

Encapsulated Plasmid DNA Treatment for Human Papillomavirus 16-associated Anal Dysplasia: A Phase I Study of ZYC101¹

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ABSTRACT

High-grade dysplasia induced by high-risk types of human papillomavirus (HPV) precedes invasive cancer in anal squamous epithelium just as it does in the cervix. A therapeutic HPV vaccine strategy as a potential treatment for anal dysplasia was tested in a standard Phase I dose escalation trial. The primary objective was to evaluate the safety of the agent; additional study aims were to evaluate the histological response, immune response, and effect on anal HPV-16 infection. Each subject was treated with four i.m. injections of 50–400 µg of ZYC101 at 3-week intervals. ZYC101 is composed of plasmid DNA encapsulated in biodegradable polymer microparticles. The plasmid DNA encodes for multiple HLA-A2-restricted epitopes derived from the HPV-16 E7 protein, one of two HPV oncoproteins consistently expressed in neoplastic cells. Fifty-six potential anal dysplasia subjects were screened to identify 12 eligible subjects with HPV-16 anal infection and a HLA-A2 haplotype. The investigational agent was well tolerated in all subjects at all dose levels tested. Three subjects experienced partial histological responses, including one of three subjects receiving the 200-µg dose and two subjects at the 400-µg dose level. Using a direct Elispot, 10 of 12 subjects demonstrated increased immune response to the peptide epitopes encoded within ZYC101; each continued to show elevated immune

responses 6 months after the initiation of therapy. These results support the continued investigation of a therapeutic vaccination strategy for anal dysplasia.

INTRODUCTION

HPV³ infections of the anogenital tract are sexually transmitted and endemic worldwide (1–3). Whereas the majority of HPV infections are self-limiting, they can cause anogenital disease. Of >100 different genetic subtypes of the virus, certain so-called high-risk types with mucosal tropism are recognized as a primary risk factor in the development of anogenital neoplasia. The majority of anogenital cancers and their precursors, high-grade dysplasia, are associated with HPV-16, HPV-18, or other closely related subtypes (2–5). The expression of two viral gene products, E6 and E7, is responsible for the oncogenic potential of these high-risk HPVs (6). The E6 viral protein interacts with a host factor, E6-AP, to target the p53 protein for ubiquitin-mediated degradation (7–9), whereas the viral protein E7 interferes with the normal function of the retinoblastoma protein (10). Collectively, E6 and E7 interfere with normal cell cycle checkpoint processes and induce cell division.

Cervical cancer is by far the most common HPV-associated cancer worldwide, but anal cancer has a strikingly similar pathophysiology and relationship with HPV (5, 11–13). Whereas the rate of anal cancer at 0.9 of 100,000 cases is nearly a log lower than the current rate of cervical cancer in this country (14), this rate has doubled over the past few decades. The rise in incidence of anal cancer in the general population has been attributed to a change in sexual behaviors (15). In contrast to the relatively low incidence of anal cancer in the general population, the incidence among certain risk groups, such as men who have sex with men, is markedly higher (16, 17). Data collected before the height of the HIV epidemic revealed an incidence of anal cancer in 35 of 100,000 men who have sex with men (16), comparable with the incidence of cervical cancer among women before routine cervical cytology screening. The effect of the HIV epidemic on the incidence of anal cancer is just now becoming appreciated. Recent data support anal cancer as HIV-related for both HIV-infected men and women and those from all HIV risk categories (18). Similarly, the probability of developing anogenital dysplasia is increased in patients with suppressed immune systems (19).

High-grade dysplasia, also known as HSIL, of the anal mucosa or perianal skin is likely the precursor of invasive anal

Received 8/27/01; revised 1/10/02; accepted 1/22/02.

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¹ Supported by ZYCOS, Inc. (Lexington, MA). The clinical trial was conducted in the General Clinical Research Center, Mt. Zion Hospital of the University of California at San Francisco, with funds provided by the National Center for Research Resources, 5 M01 RR-00079, USPHS.

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³ The abbreviations used are: HPV, human papillomavirus; pDNA, plasmid DNA; HSIL, high-grade intraepithelial lesion; LSIL, low-grade epithelial lesion; HRA, high-resolution anoscopy; PLG, polylactide co-glycolide; APC, antigen-presenting cell; UCSF, University of California at San Francisco; AIN, anal intraepithelial neoplasia; PBMC, peripheral blood mononuclear cell; Flu, influenza virus; SFC, spot-forming cell; HC, hybrid capture; PR, partial response.

cancer, just as cervical HSIL is the lesion from which cervical cancer arises. Anal HSIL is found most often in the transformation zone where columnar epithelium of the rectum transitions to squamous epithelium of the anus. A range of anal neoplasia can be detected by anal cytological screening (anal Pap) using techniques developed and validated for cervical Pap analysis (20). If an anal cytological abnormality is found, HRA, the counterpart to cervical colposcopy, is recommended (21). HRA-directed biopsies are taken from abnormal areas to exclude invasive carcinoma and grade the level of dysplasia. Although warts or anal bleeding bring some patients to medical attention, the majority of patients with recognized anal dysplasia are detected on the basis of screening because anal dysplasia is usually asymptomatic. Thus, most individuals with anal dysplasia remain undiagnosed because expertise in HRA is not widely available and screening has not yet been widely implemented, although the potential benefits of screening programs have been described (22–24).

