

II PATIENTS AND METHODS

II. 1 STRUCTURE OF OUR CLINICAL STUDIES

Our studies are small Phase I/II, nonrandomized, multicenter, open-label trials. Phase I/II studies are usually monocenter studies. However, because of the large number of patients who need to be screened for HLA- and *MAGE* expression in their tumors to identify eligible patients and because of the approximately 25% drop-off rate of patients with advanced metastatic cancers, several clinical centers were required although the number of patients to be studied in each trial was rather small.

A complete list of our clinical trials is given in Annex 1.

Institutions that participated in our clinical trials since 1993

[INSTITUTIONS' CODE(S)]

Australia

Ludwig Institute for Cancer Research, Melbourne Branch and Cancer Clinical Trials Centre, Heidelberg (*J. Cebon, M. Shackleton and I. Davis*) [LM]

Belgium

- Université catholique de Louvain, Cliniques universitaires Saint-Luc, Consultation pluridisciplinaire du mélanome (*Jean-François Baurain, André Bourlond, Vincent Brichard, Yves Humblet, Isabelle Tromme, Liliane Marot, Romain Vanwijck, Birgit Weynand*); and *Jean-Marie Collard, Roger Detry, Marc Hamoir, Jean-Marie Lachapelle, Jean-Pascal Machiels, Bertrand Tombal and Paul Van Cangh*

[UCL / CUSL / LB]

- Université catholique de Louvain, Cliniques universitaires UCL de Mont-Godinne, Yvoir, Belgium (*Patrick Weynants, Joseph Kerger, Fabrice Duplaquet, Paul-Emile Baugnée and Luc Delaunois*)

[CUMG]

- Université libre de Bruxelles, Hôpital Erasme, Brussels (*Thierry Velu and Myrto Trakatelli*)

[EB]

- Institut Jules Bordet (*Rika Deraemaeker, Joseph Kerger, François Salès and Frédéric Lehmann*)

[BB / IJB]

- Universitair Ziekenhuis Gasthuisberg, Katholieke Universiteit, Leuven (*Marguerite Stas, Ivo de Wever and Joost van den Oord*)

[KUL]

- Akademisch Ziekenhuis Vrije Universiteit, Division of Hematology & Medical Oncology, Brussels (*Bart Neyns, Jacques De Greve and Kris Thielemans*)

[VUB]

- Universitair Ziekenhuis, Ghent (*Jean-Marie Naeyaert and Véronique Cocquyt*)

[UZG]

- Université de Liège (*Thierry Lahaye, Gérard Degiovanni and Annie Ooms*)

[LG]

- Hôpital de Jolimont, Haine-Saint-Paul (*Marc Beauduin and Françoise Majois*) [LB]

France

- Hôtel Dieu, CHU de Nantes, Nantes (*Brigitte Dréno, Marie-Hélène Tessier and Amir Khammari*) [NAP]

- Institut Gustave Roussy, Villejuif (*Marie-Françoise Avril and Bernard Escudier*) [IGR]

- Institut Curie, Paris (*Pierre Pouillart, Thierry Dorval, Vincent Brichard and Sophie Piperno-Neumann*) [CP]

- Institut Paoli-Calmettes, Marseille (*Dominique Maraninchi and G. Gravis*) [MA]

- Hôpital Ste-Marguerite, Marseille (*Jean-Jacques Grob and Marie-Aleth Richard*) [MA]

- Centre Léon Bérard, Lyon (*Sylvie Négrier*) [LY]

- Hôpital Henri Mondor, Creteil (*Dominique Chopin, Jean-Jacques Patard, Martine Bagot and P. Wolkenstein*) [HM]

- Centre Jean Perrin, Clermont-Ferrand (*R. Plagne and Ph. Chollet*) [CF]

- CHU Michallon, Grenoble (*Mireille Mousseau and René Schaerer*) [EN]

- Centre Claudius Regaud, Toulouse (*Christine Chevreau*) [TOU]

Germany

- Krankenhaus Nordwest, Frankfurt am Main (*Alex Knuth and Elke Jäger*) [MZP / NW]

- University Hospital Benjamin Franklin, Free University of Berlin, Berlin (*Ulrich Keilholz and Carmen Scheibenbogen*) [UKBF]

