Tumor Rejection Antigens and Immune Surveillance

B. Van den Eynde, B. Lethé, A. Van Pel, and T. Boon [1]

The existence of specific tumor rejection antigens was first demonstrated with chemically induced mouse sarcomas: each tumor was found to express a different antigen [1]. Similar findings were made with ultraviolet-induced tumors [2]. Later, the generality of the existence of tumor rejection antigens was questioned when spontaneous mouse tumors were found to be completely incapable of eliciting an immune rejection response [3]. However, further experiments demonstrated that even these tumors express weak transplantation antigens that are potential targets for immune rejection by the syngeneic host [4].

But what is the molecular nature of tumor rejection antigens? And what is the relation between their appearance and the tumoral transformation process? These questions are still unanswered because these antigens, which elicit strong T-cell mediated immune responses, do not stimulate B cells to produce antibodies. It has therefore been impossible to isolate the antigenic molecules by immunoprecipitation. Recently, we have developed a gene transfection approach aimed at identifying directly the genes that code for this type of antigen. It was applied to "tum$^-$" transplantation antigens, which arise on mouse tumor cells when they are treated with mutagenic agents, and to a tumor rejection antigen present on mouse mastocytoma P815.

Tum$^-$ Antigens

In *vitro* mutagen treatment of mouse tumor cells generates at high frequency stable immunogenic variants that are rejected by syngeneic mice [5]. Because of their failure to form tumors, these variants were named "tum$^-$" as opposed to the original "tum$^+$" cell, which produces progressive tumors. This phenomenon has been observed on a large number of mouse tumor cell lines of various types [6]. Most tum$^-$ variants express new transplantation antigens not found on the original tum$^+$ cell. The existence of these tum$^-$ antigens was first demonstrated by transplantation experiments [7]

We have studied a series of tum$^-$ variants derived from mastocytoma P815, a tumor induced in a DBA/2 mouse with methylcholanthrene. From clonal tum$^+$ line P1, we obtained more than 30 different tum$^-$ variants, which rarely produced progressive tumors even when they were injected at very large doses. When restimulated in vitro, spleen cells of mice that had rejected these variants produced cytolytic T cells (CTL) that lysed preferentially the immunizing tum$^-$ variant [8]. From these lymphocytes, we were able to isolate stable CTL clones [9]. Some of these appeared to be directed against a tumor rejection antigen of P815: they lysed P1 and all P815-derived cells but not syngeneic control tumors. Others lysed the immunizing tum$^-$ variant, but neither the original tum$^+$ cell

[1] Ludwig Institute for Cancer Research, Brussels Branch, 74 avenue Hippocrate UCL 7459, B-1200 Brussels, Belgium and Cellular Genetics Unit, University of Louvain, 74 avenue Hippocrate UCL 7459, B-1200 Brussels.

Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

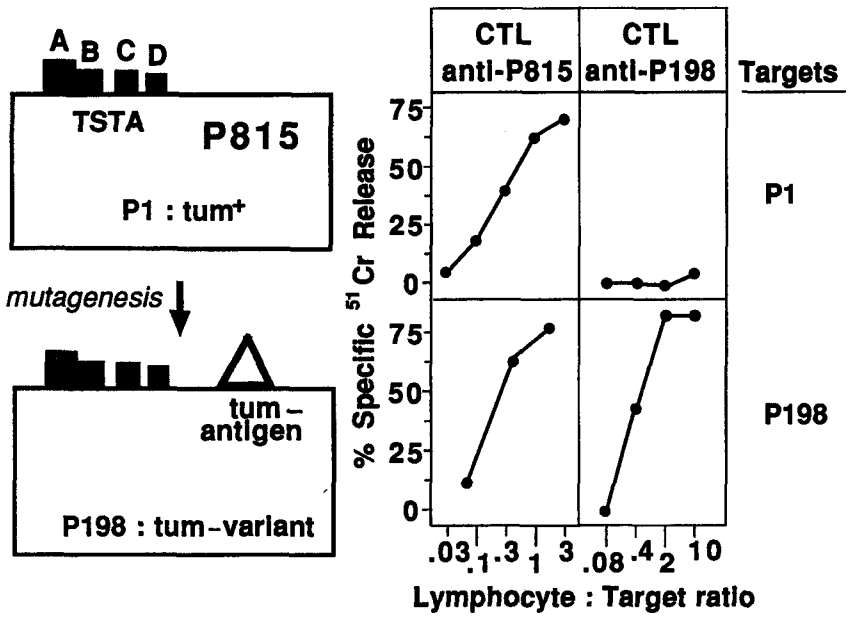

Fig. 1. Tumor rejection antigens and tum$^-$ antigen present on the original P815 line P1 and on tum$^-$ variant P198. Lysis by CTL clones directed against a tumor rejection antigen (anti-P815) or a tum$^-$ antigen (anti-P198)

