

Recurrent T Cell Receptor Rearrangements in the Cytotoxic T Lymphocyte Response In Vivo against the P815 Murine Tumor

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Summary

P815 is a murine mastocytoma of DBA/2 origin which, although immunogenic, rapidly develops as a tumor in immunocompetent syngeneic hosts. In this report, we have studied, by a molecular approach, the *in vivo* α/β T cell response to P815. Both situations of tumor growth after engraftment of naive animals or tumor rejection by preimmunized animals have been analyzed. The spectrum of T cell receptor β chain rearrangements in the tumor-infiltrating lymphocytes was found to be highly variable among individual tumor-bearing mice. However, two rearrangements, one using V β 1 and J β 1.2 segments and one using the V β 1 and J β 2.5 segments, with conserved junctional regions, reproducibly emerge in most individuals. These two rearrangements thus correspond to "public" (recurrent) T cell clones, as opposed to "private" ones, which emerge in a seemingly stochastic fashion in immunized animals. Importantly, these public cells are observed in situations of either growth or rejection of the tumor. Quantification provides a clear increase in public T cells in secondary responses, but no obvious correlation between their level and primary tumor rejection. The V β 1-J β 1.2 rearrangement is borne by CTL directed against an antigen derived from P1A, a nonmutated mouse self protein which is expressed in P815 but not in normal mouse tissues except testis. A recurrent, public T cell response can thus be observed to an antigen derived from a self protein expressed by a tumor.

CD8⁺ and CD4⁺ T cells recognize antigenic peptides presented by class I and class II MHC molecules, respectively. The α and β chains of the TCR are encoded by rearranged V α -J α -C α and V β -D β -J β -C β gene segments. The diversity of TCR is potentially as high as, if not higher than that of antibody molecules (1).

How diverse is the T cell response to a given antigen, and more precisely, to a given antigenic peptide? This question has been addressed by several authors, and various class I- and class II-restricted antigens have been used. The T cell response has been found to be very diverse in some instances or rather homogenous in others (reviewed in reference 2).

Based on recent work in our laboratory by Cibotti and co-workers (3), we make the distinction between the "private" and "public" components of a T cell response. The public arm of the response is characterized by one highly shared TCR chain rearrangement, reproducibly found in all or most individuals with identical genetic background. In contrast, the private response involves the seemingly stochastic emergence of T cell clones with distinct TCR chain

rearrangements which vary in different animals. The interest of such a distinction, besides the obvious experimental advantage of working with cells that possess a constant TCR, is that, in the case of the lysozyme system, public T cells appear to be of high affinity for their antigen, as suggested both by their recurrent selection and by their disappearance upon low expression of lysozyme expressed as a self protein in transgenic mice (3).

In this context, we have examined whether the T cell response against a tumor-associated antigen derived from a nonmutated self protein (encoded in the host genome) involves a public component. Our model was the P815 tumor grafted in syngeneic DBA/2 hosts. P815 is a methylcholanthrene induced mastocytoma (4) in which five tumor-associated antigens (A, B, C, D, E) recognized by CTL clones have been so far defined (5, 6). The A and B antigens are epitopes located in the 35-43 peptide of the P1A protein (7). The latter is not expressed in adult tissues except testis (8, 9). It is expressed but not mutated in P815 cells (8). We show here that two public β chain rearrangements, borne by CD8⁺ T cells specific for the A and E antigens, are

found in the anti-P815 T cell responses in vivo. The implications are discussed.

Materials and Methods

Mice. Male DBA/2 mice were raised in the animal facilities of the Institut Pasteur or bought from Iffa-Credo (L'Abresle, France). BALB/c, C57BL/6, C3H/OH, and SJL/J female mice from Iffa-Credo were bred with male DBA/2 to obtain the F1 mice. Mice were 4–8 wk old at the time of the first tumor inoculation.

Cell Lines. The P815 subline used in this study is the highly transfectable clone, P1-HTR (10). P1:204 is a P815 antigenic variant that does not express the P815 A and B antigens, but retains sensitivity to lysis by anti-C and anti-D CTL clones; P1.204 C⁺D⁻, C⁻D⁺, and C⁻D⁻ have lost the corresponding antigens (5). All retain sensitivity to lysis by anti-E CTL (6). L1210-P1A was generated by transfection of the L1210 lymphoma (of DBA/2 origin), which does not share tumor-specific antigens with P815, with a P1A cDNA (6). P815-IL2 was generated by transfection of P1-HTR with a plasmid containing the IL-2 cDNA (11). V2D1, V4D6, R56VT, and 15V4T2 are four DBA/2 mastocytoma cell lines related to PB-3c (12), and are a kind gift of Dr. C. Moroni (Institut für Medizinische Mikrobiologie der Universität, Basel, Switzerland). The CTL clones, kindly provided by Dr. Aline van Pel (Ludwig Institute for Cancer Research, Brussels, Belgium), have been generated as described in (5) from splenocytes or tumor-infiltrating lymphocytes (TiLs).¹

Tumor Inoculation and Immunization. Tumor graft consisted of a subcutaneous injection of 10⁶ P815-HTR cells in the right flank in 300 μ l of serum-free DMEM. Naive mice so inoculated provided the tumors indicated as growing.

Preimmunization of the mice against P815 was performed by the subcutaneous injection of 10⁶ P815-IL2 cells (13). Except where indicated, mice were challenged with 10⁶ P815-HTR cells 2–4 mo later. The nodules that appeared transiently after these challenges provided the tumors indicated as regressive.

Oligonucleotides. Oligonucleotides for repertoire analysis have been described previously (14), except:

C β 4: GCCCATGGAAGTGCACCTGGC (from 5' to 3' end)

J β 1.2far: CCTCTATTACCAAAAGCCTGG

J β 2.5far: CCTCTAACACGAGGAGCCGAG

Clonotypic oligonucleotides:

P11R:*-GGTGTAGTCGGAGTTICCGITITCTTGG

P21R:*-GTACTGGGTGTCTTGGTTIATIGTITGG

These oligonucleotides have been devised to hybridize with the CDR3 sequences shown in Table 1 (P11R for J β 1.2, P21R for J β 2.5). Since there was some variability at the third bases of codons in the junctional regions, inosine residues (indicated as I above) were incorporated at these positions. The asterisk indicates labeling with a fam fluorophore (Applied Biosystems, Inc., Foster City, CA).

Oligonucleotides were either synthesized on a DNA synthesizer (Applied Biosystems) or purchased from Genosys (Cambridge, UK).

