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MYCOTIC INFECTION IN ORAL LEUKOPLAKIA

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During the last few decades oral leukoplakias have been studied more and more intensively, both from a clinical and a histological point of view (*Hobæk* 1946, *Renstrup* 1958, *Shafer & Waldron* 1961, *Pindborg et alii* 1963). Defining the clinical condition has been the subject of a good deal of controversy, but everybody agrees in considering oral leukoplakia precancerous. Several attempts have been made to divide the leukoplakias in different clinical types (*Prinz* 1928, *McCarthy* 1936, *Hobæk* 1946, *Cooke* 1964). Recently *Pindborg et alii* (1963) drew attention to the so called "speckled" leukoplakia, which in contrast to the "common" type displays erythematous areas between hyperkeratotic patches. In this type, epithelial atypia was found in 51 per cent and invasive carcinoma in 14 per cent. These figures are considerably higher than those published on the basis of larger series of cases, where clinical differentiation was not attempted (*Sovadina* 1955, *Shafer & Waldron* 1961) and it therefore seems justified to pay special attention to the speckled type of oral leukoplakias.

The frequency of oral mycotic infection has shown a steady rise in recent years, and several authors (*Hultin* 1954, *Giunchi* 1958, *Cawson* 1963, *Lehner* 1964) have pointed out the widespread occurrence of Candida albicans in different lesions of the oral mucosa.

During an investigation on the occurrence of Candida albicans in keratotic lesions it was constantly found, that swabs or smears from speckled leukoplakias contained an abundant number of Candida albicans organisms.

Since monilial infection in oral leukoplakia has not been mentioned previously in the literature, the present investigation was carried out with the purpose of showing the possible role played by yeasts in the development of speckled leukoplakia.

PRESENT INVESTIGATION

Material

The series consists of 48 patients (9 female, 39 male) displaying a total of 64 oral leukoplakias, their ages varying between 28 and 74 years, the average being 53. As controls serve 45 patients without clinical signs of pathologic conditions of the lining oral mucosa.

In 16 patients — all men — 23 leukoplakias were of the speckled type, selected from the material according to the criteria of *Pindborg et alii* (1963), showing varying degrees of erythema in between white, hyperkeratotic areas. The remaining 41 lesions, hereafter referred to as "common type leukoplakia", all demonstrated the well-known clinical picture, comprising whitish, often wrinkled and slightly elevated mucosa without erythema. (Figures 1 a and 1 b).

The locations of the lesions were as follows:

	Common type	Speckled type
Commissures	27	21
Buccal mucosa	5	1
Lower lip	4	1
Tongue	2	
Alveolar process	2	
Palate	1	
Total	41	23

Nine of the patients with speckled leukoplakia complained of stinging or burning sensations of varying degree during food intake, especially provoked by spiced or hot food. None of the patients with common type leukoplakias had symptoms.

The common type and the speckled type leukoplakias were never found in the same patient.

Clinical and laboratory procedures

In all the patients including controls, the oral cavity was examined for yeast-like fungi. This was done in two different ways, both qualitatively by agar-plate cultivation and quantitatively (semi-quantitatively) by direct microscopic examination of oral smears.

With a steril cotton-applicator swabs from the patient's tongue, leukoplakic lesions and in case of upper denture also from the palatal mucosa, were transferred to a maltose-agar plate (Sabouraud) with penicillin and streptomycin added to suppress bacterial growth. The plate was incubated at 37° C for 72 hours, whereupon possible colonies were examined for Candida albicans, partly by tube-formation in human serum, partly by chlamydosporeformation on rice-agar. Yeasts, different from Candida albicans, were identified by fermentation tests.

At the same time scrapings were taken from the areas previously mentioned, the material was spread on slides, fixed in ether/alcohol 1: 1 and stained according to the P.A.S.-method with hematoxylin as counterstain. The slides were evaluated with reference to occurrence of hyphal structures, differentiating quantitatively between single or no hyphae contrary to colonies of hyphae. This difference is illustrated in Figures 2 a and 2 b.

To show that the hyphal structures, seen by direct microscopy, may well be interpreted as yeasts, the following experiment was carried out: A verified Candida albicans culture, taken from a Sabouraud plate, thus being in the blastospore phase, is transferred to human, yeastfree saliva, containing penicillin and streptomycin. After incubation for 18 hours at 37° C hyphal structures are seen, morphologically identical to those found in smears (see Fig. 3). In two cases Candida albicans, also in the yeast phase, was smeared on the dorsum of the tongue in yeast-

Fig. 1 a. Speckled leukoplakia behind the right commissure, showing white patches on an erythematous background.

Fig. 1 b. Common type leukoplakia behind the right commissure.