nor the other tum$^-$ variants. They therefore defined new tum$^-$ antigens specific for each variant (Fig. 1). These antigens displayed considerable diversity: no antigen was found twice among 15 tum$^-$ variants that were analyzed. By in vitro immunoselection with anti-tum$^-$ CTL clones it was possible to demonstrate that some tum$^-$ variants carry several tum$^-$ antigens [10]. These experiments also demonstrated that the tum$^-$ antigens defined by CTL are relevant to the rejection of the variants, as shown by the correlation between the loss of these antigens and the reversal of the tum$^-$ phenotype [10, 11].

To find an explanation that could reconcile the remarkably high frequency of tum$^-$ variants with their stability and to understand the source of their diversity, it appeared essential to identify the antigenic molecules. We failed in our attempts to obtain antibodies directed against tum$^-$ antigens. Therefore, we undertook to clone directly the relevant genes on the basis of their ability to produce the antigens recognized by the anti-tum$^-$ CTL.

Cloning of Genes Encoding Tum$^-$ Antigens

The procedure that we developed for the cloning of the gene coding for tum$^-$ antigen P91A is based on gene transfection. It involves the use of a highly transfectable P815 cell line called P1. HTR [12] and the detection of antigen-expressing transfectants by their ability to stimulate CTLs [13]. By transfecting P1. HTR cells with a cosmid library prepared with the DNA of a cell expressing tum$^-$ antigen P91A, we obtained transfectants expressing this antigen at a frequency of 1 per 28000 [14]. By direct encapsidation of the DNA of these transfectants into lambda phage heads, we obtained a cosmid capable of transferring the expression of the antigen. An 800-bp restriction fragment from this cosmid was found to transfer the expression of the antigen. This fragment was then used to identify cosmids containing either the normal or the antigenic allele of the entire P91A gene as well as complementary (c)DNA clones of the homologous messenger RNA.

