Phase I clinical trial of a human idiotypic p53 vaccine in patients with advanced malignancy

M. Lomas¹, W. Liauw¹,², D. Packham¹, K. Williams², A. Kelleher³, J. Zaunders³ & R. Ward¹,⁴*

¹Department of Medical Oncology, ²Clinical Trials Unit and ³Centre for Immunology, St Vincent’s Hospital, Darlinghurst; ⁴School of Medicine, University of New South Wales, Sydney, Australia

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Background: The purpose of this study was to induce immunity to p53 by using an idiotypic vaccine, composed of a pool of eight peptides derived from the complementarity determining regions (CDRs) of human anti-p53 antibodies.

Patients and methods: Subjects with advanced malignancy received up to four, monthly intradermal injections of pooled peptides (500 µg of each) admixed with granulocyte–macrophage colony-stimulating factor (GM-CSF; 100 µg). In addition, two sheep and two rabbits were also vaccinated with the pooled peptides.

Results: Fourteen subjects were enrolled into the study and six of these completed the vaccination schedule. The vaccine was well tolerated by all subjects and no major adverse events were attributable to the vaccine. All subjects mounted in vivo delayed type hypersensitivity (DTH) responses to two or more of the individual vaccine peptides. Vaccine-induced antibodies specific for peptides 2, 5 or 8 were detected in four of six subjects, and two of these had vaccine-specific, cell-mediated responses. Increasing titers of p53-specific antibodies were found in one patient. No T-cell response to p53 was observed in any of the subjects. All animals developed humoral immunity to the peptides and one of the sheep developed rising serum titers of anti-p53 antibodies.

Conclusions: Vaccination with human antibody CDR regions represents a novel method for inducing human antibodies, which may in turn serve as immunological mimics of p53.

Key words: cancer, idiotype, p53, vaccination

Introduction

The tumor suppressor protein p53 regulates the transcription of genes involved in cell cycle arrest, apoptosis and DNA repair [1]. P53 is mutated in one-half of all human cancers, and unlike wild-type p53, which is rapidly degraded, mutant p53 gene products accumulate in the nucleus and cytoplasm of tumor cells [2, 3]. The accumulation of p53 is implicated in the p53-specific B- and T-cell responses observed in cancer patients [4–6]. P53-specific cytotoxic T cells are directed against peptides in the non-mutant part of the protein, and are able to lyse tumor cells in vitro [7–9]. The presentation of p53 peptides on the surface of tumor, but not normal cells, provides a potential target for anti-cancer vaccines, as demonstrated in mouse studies [9–11]. Although several vaccines have sought to similarly augment the immune response to p53 in humans, long-lasting anti-tumor immunity and clinical responses have not yet been achieved, perhaps due to the tolerance mechanisms associated with self proteins.

Tolerance to p53 may be circumvented by employing the principles of Lindenmann [12] and Jerne’s [13] idiotypic network theory, which proposes that every antibody can itself be used as an antigen. Therefore, target specific antibodies can be used to generate further antibodies, which will mimic the target and themselves induce immunity to the target via an idiotypic cascade. An idiotypic vaccination strategy has been used in several clinical trials targeting self-antigens, including carcinoembryonic antigen [14], gp37 [15], disialoganglioside GD2 [16] and idiotypic IgM [17]. Evidence to support p53-specific vaccines has been offered by four studies in rodent cancer models, which showed that idiotypic vaccines against p53 can protect against tumor development and can reduce the number of established metastases [18–21]. Importantly, the murine studies demonstrated that the CDR of the murine p53-specific antibody was effective in vivo when used alone and that it was the specificity of the antibody that determined the host response.

We have previously described a panel of unique human N-terminal- and central-domain-specific anti-p53 antibodies derived from the regional lymph nodes of individuals with colorectal cancer [22, 23]. In this phase I clinical trial, we tested the hypothesis that vaccination with peptides derived from the CDRs of these antibodies could be used to generate a p53-specific idiotypic response in individuals with cancer. The end points of the trial were to assess the safety and the immunogenicity of the vaccine in humans.
Patients and methods

Vaccine preparation

Eight previously described peptides [22, 23], derived from the N- and C-termini of anti-p53 antibodies, were synthesized (Multiple Peptide Systems, San Diego, CA). The vaccine was prepared by admixing the peptides (500 µg of each) with granulocyte-macrophage colony-stimulating factor (GM-CSF) 100 µg (Schering-Plough, Baulkham Hills, Australia) in an aqueous solution containing 31.25% dimethyl sulfoxide (v/v), 0.05M NaCl, to a final volume of 800 µL.