One deterrent to implementation of screening programs is the lack of adequate therapeutic options for anal HSIL once it is detected. Standard therapy for anal HSIL has been surgical excision or laser ablation; however, there are no data for the long-term efficacy of surgery to prevent HSIL recurrence or progression to anal cancer. Relapse rates are high with surgical therapy, and side effects include pain and, rarely, infection, stricture, and sphincter incompetence (25, 26). Many patients refuse subsequent anal surgery when the disease recurs. Patients with widespread disease are no longer candidates for surgery when their HSIL lesions become too large for simple removal or ablation. Unlike the treatment of cervical HSIL, with anal HSIL it is not possible to remove the entire transformation zone, because this may result in significant morbidity such as anal stricture. Nonsurgical approaches, such as application of trichloroacetic acid, cryosurgery, imiquimod, or podophyllotoxin, are unproven and also probably ineffective in diffuse disease. Alternatives include watchful waiting or experimental studies of chemoprevention agents. Better treatment options are urgently needed.

The relationship between immune status and the incidence of anal neoplasia combined with the development of improved viral immunogens suggests that therapeutic vaccination could be considered as an alternative to the surgically ablative treatments (27–33). Two potential therapeutic targets are the gene products of E6 and/or E7 from high-risk HPV types because these proteins are expressed consistently throughout the range of neoplastic cells. The expression of E6 and E7 from HPV-16 or HPV-18 is sufficient to immortalize some primary cell lines, and blocking their function has been shown to reverse the transformed state (34, 35). As proteins required to maintain the transformed state and expressed in both recently infected cells and cancerous cells, E6 and E7 represent potential antiviral therapeutic drug targets (8, 10). Recently, HPV-16- and HLA-A2-positive women with cervical dysplasia were studied after receiving escalating doses of a synthetic HPV E7 peptide vaccine. This approach was successful in generating immunological response possibly associated with clinical activity (32).

In a similar effort, ZYC101 was developed for the treatment of HPV-16 infections in individuals who carry a HLA-A2 allele. This investigational agent incorporates DNA rather than

a peptide and encapsulates it within small particles allowing for better delivery to APCs (36). The DNA is selected from a region proximal to the COOH-terminal end of the HPV-16 E7 protein (codons 83–95; LLMGTLGIVCPIC) that encodes several overlapping CTL epitopes. These have been defined by peptide binding libraries, immunological screening assays (27, 29, 37, 38), and epitope elution/sequencing methods.⁴ To express this multivalent epitope region and elicit immune responses, numerous genetic constructions were investigated in mice with a molecular fusion construct to a secretory peptide chosen as the optimal format (36). These sequences encoded in a pDNA vector were then encapsulated within 1–2- μ m microparticles comprised of PLG, a biocompatible polymer used in a number of pharmaceutical products, including sutures. The activation of a T-cell response requires efficient presentation of an antigen by professional APCs, a feature thought to be lacking in many typical DNA injection techniques (39). The particulate nature and specific size of ZYC101 make it suitable for intracellular delivery of nucleic acids to the phagocytic APCs, initiating HPV-specific cellular immune responses. The primary goal of our study was to explore the safety of this new investigational agent in a dose escalation study, along with a serial analysis of viral specific immunity and cytological and histological monitoring.

PATIENTS AND METHODS

Study Design. In collaboration with ZYCOS, Inc. (Lexington, MA), UCSF performed a single-institution, open-labeled, Phase I dose escalation study to test the safety of the pDNA vaccine ZYC101 in HLA-A2-positive adults with anal HPV-16 infection and concomitant anal HSIL. The study was approved by UCSF's Institutional Review Board. B. K. received approval from the United States Food and Drug Administration by submitting an Investigational New Drug application. The study planned to test four dose levels, unless limited by toxicity. Additional study aims were to evaluate for evidence of clinical activity defined histologically and to study the cellular immune response induced by serial dosing of ZYC101.

Investigational Agent. The active component of ZYC101 is a bacterial expression plasmid (Biotope) that expresses a segment (codons 83–95; LLMGTLGIVCPIC) of the HPV-16 E7 protein fused to a 25-amino acid secretory leader sequence derived from the human HLA-DRA*0101 locus. A L83A substitution was included at the junction site between the leader sequence and the HPV E7 segment to optimize cleavage during translocation. Transcription was driven from an immediate early cytomegalovirus promoter, and the resulting mRNA was flanked on both ends with RNA-stabilizing sequences derived from the β -globin gene. Translation was facilitated by inclusion of a Kozak motif immediately upstream of the start site methionine.

Under current Good Manufacturing Practice guidelines, bulk pDNA was produced by bacterial fermentation and then purified by column chromatography. The pDNA was then en-

⁴ R. Chicz, personal communication.

capsulated into 1–2- μm particles comprised of a biocompatible polymer, PLG, formed in a double emulsion-based process. The vast majority of pDNA remains supercoiled inside the microparticles, inaccessible to solvents and nuclease. At the end of the encapsulation process, pDNA-filled microparticles were lyophilized in glass vials and stored at -20°C until use. Each manufacturing run produced material containing 3–8 μg pDNA/mg PLG, and each dose was based on pDNA content. The drug was warmed to room temperature and resuspended in United States Pharmacopeia saline just before patient injection.

