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Changes in the number and distribution of Langerhans cells in the hydantoin hyperplasic gingiva as compared with the clinically normal one

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Summary. A study was made of the number of Langerhan's cells (LCs) per mm^2 of section which express the antigens T_6 and/or HLA-DR in seriated gingival sections of diphenylhydantoine-induced hyperplasia (HG) and clinically normal gingivae (NG). NG showed histological correlation with its macroscopic appearance. In HG the classical histopathological findings were verified, as well as the epithelial maturation irregularities, conducive to the development of epithelial gaps. In the immunostained samples, LCs appear amply distributed in the epithelium in greater numbers than in NG and more branched except in the immature areas, where they mostly express HLA-DR. In HG keratinocytes, HLA-DR^+ are observed in the basal layer, except in developing epithelial gap zones. The Wilcoxon test for the NG-T6/NG-DR and HG-T_6/HG-DR was not significant; but the Mann Whitney test for NG-T_6/HG-T_6 and NG-DR/HG-DR was significant to $p < 0.05$. It is understood that the increase in LC numbers in HG is a manifestation of their active participation in local immune reactions. The presence of DR^+/T_6^- LCs in the less keratinized areas seems to indicate the relationship of LCs with epithelial proliferation and/or differentiation.

Key words: Gingiva, Hydantoin, Langerhan's cells, T_6 (CD1a), HLA-DR

Introduction

Gingival hyperplasia appears in about half the individuals that use diphenylhydantoine (DPH) as an anticonvulsant drug for long periods. This drug is blamed for a host of collateral effects and idiosyncratic reactions when it is administered therapeutically (Bascones et al., 1981; Ibáñez et al., 1986; Grant et al., 1988).

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The LCs have their place at gingival epithelium level, among the keratinocytes. Their main function is to recognize, process and present antigens to the T-lymphocytes under the HLA-DR code restriction, and it is also thought that LCs have an influence on the epithelial proliferation and/or differentiation. Recently LCs have been studied by means of anti-T_6 (CD1a) and anti HLA-DR (Ia-like) monoclonals. The level of expression of these antigens by LC varies with the pathological circumstances and can reflect the cellular activation status at the start, development and maintenance of the inflammatory pattern linked with hydantoinic hyperplasia, as well as with the growth and keratinization of the epithelium (Castelain and Sagag, 1986; Sauget et al., 1986; Williams and Daniels, 1987; Hammar, 1988; Tapia et al., 1989).

The purpose of the present study is: 1) to determine the quantitative distribution of LCs in a case of gingival hyperplasia induced by DPH, 2) to compare the T_6 and HLA-DR antigen expressions due to these cells, and 3) to relate the results with those assessed in the normal gingival mucosa.

Materials and methods

Five biopsic pieces were studied: one taken from a 37-year-old male treated with DPH for 15 years and presenting a hyperplasic aspect, the others, with normal clinical appearance, obtained from patients who required field conditioning surgical treatment prior to fixation of a prothesis. Their ages ranged from 30 to 45 (mean 34.4 years) and all had maintained good dental hygiene.

Once the pieces had been obtained, and after washing with physiological serum, most of the material was frozen in liquid nitrogen wrapped with Tissu-tek(R). Each piece was then cut down into 10 blocks, which were placed on supports and cryostat sectioned. Thus 10 μm-thick seriate sections were obtained, from which, and for each block, five were immunostained with anti-OKT_6

(Ortho-mune) 1:10 diluted in PBS, as a primary antibody, and the other alternate five were immunostained with anti OK-DR (Ortho-mune) diluted 1:10 in PBS with the peroxidase-antiperoxidase method and following the immunostaining universal kit instructions for use with murine monoclonal antibodies (Cambridge Research Laboratory). Negative controls were carried out. The reason for our choice of 10 μm sections was to be able to count the same cells in the T_6 and DR immunostainings, the diameter of LCs being estimated at 20 μm.

Each cut was projected upon a paper sheet. A Nikon Micropan 4x lens microprojector was used, and the epithelium contour was drawn. Once adjusted, the projected image was kept at a constant distance throughout all the proceedings. The drawings were digitilized upon a Schlumberger 6451 tablet, linked to an IBM computer, the areas being computed by means of correct software for morphometric data; the conversion into mm^2 of the surface units given by the computer was made after extrapolation of the measures taken in a 25 cm^2 (true) square with an Olympus calibrating microslide OBM-1/100, placed in the microprojector plate and projected in the same conditions as the samples under study.

