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IMMUNOTHERAPY IN THE TREATMENT OF LARINGEAL RECURRENT RESPIRATORY PAPILLOMATOSIS (CLINICAL STUDY)

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ABSTRACT

Introduction: Recurrent respiratory papillomatosis (RRP) is a relatively rare disease. Although histologically RRP is a benign entity, it is a major clinical problem because of its location, often dramatic presentation due to significant airway obstruction, the ongoing resistance to therapies, frequent recurrences, spread to the lower respiratory tract and esophagus (aggressive forms) and the possibility of malignant transformation into squamous cell carcinoma.
Materials and Methods*:* We present our observations on 17RRP patients undergoing transoral carbon dioxide laser microsurgery with subsequent immunotherapy. Some patients have had more than one surgery. We track treatment options of recurrent respiratory papillomatosis that gives us this combined approach.
Results and Discussion: Laser excision has palliative effect, but through it we provide released airways and improve the quality of the voice. Starting of application of immunotherapy with Calgevax BCG in RRP we observe attenuation and discharge of relapses.
Conclusion: The laser surgery can't achieve a cure and stop the spread of the lesions, but it is extremely important in restoring airway patency. By applying Calgevax BCG in laryngeal papillomatosis achieve, if not cure, then reduce the frequency of relapses and improve the quality of life of patients. Combining it with immunotherapy, we aim to maximize the therapeutic effect.

INTRODUCTION

Recurrent respiratory papillomatosis (RRP) is a relatively rare disease, characterized with proliferation of recurrent squamous papillomas in the aerodigestive tract, (Draganov *et al*., 2006; Todorov, 2009; Todorov, 2009). Although histologically RRP is a benign entity and over 90% of the cases are associated with low-risk types (6 and 11), it is a major clinical problem, (Goon *et al*., 2008; Gallagher and Derkay, 2008). It is possible a malignant degeneration to squamous cell carcinoma with very poor prognosis which could be observed in 3-5% of patients. Extension into the lower airways occurs in approximately 17% of patients. In aggressive forms time descent from the larynx into the lower respiratory tract is typically 12 years, but there are patients in which the diffusion occurs very rapidly, (Gelinas *et al*., 2008; Blumin *et al*., 2009; Avramov *et al*., 2014). Etiologic factors for RRP are human papilloma virus (HPV) that are uncovered double-stranded DNA viruses from the family Papilloma viridae. Having in mind that asymptomatic HPV infection is widely spread among the population, the low incidence of RRP raises the question about the immune mechanisms that might determine the occurrence, relapses, and malignisation (Blumin *et al*., 2009). We divide papillomatosis of juvenile (type I) and red papillomatosis in adults (type II).

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The juvenile form occurs in 4.5 per 100 000, mainly in children under 5 years of age with 25% of affected being infants, with number of boys equal to girls. It is carracterised by multiple papillomas and show a greater tendency to relapse. The red papillomatosis occurs in 2 per 100 000, after the age of 20, with men prevailing, is carracterized by multiple papillomas, frequent relapse and in 3-5% of the cases malignant transformation in squamous cell carcinoma with a very poor prognosis. Last ten years we have seen a sharp increase in the proportion of red papillomatosis or type II as opposed to juvenile, which occurs rarely. In the treatment of RRP surgical, conservative and combined approach are applied. The most commonly used therapies for the treatment of the RRP at present are: laryngeal microsurgery; electro surgery, carbon dioxide (CO_2) laser surgery; treatment with α-Interferon; therapy with Cidofovir and PDT with ALA-5-amino-levulinic acid, (Avramov *et al*., 2014). The palliative treatment effect of conventional and laser surgery has fostered the application of alternative treatment protocols. Multiple combined protocols including antivirals (Ribavirin and Cidofovir), vitamins and oligoelements, cyclooxygenase-2, EGFR or IFN-γ have been applicated without definitive success.