- Medizinische Fakultät Der Universität, Klinische Kooperationseinheit Für Dermatoonkologie, Mannheim (*Dirk Schadendorf*) [DKFZ]

- Medizinische Hochschule Hannover, Hannover (*Jens Atzpodien*) [HA]

- Johannes Gutenberg University of Mainz, Mainz (*Thomas Wölfel, Cedrik M. Britten and Ralf G. Meyer*) [MZ / MZP]

Italy

- Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano (*F. Arienti and G. Parmiani*) [MI]

- San Raffaele Hospital, Milano (*Vincenzo Russo and Catia Traversari*) [MSR]

Norway

The Norwegian Radium Hospital, Oslo (*S. Aamdal*) [NRH]

Spain

Hospital Virgen de las Nieves, Granada (*F. Garrido, S. Serrano Ortega, M. Lopez Nevot*) [GD]

Sweden

- Karolinska Institutet Radiumhemmet, Stockholm (*Giuseppe Masuccci*) [KI]

- University Hospital of Linköping, Linköping (*L. Hakansson*) [UHL]

Switzerland

- Ludwig Institute for Cancer Research, Lausanne Branch and Centre Pluridisciplinaire d'Oncologie, Centre Hospitalier Universitaire Vaudois, Lausanne
(*Danielle Liénard, Daniel Speiser and Ferdy Lejeune*) [LAU / CHUV / CPO]

- Hôpital Cantonal Universitaire de Genève, Genève (*Pierre-Yves Dietrich*) [HUG]

The Netherlands

- Erasmus Medical Center - Daniel den Hoed Cancer Center, Rotterdam, The Netherlands
(*Alexander M.M. Eggermont, Wim H.J. Kruit, Heidi H. van Ojik, Cor H.J. Lamers and Gerrit Stoter*) [DDHK]

- Antoni van Leeuwenhoek Hospital, The Netherlands Cancer Institute, Amsterdam
(*Elaine Rankin and G.C. de Gast*) [AVL]

- University Hospital Vrije Universiteit, Amsterdam (*J. Wagstaff and W. Gerritsen*) [AZVU]

- University Hospital Nijmegen, Nijmegen (*C.J.A. Punt*) [AZN]

- Leiden University Medical Center, Leiden (*Susanne Osanto*) [LE]

United Kingdom

- Weston Park Hospital, Sheffield (*BW Hancock, P Lorigan and RC Rees*)

[SH]

- ICRF Cancer Medicine Research Unit, St James' University Hospital, Leeds
(*Poulam Patel and P.J. Selby*)

[ICRL]

II. 2 ELIGIBILITY CRITERIA

Patients were required to have advanced, i.e. stage III or IV cutaneous melanoma or other tumor types, i.e. non-small-cell lung cancers (NSCLC), head and neck-, esophageal- or bladder carcinoma, with at least 1 evaluable metastasis, according to the AJCC staging system (18, 193, 194). They were also required to have tumor expression of the genes coding for the vaccinal antigen(s), as assessed by RT-PCR on messenger ribonucleic acids (mRNA) extracted from a frozen tumor biopsy and to express the appropriate presenting HLA alleles (195). Patients whose tumor sample only displayed low quantity of *MAGE* mRNA as detected by RT-PCR, i.e. less than 1% of the corresponding positive control, were not included in the studies, because under this threshold, melanoma cells expressing the appropriate HLA allele might no more be recognized by the specific CTL corresponding to the antigen (130, 196). Determination of the HLA allele(s) corresponding to the vaccine antigen(s) was assessed by serological typing on blood lymphocytes, except when MAGE-3 protein-based vaccines were used. Because the MAGE-3 protein contains multiple epitopes presented by both class I and class II HLA molecules, there is no need to select patients on the basis of their HLA type prior to vaccination with this protein.

In addition, usual criteria for phase I/II trials had to be fulfilled. Patients had to be over 18 years old, with a World Health Organization (WHO) performance status ≤ 1 (*Annex 2*), normal organ functions and without second neoplasm or other serious disease. No chemotherapy, radiotherapy or immunotherapy was allowed in the 4-week period before the first vaccination. Detailed eligibility criteria for studies LB 94-001, LUD 97-002, LUD 99-003 and LUD 02-002 are given in Annex 3.

