

Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen

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Melanoma patients have high frequencies of T cells directed against antigens of their tumor. The frequency of these antitumor T cells in the blood is usually well above that of the anti-vaccine T cells observed after vaccination with tumor antigens. In a patient vaccinated with a MAGE-3 antigen presented by HLA-A1, we measured the frequencies of anti-vaccine and antitumor T cells in several metastases to evaluate their respective potential contribution to tumor rejection. The frequency of anti-MAGE-3.A1 T cells was 1.5×10^{-5} of CD8 T cells in an invaded lymph node, sixfold higher than in the blood. An antitumor cytotoxic T lymphocyte (CTL) recognizing a MAGE-C2 antigen showed a much higher enrichment with a frequency of $\sim 10\%$, 1,000 times higher than its blood frequency. Several other antitumor T clonotypes had frequencies $> 1\%$. Similar findings were made on a regressing cutaneous metastasis. Thus, antitumor T cells were $\sim 10,000$ times more frequent than anti-vaccine T cells inside metastases, representing the majority of T cells present there. This suggests that the anti-vaccine CTLs are not the effectors that kill the bulk of the tumor cells, but that their interaction with the tumor generates conditions enabling the stimulation of large numbers of antitumor CTLs that proceed to destroy the tumor cells. Naive T cells appear to be stimulated in the course of this process as new antitumor clonotypes arise after vaccination.

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In Germeau et al. (1), which appears in this issue of *JEM*, we show that metastatic melanoma patients who receive vaccinations with defined tumor antigens have high numbers of T cells directed against tumor antigens other than the vaccine antigens in their blood. These T cells, labeled “antitumor,” are found in the blood at high frequency both before and after vaccination. This frequency usually vastly exceeds that of the “anti-vaccine” T cells observed after several injections of the vaccine. To obtain a first indication of the respective contributions of the antitumor T cells and the anti-vaccine T cells to the tumor rejections that occasionally occur after vaccination, we set out to evaluate the presence of both cells in the metastases of a well-characterized patient.

Metastatic melanoma patient EB81 received four cutaneous vaccinations with a recombinant canarypox (ALVAC) virus carrying a minigene

coding for two antigenic peptides encoded by genes *MAGE-3* and *MAGE-1*. These antigenic peptides are MAGE-3_{168–176} (MAGE-3.A1) and MAGE-1_{161–169} (MAGE-1.A1), and both are presented by HLA-A1. The ALVAC vaccinations were followed by vaccinations with the peptides in the absence of adjuvant.

At treatment onset, the patient had ~ 20 progressing cutaneous metastases in the leg, including several above 1 cm in diameter (see Fig. 1). 2 mo after the first vaccination, all of these metastases began to disappear gradually. However, a local lymph node started swelling after 4 mo. It was removed after 7 mo and found to contain melanoma cells. 1 yr after the first vaccination, all of the cutaneous metastases had completely disappeared. The patient has remained tumor-free for 3 yr.

Before the vaccinations, the frequencies of anti-MAGE-3.A1 and anti-MAGE-1.A1 CTLs

in the blood were $<10^{-7}$ of CD8 T cells, which is not higher than those detected in noncancerous individuals. After the ALVAC vaccinations, the frequency of anti-MAGE-3.A1 CTLs rose to a level of $\sim 3 \times 10^{-6}$ of CD8 T cells and remained in this range during the next year. This response was quasimonoclonal with 98% of it consisting of CTL clonotype 35. No response against MAGE-1.A1 was observed (2).

A tumor cell line was derived from the cells of the invaded lymph node. As described by Germeau et al. (1), the use of these cells as stimulators made it possible to detect in the prevaccination blood a frequency of 3×10^{-4} for CTLs that were capable of lysing specifically the tumor cell line. The frequency of these antitumor CTLs rose to 3×10^{-3} in postvaccination blood. These CTLs turned out to be directed mainly against antigens encoded by cancer germline gene *MAGE-C2* and melanocyte differentiation gene *gp100*. CTL clone 16, directed against a MAGE-C2 antigen, had a frequency of $\sim 10^{-4}$ after vaccination, i.e., 30 times higher than the anti-MAGE-3.A1 CTL 35.

Here we describe the frequency inside various metastases of anti-vaccine CTL clone 35 and antitumor CTL clone 16. We also present a frequency spectrum of the TCR sequences expressed inside metastases collected before and after vaccinations.

RESULTS

Modest enrichment of anti-vaccine T lymphocytes in metastases

The invaded lymph node of patient EB81, which was removed at a time when most cutaneous metastases had regressed (Fig. 1), presented a large circular area of living tumor cells surrounded by a fibrous shell (Fig. 2 A). There was also a large area where macrophages loaded with melanin had accumulated, presumably after destruction of tumor cells (Fig. 2 D). Such macrophages were also observed at the periphery of the area of living tumor cells (Fig. 2 C). Thus, it appeared that a large number of tumor cells had been destroyed in the lymph node, but that many living tumor cells were still present.

