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ORIGINAL ARTICLE

Correlation between Interferon-gamma Production in vitro and Clinical Response to Immunotherapy in Recalcitrant Genital Warts

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ABSTRACT

Introduction: *Genital warts are the most common sexually transmitted disease caused by human papillomavirus (HPV) with significant morbidity and discomfort. Treatment of genital warts is often challenging. Intralesional immunotherapy appears to be a hopeful, efficient, and safe treatment procedure for genital warts.* ***Objectives:*** *This study aimed to evaluate a new line of therapy for recalcitrant genital warts and compared its efficacy with already used protocols of immunotherapy. Also, we aim to assess significance of interferon gamma (IFN-γ) levels in cell culture treated with either Candida antigen, imiquimod, or both as predictor of successful response to immunotherapy.* ***Methodology:*** *30 patients with recalcitrant genital warts were randomly allocated into one of three groups. Group (A) therapy (10 patients) received intralesional injection of Candida antigen, Group (B) therapy (10 patients) received topical therapy with imiquimod 5% cream, Group (C) therapy (10 patients) received combined therapy of intralesional injection of candida antigen and topical therapy with imiquimod 5%. Response to therapy was assessed clinically by the decline in size of warts and photographic difference. Blood samples were collected from patients before therapy, cultured with either Candida antigen, Imiquimod (R837) or both for IFN-γ level assessment.* ***Results:*** *The complete therapeutic response was higher in group C (50%) than in group B (30%) and group A (20%). Although IFN-γ levels in group C and group B (28 ±10.5 IU/mL, 18.8±8.8 IU/mL respectively) were statistically highly significant compared to those in group A (2.645± 1.143 IU/mL) ($p< 0.05$), IFN-γ levels can be used as a good predictor of the therapeutic response in group A and group B but not in group C.* ***Conclusion:*** *New combination regimen of intralesional Candida immunotherapy and topical application of 5% imiquimod appears to be a promising therapy option in patients with recalcitrant genital warts.*

INTRODUCTION

Genital warts are the most common sexually transmitted disease caused by human papillomavirus with significant morbidity and discomfort[1,2]. Their incidence is increasing rapidly and have been associated intimately with cervical neoplasia and another genital tract neoplasms[3].

Treatment of genital warts is often challenging. It depends on two main therapeutic options: the first is the conventional destructive method, which includes treatment with chemical cautery, cryotherapy, electrocauterization, surgical excision, and laser ablation, and the second is immunotherapy, which is based on the activation of the immune system to suppress activity of human papillomavirus[4].

There are several methods for immunotherapy administration in warts. The first provokes a contact hypersensitivity reaction through topical application of certain immune modulators like imiquimod[5]. The efficacy of imiquimod in treating genital warts is mediated by activation of components of the innate and cell mediated immunity, as it has no direct antiviral or antiproliferative properties[6].

The second method is intralesional immunotherapy[5]. It appears to be a hopeful, effective, and safe treatment procedure for genital warts. Several authors have observed that effective intralesional antigen immunotherapy is associated with a predominant T helper (Th1) cytokines such as IFN-γ, IL-2, and IL-12, while the lack of response to intralesional immunotherapy is accompanied with high levels of Th2 cytokines such as IL-10 and IL-4[7].

It is exciting is that this immunotherapy has a potential to eradicate not only the treated wart but also distant lesions. Different immunotherapeutic antigens for intralesional injection have been used by many authors. These embrace, Candida antigen, mumps

antigen, MMR vaccine, tuberculin and BCG vaccine[8]. To our knowledge, there are no data about how to predict successful immunotherapy of genital warts. In this paper, we evaluate IFN-γ levels in cell culture treated with either Candida antigen, imiquimod, or both as predictor of response to immunotherapy. Moreover, we introduced a new protocol of immunotherapy of genital warts and compared its efficacy with already used protocols of immunotherapy.