II. 3 STUDY TREATMENT

Vaccinations are given in 2 steps. The first step, called cycle 1 or study treatment, includes 3 to 6 vaccinations at 2 to 4-week intervals. The second step, or maintenance treatment, includes additional vaccinations at increasing intervals for up to 5 years. Maintenance vaccinations are only proposed to patients whose tumor evolution does not require other systemic anti-cancer treatment.

Peptides were usually administered at a dose of 300 μg per peptide, per vaccination day and the MAGE-3 protein, at a dose of 300 μg per vaccination day. When required by the protocol, peptides or the MAGE-3 protein were mixed with an immunological adjuvant (*Section II.8.3*) (197, 198).

Vaccinations were usually given intradermally (i.d.) and subcutaneously (s.c.) at injection sites distant from the tumor. This injection route is indeed believed to lead to more effective immunization

than the intramuscular route (i.m.), possibly because of the presence of antigen presenting cells and of a rich lymphatic network in the skin. However, when the peptide(s) or the recombinant MAGE-3 protein were mixed with an immunological adjuvant, the i.m. route was used in order to avoid skin injury (198). Injections were given in the arms or in the anterior aspect of the thighs. No injection was given into limbs where draining lymph nodes were surgically removed or irradiated, or in case draining lymph nodes were known to contain metastases. In most studies, the sites were rotated upon each vaccination. Splitting the total dose of protein or peptides between several injection sites and rotating the injection sites upon each vaccination day should allow to target several draining lymph nodes corresponding to the different skin areas injected. This should increase the likelihood of inducing T cell responses (199).

Patients who were withdrawn from the study before the end of the first cycle of vaccinations, for reasons other than toxicity, were replaced. All patients were included in the analysis of results.

II. 4 STUDY ENDPOINTS AND EVALUATION OF PATIENTS

Criteria that would warrant further clinical development of the vaccine were the following: severe toxicity in less than 10% of the patients and an objective tumor response rate of $\geq 20\%$, or if the response rate was lower, the progression-free survival should be $\geq 10\%$ at one year.

II. 4.1 Safety and toxicity

Safety and toxicity were evaluated in all patients having received at least 1 vaccination by documenting adverse events and reporting serious adverse events.

An adverse event (AE) is any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and that does not necessarily have a causal relationship with the treatment. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not related to the medicinal (investigational) product. The definition above, provided the Good Clinical Practice (GCP) guidelines and the International Conference on Harmonization (ICH), GCP-ICH Guideline E6, is being extended for the purpose of LICR studies to include any events, intercurrent diseases and accidents observed while the patient is on study, i.e. during the actual treatment period, as well as during drug-free, pre- and post-treatment study periods. It also includes any such events observed in control groups of patients receiving placebo or other drugs or non-drug therapy or no treatment while on study.

For **adverse events grading**, we successively used the following **toxicity scales**:

- National Cancer Institute of Canada clinical trial group common toxicity criteria scale: “Recommendations for Grading of Acute and Subacute Toxicity”, revised version, December 1994 (Studies LB 93-001 to LUD 01-006).
- National Cancer Institute common toxicity criteria scale, version 2.0 April 30, 1999 (Study LUD 02-002).
- National Cancer Institute common toxicity criteria scale, version 3.0, December 12, 2003 (Studies LUD 02-001 and LUD 03-007).

The relationship of AE to treatment was evaluated by the clinical investigator as definitely, probably, possibly, or not related. Adverse events were considered as being related to the treatment if the relationship was reported as definite, probable or possible. Treatment-related adverse events are termed here “toxicities”. If dose-limiting toxicity occurred, i.e. grade 3 (other than skin or flu-like symptoms) or grade 4 toxicity, patients had to be removed from the study. If 2 patients had shown treatment-related grade 3 toxicity (other than skin or flu-like symptoms) or grade 4 toxicity, the study would have been temporarily interrupted for discussion between the Ludwig Institute and the investigators. In case of grade 3 toxicities, a reduction of the vaccine dose injected would have been discussed. In case of grade 4 toxicities, a delay of vaccine administration or discontinuation of the trial would have been considered.

A Serious adverse event (SAE) is any untoward medical occurrence that:

- Results in death,
- Is life-threatening,
- Requires inpatient hospitalization or prolongation of existing hospitalization,
- Results in persistent or significant disability or incapacity,
- Is a congenital abnormality / birth defect or
- Is another medically important condition.