To analyze the frequency of anti-MAGE-3.A1 CTL 35 among the CD8 T cells of this invaded lymph node, we applied clonotypic PCR amplifications detecting with high specificity the rearrangements of the sequences coding for the α and β TCR chains of this anti-vaccine CTL clone. Cryosections of the lymph node, similar to that shown in

Fig. 2 A, were used as sources of RNA. Using a quantitative PCR for the CD8 β sequence, we estimated the number of CD8 T cells in these sections to be $\sim 26,000$ based on an estimate of 40 CD8 β transcripts per CD8 T cell. Two lymph node sections out of six provided a positive signal for both the α and β chains of CTL 35, leading to a frequency estimate of $1/64,000$ (1.5×10^{-5}) of the CD8 T cells present in the invaded lymph node. At the time of resection of the lymph node, the frequency of CTL 35 in the blood was $\sim 2.5 \times 10^{-6}$. Therefore, there was a modest sixfold enrichment in the invaded lymph node.

On a cutaneous metastasis, which showed signs of regressions, a punch biopsy was performed after the fourth ALVAC vaccination (Fig. 1). The metastasis still contained tumor cells. The number of CD8 β transcripts in the biopsy was measured by quantitative PCR, leading to an estimate of $\sim 30,000$ CD8 T cells. The clonotypic PCR for the α and β chain of CTL 35 were negative, leading to a frequency estimate below 3×10^{-5} CD8 T cells. This excluded that the anti-vaccine CTLs were more enriched in this metastasis than in the invaded lymph node.

High enrichment of anti-MAGE-C2 CTL 16 in metastases

The same approach was used to evaluate the intratumoral frequency of anti-MAGE-C2 CTL clone 16, an antitumor CTL that had been found at high frequency in the postvaccination blood. All of six sections of the invaded lymph node gave a positive signal for the clonotypic PCR for both the α and β chains. Therefore, we resorted to laser microdissection to obtain subsections that constituted $\sim 1\%$ of the entire section (Fig. 2 A). Most of these also gave a positive signal. This led to a minimum CTL 16 frequency of 1.7% of the CD8 T cells present in the region where lymph node architecture was preserved (Fig. 2, A and D). In the area, which contained living tumor cells, where the density of CD8 T cells was ~ 10 times lower, the frequency of CTL 16 was estimated to be $>13\%$ of these CD8 T cells. As CTL 16 was present in the blood at a frequency of 9×10^{-5} CD8 T cells at the time the metastasis was collected, the enrichment in the invaded lymph node was >200 or $>1,500$ -fold according to the region considered.

To reduce the risk of possible technical biases, we confirmed this result with an independent approach. RNA from the invaded lymph node was reverse transcribed using a primer corresponding to the constant region of the TCR β

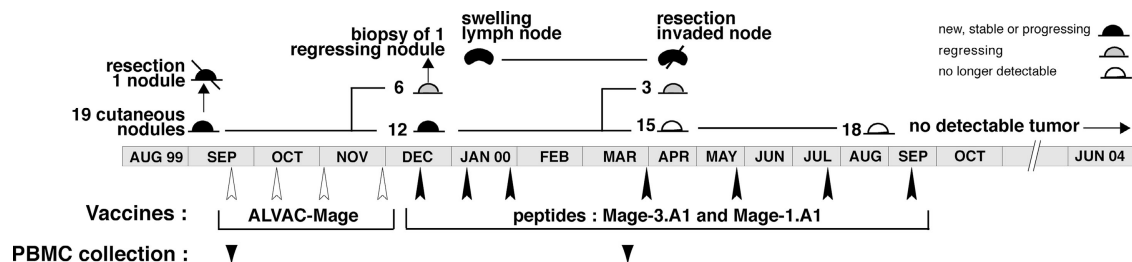


Figure 1. Clinical evolution of patient EB81 and timing of vaccinations and blood cell collections.