Direct sequences of V β 1 PCR products were made with the nested V β 1 S oligonucleotide: CTACTTTTACATATATCTGCC. Expression of P1A was tested with the two following oligo-

nucleotides: P1A-5': CCAGACAAAGCCCCACAGTGGC P1A-3': GCTTTGCCTGGCTATCCAGGC.

RNA Extraction and cDNA Synthesis. Total RNA was isolated after ultracentrifugation on a CsCl cushion according to the method described by Chirgwin et al. (15). cDNA was synthesized from 10 μ g of RNA, using a (dT)17 primer, 25 U of RNasin (Promega Corp., Madison, WI) and 10 U of AMV reverse transcriptase (Boehringer Mannheim, Indianapolis, IN) in the provided buffer.

PCR and Run-Off Procedure. All PCRs were performed with the following mixture: 25 U/ml of Promega or Bioprobe (Bioprobe Systems, Montreuil, France) Taq polymerase, with the provided buffer; MgCl₂ 2.5 mM; dNTP 0.2 mM; each primer 0.5 μ M; and template (the equivalent of 10–100 ng of reverse-transcribed RNA). A 30- μ l final vol was used. 40 rounds of amplification consisting each of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C were performed.

The run-off protocol is similar, with the following modifications: a single dye-labeled primer is used, at the concentration of 0.1 μ M; the template is 2 μ l of PCR product for a 10 μ l final vol, and one to six cycles of elongation are performed, depending on the yield of the PCR. Annealing temperature was 60°C for all primers except P11R for which it was 65°C.

Repertoire Analysis. The technique of repertoire analysis has been described in (16). Briefly, a PCR is performed on the cDNA of interest using a V β -specific primer with the C β 4 primer. (To cover the full repertoire, 21 such reactions, using the primers specific for the 21 functional V β segments of DBA/2, are made.) The PCR product is then subjected to run-off reactions using nested dye-labeled primers, specific either for C β or one of the 12 functional J β segments. This run-off product is run on an automated sequencer, together with size standards; the intensity of the various bands is recorded, then analyzed with the Immunoscope[®] (developed by Christophe Pannetier) software, which also provides the lengths of the DNA fragments. The repertoire observed with peripheral T cells of a naive mouse (for any V β -J β combination) is a highly reproducible gaussian-shaped profile with 3-bp spaced peaks corresponding to in-frame sequences (14, 16).

Direct Sequence of PCR Products. The sequence was performed using the Sequenase 2.0 kit (U.S. Biochemical Corp., Cleveland, OH) and ³⁵S-labeled dATP (Amersham Corp., Arlington Heights, IL), according to the technique described by Casanova et al. (17). The template is the product of a 100- μ l PCR performed with the relevant V β and J β primers, after purification by migration on a ethidium bromide-stained 2% agarose gel, excision of the specific band, electroelution, and ethanol precipitation.

Public Clones Quantification. We quantify the proportion of mRNAs coding for a TCR with a public CDR3 sequence in the total mRNAs coding for TCRs sharing V β and J β segments (but with any CDR3 sequence) by the following procedure. To measure the V β 1-J β 1.2 clone, the cDNA from the sample of interest is subjected to a PCR using the V β 1 and J β 1.2far primers, the product of which is separated into two equal aliquots. The total population is then revealed by performing a run-off reaction on the first aliquot with the fam-labeled J β 1.2 primer, which yields 126–147-bp-long fluorescent products. The specific sequence products are revealed in a run-off reaction on the second aliquot with the fam-labeled P11R clonotypic primer, whose result is a 113-bp-long fluorescent product. (To measure the V β 1-J β 2.5 clone, the procedure is identical, except for the following differences: in the initial PCR, the V β 1 and J β 2.5far primers are used; in the run-off on the first aliquot, the fam-labeled J β 2.5 primer, which yields 118–139-bp-long product, is used; in the run-off on

¹Abbreviations used in this paper: HEL, hen egg white lysozyme; TiL, tumor-infiltrating lymphocyte.

the second aliquot, the P2IR clonotypic primer, whose result is also a 113-bp long product, is used.) The two run-off products are mixed in equal amounts, then loaded in the automated sequencer. The ratio of the fluorescence intensity of the 113-bp band to the sum of the other bands indicates the proportion of the specific sequence in the total (after correction of the specific activities of the primers). The background, as measured either on the polyclonal population of a naive mouse or on a cloned template with a CDR3 of right size but wrong sequence, is ~3%.

To estimate the percentage of V β 1⁺ T cells in tumors, a fully quantitative PCR protocol, already described in (18) and (19), has been used. Briefly, it consists of the coamplification in the same PCR tube and with the same primers, of the cDNA of interest and of a known number of copies of a standard plasmid that contains the sequence amplified from cDNA, with a 4-bp deletion somewhere in the middle (not in the primer regions). Both sequences are amplified with the same yield up to saturation. A run-off reaction is then made on this PCR product using a dye-labeled, nested primer. This product is run on an automated sequencer: the products of the cDNA and standard plasmid are separated on account of the 4-bp size difference. The ratio of the amount of wild-type sequence with the amount of standard sequence is determined with the Immunoscope[®] software. The accuracy of the measurement is controlled by using a serial dilution of standard plasmid, checking that the yield is linear. The median result from standard/wild type ratios in the range from 0.1 to 10 is taken as the final number.

Flow Cytometry. Cell populations were analyzed on a FACS[®] using the FACScan[®] (Becton Dickinson and Co., Mountain View, CA) software. Cells were incubated with FITC-conjugated anti-CD8 mAb (YTS 169.4; Caltag Laboratories, South San Francisco, CA), PE-conjugated anti-CD4 mAb (YTS 191.1; Caltag), and dead cells were excluded by propidium iodide incorporation.

Panning. 8-cm diameter polystyrene plates were coated with mouse anti-rat Ig (H+L) polyclonal antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Cells were then added in PBS 2% FCS, after their incubation with either the anti-CD8-FITC or the anti-CD4-PE mAb indicated above. After 70

min of incubation at 4°C, the nonadherent population was harvested by three gentle washes with PBS 2% FCS. The adherent cell population was then collected using a silicone cell scraper. The cells were then incubated with the second antibody and the purification level estimated by FACS[®] analysis.

