Tumor antigens presented by major histocompatibility complex (MHC) class I molecules and recognized by CD8+ cytotoxic T lymphocytes (CTLs) may generate an efficient antitumor immune response after appropriate immunization. Antigenic peptides can be used in vivo to induce antitumor or antiviral immunity. The efficiency of naked peptides may be greatly limited by their degradation in the biological fluids. We present a rational, structure-based approach to design structurally modified, peptidase-resistant and biologically active analogues of human tumor antigen MAGE-1.A1. This approach is based on our understanding of the peptide interaction with the MHC and the T cell receptor and its precise degradation pathway. Knowledge of these mechanisms led to the design of a non-natural, minimally modified analogue of MAGE-1.A1, [Aib5,NMe-ser]MAGE-1.A1, which was highly peptidase-resistant and bound to MHC and activated MAGE-1.A1-specific anti-melanoma CTLs. Thus, we showed that it is possible to structurally modify peptide epitopes to obtain analogues that are still specifically recognized by CTLs. Such analogues may represent interesting leads for antitumor synthetic vaccines.

However, the results obtained with such peptide vaccines have been variable, dependent on a number of factors (6). Successful immunization was obtained after vaccination with MUT1 and MUT2 antigens expressed on murine carcinoma with regression of established metastases (8). Tumor regression was also observed after vaccination with a synthetic peptide with enhanced MHC binding derived from human melanoma antigen gp100 (9). Protective immune responses were obtained against viruses after peptide vaccination (10), but in other models peptide vaccines gave limited or no results (7, 11, 12).

Indeed, the major factor limiting the efficiency of peptide vaccines in vivo is, in most cases, their rapid degradation in serum and other biological fluids (13, 14). Peptides presented naturally by MHC class I molecules after intracellular processing of endogenous proteins were shown to be protected, by the MHC, against further protease degradation (15, 16). When exogenous peptides are used for vaccination, they may be degraded by extracellular proteases before reaching, and binding to, MHC molecules. Presentation of exogenous peptides by MHC molecules might be altered by the presence of serum proteases (14, 17, 18). A correlation was found between peptide stability in vitro in serum and peptide persistence in vivo, in ascorbic acid as well as on the surface of cells presenting the peptide (18). The stability of peptides in biological media must therefore be taken into account in the design of synthetic peptide vaccines, and the development of non-natural peptide antigens may be of great interest. However, the major difficulty in the design of such modified peptides lies in the fact that the structural modifications introduced must not induce a loss in the antigenicity and immunogenicity of the peptide. Indeed, the activity of an antigenic peptide depends upon two critical steps: (i) its presentation by the MHC, determined by the anchor positions (19–21) and (ii) the recognition of the peptide-MHC complex by a specific T cell receptor (TcR) (22, 23). These two mechanisms depend very closely on the structure of the antigen (24). Examples of non-natural (25–29) or altered peptides (30) that specifically bind MHC molecules have been described. Many of these peptides were MHC blockers and did not therefore activate CTLs (25, 28). In the case of cancer vaccines, peptide analogues should not only bind to MHC but must also activate specific antitumor CTLs.

In this report, we present the rational design of a non-natural, peptidase-resistant, and antigenic analogue of human tumor antigen MAGE-1.A1. Encoded by the MAGE-1 gene expressed in 40% of melanomas but not in normal tissues (testis excepted) (2), MAGE-1.A1 (EADPTGHSY) is presented by the MHC class I molecule HLA-A1 (3). Its anchor positions are Asp (D)3 and Tyr (Y)9 (31). The residues of MAGE-1.A1 recognized by the different anti-MAGE-1.A1 CTL clones are located at P1 and in the P4–P8 domain (32). Analysis of the structural parameters involved in the degradation mechanism in serum of
MAGE-1.A1 and in its interactions with the MHC on one side and the TcR on the other allowed us to design stable and biologically active non natural MAGE-1.A1 analogues.

