Brief Definitive Report

Suboptimal Activation of Melanoma Infiltrating Lymphocytes (TIL) due to Low Avidity of TCR/MHC-Tumor Peptide Interactions

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Summary

Coculture of melanoma cells and T cell clones derived from tumor-infiltrating lymphocytes (TIL) generally results in lysis of the antigen-bearing tumor cells but to inefficient proliferation and IL-2 secretion by responder T cells. This suboptimal activation is classically explained by an inability of tumor cells to provide costimulatory signals. Here we analyzed the responses to synthetic peptides of HLA-A2.1-restricted CTL clones specific for melanoma antigens MART-1 and NA17-A. We showed that peptide concentrations ranging from 1 pM to 10 nM efficiently sensitized the peptide transporter-deficient T2 cells to lysis. T2 cells pulsed with melanoma peptides also induced TIL proliferation and detectable secretion of IL-2, IFN-γ and GM-CSF, but only for peptide concentrations 10- to 10,000-fold higher than those required for lysis. Hence this suggests that partial triggering of TIL clones by melanoma cells could be due to expression of appropriate MHC-peptide complexes at subthreshold levels. In support of this, we showed that melanoma cells, unable to trigger IL-2 secretion, developed this ability when incubated with the appropriate peptide. These results indicate that the level of antigens expressed on melanoma tumors critically affects TIL activation status and thus, the efficiency of specific immune reactions mediated by these cells.

A number of melanoma-derived peptides, recognized in vitro by autologous T lymphocytes, have been identified recently (1). Little is known, however, about the capacity of these epitopes to trigger efficient T cell activation and immune reactions in vivo. It is widely assumed that T cell activation requires two signals. Signal one originates from the ligation of the T cell receptor and its coreceptors. Signal two, which is not antigen specific, is generated by either soluble factors such as IL-2 or by interactions with surface molecules expressed by antigen presenting cells (see reference 2 for a review). The best characterized costimulatory signal for T lymphocytes is mediated by CD28-B7 interactions (see reference 3 for a review). Classically, T cells that bind antigen but do not receive a costimulatory signal become anergic. Since melanoma cells, as most nonhemopoietic tumors, do not express costimulatory molecule, such as B7-1 or B7-2, it has been speculated that they could anergize tumor-specific TIL[1] by delivering an antigenic signal only (3). To address this issue we have derived a number of melanoma TIL clones and extensively analyzed their reactivity against tumor cells in vitro. We reported recently that most melanoma infiltrating T cell clones (TIL) stimulated by melanoma cells efficiently killed these cells and secreted TNF, but few of them produced detectable levels of IL-2 and IFN-γ (4). However, suggesting that defective costimulation was not the main reason for partial TIL triggering, we had shown that melanoma cells induced to express B7-1 by gene transfection, did not trigger either a full TIL activation (5). This did not appear to be due to a functional defect of TIL clones as they secreted large amounts of IL-2 following CD3 cross-linking (4). Furthermore, melanoma lines unable to stimulate IL-2 secretion by TIL efficiently triggered this response once induced to express high levels of LFA-3, after transfection (reference 4 and unpublished data). Together these data suggested that defective TIL activation by melanoma lines was due to delivery of a suboptimal antigenic signal. This led us to investigate whether the nature, agonist or partial agonist, or the density of melanoma peptides expressed on tumor cells could be responsible for partial TIL activation. To address this, we analyzed the ability of HLA-A2.1-restricted epitopes from melanoma antigens, MART-1/Melan-A (6–8) and NA17-A (9) to induce secretion of IL-2, IFN-γ, GM-CSF, and TNF by specific TIL clones.

[1] *Abbreviation used in this paper:* TIL, tumor infiltrating lymphocytes.

Drs. Gervois and Guilloux contributed equally to this work.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/05/2403/05 $2.00
Volume 183 May 1996 2403–2407

We measured the various responses of TIL clones to increasing amounts of their specific peptides, presented by the (TAP-) peptide transporter-deficient T2 cell line or by melanoma cells. We show that human melanoma peptides recognized by TIL are full agonists and that hyporesponsiveness of MART-1- or NA17-A-specific TIL clones to melanoma cells in vitro is not due to a defective TIL function but to expression of suboptimal levels of specific MHC-peptide complexes on melanoma cells in vitro. Relevance of these data to melanoma vaccine development are discussed.