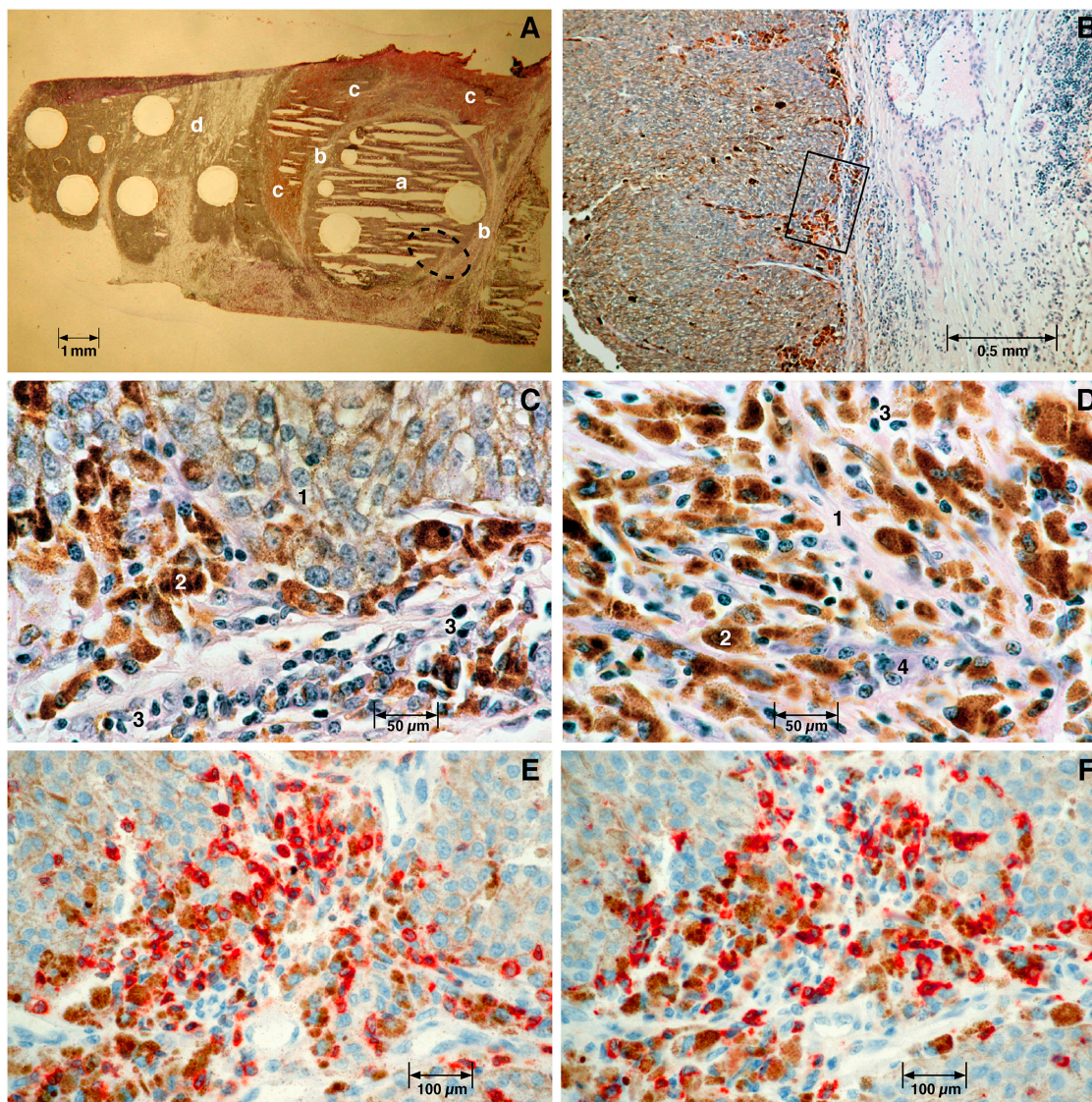


Figure 2. Analysis of the invaded lymph node resected in April 2000.

(A) Overall structure presented on a cryosection stained with hematoxylin. A circular area of living tumor cells (a) is surrounded by a fibrous band (b). Above, a crescent-shaped region (c) is devoid of tumor cells. Numerous macrophages containing melanin and other inflammatory cells are present in this region (see D). The area on the left part of the section (d) represents the uninvaded part of the lymph node. The stippled ellipse indicates the localization of the area shown in B. The 1-mm holes represent samples collected by laser microdissections for gene expression analysis. (B–F) Paraffin-embedded sections stained with hematoxylin and (B–D) eosin. (B) Higher magnification of an area straddling the living tumor cell region

chain. This β chain cDNA was obtained under conditions that made it suitable for PCR amplification and cloning. Thus, a library of TCR β sequences was obtained under conditions ensuring an unbiased representation of the TCR β sequences present in the invaded lymph node. We sequenced 110 clones of this library. 10 of these sequences turned out to be the β sequence of CTL 16, leading to a frequency estimate of 9% of the TCR present in the lymph node. The ratio of CD4 to CD8 T cells in the node was

and the fibrous band, which is infiltrated with lymphocytes. Macrophages containing melanin are located at the outer rim of the tumor cell region. The rectangle delineates the area shown in C. (C) Further enlargement of the border between tumor cells (1) and the fibrous band, which contains macrophages with melanin accumulation (2) and lymphocytes (3). (D) Higher magnification of region c (A) with fibroblasts (1), macrophages with melanin (brown-colored; 2), lymphocytes (3), and plasmacytes (4). (E and F) Adjacent sections treated with anti-CD3 (E) and anti-CD8 α antibodies (F). T cells infiltrating the tumor are stained in red (immunoperoxidase). Macrophages with melanin are brownish.

$\sim 2:1$, as in the blood. Therefore, the CTL 16 TCR β represented as much as 27% of the TCR chains of the CD8 T cells present in this invaded lymph node. Admittedly, this approach provides proportions of the TCR β transcripts and not proportions of T cells. And because CTL 16 might have been in a more activated state than the average CD8 T cell present in the lymph node, it might have contained a higher amount of TCR mRNA (3). Nevertheless, even allowing for a correction by a factor of 10, this approach confirmed

Table I. Frequency of anti-vaccine and antitumor CD8 T cell clonotypes in the blood and in metastases

T cells ^a	Before vaccination		After vaccination		
	Blood ^b	Cutaneous metastasis ^c	Blood ^d	Regressing cutaneous metastasis ^e	Invaded lymph node ^e
Anti-MAGE-3.A1 (Anti-vaccine)					
168-176 CTL 35	<10 ⁻⁷	<0.001%	2.5 × 10 ⁻⁶	<0.003%	0.0015%
Anti-MAGE-C2					
336-344 CTL 16	<7 × 10 ⁻⁷	<0.001%	9 × 10 ⁻⁵	3%	9%
336-344 CTL 40	4 × 10 ⁻⁵	0.03%	10 ⁻⁴	1%	2%
191-200 CTL 11	9 × 10 ⁻⁶	0.1%	3 × 10 ⁻⁵ ^o	0.5%	0.5%
191-200 CTL 41	2 × 10 ⁻⁶	0.01%	6 × 10 ⁻⁵	2%	6%
42-50 CTL 1	2 × 10 ⁻⁵	0.1%	2 × 10 ⁻⁴	1.5%	6%
Anti-gp100 ₂₀₉₋₂₁₇					
CTL 7	<7 × 10 ⁻⁷	<0.001%	10 ⁻⁴	2%	0.4%
Anti-mutated caseinolytic protease					
CTL 101	<7 × 10 ⁻⁷	<0.001%	10 ⁻⁴	3%	7%
Other antitumor					
CTL 10	4 × 10 ⁻⁵	3%	8 × 10 ⁻⁵	1%	0.2%

^aAntitumor clonotypes that appeared only after vaccination are in bold. Small numbers define the antigenic peptides.

