *Mycoplasma*, and viruses (Charles River Professional Services, Wilmington, Mass).

T-cell-deficient mice. Mice were made T-cell-deficient by thymectomy at 4 weeks of age, followed in 1 week by 1000 rad whole-body gamma irradiation from a ^{137}Cs source. Immediately following irradiation, the mice were reconstituted with 2×10^7 syngeneic bone marrow cells. The thymectomized, irradiated, bone-marrow-reconstituted (TXB) mice were rested for at least 4 weeks before use.

Tumors. The P815 mastocytoma, syngeneic in DBA/2 mice, was originally obtained from Dr. Virginia Evans, Tissue Culture Section, National Cancer Institute. The tumor is passaged weekly in B6D2F1 mice as an ascites. A new passage is initiated every 3 months from tumor stocks that are cryopreserved over liquid nitrogen in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 20% fetal calf serum (FCS, Gibco) and 10% dimethyl sulfoxide (Mallinckrodt Inc., St. Louis, Miss). For implantation, tumor cells were harvested from peritoneal ascites in sterile phosphate-buffered saline (PBS) containing 10 units/ml heparin. Cells were washed and resuspended to the appropriate concentration in PBS.

Therapy-failure tumors (TF-1 through TF-10) were obtained from mice in which adoptive immunotherapy had failed. Animals with recurrent tumors were sacrificed and first examined for evidence of incomplete thymectomy. Animals in which any possible thymic remnant was identified were excluded from further study since incomplete thymectomy may have permitted the development of tumor-induced immunosuppression that could prevent expression of transferred immunity [1, 8]. If no thymus remnants were observed, the recurrent tumor tissue was removed, and a tumor cell suspension was prepared by mechanical disruption by dicing and teasing in PBS with 1% FCS. The tumor cell suspension was injected intraperitoneally into TXB B6D2F1 mice. The resultant ascites was removed and cryopreserved as described above.

Immunizations. B6D2F1 mice were immunized for use as donors of P815-immune spleen cells for adoptive immunotherapy by intradermal injection, on the midline of the abdomen, of 2×10^6 P815 cells admixed with 50 μg heat-killed *Propionibacterium acnes* (Trudeau Institute). Injection of this admixture intradermally results in an initial 8–10-day period of tumor growth, followed, in most animals, by total tumor regression in about 3–4 weeks [10].

Effector cells for use in CTL assays were obtained from B6D2F1 mice given intradermal injections of 2×10^6 P815 cells admixed with *P. acnes* (P815-specific), or from AB6F1 mice that were given 2×10^6 tumor cells alone (DBA/2-specific). The effector cells were obtained between 7 days and 10 days after tumor implantation from axillary lymph nodes draining the site of tumor rejection on the lateral thorax. Previous studies from this Institute with this P815 tumor have shown that the cytolytic cells present in the draining lymph nodes of animals immunized by this protocol are $Thy1^+$, $CD8^+$ lymphocytes (CTL) [10, 20, 21].

Adoptive immunotherapy. Spleens were removed from immune mice 10 days after intradermal immunization with tumor cells admixed with *P. acnes* as described above. A single-cell suspension was prepared by dicing the spleens into small pieces and pushing the pieces through a 60-mesh stainless steel screen into PBS containing 1% FCS. The cell suspension was repeatedly pipetted with a pasteur pipet and then filtered through sterile surgical gauze to remove debris. The cells were then washed, resuspended in PBS, and infused via a lateral tail vein into TXB B6D2F1 mice that had received 10^6 tumor cells intradermally 4 days before. Tumor growth was followed by measuring two perpendicular diameters of the tumor at regular intervals and plotting the mean diameter against time. Mice without obvious tumors at 100 days after the initiation of therapy were considered cured.

CTL and cold-target inhibition assays. Tumor cells used as targets were obtained either from tissue culture during log-phase growth in RPMI 1640 medium containing 10% FCS and 5 μg/ml gentamicin, or from peritoneal ascites. Tumor target cells were labeled for 1 h at 37° C with 100 μCi sodium [^{51}Cr]chromate (CJS.11; Amersham Corp., Arlington Heights, Ill) per 10^6 cells in RPMI 1640 medium containing 10% FCS.