All serious adverse events - irrespective of suspected causation - which occurred while the patient was on study, were reported by the clinical investigator to LICR within one working day of discovery, using the LICR Initial Serious Adverse Event Report form. This included any death that occurred within 6 weeks of the last day on which an investigational agent was administered (early deaths). Reports were directed to the Office of Clinical Trials Management of the Ludwig Institute, to the Institutional Review Boards of the participating institutions and - when required - to the National Health Authorities.

II. 4.2 Tumor response

Because we did not expect effective immunization of the patients before several injections, patients were only considered evaluable for tumor response if they had completed the first cycle of vaccination, including 3 to 6 vaccinations at 2 to 4 week intervals.

Tumor response was assessed 2 weeks after the first cycle of vaccination, as compared to baseline tumor measurements performed in the 4-week period preceding the first vaccination. At each time point, tumor staging included physical examination, chest-, complete abdomen-CT-scans, as well as brain CT-scan or magnetic resonance imaging (MRI). Cutaneous and subcutaneous metastases were documented by pictures and by ultrasound, respectively. A long-term follow-up of all patients enrolled in the studies was performed every six months or until death.

Tumor response evaluation was performed according either to the WHO criteria based on the bidimensional measurement of target lesions or, to the more recently defined “response evaluation criteria in solid tumors” (RECIST), based on the unidimensional measurement of tumor lesions (200-202). From study LUD 02-001, the RECIST system replaced the WHO criteria.

II. 4.2.1 Definition of tumor response according to the WHO criteria

These criteria were used for Studies LB 93-001 to LUD 01-006 (*Annex 1*).

Complete response (CR): Disappearance of all signs, symptoms, biochemical and imaging evidence of all tumor lesions for a period of at least 4 weeks.

Partial response (PR): Decrease in size of all measurable tumors by at least 50% of the sum of the products of the greatest and perpendicular diameters, for at least 4 weeks, without appearance of new lesions or progression of any lesion.

Progressive disease (PD): Appearance of any new tumor lesion, or increase in size of any measurable tumor lesion by at least 25% of the product of the greatest and perpendicular diameters.

Stable disease (SD): A <25% increase or <50% decrease of the sum of the products of the greatest and perpendicular diameters of all measurable lesions, without appearance of any new lesion or progression of any lesion.

II. 4.2.2 Definition of tumor response according to the RECIST

Criteria used from study LUD 02-001 (*Annex 1*).

In the RECIST system, response of target- and non-target-lesions, as well as new lesions, are first reported separately. The “best overall response” integrates the analysis of the 3 categories of lesions (*Annex 4*).

- For target lesions:

Complete response (CR): Disappearance of all target lesions.

Partial response (PR): At least a 30% decrease in the sum of the longest diameter (LD) of target lesions, taking as reference the baseline sum LD.

Progressive disease (PD): At least a 20% increase in the sum of the LD of target lesions, taking as reference the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions.

Stable disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum LD since the treatment started.

- The “best overall response” is the best response recorded from the start of treatment until disease progression or recurrence, taking in consideration all the detectable metastatic lesions, i.e, target-, non-target lesions and new lesions.

Target lesions	Non-Target lesions	New Lesions	Overall response
CR	CR	No	CR
CR	Incomplete response / SD	No	PR
PR	Non-PD	No	PR
SD	Non-PD	No	SD
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

II. 4.2.3 Objective tumor responses (OR) are CR and/or PR.

II. 4.2.4 Other tumor responses

For the purpose of our biological understanding of anticancer therapeutic vaccines, we also evaluated descriptively patients who presented tumor regression not qualifying for an OR according to the WHO or the RECIST systems. These patients are the following:

1) Patients who had only non-measurable cutaneous or superficial lesions at study entry and who achieved complete or partial regression or stabilization of these lesions (typically, small in-transit metastases in melanoma). Such observations need to be documented by color photographs with a ruler in the picture at each physical examination during the study or by ultrasound examination performed at each tumor staging. Measurement of these lesions with a caliper should be performed at each physical examination. According to the WHO or the RECIST systems, patients belonging to the first category are not evaluable for tumor response, which, in my opinion, constitutes a gap of both systems.