^bFrequencies among CD8 T cells obtained by clonotypic PCR applied to DNA of limiting dilution groups of PBMCs collected in September 1999.

^cValues <0.001% are based on a negative clonotypic PCR result performed on cDNA extracted from a fragment of metastasis estimated to contain 10⁵ CD8 T cells. Other values represent frequencies of the TCRβ chain sequence in the total set of TCRβ cDNAs. These values were obtained either by sequencing or by clonotypic PCR.

^dFrequencies among CD8 T cells. The value marked by ^o was obtained by limiting dilution stimulation of PBMCs with peptide. The other values were obtained by clonotypic PCR of cDNA obtained from limiting dilution groups of PBMCs collected in March 2000.

^eExcept for CTL35 where the frequency was obtained as described in Results, all values represent frequencies of the relevant TCRβ chain sequence among the total set of TCRβ cDNAs. These values were obtained either by sequencing or by clonotypic PCR.

the considerable enrichment of CTL 16 demonstrated by the first approach.

The TCRβ library approach was also applied to the regressing cutaneous metastasis. Out of 228 TCRβ sequences that were obtained, 6 represented the CTL 16 sequence, indicating a very high enrichment in this metastasis also.

Adjacent to the invaded lymph node, another lymph node was resected and found to be free of tumor cells. Quantitative PCR amplification of CD8β sequences and TCR16β sequences indicated that the proportion of TCR16 mRNA per CD8β mRNA was 200 times lower in this non-invaded lymph node relative to the invaded lymph node, indicating little or no enrichment in the noninvaded lymph node relative to the blood.

High frequency of other antitumor T cells in metastases

The TCR library approach provided a global view of the frequent TCR mRNA present in the invaded lymph node. Besides CTL 16, several other TCR clonotypes directed against MAGE-C2 antigens had been identified in the blood of patient EB81 as described by Germeau et al. (1). These clonotypes were also found to be very frequent in the invaded lymph node (Table I). Their enrichment relative to the blood ranged from 150 to 1,000 (Table I). Thus, anti-MAGE-C2 TCR message represented >20% of the TCR message found in the invaded lymph node. The TCR sequences of all these anti-MAGE-C2 clonotypes were also found to be very frequent in the regressing cutaneous metastasis (Table I).

A number of clonotypes directed against other antigens were also very frequent in the invaded lymph node. A clono-

type directed against a gp100 antigen was present at a frequency of 0.4% of all TCR. T cell clonotype 101, which had not been identified after stimulation of blood lymphocytes, was present at a frequency of 7% (Table I). A CTL clone bearing this TCR was derived by stimulation with tumor cells of T cells collected from the invaded lymph node. The target antigen proved to be a peptide encoded by the gene coding for caseinolytic protease, which bears a mutation in the tumor, resulting in a new antigen. Several other antitumor clonotypes directed against uncharacterized antigens also had frequencies in the 1% range.

The sets of dominant TCR clonotypes found in the two metastatic sites collected after vaccination were found to have considerable overlap. 30 of the 110 TCR sequences found in the lymph node were also found among the 86 TCR sequences analyzed in the cutaneous metastasis.

Ratio of T cells to tumor cells in a cutaneous metastasis

Antitumor T lymphocytes are present in metastases at a very high frequency among the CD8 T cells. But what about their frequency relative to the tumor cells? In the regressing cutaneous metastasis, the proportion of tumor cells was evaluated visually to be ~75%. An independent evaluation was made as follows. The melanoma of patient EB81 carries the BRAF-1 mutation, which is present in most melanomas (4). Sequencing of PCR amplification products of the tumor cell line indicated a ratio of mutated to normal sequence of 5:1, presumably due to gene amplification of the mutated allele. The same ratio was observed for the encapsulated region of the invaded lymph node, which, on visual examination, appeared to contain almost only tumor cells.

For the regressing cutaneous metastasis, the ratio was 1.3:1, leading to the estimate of 42% of tumor cells in this metastasis. Using this estimate and assuming 10 pg total RNA per tumor cell and 2 pg per normal cell, our sample of 19 μ g RNA originated from a piece of metastasis containing $\sim 1.5 \times 10^6$ tumor cells. As stated above, this biopsy was estimated to contain 30,000 CD8 T cells. The ratio of CD8 T cells to tumor cells was therefore 2%. On the basis of the considerations presented above, antitumor TCR sequences represent $\sim 40\%$ of CD8 TCR sequences in this metastasis. Accordingly, the antitumor T cells therefore amounted to no more than 0.8% of the tumor cells in the regressing metastasis. The anti-vaccine T cells amounted to $< 6 \times 10^{-7}$ of tumor cells.

However, the low global ratio of antitumor T cells to tumor cells may have limited relevance because the T cells do not appear to be distributed at random among the tumor cells. Instead, they appear to be concentrated on the border of large compact regions containing tumor cells. For the cutaneous metastasis there was such a front of T lymphocytes at the inner tumor border. A similar concentration of T cells accompanied by macrophages was also observed on the inner border of the collagen shell located around the tumor cells in the invaded lymph node (Fig. 2, B and C). Thus, the local ratio of antitumor T cells to tumor cells in the front where interaction occurs might be close to 1:1.

