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Adjuvant treatment with a dialyzable leukocytes extract contributes to maintain HPV-infected women free of low-grade cervical lesions

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Summary

Purpose of investigation: To investigate if adjuvant treatment with a dialyzable extract of leukocytes (DLE), may help HPV-infected patients with low-grade intraepithelial squamous cervical lesions (LIS) to get free of HPV infection and cervical lesions. *Materials and Methods:* Patients with untreated, low-grade cervical lesions were treated either with surgery (Group A) or with DLE (Group B). Patients with low-grade but recurrent cervical lesions were newly treated with surgery plus DLE (Group C). *Results:* A decreased or absent cervical lesion correlated with a diminished or absent HPV viral load at one year of treatment ($r = 0.6$, $p < 0.05$). Seventy-nine percent of Group B but only 50 % of Group C and 38 % of Group A patients were free of cervical lesion after 24 months of treatment ($p < 0.05$). *Conclusion:* The present data support the benefit of adding DLE as adjuvant for treating HPV-infected women with LIS.

Key words: Dialyzable leukocytes extract, low-grade cervical lesions, HPV.

Introduction

High-risk human papilloma virus (HR-HPV) infection is the prevalent risk factor for development of cervical cancer [1, 2]. A global HPV burden as high as 10.4% of the world population has contributed to establish cervical cancer as the second cause of cancer death of women worldwide [1, 3]. Eighteen of the 200 known HPV subtypes are high-risk subtypes HPV that may alter the uterine cervix transitional epithelium cells' growth and favour the appearance of squamous epithelial lesions and cervical cancer [4 - 7]. In Mexico, up to 43.6% of the adult female population may be HPV-infected and most of them are infected with high-risk (16, 18, 31, 33, 35, 52, 58) HPV subtypes [4, 8-10].

Most HPV infections are cleared without treatment, even if they are caused by high-risk HPV subtypes [11]. However, up to 15 % of women with high-risk HPV infections may be unable to remove the virus from the cervix, which facilitates epithelial squamous lesions' progression from low- to high-grade and to cancerous lesions [12]. Cervical intraepithelial neoplasias (CIN) grade 1, 2 or 3 pre-cancerous lesions are currently treated with different approaches. A simple follow-up is often exercised without any treatment for mild pre-cancerous cervical lesions such as CIN 1, until further evaluation [13]. However, moderate dysplasia (CIN 2) and severe pre-cancerous cervical lesions (CIN 3) usually associated with high-risk HPV infection require either cryo- or laser-surgical removal of the area of abnormality. Although recurrence rates vary, patients who have been treated for CIN are considered to be at high-risk of developing invasive cervical cancer for many years after treatment, particularly in association with high-risk HPV persistence [14 - 16]. Whereas a Th1 immune response could be important to achieve regression of HPV infection, recurrence may be facilitated by preoperative factors negatively influencing the immune status such as HIV infection or pregnancy and postoperative factors, such as positive surgical margins and high-grade pathology on the excision specimens [17 - 19].

Dysplasia and tumour progression are often accompanied by a poor immune response, viral evasion mechanisms or both [2]. CD3+ CD4+, CD3+ CD8+, and CD3+ CD4+ CD25+ peripheral blood T cells may not be capable of containing HPV infection in spite of its normal or even high numbers in HPV-infected women [3, 12, 20]. IL-10 and IL-10 producer cells may also be in high numbers in these patients, contributing to a decreased Th1 cells function and lesion transformation into cervical neoplasia [21 -22]. These alterations strongly suggest the need to evaluate the immune status of HPV-infected women with low-grade cervical lesions and to test new means to restore a more suitable cell immunity balance in these patients [3, 4, 23].