**Experimental Procedures**

**Peptide Synthesis**—Peptides were synthesized by the solid-phase method using Fmoc chemistry. Fmoc aa derivatives were purchased from Bachem, France. The non natural N- or α-methylated aa derivatives were coupled using O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate instead of 2-(1H-benzo triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate used for natural aa derivatives. Peptides were purified by high pressure liquid chromatography (HPLC) on reversed-phase columns (RP300-C8 Brownlee Lab and an Applied Biosystems 130 apparatus). The purity of all peptides was >98%. The identity of the purified peptides was confirmed by electrospray ionization-mass spectrometry (ESI-MS) analysis. Stock solutions (2 × 10⁻³ M in H2O) were prepared and stored at −20 °C.

Human Cell Lines and CTL Clones—The TAP⁺ BM36.1 (45) and the TAP⁺ BM21 EBV-transformed lymphomas (HLA-A1, B35, C4w), were used in MHC stabilization experiments and CTL assays, respectively. They were grown in RPMI medium supplemented with 8% heat-inactivated fetal calf serum and antibiotics. Melanoma cell line MZ2-MEL was maintained in RPMI medium supplemented with 8% heat-inactivated fetal calf serum and antibiotics. Melanoma cell line MZ2-MEL was activated fetal calf serum and antibiotics. Melanoma cell line MZ2-MEL was grown in RPMI medium supplemented with 8% heat-inactivated fetal calf serum and antibiotics. Melanoma cell line MZ2-MEL was used in MHC stabilization experiments and CTL assays, respectively.

**Peptide Degradation Assay**—Peptides were added to preheated (15 min at 37 °C before the assay) human serum to a final concentration of 10⁻³ M and incubated at 37 °C. Human sera were obtained by centrifugation of all 10 ml of human serum, repeated three times. Target cells were then incubated in V-bottom microwells (10⁵ cells in 50 μl) in the presence of different concentrations of peptide (50 μl) for 30 min at 37 °C. CTLs were then added at an effectortarget ratio of 10:1 (10⁵ cells in 100 μl), and ⁵¹Cr release was measured after 4 h at 37 °C.

**Molecular Modeling of HLA-A1-Peptide Interactions**—HLA-A1 (a1 and a2 domains) structure was obtained by modeling the crystallographic data of the HLA-A2 3D structure (48) (Brookhaven Protein Data Bank, Brookhaven National Laboratory, Upton, NY), both molecules sharing more than 85% sequence homology in their peptide binding grooves. The HLA-A2/Tax nonamer to HLA-A1/MAGE-1.A1 mutations were introduced manually using the program g. Side chain conformation was based on the most probable rotamer and the probable heavy chain conformation was taken as that of the corresponding MHC alone. Additional minimization, 1000 iterations without constraints using Steepest Descent minimization, was performed after transfer of HLA-A1/MAGE-1.A1 data to insightII (Biosym Technologies), and further studies were performed using insightII. MAGE-1.A1 residues at P2 and P8 were then substituted by their non-natural analogues using HOMOLOGY (for η-isomers and Aib) and BIOPOLYMER (for NMe-aa) modules of insightII (Biosym Technologies) followed by minimization using VA09A (500 iterations without constraints) and STEEPEST DECENT (1000 iterations without constraints) algorithms.

**RESULTS**

**MAGE-1.A1 Is Rapidly Degraded in Human Serum in Vitro by Amino- and Dipeptidylcarboxypeptidases**—MAGE-1.A1 degradation fragments generated at 37 °C were analyzed by online HPLC/MS. UV analysis (Fig. 1, panel a) facilitated the quantification and determination of the kinetics for the appearance and disappearance of each peak (peaks A–F). In the experimental conditions used, the MAGE-1.A1 half-life (incubation time necessary for the degradation to half of the initial quantity of peptide) was less than 30 min (Fig. 2, panel a). MS analysis (Fig. 1, panel b) of each peak facilitated the determination of the corresponding MAGE-1.A1 degradation fragment. The mass spectrum of peak D (m/z = 269) is given as an example in Fig. 1b (inset). In the MAGE-1.A1 sequence, only the fragment corresponding to a m/z value of 269 is the dipeptide SY. Similar analysis of peaks A–F led to the identification of all MAGE-1.A1 degradation fragments (Table I). The kinetics studies (Fig. 2, panels a, e, and i) allowed us to establish the degradation pathway of MAGE-1.A1 (Fig. 1c); MAGE-1.A1 was sensitive to two exopeptidase activities, aminopeptidase and dipeptidyl carboxypeptidase (or angiotensin-converting enzyme, ACE). The major pathway was initiated by two successive aminopeptidase cleavages, leading to the dipeptide DPTGH (peak E), then cleaved by ACE to give the pentapeptide DPTGHS (peak F), and the dipeptide SY (peak D) degraded itself to amino acids). Direct action of ACE on MAGE-1.A1 represented a minor degradation pathway.