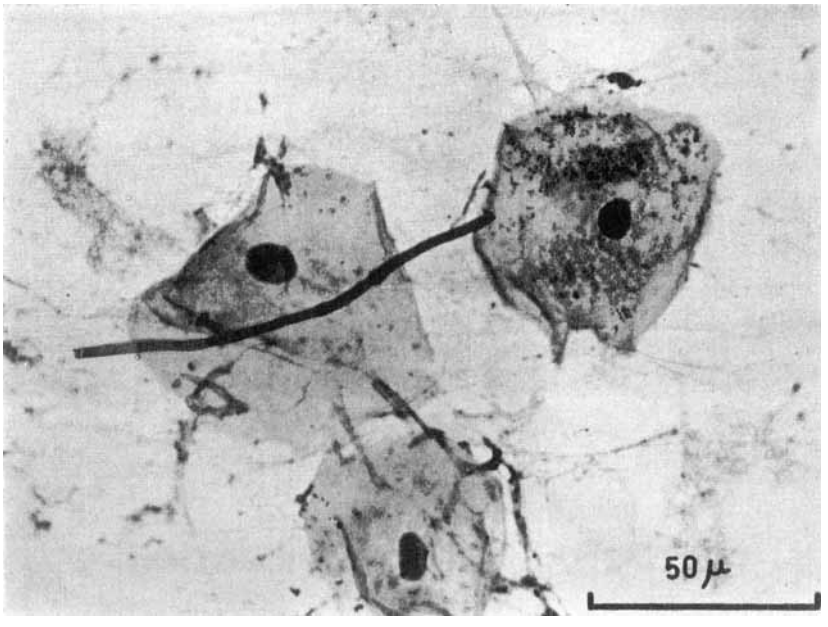

Fig. 2 a. Single hypha as found in smear from a control patient. P. A. S.-hematoxylin.

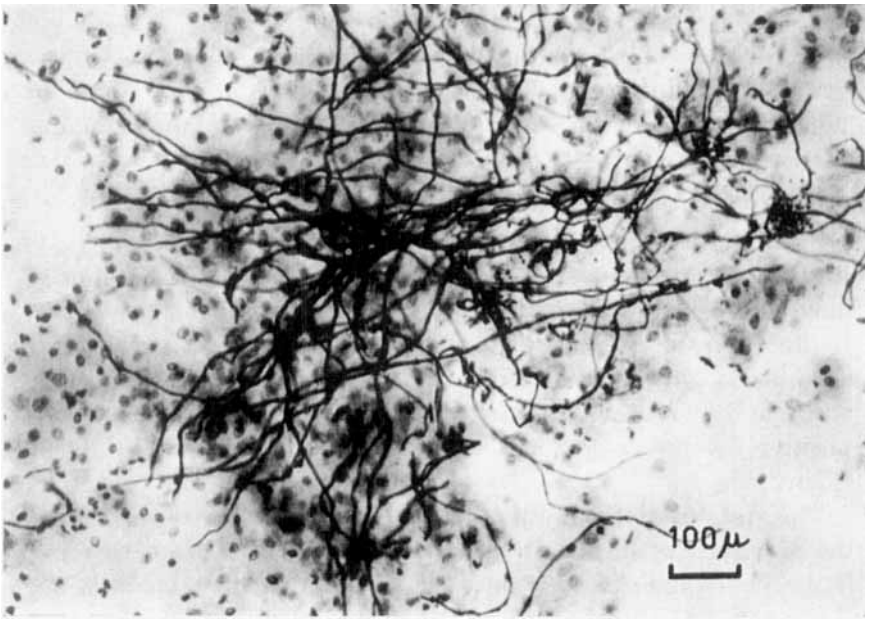

Fig. 2 b. Colony of hyphae as found in smear from a speckled leukoplakia. P. A. S.-hematoxylin.

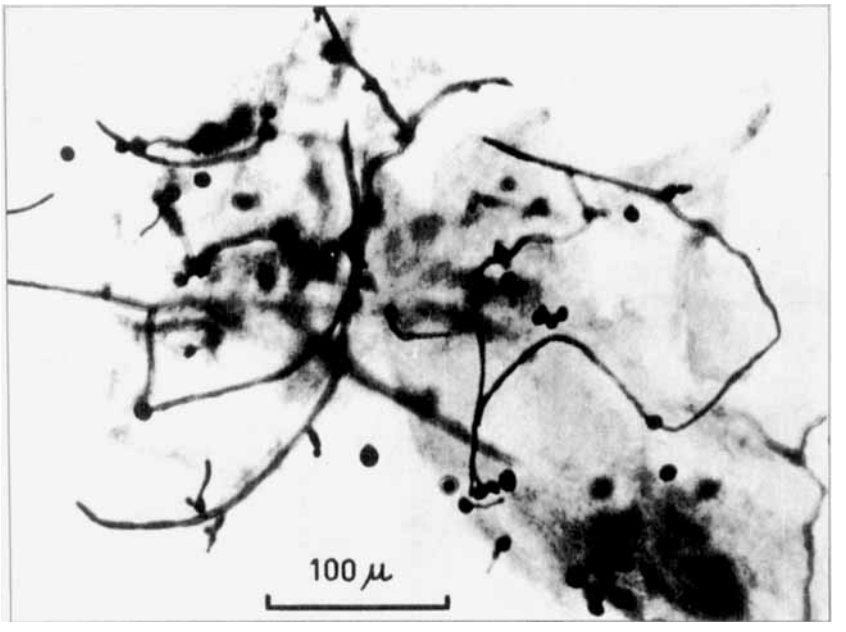

Fig. 3. Hyphal structures developed from blastospores of Candida albicans, incubated in human saliva for 18 hours at 37°C.

free volunteers, who immediately started mouthwashes with a penicillin solution, 150,000 units three times a day. After 48 hours the characteristic hyphal structures were abundantly found in scrapings from both cases.