2) Mixed response or dissociated response (MxR) are frequently observed in melanoma patients. They associate regression of some metastases while others either remain stable, progress or appear simultaneously. Such responses are formally classified as stable or progressive disease in the WHO and RECIST evaluation systems.

3) Minor response (MR): where the WHO system was used, MR is defined as the decrease in size of all measurable tumors by at least 25% of the sum of the products of the greatest and perpendicular diameters, for at least 30 days, without appearance of new lesions or progression of any lesion. Patients belonging to this category are formally considered as having PD.

II. 4.3 Time to progression

Time to progression had to be evaluated in all patients having received at least one vaccination. The starting time point to calculate the time to progression was the time point at which the disease was the most advanced during the treatment phase, which did not necessarily correspond with the start of treatment. In patients with initial tumor progression followed by stabilization or regression, time to progression was calculated from the date of maximal initial progression under treatment, whereas in all other patients, it was calculated from the date of the first vaccination. Patients with initial tumor progression followed by stabilization or regression would otherwise be considered as having progressive disease.

II. 4.4 Immunological responses

Patients were only considered evaluable for immune responses if they had completed the first cycle of vaccination, including 3 to 6 vaccinations at 2 to 4 week intervals (*Section 4.2*).

II. 4.4.1 Detection of CD8⁺ T lymphocyte responses.

The presence of anti-MAGE-3.A1 CTL was evaluated as follows.

Method A : Mixed lymphocyte-peptide culture (MLPC) / lysis assay n° 1:

Autologous PBMC were thawed, resuspended in serum-free X-VIVO 10 medium (Whittaker Bioproducts, Walkersville, MD) and incubated over 40 min at 4°C with magnetic microbeads coated with an anti-CD8 antibody (MACS, Miltenyi Biotech, Bergisch Gladbach, Germany). The CD8⁺ cells were sorted with a magnet, washed, resuspended in stimulation medium consisting of DMEM (Gibco) supplemented with 5% human serum, L-arginine (116 mg/L), L-asparagine (36 mg/L), L-glutamine (216 mg/L) and 2-mercaptoethanol (5×10^{-5} M) and seeded at 10^5 cells/well in 1 ml wells (Falcon 3078, Becton Dickinson, Mountain View, CA). The CD8⁺ cells were washed, resuspended in X-VIVO 10 medium containing MAGE-3.A1 peptide EVDPIGHLHY or influenza.A1 peptide CTELKLSYD (100 μ M) and incubated at room temperature for 60 min, during which they were irradiated (30 Gy from a ¹³⁷Cs source), washed and resuspended in stimulation medium. These stimulator cells were added (10^6 cells/well) to the CD8⁺ lymphocytes and human rIL-2 (Proleukin, Chiron, Amsterdam) was added at 30 Unit/ml (one Unit/ml is the concentration that gives half-maximal proliferation of CTLL-2 cells). On day 7, the responder lymphocytes of each well were washed, resuspended in medium containing the same concentration of rIL-2 in 2 ml wells, restimulated with 10^6 autologous PBMC pulsed with the relevant peptide and irradiated as on day 0. On day 14, restimulation was performed under the same conditions, except that peptide-pulsed C1R cells (203) transfected with an HLA-A1 construct were used as stimulators. On day 21, aliquots of the cultures were tested in a chromium release assay against Epstein-Barr virus (EBV)-transformed B lymphocytes BM21, in the presence or absence of the relevant antigenic peptides and in the presence of a 50-fold excess of unlabelled K562 cells to inhibit the activity of NK-like lytic effectors.

Method B : MLPC / lysis assay n° 2:

Another approach involving a large number of CD8⁺ lymphocytes (more than $2 \cdot 10^7$) was carried out as described elsewhere (204). In brief, CD8⁺ T cells were distributed amongst 96 microcultures (226,000 cells/well). Autologous monocyte-derived dendritic cells were irradiated, incubated with 50 μ M of the MAGE-3.A1 peptide EVDPIGHLHY and distributed in the microcultures (85 000 cells/well) to stimulate the autologous CD8⁺ T cells. IL-6 (1,000

U/ml) and IL-12 (10 ng/ml) were added during the first week, followed by IL-2 (10 U/ml) and IL-7 (5 ng/ml) from day 7. The microcultures were restimulated each week with autologous phytohemagglutinin (PHA)-activated T cells (150,000 cells/well), incubated with the peptide and irradiated. On day 25, aliquots of the cultures were tested in a chromium release assay against the HLA-A1 EBV-transformed B cell line BM21 in the presence or absence of the MAGE-3.A1 peptide and in the presence of a 50-fold excess of unlabelled K562 cells to inhibit the activity of NK-like lytic effectors.

