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Phenotypic analysis of peripheral blood cell immunity in Italian patients with different varieties of oral lichen planus

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SUMMARY

Quantitative analysis of peripheral blood lymphocytes was carried out in 25 patients with atrophic-erosive type of oral lichen planus (OLP) (Group 1), in 28 patients with reticular-plaque like lesions of OLP (Group 2) and in 21 healthy patients (Group 3) by using flow cytometry. CD4+ subsets decreased significantly in patients with reticular-plaque like varieties when compared with healthy patients (Group 3) (One way analysis of variance $p=0.039$; t-test with Bonferroni correction $p<0.05$). Moreover, in patients with hyperkeratosic forms of OLP (Group 2) CD8+ cell populations were significantly higher than in controls (Group 3) (Kruskal-Wallis test $p=0.035$; Mann-Whitney test with Bonferroni's correction $p<0.0001$) and consequently CD4/CD8 ratio was significantly lower in patients with reticular-plaque like lesions than in controls (Kruskal-Wallis test $p=0.01$; Mann-Whitney test with Bonferroni's correction $p=0.013$). No statistical differences between patients of Group 1 (atrophic-erosive OLP) and the other two Groups (hyperkeratosic OLP and healthy controls) were detected. 40% of the patients of Group 1 were affected by chronic hepatopathies, most of which were related to hepatitis C virus (HCV), but the data were not substantially modified after adjustment for the patients with chronic liver disease HCV positive. There is no clear evidence that these results indicate the existence of a different pathogenetic mechanism between erosive-atrophic and hyperkeratosic types of OLP. On the other hand, these results and the previously reported immunohistochemical findings suggest that quantitative alterations of peripheral blood lymphocytes in hyperkeratosic varieties of OLP could represent a shift of CD4+ cells from the vascular to the oral mucosa compartment.

KEY WORDS:

Oral lichen planus, T lymphocytes, flow citometry.

RÉSUMÉ

Les lymphocytes du sang périphérique ont été évalués par cytométrie de flux dans deux groupes de malades porteurs d'un lichen plan de la muqueuse buccale: 25 à forme atrophique-érosive (Groupe 1), 28 à forme en réseaux ou en plaques blanches (Groupe 2), et chez 21 sujets sains (Groupe 3). Au terme de cette

étude les différences les plus remarquables ont été les suivantes: diminution de la fraction CD4+ et une augmentation de la fraction CD8+ dans le Groupe 2 (réseaux et plaques blanches) comparés au Groupe 3 (contrôle). la différence est statistiquement significative (One ways analysis of variance $p=0.039$, t test corrigé par Bonferroni $p<0.05$ pour CD4+ et Kruskal-Wallis test $p=0.035$, Mann-Whitney test corrigé par Bonferroni $p<0.001$ pour CD8+) par conséquent le rapport CD4/CD8 du Groupe 2 a été significativement plus bas par rapport au Groupe 3 (Kruskal-Wallis test $p=0.014$; Mann-Whitney test corrigé par Bonferroni $p=0.013$). Aucune autre différence significative entre les trois groupes n'a été observée, en particulier avec le Groupe 1 (formes atrophiques-érosives) dont il faut signaler que le 40% des sujets sont porteurs d'une hépatopathie chronique souvent due au virus de l'hépatite C. En conclusion la différence des résultats entre les groupes 1 et 2 ne permet pas d'affirmer l'existence d'une pathogénie différente entre les formes atrophiques-érosives et les formes en réseaux ou en plaques, elle est en accord avec les précédentes études en histo-immunochimie. Il est possible que la diminution des lymphocytes CD4+ soit secondaire au déplacement de cette population cellulaire du compartement vasculaire de la muqueuse affectée par le lichen plan.

MOTS CLÉS:

Lichen plan, bouche, lymphocytes T, cystométrie de flux.