Axillary lymph node cells for use as effector cells were obtained 8–10 days following immunization of B6D2F1 mice (P815-specific), or 7–9 days after immunization of AB6F1 mice (DBA/2-specific). Effector cells were prepared by pressing finely diced pieces of lymph nodes through a stainless-steel screen into 4° C RPMI 1640 medium containing 10% FCS, 10 mM morpholinepropanesulfonic acid (Sigma Chemical Co., St. Louis, Miss), and gentamicin, 5 μg/ml (Gibco). The cells were then centrifuged and resuspended to 10^7/ml. The assay was performed in quadruplicate wells in plates containing 96 round-bottomed wells (Costar Corp., Cambridge, Mass). Each well contained 10^6 effector cells and 10^4 ^{51}Cr-labeled target cells (100:1, effector:target) in a total volume of 0.2 ml. The assay was initiated by centrifugation of the microtiter plate at 300 *g* for 5 min. After 6 h of incubation at 37° C in an atmosphere of 5% CO_2 in air, 0.075 ml medium was removed from each well and counted in a 1282 Compugamma gamma counter (LKB Instruments Inc., Gaithersburg, Md). Controls for the assay included labeled target cells incubated alone (spontaneous release), and treatment of labeled target cells with 0.5% Triton X-100 (maximum release). The maximum release was approximately 90% of the incorporated label. Spontaneous release was between 5% and 9% of the maximum release after 6 h of incubation. The percentage of specific ^{51}Cr release was calculated as [(experimental release − sponteneous release)/(maximum release − spontaneous release)] × 100.

The ability of unlabeled tumor cells to inhibit cell-mediated cytotoxicity, i.e., the cold-target inhibition assay, was measured using a modification of the cell-mediated cytotoxicity assay described above. Unlabeled tumor cells (10^4, 4×10^4, or 1.6×10^5) were added to each well of a microtiter plate that already contained 10^6 effector cells and 10^4 ^{51}Cr-labeled P815 cells to give ratios of unlabeled tumor cells to labeled target cells of 1:1, 4:1, and 16:1 respectively. The assays were then performed as described above for the cell-mediated cytotoxicity assay. Controls for specific and nonspecific inhibition were run simulta-

neously. The specific inhibition control consisted of adding unlabeled P815 cells to wells that contained ^{51}Cr-labeled P815 target cells. Unlabeled AC8 lymphoma cells, syngeneic in Balb/c mice [15], were used as a control for nonspecific inhibition due to crowding.

Analysis of major histocompatibility complex class I antigen expression by flow cytometry. Serological analysis of class I antigen expression on the surface of tumor cells was performed by indirect immunofluorescence with monoclonal antibodies directed against H-2K^d and H-2D^d determinants. The monoclonal antibodies were produced by hybridoma cell lines obtained from the American Type Culture Collection (Rockville, Md). The hybridomas used were: (a) HB102 (34-5-85), which produces a mouse IgG2a immunoglobulin that recognizes H-2D^d; (b) HB77 (31-3-45), which produces a mouse IgM that recognizes H-2K^d; and (c) HB79 (34-1-25), which produces a mouse IgG2a that recognizes public specifities on both H-2K^d and H-2D^d. The secondary reagents were fluorescein-conjugated F(ab')^2 fragments of goat anti-(mouse IgM) and goat anti-(Mouse IgG) (Cappel Laboratories).

Immunofluorescence was performed using ascites tumor cells obtained during log-phase growth in normal B6D2F1 mice. Tumor cells (2×10^6) were suspended in 1.0 ml antibody in PBS containing 1% bovine serum albumin and 10 mM sodium azide (buffer A) at 4° C for 30 min. After three washes in buffer A the cells were incubated in 0.5 ml fluorescein-labeled secondary reagent, diluted with buffer A containing 2% normal goat serum, for 30 min at 4° C. The cells were then washed three times, resuspended in 2.0 ml, and kept on ice. Immunofluorescence was analyzed using a FASCan flow cytometer (Becton Dickinson, Mt. View, Calif).