A simple count of the LCs was made on all the immunostained sections, only those somas presenting at least two dendrites being taken into account. This tally was always made by the same worker. Once the number of LCs/mm^2 of epithelium section in the immunostainings with both anti-T_6 and anti-DR was assessed, the simple arithmetic mean of the number of LCs/mm^2 of epithelium section was obtained for each of the five seriate sections from each of the ten processed pieces taken from the hyperplasic and healthy gingivae.

The values found were statistically analyzed by computer with the aid of the Sigma(R) program. The mean, standard deviation, variability coefficient, standard error of the mean (SEM) and each mean estimate were obtained in order to represent the conditions attributable to the non-accesible LC population group. Likewise, Wilcoxon tests were made (paired data between the normal-T_6/normal-DR and hydantoin-T_6/hydantoin-DR variables), and Mann Whitney tests (bilateral contrast between the normal-T_6/hydantoin-T_6 and normal-DR/hydantoin-DR variables). Simple and comparative graphs were computer drawn.

For their histopathological study, fragments of normal and hyperplasic gingivae, previously fixed in formol, were embedded in TAAB-transmit resin. The 3μm sections, deplastified in a NaOH-saturated alcoholic solution, were stained with H/E, PAS and Masson's trichrome method.

A Zeiss Universal photomicroscope was used for the observation of all samples, the images being recorded on Kodak Ektachrome 50 DX professional film.

Results

Observation of the sections taken from the healthy gingivae, embedded in TAAB-transmit resin and stained with H/E, PAS and Masson's trichrome method, revealed a characteristic masticatory epithelium, compounded of several cellular strands which traced deep crests towards the chorion, from which they were separated by a basal membrane. The elements of the germinative stratum showed basophilia and characteristic cubic-cylindrical morphology. Stratum spinosum was compounded by several cellular strands, formed by polygonal cells which flattened as they neared the surface. Overlaying stratum granulosum, contained 3-4 layers of flat cells, with dense cytoplasms and picnotic nuclei; lastly there was the parakeratosic stratum, integrated by keratinized cells which sometimes retained their nuclei. Some sections emcompassed the epithelium of the sulcus, with a thinner epithelium now appearing which lacked parakeratosic stratum and which had a less prominent epithelial crest. In no section was there junctional epithelium.

The chorion appeared with the characteristic aspect of a dense connective tissue, with thick collagen fascicles traversing it in an undulating way and, mostly, perpendicular to the epithelium. Among the collagen fibers there was a rich vascular bed, some nervous fascicles and a cellular population compounded mainly of fibroblasts, some isolated mastocytes and a few cells of an inflammatory nature. Those which indicated a subclinical inflammation, we considered as normal; even more so those located in the proximity of the gingival sulcus, on account of the septic environment, and the presence of the bacterial plaque or simply the buccal flora.

The study of the sections taken from the hydantoin hyperplasic gingiva (embedded in plastic resin, stained with H/E, PAS and Masson's trichrome method) showed a typical gingival hyperplasic histopathological pattern, to which the appearance of gaps extending through all the mucous epithelium thickness can be added. Alterations at epithelium level consisted of acanthosis, papilomatosis and a discrete hyperparakeratosis. Also, irregularities in the cellular maturing were noticeable, such as thinning and even sudden interruption of the parakeratosic stratum, formation of horny pearls and modifications in the aspect and staining properties of the cellular elements; these last can occur either in isolated cells or in rounded morphology areas, more frequently in those with conical upper base. It should be said here that the cells with retarded maturity showed PAS positivity, vacuolated cytoplasms and compressed and misshapen nuclei. Such alterations in maturity led to the development of epithelial gaps (Fig. 1).

At chorion level an intense development was notice, due to the increase in the collagen fibers, which form an array of fine crisscrossed fascicles; these fascicles were interrupted by focal areas of inflammatory infiltrate, of very variable amplitude, in which numerous plasmatic cells stood out loaded with Russell's bodies as well as lymphocytic and histiocytic elements.