The procedure that led to the isolation of tum$^-$ gene P91A was applied with

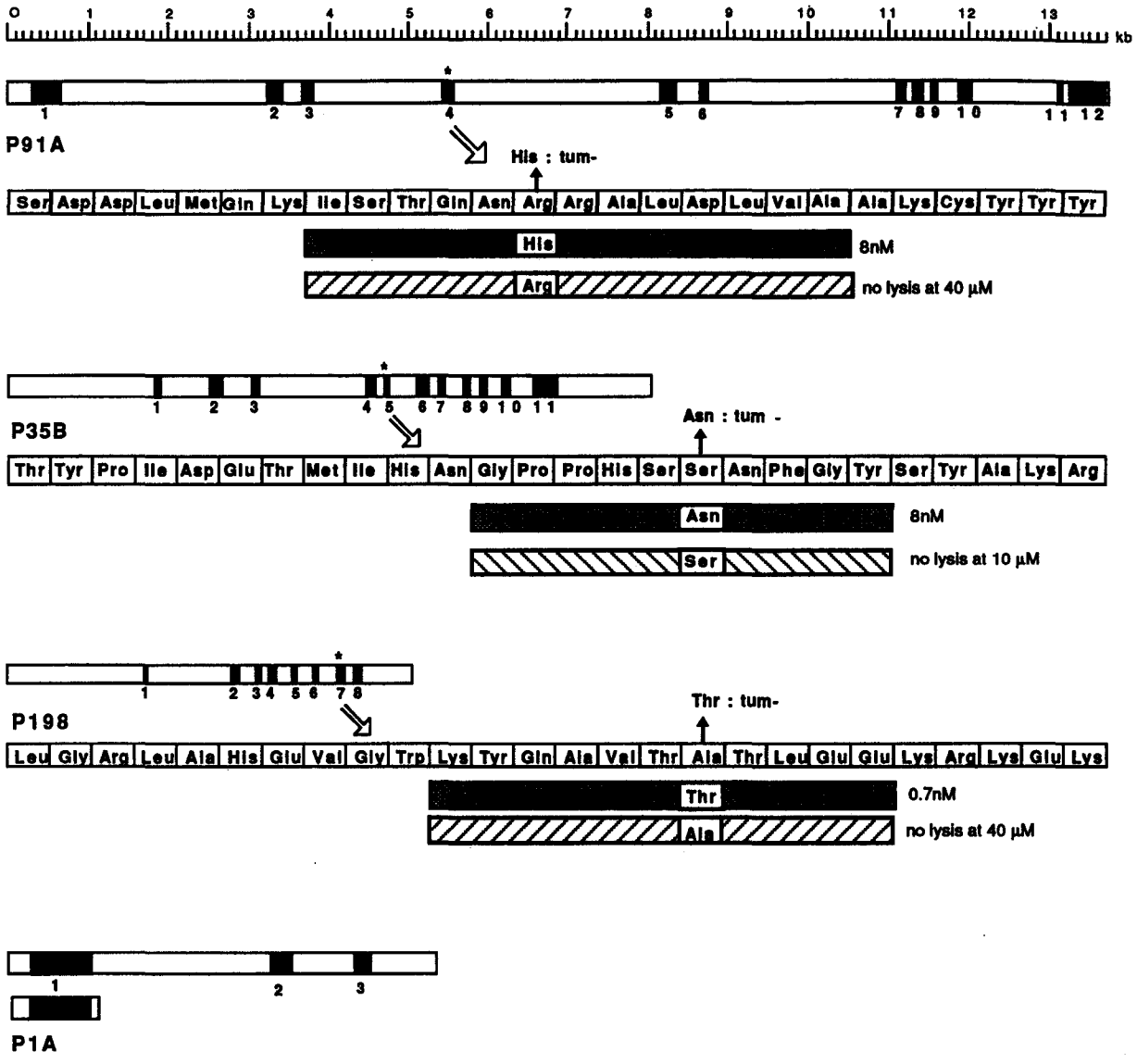

Fig. 2. Structure of genes P91A, P35B, P198, and P1A and antigenic peptides. *Dark regions* represent exons. The exon containing the tum$^-$ mutation is marked by an *asterisk*. Sections of the proteins located around the mutated amino acid are indicated. Synthetic peptides corresponding to the mutant and normal sequences of the genes are represented by *boxes*. They were tested for their ability to render P1. HTR cells susceptible to lysis by anti-tum$^-$ CTL. The concentration indicated to the right of each peptide provided 50% of the lysis obtained at saturating concentration of peptide. For P1A, the box indicates the subgenic fragment capable of transferring the expression of antigens P1A and P1B. The antigenic peptides for P1A and P1B are not yet identified

success to the cloning of tum$^-$ genes P35B and P198, which encode antigens expressed by other tum$^-$ variants derived from P815 [15, 16].

Tum$^-$ Mutations

Northern blots probed with the 800-bp fragment of gene P91A revealed a single messenger RNA species of 2.2 kb. The band was of equal intensity for tum$^-$ variant P91 and for P1, which does not express the antigen. The expression of antigen P91A is therefore not due to the activation of a silent gene.

The structure of gene P91A is shown in Fig. 2. It comprises 12 exons spread over 14 kb [17]. It does not show any similarity with Ig, T cell receptor or MHC genes. The complete sequence was obtained. It is unrelated to any sequence presently recorded in the main data banks.

A sequence comparison of the normal and tum$^-$ alleles of gene P91A indicated that they differ by a point mutation in the exon which is present in the transfecting 800-bp fragment (Fig. 2). This tum$^-$ mutation is a G to A transition that changes an arginine into a histidine in the main open reading frame of the gene [14]. This mutation appears to be the only difference distinguishing the normal from the antigenic allele.

The study of the tum$^-$ alleles of genes P35B and P198 also revealed that they differ from the normal alleles by a point mutation in an exon (Fig. 2). The general structures and the sequences of the three tum$^-$ genes isolated so far are completely unrelated.