Results

Analysis of TiLs. Two groups of DBA/2 mice were injected subcutaneously with 10⁶ P815 cells. In the first group, composed of initially naive mice, steadily growing tumors developed, causing death after ~30 d. The second group contained DBA/2 mice preimmunized with 10⁶ P815-IL2 cells, a procedure known to vaccinate mice against a subsequent challenge with P815 cells (11). When P815 cells were similarly grafted onto these immunized mice, a small tumoral nodule developed for ~1 wk, then stabilized and disappeared in ~3 wk.

Tumors and spleens of naive and preimmunized individual mice were taken 2 wk after P815 tumor inoculation. RNA was extracted, reverse-transcribed into cDNA, and T cell repertoire analyses were carried out as described previously (16). Briefly, PCR reactions were performed with a C β -specific and each V β -specific oligonucleotide, and aliquots were subjected to run-off reactions initiated with fluorescent J β or C β -specific oligonucleotide primers. The fluorescent elongation products display different lengths because of differences in their CDR3 regions. They were size-fractionated in an automated DNA sequencer and the fluorescence intensity in each peak was recorded.

Results are shown in Fig. 1 for four representative V β s and three mice from each group. The peripheral (spleen) repertoire of the tumor-bearing mice does not notably differ from that of control mice, with rare exceptions of minor peaks (Fig. 1, mouse 4, V β 8.1). The repertoire of TiLs is strikingly different from this steady pattern. For most

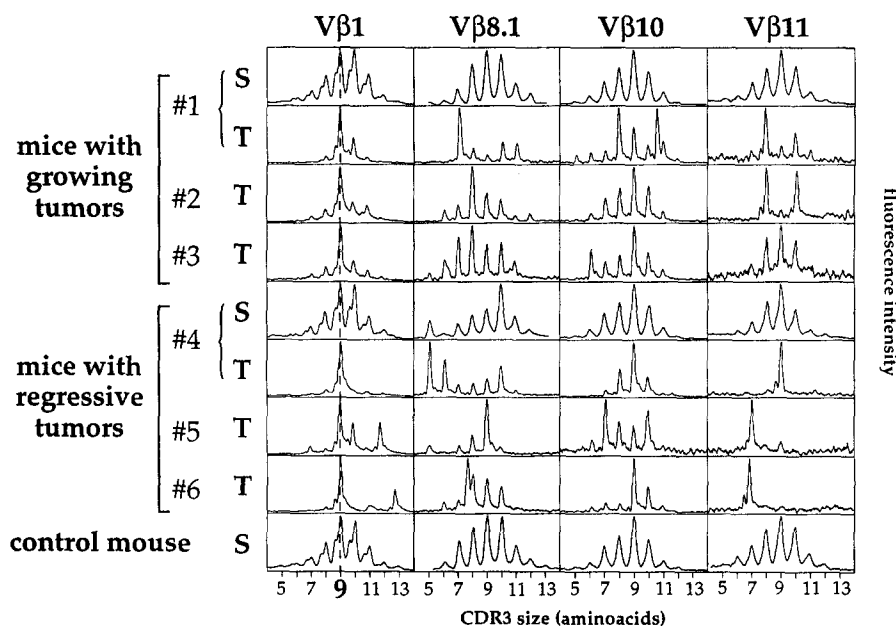


Figure 1. Repertoire of α/β T cells in the tumors or the spleens of individual tumor-bearing mice, for a few representative V β segments. Total RNA was extracted from spleen (S) or tumor (T) of individual mice bearing growing or rejected tumors (see text). After reverse transcription into cDNA, V β -C β PCR reactions were performed with the various V β -specific primers, labeled with a nested fluorescent C β -specific primer, and size-fractionated in an automated DNA sequencer. (Horizontal axis) Size in amino-acids of the CDR3 junctional region as defined by Chothia et al. (20) deduced from the fragment size. (Vertical axis) Fluorescence intensity, in arbitrary units. A naive DBA/2 mouse has been included as a control.

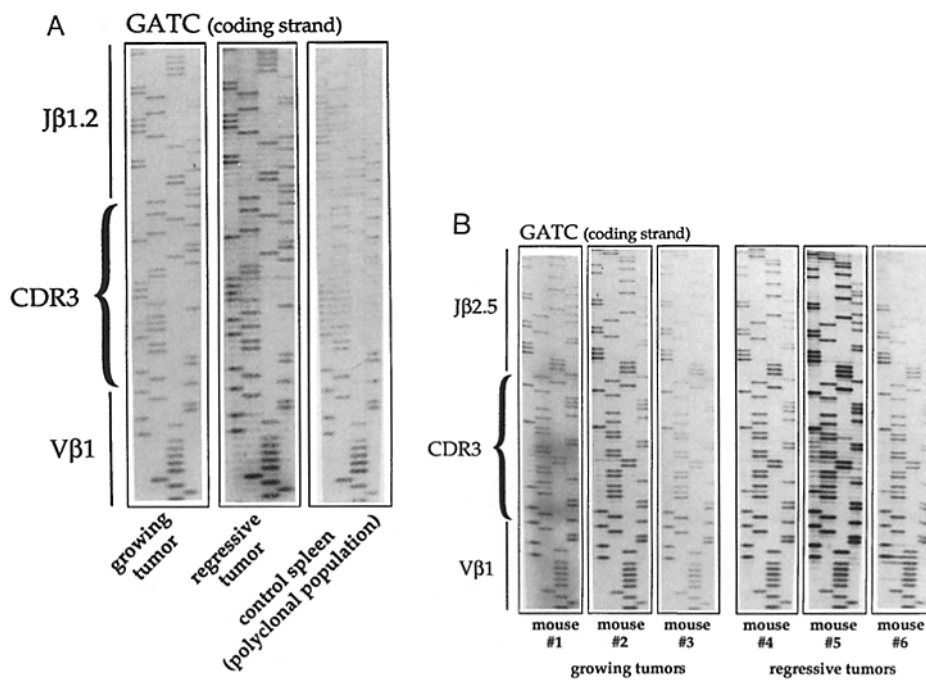


Figure 2. Direct sequences of V β 1-J β 1.2 (A) or V β 1-J β 2.5 (B) PCR products from tumor samples or a control DBA/2 spleen.

V β s, only a few peaks are observed. We directly sequenced several PCR products, which yielded single peaks. The junctional region was readable and unambiguous, strongly suggesting that such peaks correspond to clonal expansions. Thus, as previously observed in humans (21–24), TiLs appear to be oligoclonal. In the case of P815, our data (Fig. 1

and not shown) lead to the estimate that the TiL population in one mouse contains 100–300 T cell clones.