Microscopic observation of normal gingivae sections immunostained with the anti-T_6 allows a description of the LCs as elements placed, mainly, in the epithelium suprabasal zone, whose dendritic processes wind among

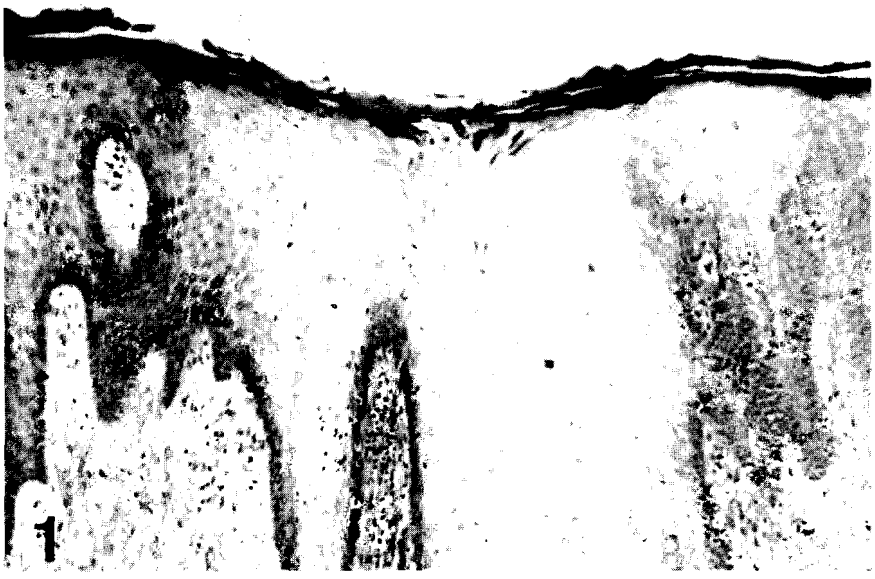


Fig. 1. Hyperplasic gingiva. Zonal interruption of parakeratosic layer and altered staining properties of underlying epithelial cells whose cytoplasms are pale and wide. TAAB resin. Masson trichrome method. × 100

Fig. 2. Hypeplasic gingiva. Numerous LCs are seen at suprabasal and intermediate layers. But they are more densely arranged in the suprapapilar zones where epithelium thickness is smaller. Cryostat section. Anti-OK-T_6 (PAP) × 50

the keratinocytes, branching and following curving paths, some of which almost reach the epithelial surface.

The LC distribution was far from being uniform, varying in each section and tending to concentrate in the suprapapilar zones, where the epithelium thickness was less. Observing the alternate anti-DR immunostained sections, morphologic or distribution differences in relation to T_6 could not be found, although positivity for this antibody in macrophages and endothelial cells of the chorion was distinguishable, as could be expected.

The mean for LC T_6^+/mm^2 of epithelium section in the normal gingivae was 59.61, standard error and deviation being 2.43 and 7.71 respectively. LCs shown by anti-DR reach a mean value of $62.63/mm^2$ section, with standard error and deviation being 1.71 and 5.42 respectively (Table 1). Wilcoxon test between normal-T_6 and normal-DR variables was not significant.

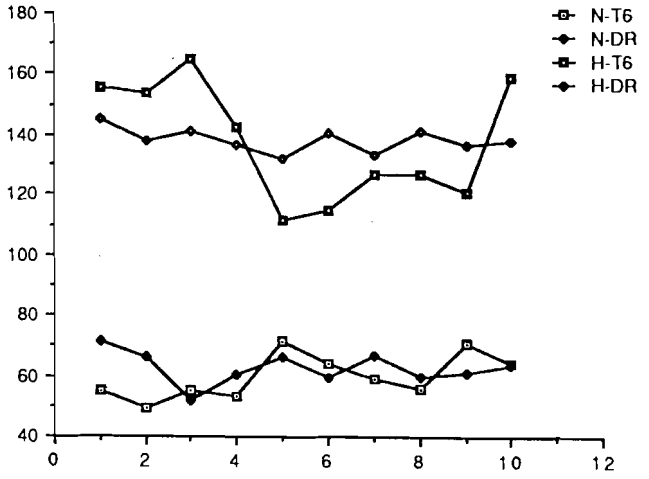
In the samples taken from the DPH hyperplasic gingiva, LCs immunostained with anti-T_6 appeared, subjectively, in greater number than in the healthy ones situated in the epithelium, the suprabasal and intermediate strata and with a greatly developed dendritic component; notwithstanding the highly random LC-T_6^+ distribution, they appeared more concentrated in the zones of lower epithelial height and very scantily in the areas with maturity alterations by defect. Likewise, immunostaining with anti-DR revealed a greater LC ratio than in the healthy gingivae; also, LCs appeared somewhat more branched than when exposed to anti-T_6 and their distribution varied in the zones with lesser staining capacity inasmuch as they populated the said zones in greater proportion than LCS-T_6+. Also, the occurrence of positivity for HLA-DR antigen at basal stratum keratinocytes level was notable, and, to a lesser degree and isolated, in keratinocytes groups of the lower part of the spinosum stratum. Immunostaining with anti-DR located this antigen on the surface of the aforesaid epithelial cells, intracytoplasmically in the basal ones, and, occasionally in the suprabasal intercellular spaces. Finally abundat DR^+ macrophages were outstanding in the chorion, which tended to concentrate in the basal lamina region, mainly in the zones located immediately under the immature areas (Figs. 2-5).