Presence of antitumor T cells in metastases before vaccination

Antitumor T cells, including some anti-MAGE-C2, were already present at a high frequency among the blood lymphocytes of patient EB81 before she was vaccinated (Table I). At that time, the cutaneous metastases of the patient were clearly progressing. Was this due to a lack of presence of antitumor T cells inside these cutaneous metastases? Apparently not. Anti-MAGE-C2 clonotypes 1, 11, 40, and 41 were detected in a prevaccination metastasis, albeit at a lower frequency than in the metastases collected after vaccination (Table I). But another antitumor CTL was present at a 3% frequency. Somehow, the antitumor T cells found in the progressing prevaccination metastasis must have become incapable of attacking the tumor cells.

Out of 100 sequences analyzed in a TCR β library derived from the prevaccination metastasis, 17 were also represented in at least one of the two groups of ~ 100 TCR sequences obtained from the two metastatic sites resected after vaccination. Thus, a number of very frequent antitumor T cell clonotypes stably occupied the tumor sites, being found at a high frequency in metastases that were resected at a distance of several months.

Emergence of new antitumor CTL clonotypes after vaccination

The contrast observed between the very high frequency of antitumor T cells and the much lower frequency of anti-vaccine T cells in the metastases collected after vaccination, suggests the possibility that after vaccination, antitumor T cells are activated and become the main effectors that eliminate the tumor cells. This could occur through the reactivation of

T cells that are already present in the tumor or through the activation of naive antitumor T cell precursors. The latter implies that new antitumor T cell clonotypes ought to be detected after vaccination. This is indeed what we observed. The most frequent anti-MAGE-C2 clonotype found in the invaded lymph node, namely CTL 16, was not detected in the blood or in the cutaneous metastasis collected before vaccination. In addition, the highly frequent anti-gp100 clonotype 7 and clonotype 101 directed against mutated caseinolytic protease (Table I) were also not detected. But these findings do not exclude that in addition, previously present antitumor T cells were reactivated. Moreover, even though our observations are compatible with the notion that the anti-vaccine CTLs occasionally promote the stimulation of new effective antitumor CTLs, they remain short of proving this point, as it cannot be excluded that new waves of antitumor clonotypes appear continuously in the natural course of the disease.

DISCUSSION

What led to the analysis reported here was the observation that patients who show evidence of tumor regression after vaccination usually have a very low frequency of anti-vaccine T cells in the blood. Our results indicate that this paradox appears to extend to the tumor. In metastases of patient EB81, including one that clearly regressed, the single responder CTL clonotype directed against the MAGE-3.A1 vaccine antigen showed little enrichment relative to the blood. As a consequence the ratio of these CTLs to tumor cells was as low as 6×10^{-7} . This reinforces the probability that other cells might be the main effectors for the regression.

The findings reported here and in Germeau et al. (1) make a strong case for a possible involvement of T cells directed against tumor antigens other than the vaccine antigen. Such antitumor T cells were found at high frequencies in the blood. In patient EB81, who showed a nearly complete tumor response, many of these antitumor T cells were highly enriched in the metastases. A few dominant T cell clonotypes directed against tumor-specific antigens accounted for a large fraction, possibly for the majority of the CD8 T cells present in the metastases after vaccination. Because of the low proportion of CD8 T cells in the metastases, the global ratio of the number of antitumor T cells relative to tumor cells did not exceed 1%, but they appeared to be concentrated on certain fronts where this ratio might be close to 1:1. Finally, we observed after vaccination the appearance of a wave of new antitumor CD8 T cell clonotypes that were highly concentrated in the metastases.

These findings made with patient EB81 do not appear to represent a unique observation. Recently, we have initiated a similar analysis of a melanoma patient who showed tumor regression after vaccination with the MAGE-3.A1 peptide pulsed on dendritic cells. The findings are very similar. There is a low frequency of anti-vaccine T cells and there is a high frequency of antitumor CTLs with high enrichment in metastases. Remarkably, the most frequent antitumor

CTL is also directed against an antigen encoded by *MAGE-C2* (unpublished data).

Antitumor T cells were already present in patient EB81 before vaccination not only in the blood, but also in metastases, where they showed considerable enrichment and some highly dominant clonotypes. Therefore, the progression of the tumor before vaccination was not due to a lack of migration of these T cells into the tumor site. Nor was there a resistance of the tumor cells due to loss of presentation of tumor antigens. The metastases regressed after vaccination and the tumor cell line derived from these metastases was lysed by the antitumor T cells. Therefore, it appears that at one point the antitumor T cells became ineffective in the tumor. As these antitumor T cells become active again upon in vitro restimulation by tumor cells in the presence of cytokines, their inactivation may also be reversible in the tumor.