**Point Amino Acid Modifications Can Confer Peptidase Resistance to MAGE-1.A1 Analogues**—Series of MAGE-1.A1 analogues modified at positions engaged in one or both of the peptidase-sensitive bonds Glu³Ala⁵ and His⁹Ser⁵ were synthesized and then studied for their degradation properties (Table II). Peptides substituted at either position 1 or 2 (analogues 1 to 5) showed increased half-lives but this effect remained poorly effective since it varied from very weak (analogue 5) to moderate (analogue 3) in a range of 5–25 min increase as compared with MAGE-1.A1 half-life. However, all these substitutions with ice-cold 1% bovine serum albumin-phosphate-buffered saline, cells were incubated for 1 h with fluorescein isothiocyanate-conjugated goat anti-mouse IgG secondary antibody (Sigma). Cells were then washed twice, fixed with 1% formaldehyde in bovine serum albumin-phosphate-buffered saline, and analyzed by flow cytometry (Becton Dickinson FACScan) for each peptide (staining concentration of peptide giving half of the maximal stabilization effect) was determined, which reflects its affinity for the studied MHC allele.

**CTL Assays**—Lysis of target cells by CTL clones was measured by chromium release assay. Target cells were ⁵¹Cr-labeled for 1 h at 37 °C in the presence of monoclonal anti-human MHC class I antibody W6.32 and washed three times. Target cells were then incubated in V-bottom microwells (10⁵ cells in 50 μl) in the presence of different concentrations of peptide (50 μl) for 30 min at 37 °C. CTLs were then added at an effectortarget ratio of 10:1 (10⁵ cells in 100 μl), and ⁵¹Cr release was measured after 4 h at 37 °C.
were efficient against aminopeptidases, since none of the fragments 2–9 and 3–9 expected from aminopeptidase cleavage of the nonamer were detectable by UV (see for example analogue 5 in Fig. 2, panel f) or on-line HPLC/MS (data not shown) analysis. In contrast, in all cases, the fragment 1–7, generated from ACE cleavage, was more intense than for unmodified MAGE-1.A1 degradation, showing that NH₂-terminal protection of the peptide rendered its COOH terminus more sensitive (see analogue 5 in Fig. 2, panel j). This explains the poor overall gain in half-life for these analogues. At the COOH terminus, substitutions at positions 7 or 8 (analogue 6–8) did not significantly improve MAGE-1.A1 half-life, as expected, since ACE
Cleavage represents the minor degradation pathway. However, they efficiently protected MAGE-1.A1 against ACE, since fragments 1–7 and 8–9 were totally undetectable among the degradation products of the COOH terminus-protected analogues by UV (see analogue 8, Fig. 2, panel k) or by on-line HPLC-MS analysis. However, analogues were degraded by aminopeptidases giving fragments 2–9, 3–9 and 5–9 (see for example analogue 8 in Fig. 2, panel g). As protection of one extremity sensitized the opposite one to peptidase activity, we synthesized four analogues (9–12) in which substitutions at NH₂ and COOH terminus were combined. All four disubstituted peptides showed no detectable degradation even after a 24-h incubation in serum (Table II) (analogue 10 is shown in Fig. 2, panels d, h, and l). This shows that disubstitution conferred long term resistance to both aminopeptidase and ACE and, importantly, that MAGE-1.A1 was not sensitive to other peptidase activities in serum.