Method C : Anti-MAGE-3.A1 T-cell responses detected with tetramer (205)

Tetramers or multimers are soluble complexes of recombinant MHC molecules folded in the presence of an antigenic peptide and biotinylated (206). Tetramers allow to enumerate and sort living T cells of a given specificity present at a low frequency (0.01 to 0.1%), irrespective of their functional properties. We used soluble complexes of HLA-A1 molecules folded with peptide MAGE-3.A1 and β 2-microglobulin, biotinylated and multimerized with avidin conjugated to phycoerythrin. We wished to use tetramers so as to detect safely an increase of at least 10-fold of the naive frequency of anti-MAGE-3.A1 T cells, that was estimated to approximately 3×10^{-7} of the blood CD8⁺ cells (204). This increased frequency, about 3×10^{-6} , was clearly too low for a direct detection with the MAGE-3.A1 tetramers, which had a sensitivity threshold that was about 100-fold higher (0.03% of CD8⁺ cells). We resorted to an *in vitro* restimulation with the antigenic peptide in the presence of IL-2, IL-4 and IL-7, before labeling with tetramers. In order to evaluate precursor frequencies, these MLPC were carried out in limiting dilution condition with about 2×10^5 PBMC/well. The cultures were restimulated on day 7 under the same conditions and aliquots of each culture were labeled with the tetramer around day 14. To assess the specificity of the tetramer staining, the positive CD8⁺ cells from several cultures were sorted, cloned and their specific anti-MAGE-3.A1 lytic activity was verified against either HLA-A1 Epstein-Barr-virus-transformed B lymphocytes (EBV-B) target cells incubated with the antigenic peptide, or melanoma target cells that naturally expressed the *HLA-A1* and *MAGE-3* genes. The TCR V α and V β gene expression of specific CTL clones derived from cells stained with the A1/MAGE-3 tetramer was then analyzed. "Clonotypic" PCR amplifications specific for V α or V β rearrangement of the specific TCR identified, enabled us to analyze directly the frequencies of blood T cells expressing the corresponding TCR(s).

II. 4.4.2 Detection of CD4⁺ T lymphocyte responses

The method used in study LUD 99-003 was recently described (207). Briefly, van der Bruggen and colleagues developed an approach which involves overnight stimulation of blood T cells with

autologous dendritic cells loaded with MAGE-3 protein, sorting by flow cytometry of the CD4⁺ T cells that produce IFN- γ , cloning of these cells and evaluation of the number of T cell clones that secrete IFN- γ upon stimulation with the antigen. An important criterion is that T cell clones must recognize not only stimulator cells loaded with the protein but also stimulator cells transduced with the *MAGE-3* gene, so as to exclude the T cells that recognize contaminants generated by the protein production system. By using this approach, it is possible to measure T cell frequencies as low as 10⁻⁶. TCR of the anti-MAGE-3 CD4⁺ T cell clones were then characterized and the frequencies of one anti-MAGE-3 CD4⁺ T cell clonotype were confirmed by PCR analysis on blood lymphocytes. In study LUD 99-003, patients' PBMC were collected and frozen before the first and after the 4th and the 6th vaccinations. Patients who received additional vaccinations had a PBMC collection after the 9th and 12th vaccinations. The anti-MAGE-3 CD4⁺ T cell response was considered positive if the post-vaccination/pre-vaccination frequencies ratio was ≥ 10 .

II. 4.4.3 Detection of antibody responses

Anti-MAGE-3 and anti-ProtD antibody responses were assessed by enzyme-linked immunosorbent assay (ELISA) as previously described. In both studies, patients were considered evaluable for antibody response if they had received at least 4 vaccinations.

Method A, study LUD 97-002 (208):

To quantify the antibodies, 2 specific IgG ELISAs were developed. A patient was considered a MAGE-3 or ProtD vaccine responder when the postvaccination titer was above the respective value and at least twice the patient's own baseline value.