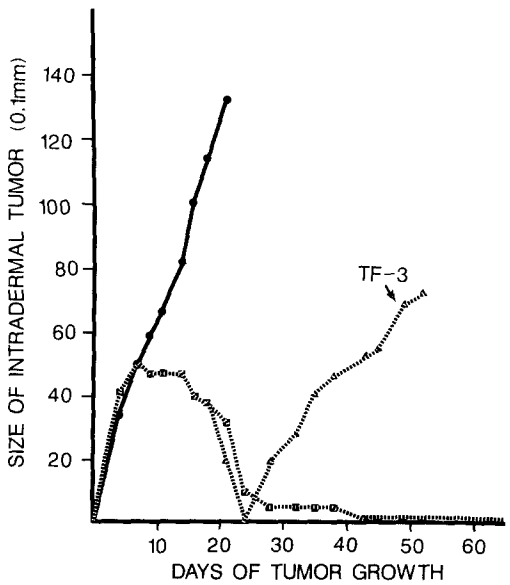

Fig. 1. An example of failure of adoptive immunotherapy of the P815 mastocytoma. Groups of five TXB B6D2F1 mice were infused intravenously with 1.5×10^8 normal spleen cells (——), or P815-immune spleen cells (. . . .) 4 days after receiving an intradermal implant of 10^6 P815 cells. The tumors in all five of the mice receiving P815 immune cells underwent apparent complete regression, but the primary tumor in one mouse subsequently regrew (TF-3)

Results

Incidence of failure of adoptive immunotherapy of the P815 mastocytoma

Previous studies from this Institute had shown transfer of spleen cells from mice immunized by the intradermal implantation of 10^6 P815 cells, admixed with *P. acnes* 10 days earlier, to immunodeficient TXB mice with an intradermal P815 tumor initiated by the implantation of 10^6 cells 4 days earlier, to be a successful model of adoptive immunotherapy of metastatic disease [8]. Tumor regression in this system was correlated with the generation of CD8$^+$ effector T cells [20], and was tumor-specific [9]. Although the above protocol is curative in most instances, occasional therapy failures are encountered in which either the primary or a metastatic tumor regrows (Fig. 1). The overall frequency of therapy failure observed in this study was 10 out of 112 mice (9%). Therapy failure due to regrowth of tumors were found in 4 out of 20 mice (20%) receiving 7.5×10^7 immune spleen cells, 5 out of 65 mice (8%) receiving 1.5×10^8 immune spleen cells, and 1 out of 27 mice (4%) receiving 3×10^8 immune spleen cells. Although these differences are not statistically significant, they suggest that the frequency of therapy failures decreases as the number of immune spleen cells transferred increases. Each of these ten therapy-failure tumors was removed, a single-cell suspension was prepared, and was passaged once as an ascites in TXB mice before cryopreservation in liquid nitrogen.

Therapy failure may result from survival of cells with a reduced susceptibility to rejection by transferred P815-immune cells

To determine whether failure of adoptive immunotherapy occurred as the result of survival of cells present within the original tumor that had a low susceptibility to rejection by P815-immune effector cells, the susceptibility of the ten therapy-failure tumors to rejection *in vivo* by transferred P815-immune cells was determined. Table 1 shows that for all ten therapy-failure tumors, transfer of 1.5×10^8 P815-imune spleen cells significantly prolonged the survival of tumor-bearing TXB mice. However, seven of the ten therapy-failure tumors were significantly less susceptible to rejection by P815-immune cells than was the P815 tumor. The decreased susceptibility of the seven tumors to rejection by P815-immune cells was manifest both as a decrease in survival time, and as a decrease in cure rate. The decreased susceptibility of the therapy-failure tumors did not correlate with either an overall faster growth rate of the primary intradermal tumor in TXB mice (data not shown), or with increased rate of metastases, as reflected in a shorter survival time (Table 1). These data were interpreted as indicating that seven of the ten therapy failures occurring in 112 mice (6% overall), could have been due to immunoselection of a phenotypically stable population of cells with a reduced susceptibility to rejection by anti-P815 immunity.

The three tumors that were not significantly different from the P815 tumor (TF-4, TF-8, TF-9) all recurred in animals that received 1.5×10^8 P815-specific immune spleen cells. Thus, the frequency of therapy failure that may have been due to immunoselection in mice receiving 1.5×10^8 immune cells (2 out of 65) was significantly less ($P < 0.05$, $\chi^2 = 4.34$) than that in mice receiving 7.5×10^7 immune spleen cells (4 out of 20).