METHODOLOGY

Study design and Subjects

This study was an open label, randomized, uncontrolled, clinical trial. 30 patients with recalcitrant genital warts were recruited. All the patients were referred to the Immunology Research Laboratory at Microbiology and Immunology Department, Zagazig University Hospitals, Zagazig, from June 2016 to 2017. The study was explained to each patient and the eligibility was determined. An informed consent was taken from the patients. This study was approved by IRB of Medical School at Zagazig University. Recalcitrant warts were defined as warts persistent for more than two years despite therapy with at least two dissimilar modalities[9]. Patients with acute febrile illness, previous intralesional immunotherapy with BCG, MMR or candida antigen, hypersensitivity to intradermally Candida antigen injection, known active or recent yeast infection, pregnant or lactating females and immunocompromised patients were excluded. All patients were subjected to full history taking; clinical examination 72 hours before the first treatment session; photography of the lesions; intralesional injection of candida antigen, topical therapy with imiquimod 5% (Aldara) or combined therapy according to the group allocation and blood sampling 72 hours before starting treatment.

Patients

The patients were randomly allocated into one of three groups. Group (A) therapy: included 10 patients received intralesional injection of 0.3 ml of 1/1000 solution of Candida antigen without pre-sensitization (Candida albicans 1:20 w/v10 ml vial, Allergy Laboratories, INC. Oklahoma City, USA) into the largest wart using an insulin syringe. Injections were performed every 2 weeks until the warts completely cleared or up to 3 treatment sessions[10]. Group (B) therapy: included 10 patients received topical therapy with imiquimod 5% cream for application overnight (for a period of 6 to 10 hours), 3 times per week. Until the warts completely cleared, or for up to 3 months. Patients were informed with the use of the topical therapy and the first dose was applied by the patient under supervision. Patients were instructed to shower before topical application of imiquimod 5% cream (but not during day period when the cream was on the skin. Group (C) therapy: included 10 patients received both therapy with intralesional injection of candida antigen and topical therapy with imiquimod 5%. Follow-up of patients was performed every 3 weeks for 3 months for clinical evaluation of results and any side effect. Response to treatment was assessed by the decrease in size of warts and photographic difference. The complete disappearance of the warts and return of normal skin was considered complete response, while partial response was decrease in wart size by 50–99%, and no response was 0–49% decrease in wart size.

Isolation, Culture, and Stimulation of PBMCs

Heparinized whole blood was obtained from all patients in three groups before initiating therapy. PBMCs were isolated by centrifugation over a Histopaque 1077 density gradient (Sigma Diagnostic, St.Louis, MO) and washed twice with PBS/2 % fetal calf serum (FCS). After washing, the cells were cultured at 2×10^6cells/ml in RPMI 1640 (Sigma Diagnostic, St. Louis, MO) supplemented with L-glutamine, 100 U/ml penicillin, 100µg/ml streptomycin (Invitrogen Cooperation, Grand Island,NY), and 10 % FCS in the presence of either 10 µl of Candida antigen, Imiquimod (R837) (1µg/ml) (R837 was made up at a concentration of 1 mg/ml in sterile endotoxin-free water, InvivoGen, San Diego, CA) or 10 µl of Candida antigen + Imiquimod (R837)(1 µg/ml) for 48 hours at 37˚C in humidified 5% CO2. At the end of the 48 hours culture period, PBMCs were tested for viability by Trypan blue exclusion and counted. Culture supernatants were harvested, collected, and stored at -20°C for IFN-γ measurement.

Measurement of IFN-γ

The levels of IFN-γ in the culture supernatants were measured by sandwich enzyme-linked immunosorbent assay (ELISA) (Invitrogen, USA, Catalog no. KAC1231) according to the manufacturer's protocols. The minimal detection level of IFN-γ was 0.03 IU/mL.

Statistical analysis

Analysis was performed using IBM SPSS (Statistical Program for Social Science Version 20; SPSS Inc., Chicago, IL, USA). Quantitative variables were expressed as means (±SD) and qualitative ones as percentages. Chi square test was used for comparison of qualitative data and ANOVA test for comparison between three groups. The correlation between IFN-γ level and response to therapy in all groups was assessed using Pearson's correlation test. P-values <0.05 were considered significant.