The mean number of LCs-T_6^+/mm^2 of epithelial section in the hyperplasic gingiva was 137.63 with a standard error and deviation of 6.25 and 19.79 respectively. LCs revealed by anti-DR amounted to a mean of $138.26/mm^2$ and 1.24 and 3.93 standard error and deviation (Table 1). Wilcoxon's test between the hydantoin-T_6/hydantoin-DR variables was not significant on account of a similar number of $T6^+$ and

Table 1. Number LC_S/mm^2 of epithelial section

BLOCK No. ($\bar{x}$ IN 5 SECTIONS)	T_6 CONTROL $\bar{x}$ IN 4 SAMPLES	DR CONTROL $\bar{x}$ IN 4 SAMPLES	T_6 HYDANTOIN	DR HYDANTOIN
1	55.27	71.45	155.38	145.29
2	49.08	66.00	153.69	137.72
3	55.20	51.86	164.82	140.92
4	52.89	60.13	142.53	136.53
5	71.73	66.22	111.33	132.26
6	64.13	59.76	114.73	140.80
7	59.22	66.81	127.01	133.21
8	55.80	59.43	126.93	141.27
9	70.65	60.80	120.86	136.55
10	64.58	63.82	159.25	137.99
TOTAL MEAN	59.62	62.61	137.63	138.26
SEM	2.43	1.71	6.25	1.24
σ	7.71	5.42	19.79	3.93

Graph. 1



DR^+ elements. Nevertheless, on comparing the normal-T_6/hydantoin-T_6 and normal-DR hydantoin-DR variables by means of Mann-Whitney's test, significance level reached $p < 0.05$; the theoretical limit for U being equal to 23.

In graph 1 the differences between the mean of the T_6 and DR variables for the normal and hyperplasic gingiva are displayed.

Discussion

These results pose several questions for discussion, such as: 1) the presence of epithelial gaps, 2) the influence upon the number of LCs expressing T_6 and DR-antigens of the process activity level, 3) HLA-DR expression on the part of keratinocytes.

Relative to the first question, we have already pointed out in an earlier work (Pino et al., 1985) that the absence of parakeratotic stratum exhibited by

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Fig. 3. Early epithelial gap with altered staining properties and almost devoid of T_6-positive Langerhans cells. Cryostat section. Anti-OK-T_6 (PAP). × 50

some epithelium zones coincides precisely with those areas where there are epithelial gaps. This causes us to think that in those zones a focal reepithelization

Fig. 4. Same field as Fig. 3 with DR-positive Langerhans cells and numerous macrophages in the chorion. Cryostat section. Anti OK-DR (PAP). × 50

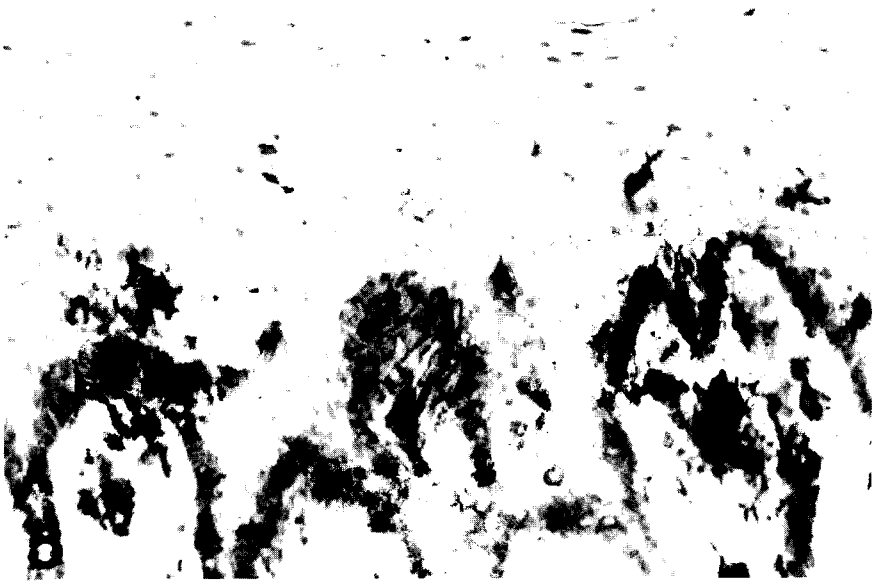

Fig. 5. Hyperplasic gingiva. DR-positive keratinocytes in the basal layer. Cryostat section. Anti-OK-DR (PAP). × 126

phenomenon takes place, which in its first phases retards maturation.