These oligoclonal patterns are highly variable in distinct animals. Dominant peaks on V β -C β profiles are not observed at the same CDR3 length in all animals and recurrent peaks in two or more animals correspond most often

Table 1. Sequences of V β 1-J β 1.2 and V β 1-J β 2.5 TCR Junctional Regions Read from PCR Products Obtained from Tumor Samples, and Deduced Amino Acid Sequences of the CDR3.

Mouse	V β	J β	Length	V β End	Junctional region	J β Beginning	Amino acid sequence
1	1	1.2	9	AGC CA	A GAG ACG GGS	AAC TCC GAC TAC	SQETGNSDY
2	1	1.2	9	AGC CA	A GAR ACG GGG	AAC TCC GAC TAC	SQETGNSDY
3	1	1.2	9	AGC CA	A GAA ACR GGS	AAC TCC GAC TAC	SQETGNSDY
4	1	1.2	9	AGC CA	A GAG ACR GGR	AAC TCC GAC TAC	SQETGNSDY
5	1	1.2	9	AGC CA	A GAG ACS SGG	AAC TCC GAC TAC	SQETGNSDY and SQETRNSDY
6	1	1.2	9	AGC CA	Unreadable	Unreadable	Mix
Spleen	1	1.2	9	AGC CA	Unreadable	Unreadable	Mix
1	1	2.5	9	AGC CA	G ACA ATT	AAC CAA GAC ACC CAG	SQTINQDTQ
2	1	2.5	9	AGC CA	A ACA ATT	AAC CAA GAC ACC CAG	SQTINQDTQ
3	1	2.5	9	AGC CA	A ACA ATT	AAC CAA GAC ACC CAG	SQTINQDTQ
4	1	2.5	9	AGC CA	R ACA ATT	AAC CAA GAC ACC CAG	SQTINQDTQ
5	1	2.5	9	AGC CA	R ACA ATT	AAC CAA GAC ACC CAG	SQTINQDTQ
6	1	2.5	9	AGC CA	R ACA ATT	AAC CAA GAC ACC CAG	SQTINQDTQ
Spleen	1	2.5	9	AGC CA	Unreadable	Unreadable	Mix

Positions where two nucleotides could be read are indicated as follows: R = A or G; S = C or G. Sequences are available in Genbank under accession numbers U44406 to U44416.

to different J β segments (not shown). There is, however, one exception: as can be seen in Fig. 1, in all analyzed tumors, a prominent peak is found in the V β 1 profile with a CDR3 length of nine amino acids. J β analysis revealed the usage of two J β s in this peak: J β 2.5 in all samples and J β 1.2 in five out of six.

Recurrent V β 1-J β 1.2 and V β 1-J β 2.5 Sequences in Different Individuals. We directly sequenced the PCR products obtained with oligonucleotides specific for V β 1 and J β 1.2 or J β 2.5 from a number of TiL samples (Fig. 2, Table 1). In all samples but one (mouse 6, J β 1.2), sequences were readable in the CDR3 region and, as anticipated, in-frame TCR sequences with a CDR3 length of nine amino acids could be deduced from the sequences. In some cases, two nucleotides could be read at the same position. This was no PCR artifact since the same phenomenon was observed with two independent PCR products. It indicates that a few clones, with almost identical TCR β sequences, are present together in the same tumor. Amino acid sequences were identical among different individuals, or among different sequences in the same individual (with one partial exception, mouse 5, J β 1.2) despite a few differences in the nucleotide sequences. These amino acid sequences correspond to those of CTL clones obtained by one of us after *in vitro* expansion of splenocytes from a single individual (6).

These two recurrent rearrangements will, from now on, be called public rearrangements, and it will be assumed that each correspond to one or a few public T cell clones, even though the corresponding α chain rearrangements remain unknown.

Increased Frequency of T Cells Using the Public TCR β Chains Is Observed in Peripheral T Cells of Tumor-bearing Mice. As mentioned above, the peripheral repertoire of tumor-bearing animals and of controls look similar (Fig. 1). This indicated that analysis of V β -J β profiles was not quite sensitive enough to detect the clonal expansion or the accumulation of public T cells in the periphery of tumor-bearing animals. This problem, however, could be overcome by the use of "clonotypic" oligonucleotides. Knowing the β chain sequence of public clones, we devised more specific oligonucleotides, penetrating within the CDR3 region, as described by Cochet et al. (16). To cope with the previously mentioned nucleotide variations, inosine residues were incorporated at certain positions, as described in Materials and Methods. Using two such primers, we found both public β rearrangements in splenocytes, PBL, LN cells, but not thymocytes (Fig. 3 represents the results with the V β 1-J β 2.5 rearrangement for a mouse in which these public cells were particularly frequent in the periphery). Thus, public T cells observed among TiLs can also be found in the periphery.

The Frequency of Public T Cells in Either Spleen or Tumor Is Higher in Secondary than in Primary Responses, Although No Obvious Correlation Can Be Made with Tumor Rejection. As we did not anticipate finding a similar response in mice with growing and rejected tumors, we used quantitative PCR techniques to measure the size of the public clones in these mice, using the same cDNAs as above. Results, dis-

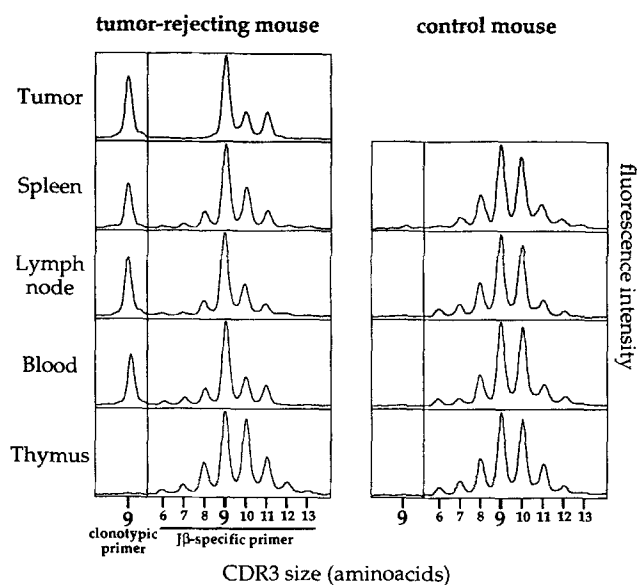


Figure 3. Detection of the tumor-specific T cells in various organs of a tumor-bearing mouse. The tumor-rejecting mouse has been immunized with P815-IL2, challenged with P815 6 wk later, and analyzed 14 d after the challenge. The control mouse is a naive DBA/2 mouse. A PCR with the V β 1 and J β 2.5-far primers has been performed on the cDNA obtained from the organ. This product was separated into two equal aliquots: one was used as a template for the elongation of a fluorescent J β 2.5-specific primer, providing the peaks on the right part of each panel. The other was used in the elongation of the clonotypic P2IR primer, providing the single peak on the left. These two run-off products were then mixed and size-fractionated in an automated sequencer (see Materials and Methods, *Public Clones Quantification*, for more details).

played in Fig. 4, show that, either in the spleen or in the tumor itself, the public T cells are more frequent in tumor-rejecting mice than in those bearing growing tumors. The sum of the populations of both clones has to be considered, since the response can be strongly biased toward one or the other kind of clone.