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Lawrence and Borkowsky were the first to report that a dialyzable extract of leucocytes (DLE) from donors that were sensitized and immune responsive to specific antigens could be administered to unresponsive recipients and make them become responsive to those particular antigens [24]. Kirkpatrick *et al*. determined that DLE contains more than 200 molecules with < 10 kDa MW and proposed that the DLE specificity might be due not to one, but many small peptides [25]. Numerous investigators have used DLE to treat various kinds of diseases with variable success and the present authors have earlier demonstrated that DLE may increase *in vivo* production of TNF_α and IFN_γ in experimental tuberculosis and experimental glioma [26 - 32]. Moreover, they have also shown that treatment with DLE may increase the number of CD4+ circulating T cells and serum concentrations of IFNγ, helping patients affected by acute infection of herpes zoster to achieve a more favourable clinical course [33]. Now, the present authors report on a group of HPV-infected women having low-grade cervical lesions that were treated with surgery, DLE, or surgery plus DLE, to determine if DLE may modify their numbers of circulating regulatory T cells and their cytokine profile in order to achieve a proper immune cell response against HPV, and to prevent their lesions' progression to cervical cancer.

Table 1. — *Study groups' features.*

		A	B	C	D
Age*		32 ± 7.4	31 ± 10.2	31 ± 6.9	30 ± 8.2
Sex partners	1	9	15	7	7
	2	3	2	4	5
	≥ 3	5	1	8	8
HR-HPV		9	13	11	0
LR-HPV		2	3	8	0
NDR		5	2	0	0
Negative		1	0	0	20
LIS		17	18	19	0
HIS		0	0	0	0
Cervicitis**		0	1	3	0
Lesion size***		3.2 ± 2.2	4.8 ± 2.1	4.04 ± 1.8	None

* Mean ± SD years old, ** Inflammatory process in addition to the cervical lesion, *** diameter in cm. Group A (n = 17) had first-time lesion and received surgical treatment, Group B (n = 18) had first-time lesions and was treated only with dialyzable extract of leukocytes (DLE), Group C (n = 19) had recurrent lesions and was treated both with surgery and DLE. Group D was a control group of non-HPV-infected, age-matched, healthy volunteers. HR = high-risk HPV 16, 18, 33, 39, 51, 52, 56, 58, 59 or 66. LR = low-risk HPV 11, 43, 44, 81 or 61, NDR = non-determined-risk HPV 20, 69, 90 or 102, Negative = non-viral protein detection. LIS = low-grade intraepithelial squamous lesion. HIS = high-grade intraepithelial squamous lesion.

Materials and Methods

Study subjects

The protocol of this project was evaluated and approved by the Hospital Ethical Committee. All the women included in the study signed informed consent before initiating the study and treatment protocols. Patients receiving any kind of immune modifier therapy were excluded from the study. Pregnant, menopausal, chronically ill women suffering diabetes, allergies, autoimmunity, AIDS or other sex transmitted diseases were also excluded. A cervical biopsy was taken from the affected tissue in every patient to determine the type of intraepithelial cervical lesion they had (Table 1). Women with high-grade cervical cell abnormalities were also excluded. Fifty-four sexually active women with a mean age of 29.2 ± 6.8 (range 18 to 45) years, diagnosed with low-grade intraepithelial squamous lesion (LIS) formed three study groups. Sixty-one percent of the patients were infected by high-risk (16, 18 or 33), whereas 24 % were infected by low-risk (11, 44 or 81) HPV subtypes. One patient was negative and seven of them were infected with non-determined-risk HPV subtypes (Table 1). Patients diagnosed for the first time with non-treated cervical lesions were randomly separated in two groups, including 17 women that were treated with cryo- or electro-surgery (Group A) and 18 women that were treated only with oral and topical DLE (Group B). Nineteen women with recurrent lesions formed group C and were treated both with surgery and oral and topical DLE. Patients were examined by Papanicolaou and colposcopy and followed-up with periodical cervix and laboratory examinations up until 24 months. Twenty non-HPV-infected and lesion-free, age-matched, healthy women formed the control group.

Dialyzable extract of leukocytes (DLE)

DLE was prepared by repeated freezing and thawing of leukocytes isolated from buffy coats of healthy blood donors. The dialyzed extract of leukocytes was adjusted by protein content and stored frozen until use. One unit (2.2 mg of protein in five ml of bi-distilled sterile water) was orally administered to patients from Group B and C during five weeks. One more unit was at the same time, topically applied to these patients, every 72 hours during the first two weeks. This schedule of treatment was repeated when a persistent lesion was found during colposcopy examination at three, six, nine, and 12 months of the study. Additional schedules of DLE were orally and topically administered to eight patients of Group B and 12 patients from Group C during the first year.