Eligible Patients. Consecutive potential subjects 18 years of age or older were identified as their anal biopsies revealed HSIL of the intra-anal or perianal region, if they had no standard treatment options available and exhibited measurable dysplasia on HRA. Subjects with disease amenable to surgical excision (where technically feasible) were not recruited for this study. Subjects were recruited from a base of several hundred individuals at UCSF, including men already enrolled in a long-term natural history cohort study of anal dysplasia, women participating in the Women's Intra-agency HIV Study substudy of anal neoplasia, and patients being followed for clinical reasons in the UCSF Dysplasia Clinic. Signed informed consent was required before study-specific screening procedures were performed.

Inclusion into the study required patients to have a HLA-A2 haplotype based on phenotypic testing and evidence of anal HPV-16 infection. Type-specific HPV testing was performed using a nested PCR on an anal swab. It was not required that HPV-16 be found exclusive of other high-risk HPV types; although concurrent infection with other high-risk HPV types might be expected to reduce the clinical activity of the agent, the primary objective of this study was safety of the agent. Subjects were otherwise healthy, with no known immunological disorders. They were excluded if they had received systemic corticosteroid therapy within 30 days before enrollment or were anergic to a panel of common antigens (mumps, candida) on skin testing for delayed-type hypersensitivity. A negative HIV test result using an approved antibody detection method was required for enrollment. Subjects with abnormal hepatic, renal, or hematological bone marrow function were excluded, as were those with significant concurrent medical or psychiatric illness. Both men and women with childbearing potential agreed to use effective contraception, and pregnancy or breastfeeding were not allowed. Subjects were enrolled sequentially in the trial as they were identified and confirmed to meet all criteria for study participation.

Treatment. Treatment consisted of a series of four vaccine injections administered i.m. to alternating anterior thigh muscles every 3 weeks at weeks 0, 3, 6, and 9. A standard dose escalation scheme was followed. Dose levels of 50, 100, 200, and 400 μg of DNA were tested. There was no dose escalation within individual subjects. A minimum of three subjects were treated at each dose level and observed closely for toxicity for a minimum of 28 days before enrollment to the next higher dose level was allowed.

Patient Procedures. Subjects were evaluated by history, physical examination, and laboratory testing every 3 weeks for 12 weeks and again at week 24. Standard chemistry and hematology panels were sent to the UCSF clinical laboratory for routine analysis. An additional 80 ml of heparinized venous

blood was sent to ZYCOS, Inc. for immunological assays at each study visit. All symptoms and abnormal physical or laboratory findings were graded using the National Cancer Institute's Common Toxicity Criteria version 2.0 and reported as potential adverse events. A thorough anogenital examination at baseline and weeks 6, 12, and 24 included palpation and inspection using routine anoscopy of the anal mucosal surface and perianal skin. Subjects also underwent anal cytology testing at baseline and weeks 12 and 24. This anal Pap evaluation required a moistened Dacron swab to be inserted approximately 6–8 cm into the anal canal, past the squamous-columnar junction. In addition, HRA examinations were performed using a colposcope to visualize areas of aceto-whitening (areas that appear abnormal under HRA after the application of dilute acetic acid) and/or areas that excluded uptake of Lugol's stain. Each lesion was characterized clinically, based on the appearance of the vascular and mucosal surface patterns under HRA as HSIL or LSIL, using criteria developed for cervical colposcopy and previously validated for anal examinations (21). Computerized storage of digital images allowed all areas of dysplasia to be recorded and retrieved easily at the time of subsequent examinations. The primary purpose of identifying lesions was to identify the most appropriate sites to biopsy rather than to classify the degree of response to the therapy. Areas that were suspicious for HSIL were biopsied at baseline and weeks 12 and 24. Up to three lesions were sampled at each time point using endoscopic forceps to obtain 2–3 mm^3 epithelial and submucosal tissue/biopsy. Each sample was placed in 10% neutral buffered formalin for routine histological evaluation.

Histological Response Criteria. Response to therapy was judged using the histology results. Anal cytology and histology were evaluated by a single UCSF cytopathologist (T. D.), an expert in the recognition and evaluation of HPV-related anogenital disease, who was blinded to the clinical information, including treatment received. Dysplasias are graded as LSIL or HSIL on the basis of histological criteria used for grading other HPV-related lesions of the lower genital tract. Patients with either moderate or severe AIN (AIN2 or AIN3) are categorized as having HSIL. Those with mild AIN (AIN1) are categorized as having LSIL. Specimens that contained scattered atypical cells that could not be characterized as squamous intraepithelial lesions were categorized as atypia.

Responses were assessed by use of week 12 and week 24 cytology and histology results. As many as three lesions were biopsied at each time point. The highest grade of pathology found in any lesion was used to categorize the response. A subject with LSIL but no HSIL in any anal biopsy specimen or on anal cytology was characterized as having a PR. A complete response required the complete absence of dysplastic cells in the biopsy specimens at two consecutive study evaluation time points as defined prospectively by the investigators for this study protocol. Atypia was included in the PR category because the definition of a complete response could not be met. To achieve a histological response, the absence of HSIL on anal Pap was also required. The cytology test was used as an adjunct to histology because it occasionally indicates the presence of HSIL in the absence of HSIL on clinical examination or in the biopsy. If HSIL was found on cytology, a repeat biopsy would

then be indicated to be certain that a sampling error had not occurred.

Immunological Assays. Quantitative analysis of enhanced HPV-specific PBMCs from subjects before, during, and well after drug administration was performed to access the immunological activity of the test article, ZYC101. An Elispot assay for single-cell IFN- γ secretion can detect low frequencies of antigen-specific CD8+ T lymphocytes isolated from peripheral blood (40–48). IFN- γ Elispot was used to detect enhanced antiviral immune activity after ZYC101 treatment. All assays were performed in duplicate and run with appropriate controls.