Since public T cells arise and are detectable during the primary response to the P815 tumor, we tested whether the presence of one or the other clone had an effect on the growth of the tumor. To do so, we took advantage of the fact that in a few occurrences, naive DBA/2 mice reject a usually lethal P815 graft. We bled a group of initially naive mice inoculated subcutaneously with 10^6 P815 cells at day 14 after the graft, when it was not yet possible to know whether they would reject the tumor or not. The tumors were then regularly followed. We compared the amount of public cells found in the blood of three mice that finally rejected their tumor, one with only a transient rejection, and six with the usual steady tumor growth. Results are displayed in Fig. 5: as can be observed, no correlation can be established between subsequent rejection and amount of public cells at day 14 in blood.

Public Clones Are Specific for Two Antigens of P815 *In Vivo*. CTL clones derived *in vitro* by one of us, with the two public TCRs, are specific for the P815 A and E antigens (6). To check if this specificity is the same for the public cells observed *in vivo*, we investigated whether they

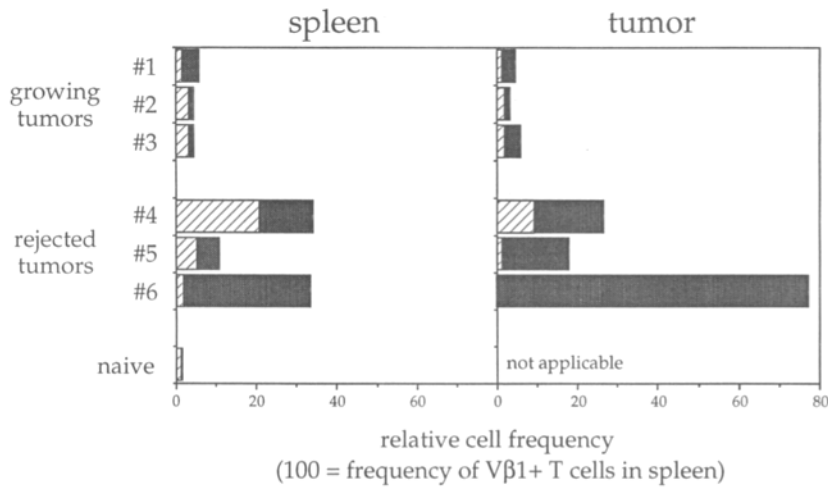


Figure 4. Frequencies of public cells in the spleen and tumors of tumor-bearing mice. The percentage of public T cells among the Vβ1-Jβ1.2 or Vβ1-Jβ2.5 populations was measured as explained in the legend to Fig. 3. The percentage of Vβ1-Jβ1.2 and Vβ1-Jβ2.5 among total Vβ1+ cells was measured in a similar way, with a Vβ1-Cβ PCR followed by run-offs with Cβ-, Jβ1.2-, and Jβ2.5-specific fluorescent primers of known specific activities. Finally, the frequency of Vβ1+ T cells in the tumors was estimated by the measure of Vβ1 and hypoxanthine guanine ribosyl transferase transcripts by quantitative PCR as described in Materials and Methods. Public clone: ▨, Vβ1-Jβ1.2; ■, Vβ1-Jβ2.5.

emerge in the repertoire of mice immunized with either P815 variants, a P1A transfectant, or other mastocytoma lines (Table 2).

The public Vβ1-Jβ1.2 rearrangement was observed after immunization by P815 but not by P1:204, a variant of P815 that expresses antigens C, D, and E, but has lost AB expression (5, 6). The Vβ1-Jβ1.2 public clones are thus specific in vivo for a P1A-derived antigen, as confirmed by the fact that it is elicited by the L1210-P1A transfectant. It is worth noting that this public response was observed only in two-thirds of the mice responding to P815, even upon secondary immunization.

Cells harboring the Vβ1-Jβ2.5 TCR are not specific for a P1A product since they arise in response to P1:204. They are specific for neither the P815 C nor D antigens because they also arise in response to the corresponding antigen-loss variants. Since they are not elicited by L1210-P1A or the other DBA/2 mastocytoma cell lines, they are

specific for a P815 antigen not shared with these tumor lines (thus excluding a mast cell differentiation antigen). The only characterized antigen of P815 that remains is the E antigen for which the corresponding loss variant was not available.

Since we found one occurrence of a response with the Vβ1-Jβ1.2 public TCR against the 15V4T2 line, we checked whether the four PB-3c-derived cell lines (12) expressed P1A in vivo, by PCR with P1A-specific oligonucleotides on cDNA obtained from the solid tumors. V4D6 and 15V4T2 were found to express P1A mRNA (in three samples out of three), in contrast to V2D1 and R56VT. Although three samples are not statistically significant, our data suggest that 15V4T2 and V4D6, although they express P1A mRNA, are less efficient than P815 or L1210-P1A to generate anti-P815A CTL responses in vivo.