The HLA-A1 Binding Groove Tolerates Structural Modifications in the MAGE-1.A1 Sequence—Binding of the structurally modified analogues to HLA-A1 was then tested and compared with that of the parent peptide MAGE-1.A1 (examples are shown in Fig. 3, panels a, d, g, and j; results are presented in Table II, right column). The effect of a substitution on MHC binding depended on both the nature of the chemical modification and its position in the peptide sequence. Introduction of Aib at position 2 (analogue 5), a D-isomer at position 7 (analogue 6), or a NMe group at position 8 (analogue 8) did not alter dramatically HLA-A1 binding of MAGE-1.A1 (Table II). When two of these substitutions were introduced in the same sequence, their effects accumulated; the disubstituted analogues 9 and 10 bound to HLA-A1 with lowered but still good affinities (Fig. 3, panels a and d; Table II). Unlike the previous substitutions, D isomerization at position 2 (analogue 3) or 8 (analogue 7) dramatically decreased HLA-A1 binding. Consequently, the disubstituted [D-Ala₂,D-Ser₈] peptide (analogue 12) did not bind to HLA-A1. Finally, the monosubstituted analogues 1 and 4 and disubstituted analogue 11 exhibited intermediate to weak (i.e. 30–100 times lower than that of MAGE-1.A1) binding to HLA-A1 (Fig. 3, panel g; Table II). Thus, out of the 12 non-natural MAGE-1.A1 analogues, 2 (anallogues 9 and...
Stable MAGE-1.A1 Analogues Can Activate MAGE-1.A1-specific CTL—

MAGE-1.A1 mono- and disubstituted analogues were tested for their capacity to activate anti-melanoma MAGE-1.A1-specific CTL clones 82/30 and 258/8. Residues Gly³, Thr⁶, and His⁷ are recognized by both clones while residues Pro⁴ and Ser⁸ are selectively recognized by clones 82/30 and 258/8, respectively (32). Results are summarized in Table III (CTL assay experiments for four series of mono- and corre-

**TABLE II**

Biochemical properties of MAGE-1.A1 and its substituted analogues

Part a, half-lives were determined as follows: after different incubation times in human serum, the parent peptide was separated from its degradation fragments by HPLC, and the corresponding peak was quantified (Fig. 2, upper panels). Part b, binding affinities (MHC stabilization assay) are the mean of at least three independent experiments.

| Analogue no. | Substituted position(s) | Sequence | Stability (half-life) | Binding affinity (SC₅₀) |
|--------------|-------------------------|----------|----------------------|------------------------|
| 1            | 1                       | Glu Ala Asp Pro Thr Gly His Ser Tyr | 30 min | 0.2 ± 0.02 |
| 2            | 1                       | NMe D - - - - - - -           | 45 min | 7 ± 3 |
| 3            | 2                       | - D - - - - - -              | 55 min | 20 ± 2 |
| 4            | 2                       | - NMe - - - - - -            | 40 min | 8 ± 2 |
| 5            | 2                       | - Aib - - - - - -            | 35 min | 1.2 ± 0.3 |
| 6            | 7                       | - - - - - - - - D            | 35 min | 3 ± 1 |
| 7            | 8                       | - - - - - - - - D            | 30 min | 50 ± 5 |
| 8            | 8                       | - - - - - - - - NMe           | 35 min | 1.1 ± 0.4 |
| 9            | 2, 7                    | - Aib - - - - - - D          | >24 h  | 9 ± 2 |
| 10           | 2, 8                    | - Aib - - - - - - NMe         | >24 h  | 4 ± 1 |
| 11           | 2, 8                    | - NMe - - - - - - NMe         | >24 h  | 20 ± 3 |
| 12           | 2, 8                    | - D - - - - - - D            | >24 h  | >100 |

**FIG. 3.** HLA-A1 binding and CTL activation properties of MAGE-1.A1 and its structurally modified analogues. HLA-A1 binding (left panels) was determined using the peptide-induced MHC stabilization procedure. Activation of CTL clones 82/30 (middle panels) and 258/8 (right panels) was measured by ⁵¹Cr assay. The disubstituted analogues 9 [Aib², d-His⁷], 10 [Aib², NMe-Ser⁸], 11 [NMe-Ala², NMe-Ser⁸], and 12 [d-Ala², d-Ser⁸] (closed circles) and their respective amino-terminal (open squares) and carboxyl-terminal (open triangles) monosubstituted analogues are shown in comparison to MAGE-1.A1 (open circles).
The recognition of MAGE-1.A1 or its substituted analogues by CTL clones 82/30 and 258/8 was determined by $^{51}$Cr release assay after incubation of $^{35}$S-labeled BM21 target cells with the studied peptides. EC$_{50}$ are the results of at least two independent experiments.