Receiver operating characteristic (ROC) curve analysis was used to identify optimal cut-off values of IFN-γ of maximum sensitivity and specificity for prediction of response to each therapy. ROC curve was performed using Microsoft® Excel version 16.0, 2016/XLSTAT (Version 19.4, Addinsoft, Inc., Brooklyn, NY, USA**;** http://www.xlstat.com; 2017).

RESULTS

In the present study, 30 patients with recalcitrant genital warts were enrolled. They were divided into three groups, each included 10 patients. The group (A) was treated by intralesional injection of Candida antigen, the group (B) was treated by topical therapy with imiquimod 5% cream, while the group (C) received both therapies. Table (1) summarizes baseline characteristics of the studied patients in the three groups. No statistically significant difference ($p > 0.05$) was seen between the study groups concerning age and gender.

Clinical response

The results revealed that complete therapeutic response was higher in group C (50%) than in group A (20%) and group B (30%). Partial therapeutic response was higher in group A (60%) than in group B (30%) and group C (20%). Absence of therapeutic response in group A, group B and group C was 20%, 40% and 30%, respectively as shown in Figure (1). No correlation was found between gender and the therapeutic response in group A, group B and group C ($p = 0.258$, $p = 0.611$ and $p = 0.679$, respectively). Patient age did not show any relation to the therapeutic response in group A and group C ($p = 0.593$ and $p = 0.764$, respectively). But, there was a statistically significant positive correlation between age and the therapeutic response in group B ($p < 0.05$).

IFN-γ Levels

The results are shown in Table (2). The IFN-γ levels in group C and group B (28 ±10.5 IU/mL, 18.8±8.8 IU/mL respectively) were statistically highly significant compared to those in group A (2.645± 1.143 IU/mL) ($p< 0.05$). Moreover, the IFN-γ levels in group C was statistically significant different from group B.

No statistically significant association was found between the levels of IFN-γ and the different clinical variables, including age, gender. There was a statistically significant positive correlation between IFN-γ levels and therapeutic response in group A and group B ($p< 0.05$). While there was a statistically significant negative correlation between IFN-γ levels and therapeutic response in group C ($p< 0.05$) as shown in Figure (2).

Our results revealed that IFN-γ at cut off point 0.6 had a sensitivity of 100%, specificity of 50% and accuracy of 88.9% in group A, indicating that it can be used as a good predictor of the therapeutic response to intralesional injection of Candida antigen. Also in group B, IFN-γ can be used as a good predictor of the therapeutic response to Aldara topical therapy as IFN-γ at cut off point 10.1 had a sensitivity of 100%, specificity of 75% and accuracy of 88.9%. On the other hand, IFN-γ was not a good predictor of the therapeutic response to combined therapy in group C (Table 3) and Figure 3).

Table 1: Baseline characteristics of patients in three groups

	Group A	*Group B*	*Group C*	P *value*
Number of patients	10	10	10	
Age (mean± SD) years	31.64 ±6.55	31.00 ±9.71	30.70 ±8.42	0.971
Gender (N%)				
Male	2 (20%)	3 (30%)	3 (30%)	0.843
Female	8 (80%)	7 (70%)	7 (70%)	