Table 1. Comparison between P815 and therapy-failure tumors for susceptibility to rejection by transferred P815-immune spleen cells[a]

Tumor	Median survival time (days) (range)		Number cured
	Non immune spleen cells	P815-immune spleen cells	Number treated
P815	25 (18–43)	100 (43–100)	56/65
TF-1	20 (18–27)	54 (25–100)[b]	3/24[c]
TF-2	20 (18–29)	34 (18–100)[b]	2/17[c]
TF-3	22 (18–39)	46 (32–100)[b]	3/18[c]
TF-4	27 (20–37)	100 (36–100)	7/12
TF-5	21 (18–26)	49 (39–100)[b]	1/10[c]
TF-6	21 (18–23)	37 (26– 43)[b]	0/12[c]
TF-7	21 (20–25)	49 (38–100)[b]	1/12[c]
TF-8	19 (18–21)	100 (38–100)	4/6
TF-9	22 (21–23)	85 (56–100)	3/6
TF-10	22 (21–23)	53 (38– 56)[b]	0/5[c]

[a] Immunodeficient TXB B6D2F1 mice that had received 10^6 tumor cells intradermally 4 days earlier were injected intravenously with 1.5×10^8 spleen cells from normal B6D2F1 mice or mice immunized with P815 admixed with *P. acnes*. Animals were observed for 100 days, and animals surviving for 100 days without evidence of tumor were considered cured

[b] Statistically significant shorter survival time ($P \leq 0.05$) compared to the P815 tumor using the Mann-Whitney *U* test

[c] Statistically fewer cures compared to the P815 tumor using the χ^2 test

Table 2 shows that against three representative tumors having a reduced susceptibility to rejection by 1.5×10^8 immune spleen cells (TF-1, TF-2, TF-3), increasing the number of spleen cells transferred to 3×10^8 (the maximum tolerated dose) failed to produce a gain in therapeutic effect. Transfer of the larger number of cells neither produced more cures, nor did it extend the survival time of the mice that died. These results suggest that once selection and outgrowth of a less susceptible population has occurred, additional attempts at adoptive immunotherapy using similar immune cell populations is unlikely to be beneficial.

Therapy-failure tumors have a reduced sensitivity to lysis by P815-specific cytotoxic T lymphocytes

Previous investigations of adoptive immunotherapy with the P815 tumor used in these studies had indicated that tumor rejection was correlated with the development of $CD8^+$ anti-P815 cytotoxic T cells in the lymph node draining the tumor bed in the recipient animal [20]. When the ten therapy-failure tumors were compared to the P815 tumor for susceptibility to lysis by CTL, nine of the ten were found to be significantly less susceptible to lysis by P815-specific CTL (Table 3). The reduced susceptibility to lysis by anti-tumor CTL was not, however, due to a general resistance to CTL lysis by the therapy-failure tumor, because eight of the ten tumors were at least as susceptible as the P815 tumor to lysis by DBA/2-specific CTL (Table 3).

Table 3. Comparison between P815 and therapy-failure tumors for susceptibility to lysis by P815 and DBA/2-specific cytotoxic T lymphocytes (CTL)

Tumor	Relative susceptibility to lysis compared to P815[a] (%)	
	P815-specific CTL	DBA/2-specific CTL
TF-1	5 (± 3)[b]	63
TF-2	10 (± 3)[b]	73
TF-3	12 (±10)[b]	99
TF-4	95 (±24)	101
TF-5	74 (± 7)[b]	96
TF-6	16 (±17)[b]	96
TF-7	81 (± 1)[b]	94
TF-8	43 (±12)[b]	104
TF-9	66 (± 1)[b]	93
TF-10	15 (± 9)[b]	91

[a] Relative susceptibility compared to P815 was calculated as 100 × (% specific lysis of therapy-failure tumor/% specific lysis of P815). The values represent the mean (±SD) of three or four replicate assays for P815-specific CTL, and the means of duplicate tests for DBA/2-specific CTL

[b] Significantly less susceptible than P815 ($P < 0.05$) using Student's *t*-test