Public Clones Are CD8+ In Vivo. To determine whether the two types of public T cell clones express CD4 or CD8

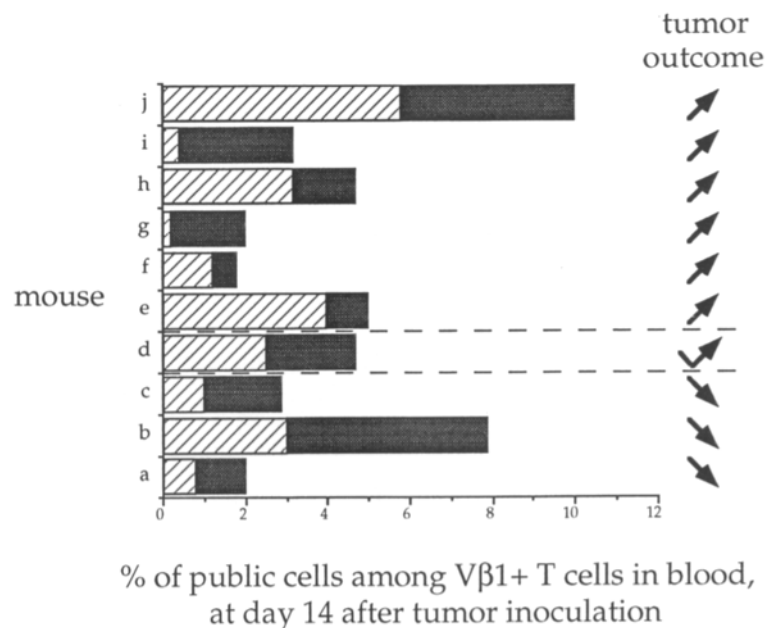


Figure 5. Measurement of the size of public clones in the blood of mice 14 d after graft of a P815 tumor and subsequent outcome of the graft. Naive DBA/2 mice were injected with 10^6 P815 cells subcutaneously. At day 14, when all possessed a tumor nodule, they were bled at the base of the tail, and cDNA was synthesized from these blood samples. Public clone quantification was then performed as explained in the legend to Fig. 3. The size of the tumor nodule was then regularly checked. Although most mice were dead at day 30 after a steady growth of the tumor (indicated by the up-pointing arrow), the nodule spontaneously regressed in four mice, three up to disappearance (down arrow), one only transiently (down and up). ▨, Vβ1-Jβ1.2; ■, Vβ1-Jβ2.5.

Table 2. Occurrences of Public T Cells in Response to Various Tumors.

Cell line	Type of response	Tested tissue	Occurrences of public clone	
			V β 1-J β 1.2	V β 1-J β 2.5
P815	Primary	Tumor	5/6	6/6
	Primary	Blood	7/11	11/11
	Secondary	Tumor	4/6	6/6
P1.204	Primary	Tumor	0/4	4/4
P1.204 C+D-	Primary	Tumor	0/3	3/3
P1.204 C-D+	Primary	Tumor	0/3	3/3
P1.204 C-D-	Primary	Tumor	0/3	3/3
L1210-P1A	Primary	Tumor	5/6	0/6
V2D1	Primary	Tumor	0/3	0/3
V4D6	Primary	Tumor	0/3	0/3
R56VT	Primary	Tumor	0/3	0/3
15V4T2	Primary	Tumor	1/3	0/3

Mice were analyzed 14 d after the subcutaneous inoculation of 10^6 tumor cells in naive (primary responses) or P815-IL2-vaccinated (secondary responses) DBA/2 mice. Public clone quantification (see Materials and Methods) was performed on cDNA from TiL or PBL of individual mice as indicated. α/β T cell infiltration was observed in all cases. In a given V β -J β subpopulation, the mean value and standard deviation of the background level of public/total TCR ratio measure has been calculated on three naive DBA/2 PBL samples. Measures falling within the range of this mean value \pm 1 SD were considered as negative. All positive measures were above mean value + 3 SD.

coreceptor molecules in vivo, we immunized mice with P815 IL-2 and inoculated them several times with P815 to expand the specific T cells. Splenocytes taken 14 d after the last challenge were enriched in CD4- or CD8-positive cells by panning. The degree of enrichment was measured by flow cytometry, and public clones were quantified in the samples as above. Fig. 6 shows that T cells with the public V β 1-J β 2.5 rearrangement segregate with the CD8 marker, demonstrating that at least most of these cells are CD4⁻CD8⁺ in vivo. Comparable results have been obtained with both public clones and in several mice. This result is consistent with the specificity of these T cells, since CTL are usually CD8⁺ and P815 is MHC class II-negative (not shown).

Presence of Public and Private T Cells in a Panel of Anti-P815 CTL Clones. We also sequenced the TCR β chain in a panel of in vitro-derived P815-specific CTL clones (Table 3). Two clones, P1:5 and 3I(5), obtained from different mice, both specific for a P1A product, used the V β 1 and J β 1.2 segments, with the CDR3 sequence of public cells.

The other sequences from this panel were found only once (TiL3III(16) and -3III(18) share exactly the same sequence but arose in the same mouse and are likely to be sister clones). We looked in the TiL repertoires of several P815 tumor-bearing mice for prominent peaks sharing the same V β segment and CDR3 length, but we did not find any. These CTL clones are thus very likely to be private clones.

The Public Responses Are Observed in F1 Animals with a DBA/2 Parent. Humans are usually heterozygous at their different MHC loci. The presence of these other alleles may greatly influence the available repertoire restricted to one given allele, precluding the emergence of a public re-

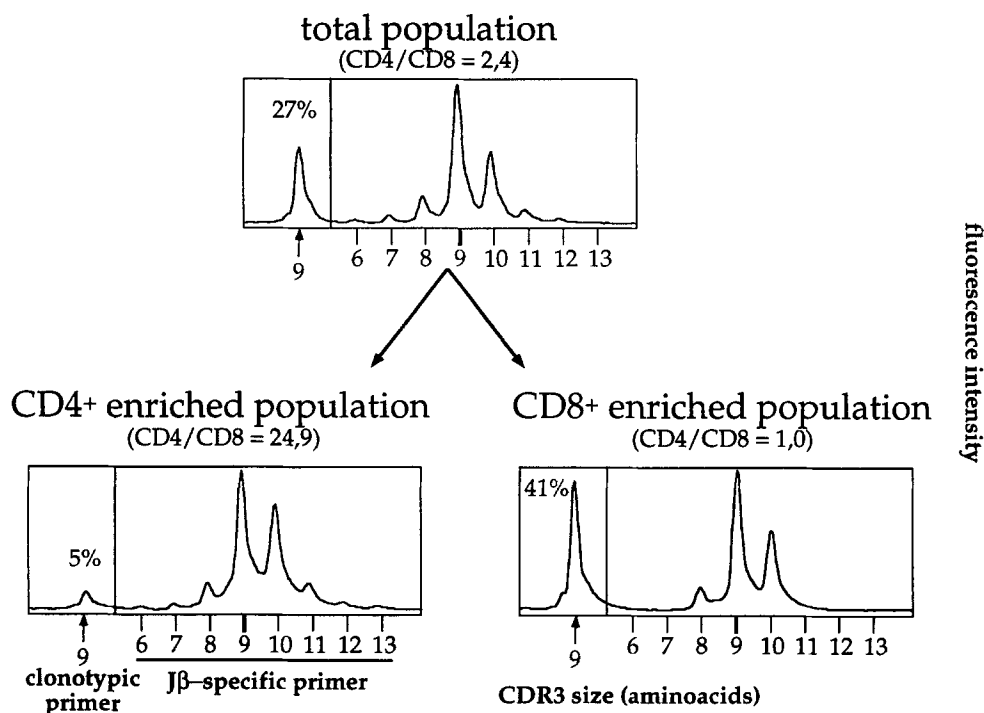


Figure 6. Public T cells segregate with the CD8 marker. Public clone quantification has been performed, as explained in the legend to Fig. 3, on splenocytes from an immunized mice sorted by panning (see details in text). Results for the V β 1-J β 2.5 public sequence are shown here. The CD4/CD8 ratio, as measured by flow cytometry before and after the panning, are indicated above each panel. Indicated is the ratio of the area of the sequence-specific peak indicated by the arrow with the sum of the areas of the other peaks for the whole population.