HPV subtypes and viral load

Cervicovaginal exudates were collected by brushing and sent to the Investigation and Molecular Analysis Laboratory (LIAM) for PCR determination of HPV infection and viral subtype. The MY09/MY11 consensus-primer set served to obtain a 450 bp L1 gene fragment that was automatic sequenced. The highly conserved MY09/MY11 primer set allowed detection of 42 HPV subtypes, including high-risk 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 and 73 HPV subtypes and low-risk 2a, 3, 6, 7, 10, 11, 13, 26, 27, 28, 29, 30, 32, 34, 40, 42, 44, 53, 54, 55, 57, 61, 62, 66, 67, 70, 72, and 74 HPV subtypes. Cervicovaginal samples were also obtained by scraping and brushing and analysed by the hybrid capture 2 assay at LIAM as complementary analysis for detection of high-risk 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 HPV subtypes. Results of the hybrid capture two assays are given in relative light units (RLU), which provide a semi-quantitative estimate of viral load in the specimen, indicating also if the HPV is in replicative or latent phase [34].

Flow cytometry

Venous blood was collected in vacutainer tubes with EDTA at 0, 15, 30, 90, 180, and 365 days of study. Peripheral blood cells were phenotyped by single or double immunofluorescence staining with fluoresceinated anti-CD 3, -CD 4, -CD 8, -CD 16, -CD 56, -CD 25, or -CD 19 monoclonal antibodies (Mab) to identify total T, helper T and cytotoxic T cells, NK cells, and B cells. Non-stained cells and cells treated with FITC- or PE-conjugates with matched isotypes

Table 2. — *Absolute numbers of cell subpopulations ($\bar{x}$ ± S.E.) and size of the cervical lesions before treatment.*

	Group A	Group B	Group C	Group D
Total leukocytes	6818 ± 600	5673 ± 426	6463 ± 479	6297 ± 421
Total lymphocytes	2487 ± 322	2505 ± 289	2559 ± 269	2160 ± 162
CD 3 +	1595 ± 232	1693 ± 207	1849 ± 201*	1350 ± 118
CD 19 +	289 ± 44 *	321 ± 57 *	426 ± 99	477 ± 64
CD3+CD4+	543 ± 80	708 ± 110	777 ± 107 *	507 ± 71
CD3+CD8+	416 ± 82	336 ± 123	439 ± 68	317 ± 41
CD3+ CD4+ CD25+	72 ± 36 *	107 ± 30 *	144 ± 28 *	30 ± 6
CD3+CD56+	497 ± 78	575 ± 89	291 ± 56	414 ± 76
CD3+ IL10+	7 ± 4	11.8 ± 4	31 ± 10 *	5 ± 0.8
CD3+ IFN-γ+	0.7 ± 0.2	0.63 ± 0.3	0.96 ± 0.4	1.08 ± 0.5
Number of sex partners	3 ± 0.5	2 ± 0.2	3 ± 0.5	3 ± 0.5
Lesion size	3 ± 2.0	5 ± 2	4 ± 2	N.A.

N.A.= not applicable. * $p < 0.05$ *vs* Group D.

were used as controls. Thirty µl of leukocytes-containing plasma were incubated 20 minutes at room temperature in the dark with five µl of one or two of the above listed monoclonal antibodies for determination of cell surface markers. Erythrocytes were lysed by 12 minutes incubation with FACS lysing solution. Cells were then washed with PBS, resuspended in 400 µl paraformaldehyde 2% and analysed by flow cytometry. In addition, 100 µl heparinized blood were incubated for 20 minutes with five µl anti-CD3-PercP. After lysing erythrocytes, these cells were washed and incubated for 20 minutes with permeabilizing solution and ten µg/ml brefeldin A, washed, further incubated for 30 minutes more with five µl anti-IL-10-PE, anti-IFN-γ-FITC, anti-IL-4-PE or their respective isotype-matched FITC- or PE-conjugated Mab, washed and PFA fixed. Stained samples were analysed by flow cytometry, acquiring 10,000 cells for each surface marker or 20,000 cells for cytokines intracellular determination with an acquisition rate < 400 events/sec in a system equipped with an argon laser and CELLQuest pro software.