The reduced ability of P815-specific CTL to lyse therapy-failure tumors suggested that less sensitive tumors may have a reduced expression of a tumor-associated antigen(s) recognized by P815-specific CTL. This possibility was further examined by comparing the ability of unlabeled therapy-failure tumor cells to inhibit the lysis of ^{51}Cr-labeled P815 tumor cells by P815-specific CTL. The data in Fig. 2 indicate that the relative ability of the therapy-failure tumors to inhibit P815-specific CTL lysis of P815

Table 2. Comparison of the therapeutic effect of transfer of different numbers of spleen cells from P815-immunized mice to thymectomized, irradiated, bone-marrow reconstituted mice with therapy-failure tumors[a]

Tumor	Number of P815-immune spleen cells transferred			
	1.5×10^8		3×10^8	
	Number cured / Number treated	Mean survival time (±SD) of mice that died (days)	Number cured / Number treated	Mean survival time (±SD) of mice that died (days)
P815	12/12	–	16/16	–
TF-1	1/13	48 (±13.3)	1/11	51 (±8.7)
TF-2	0/5	32 (± 7.6)	0/6	38 (±4.9)
TF-3	0/6	44 (± 9.4)	0/6	42 (±8.7)
TF-4	4/6	45 (±12.7)	5/5	–

[a] TXB B6D2F1 mice that had received 10^6 tumor cells intradermally 4 days earlier were injected intravenously with either 1.5×10^8 or 3×10^8 spleen cells from B6D2F1 mice immunized by the intradermal implantation of P815 cells admixed with *P. acnes* 10 days earlier

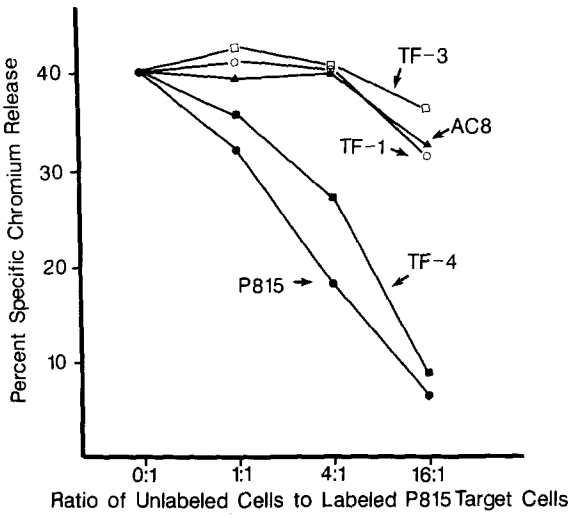

Fig. 2. Evidence that the reduced susceptibility of therapy-failure tumors to lysis by P815-specific CTL is due to a reduced expression of the tumor-associated antigens recognized by P815-specific CTL. Unlabeled TF-4 cells, which were not significantly different from P815 in their susceptibility to lysis by P815-specific CTL, were almost as efficient as unlabeled P815 in inhibiting the lysis of ^{51}Cr-labeled P815 by P815-specific CTL. In contrast, unlabeled TF-1 and TF-3 tumor cells were as inefficient in inhibiting the P815-specific lysis of ^{51}Cr-labeled P815 target cells as were cells of the completely unrelated AC8 leukemia

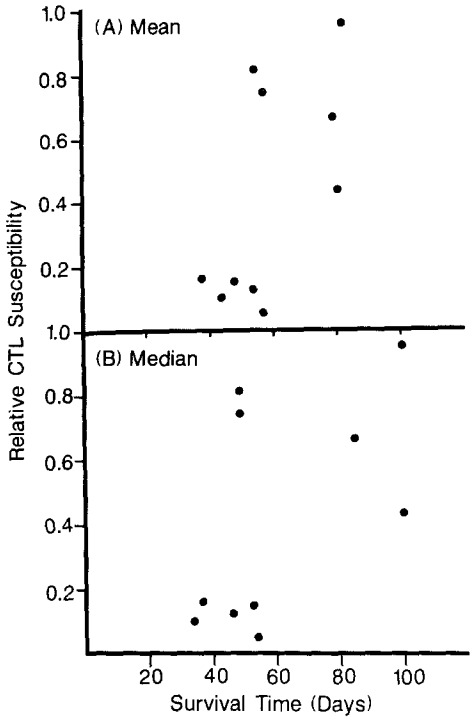

Fig. 3. Evidence that the relative susceptibility of the ten therapy-failure tumors to lysis by P815-specific CTL is not correlated with the relative susceptibility of the same tumors to rejection in vivo by adoptively transferred P815-immune spleen cells. **A** Plot based on mean survival time, **B** plot based on median survival time from Table 1