Many interplaying factors may produce in the tumor a negative environment that leads to a local paralysis of the antitumor T cells. Mechanisms of inhibition of T cells that appear to be inherent to the functioning of the immune system have received considerable attention in recent years. Analysis of mouse systems have demonstrated an inhibitory role for CD4⁺ CD25⁺ regulatory T cells (5). Recently, a CD4⁺ CD25⁺ suppressor T cell clone directed against an antigen encoded by *LAGE-1*, a cancer germline gene, has been isolated from a melanoma metastasis (6). Another inhibitory process described in a mouse system involves NK T cells producing IL-13, which stimulates the production of TGFβ by immature myeloid cells (7). Diversion of the response toward a Th2 cell response with IL-10 could also render the T cells ineffective against the tumor. TGFβ appears to be a central inhibitory agent (8). The inhibitory processes may involve positive feedback not only incapacitating the lymphocytes, but also rendering them more sensitive to further inhibitory agents and even transforming them into inhibitory lymphocytes themselves (9, 10). Indoleamine dioxygenase, which is produced by tumor cells and dendritic cells, possibly under the influence of regulatory T cells, might play an important role in inhibiting T cells either in the tumor or in the draining lymph node by depleting the local tryptophan supply (11–13). Several other inhibitory factors have been reported to be released by tumors. It will be very important to find out which of these mechanisms constitute the main inhibitory processes operating in human tumors. Possibly, the prevailing mechanism may vary from one tumor to another.

How can we envision the process that leads to the rejection of tumor cells that is observed occasionally after vaccination? We feel that the following, admittedly hypothetical, sequence of events would be in line with our findings. The patients would respond to the peptide or ALVAC vaccination by expanding one or a few CTL clonotypes to a level below or barely above our present level of detection. In this regard it is worth remembering that if a single precursor cell expands 10,000-fold, in our view a significant expansion, this still leaves its frequency $<4 \times 10^{-7}$ CD8 T cells, the global base level frequency of the naive T cells directed

against the antigen (14). Some anti-vaccine T cells would then be capable of migrating to the tumor. They would resist the immunosuppressive environment for a while. They would be stimulated by the tumor cells and could destroy a number of them. This would lead to a local reversion of the inhibition, probably due to the release of activatory factors by the stimulated anti-vaccine T cells. This positive environment would extend focally in a way that might involve a positive feedback. Activatory factors could render lymphocytes less sensitive to the inhibitory factors and might also block suppressor T cells. As a result, new naive precursors of antitumor T cells would be activated by the tumor antigens instead of succumbing under the local suppression as they did before. Possibly, some antitumor T cells already present in the tumor, in the activation focus, would also become re-activated. In the favorable instances, the activated region would advance slowly throughout the tumor. If this type of scenario is correct, it might not be the number of CTLs elicited by the vaccine that would be of crucial importance, it might be their capacity to migrate to the tumor and foremost to resist to the local inhibitory conditions long enough to be stimulated by the tumor cells and generate an activation focus.

A process of local activation, similar to the process we postulate to be triggered by the anti-vaccine CTL, may have occurred spontaneously at a previous moment in the evolution of the tumor, generating the high level of antitumor T cells observed in melanoma patients. It is possible that in the course of tumor progression, occasional events such as local inflammation or destruction of some tumor cells create conditions allowing a focal stimulation of antitumor T cells that eliminate a number of tumor cells. Sometimes the elimination would be complete, leading to the spontaneous regressions that are observed occasionally with cutaneous melanoma. In other instances, the regression would be incomplete because local inhibitory immunological processes would prevail. Possibly, such immunosurveillance events might occur repeatedly, leading to the accumulation of successive waves of antitumor T cells and slowing down tumor progression. The effect of vaccination would be to trigger one more such event. All this implies that we vaccinate under circumstances where a “spent” immunosurveillance process has left the tumor in a stance of immunological inhibition that renders the task of the anti-vaccine CTL much more arduous than it would be if previous immunosurveillance processes had not occurred (15).

Insofar as the focal release of inhibition by anti-vaccine T cells promotes the expansion of other antitumor T cell clones, it could also enable the anti-vaccine T cell clonotype itself to expand further. This puts into a new light the correlation between detectable anti-vaccine T cell response and tumor regression, which has been observed on patients vaccinated with an ALVAC–MAGE construct (2, 14). Perhaps this correlation is not due to the fact that only those patients who make a sizeable T cell response to the vaccine have enough T cells to successfully attack the tumor. Instead, it might be due to the fact that only in those patients where a

rejection of the tumor is triggered by anti-vaccine T cells there occurs a further expansion of anti-vaccine T cells, which raises their frequency above the detection threshold.

On the basis of these considerations, what could be the reasons for the failure of therapeutic vaccination in a large majority of the patients? First, there could be a complete lack of response to the vaccine. The problem could be of a stochastic nature as there may be only a small chance that a CTL precursor is activated after each injection of the vaccine. We cannot exclude this possibility, but it is not favored by the observations of treatment failure in patients who receive a very large number of vaccinations, whereas some patients respond after a few. The alternative possibility that some patients may not have T cell precursors directed against the vaccine is rendered very unlikely by the large number of precursor clonotypes for antigen MAGE-3.A1 found in noncancerous individuals (14). Another possible explanation could be the overall immunodepression reported in very advanced cancer patients, but we do not believe that it applies on our group of patients. In conclusion, we doubt that the complete absence of response to the ALVAC or peptide vaccine is the major cause of failure. A second hypothesis is that the limiting factor is the occurrence of an anti-vaccine T cell that has the required functional properties to migrate to the tumor, resist the inhibitory environment of the tumor, and initiate focal activation. Only a subset of anti-vaccine T cell clones might have this property and such a T cell clone might not be generated in most patients by current vaccination procedures. The response to any given antigen might be "locked" into a few predominant but unfit clones that prevent others to appear regardless of how repeatedly one vaccinates. We feel that this hypothesis deserves careful consideration. A third possibility is that the severity of the intratumoral inhibition varies considerably from one patient to another and that in the majority of patients, this level of inhibition would prevent any anti-vaccine CTL from being effective in the tumor.