INTRODUCTION

Oral lichen planus (OLP) is a relatively common chronic inflammatory disease of unknown etiology in which the cell mediated arm of the immune system probably plays an important role in the initiation and progression of lesions (Walsh *et al.*, 1990). Immunohistochemical studies have shown that OLP can be considered as a clinical manifestation of the response of infiltrated T cells to modified autologous epithelial cells (Matthews *et al.*, 1984; Hirota *et al.*, 1992; Boisnic *et al.*, 1992).

These consistent lesional signs of cell-mediated immunity led in the past to phenotypic and functional investigations of systemic cellular immunity in OLP patients, but the results are controversial (Lin *et al.*, 1988; Malmstrom *et al.*, 1989; Konttinen *et al.*, 1989; Yamamoto *et al.*, 1990; Sugerman *et al.*, 1992).

However, most of these studies investigated mainly hyperkeratosic forms of OLP and rarely was the clinical variety of the oral lesions related with systemic cellular immunity. Several recent studies have confirmed the existence of some differences in genetic predispositions and clinical course between hyperkeratosic forms and atrophic-erosive forms of OLP (Thorn *et al.*, 1988; Cottoni *et al.*, 1988; Robertson & Wray, 1992; Bagan *et al.*, 1993; Gandolfo *et al.*, 1994; Porter *et al.*, 1993). Atrophic-erosive lesions seem more common in patients with diabetes (Bagan *et al.*, 1993), with chronic liver disease (Gandolfo *et al.*, 1992), with immunological disturbance (Cottoni *et al.*, 1988) and also in patients treated with nonsteroidal anti-inflammatory drugs (Robertson & Wray, 1992), but the biological significance of these associations is at the moment not clear. It is known too, that erosive or atrophic types of OLP seem more frequently associated with malignant evolution than hyperkeratosic forms (WHO, 1978). Moreover, some authors have suggested that OLP could include several pathologic entities with a certain geographic variability (Valsecchi *et al.*, 1988; Lin & Sun, 1990; Porter *et al.*, 1993).

As far as we know there are only few functional or phenotypical studies comparing systematically peripheral cell-immunity in patients with different varieties of OLP.

The purpose of this study was therefore: to establish if changes in systemic cellular immunity exist in italian patients with OLP and to identify some phenotypical differences in cell-mediated immunity between patients with atrophic-erosive lesions and patients with reticular-plaque-like lesions.

MATERIAL AND METHODS

Patients and Controls

The study population was comprised of 3 group of patients sex and age matched:
A) Group 1 consisting of 25 patients (14 men and

11 women) whose average age was 62 (range 28-81) affected by atrophic-erosive varieties of OLP;

B) Group 2 consisting of 28 OLP patients (13 men and 15 women) whose average age was 52 (range 24-80) with only reticular-plaque-like lesions of OLP;

C) Group 3 consisting of 21 healthy patients (9 men and 12 women) whose average age was 59 (range 24-81) without any clinical sign of oral disease.

In all the OLP patients the clinical diagnosis were confirmed histologically following the WHO (1978) recommendation. None of the patients has ever been treated for OLP; neither they or the control group had taken any drugs that may have influenced results during the 3 months before the analyses. No patient was suspected of having drug-induced or restoration related OLP. A full medical history was obtained from each patient. Peripheral blood samples were collected by venipuncture from each patient at the same time (9 a.m.) to control for circadian variations in endogenous cortisol levels.