Statistical analysis

Mean values were compared using the Student's t-test for paired and unpaired samples. Pearson's coefficient of correlation and ANOVA test were used to analyse the correlation between independent observations and two-tailed statistical significances were determined. Values without normal distribution were analysed with the non-parametric Mann Whitney U test.

Results

Analysis before treatment

Age-matched healthy controls were not different to the patients in respect to number of sex partners and socioeconomic status. None of the healthy controls were HPV-infected whereas all the patients but one were HPV-infected (Table 1). Lesion size was also similar in the different groups of patients and there was no significant difference either between the numbers of total leukocytes and total lymphocytes from patients and healthy controls (Table 2). Patients from Groups A and B had lower amounts of B lymphocytes (289 ± 44 and 321 ± 57, respectively) than healthy controls (477 ± 64, $p < 0.05$), whereas the numbers of T lymphocytes (1849 ± 201) and CD3+ CD4+ (777 ± 107) were slightly higher in patients from the Group C than in healthy controls (1350 ± 118 and 507 ±71, $p < 0.05$). In addition, numbers of CD3+ CD4+ CD25+ peripheral blood cells in the three groups of patients

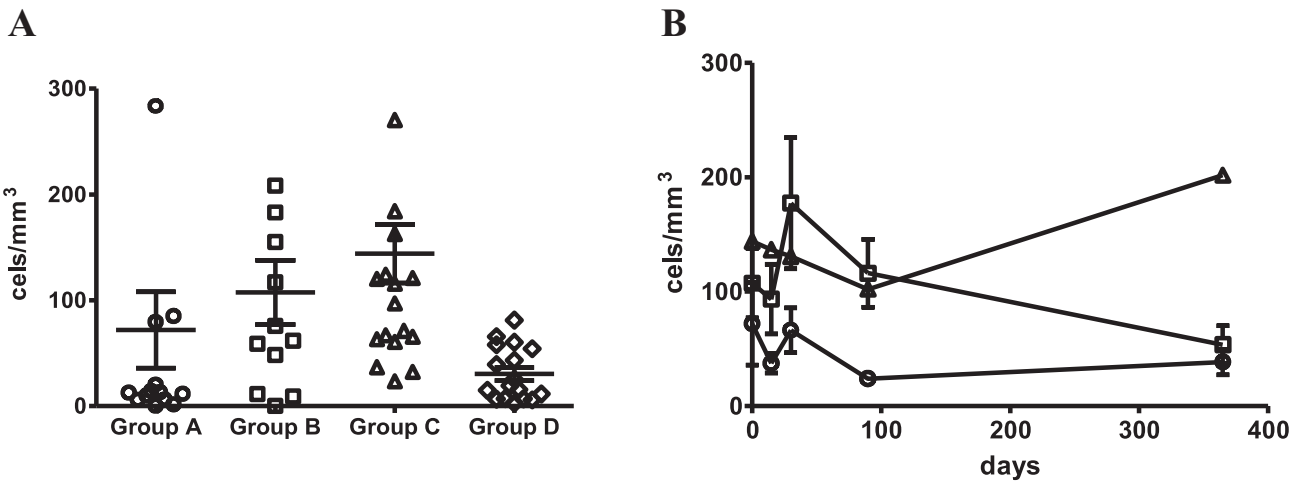

Figure 1. — A) absolute numbers of CD3 + CD4 + CD25 + peripheral blood cells of patients with first-time diagnosed low grade cervical lesions before treatment with surgery (Group A), patients with first-time diagnosed low grade cervical lesion before treatment with the dialyzable extract of leukocytes (DLE, Group B), and recurrent patients before treatment with surgery plus DLE (Group C) and non-HPV infected, age-matched, healthy women (Group D). B) absolute numbers of CD3 + CD4 + CD25 + peripheral blood cells during a 365 days follow up of patients in Group A (○), Group B ( ), and Group C (Δ). CD3+ CD4+ CD25+ cells were highest in the group C before and after treatment ($p < 0.05$) as compared to Groups A, B, and D.