Peptides. Six distinct HLA-A2-restricted class I E7 epitopes were encoded by the investigational agent, but because of limits on the availability of PBMCs, only three (2.1, 2.4, and 2.4-C) were selected for analysis. Peptides for these assays were obtained from Multiple Peptide Systems (San Diego, CA). In addition to the three class I, A2-restricted HPV-16 E7 peptides [2.1 (LLMGTGLGIV), 2.4 (TLGIVCPIC), and 2.4-C (TLGIVCPI)], a class I, A2-restricted Flu matrix peptide (GILG-FVFTL) was used as a positive control, and a class I, A2-restricted *Plasmodium falciparum* (cp36) peptide (YLKTIQNSL) was used as a negative control. The peptides were prepared by Boc/benzyl-based chemistry and then side-chain deprotected and cleaved from the solid support with hydrofluoric acid. Peptide purification consisted of reverse phase high-performance liquid chromatography with an Advantage 300 C18 column to attain >95% purity. The peptides were eluted from the column with 0.1% (v/v) trifluoroacetic acid/H₂O buffer and verified by mass spectral analysis.

Isolation of PBMCs. Approximately 80 ml of heparinized venous blood samples were drawn during each visit and shipped overnight at ambient temperature from UCSF to ZY-COS, Inc. for PBMC processing. PBMCs were isolated by centrifugation on a Ficoll-Paque gradient (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and stored in a freezing medium containing 90% FCS (JRH Biosciences, Lenexa, KS) and 10% DMSO (Sigma Chemical Co. Aldrich, St. Louis, MO). All PBMC samples were aliquoted at a concentration of 5×10^6 cells/ml and stored frozen overnight at -80°C before transfer to liquid nitrogen, where they were maintained in the vapor phase. On the day of the assay, sufficient numbers of vials of PBMCs were thawed and washed twice with PBMC wash medium [RPMI 1640 (JRH Biosciences) containing 1% penicillin-streptomycin (Life Technologies, Inc.) and 1% HEPES buffer (Life Technologies, Inc.)]. PBMC samples were then counted in trypan blue (Life Technologies, Inc.). The samples were adjusted to the appropriate cell concentration in PBMC medium and used in the Elispot assay.

Elispot Assay. To enumerate the frequency of HPV-specific effector cells within PBMC populations at each time point, an IFN- γ Elispot was performed using kits purchased from R&D Systems, Inc. (Minneapolis, MN). All steps involving plate development were performed according to the manufacturer's instructions. The assays were performed with 2×10^5 PBMCs and 2×10^5 peptide-pulsed APCs/well. The T2 cell line (49) served as the APC in all Elispot assays, and in each instance, the cells were incubated with a final concentration of 150 $\mu\text{g/ml}$ peptide for 3 h at 37°C before being mixed with effector T cells. All samples were tested with the three HPV peptides, the Flu peptide, and the irrelevant peptide.

Elispot plates were blocked with complete PBMC medium [RPMI 1640 (JRH Biosciences) with 10% human AB serum (C-Six Diagnostics, Germantown, WI), 1% HEPES buffer (Life Technologies, Inc.), 1% L-glutamine (Life Technologies, Inc.), 1% penicillin-streptomycin (Life Technologies, Inc.), and 0.1% 2-mercaptoethanol (Life Technologies, Inc.)] for 20 min at room temperature. Blocking medium was removed before the addition of cells to the plates. Duplicate test and control wells were set up for all assays performed in this trial. Cells were incubated in plates for 24 h at 37°C and removed, and then the plates were washed five times with wash buffer (prepared as per the manufacturer's instructions). Detection antibodies were added, followed by an overnight incubation at 4°C . The plates were washed three times, followed by the addition of streptavidin horseradish peroxidase. Plates were incubated for 2 h at room temperature, followed by three wash cycles, and then a 5-bromo-4-chloro-3'-indolyl phosphate p-toluidine salt and nitroblue tetrazolium chloride substrate was added to all wells for spot development. Plates were incubated for 1 h at room temperature in the dark and then rinsed with distilled water. Plates were dried at room temperature and then shipped to Zellnet Consulting, Inc. (New York, NY), for counting via the Elispot reader system (Carl Zeiss Vision, Munich-Hallbergmoos, Germany) with KS Elispot 4.0 software. Results are read as SFCs/million PBMCs. On the basis of the performance of the assays, the limit of detection was ≥ 20 SFCs/ 10^6 PBMCs.

Statistical Analysis. Descriptive statistics (e.g., mean, median, SD, and proportions) were calculated to characterize the toxicity of the HPV vaccine as well as any histological and immunological response to therapy. The HPV response of the HC results for paired data were analyzed using both the parametric ANOVA method for repeated measures and the nonparametric Wilcoxon matched pairs test. The immunological response was analyzed in two ways, with the response to each peptide at each time point considered first as a continuous variable and then as a categorical variable. To evaluate the change in mean response over time to each peptide, ANOVA methods for repeated measures were performed. In addition, the specific comparison between the paired baseline and week 24 values was carried out to assess the 6-month change. The immunological response was also treated as a categorical variable to indicate whether a 2-fold increase over baseline was recognized. For each peptide, the outcome at each of the five time points (3, 6, 9, 12, and 24 weeks) is coded as a binary random variable, indicating a value of $\geq 2\times$ baseline or $< 2\times$ baseline.