**TABLE III**  
Biological activities of MAGE-1.A1 and its stable substituted analogues

| Analogue no. | Substituted position(s) | Sequence | Biological activity (EC$_{50}$) |
|--------------|-------------------------|----------|-------------------------------|
|              |                         |          | CTL 82/30                         |
|              |                         |          | CTL 258/8                        |
| 1            | 1                       | D       | Glu Ala Asp Pro Thr Gly His Ser Tyr | 0.3 ± 0.1 | 1.9 ± 1.6 |
| 2            | 1                       | NMe     | - - - - - -                        | 40 ± 10   | 130 ± 100 |
| 3            | 2                       | D       | - - - - - -                        | 120 ± 50  | 9000 ± 1400 |
| 4            | 2                       | NMe     | - - - - - -                        | 900 ± 140 | 2650 ± 200 |
| 5            | 2                       | Aib     | - - - - - -                        | 30 ± 10   | 7.5 ± 0.7 |
| 6            | 7                       | -       | - - - - - -                        | 3 ± 0.5   | 90 ± 14  |
| 7            | 8                       | -       | - - - - - -                        | 667 ± 300 | >10000  |
| 8            | 8                       | -       | - - - - - -                        | 1 ± 0.1   | >10000  |
| 9            | 2, 7                    | Aib     | - - - - - -                        | 33 ± 6    | 590 ± 400 |
| 10           | 2, 8                    | - Aib   | - - - - - -                        | 3 ± 0.7   | >10000  |
| 11           | 2, 8                    | - NMe   | - - - - - -                        | 2750 ± 1700 | >10000  |
| 12           | 2, 8                    | - D     | - - - - - -                        | >10000    | >10000  |

**DISCUSSION**

In a perspective of design of synthetic peptide vaccines, we showed in this study that non-natural, peptidase-resistant tumor antigen analogues might specifically activate antitumor CTLs. The rational design of these analogues was based on: (i) the understanding of the interactions of the antigenic peptide with the MHC and the TcR and (ii) the precise knowledge of the degradation mechanism of the peptide.

On-line HPLC/MS analysis led to the identification and quantification of degradation fragments and to the determination of the peptidase-sensitive bonds. Despite the diversity of peptidases present in serum, we found that MAGE-1.A1 was degraded by exopeptidases (mainly aminopeptidases, then by ACE, which are both exopeptidases). Indeed, short peptides are often degraded by exopeptidases rather than endopeptidases (13, 33). This structure-based approach combined with the knowledge of the interactions of MAGE-1.A1 with HLA-A1 and TcR obtained in the present work and from previous structure-activity relationship studies (32, 34) allowed us to introduce precise and local substitutions at the sensitive site as opposed to systematic modifications of all of the peptide positions. Indeed, this point is of crucial importance for the use of biologically active peptides in the MHC-peptide-TcR system (Ref. 24 and see below). The non-natural residues we used (D-amino acids, N-methyl-amino acids, and Aib) are efficient inhibitors of exopeptidase (aminopeptidase and ACE) activities (35). We may reasonably postulate that the disubstituted analogues should also be peptidase-resistant in vivo (18).