Materials and Methods

Cell Lines. Tumor cell line M17 was derived from the metastatic melanoma of patient M17. The human mutant cell line CEMx721.174.T2 (T2) (10) used as presenting cells and the mouse fibrosarcoma WEHI 164 clone 13 cells, used for TNF assays, were obtained from Dr. P. Coulie (Ludwig Institute for Cancer Research, Brussels, Belgium). All these cell lines are cultured in RPMI containing 10% FCS.

Generation of Melanoma-specific TIL Clones. Production, characterization and culture of human TIL derived clones have been described in detail previously (11). Specificity and restriction were investigated using various functional assays including proliferation, cytolysis, and lymphokine secretion (4, 5).

Synthetic Peptides. Peptides were synthetized on solid phase using F-moc for transient NH_2-terminal protection as described (12), and were characterized by mass spectrometry. Purity was controlled by analytic HPLC. Lyophilized peptides were dissolved at 10 mg/ml in DMSO and stored at −80°C.

^{51}Chromium Microcytotoxicity Assay. 1,000 ^{51}Cr-labeled target cells (T2 or melanoma cells) were incubated for 30 min at room temperature with various concentrations of peptide before addition of 10,000 lymphocytes. After 4 h of incubation at 37°C, 100 μl of supernatant were harvested and radioactivity was determined in a gamma counter.

Cytokine Production Assays. At least 10 d after the last stimulation, T cells were washed thoroughly. 10^4 T lymphocytes were mixed with 3×10^4 target cells pulsed with different concentrations of peptide, in 200 μl of culture medium. Control assays were done in which the lymphocytes were incubated with peptides in the absence of target cells. Supernatants were harvested 24 h later. The IL-2 dosage was done using the CTLL-2 cell line proliferation assay, compared to a standard curve with rIL-2 (Cetus). No IL-2 production was detected in the absence of target cells (data not shown), showing that T lymphocytes did not efficiently present these peptides. TNF determinations were done by a biologic assay using cytotoxicity on the highly sensitive WEHI 164 clone 13 cells, compared to a standard curve with rTNF-β (Genzyme Corp., Cambridge, MA) (13). IFN-γ and GM-CSF concentrations were assessed by immunoenzymatic assays (Medgenix Diagnostics and Immunotech International): limits of detection were 0.03 IU/ml and 1.5 pg/ml, respectively. The relative peptide index is calculated by dividing peptide concentrations inducing half maximum responses (lysis, TNF, IFN-γ and GM-CSF secretion) by the concentration inducing, in the same experiment, half maximum IL-2 secretion.

Proliferation Assays. Microcultures were set up in triplicate in 96-well culture plates. Briefly, 10^4 T cells were cultured for 3 d with 3×10^4 irradiated peptide-pulsed melanoma cells in 200 μl of medium without IL-2. Proliferation was determined by adding 1 μCi of ^{3}H thymidine (3HT) to each well for the last 18 h of culture. 3HT uptake was measured in a liquid scintillation counter.

Results and Discussion

Lytic and Cytokine Responses of TIL Clones Are Triggered by Peptide-loaded T2 Cells. CTL clones used here have been described elsewhere (11). They were derived from two melanoma tumors M17 and M77. They all recognized their epitope in the HLA-A2.1 context. Clone M17.2 was shown to recognize a peptide shared by a high proportion of melanoma lines (14). Recently the antigen recognized by this CTL clone was identified and called NA17-A. Expression of this antigen was shown to be restricted essentially to melanoma tumors (9). Only two overlapping peptidic sequences deduced from NA17-A gene sequence, Ac1-9 and 1-10 were recognized, at about the same levels, by clone M17.2 (9). MART-1-specific clones, M77.80, 84, 86 and 25, were shown to be distinct by CDR3 sequencing (Pannetier, C., unpublished). Analysis of their reactivity to MART-1 sequences, Ac27-35, 26-35, 27-36, and 32-40 previously reported to be the HLA-A2.1 restricted epitopes recognized by TIL (8) revealed that all clones recognized MART-1 Ac26-35 and/or 27-35. Whereas clone M77-80 responded at about the same level to both epitopes, the other three clones (84, 86, and 25) responded much better to the decamer Ac26-35 (our unpublished data). Therefore, one or both of these peptides probably corresponded to the naturally presented epitope on HLA-A2.1-expressing melanoma cells. The T2 cell line was used to titrate the responses of TIL clones to MART-1 and NA17-A peptides. As shown in Fig. 1, peptide concentrations between 0.6 pM and 10 nM efficiently sensitized T2 cells to lysis by TIL clones. These concentrations are close to or lower than commonly described stimulatory concentrations of T cell epitopes recognized by high affinity TCR (15). These peptides also induced IL-2 secretion but at concentrations fifteen to ten thousand-fold higher than those inducing lytic activity, (Fig. 1). This difference is unlikely to be due to different costimulation requirements for these two T cell functions as it was observed in the context of peptide presentation by B7 expressing APC (T2 cells [Fig. 1] and B7-1 transfected melanoma cells [data not shown]). Similar differences between peptide concentrations required for IFN-γ and lysis triggering was also observed recently using influenza-specific CTL clones (Valitutti, S., personal communication). Together these data would suggest that many CTL clones require a higher antigen density for significant lymphokine release than for cytolysis.