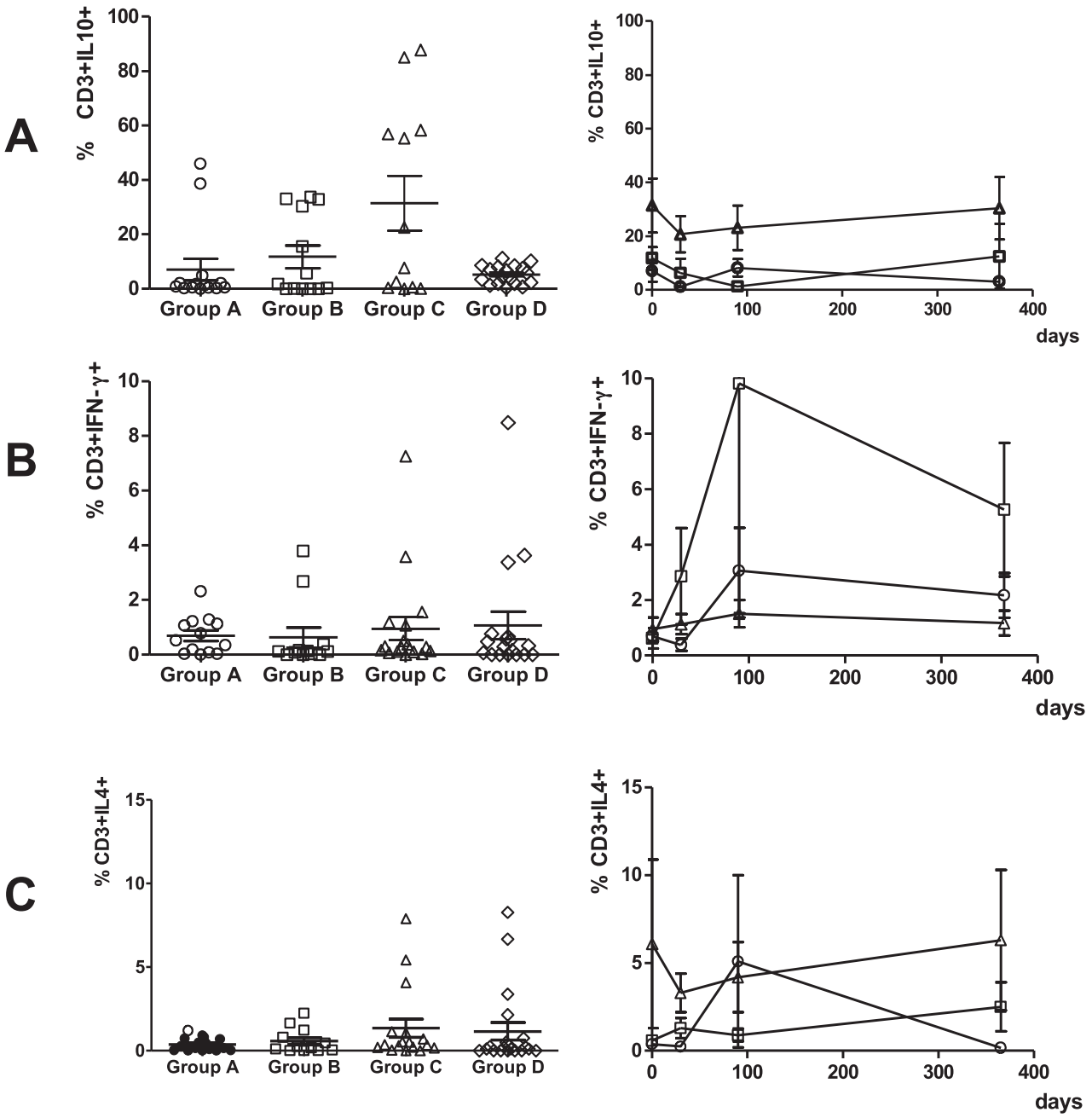

Figure 2. — Percentages of CD3+ IL-10+ (2A), CD3+ IFNγ + (2B), and CD3+ IL4+ (2C) cells in peripheral blood of HPV-infected women, before (left hand side) and during treatment (right hand side). Patients with first time lesions that were treated with surgery (○, Group A), patients with first time lesions that were treated only with DLE (, Group B), and recurrent patients that were treated with surgery plus DLE (Δ, Group C). Group D (◊) was formed by non-HPV infected, age-matched, healthy women. CD3+ IL-10+ cells were highest in Group C before and after treatment ($p < 0.05$).