HPV Testing. HPV-16-specific testing was performed on material from an anal swab obtained at study entry and at week 24 on all subjects using a nested type HPV-16-specific PCR technique to detect and amplify HPV-16 DNA as the primary method of analysis. In addition, a HPV-16-specific HC test (Digene Corp., Beltsville, MD) was performed after the completion of the study using the same anal swabs taken at baseline and the final study evaluation. All subjects had detectable HPV-16 at study entry as a requirement for enrollment in the trial. HPV response was defined as the absence of HPV-16 detection in the week 24 specimen by nested PCR. The HPV laboratory was blinded to all other study results.

For the nested PCR test, a crude DNA preparation was

made from the Digene virapap specimen. The specimen was incubated at 56°C for 1 h before proteinase K (Invitrogen, Carlsbad, CA) was added to a concentration of 250 µg/ml and incubated at 56°C overnight. In the morning, the proteinase K was heat inactivated, and 200 µl of the specimen were ethanol precipitated and resuspended in 25 µl of Tris-EDTA buffer. One µl of this suspension was used in the HPV-16-specific nested PCR. Samples were subjected to a two-tube nested PCR to determine the presence of HPV-16. External primers were CAT-TTG-TTG-GGG-TAA-CCA-AC and CAG-TTG-TAG-AGG-TAG-ATG-AG, and the internal primers were TAG-GTC-TGC-AGA-AAA-CTT-TTC and GTC-ATT-ATG-TGC-TGC-CAT-ATC. PCR conditions for both reactions used 1× PCR buffer (3.0 mM MgCl₂, 0.2 mM of each deoxynucleotide triphosphate, and 5 µM of each primer; PE Applied Biosystems, Foster City, CA), 1.25 units of Taq polymerase, and 1 µl of template in a 50-µl reaction. The reaction times were a 2-min 95°C hot start, 30 cycles of 30 s at 95°C, 1 min at 60°C, and 1 min at 72°C, followed by a 7-min 72°C elongation step. For the internal PCR, the reaction time was repeated for 20 cycles. Ten µl of both reactions were analyzed on a 2.0% agarose gel, and if a visible band was seen at the same size as in the positive control (SiHA cell culture DNA carrying 1–2 copies of HPV-16/cell), the sample was considered positive for HPV-16.

HC was used to detect and quantify HPV DNA in the anal swab (50). HC was performed according to the manufacturer’s recommendations, using only the HPV-16-specific probe. The results of HC were expressed as a relative light unit ratio, determined by dividing the chemiluminescent signal of the test sample by that obtained with a control sample containing 1 pg/ml HPV-16 DNA or 10 pg/ml HPV-16 DNA. The magnitude of the relative light unit ratio increases with increasing quantity of HPV in the specimen. Whereas HC is semiquantitative, the results are not calibrated for the quantity of human cells picked up by the swab and present in the specimen.

RESULTS

Patient Characteristics. In a period of 14 months from November 1999 through December 2000, 56 HIV-negative patients were screened sequentially for participation in this Phase I clinical trial. Consecutive individuals with biopsy-confirmed HSIL and HRA evidence of disease for whom surgical therapy was not appropriate were informed of the study. Informed consent was obtained from those who agreed to participate. The screened population included 55 men (52 Caucasians, 2 Hispanics, and 1 American Indian) and 1 Caucasian woman with a mean age of 45 years (range, 27–68 years). A total of 25% or 14 of these 56 screened patients were potentially eligible after undergoing HLA and HPV testing. This included 49% or 27 of the 55 patients who were positive for HPV-16. Of the 37 who were HLA typed, 20 or 54% were found to be HLA-A2 positive. Of the 14 potential subjects identified, 2 were excluded (1 had a lower than normal platelet count and 1 had no residual clinical evidence of HSIL at the screening examination). Both of these excluded patients had only had one prior HSIL biopsy each, obtained at 4 and 7 months before the screening biopsy.

The characteristics of the 12 subjects selected for study participation appear to be representative of the initial screened

Table 1 Toxicity

Toxicity	Grade 1	Grade 2
Injection site pain	8	1
Injection site hypesthesia	4	0
Thigh warmth and erythema	3	0
Fever	3	1
Sweats	2	0
Malaise	2	0
Fatigue	4	0
Headache	3	0

population as shown in Table 3. Their mean age was 47 years (range, 27–68 years), and all 12 were male Caucasians. The mean pre-enrollment duration of HSIL diagnosis was 26 months (range, 1–81 months), with a median of 12.5 months. Five patients were relatively newly diagnosed (they had been confirmed to have HSIL only 1–4 months before enrollment). Four others repeatedly had HSIL documented over a period of 52, 53, 70, and 81 months. All were undergoing examinations and biopsies approximately every 3 months as part of their participation in prospective cohort studies. Because of the widespread nature of their disease and lack of effective treatments, most subjects had received no prior therapy, and those who were treated previously (subjects 5 and 10) underwent HSIL treatment 4 or more years before enrollment in the current study.

Toxicity. Enrollment proceeded in a stepwise fashion through the four planned dose levels. All therapy and planned study evaluations were delivered and performed on schedule with the exception of subject 12, who underwent the final study evaluation procedures at week 15 instead of week 24 because of his relocation out of the area. No subject required a dose reduction. Subjects 1–3 received 50 µg pDNA/injection, subjects 4–6 received 100 µg pDNA/injection, subjects 7–9 received 200 µg pDNA/injection, and subjects 10–12 received 400 µg pDNA/injection. All adverse events were recorded, scored according to the National Cancer Institute’s Common Toxicity Criteria version 2.0, and tabulated. No serious (grade 3 or higher) adverse events were experienced during the course of the study. Any toxicity possibly attributable to the investigational agent was mild and transient, as shown in Table 1, where each episode represents the occurrence of an adverse event after a single injection. Thus, for 12 subjects, each of whom received four injections, the potential for 48 episodes existed.