Because the vaccine contained a recombinant MAGE-3 protein produced in *E. coli*, the MAGE-3 ELISA was carried out with a MAGE-3 protein produced in a different system, i.e. insect cells, using the baculovirus expression system (MAGE-3^{baculo}), to allow a MAGE-3 protein specific response. Plates were coated with MAGE-3^{baculo} protein. Plasma from MAGE-3-vaccinated patients, whose sera were shown to be positive in a Western blot, were included on each plate and served as standard and positive control. Twofold serial dilutions of patients' sera starting at 1/20 dilution were tested. The titers of the sera pre- and postvaccination were determined by comparison with the standard plasma of known titer value and are expressed as ELISA Units per milliliter (EU/mL). The cutoff value was 27 EU/mL and corresponded to 3 standard deviations (SD) above the mean titer of a healthy control population (n = 60).

ProtD ELISA was carried out with plates coated with the whole ProtD (± 300 AA). The cutoff was 100 EU/mL for antibody response.

Method B, Study LUD 99-003 (209):

The IgG antibodies against the MAGE-3 protein were evaluated by ELISA as previously described, using *E. coli*-derived MAGE-3 protein (AA 57-219)-coated plates and uncoated plates (209). The serum of a cancer patient identified prior to this study as containing specific anti-MAGE-3 IgG antibodies was used as a positive control in this assay. It was positive at a serum dilution of 1:16,000. Plates coated with non-relevant proteins, i.e. MAGE-1 and NY-ESO-1, were used as negative controls. Sera were tested over a range of serial fourfold dilutions from 1:100 to 1:100,000. A serum was considered positive against MAGE-3 protein if the OD reading at a serum dilution of 1:400 exceeded the cutoff value of 458 (median + 3 SD of 25 pre-vaccination sera). Above the cutoff, the titers were considered positive and below the cutoff, the titers were considered negative. An antibody response was considered positive if the post-vaccination/pre-vaccination titers ratio was ≥ 2 . Patients' sera were collected before the first and after the 4th vaccination. Patients who received additional vaccinations had serum collection after the 6th, 9th and 12th vaccinations.

II. 4.5 Analysis of the antigen presentation capability by the tumor

Patients who received at least one vaccination were considered evaluable for this analysis. Biopsies of superficial metastases were performed before, during and after treatment to assess *MAGE-3* and HLA class I expression by the tumor. Each metastasis was evaluated as progressive or regressive by physical examination. *MAGE-3* RT-PCR was performed as previously described (195). HLA class I expression was assessed by immunohistochemistry, using an anti-MHC class I conformational antibody (W6/32) and an anti- β 2-microglobulin antibody (BM-63) (210).

II. 5 REGULATORY AND ETHICAL OBLIGATIONS

All studies were conducted in full conformity with the current revision of the Declaration of Helsinki, the ICH, the GCP guidelines and the applicable local laws and regulations. All patients provided signed informed consent prior to participation. The studies were approved by the Protocol Review Committee of the Ludwig Institute for Cancer Research (LICR), Office for Clinical Trial Management (OCTM), New York (NY, USA) and by the regulatory boards of the participating institutions.

II. 6 SPONSORING

All studies presented in Section III were sponsored by the Ludwig Institute for Cancer Research, except for study LUD 97-002 that was sponsored by GlaxoSmithKline Biologicals (GSK), Rixensart

(Belgium) and performed in collaboration with the MCG and Biological Treatments Development Group (BTDG) of the EORTC.

II. 7 MONITORING

Our very first studies, LB 93-001 and LB 94-001, were non-monitored studies (*Annex 1*).

From study LUD 94-002, our studies were monitored by the LICR Office of Clinical Trials Management, New York (NY USA) either by Dr Christian Verfaillie from the LICR Brussels Branch, or by a monitor from the Contract Research Organization Omnicare, Germany.

Upon request of GSK, study LUD 97-002 was monitored by the EORTC New Drug Development Office (NDDO), Amsterdam, The Netherlands.

II. 8 VACCINES PRODUCTION

All synthesis, production, formulation and packaging of the investigational agent were in accordance with applicable current Good Manufacturing Practices and met applicable criteria for use in humans.