TNF, IFN-γ and GM-CSF secretion were also measured. Since all these responses were not simultaneously analyzed, data are expressed as a relative peptide index (as defined in Material and Methods). Data in Table 1 confirmed differences in peptide concentrations required to induce lysis and IL-2 by NA17-A and MART-1-specific T cell clones. Furthermore, peptide concentrations needed to induce IFN-γ and GM-CSF secretion by these clones were

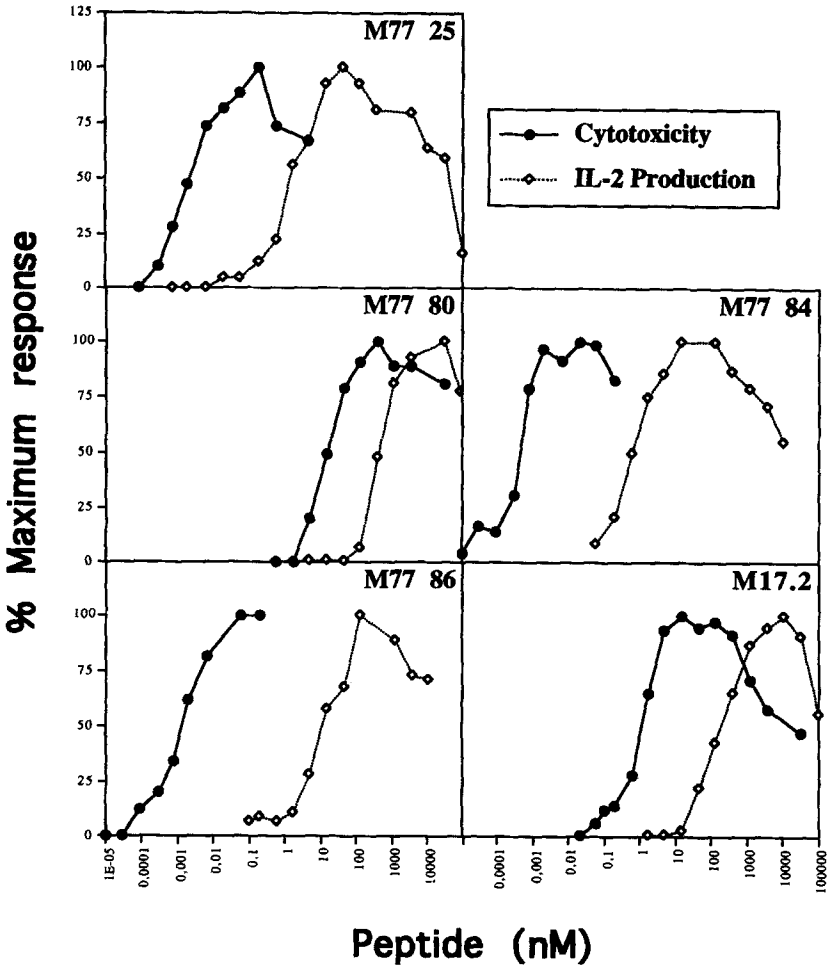

Figure 1. Lytic and IL-2 responses of four MART-1-specific clones (M77) and one NA17-A-specific clone (M17.2) induced in the same stimulation experiment by the optimal melanoma peptide presented by T2 cells.