tumor cells was inversely correlated to the susceptibility of the therapy-failure tumor cells to P815-specific CTL lysis. Therefore, the results of the cold-target inhibition assays were consistent with the hypothesis that the reduced susceptibility of the therapy-failure tumors to lysis by P815-specific CTL was due to a reduced expression of tumor-associated antigens. The inability to immunize B6D2F1 mice with the TF-1, TF-2, or TF-3 tumor to produce either P815-specific or therapy-failure-specific CTL, using the same protocol as was effective for the production of the P815-specific CTL (data not shown), was also consistent with the above interpretation.

Relative susceptibility of therapy-failure tumors to rejection by transferred P815-immune cells is not strongly correlated with relative susceptibility to lysis by P815-specific CTL

The preceding results show that, compared to the P815 tumor, seven of the ten therapy-failure tumors are less susceptible to rejection by transferred P815 immunity, and that nine of the ten therapy-failure tumors are less susceptible to lysis by P815-specific CTL. The data shown in Fig. 3 indicate, however, that within the group of therapy-failure tumors relative susceptibility to rejection, as indicated by survival time, was not strongly correlated with relative CTL sensitivity. Correlation coefficients, calculated for median survival time and relative CTL sensitivity using the non-parametric Spearman method (0.4146) or linear regression (0.5524), are not significant at the 0.05 level. Similarly the correlation coefficient for mean survival time and relative CTL sensitivity calculated using linear regression (0.6277) is not significant at the 0.05 level. The correlation coefficient for relative CTL sensitivity and median survival time by the Spearman method (0.5758) is only weakly significant ($P < 0.045$). These results have been interpreted as indicating that tumor-associated antigens other than those recognized by CTL are important in tumor rejection in vivo, a conclusion in agreement with that of a previous study by this laboratory of two different P815 sublines [16].

Neither the relative susceptibility of therapy-failure tumors to rejection by transferred P815-immune cells nor to lysis by P815-specific CTL is correlated with expression of cell surface class I histocompatibility antigens

Because cell-surface major histocompatibility complex (MHC) antigens are important for the recognition of tumor cell-surface antigens by T cells, expression of class I MHC antigens by P815 and the therapy-failure tumors was compared. The P815 tumor does not express class II MHC antigens [18].

Ascites tumor cells from log-phase growth were reacted with monoclonal antibodies directed against H-2K^d and H-2D^d determinants, and were counterstained with a fluorescein-conjugated goat anti-(mouse immunoglobulin) F(ab')$_2$ fragments. Antigen expression was then analyzed by flow cytometry. Compared to P815, both H-2K^d and H-2D^d expression was the same for three therapy-failure tumors (TF-2, TF-9, and TF-10), decreased for two therapy-failure tumors (TF-1, TF-6), and increased for five therapy-failure tumors (TF-3, TF-4, TF-5, TF-7, and TF-8). Neither the direction nor magnitude of these changes was correlated with relative susceptibility to either rejection by transferred P815-immune cells or to lysis by P815-specific CTL (data not shown). These results indicate that failure of adoptive immunotherapy of the

P815 tumor did not occur consistently as the result of selection for cells with altered class I MHC expression, although the reduced susceptibility of the TF-1 and TF-6 tumor to P815 effector cells and tumor-specific CTL may have been partly attributable to reduced class I antigen expression.

Discussion

The observation that seven of the ten tumors recovered from mice in which adoptive immunotherapy had failed were significantly less susceptible to rejection by P815-specific effector cells than was the original P815 tumor suggests that survival and outgrowth in vivo of immunologically variant cells frequently contributed to the failure of specific adoptive immunotherapy. However, since the overall rate of therapy failure in this model was low (9%), selection and outgrowth of variant cells actually occurred in only 6% of treated animals. The probability of selection and outgrowth of variant cells would presumably be a function of the extent of heterogeneity within a tumor. Unfortunately, neither the extent of immunological heterogeneity within the P815 tumor line, nor how the immunological heterogeneity of the P815 tumor line relates to the heterogeneity of other tumors is currently known. Thus, whether selection and outgrowth of variant cells should be expected to occur at a different frequency with other tumors is not clear. This laboratory has shown, however, that expression of tumor-rejection antigens by the P815 tumor is a remarkably consistent property [16], and thus a 6% failure frequency for the P815 tumor may indicate that immunoselection may be a much more serious obstacle for specific adoptive immunotherapy of phenotypically less stable and more heterogeneous tumors.