All therapies listed above have their place in the treatment of RRP, but none of them alone can cope with this disease. Different antiviral drugs used have a contingent effect in certain patients, but in other patients without significant differences in clinical manifestations no effect is observed, (Avidano and Singleton, 1995; Chadha and James, 2007; Ciardiello and Tortora, 2008; DeVoti *et al*., 2004; Klebe and Henderson, 2013; Leventhal *et al*., 1988). Low incidence of disease amid widespread carrier HPV raises the question of imunopatogenetic mechanisms of occurrence, recurrence and malignant progression of RRP. HPV is controlled by the natural and adaptive mechanisms of cell-mediated immunity. Established leadership Th1 CD4 and CD8 cytotoxic T lymphocytes. CD4 T lymphocytes differentiate into several effector and regulatory subpopulations: Th1/Th2/Th17/Treg, which in turn affect CD8 T response. Violation of this balance leads to persistence and expression of infection. Th1 cytokines potentiate the antiviralimmunity versus Th2. Unsatisfactory results of conventional treatment provokes necessity to consider a possible link to the frequent recurrence of ineffective anti-viral immune response, (Goon *et al*., 2008).

Recurrent respiratory papillomatosis is a chronic viral inflammation. Persistent infections are usually characterized with imbalanced differentiation of effector, memory and regulatory subsets. Modulation of the immune system with therapeutic lines started in 1796. Therefore, we focusour interest in immunotherapy of this disease and our efforts on the monitoring of the immune response and in particular to T-cell immunity in patients. We assume that inefficient antiviral response is characterized by: abnormal differentiation of CD4 and CD8 effector T cells and imbalance of memory/effector subpopulations. We chose Bulgarian immunomodulator Calgevax® (BCG), which is a potent stimulator of Th1 response. The medicament has established therapeutic effect in superficial bladder carcinoma and melanoma. Data of BCG effects in chronic HPV infection are limited, (Damyanov *et al*., 1994; Conti-Freitas *et al*., 2009). The aim of our studyis to follow the results of the subsequent surgery immunotherapy with Calgevax® (BCG) in Bulgarian RRP patients in order to identify the activation of natural cellular mechanisms combined with differentiation of IFN-γ, CD4 (Th1) and CD8 T cells, while a regulatory CD4+FoxP3+ T subset prevents the antigen-specific clones from exhaustion, and potentiates formation of memory.

MATERIAL AND METHODS

We present our observations onRRP patients for 5 years period (2010-2014). The followedgroup consist of 17 patients (11men and 6 women)undergoing minimally invasive transoral carbon dioxide laser microsurgery (TLM) with Sharplan - 40C, Izrael. Before deciding to initiate immunotherapy with Calgevax® (BCG) in these patients they were followed for a period of 2 to 12 years. In some of them we have seen up to 12 relapses per year in different periods,enforced to perform a tracheotomy to ensure airway patency.The average number of previous surgical interventions is 21.71±59.23. In some patients the lesions have been asymptomatic at the beginning and are diagnosed incidentaly during examination about complaints for a recent change in voice or hoarseness. Those symptoms can be divided in: change the voice (mild, moderate, strong) and difficulty in breathing (slightly marked, moderate, severe). RRP diagnostic examination consist in: indirect laryngoscopy, scopy with flexible laryngoscope and rigid bronchoscope or microlaryngoscopy. Typically multiple lesions are observed, the most affected parts of the larynx being the vocal cords. RRP can be diagnosed anywhere in the larynx.

We perform aminimally invasive surgical treatment of laryngeal papillomatosis under endotracheal anesthesia, transient apnea or high frequency jet ventilation using a microscope and endoscopes. We recommend the last one for disseminated forms of papillomatosis, to ensure maximum visibility in the operational area. Laser beam with low power, which allows to controlled remove of papillomasis used for treatment the laryngeal lesions. Our goal is adequately removing lesions to leave islands of healthy mucosa between them, and thereby achieve faster reepithelialization of the endolarynx. (Conti-Freitas *et al*., 2009; Tchalakov *et al*., 2011) Great convenience of TLM is that even at very advanced form of papillomatosis, after thelaser intervention patients can be extubated. Removed papillomas are sent for pathologic examination. Polymerase chain reaction (PCR) for viral typization is done in the Laboratory of Molecular Virology at the Military Medical Academy Sofia, (Draganov *et al*., 2006). Before starting treatment, we perform immunologic assay (peripheral blood - 12 ml with anticoagulant heparin Na) for exclusion severe immune deficiency and tuberculin skin test (Mantoux). We use following methods for assessing cellular immune response - immunophenotyping of leukocyte populations and quantification of cytokines in the supernatant.