SD, standard deviation

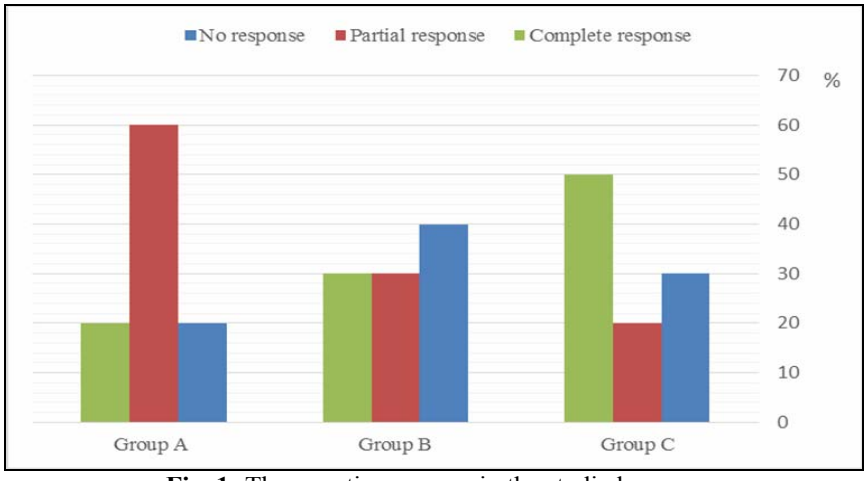

Fig. 1: Therapeutic response in the studied groups

Table 2: IFN-γ levels in the studied groups

IFN-γ IU/mL	*Group A*	*Group B*	*Group C*
Mean	2.645± 1.14	18.8±8.8*	28.±10.5*, **
Range	0.6- 5.0	6-35	14-42

* Significant differences from group A, **Significant differences from group C

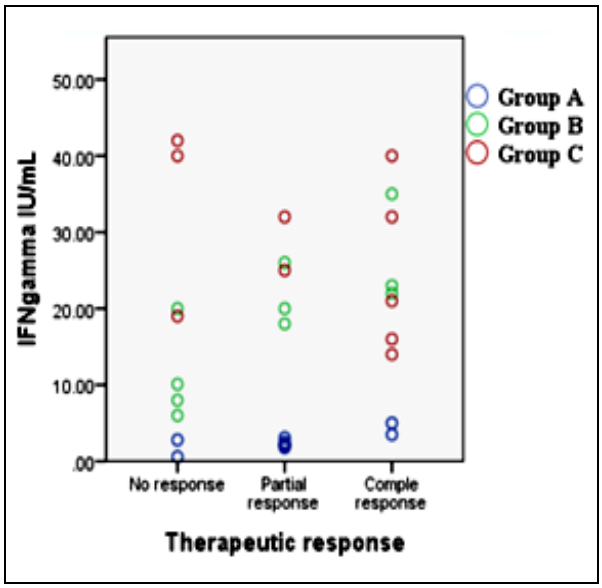

Fig. 2: Relation between IFNγ levels and therapeutic response in the studied groups

Table 3: Validity of IFN-γ in prediction of therapeutic response

	Cutoff	*AUC*	*Sensitivity*	*Specificity*	*+PV*	*-PV*	*Accuracy*	p-*value*
Group A	>0.6	0.643	100	50	87.5	100	88.9	0.689
Group B	>10.1	0.925	100	75	75	83.3	88.9	<0.0001
Group C	>19	0.25	66.6	33.3	66.6	33.3	55.6	0.243