Clinical Response. A total of three subjects achieved a PR (subjects 8, 10, and 11; Table 2). No subject in dose level 1 or 2 (50 or 100 µg pDNA/injection) showed evidence of histological response or improvement of clinical impression by HRA. In the third dose level, one subject (subject 8) had atypia as the highest level of histological abnormality from either of his two biopsy specimens at week 24. He had no evidence of dysplasia on Pap for that same time point. In the highest dose cohort, another two subjects experienced PRs, one of whom had atypia as the highest level of histological abnormality in his biopsy specimens at week 12; this persisted until at least week 24. He had normal Pap studies at each time point. The other responder in cohort 4 had benign histology at week 12 and histological evidence of LSIL at week 24, with normal cytology on anal Pap. His response was not characterized as complete because the

Table 2 Histology results on anal biopsy specimens

Subject-dose level	Baseline	Week 12	Week 24	Response
1-50 μg	HSIL (AIN 2)	HSIL (AIN 3)	HSIL (AIN 3)	
2-50 μg	HSIL (AIN 2)	HSIL (AIN 3)	HSIL (AIN 2)	
3-50 μg	HSIL (AIN 3)	HSIL (AIN 2)	HSIL (AIN 3)	
4-100 μg	HSIL (AIN 2)	HSIL (AIN 3)	HSIL (AIN 3)	
5-100 μg	HSIL (AIN 2)	HSIL (AIN 3)	HSIL (AIN 3)	
6-100 μg	HSIL (AIN 3)	HSIL (AIN 3)	HSIL (AIN 3)	
7-200 μg	HSIL (AIN 2)	HSIL (AIN 2)	HSIL (AIN 2)	
8-200 μg	HSIL (AIN 2)	HSIL (AIN 3)	Atypia	Partial
9-200 μg	HSIL (AIN 3)	HSIL (AIN 3)	HSIL (AIN 3)	
10-400 μg	HSIL (AIN 3)	Atypia	Atypia	Partial
11-400 μg	HSIL (AIN 3)	Benign	LSIL (AIN 1)	Partial
12-400 μg	HSIL (AIN 3)	HSIL (AIN 3)	HSIL (AIN 3) (Week 15)	

benign result at week 12 was not maintained at week 24, when atypical cells were found in the biopsy specimen.

Immune Competence of Study Cohort. To assess the general immune status of each subject, potential subjects were required to show reactivity to common recall antigens (mumps, candida) with a standard delayed-type hypersensitivity skin test before study enrollment. All subjects were HIV negative as confirmed by a standard ELISA antibody assay within 30 days of the start of therapy. In addition, recall T-cell responses to a known HLA-A2-restricted epitope derived from the Flu virus were measured. All 12 subjects mounted a vigorous recall response to Flu as measured by direct Elispot. The overall mean value for the positive control peptide (Flu) at baseline was 2009 SFCs/ 10^6 PBMCs with a SD of 766 and a median value of 2224 SFCs/ 10^6 PBMCs. The overall mean value for the negative control peptide (*P. falciparum*) was 220 ± 155 SFCs/ 10^6 PBMCs with a median value of 235 SFCs/ 10^6 PBMCs. These data support the immune competency of this study cohort.

Biological Responses: HPV-specific Direct Elispot.

Direct Elispot data are presented for all three test peptides from six time points (baseline and weeks 3, 6, 9, 12, and 24) from all 12 subjects with the exception of the final time point for subject 12, who withdrew from the study at week 15 for personal reasons. The week 15 data for this subject was evaluated as a week 24 observation so as not to exclude this subject from the analysis because all end-of-study evaluations were performed at that study visit.

When examining the results of the group as a whole, there is a significant difference over time in mean response to each peptide ($P = 0.008, 0.02,$ and 0.03 for test peptides 2.1, 2.4, and 2.4-C, respectively). There is a significant increase in the mean response at week 24 compared with a baseline for epitopes 2.1 ($P = 0.04$) and 2.4 ($P = 0.02$) and a borderline increase for 2.4-C ($P = 0.08$). There is a significantly lower response with epitope 2.4 compared with each of the other epitopes at week 24, regardless of the baseline value (2.4 versus 2.1, $P = 0.005$; 2.4 versus 2.4-C, $P = 0.02$). The mean values at week 24 increased >6 -fold over baseline (mean percentage increases for epitope 2.1, 2.4, and 2.4-C were 7.73%, 9.26%, and 6.93%, respectively), although this was of borderline significance ($P = 0.06$) due in part to the large SD and the small sample size.