A possible remedy for the present lack of success could be vaccination against several antigens, so that at least one or two responder T cell clones with proper migration capability and resistance to inhibitory factors are obtained. A crucial adjunct to vaccination might be a treatment that alleviates the immunosuppressive environment of the tumor. One possible approach is the depletion of some subsets of T cells, particularly CD4⁺ CD25⁺ suppressor T cells. Recent successes of protocols of adoptive therapy preceded by depletion of T lymphocytes by chemotherapy might be due to the removal of suppressor T cells (16). Another approach could involve the use of agents counteracting inhibitory factors such as TGF β , indoleamine dioxygenase, and cyclooxygenase 2. In addition, activatory agents such as IFN- α , IL-2, and their combination could be administered not at the time of vaccination, but later, to increase the resistance of the anti-vaccine T cells to the negative environment of the tumor.

Our findings have important implications regarding the possibility of tumor escape from the T cell response induced by vaccination. The chance that a very small fraction of the

tumor cells lose the expression of the gene that codes for an antigen targeted by T cell responses appears to be high (17, 18). It is often stated that it is therefore important to vaccinate against antigens that the tumor cannot afford to lose, such as telomerase or various oncogenes. However, if our findings prove to be general, if successful vaccination involves the induction of many effective T cell clones directed against several tumor antigens other than the vaccine antigen, then the probability of tumor escape decreases significantly and may not be a limiting factor for the success of the therapeutic vaccination of cancer patients.

MATERIALS AND METHODS

Patients. See Germeau et al. (1) for description of patient and vaccination. This experimental protocol was approved by the Ethics Review Committee of the Faculty of Medicine of the Catholic University of Louvain.

Histology. Immunostaining was performed on formalin-fixed paraffin sections according to the multistep streptavidin-biotin peroxidase system revealed with 3-amino-9-ethylcarbazole. Anti-CD3 ϵ polyclonal antibody and anti-CD8 α monoclonal antibody C8/144B were from DakoCytomation.

RNA and DNA extraction. Total RNA was extracted from PBMCs, frozen samples of metastases, and tumor cryosections with the TriPure reagent (Roche). 7- μ m cryosections were laid on polyethylene-naphthalate membranes and quickly stained with half-diluted RNase-free hematoxylin. RNA was then extracted from whole sections, parts of sections dissected with a lancet, or smaller areas of ~ 1 mm² obtained by laser-assisted microdissection and laser pressure catapulting (P.A.L.M.; Microlaser Technologies AG). RNA from laser-microdissected fragments was extracted in 200 μ l TriPure reagent in the presence of 5 ng synthetic poly-A RNA and 5 μ g glycogen. For some tumor sections, which contained a high concentration of melanin, the RNA was extracted using the RNeasy procedure (QIAGEN), and in these cases, the reverse transcription was performed in the presence of 1 μ g/ μ l bovine serum albumin (Roche). Reverse transcription was applied to all the RNA using the SuperScript II RNase H⁻ reverse transcriptase (Invitrogen) and an anchored oligo(dT)₂₁ primer with a T7 promoter sequence at its 5' end. The cDNA was then treated with 0.2 U/ μ l Ribonuclease H (Invitrogen). DNA was extracted from PBMCs with QIAamp columns (QIAGEN).

Measurement of clonotype frequency in the blood Clonotypic PCR was applied on cDNA prepared from groups of 5×10^4 – 10^6 PBMCs as described previously (2). The proportion of positive groups for the clonotypic PCR and the proportion of CD8 T cells in the PBMCs were used to estimate the frequency of the CTL clone. The cDNA was used as a template for a first low stringency PCR (22 cycles) aimed at amplifying with certainty all V α and V β products of up to three clonotypes. A set of forward primers corresponding to all the relevant V α and V β chains was used, with a reverse primer matching the T7 sequence of the RT primer. A second, more stringent, PCR amplification (35 cycles) was applied to 1% of the first product with primers corresponding to the V β and J β -C β regions of the evaluated clonotype. Finally, 0.001% of this product was used for a third amplification (35 cycles), highly specific for a precise clonotype, usually with a V primer different from those used in the previous amplifications and a primer straddling the CDR3/J junction without covering it completely to check the CDR3 sequence of the amplified product.

Because the RNA of prevaccination PBMCs was found to be degraded, the frequencies of TCR clonotypes were evaluated by applying PCR to DNA extracted from 8×10^6 PBMCs containing 17% of CD8⁺ T cells and aliquoted into 192 microwells. A first PCR amplification used a forward V β primer and a reverse primer matching an intronic sequence downstream to the relevant J β coding sequence. 1% of this product was used in a second, more stringent amplification with another V β primer and a CDR3/J β primer.