Table 3. Sequences of the CDR3 Regions of P815-specific CTL Clones

CTL clone	Antigen	V β	J β	CDR 3 length	V β 3'	Junctional region	J β 5'	Amino acid sequence
P1:5	A	1	1.2	9	GCC AGC AGC CA	A GAG ACG GGA	AAC TCC GAC TAC	SQETGNSDY
P35:10	B	2	1.1	5	AGT GCC	ACA GGG C	CA GAA GTC	TGPEV
TiL 3I (4)	AB	5.2	2.7	7	GCC AGC TCT	CCC GGG G	CC TAT GAA CAG	SPGAYEQ
TiL 3I (5)	AB	1	1.2	9	GCC AGC AGC CA	A GAA ACA GGG	AAC TCC GAC TAC	SQETGNSDY
TiL 3III (16)	AB	10	1.1	8	GCC AGC AGC	CAC AAT G	CA AAC ACA GAA GTC	SHNANTEV
TiL 3III (18)	AB	10	1.1	8	GCC AGC AGC	CAC AAT G	CA AAC ACA GAA GTC	SHNANTEV
P1.515	C	6	1.3	10	GCC AGC A	TC TCC GGG ACA TT	T TCT GGA AAT ACG GTC	ISGTFSGNTV
P1.204:8	D	10	2.7	7	GCC AGC AGC	TCG CAA AAA	TAT GAA CAG	SSQKYEQ

RNA has been extracted directly from a frozen vial of cells, cDNA synthesized, and V β -C β PCR reactions performed with the functional V β -specific primers. Positive reactions were confirmed, and their product directly sequenced with the C β 5' primer. Only the productive rearrangements obtained are displayed. The two first codons displayed under V β 3' do not belong to the CDR3 region as defined in (20) but have been shown for clarity; they are not included in the amino acid sequence. Antigen indicated as AB means specificity for a P1A product (i.e., lysis of L1210-P1A but not of L1210). Sequences are available in Genbank under accession numbers U44417 to U44424.

sponse observed in homozygous individuals. To mimic this situation, we analyzed whether the public response against P815 is maintained in F1 animals, derived from a cross between DBA/2 and either BALB/c (H-2^d), C57Bl/6 (H-2^b), C3H/OH (H-2^k), or SJL/J (H-2^s) mice. As expected, tumors grew in F1 mice, but not in parental strains other than DBA/2. The tumors of F1 mice were taken at day 14 and the repertoire of TiLs searched for the public TCRs, using the clonotypic primers. The two rearrangements were found in all F1 hybrids. Thus, there is no negative effect of the other MHC alleles on the public response against P815.

We wondered whether the two public T cell clones seen in DBA/2 would be detectable in BALB/c mice, which are also of H-2^d MHC haplotype. In BALB/c mice, the tumor does not grow, implying that an immune response is directed against minor histocompatibility differences with DBA/2. It may thus not be surprising that the public TCRs were not detected in the spleens of BALB/c mice 14 d after P815 inoculation.

Discussion

We have studied the repertoire of TiLs in DBA/2 mice grafted with the P815 mastocytoma tumor cell line in which five tumor-specific antigens, A, B, C, D, and E, have been identified (5, 6). We found that the TiL population is oligoclonal, containing a few hundred clones, most of which do not reproducibly emerge in all tumors. However, two TCR β chain rearrangements, with CDR3 sequences entirely conserved at the amino acid level, occurred in most or all animals. A V β 1-J β 1.2 rearrangement was found in ~70% of the P815-bearing mice, in primary or secondary responses. A V β 1-J β 2.5 rearrangement was detected in 100% of the samples. Cells with both TCR types were of CD8 phenotype and could be detected among TiL and in the peripheral lymphoid compartments of tumor-bearing mice. CTL clones with corresponding

TCR rearrangements, derived from splenocytes of a single mouse, have been recently described by one of us; the specificity of the V β 1-J β 1.2 cells was attributed to a product of the P1A gene, and that of the V β 1-J β 2.5 cells to the P815E antigen (6). Using antigen-loss variants and other tumor lines, we find a fully compatible specificity of our public T cell clones in vivo. Furthermore, the V β 1-J β 1.2 public rearrangement was found to be borne also by the P1:5 CTL clone, the epitope of which has been extensively characterized as being the 35–43 peptide of P1A presented by L^d (7), particularly its Leu⁷-Val⁸ residues (25). It is highly likely that public V β 1-J β 1.2 cells, even though their TCR α chain is not known, recognize the same epitope in vivo.

Analyzing TCR use in vivo, at the tumor site or in the periphery, we found T cells specific for P815A in 70% of mice, specific for P815E in 100% of the mice. Some mice displayed a substantial amount of both types of clones, whereas others were strongly biased in favor of only one. Brichard et al., measuring cytotoxic activity of splenocytes after 7 d of in vitro culture, detected activity against the AB antigens in 50% of the mice, against CDE in 85% (6). There again, the response could be evenly distributed between the two types of effectors or strongly biased. Thus, similar conclusions can be reached with two independent sets of data obtained with very different techniques. The larger amount of responsive individuals in this report can be explained in several ways. First, the two techniques may differ in sensitivity. Second, T cells with the public TCR β chain in vivo may not always be specific for the A or E antigens, since their α chain is not known; however, given TCR diversity, this is extremely improbable. Nevertheless, some of these public T cells detected by PCR may be non-cytolytic (as discussed later). Third, immunization protocols are not exactly the same, but this is not the only reason, since we observed that the protocol used in (6) yielded too a 100% occurrence of public anti-E T cells (on 16 sam-

ples, not shown). Finally, it is possible that *in vitro* culture somehow modifies the response, perhaps increasing the initial bias between the clones.