Phenotypical cell immunity analysis by flow cytometry

The following monoclonal antibodies (AbMo) were used: anti-Leu4 (CD3), anti-Leu3 (CD4); anti-Leu2 (CD8); anti Leu12 (CD19); anti-HLA-DR; anti-Leu11 (CD16) (Becton-Dickison, Mountain View, Calif). For each AbMo 100 micronl of heparinized blood and 10 micronl of AbMo was used. The erytrocytes were removed with 2 ml of lysing solution diluited 1/10($<50\%$ of etilenic glicol and $<15\%$ of formaldehyde). After incubating for 10 minutes the sample was centrifuged at 1500 rpm for 10 minutes. The supernatant was removed and the plate was resuspended in 2 ml of phosphate buffered saline solution containing 0.1% azide. After a second centrifuge (1500 rpm for 10 minutes), the supernatant was removed and the isolated lymphocytes were resuspended in 1 ml of Sheath-Fluid. Labelled cells were analyzed then by flow-cytometry using a fluorescence-activated cell sorter analyzer (FACstar, Becton-Dickison, Mountain View, Calif). The data were filed in computerized archives by means of modified database which was processed with a hardware PC-AT IBM (Gandolfo *et al.*, 1993).

Statistical Analysis

The data were analyzed by one-way parametric analysis of variance if the variance was homogeneous with the Bartlett test. When the variance was dyshomogeneous it was used the non parametric Kruskal-Wallis test. This analysis was performed by means of EPI-INFO version 5 (A world processing data-base and static program for epidemiology on microcomputers, 1990). For multiple comparisons between group the t-test or the Mann-Whitney test with Bonferroni's correction was used as appropriate. P-values <0.05 wee considered significant.

RESULTS

The mean percentage of CD4+ (helper/inducer) subsets was significantly lower in patients with the reticular-plaque-like (37.2 ± 7.32) (Group 2) type of OLP than in healthy controls (Group 3) (43.81 ± 9.416) (One way analysis of variance $p=0.039$; t-test with Bonferroni correction $p<0.05$), while no difference in CD4+cells was detected between patients with atrophic-erosive lesions (Group 2) and controls or between the various forms of OLP studied (Table I and II).

In Group 2 the mean CD8+ (cytotoxic/suppressor) cell level (36.84 ± 10.106) was significantly higher than in healthy controls (Group 3) (30.348 ± 5.509) (Kruskal-Wallis test $p=0.03$; Mann-Whitney test with Bonferroni's correction $p<0.0001$) (Table I-II). No statistical differences in CD8+ mean were shown between patients affected by atrophic-erosive variety of OLP (Group 1) and controls (Group 3) or between patients with atrophic-erosive OLP and hyperkeratosic forms of OLP (Group 2) (Table II). Consequently, the CD4/CD8 ratio was significantly lower in patients with reticular-plaque-like lesions (1.108 ± 0.391) than in controls (1.424 ± 0.333) (Kruskal-Wallis test $p=0.01$; Mann-Whitney test with Bonferroni's correction $p=0.013$), but not in patients with atrophic-erosive varieties (Table I-II). Neither was there any significant difference in the CD4/CD8 ratio between patients affected by reticular-plaque-like forms and patients with atrophic-erosive forms (Table I-II). No other differences were detected in mean percentage of CD3+ (total T) populations, CD19+ (B-cells), HLA-DR+ cells or NK+ (natural killer [CD16]) cells among the three groups studied. 40% of patients of Group 1 were affected by chronic hepatopathies, most of which were related to hepatitis C virus (these patients have been described in details elsewhere (Gandolfo *et al.*, 1992), but there was no evidence of any influence of liver disease on T-cell subsets (data not shown).

TABLE I: Mean values of peripheral blood lymphocytes, lymphocytes B, lymphocytes T, subpopulations T, HLA-DR and natural killer (NK).
TABLEAU I: Valeur moyennes des lymphocytes sanguines périphériques, lymphocytes B, lymphocytes T, sous-population T, HLA-DR et natural killer (NK).