Subjects and clinical protocol

Between 6 November 2001 and 16 October 2002, 14 subjects with solid tumors were enrolled into the study (median age 52.5 years, range 39–70 years; seven males). Ten had metastatic malignancy (one breast, five colorectal, one non-small-cell lung, one hemangioepitcytoma, one renal and one prostate carcinoma), one had local recurrence (squamous cell carcinoma of the head and neck) and three were disease free after local therapy (one esophageal, one renal and one non-small-cell lung carcinoma). Informed consent was obtained from all of the patients in accordance with the St Vincents Hospital Human Research Ethics Committee.

Eligible subjects were required to have an Eastern Co-operative Oncology Group (ECOG) performance status of 0 (11 subjects) or 1 (three subjects), a life expectancy of ≥26 months, overexpression of p53 in either the primary or metastatic tumor and a positive response to recall antigen as determined by the cell mediated immunity (CMI) multitest (Pasteur Merieux, Lyon, France). Subjects were excluded if they had undergone chemotherapy, radiotherapy or surgery, or had received immunosuppressive therapy in the preceding 6 weeks.

The first four subjects received one intradermal vaccination of the peptide mixture and GM-CSF delivered as four separate injections of 200 µL. Further vaccinations were available to this group on compassionate grounds, although they were not assessed for an immunological response to the vaccine. The second group then received four vaccinations at monthly intervals, delivered as for the first group. The vaccine was delivered intradermally as four separate injections of 200 µL. Blood for immunologic assays was drawn before each immunization, and at 1 and 2 months post-vaccination 4. Clinical observations, including temperature, blood pressure, heart rate and respiratory rate, were recorded at the time of injection and at 24 and 48 h. Adverse events were graded according to the National Cancer Institute (NCI)-Common Toxicity Criteria Version 2 [24]. Subjects who completed all four vaccinations were assessed for vaccine-specific immune responses. All patients were assessed for safety and toxicity. Tumor response was not assessed as part of this study.

Delayed type hypersensitivity (DTH) testing was carried out 1 month after the final vaccination. The individual vaccine peptides (100 µg), a negative control peptide derived from the light-chain CDR2 sequence of a human HIV gp41-specific antibody (H-PKLL1YKASSLESGVPSPR-OH) and a vehicle-only control were injected intradermally to the interscapular area. Dermal induration of ≥10 mm² at 48 h post-injection was considered positive.

Immunization of sheep and rabbits with the vaccine

Polyclonal sera specific for each of the peptides were generated by immunizing two sheep and two rabbits. Sheep were primed with 200 µg of the peptide mixture in a CpG DNA adjuvant (ImmunEasy Adjuvant, Qiagen, Hilden, Germany), boosted 6 weeks later, and bled on the day of priming and 10 days after the boost. Polyclonal sera reactive with peptides 3 and 7 could not be generated in sheep and therefore rabbits were primed with a mixture of peptides 3 and 7 (375 µg each) in a QuilA/DEAE Dextran/Montanide ISA 50V adjuvant mixture (Bioquest, Sydney, Australia) and boosted 6 weeks later.