In the whole of the process of development and perpetuation of gingival hyperplasia caused by hydantoin drugs, LCs do not remain quiescent, if we bear in mind their participation in the local immune reactions and, possibly, in the epithelial proliferation and/or maturation control. In fact, the LC population existing in any given moment in the hyperplasic gingival epithelium should be the result of a balance between those factors that stimulate their proliferation and those that inhibit it. To assess exactly the LC population it is necessary to bear in mind the existence of the subpopulations: $T6^+/DR^+$; $T6^-/DR^+$ and T_6^+/DR^-, the first of which is predominant in normal conditions, and balanced in the immune response; the second testifies to the presence of LCs recently incorporated from the chorion, whereas the third —as Van Loon (1988) points out— signifies, perhaps, a homeostatic mechanism to prevent hyperstimulation in immune response (Cruchley et al., 1987; Baker et al., 1988; Markopoulos and Konstantinidis, 1988; van Loon et al., 1989).

In the current phase of our work, we have conclusively shown that the LC population, in the case of gingival hyperplasia caused by hydantoin, rises to more than twice that of the normal gingiva, practically all of it being T_6^+/DR^+. Nevertheless, there is a small proportion of T_6^-/DR^+, which is predominantly found in the immature epithelium areas, or, which represents LCs recently incorporated from the chorion that probably require a greater level of keratinization for T_6-antigen expression.

In any case, there are many factors that can have an influence upon the LC numbers in the hydantoinic gingival hyperplasia-developing process, such as immunitary, humoral and cellular depression which occurs in patients treated with hydantoin drugs for long periods of time. The greater susceptibility that these patients have for the development of infections would stimulate, in principle, the antigen presenting capability of LCs, though, in the long run, the inflammatory reaction products would have a negative effect upon these. The proportion of glucocorticoids should also be taken into account as another mechanism implied in the regulation of LCs since, as is well known, hydantoin drugs quicken steroid catabolism. Bearing in mind that corticoids inhibit LC proliferation, their influence would be positive upon the balance (Ibáñez et al., 1986).

We should state here that the LC number/mm^2 of section found by us in the clinically normal gingivae coincides with the values recorded by other authors (Daniels, 1984; Ahlfors et al., 1985; Juhl et al., 1988; van Loon, 1989)

if the appropriate correction is made, because these last values refer to LC density relative to the outer surface of the epithelium instead of the section.

In our work, the immunostaining of the hyperplasic gingiva sections with HLA-DR, revealed, besides the LCs, a clear positivity for this antigen at keratinocyte level. Forson et al. (1987) have recently reviewed the circumstances under which the epithelial cells can express HLA-DR antigens. Although the significance of this finding remains obscure, it seems to be a function that could be induced by certain cytokines, particularly interferon, lymphokines and tumoral necrosis factor. Thus HLA-DR expression in the keratinocytes should appear in a host of circumstances in which the immune response is directed against an antigen, either unknown, bacterial, tumoral or with the DPH itself acting as haptene (Auböck et al., 1986; Walsh et al., 1987). The notion that keratinocytes relieve or cooperate with LCs in their antigen-presenting function seems suggestive. The importance and meaning of these findings should be analysed in greater depth.

In summary, the following conclusions can be made:

1.– The increase in the numbers of LCs in the gingiva with hydantoin hyperplasia as compared with the clinically normal gingivae, reveals the active participation of LCs in the local immune reactions.

2.– Hydantoin drugs acting as haptens, or antigens of a probable bacterial nature —located upon the epithelial surface or carried away by the sulcular fluid— are presented by the LCs to T-lymphocytes in order to initiate the corresponding immune response.

3.— Acquisition of T_6 surface antigen on the part of LCs is in relation to the degree of epithelium maturation.

4.— The point demonstration of T_6- and DR-antigens allows assessement of the evolution of the gingival condition.

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