Table 1. *Relative Concentrations of MART-1 and NA17-A Peptides Required to Activate the Different Functions of Two Specific CTL Clones**

Peptides	Cytotoxicity	IL-2	TNF	IFNγ	GM-CSF
MART-1					
expt 1	0.15‡	1	1.1		
expt 2		1		1.9	
expt 3	0.03	1	1.4		0.53
expt 4		1	0.37		
NA17-A					
expt 1	0.005	1	0.08	1.7	
expt 2	0.005	1			
expt 3		1			0.15
expt 4	0.15	1	0.02		

*Responses to MART-1 and NA17-A are those from clone M77.80 and M17.2, respectively.

‡Data are expressed as the relative peptide index (RPI) required to induce lysis or lymphokine secretion, compared to the IL-2 response. RPI is calculated by dividing the concentration of peptide inducing 50% of maximal lysis or lymphokine production by the concentration of peptide inducing 50% IL-2 production. IL-2 and TNF productions were determined using biological assays with the IL-2-dependent cell line CTLL2 and the TNF susceptible cell line WEHI 164 clone 13. IFNγ and GM-CSF measurements were done with imunoenzymatic tests.

in the same range than those required for IL-2 secretion. This was also the case for the induction of TNF secretion by MART-1-specific clones whereas concentrations of NA17-A peptide inducing IL-2-were significantly higher than those inducing TNF secretion by the specific clone.

Data reported here established that melanoma peptides are true agonists, since they efficiently stimulated all the CTL clone functions.

Melanoma Cells Unable to Trigger IL-2 Secretion by TIL Clones Acquire This Capacity Once Loaded with the Proper Peptide. Although a few melanoma lines expressing NA17-A and MART-1 antigens (either autologous or allogeneic) induced a full activation (i.e., IL-2 secretion and proliferation) of specific TIL clones in vitro (4, 5, and unpublished data), many of them induced only lysis and TNF secretion by responding cells. This was typically the case for the M17 melanoma cell line which was recognized by both MART-1

and NA17-A-specific clones (data not shown). In behalf of data obtained with T2 cells suggesting requirement for higher avidity TCR interactions to activate IL-2 secretion, we hypothesized that some melanoma cells failed to trigger full TIL activation because they did not express a sufficient density of antigenic peptides. To address this directly, we studied the effect of incubating M17 melanoma cells with antigen-derived synthetic peptides on IL-2 secretion by tumor-specific T cells. As shown on Figs. 2 and 3, M17 melanoma cells pulsed with NA17-A and MART-1 peptides stimulated both TIL proliferation and IL-2 secretion by TIL clones. Therefore preincubation of tumor cells with adequate concentrations of tumor peptides rendered these cells capable to fully activate specific TIL clones. Although, in the case of MART-1-specific T cell clones, it is difficult to formally prove that this result was not due to differences between MART-1 peptides naturally processed by M17 melanoma cells and the synthetic peptides used here, such an explanation could be ruled out for the NA17-A response, since transfection of the cDNA coding for the NA17-A peptide into M17 and other HLA-A2.1+ melanoma lines led to efficient triggering of IL-2 secretion by TIL responders otherwise incapable of this response (data not shown). Therefore, our data strongly suggest that partial activation of TIL by antigen-presenting melanoma cells resulted from low expression levels of MHC-melanoma peptide complexes.