Humoral immune response to the vaccine and p53

Enzyme-linked immunosorbent assays (ELISAs) were used to detect serum antibodies specific for either the individual vaccine peptides or recombinant p53. Each biotinylated peptide (5 µg/ml; Auspep, Melbourne, Australia) was captured separately onto plates coated with 5 µg/ml of streptavidin (Sigma, St Louis, MO), and the purified recombinant p53 (10 µg/ml) was plated directly as described previously [22]. Sera were applied in triplicate and tested over a range of dilutions (1:25 to 1:800). Binding antibodies were detected with an alkaline phosphatase-conjugated goat anti-human IgA+IgG+IgM (H+L) antibody (0.12 µg/ml; Jackson Immunoresearch, West Grove, PA). Samples with a mean change in Abs410 nm (Abs410 nm with peptide minus Abs410 nm without peptide) of >0.3 were considered positive. Positive responses were isotyped by detecting binding antibodies with a panel of murine anti-human IgA-, IgG1-, IgG2-, IgG3-, IgG4- and IgM-specific antibodies (0.25 µg/ml; Zymed, San Francisco, CA) followed by a goat anti-mouse IgG alkaline phosphatase conjugate (0.12 µg/ml; Jackson Immunoresearch). Detection of anti-peptide and anti-p53 responses in the rabbits and sheep were performed using ELISAs as described above, but serum binding antibodies were detected using alkaline phosphatase-conjugated donkey anti-sheep or anti-rabbit IgG antibodies (0.12 µg/ml; Jackson Immunoresearch). Sera from 54 cancer controls (median age 66.3 years, range 40.9–84.0 years; 36 males) and 30 normal controls (median age 34.5 years, range 22.7–61.6 years; 14 males) were also tested.

Cell-mediated response to the vaccine and p53

Proliferation in response to stimulation by vaccine (individual peptides or vaccine pool) or p53 was measured by [3H]thyidine and carboxyfluorescein succinimidyl ester (CFSE) proliferation assays, and the secretion of IFN-γ was measured by IFN-γ-ELISPOT assay (Diaclone, France) according to manufacturer protocol. Peripheral blood mononuclear cells (PBMCs) were plated at a concentration of 1 × 10³ cells/well for thyamine proliferation and ELISPOT assays, and at 1 × 10⁴ cells/well (24-well plates) for the CFSE proliferation assay. Cells for CFSE assay were stained with 5 µM CFSE-FITC (fluorescein isothiocyanate) (Molecular Probes, Eugene, OR). All PBMCs were RPMI supplemented with 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin/ streptomycin and 10% human AB serum (Australian Red Cross Blood Service). Cells were stimulated with the pooled vaccine (50 µg/ml), individual vaccine peptides (10 µg/ml) and recombinant p53 (5 or 10 µg/ml), and incubated at 37°C in a 5% CO2 atmosphere for 20 h (ELISPot) or 6 days (proliferation assays).

In the CFSE proliferation assay, cells were stained with the following conjugated monoclonal antibodies: CD3-PerCp (peridinin chlorophyll), CD71-PE (phycoerythrin) (both from BD Biosciences, San Jose, CA) and CD8-ECD (energy-coupled dye) (Beckman Coulter, Miami, FL), and analyzed using an EPICS-XL flow cytometer [25]. Staining was considered positive when the percentage of cells that were both CD71-positive and had moved out of the undivided (CFSE dim) population was at least twice that observed in the absence of stimulation.

In the thyamine proliferation assay, cells were pulsed with 0.5 µCi/well methyl-[3H]thyidine (Amersham, Buckinghamshire, UK) and harvested 18 h later. [3H]thyidine incorporation was measured by liquid scintillation counting. A sample was considered to be positive when the stimulation index [14] for each well in the quadruplicate sample exceeded the mean of unstimulated cells by at least three standard deviations.

Results

Clinical outcomes

A total of 102 individuals were screened for entry into this study. Forty-two individuals were excluded on clinical grounds, 26 individuals...
Individuals were excluded because their tumor did not over-express p53 and a further 13 were excluded because their disease progressed or they elected not to continue with the study during the screening phase. Twenty-one subjects were subjected to CMI testing, 14 of whom participated in the trial, although only six completed all four vaccinations. Four of 14 subjects participated in a single-dose toxicity assessment. Toxicity for all subjects that received vaccine, and immunogenicity for subjects completing four vaccinations is reported.

Over the total of 49 separate occasions of vaccination, the vaccine was well tolerated and the majority of systemic adverse events recorded were attributable to the underlying disease. Local reactions were the most common adverse event, with subjects developing an increasing area of post-vaccination induration after each vaccination.

Toxicities that were probably or possibly attributed to the vaccine were limited to grades 1 or 2 in severity and included arthralgia (n = 10), nausea (n = 4) and febrile reactions (n = 16). These effects have all been described with GM-CSF alone [26]. Grade 3 or 4 toxicities, including jaundice (n = 1), motor neuropathy (n = 2), vomiting (n = 1), tumor pain (n = 3), neuropathic pain (n = 1) and non-neutropenic sepsis (n = 1), and two serious adverse events, cord compression and death from pneumonia, were attributable to disease progression.