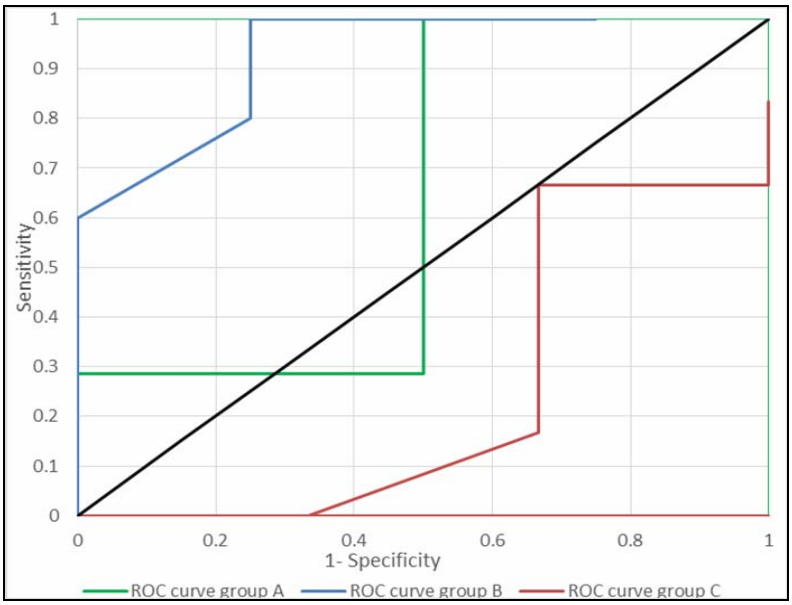

Fig. 3: ROC curves for IFN-γ levels in the studied groups

DISCUSSION

Genital warts are the most prevalent form of viral genital mucosal lesions and are caused by infection with several types of HPV most commonly (types 6 and 11)[11]. Treatment of genital warts is not always a simple affair as many patients fail to respond to all conventional modes of treatment. In addition, there are patients in whom warts do respond but there are multiple recurrences. Recalcitrant warts may due to a deficiency in cellular immunity to HPV. Various explanations like inability of T lymphocytes to recruit to sites of HPV infection, failure of clonal expansion of lymphocytes to adequate stimulus, defect in memory T cells to HPV, and weak effector response have been hypothesized[8]. So, immunotherapy is a promising modality option for recalcitrant warts that principally stimulate the immunologic response to HPV, thereby leading to complete resolution and decreased recurrences[5].

Our paper introduces a new therapeutic modality for recalcitrant genital warts which is an immunotherapy combination of intralesional candida injection and topical application of 5% imiquimod. The results revealed that complete therapeutic response was higher in group C (50%) that was treated with combination immunotherapy than in group B (30%) who received topical application of 5% imiquimod alone and group A (20%) who received intralesional candida immunotherapy alone. Thus, we proposed that the immunity against HPV acquired by intralesional immunotherapy and enhanced by imiquimod could exert a positive effect on treatment of recalcitrant genital warts.

Similarly, King et al. further discussed the idea of combination immunotherapy for genital warts therapy. But in their study thirteen patients received either 0.3 mL of single antigen treatment (mumps, Candida, or Trichophyton), or combination immunotherapy with 0.1 mL each of mumps, Candida, and Trichophyton. Complete resolution rate in the combination therapy group was 60%. They concluded that combination therapy may further enhances outcomes by addressing multiple immune pathways simultaneously[12].

Concerning our results in group A that received intralesional candida immunotherapy alone (20% complete response) were slightly lower than those reported by Majid & Imran (56%)[5], Horn et al. (54%)[13], Signore (51%)[14], Clifton et al. (47%)[15] and Alikhan et al. (39%)[16] and much lower than that reported by Maroon et al. (87%)[17], Kim et al. (82%)[18], Khozeimeh et al. (76.7%)[19]. All previous studies of intralesional candida immunotherapy were done in patients with common and extragenital warts. However, studies on recalcitrant genital warts are still lacking.

Our protocol of intralesional candida immunotherapy was without pre-sensitization. In contrast to most studies, a pre-sensitization test was performed by intradermal injection of 0.1 ml of the antigen before the initiation of the trial, where responder to test were joined in the study, and the non-responders were excluded. Our protocol would be more applicable in terms of time, cost and patient compliance. This has been strengthened by the absence of a significant association between the clinical response and the size of sensitization reaction as observed by several studies[7].

Imiquimod is a topical immunomodulator that was US FDA approved for the treatment of external genital warts in 1997 with antiviral and antitumor activity[8]. Regarding our results in imiquimod therapy in group B complete clearance was 30% in accordance with Hengge et al. study[20].However, higher complete clearance rates of 40%, 50%, 52% 59% and 67% were reported in placebo controlled trials of imiquimod in patients with anogenital warts[1,21-24]. The divergence of the results of these studies compared to our study may be due to differences in the study population selected for treatment, number of the studied patients, type, duration and resistance of warts, protocol and number of treatment sessions and score for evaluation of complete response. Also, Arany et al.[25] demonstrated that there is a correlation between of complete response to imiquimod therapy and high levels of signal transducer and activator of transcription 1 (STAT1) and interferon response factor 1 (IRF1).