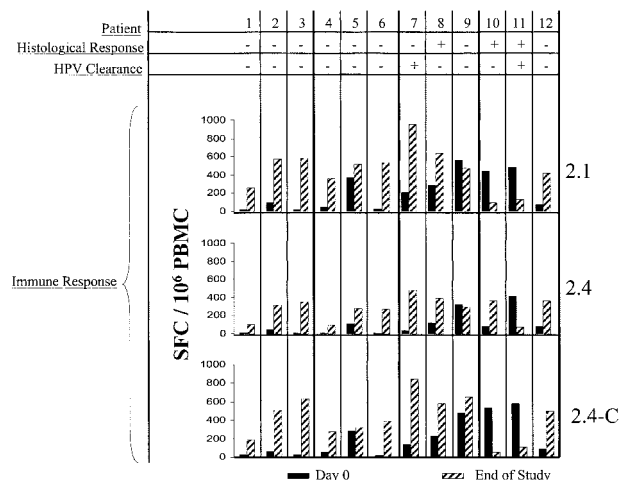


Fig. 1 Elispot results. Elispot results (measured in SFC/ 10^6 PBMC) from baseline and the final evaluation time point for each subject for each of the three test peptides are shown. The final time point occurred at week 24 for subjects 1–11 and week 15 for subject 12. A 2-fold rise was required for a positive result.

When an individual subject's data are analyzed relative to his own baseline with response defined as at least a 2-fold increase over baseline, the direct Elispot demonstrates an increase in HPV-specific, T-cell response levels over the course of the trial in 10 of 12 subjects (all subjects except subjects 9 and 11; Fig. 1). The time point of first response (defined as at least a 2-fold increase over baseline response) for each subject by peptide is shown in Table 3. A – indicates that no response was noted at any of the week 3, 6, 9, 12, or 24 time points. Increases at the final time point (week 24 for subjects 1–11 and week 15 for subject 12) are also observed in these 10 responding subjects (Fig. 1). For subjects 9 and 11, no response is observed for any of the three peptides at week 24. Eight subjects (all subjects except subjects 5, 9, 10, and 11; Fig. 1) display a response to all three HPV peptides at week 24. Baseline values for all three peptides for these four subjects are 200–300 SFCs higher than the values for the eight subjects that responded to all three

Table 3 Patient characteristics and response summary

Subject-dose level	Age (yrs)	Duration of prior HSIL at enrollment (mo)	Week on study that the Elispot first detected a response			HPV response/histological response
			2.1	2.4	2.4-C	
1-50 µg	68	3	12	12	12	-/-
2-50 µg	42	3	3	3	3	-/-
3-50 µg	56	52	6	9	9	-/-
4-100 µg	50	53	12	12	24	-/-
5-100 µg	68	18	— ^a	9	6	-/-
6-100 µg	45	70	12	12	12	-/-
7-200 µg	27	1	24	6	24	+ by PCR/-
8-200 µg	46	12	24	3	3	-/+ (PR)
9-200 µg	62	4	—	—	—	-/-
10-400 µg	40	81	—	6	—	-/+ (PR)
11-400 µg	34	2	—	—	—	+ by PCR/+ (PR)
12-400 µg	30	13	6	6	12	NA/-

^a —, no response noted at any of the week 3, 6, 9, 12, or 24 time points.

peptides, and thus an enhancement of the immune response may have been technically difficult to detect with this assay. Two other subjects, subjects 5 and 10, demonstrate an increase for only one (2.4) of the three peptides. In these cases, the baseline values for this 2.4 peptide were 100–200 SFCs lower than the baseline values for the other two peptides, for which no increase was detected.

HPV Data. The subject who withdrew from the study early (subject 12) did not have an anal swab for HPV testing submitted at his final study evaluation point. Of the remaining 11 subjects, two (subjects 7 and 11) had no detectable HPV-16 at week 24 using the nested PCR assay. Analysis of the semi-quantitative HC data reveals no statistical difference in the results when comparing baseline with the week 24 measurements using either a parametric method (ANOVA for repeated measures) or nonparametric method (Wilcoxon matched pairs) when examining the group as a whole. Each control group (1 and 10 pg/ml) was compared separately, but differences were not statistically significant. Similarly, there is no evidence of a dose-response relationship because no significant decline was seen when looking only at those receiving 200-µg and 400-µg doses or only 400-µg doses. Using this method, substantial variability in the quantity of HPV-16 at baseline as quantified by the 1 pg/ml test is observed. Because the HC assay does not control for the amount of total DNA in the specimen, the variable degree of cellularity expected among anal swabs can confound the analysis.

DISCUSSION

As the incidence of anal cancer rises steadily in the population as a whole and more sharply in specific risk groups, anal cancer and anal HSIL are receiving greater attention from the medical community as well as from at-risk patients. Because of the lack of standard and effective treatment options for the increasingly well-recognized condition of anal dysplasia, new approaches are greatly needed. The E6 or E7 gene products from the more common high-risk HPV types such as HPV-16 and HPV-18 are attractive chemotherapeutic targets because they

are responsible for the oncogenic transformation of the infected cells and are consistently expressed throughout the neoplastic process.

Conventional vaccination most often stimulates a humoral immune response and is typically used prophylactically to prevent disease. A therapeutic vaccination that attempts to clear disease already in place (HPV infection or HPV-related disease such as dysplasia or invasive cancer) is thought to primarily require the induction of a cellular immune response, a requirement not easily accomplished with traditional vaccination strategies. ZYC101 is formulated to invoke such a response.

Reported here are data that demonstrate the ability of encapsulated pDNA (ZYC101)-delivered antigens to activate multivalent HPV-specific immune responses at a cumulative dose as low as 200 µg and to induce histological regression of HSIL at a cumulative dose of ≥800 µg. In the 12–24 weeks after vaccination, three subjects achieved a partial histological response, including one of three subjects treated at the 200-µg dose level and two of three subjects treated at the 400-µg dose level. The investigational agent was well tolerated in all subjects at all dose levels. These encouraging results may suggest a dose-response relationship and certainly support the continued investigation of a therapeutic vaccination strategy for anal dysplasia.