Parameters	G@	Mean	Variance	Std Dev	#F(*H)	P
CD3(%)	1	70.560	120.590	10.981	#0.022	0.978
	2	70.240	95.690	9.782		
	3	70.857	76.229	8.731		
CD4(%)	1	41.720	114.543	10.702	#3.400	0.039
	2	37.200	53.583	7.320		
	3	43.810	88.662	9.416		
CD8(%)	1	31.280	54.127	7.357	*6.682	0.035
	2	36.840	102.140	10.106		
	3	30.348	30.348	5.509		
CD4/CD8	1	1.408	0.356	0.596	*8.470	0.014
	2	1.080	0.153	0.391		
	3	1.424	0.111	0.333		
HLA-DR (%)	1	22.043	60.273	7.778	#0.506	0.610
	2	23.840	66.223	8.138		
	3	22.000	25.700	5.070		
NK(%)	1	13.077	77.910	8.827	#0.572	0.573
	2	13.000	33.077	5.751		
	3	15.333	54.333	7.371		
CD19(%)	1	8.000	16.500	4.062	#0.737	0.511
	2	8.813	7.763	2.786		
	3	9.143	22.229	4.715		

@G: Group
#F : One way analysis of variance (variance homogeneous at 95% with Bartlett test)
*H : Kruskal-Wallis test (variance not homogeneous)

TABLE II: Multiple comparisons between mean values of CD4, CD8 cell and CD4/CD8 ratio in 3 studied Group.
TABLEAU II: Comparaisons multiples entre les valeurs moyennes des CD4, CD8 et du rapport CD4/CD8 dans 3 groupes étudiés.

	Comparisons between Groups	Mean	Z*	P
CD4	1 vs 2	41.720 vs 37.200	#1.792	NS
	2 vs 3	37.200 vs 43.810	#2.497	<0.05
	1 vs 3	41.720 vs 43.810	#0.770	NS
CD8	1 vs 2	31.280 vs 36.840	2.030	NS
	2 vs 3	36.840 vs 30.348	3.335	<0.0001
	1 vs 3	31.280 vs 30.348	0.121	NS
CD4/CD8	1 vs 2	1.408 vs 1.080	0.376	NS
	2 vs 3	1.080 vs 1.424	2.487	0.013
	1 vs 3	1.408 vs 1.424	0.752	NS

t-test with Bonferroni correction
* Mann-Whitney test with Bonferroni correction

DISCUSSION

Early studies on T-cell subsets in peripheral blood of patients with LP showed decreased CD8+ fraction suggesting a possible defective suppressor activity, but most of these investigations regarded patients with cutaneous LP (Gomes *et al.*, 1982; Simon & Keller, 1984; Maduit *et al.*, 84). Subsequently, Lin *et al.* (1988) did not find significant difference in peripheral blood T-subsets between patient with OLP and healthy controls. However, the small numbers of healthy controls in this study preclude any significant conclusion from being drawn. Recently, flow cytometric analysis of peripheral cellular immunity in OLP have been made but the results have been equivocal. Yamamoto *et al.* (1990) reported an increase in the percentage of CD8 CD11b+ (suppressor T) cells in OLP patients as compared with controls and suggested that cellular immunosuppression is a pathologic charactaristic of OLP, whereas Sugerman *et al.* (1992) did not find changes in CD8+ subpopulations but a significantly less proportion of CD4+CD45 RA+ (suppressor-inducer) cells in OLP. Moreover, in the latter study functional analysis of peripheral blood lymphocytes suggested the possible association between non-reticular OLP and defective cell-mediated suppressor circuits, although also in this work the number of the patients was too limited to permit any definitive conclusion. As far as we know there are not other studies analizing systematically peripheral blood cell immunity in patients with different varieties of OLP, although some pathogenetic difference could exist especially between erosive and hyperkeratosic type of OLP (Jungell *et al.*, 1990).

The present study concurs with the results of Yamamoto *et al.* (1990) demonstrating an imbalance of T-lymphocyte subsets with an increased of CD8+ fraction and a lower CD4+/CD8+ cell ratio in patients with OLP. The CD4+ cell population was also decreased in OLP patients in partial agreement with Sugerman *et al.* (1992). However, all these alterations were seen only in patients with hyperkeratosic forms of OLP, while in patients with atrophic-erosive varieties there was no indication of significantly different T-cell subset proportions from healthy subjects. On the other hand, no differences regarding cellular immunity were shown among patients with different forms of OLP.