Antigenic Peptides

The main open reading frame of gene P91A encodes a protein of 60 kDa, which does not have a typical N-terminal signal sequence [17]. In vitro translation experiments suggest that the two potential N-glycosylation sites present in the sequence are unused (Godelaine, Amar-Costesec, De Plaen, Beaufay, unpublished results). Antigen P91A is therefore unlikely to be borne by a membrane protein. This is however hardly surprising, considering the recent demonstration that CTL can recognize influenza antigens corresponding to endogenous proteins remaining inside the cell and considering the observation that CTL recognize small peptides that bind to surface class I MHC molecules [18–20]. On the basis of this evidence, we examined whether we could also identify a small peptide that would trigger the lysis of P815 cells by anti-P91A CTL. In our search for this peptide we were guided by the location of the tum$^-$ mutation. A short peptide (Fig. 2) corresponding to the mutant sequence induced the lysis of P1 by anti-P91A CTL. Transfection and peptide studies with H-2k fibroblasts, which expressed also either Kd, Dd or Ld, demonstrated that antigen P91A is associative with Ld. Antigenic peptides corresponding to the sequence surrounding the tum$^-$ mutation were also obtained for genes P35B and P198. They associate with Dd and Kd respectively.

Studies with P91A peptides enabled us to understand the role of the tum$^-$ mutation. A priori, the mutation could influence either the production of the antigenic peptide or its ability to associate with the Ld molecule (i.e., the aggretope) or also the epitope presented to T cells by the peptide-MHC complex. Having the antigenic P91A peptide, we prepared the homologous peptide corresponding to the normal allele of the gene. This normal peptide did not induce lysis by anti-P91A CTL, nor did it compete with the mutant peptide. Moreover, we found that the mutant peptide competed effectively to prevent a cytomegalovirus-derived peptide from inducing lysis by CTL directed against a Ld-associative cytomegalovirus antigen. The normal peptide did not compete [17] and we concluded that it does not bind to Ld. This indicates that the P91A tum$^-$ mutation generates the aggretope of the antigen, but does not exclude that it also influences the epitope. For antigen P198, the effect of the mutation appears to be different: here a new epitope is introduced on a normal peptide that is already capable of binding to the Kd presenting molecule.

Cloning of the Gene Encoding a Mouse Tumor Rejection Antigen

We have applied the same cloning procedure to the isolation of the gene coding for a tumor rejection antigen expressed by tumor P815 [21]. As opposed to the tum$^-$ antigens, these antigens are present on all P815 cells, whether they are mutagenized or not. The study of antigen-loss variants enabled us to identify four distinct antigens recognized by different syngeneic CTL clones. They were called P1A, B, C, and D (Fig. 1) [22]. Antigens P1A and P1B thus defined *in vitro* are

relevant *in vivo*, because P815 tumor cells that progressed in mice after nearly complete initial rejection were found to have lost the expression of one or both these antigens. Antigens P1A and P1B showed linkage, since several antigen-loss variants for P1A were found to have lost P1B concurrently.

For the transfection of antigen P1A, we used as recipient cell a $P1A^{-}$ B^{-} antigen-loss variant selected from line P1. HTR with an anti-P1A CTL clone. Transfectants expressing both antigens P1A and P1B were obtained with the genomic DNA of P1. HTR. This confirmed the close link between these two antigens. Transfectants were then obtained with a cosmid library made with the DNA of a genomic transfectant. By directly packaging the DNA of one of these cosmid transfectants, we obtained a cosmid that was able to transfect both antigens P1A and P1B.

The structure and the complete sequence of gene P1A were then obtained (Fig. 2). They proved completely different from those of the tum^{-} genes and of any known gene reported in data banks.

Transfection studies in H2-k fibroblasts previously transfected with either Kd, Dd, or Ld demonstrated that both P1A and P1B were presented to the CTL by the Ld molecule.

We compared the sequence of this gene, cloned from tumor cells, to the sequence of the equivalent gene cloned from normal cells of the same mouse strain. From a genomic library made with the DNA of normal DBA/2 mouse kidney we isolated the gene homologous to gene P1A. The analysis of this gene revealed that its sequence was identical to the sequence of the tumoral gene. To confirm this, we transfected this normal gene and found that it transferred the expression of antigens P1A and P1B as efficiently as the gene cloned from P815 cells (Fig. 3). The antigenicity is therefore not the result of a mutation in the tumoral gene, and P1A and P1B are presumably two different peptides derived from the same protein.