The observations in this study were consistent with those of previous studies that showed successful adoptive immunotherapy depends upon the relationship between tumor burden and the number of immune effector cells transferred [8]. Both the overall frequency of therapy failure and the frequency of failure due to immunoselection increased as the number of immune cells transferred decreased. Therefore, the probability of immunoselection leading to therapy failure was inversely related to the number of immune cells transferred. This result would suggest that the significance of immunoselection as a cause of failure of adoptive immunotherapy might be reduced by increasing the level of immunity initially transferred. These results also showed, however, that once selection and outgrowth of a less susceptible population have occurred, increasing the level of immunity transferred provided no increase in therapeutic effect (Table 2), despite the fact that within all of the therapy-failure tumors at least some cells continued to share some tumor antigens in common with the original P815 tumor.

Even though nine of the ten therapy-failure tumors were less susceptible to lysis by P815-specific CTL, susceptibility to rejection in vivo and susceptibility to lysis by CTL were not strongly correlated. Tumors with a reduced susceptibility to either CTL lysis or to in vivo rejection, but without a corresponding reduction in the other sensitivity, were found. Reduced CTL sensitivity did appear, however, to correlate with a reduced expression of tumor antigens recognized by P815-specific CTL, and not with either a decreased susceptibility to CTL lytic mechanisms in general, nor with alterations in class I MHC antigen expression. These results suggest that the P815 tumor expresses multiple tumor-specific antigens, only some of which may be recognized by CTL, a conclusion in agreement with that from other studies of this tumor [16, 29].

Using CTL clones, Uyttenhove et al. have characterized a series of stable, antigen-loss variants of P815 selected in vivo that express at least four distinct antigenic specificities [29]. Furthermore, the loss of some of these specificities was correlated with escape from in vivo rejection. In addition, this laboratory has shown that a subline of the P815 tumor used in these studies, but which had been passaged separately in vivo for over a decade, no longer expressed P815-specific antigens recognized by CTL generated in vivo, yet retained the ability to induce reciprocal cross-protection in vivo with the subline expressing P815-specific antigens recognized by CTL [16]. In aggregate, these results suggest that recognition of tumor antigens by CTL may be neither necessary nor sufficient for in vivo rejection. These conclusions do not imply, however, that CTL, when present, do not participate in tumor rejection.

One possible explanation for the observation that sensitivity to CTL killing does not correlate with in vivo sensitivity to rejection may be that only some of the tumor-associated antigens relevant to rejection are recognized by class-I-MHC-restricted CTL, and that others are recognized by class-II-restricted T cells. Therefore, some of the tumor recurrences may have been due to selection for variant cells that no longer express tumor-associated antigens critical for rejection that are recognized by class-II-restricted T cells. Greenberg et al. have demonstrated that class-II-restricted T cells can be major effector cells in the adoptive immunotherapy of the FBL-3 leukemia even though, like the P815, it does not express class II MHC antigens [14].

Other studies have shown that factors such as the ability of transferred effector cells to migrate into the tumor can be important to the success of specific adoptive immunotherapy [7]. In addition, tumor burden at the time of immunotherapy undoubtedly affects the likelihood of success, just as it does with chemotherapy [13, 25]. Indeed, in the present study TF-4, which is not significantly different from the original P815 tumor in its susceptibility to P815 effector cells, may represent an example of a tumor that recurred because the number of effector cells transferred was simply inadequate to kill all the original tumor cells present, and ultimately the tumor regrew. However, notwithstanding the above considerations, the results of this study indicate that 70% of the time failure of specific adoptive immunotherapy of the P815 tumor involved immunoselection of variant cells. It is hoped that understanding the reasons for therapy failure may suggest ways to improve the therapeutic efficiency of specific adoptive immunotherapy.

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