II. 8.1 Peptides: we successively used 3 main sources of peptides for our clinical trials:

- UCB Bioproducts, Braine l'Alleud, Belgium
- Multiple Peptide Systems (MPS), San Diego, USA
- Clinalfa, Läufelfingen, Switzerland

Peptides were formulated as single use preparations. Each vial contained 300 ± 30 µg of lyophilized peptide. Some of the peptides were formulated in phosphate buffered saline (PBS) pH 7.4 or in 100% dimethyl sulfoxide (DMSO). The peptide dose per injection day ranged from 10 to 300 µg.

Peptides used in our clinical studies

<i>Name</i>	<i>AA sequence</i>	<i>AA position</i>	<i>Reference</i>
HLA-class I presenting molecules			
<u>HLA-A1</u>			
MAGE-1.A1/B35	E A D P T G H S Y	161-169	(110)
			(151)
MAGE-3.A1/B35	E V D P I G H L Y	168-176	(132)
			(137)
<u>HLA-A2</u>			
MAGE-1.A2	K V L E Y V I K V	278-286	(211, 212)
MAGE-3.A2	F L W G P R A L V	271-279	(133)
MAGE-3.A2	K V A E L V H F L	112-120	(134)
MAGE-4.A2	G V Y D G R E H T V	230-239	(213)
MAGE-10.A2	G L Y D G M E H L	254-262	(214)
NA-17.A2	V L P D V F I R C	intron	(163)
MAGE-C2.A2	A L K D V E E R V	336-344	(215)
NY-ESO-1.A2	S L L M W I T Q V	157-165	(182)
NA-17.A2	V L P D V F I R C	intron	(163)
Tyrosinase.A2	Y M D G T M S Q V	369-377	(112)
<u>Other HLA-class I presenting molecules</u>			
MAGE-1.Cw*16	S A Y G E P R K L	230-238	(216)
MAGE-3.A24	I M P K A G L L I	195-203	(217)
MAGE-3.B44/B18	M E V D P I G H L Y	167-176	(136, 140)
MAGE-12.Cw7	V R I G H L Y I L	170-178	(218, 219)
HLA-class II presenting molecules			
MAGE-3.DP4	K K L L T Q H F V Q E N Y L E Y	243-258	(143)
MAGE-3.DR13	A E L V H F L L L K Y R A R	114-127	(149)

II. 8.2 Recombinant MAGE-3 protein

The MAGE-3 recombinant protein was provided by GSK, Rixensart, Belgium. The DNA recombinant MAGE-3 protein was expressed in *E. coli* as a fusion protein with a protein D derived from *H. influenzae* at the N-terminus and a sequence of several histidine residues at the C-terminus of the protein. The inclusion of the first 109 residues of the protein D as a fusion partner was expected to improve the immunogenicity and to provide the vaccine protein with additional bystander help properties, whereas the inclusion of a His affinity tail facilitated the purification of the fusion protein (Fig. 8). The encoding plasmid was designed in such a way to express a precursor protein consisting of the 18 AA signal sequence and the first 109 residues of the processed protein D, 2 unrelated AA (Met and Asp), the AA residues 2 to 314 of the MAGE-3 protein and 2 Gly residues functioning as a hinge region to expose the subsequent 7 His residues. Thus, the secreted fusion protein, Protein-D-MAGE-3-Histidine (ProtD-MAGE-3-His), comprises 433 AA residues. Before purification, the intra- and interprotein disulfide bonds of the protein were chemically reduced and the thiol groups were blocked with iodoacetamide (carboxyamidation of the protein). The protein was then extensively purified to eliminate bacterial contaminants.

Figure 8: Structure of ProtD-MAGE-3-His



II. 8.3 Immunological adjuvants

II. 8.3.1 AS02B (study LUD 97-002)

The adjuvant used in study LUD 97-002 is an oil-in-water emulsion MPL/QS21-based adjuvant system, presently referred to by GlaxoSmithKline Beecham as AS02B. The latter abbreviation will be used throughout the manuscript.

II. 8.3.2 AS15 (study LUD 02-002)

AS15 is a liposome-based adjuvant containing MPL/QS21/CpG 7909 provided by GSK Biologicals. CpG 7909 is a synthetic, single stranded oligodeoxyribonucleotide optimized for human use.