We perform immune modulation with Calgevax® included 6 to 12 applications of 2,56 x 10^8 clony forming unit (CFU) transdermally for the period of 45 ±5days, according to manufacturer's instructions for transdermal administration by scarification, without causing excessive bleeding, on an area of 25 cm² on the shoulder of the patient, local BCG application is impossible. We made a grid - 10 horizontal x 10 vertical

lines, on the skin of the first four patients at the beginning, according to the manufacturer's instructions. The next patients had a grid with 25 horizontal and 25 vertical lines at a distance of 2 mm, and we founda better absorption of the medication in the skin (Avramov *et al*., 2014). The parameters of cellular immunity (as a percentage and absolute numbers of lymphocyte subsets) and cytokine expression before and during treatment are used for evaluationof the effect of immunotherapy, done in National Referral Laboratory on Immunology, NCIPD - Sofia. The study met the ethical principles of the Helsinki Declaration, and was carried out after approval by the University hospital "Tsaritza Yoanna" review committee; a written informed consent was obtained from all patients.

RESULTS AND DISCUSSION

Analysis of demographic characteristics of the patientsshows that the mean age of the patients is 35.41 ± 11.74 years ranged from 15 to 58 years. More than half of them (52,94%) are under the age of 33. The average weight of patients is 69.00±15.09 kg, ranged from 43.0 to 99.0 kg. Medical history of the patients is not givingany evidence of a link between occupational hazards and disease onset and progression. HPV6 is detected in 13 cases (76.47%), HPV11 - in 2 (11,76%).In 2 patients (11,76%) the infection is mixed (HPV6/HPV11). Were observethe following locations of the lesions: true vocal cords - unilaterally (n=1), true vocal cords - bilaterally (n=6), glottis and supraglottic (n=3), glottis and subglottic (n=6), entire larynx (n=1). Patients are monitored for average 42 months. We didn't observe side effects or complications related to Calgevax treatment. During treatment more patients are strictly observed for a period of 45 ± 5-days between visits. In the 20 months period of immunotherapy, relapse occurred in 2 patients - between the 4th and 5th month from the beginning of therapy. They are reoperated and continue with the immunotherapy thereafter. The results of the therapy are followed for examination of the averages of key indicators of T cell immunity. They are presented to Figures 1, 2 and 3. With gray marked values of these parameters before initiating therapy, with green - after 20 months of immunomodulation and a dotted line - levels in healthy controls. Figure 1 tracks values Th1 lymphocytic subpopulation. Figure 2 tracks values Tc1 lymphocytic subpopulation. Figure 3 tracks values Th1/Th2cytokine background - ratio between the levels of IFN-γ and IL-4.

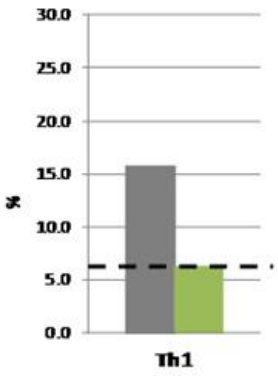

Fig. 1. Recovery of the key indicators of T-cell immune response after 20 months of treatment with Calgevax: averages of Th1

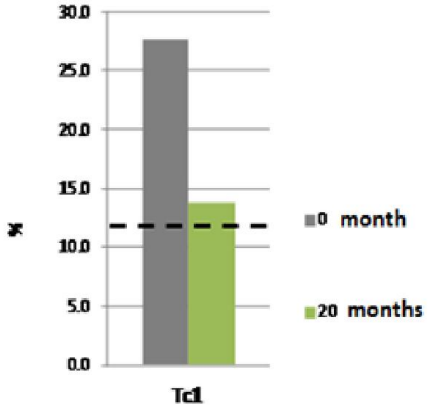

Fig. 2. Tc1 lymphocytes after the 20th month of immunomodulation, compared with levels in healthy controls marked with a dotted line.