The definite mechanism of action of combination immunotherapy, including intralesional Candida injection and topical imiquimod is still unclear. They may be mediated through induction of helper T (Th) 1 cytokines that stimulate a strong immune response against HPV and cure the warts[26]. Horn et al. have noticed that the intralesional immunotherapy is accompanied with proliferation of peripheral blood mononuclear cells that stimulates Th1 cytokine responses, which in turn activate cytotoxic T cells and natural killer cells to eradicate HPV-infected cells[13]. Furthermore, it has also been observed that wart resolution after intralesional Candida immunotherapy was accompanied with detection of specific T cells to HPV-57 Ll-peptide, indicating that these Ll-specific T cells may be responsible for wart regression. Moreover, the deliberate activation of TLRs through administration of a TLR7 agonist imiquimod improves presentation of HPV to CD8+ T cells in HPV-associated malignancy, suggesting that therapeutic intervention with imiquimod may be a viable treatment strategy[27].

No correlation was found between gender and the therapeutic response in immunotherapy. These results are in good agreement with other studies which have shown that gender had no effect on the therapeutic response in immunotherapy-treated warts[13,15,19,28]. Patient age did not show any relation to the therapeutic response in immunotherapy. Similarly, Gamil et al.[28] stated that age had no effect on the therapeutic response

in immunotherapy treated warts. In another study performed in 2001, no significant difference was observed between the immunotherapy and cryotherapy methods regarding age and therapeutic response[15]. In contrast, other authors have observed a significant association between the response for intralesional immunotherapy and the younger age less than 40 years who has robust immune response[13].

IFN-γ, which is secreted by TH1 cells, cytotoxic T cells, and stimulated natural killer cells, is known to be a main contributor to an efficient TH1-type immune response against HPV infection. Several authors reported that IFN-γ concentrations either in plasma or serum was not detectable in patients with cervical cancer and healthy controls and serum positive for IFN-γ did not have an association with clearance or persistence of HPV infection[29].That is why we investigated IFN-γ production in human PBMC cultures treated with either Candida antigen or imiquimod or both. We observed that both imiquimod and Candida antigen were capable of augmenting IFN-γ concentrations (28±10.5 IU/mL) in human PBMC cultures. Interestingly, both compounds together were statistically highly significant ($p< 0.05$) in inducing IFN-γ compared to imiquimod alone (18.8±8.8 IU/mL) or Candida antigen alone (2.645± 1.143 IU/mL).

There was a statistically significant ($p< 0.05$) positive correlation between IFN-γ production in cell culture treated with Candida antigen or imiquimod and therapeutic response in group A and group B. However, there was a statistically significant negative correlation between IFN-γ production in cell culture treated with both and therapeutic response in group C ($p< 0.05$). Therefore, IFN-γ can be used as a good predictor of the therapeutic response to either intralesional injection of Candida antigen or Aldara topical therapy. On the other hand, IFN-γ level was not a good predictor of the therapeutic response to combined therapy in group C. These results were explained by Minguela et al.[30] who observed that low-level secretion of IFN-γ is pro-inflammatory, but higher levels are anti-inflammatory in an experimental autoimmune encephalomyelitis model.

HPV infections are entirely intra-epithelial and HPV attack should be detected by the Langerhans cell (LC) which is responsible for processing and presentation of HPV antigens to naïve T cells in the draining lymph node[31]. Interleukin-12 is an essential element for generation of a Th1 response and IFN-γ secretion. The evidence came from a study on genital warts patients on imiquimod therapy demonstrated a significant association between IFN-γ and IL-12 p40 mRNA levels in biopsies taken from these patients. Additionally, the importance of IFN-γ and IL-12 in the clearance of these patients was observed[32]. In contrast, the natural killer T (NKT) cells can suppress cellular immune responses to HPV, either in the priming phase in secondary lymphoid organs, or during the effector phase in the skin[28]. Collectively, IFN-γ production depends on many factors including antigen delivery, antigen processing and presentation, the responding T-cell repertoire, IL-12, T regulatory cells and NKT cells and adhesion molecules required for lymphocyte recruitment.

CONCLUSION

New combination regimen of intralesional Candida immunotherapy and topical application of 5% imiquimod appears to be a promising therapy option in patients with recalcitrant genital warts.

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