The tumor specificity of antigens P1A and P1B can nevertheless be partially explained by the pattern of expression of the gene. Northern blot analysis revealed

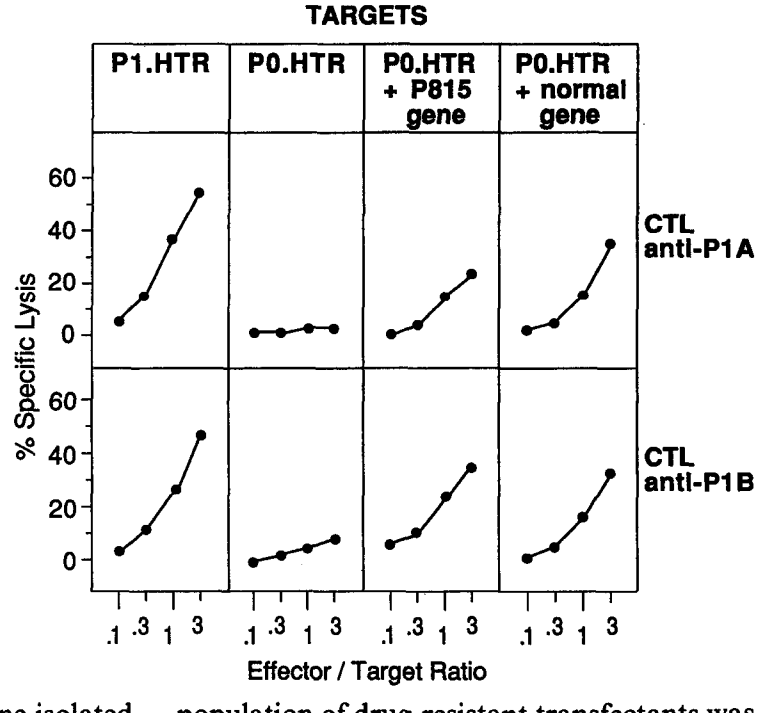

Fig. 3. Transfection of the P1A gene isolated from normal cells. The P1A gene isolated from a genomic library from normal DBA/2 mouse kidney was transfected in P0. HTR cells. The population of drug-resistant transfectants was tested with the anti-P1A and anti-P1B CTL clones

that the gene was silent in most normal tissues. However, one mast cell line (L138.8A) was found to strongly express messenger (m)RNA for P1A. This cell line, derived by Hültner et al. [28] from bone marrow of BALB/c mice, is cultivated in medium supplemented with interleukin 3. It grew as a permanent line and became spontaneously tumorigenic. Because BALB/c mice and DBA/2 mice express the same H2 haplotype, we were able to confirm the expression of gene P1A in L138.8A cells by lysis with the anti-P1A and anti-P1B CTL clones: we observed a significant lysis. Other nontransformed mast cell lines on the other hand were negative for P1A expression, so that we do not known whether the expression of the gene is associated to the mast cell lineage at a given stage of its differentiation, or whether it is related to the tumoral transformation. We failed to identify other tumor cell lines expressing mRNA for P1A.

Immune Surveillance, Tolerance, and Tumor Rejection Antigens

A first conclusion based on the results obtained with the tum$^-$ antigens is that mutations throughout all the mammalian genome generate at high efficiency antigenic peptides recognized by T cells, and that this mechanism could account for the presence of specific tumor rejection antigens on carcinogen-induced tumors.

However, the study of antigen P1A clearly showed that gene P1A is identical to its normal counterpart. The apparent tumor specificity of antigen P1A seems to be due to a specific regulation of the transcription of the gene rather than to a mutation generating an antigenic peptide. We now have to understand how the immune system may be sensitized against normal peptides to which is should be tolerant, and this without generating an obvious autoimmune pathology. Several hypotheses can be suggested. If the gene encodes an embryonic or oncofetal protein, then the antigen might have disappeared before the establishment of tolerance. If it codes for a differentiation or activation antigen, we can imagine that it is expressed very transiently by a small number of cells, so that tolerance does not develop and that an immune reaction directed against this antigen does not impair normal differentiation or activation. Lastly, if tolerance is actually present for P1A, then it must have been broken, and the simultaneous presence on the P815 tumor of other antigens like C and D may be important in that respect. These antigens could indeed be the result of a mutation and therefore be strongly immunogenic like tum$^-$ antigens. They could possibly trigger an immune response that would facilitate a response against P1A [4].