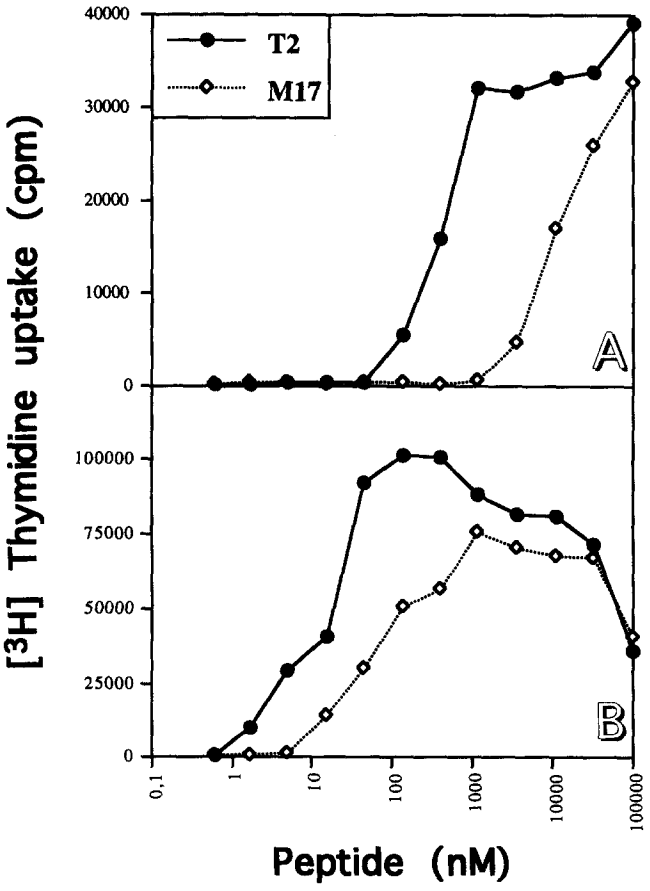

Figure 2. IL-2 secretion by a MART-1-specific clone, M77.80 (*A*) and a NA17-A-specific clone, M17.2 (*B*) induced by various concentrations of the optimal peptide presented by M17 melanoma cells or T2 cells.

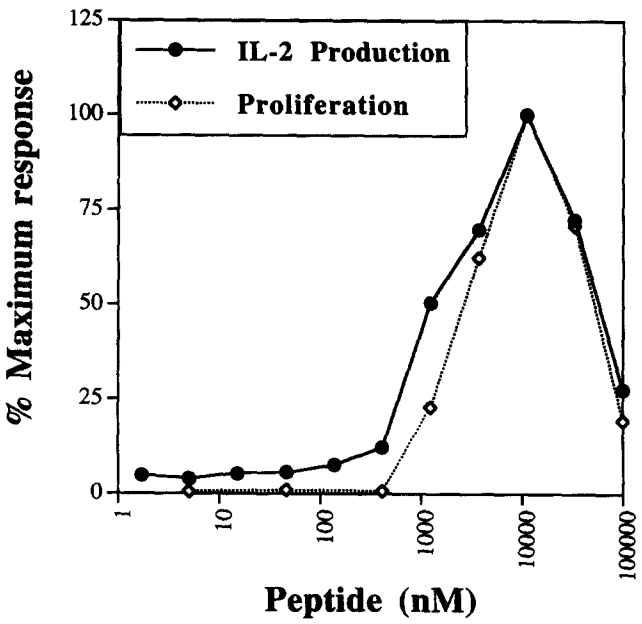

Figure 3. IL-2 secretion and proliferation of the NA17-A-specific clone to M17 melanoma cells pulsed with the appropriate peptide.

All CD8+ TIL clones secreted large amounts of IL-2 and IFN-γ when stimulated by optimal concentrations of the antigenic peptides. Since IL-2 secretion is not a frequent property of cytotoxic T lymphocytes in other situations, this suggests that intratumor T cell priming by melanoma antigens favors the expansion of CD8 clones with a good IL-2 secretion potential and expressing high affinity TCR. The IL-2 amounts produced by TIL clones used here was sufficient to induce a significant autocrine proliferation in vitro. This suggests that the expression, even transient, of sufficient levels of melanoma antigens in vivo by either tumor cells or antigen presenting cells, could trigger the expansion of specific IL-2-producing CTL clones carrying high affinity TCR and activated in an autocrine fashion. Data presented here further showed that despite lack of B7 molecules, melanoma cells efficiently activated presensitized CTL clones, provided that they expressed antigens at high enough levels. This does not support the widely accepted model according to which TIL are anergized following recognition of antigen expressing tumor cells in the absence of costimulation (3). Together our data would rather suggest that suboptimal TIL activation by tumor cells could result, at least in part, from a too low expression of the corresponding melanoma antigens, by at least a fraction of the tumor cells.

The present study stresses that lymphokine responses of specific T cells to melanoma antigens, which likely determines the efficiency of the antitumor response, critically depends on MHC-peptide density on target cells. This suggests that the efficiency of immunization protocols with

CTL epitopes, will tightly depend on the expression levels of the corresponding antigen on tumor cells. We are currently analyzing whether the levels of MART-1 and NA17-A expression by melanoma lines, measured by semi-quantitative RT/PCR, correlates with the capacity of these cells to induce IL-2 and IFN-γ secretion. If so, only melanoma patients expressing antigens on their tumor cells above a threshold that remains to be defined could be eligible for antigen-specific immunization.

We thank Marc Bonneville for critical discussion of the manuscript.

This work was supported by funds from the Institut National de la Santé et de la Recherche Médicale, by grant no. 6494 from the Association pour la Recherche contre le Cancer and funds from the Ligue Nationale et Ligue de Loire-Atlantique contre le Cancer, and from GEFLUC.