**Humoral response to the vaccine**

Sera from four of the six assessable trial subjects were found to be reactive with peptide 2, two were reactive with peptide 8 and two were reactive with peptide 5 (Figure 1A–D and data not shown). However, one of these responses was observed at the baseline time point and was not augmented by vaccination (Figure 1B). The vaccine-specific humoral responses were detected after at least two vaccinations had been administered and titers of peptide-specific serum antibodies rose to maximal levels 1 month after vaccination 4, before falling.

Isotyping identified that the serum anti-vaccine antibodies were predominantly of the IgG class (Figure 1E), indicative of an...
antigen-stimulated secondary immune response. Serum titers of the IgG antibodies rose over the period of vaccination in a similar fashion to the combined IgA, IgG and IgM responses. (data not shown).

One subject also had rising titers of serum anti-p53 antibodies (Figure 1F). Isotype-specific antibodies showed that the level of p53-specific IgG1 serum antibodies in this subject rose with increasing vaccinations, but the level of IgM antibodies remained at a constant level over the course of the vaccination schedule (data not shown).

A small proportion of sera from 30 normal individuals and 54 cancer controls demonstrated weak reactivity to peptides in the vaccine, although the absorbance in each case was less than that seen in the trial patients. The p53 mutation status of the cancer controls was unknown.

Polyclonal sera with reactivity to peptides 2, 4, 5, 6 and 8 were obtained from both sheep, while one animal did not respond to peptide 1 and neither animal responded to peptides 3 and 7 (data not shown). While serum antibody titers were equivalent for peptides 5 and 8, the responses to peptides 2, 4 and 6 were on average 50% higher in the first immunized sheep. Given the method of immunization, it was not surprising that the serum anti-peptide titers in sheep were significantly higher than those found in the trial patients. For instance, the sheep sera response to peptide 5 was still detectable at serum dilutions of 1:3200, while the highest serum titer in a trial subject was 1:200 to peptide 2 (Figure 1A).

Interestingly, the sheep with the highest titers of anti-peptide antibodies also had detectable and rising levels of vaccine-induced p53-specific antibodies (Figure 1G). The p53-specific antibodies were measurable at serum dilutions of 1:50 and 1:100, but then fell to background levels at higher dilutions. Polyclonal sera against peptides 3 and 7 were successfully isolated from the immunized rabbits, but neither of the rabbits generated anti-p53 antibodies.

**Cell-mediated response to the vaccine**

All six of the trial patients who underwent in vivo DTH testing were found to have responses to the individual vaccine peptides (Table 1). Two of the six assessable trial patients (016 and 017) were found to have T cells specific for the vaccine, as measured by the proliferation assays. Patient 016 showed responses in the thymidine proliferation assay to peptide 1 (visit 5, stimulation index = 10.4; data not shown) and peptide 5 (visits 3, 4 and 5, stimulation index = 8.88, 64.6 and 22.2, respectively; Figure 2A). The proliferative response to the vaccine pool was confirmed in subject 016 by the CFSE proliferation assay (visits 4, 8.9% CD3- and CD4-positive cells; Figure 2B and C), as was the response to peptide 5 (visit 5, 5.5% CD3- and CD4-positive cells; data not shown). The other peptides were not tested individually due to limiting cell numbers. Subject 017 also had a specific proliferative response to the vaccine peptide pool, measured by CFSE proliferation assay (visit 5, 3.9% CD3- and CD4-positive cells; data not shown). Both of these vaccine-specific proliferation assays can be attributed to the vaccination schedule, as they were not present at baseline time points (Figure 2 and data not shown). Also, the PBMCs from patients 016 and 017 did not proliferate in the absence of stimulation with either the vaccine pool or peptide 5 (data not shown). Due to limiting cell numbers, only two other patients were tested by CFSE proliferation assay (012 and 015) and neither of them had a measurable, vaccine-specific proliferative response.

In contrast to the proliferation assays, none of the trial patients was found to have vaccine-specific, IFN-γ-secreting T cells as assessed by ELISpot assay (data not shown). There was no measurable cell-mediated response to p53 in any of the trial subjects as assessed by thymidine proliferation assay, CFSE proliferation assay or IFN-γ ELISpot assay.