and those of CD3+ IL-10+ cells in the group C were significantly ($p < 0.05$) elevated as compared to normal values from Group D (Table 2).

Lymphocyte populations after treatment

CD3+ CD4+ CD25+ and CD3+ IL-10+ cells were highest in the Group C before treatment (144 ± 28 *vs* 30 ± 6 and 31 ± 10 *vs* 5 ± 0.8, $p < 0.05$) and remained elevated after treatment (Table 2 and Figure 1). The amount of CD3+ IFNγ + cells increased from 0.63 ± 1.2 to 5.3 ± 6.4 in patients from group B ($p < 0.05$, Figure 2) but not in patients from the other groups. The remaining cell populations had minor variations during the follow up period of 365 days (Figure 2).

Table 3. — *Percentages of HPV-infected patients free of low-grade cervical lesion during two years follow up. Group B was significantly different ($p < 0.05$) from Groups A and C at 24 months from initiation of treatment.*

Patient groups	Months after treatment			
	0	6	12	24
A (n = 17)	0%	78%	61%	38%
B (n = 18)	0%	78%	70%	79%
C (n = 19)	0%	64%	79%	50%

Lesion size and viral load

Thirty-eight percent of the patients from Group A, 79 % of the patients from group B, and 50 % of the patients from Group C were free of cervical lesion after two years of treatment, all of which were significantly different from their initial conditions ($p < 0.05$, Table 3). The difference in success of keeping the patients without cervical lesion was also significant between Groups B and A ($p < 0.05$, Table 3), indicating that the treatment with the DLE alone was at least as good as surgical treatment for women with LIS, diagnosed for the first time. The outcome of half of the patients from Group C also suggests that even recurrent patients, originally treated with surgery may benefit from receiving DLE as adjuvant treatment, together with a surgical removal of the new cervical lesion (Tables 3 and 4).

The predominance of HR-HPV subtypes was confirmed by the presence of the subtype 16 in most of the patients (not shown). Viral load was quantified by the hybrid capture 2 assay and reported as RLU. Viral load decreased significantly in most of the 20 patients that were tested after one year of treatment and seven of these patients were at the same time, entirely free of cervical lesion and viral load. Moreover, a decreased or absent cervical lesion correlated with a diminished or absent HPV viral load at one year of treatment ($r = 0.6$, $p < 0.05$, Table 4).

Table 4. — *HPV viral load and lesion size in patients with low-grade cervical lesion diagnosed by the first time, before and after treatment with surgery (Group A patients) or dialyzable leukocytes extract (DLE, Group B patients). HPV viral load and lesion size in recurrent patients (Group C), before and after treatment with surgery plus DLE.*

Patient	RLU *		Lesion size **	
	Initial	One year later	Initial	One year later
A1	2.1	Negative	1.9	Negative
A2	22.5	Negative	3.8	1.9
A3	1521	1408	1.9	1.9
A4	20	125	3.8	3.8
A5	1.6	Negative	3.8	Negative
A6	429	216	1.9	1.9
A7	Negative	Negative	1.9	Negative
B1	1784	ND	7.6	1.9
B2	449	Negative	5.7	1.9
B3	ND	0.87	5.7	Negative
B4	1109	Negative	5.7	Negative
B5	2.7	Negative	3.8	1.9
B6	492	Negative	7.6	1.9
C1	1769	Negative	3.8	1.9
C2	2222	121	5.7	Negative
C3	20	Negative	1.9	Negative
C4	52	Negative	5.7	Negative
C5	51	82	1.9	Negative
C6	ND	6	1.9	Negative
C7	167	Negative	1.9	Negative

* Relative light units (RLU), as determined with the hybrid capture 2 assay. ** diameter in cm, ND = not determined. Differences were significant ($p < 0.05$) between lesion size after treatment and lesion size before treatment of every group of patients.