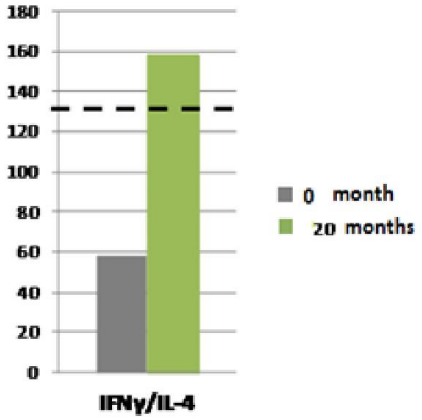

Fig. 3. Normalization of the Th1/Th2 cytokine profile after 20 months of treatment with Calgevax: ratio between the levels of IFN-γ and IL-4 after 24 hours non-specific stimulation of peripheral blood lymphocytes with phytohemagglutinin at 0 and 20 months of therapy as compared to the levels in healthy controls, marked with a dotted line

The results of immunological study on the 20-th month from the beginning of the immunotherapy with Calgevax (BCG) are:

- normalized levels of effector Th1 and Tc1 lymphocyte subpopulations;
- stimulated secretion of Th1 cytokines (IFN-γ, IL-2);
- restored Th1/ Th2 cytokine background.

These results strongly support the application of Calgevax as immune modulation therapy for RPR patients after surgery. Unfortunately, up to the moment there is no specific antiviral treatment, allowing the complete elimination of chronic HPV infection. Surgery remains the only therapeutic approach currently in RRP which maintain airway patency. Transoral laser microsurgery does not reduce the number of relapses, (Goon *et al*., 2008; Tchalakov *et al*., 2011; Tchalakov *et al*., 2010). Controlled trials failed to provide sufficient evidence for reliable conclusions about the effectiveness of antivirals (Ribavirin, Cidofovir, Indole-3-carbinol) as adjuvant therapy. According to our data, adjuvant therapy with IFN-α, does not completely prevent relapse in RRP, (Chadha and James, 2007). BCG is currently the most successful agent used for

cancer immunotherapy. The intravesical BCG immunotherapy of superficial bladder cancer is considered a "golden standard", (Nikolova *et al*., 2009; Shintani *et al*., 2007; Brandau *et al*., 2001). However, the mechanism of BCG beneficial anti-tumor effects are not yet fully understood. It is suggested that BCG helpsstrengthen cellular immune response in a non-specific manner, by activation of macrophages and lymphocytes, which point to its use, (Razack, 2007). Limited experience with BCG-therapy in laryngeal carcinoma has increased TNF-α and IL-6 secretion by macrophages as well as activation of cytotoxic T lymphocytes with NK (CD16+CD56+ CD3-phenotype. (Conti-Freitas *et al*., 2009)

The presence of recurrence in two of the treated cases showed that BCG immune modulation may not be effective consistently. After the completion of treatment patients have been monitored every three months. In the range between 6-th and 9-th months fromthe end of the therapy,relapse occurred in five patients. After re-operation the immunotherapy has been renewed. In five of patients with frequent recurrences before starting immunotherapy we observed no recurrences during follow-up period. In all observed patients we don't find clinical and immunological characteristics which can help to identify the duration of treatment. Therefore additional experience and eventualy longer period of monitoring are warranted to clarify the exact duration of immunotherapy. Our opinion is that separately nor surgical or conservative treatment can solve this complex medical problem. Only their combination will give us improving the therapeutic results.

Conclusion

Transoral CO_2 laser microsurgery still remains one of the main applications of CO_2 laser in ENT, but can't achieve a cure and the ability to stop the spread of the lesions. Reducing the incidence of recurrences is not as significant as expected. Although there are a palliative effect, it is extremely important in restoring airway patency and improve the quality of the voice. Separately conservative (α-Interferon; therapy with Cidofovir and PDT with ALA-5-amino-levulinic acid and etc.) and surgical treatments do not give satisfactory results. Our goal with the introduction of immunotherapy was not to show just one new additional therapy, but to to show a therapy that recover the protective T-cell immune. By immunotherapy with Calgevax (BCG) through immune modulation and stimulation of cellular immune effector mechanisms we observe a significant reduction of the frequency of relapses. With immunotherapy in RRP patients we achieve increasing of the therapeutic effect and can accomplish if not a permanent cure, at least reduction of the frequency of relapses and improvement of the quality of life of patients.

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