Significantly, most of the patients (70%) studied by Yamamoto *et al.* (1990) and all the cases analyzed by Sugerman *et al.* (1992) were affected by hyperkeratosic forms of OLP.

Whether these data reflect possible different immunopathogenesis between atrophic-erosive type and hyperkeratosic type of OLP is unclear. Some immunogenetic, ultrastructural and clinical differences between the two forms of OLP have been shown (Jungel *et al.*, 1990; Robertson & Wray, 1992; Porter *et al.*, 1993; Bagan *et al.*, 1993). Several authors have also found significant alterations in humoral immunity parameters in patients with erosive OLP, but these data could have been influenced by the frequent association of medical disorders (especially liver disease) with erosive OLP (Gandolfo *et al.*, 1994). Erosive OLP is probably the result of a particularly aggressive behaviour of the lichenoid infiltrate against the basement membrane zone and could reflect the behaviour of lymphocytes in tissues other than oral mucosa (Cottoni *et al.*, 1988). In our study 40% of the patients with atrophic-erosive lesions were affected by chronic liver diseases most of which were related to hepatitis C virus. The CD4+ T-cell response to viral proteins is usually essential for protection and more recently CD4+ cells responsive to HCV proteins have been shown frequently in patients with HCV infection (Botarelli *et al*, 1993). It was possible that the immune response to HCV could have influenced our results and led to the discrepancy shown in T-cells subsets between atrophic-erosive type and hyperkeratosic type of OLP, but the data were not substantially modified after adjustment for the patients with chronic liver disease AbHCV positive (data not shown).

Unlike Yamamoto *et al.* (1990), we do not think that cellular immunosuppression is a pathologic characteristic of OLP. Instead, our peripheral data suggest that a compartmentalization of T-cells in oral mucosa affected by OLP could lead to an alteration in T-subsets proportions and CD4/CD8 ratio. The characteristic intense stromal inflammatory infiltrate of OLP, mainly composed of CD4+ cells (Matthews *et al.*, 1984; Boisnic *et al.*, 1990; Hirota *et al.*, 1990), could alter the CD4/CD8 ratios due to sequestration of T-helper (CD4+) cells in the oral mucous and skin compartment. This phenomenon has been demonstrated in other inflammatory conditions such as sarcoidosis (Crystal *et al.*, 1981). The normal value of the mean T cell population (CD3+) in our OLP patients seems confirm this suggestion and furthermore the migration of T lymphocytes from the altered microvascular endothelium into the connective tissue has been demonstrated to be a critical event in the pathogenesis of LP (Gilhar *et al.*, 1989; Shiohara *et al.*, 1986). On the other hand, there are no conclusive data on functional alterations in vitro of peripheral blood mononuclear cells (PBMC) in OLP (Konttinen *et al.*, 1989; Malmstrom *et al.*, 1989; Yamamoto *et al.*, 1990; Sugerman *et al.*, 1992) and the cell-mediated immune responses of PBMC in OLP patients are normal in terms of delayed hypersensitivity reactions in vivo (Griffith *et al.*, 1974). Also the decreased natural killer (NK) cell activity reported by Hunyadi *et al.* (1986) was not confirmed by Yamamoto *et al.* (1990) and there are not different NK subset proportions in OLP patients, as confirmed by our data.

In conclusion, the current investigation indicates that quantitative alterations of PBMC in hyperkeratotic forms of OLP could represent a shift of CD4+ cells from the vascular to the oral mucosa (and skin) compartment rather than indicate cellular immunosuppression as previously supported by Yamamoto *et al.* (1990). Analogous phenotypical alterations were not seen in patients affected by atrophic-erosive OLP, but there is no evidence that these data indicate a different pathogenetic mechanism and the reasons for these discrepancies are not clear. Further studies (especially in situ) are required to clarify if some pathogenetical differences exist between the various types of OLP.

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