Infiltration by T cells bearing these recurrent TCRs can be observed in growing as well as in regressing P815 tumors. In other terms, there is no obvious correlation between tumor rejection and the occurrence of the two public clones known to correspond to cells with a demonstrable specific cytolytic activity *in vitro*. Quantitatively, however, there is a significant difference between regressing tumors grafted onto preimmunized mice and tumors growing onto naive mice, if one considers the frequency of the public cells either in the tumor itself or in the peripheral organs. This, however, does not prove that the stronger proliferation of such cells is associated with tumor regression, since it is a usual feature of a secondary T cell response that it is stronger than the primary one. It does indeed validate, in the context of the anti-P815 response, a quantification tool already put to the test in other systems like the response to pigeon cytochrome C (16). Contrary to our expectations, our attempts to find a link between the amount of public T cells among PBL in a primary response and the future evolution of the tumor have so far failed to reveal any obvious correlation. Three interpretations can be proposed:

First, public cells may be elicited by the tumor cells, but have no relevance for tumor rejection *in vivo*, the cytotoxicity of corresponding CTL clones being mainly a consequence of *in vitro* culture conditions. Arguing against this possibility is the crucial role exerted by anti-P1A CTLs *in vivo*, evidenced by the emergence of a P1A-loss variant *in vivo* after nearly total tumor rejection (5). It remains possible that the anti-P1A CTLs active *in vivo* bear TCRs different from the public one, but it would be hardly explainable. The public cells may also constitute only a very small fraction of the anti-P815 CTLs *in vitro*, which would be subject to stochastic fluctuations. Although this is possible, in the one mouse analyzed by Brichard et al. for which this question can be answered, such cells constituted all the clones obtained (6).

A second hypothesis, frequently put forward to explain the growth of immunogenic tumors, is that CTLs are elicited normally by the tumor cells, but come too late, at too slow a division rate to cope with a fast-growing tumor. Although this may explain why public T cells are present in growing tumors, this hypothesis seems ruled out by our experiment in which the early population of public cells was compared to the tumor outcome (Fig. 5). Furthermore, previous data provide some evidence that other mechanisms take place at later stages. Indeed, although it may not be an accurate picture of the events taking place at the tumor site, it has been observed that P815-specific immunity in splenocytes increases in the first 10 d or so after inoculation of P815 in syngeneic hosts, then decreases as tumor growth proceeds (26, 27).

The third interpretation, which we favor, is that the biological activities of P815-specific T cells are different in mice bearing growing or regressive tumors. For instance, these cells, primed under optimal conditions during the

immunization step with P815-IL2, could be effector CTLs in rejected tumors. In growing tumors, their first encounter with their antigen in the absence of suitable costimulation and/or growth factors may have induced anergy, or even negative regulatory functions. We are currently testing these hypotheses.

A recent study from our group of the T cell response to hen egg white lysozyme (HEL) led us to this distinction between public (i.e., recurrent) and private (i.e., nonrecurrent and apparently stochastic) clones (3). We have been able to find a public response, characterized by the V β 1-J β 1.2 rearrangement, against a self protein, namely P1A, which has been shown to be a genuine, nonmutated self protein (8). The second rearrangement (V β 1-J β 2.5) involves the E antigen which has yet not been cloned.

In addition to the two public responses, are there private responses against P815, and more precisely against products of P1A? We have sequenced six *in vitro*-isolated CTL clones which are specific for a P1A-derived peptide, out of which two display the public rearrangement and four do not (Table 3). We searched the sequences of the latter with clonotypic primers in several TiL samples and did not find them (data not shown). These sequences are thus very likely to belong to private clones. The TiL are oligoclonal and their pattern is, for most of the peaks, nonrecurrent in different samples. Whether all nonrecurrent peaks correspond to private specificities or whether some (or all) result from some random, nonspecific local expansions, cannot be assessed without further experiments.

We propose to divide tumor T cell epitopes derived from self proteins into two categories: those which, like P815A or E antigens, elicit public responses, and those which elicit only private responses (like, maybe, P815C and D). A plausible reason for the absence of a public arm against the latter may be the fact that they are expressed at low levels in the thymus or in tissues normally accessible to T cells. This would fit with the observation that, in HEL-transgenic mice, the public, but not the private, arm of the anti-HEL T cell response is deleted when HEL is expressed at low levels (3). Similarly, the self antigens for which a public response has been observed so far are derived from proteins expressed only in tissues isolated from T cell circulation, like the testis for P1A (8, 9) or the central nervous system for the myelin basic protein (28).

Along this hypothesis, one can hope to find a public component in the response to human melanoma antigens derived from nonmutated self proteins like members of the MAGE family or BAGE, which have an expression pattern similar to P1A (i.e., in testis and placenta) (9, 29–31). Conversely, antigens derived from proteins like tyrosinase or melan-A, which are expressed by normal melanocytes (32, 33) might elicit only private, rather than public, T cell responses. We are currently searching public responses in human melanoma infiltrating lymphocytes.

We wondered whether the occurrence of public T cell responses would be more easily observable in a homozygous as opposed to a heterozygous MHC background, the latter being the usual rule in humans. This was not the case

since the two public anti-P815 clones were observed in all tested combinations. Furthermore, public T cell responses against viral antigens have been reported in humans (34–36). It is thus not unlikely that our speculations hold true in the human situation.

Conversely, no public anti-A or anti-E cells have been observed when P815 was grafted onto BALB/c mice. This is interesting in regard to the question whether tumor-specific responses may be elicited with semi-allogeneic tumors as APCs. Indeed, if differences at minor histocompatibility loci, as is the case in our experiment, induce a T cell response powerful enough to smother the response to tumor-specific antigens, it is likely that allogeneic responses would have the same outcome.

There are reasons to suspect that public T cell responses might be biologically and practically significant. Their re-

current character indicates that they have been strongly selected. Furthermore, in the HEL transgenic model, the deletion of public cells by low amounts of self protein suggests that they may be of high avidity (3). If this interpretation is proven a more general rule, it may influence future immunotherapy protocols in humans: first, public rearrangements, if found in humans sharing HLA alleles, might provide an easier and possibly predictable index for therapeutic purposes. Secondly, it might be desirable to elicit and/or expand (for ex vivo immunotherapy) high avidity T cell (CTL) clones, and the identification of public specificities might be a means to access and characterize at least some of them. We are actively investigating whether the above concepts may serve as a foundation for useful therapeutic principles.

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