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Received for publication 15 January 1996 and in revised form 27 February 1996.

References

1. Boon, T., T.F. Gajewski, and P.G. Coulie. 1995. From defined human tumor antigens to effective immunization? *Immunol. Today.* 16:334–336.
2. Bluestone, J. 1995. New perspectives of CD28-B7-mediated T cell costimulation. *Immunity.* 2:555–559.
3. June, C.H., J.A. Bluestone, L.M. Nadler, and C.B. Thompson. 1994. The B7 and CD28 receptor families. *Immunol. Today.* 15:321–331.
4. Guilloux, Y., C. Viret, N. Gervois, E. Le Dréan, M.C. Pandolfino, E. Diez, and F. Jotereau. 1994. Defective lymphokine production by most CD8+ and CD4+ tumor-specific T cell clones derived from human melanoma-infiltrating lymphocytes in response to autologous tumor cells in vitro. *Eur. J. Immunol.* 24:1966–1973.
5. Viret, C., N. Gervois, Y. Guilloux, E. Le Drean, E. Diez, and F. Jotereau. 1995. T cell activation by antigens on melanoma cells 1- costimulation by B7-1 is neither sufficient nor necessary to stimulate IL-2 secretion by melanoma-specific T cell clones in vitro. *Int. Immunol.* 7:1535–1543.
6. Coulie, P.G., V. Brichard, A. Van Pel, T. Wölfel, J. Schneider, C. Traversari, E. De Plaen, C. Lurquin, J.-P. Szikora, J.-C. Renauld, and T. Boon. 1994. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 180:35–42.
7. Kawakami, Y., S. Eliyahu, C.H. Delgado, P.F. Robbins, L. Rivoltini, S.L. Topalian, T. Miki, and S.A. Rosenberg. 1994. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc. Natl. Acad. Sci. USA.* 91:3515–3519.
8. Kawakami, Y., S. Eliyahu, K. Sakaguchi, P.F. Robbins, L. Rivoltini, J.R. Yannelli, E. Appella, and S.A. Rosenberg. 1994. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J. Exp. Med.* 180:347–352.
9. Guilloux, Y., S. Lucas, V. Brichard, A. Van Pel, C. Viret, E. De Plaen, F. Brasseur, B. Lethé, F. Jotereau, and T. Boon. A peptide recognized by human cytolytic T lymphocytes on HLA-A2 melanomas is encoded by an intron sequence of the N-Acetylglucosaminyltransferase V gene. *J. Exp. Med.* 183: 1173–1183.
10. Salter, R.D., D.N. Howell, and P. Cresswell. 1985. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics.* 21:235–246.
11. Pandolfino, M.C., C. Viret, N. Gervois, Y. Guilloux, F. Davodeau, E. Diez, and F. Jotereau. 1992. Specificity, T cell receptor diversity and activation requirement of CD4+ and CD8+ derived from human melanoma-infiltrating lymphocytes. *Eur. J. Immunol.* 22:1795–1802.
12. Atherton, E., C.J. Logan, and R.C. Sheppard. 1981. Peptide synthesis. Part 2. Procedures for solid phase synthesis using Nα-fluorenylmethysoxycarbamylamino-acid on polymide supports. Synthesis of substance P and of acyl carrier protein 65-74 decapeptide. *J. Chem. Soc. Lond. Perkin Trans.* 1:538.
13. Traversari, C., P. Van der Bruggen, B. Van den Eynde, P. Hainaut, C. Lemoine, N. Otha, L. Old, and T. Boon. 1992. Transfection and expression of a gene coding for a human melanoma antigen recognized by autologous cytolytic T lymphocytes. *Immunogenetics.* 35:145–152.
14. Viret, C., F. Davodeau, Y. Guilloux, J.D. Bignon, G. Semana, R. Breathnach, and F. Jotereau. 1993. Recognition of shared melanoma antigen by HLA-A2-restricted cytolytic T cell clones derived from human tumor-infiltrating lymphocytes. *Eur. J. Immunol.* 23:141–146.
15. Sykuly, Y., A. Brunmark, T. Tsomides, S. Kageyama, M. Jackson, P.A. Peterson, and H. Eisen. 1994. High-affinity reactions between antigen-specific T-cell receptors and peptides associated with allogeneic and syngeneic major histocompatibility complex class I proteins. *Proc. Natl. Acad. Sci. USA.* 91:1187–1191.