Future studies will need to further explore the dose responsiveness suggested by these findings. Larger, placebo-controlled studies will be required to conclude that spontaneous reversion of HSIL is not responsible. Given the long duration of HSIL in at least some of the responders, this explanation is felt to be highly unlikely. Responding subjects (subjects 8, 10, and 11) had carried a HSIL diagnosis for 12, 81, and 2 months, respectively. Because all of these subjects were also participating in long-term natural history cohort studies, their prior serial anal exams by HRA and histology were well documented. It will also be important for future studies to evaluate the duration of any potential response. For a disease with such a long natural history, durable remissions would be critical to the goal of reduc-

tion in cancer incidence. This study was not designed to evaluate duration of response.

The majority of subjects (10 of 12 subjects tested) mounted an antigen-specific immune response after receiving ZYC101 when tested using a direct IFN- γ Elispot on unexpanded PBMCs and continued to show significant responsiveness at the final follow-up visit 6 months after the initiation of therapy. The group's mean response to each peptide increased over time. The frequent finding of immune activity at week 24, nearly 4 months after the final vaccine dose was delivered, was quite encouraging. Longer-term studies will be required to more fully assess the persistence of the immune response and monitor the durability of any clinical activity. It is unlikely that the frequency of biopsy procedures led to the induction of the immune response in these subjects because all had been receiving exams and biopsies every 3 months before their enrollment in this trial as part of their ongoing care.

Whereas subjects 9 and 11 did not demonstrate a statistically significant increase when using the direct IFN- γ Elispot, both were positive when tested using an Elispot method that involved short-term PBMC culture before setting up the assay. Thus, all subjects show an immune response to the HPV peptides, with 10 of 12 subjects showing increases over baseline at week 24 as demonstrated by the direct Elispot assay. As discussed above and demonstrated in Fig. 1, responses to the test peptides were not commonly seen in those with higher levels of baseline activity. In general, when subjects enter the study with elevated levels of preexisting T-cell responses, it becomes difficult to demonstrate a drug-induced 2-fold elevation to score the subject as a responder. The significance of higher baseline levels of immune activity to one or more test peptides in several subjects, all of whom had HSIL at study entry, remains unclear. The disappearance of HSIL, combined with the clearance of anal HPV-16 infection by PCR and a positive expanded Elispot assay result for subject 11, supports the presumption that the lack of detectable increase in the direct Elispot assay is most likely a false negative in this case. Using a panel of assay formats helps to address these situations when they arise.

The small sample size of a Phase I safety study precludes us from demonstrating a statistical correlation among the development of an immune response, a histological response, and clearance of anal HPV-16 infection, if such a correlation exists. No significant trends were noted when comparing results from these three different end points. Nor do we see anything remarkable about the immune assay results for the responders (subjects 8, 10, or 11) in terms of the strength of the immune response (either the absolute or relative rise), time to response, or consistency of results among assays. For these assays, only three of the six known epitopes were studied because of constraints on available PBMCs. It remains possible that immune responses to peptides that were not tested here could be better predictors of clinical outcome. There may be other explanations for the lack of a correlation between immune response and histological response. For example, some subjects may have had concurrent anal infection with other high-risk HPV types. Because the antigens encoded by ZYC101 are highly specific to HPV-16, minimal cross-reactivity may be expected outside the subtypes closely related to HPV-16. Although we did not look for anal infection with other high-risk HPV types, it is known that infection with multiple HPV types can occur. If concurrent infection with

other high-risk HPV types occurred, persistence of neoplasia might have been expected, even if all HPV-16-induced neoplastic cells had been eradicated. The low rate of HPV-16 clearance, however, would suggest that this is not a primary contributing factor. Another explanation is that a histological or HPV response could take longer than 6 months in some patients. If the time to best response is longer than the duration of our study, then the abbreviated follow-up for the third subject in the 400- μ g dose cohort may have been important. His time on study ended at week 15 because of personal reasons, although by week 15 he still had clinical and histological evidence of HSIL. Longer follow-up will be important in future studies not only to assess the durability of responses but also to determine the time to maximum response.

This investigational drug was specifically designed as a proof of concept agent for testing in those with HPV-16 infection and a HLA-A2 haplotype. A second generation product extended to target a wider range of HPV and HLA haplotypes has been developed, which will be much more applicable. Only 25% of those screened for inclusion in this study were found to be eligible candidates for the ZYC101 agent based on HLA and HPV testing. The new agent, ZYC101a, is currently being tested in a Phase II clinical study for cervical dysplasia. Any effects of concurrent infection with other high-risk HPV types is thus likely to be less important for patients treated with the new formulation. Subsequent Phase II and Phase III studies will be required to further evaluate the efficacy of either agent in the treatment of anal HSIL and the prevention of invasive anal cancer. Longer follow-up to clarify the time to response in all subjects and to measure the duration of response will be important in subsequent studies. Although the Elispot data do not suggest a dose-response relationship, the histology results might. Because there was minimal toxicity seen at any dose level, subsequent Phase II studies are likely to further explore one of the two higher dose levels.

ACKNOWLEDGMENTS

We thank Niki Nibbe for editorial assistance. We also thank Joe Thatcher, now deceased, for his generous nature, his wonderful care of participating subjects and other patients under his direction, and his meticulous oversight of this clinical trial.

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