The main successful examples of antigenic mimicry and cross-reactivity between natural peptide antigens and their non-natural analogues (retro-, retro-inverso-, or D-peptides) have been described for antibody recognition (36, 37). A major difficulty encountered in the design of biologically active analogues of MHC-restricted epitopes is that their activity depends upon the accomplishment of two critical steps: MHC binding on the one hand and TcR signal triggering on the other. Several examples of MHC-restricted epitope analogues have been described. All-retro-inverso analogues (peptides in which all bonds have been retro-inverted) of MHC class II-restricted epitopes lost their MHC binding affinity (38). In other studies, binding to MHC class I was altered for some peptides and not for others following partial modifications, retro-inverso or re-
duced bonds, depending on the position of the modification (26, 27). However, in two studies on MHC class II-restricted peptides, even when only partial structural modifications that did not alter MHC binding were introduced, recognition of non-natural analogues by CTL was dramatically affected (28, 29). Despite the strong structural analogies between the Fab antibody fragment and the Vα-Vβ TcR domain (39), the rather common cross-reactivity found between parent peptides recognized by antibodies and their non-natural analogues (36) appears to be much less applicable to MHC-restricted TcR ligands. In fact, this observation is likely to be explained by the higher stringency of the TcR-epitope interaction in comparison with the antibody-epitope, since the TcR has dual functions that must both be fulfilled: (i) the specific recognition of the antigen and (ii) the activation of the lymphocyte functions. Indeed, structurally altered ligands, partial agonists or antagonists, that can only fulfill the first TcR function, but not the second one, may then lead to partial activation or anergy of the lymphocyte (24). Thus, great care must be taken in the development of antitumor vaccine strategies to avoid specific inactivation or anergy of antitumor CTL.

Of the three stabilized analogues (9, 10, and 11) with good HLA-A1 binding, only analogue 10, [Aib₂, NMe-Ser₈]MAGE-1.A1, activated CTL in the same range of concentrations as the parent MAGE-1.A1 peptide. In this analogue, the side chain orientations of the anchoring residues Asp⁹ and Tyr¹⁰ and, more importantly, that of the residues pointing out toward the TcR (mainly Glu¹, Pro⁴, Thr⁵, His⁷, Ser⁸) were left unmodified by the structural modifications at P2 and P₈ (see Fig. 4). This observation has a dual consequence in terms of MHC binding, only analogue 10 is able to engage the MAGE-1.A1-specific TcR as the natural ligand does. We can thus postulate that this synthetic peptide can fully activate the anti-melanoma CTL functions and therefore acts as a full agonist, an important parameter in terms of successful vaccine strategies (see above). Analogue 10, stable after 24 h in serum and able to activate two of three known MAGE-1.A1-specific CTL clones, may therefore be considered as a potential activator of anti-melanoma CTL response in vivo. To overcome the ethical problems encountered when testing non-natural molecules such as ours in human cancer vaccine trials, an alternative and attractive approach to follow would be to use these molecules in ex vivo or in vitro procedures to generate potent MAGE-1.A1-specific CTL (41).

Even though promising, T cell-based immunotherapies may face limitations. First, unlike B cell (antibody)-based vaccines, T cell immunotherapeutics are not global but are restricted, by definition, to a fraction of individuals sharing a given HLA allele (for example, HLA-A1 is carried by 26% of the caucasian population). Second, the use of peptide vaccines to induce antitumor or antiviral protection may result in a wide range of effects, ranging from effective protection, through non-response and even to the induction of tolerance. The factors influencing the in vivo immune response and leading to protection or tolerance are not yet clearly understood. Priming or tolerance toward a viral antigen could be induced by injecting the peptide subcutaneously or intraperitoneally, respectively (42). The induction of protection seemed favored by the slow liberation of the peptide when administrated subcutaneously. However, tolerance was induced toward an Ad5E1A-transfected tumor by a single subcutaneous injection of epitope Ad5E1A234–243 (43). In this case, tolerance depended on the peptide used rather than on the administration protocol. Immunization of mice with tumor peptides MUT-1 and MUT-2 injected subcutaneously or intradermally in IFA led to regression of established metastasis (8). The use of stabilized analogues may thus be of particular interest when administrated subcutaneously in order to be liberated slowly. They will not be degraded in situ...
Activation of Antitumor CTL by Non-natural Antigen

before reaching MHC molecules on professional antigen-presenting cells. It will be important, however, to verify that the use of highly stable peptides will not mimic the use of high doses of peptide that lead to CTL tolerance. Tumor antigens that are not derived from viral proteins, as opposed to Ad5E1A234–243, have low MHC binding affinity (44), and the use of stabilized analogues in these cases might be of high interest.

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