References

1. Prehn RT, Main JM (1957) Immunity to methylcholanthrene-induced sarcomas. INCI 18:769
2. Kripke MI (1981) Immunologic mechanism in UV radiation carcinogenesis. Adv Cancer Res 34:69
3. Hewitt HB, Blake ER, Walder AS (1976) A critique of the evidence for active host defense against cancer based on personal studies of 27 murine tumors of spontaneous origin. Br J Cancer 33:241
4. Van Pel A, Vessière F, Boon T (1983) Protection against two spontaneous mouse leukemias conferred by immunogenic variants obtained by mutagenesis. J Exp Med 157:1992
5. Boon T, Kellerman O (1977) Rejection by syngeneic mice of cell variants obtained by mutagenesis of a malignant teratocarcinoma cell line. Proc Natl Acad Sci USA 74:272
6. Frost P, Kerbel R, Bauer E, Tartamella-Biondo R, Cefalu W (1983) Mutagen treatments as a means for selecting immunogenic variants from otherwise poorly immunogenic malignant murine tumors. Cancer Res 43:125
7. Boon T, van Pel A (1978) Teratocarcinoma cell variants rejected by syngeneic mice: protection of mice immunized

with these variants against other variants and against the original malignant cell line. Proc Natl Acad Sci USA 75:1519
8. Boon T, van Snick J, van Pel A, Uyttenhove C, Marchand M (1980) Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815. T lymphocyte-mediated cytolysis. J Exp Med 152:1184
9. Maryanski JL, van Snick J, Cerottini JC, Boon T (1982) Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815. Clonal analysis of the syngeneic cytolytic T lymphocyte response. Eur J Immunol 12:401
10. Maryanski JL, Boon T (1982) Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815. Analysis of variants-specific antigens by selection of antigen-loss variants with cytolytic T-cell clones. Eur J Immunol 12:406
11. Maryanski J, Marchand M, Uyttenhove C, Boon T (1983) Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815. Occasional escape from host rejection due to antigen-loss secondary variants. Int J Cancer 31:119
12. Van Pel A, de Plaen E, Boon T (1985) Selection of highly transfectable variant from mouse mastocytoma P815. Somatic Cell Mol Genet 11:467
13. Wölfel T, van Pel A, de Plaen E, Lurquin C, Maryanski JL, Boon T (1987) Immunogenic (tum$^-$) variants obtained by mutagenesis of mouse mastocytoma P815. Detection of stable transfectants expressing a tum$^-$ antigen with a cytolytic T cell stimulation assay. Immunogenetics 26:178
14. De Plaen E, Lurquin C, van Pel A, Mariamé B, Szikora JP, Wölfel T, Sibille C, Chomez P, Boon T (1988) Immunogenic (tum$^-$) variants of mouse tumor P815: cloning of the gene of tum$^-$ antigen P91A and identification of the tum$^-$ mutation. Proc Natl Acad Sci USA 85:2274
15. Szikora JP, van Pel A, Brichard V, André M, van Baren N, Henry P, de Plaen E, Boon T (1990) Structure of the gene of tum$^-$ transplantation antigen P35B: presence of a point mutation in the antigenic allele. EMBO J 9:1041
16. Sibille C, Chomez P, Wildmann C, van Pel A, de Plaen E, Maryanski JL, de Bergeyck V, Boon T (1990) Structure of the gene of tum$^-$ transplantation antigen P198: a point mutation generates a new antigenic peptide. J Exp Med 172:35
17. Lurquin C, van Pel A, Mariamé B, de Plaen E, Szikora JP, Janssens C, Reddehase MJ, Lejeune J, Boon T (1989) Structure of the gene of tum$^-$ transplantation antigen P91A: the mutated exon encodes a peptide recognized with Ld by cytolytic T cells. Cell 58:293
18. Townsend ARM, Gotch FM, Davey J (1985) Cytotoxic T cells recognize fragments of the influenza nucleoprotein. Cell 42:457
19. Townsend ARM, Rothbard J, Gotch FM, Bahadur G, Wraith D, McMichael AJ (1986) The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell 44:959
20. Townsend ARM, Bastin J, Gould K, Brownlee GG (1986) Cytotoxic T lymphocytes recognize influenza haemagglutinin that lacks a signal sequence. Nature 324:575
21. Van den Eynde B, Lethé B, van Pel A, de Plaen E, Boon T (1991) The gene coding for the main tumor rejection antigen of mouse tumor P815 is identical to the normal gene of the syngeneic DBA/2 mice. J Exp Med 173:1373
22. Uyttenhove C, Maryanski J, Boon T (1983) Escape of mouse mastocytoma P815 after nearly complete rejection is due to antigen-loss variants rather than immunosuppression. J Exp Med 157:1040
23. Hültner L, Moeller J, Schmitt E, Jäger G, Reisbach G, Ring J, Dörmer P (1989) Thiol-sensitive mast cell lines derived from mouse bone marrow respond to a mast cell growth-enhancing activity different from both IL-3 and IL-4. J Immunol 142:3440