Discussion

The high prevalence of HPV-infection is a global public health concern that relates both to cervical cancer and precancerous lesions world incidence [1, 3]. Only 1% from the 10% of patients that become chronically infected with HPV develops cervical cancer, apparently because there usually is a pro-inflammatory microenvironment that leads to an effective immune response capable of eliminating HPV-infected cells [17]. However, HPV viruses have developed strategies to achieve host immunity suppression and viral DNA integration into the host DNA [35 -36]. In such cases, stimulation of the immune response with adjuvants may be helpful to reverse the anti-inflammatory microenvironment.

HPV association with cervical cancer is matter of particularly great concern in Mexico, since half of the Mexican female population may be infected with high-risk (16, 31, 18, 35, 52, 33, and 56) HPV subtypes [4, 8 – 10, 37]. Additional risk factors such as low nutritional and socioeconomic status, long-term intake of contraceptives, sex activity at early age, sex transmitted diseases, high number of pregnancies, and the expression of certain HLA Class II antigens are features that characterize a large number of Mexican women and contribute to maintain the high cervical cancer prevalence that afflicts a country where one woman may be dying of cervical cancer every two hours [37 - 38]. HPV persistence and additional risk factors most probably also contribute to the elevated recurrence observed in our low-income group of patients [38]. Nearly half of our patients were positive for viral load one year after diagnosis and this percentage most probably augments with longer follow up after treatment, which may account for the high recurrence of LIS observed in patients followed up to two to five years of diagnosis [39]. The lack of an appropriate immune response against infectious agents, often associated with dietary deficiencies and inadequate healthcare also make HPV-infection become a first priority. Sex education, national screenings, and other preventive measurements such as HPV vaccine applications are therefore, as important as the availability of inexpensive and effica-

cious measures for early treatment of women with cervical lesions [10].

Increased levels of CD3+ CD4+ CD25+, and IL-10 as observed in the present recurrent patients are in agreement with other reports showing an increased activity of regulatory T cells and generation of an anti-inflammatory profile [40 – 42]. IL-10 production and other Th2 cytokines are associated with significantly longer times to HPV clearance [43]. High percentages of infection with high-risk subtypes HPV and the predominance of subtypes 16, 18, 31, 33, 35, and 58 in the present patients are in agreement with data reported on prevalent HPV subtypes infection in Mexican women with cervical intraepithelial lesions [44].

The hybrid capture 2 assay has higher negative predictive value than the Papanicolaou test for detection of CIN. A correlation exists between RLU values and the severity of cervical lesions, with lower RLU values for patients without lesion and increasingly higher values for patients with low-grade and high-grade lesions [45]. In the present work, a diminished or negative viral load after one year from initiation of the treatment of all the patients correlated to a simultaneously diminished or absent cervical lesion. The cervical lesion had also a decreased size or was totally absent in many patients from the three study groups after two years of treatment. However, recurrence may come along with HPV reinfection and the highest success in keeping more patients free of cervical lesion at two years of treatment initiation was observed in patients treated with DLE either alone or as adjuvant to surgery during the first year after diagnosis.

Conclusion

Treatment with DLE as adjuvant may provide an important stimulus to incline the balance towards a more favourable immune response from HPV-infected patients having low-grade cervical lesions, who live in permanent risk due to their poor socioeconomic status and persistent HPV infection or continuous reinfection. Results of this pilot study suggest that DLE has a therapeutic potential for HPV-infected women. Since no negative side effects have been reported following the administration of DLE and because it is a biological product with very low cost of production, the authors suggest that the administration of DLE should be considered a suitable addition to the currently established treatment for HPV-infected women, either with first or recurrent low-grade cervical lesions. Although many unanswered questions exist regarding DLE composition and mechanisms of action, its beneficial effects deserve further investigation.

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