Measurement of clonotype frequency in tumor samples. A first approach used 20–30 μg RNA extracted from each sample. 1 μg was reverse transcribed and used for the clonotypic PCR described above. The number of CD8⁺ T cells in these samples was estimated by quantitative PCR for the CD8 β cDNA, using 5'-CTTCTgCATgATCgTCgggA and 5'-ggCCCT-TCTgggTCTCTg as primers, 6-FAM-5'-CCCACCACTgCCCAgC-CCAC-TAMRA as probe, and an estimate of 40 CD8 β mRNA per cell, based on our observations with PBMC and CTL clones. A positive clonotypic PCR indicated that at least one cell of the relevant CTLs was present among the CD8 T cells present in the sample, namely 1.6×10^5 CD8 cells in the prevaccination metastasis and 3×10^4 CD8 cells in the postvaccination cutaneous metastasis. When no signal was obtained for the clonotypic PCR, we considered that fewer than three cells were present, on the basis of 100 TCR β mRNA per cell or 25 cDNA as the efficiency of reverse transcription was only 25%. Considering that 1/20 to 1/30 of the RNA was used for reverse transcription, the average recovery was about one molecule of TCR β cDNA per CTL.

When a tumor sample was positive for a given clonotype, the nested clonotypic PCR procedure described above was applied on cDNA obtained from either complete tissue sections or smaller fragments so as to obtain limiting dilution for the relevant clonotype. When all fragments were positive, we resorted to the evaluation of the frequency of the TCR β sequence in a TCR β cDNA library.

TCR β cDNA library. For each library, 1 μg total RNA was reverse transcribed using Powerscript (CLONTECH Laboratories, Inc.) and 1 μM of primer 5'-AgATCTCTgCTTCTgAT matching the C β 1 and C β 2 sequences in the presence of 1 μM of the SMART II oligonucleotide (CLONTECH Laboratories, Inc.). cDNA synthesis was performed in 10 μl according to the manufacturer's specifications except for the addition of 1.5 μg of T4 gene 32 protein (Roche). When the reverse transcriptase reaches the 5' end of the TCR β mRNA, it adds a few (dC) nucleotides to the cDNA. The SMART oligonucleotide contains a poly(G) sequence at its 3' end, which anneals to this poly(dC) sequence and serves as an extended template for the reverse transcriptase. The resulting single-stranded cDNA contains a complete TCR β transcript with a 5' extension that is complementary to the SMART oligonucleotide. The cDNA was treated with 1 U Ribonuclease H (Invitrogen), and one tenth was used as a template in a PCR amplification with an internal sequence of the SMART primer (5'-gCAGTggTAACAACgCAGAgTA) and a C β primer (5'-CgACCTCggg-TgggAACA) for 31 cycles with annealing at 60°C. The amplified product was purified on a sepharose CL-6B size-exclusion column (Amersham Biosciences) and cloned into the pcDNA3.1/V5/His-TOPO plasmid (Invitrogen). The constructs were used to transform *Escherichia coli* TOP10. Sequencing indicated that >80% of the cDNA clones represented TCR β sequences. About 50% of these corresponded to functional TCR β transcripts. The others were either truncated, aberrantly rearranged, or incompletely spliced. About 100 relevant TCR β sequences were obtained from each library. In addition, 200–800 cDNA clones, depending on the tumor sample, were screened by clonotypic PCR amplifications under limiting dilution conditions.

To confirm or precise the frequencies of some clonotypes, a fraction of the TCR β cDNA obtained for the library was also amplified by PCR with the SMART and C β primers. This amplified product was titrated by PCR for its content of fragments larger than 200 bp and aliquoted into 96 groups so as to obtain 200 such molecules per group. Clonotypic PCR was applied to these groups.

Estimation of the number of tumor cells with a BRAF genomic PCR. A segment of the exon 15 of gene *BRAF*, containing the V599E mutation that is frequently present in melanomas (4), was amplified by PCR, using primers that have been described previously (4), and cloned. A set of clones were sequenced to establish the proportion of mutated sequences. In the lymphoid tissue of the invaded lymph node (Fig. 2 A), only wild-type sequences (48/48) were found. In the tumoral part of this me-

tastasis, 50/60 (83%) of the sequences were mutated. In the cutaneous metastasis, 32/56 (57%) of the sequences were mutated.

Derivation of CTL clone 101. The TCR β sequence 101, with a V β 5.5-J β 2.7 rearrangement, was found repeatedly in the TCR β cDNA library prepared from the invaded lymph node resected in April 2000. Tumor-infiltrating lymphocytes from this metastasis were labeled with an anti-CD8 antibody coupled to phycoerythrin and an anti-V β 5.5 antibody coupled to fluorescein (BD Biosciences). The CD8⁺ V β 5.5⁺ cells, corresponding to 4% of all CD8 cells, were sorted, cloned by limiting dilution, and stimulated with irradiated (100 Gy) IFN- γ -treated (50 U/ml for 48 h; PreproTech) autologous tumor cells EB81-MEL (5,000 cells/microwell) and irradiated allogeneic EBV-transformed B cells (LG2-EBV; 50,000 cells/well) in Iscove's medium (Life Technologies) supplemented with 10% human serum, 116 mg/L L-arginine, 36 mg/L L-asparagine, 216 mg/L L-glutamine, 100 μM 1-methyl-L-tryptophan, 50 U/ml IL-2, 0.5 ng/ml IL-4, and 10 ng/ml IL-7. The lymphocytes were restimulated each week with tumor cells and feeder cells, and the resulting T cell clones were screened with a clonotypic PCR for TCR β 101. CTL clone EB81-CTL-734A/1 was obtained, which expressed TCR β 101, and specifically lysed the autologous melanoma cells. The identification of the antigen recognized by CTL clone 101 will be described elsewhere. The antigenic peptide is presented by HLA-A2 molecules and encoded by the *casein lytic protease* gene, which has undergone a point mutation in the EB81-MEL cells.

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