None of the cancer controls had a measurable cell-mediated response to the vaccine peptides assayed by thymidine proliferation assay (nine subjects), CFSE proliferation assay (four subjects) or IFN-γ ELISpot (nine subjects). Similarly, none of the normal controls had a measurable response to p53 assayed by thymidine proliferation assay (three subjects) or CFSE proliferation assay (two subjects).

**Discussion**

In this study we have demonstrated that the CDR regions of human anti-p53 antibodies are capable of initiating humoral and cellular immune responses in animals and in individuals with advanced malignancy. Anti-p53 antibodies were observed in a sheep and in one human, suggesting that the induction of anti-idiotypic immune cascade may have been triggered by the vaccinations. However, the p53-specific antibodies seen in the trial subject were also observed at the baseline time point, so it is not clear whether they were vaccine-induced or were the result of an increased antigen load following disease progression.

We sought to elicit a specific cytotoxic T-cell response to the self-protein p53 by immunizing with Ab1 (CDR regions from anti-p53 antibodies), thereby inducing the production of anti-idiotypic antibodies (anti-peptide antibodies or Ab2) that are an immunological mimic of p53. Our strategy differs from previous human idiotype trials in that we have vaccinated subjects with

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Induration area (mm²) was measured at 48 h following injection with the individual vaccine peptides (100 µg). All results with the HIV L2 peptide (100 µg) and vehicle alone 31.25% dimethyl sulfoxide (v/v), 0.05 M NaCl, were negative.

Neg., induration (if any) was <10 mm².
Ab1 rather than anti-idiotype antibodies (Ab2) isolated from immunized animals [15, 16, 27, 28]. Clearly, the vaccine did induce a CD4+ T helper cell response in four of the six evaluable subjects in this study, which led to the production of a panel of anti-idiotype (Ab2) antibodies. By inference, the T helper cell response is likely to be of the Th2 subtype; anti-peptide antibodies were predominantly of the IgG1 and IgG3 isotypes and the T cells failed to produce IFN-γ. Interestingly, only three of the eight peptides (2, 5 and 8) induced humoral and cell-mediated immune responses. There are a number of possible explanations for the lack of immunogenicity of the remaining peptides, including their human origin, the mixing of the peptides, the adjuvant used, the format and timing of vaccinations, and their human leukocyte antigen compatibility with the subjects in this study [18, 29].

Taken together, the results suggest that the current vaccination strategy was relatively ineffective in inducing detectable anti-p53 immune responses, at least in humans. It is, however, possible that the in vitro assays used in this study failed to detect humoral and CD8+ T cell responses to p53. Certainly reactivity to conformational epitopes on recombinant p53 would not be detected in the ELISA, and in the absence of cross priming the T-cell assays would only identify CD4+ responses, since p53 was added exogenously as a whole protein [30].

These technical issues aside, we suggest that the absolute amount of Ab2 available for antigen presentation may impact on its potential role as an immunogen. Notably, the highest titers of Ab2 were observed in the sheep with an anti-p53 antibody response. The second sheep, which had much lower titers of vaccine-specific antibodies, correspondingly showed no detectable p53-specific humoral immunity. While the titers of Ab2 in the trial patients were many fold lower than those seen in either sheep, it is interesting to note that the patient with the highest Ab2 response was also the individual in whom there was demonstrable anti-p53 reactivity.

Another factor that may have influenced the development of Ab3 was the time taken to develop anti-peptide or Ab2 responses. Evaluable patients did not develop peptide immunity until after the third or fourth vaccination, and the titer of antibody fell once vaccinations were discontinued. It is therefore possible that anti-p53 antibodies may only be generated following repeated vaccinations over a sustained period of time [31–33]. Irrespective of the mechanisms involved, it is clear that if the levels of vaccine-specific antibodies can not be boosted to yield a p53-specific response, it may be necessary to isolate and immunize with vaccine-specific antibodies (Ab2), thereby eliminating one step in the idiotype cascade.

In conclusion, this study demonstrates that vaccinating with human antibody CDR regions represents a novel method for inducing anti-idiotype antibodies, which may in turn represent immunological mimics of p53.

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References