Results

The qualitative examination of the 45 controls revealed yeast-like fungi in 21 cases or 47 per cent. Cultivation from the 64 leukoplakias showed positive growth in 35 cases (55 per cent). If the group of patients with speckled leukoplakias is evaluated separately, growth was found in 21 of 23 cases (91 per cent), while in the group of common leukoplakias 14 of 41 lesions were positive (34 per cent). These results are recorded graphically in Figure 4 a.

The quantitative examination of P.A.S.-stained smears from the 45 controls showed single or no hyphae, but in no case colonies of hyphae. In the group of patients with leukoplakia, colonies of hyphae were found in 24 out of 64 lesions (39 per cent). The group of speckled leukoplakias taken separately revealed,

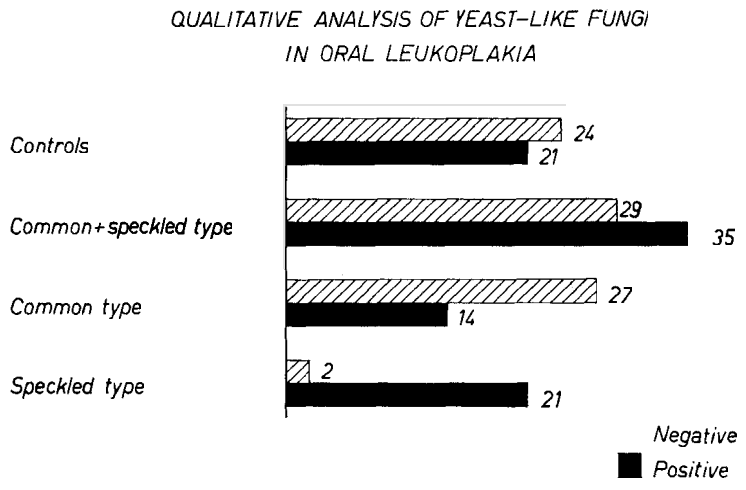

Fig. 4 a. Graphical recording of results of agar plate cultivation.

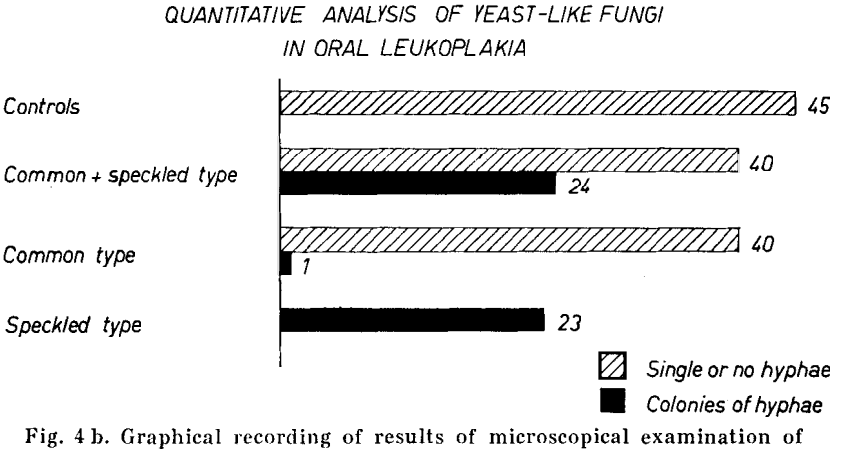

Fig. 4 b. Graphical recording of results of microscopical examination of smears.

however, colonies of hyphae in all scrapings (23 of 23), while this picture was seen in only one out of 41 common type leukoplakias. (Fig. 4 b). This single positive case was re-examined after 2 months and at that time found negative without any treatment having been performed.

The two negative results in the qualitative analysis of the speckled group are derived from the same patient and thus from the same, bipartited agar-plate. In the smears from the same two lesions, heavy occurrence of hyphal structures was seen, and the

negative result in cultivation is therefore considered a laboratory failure.

The laboratory identification of the cultivated yeasts showed the following distribution:

	Negative	Candida albicans	Other yeast-like fungi	Total
Controls	24	16	5	45
Common type leukoplakia	27	5	9	41
Speckled type leukoplakia	2	21		23

"Other yeast-like fungi" comprise the following species: Torulopsis glabrata, Candida krusei, Candida guilliermondii, Candida tropicalis, Saccharomyces cerevisiae and Torulopsis inconspicua.

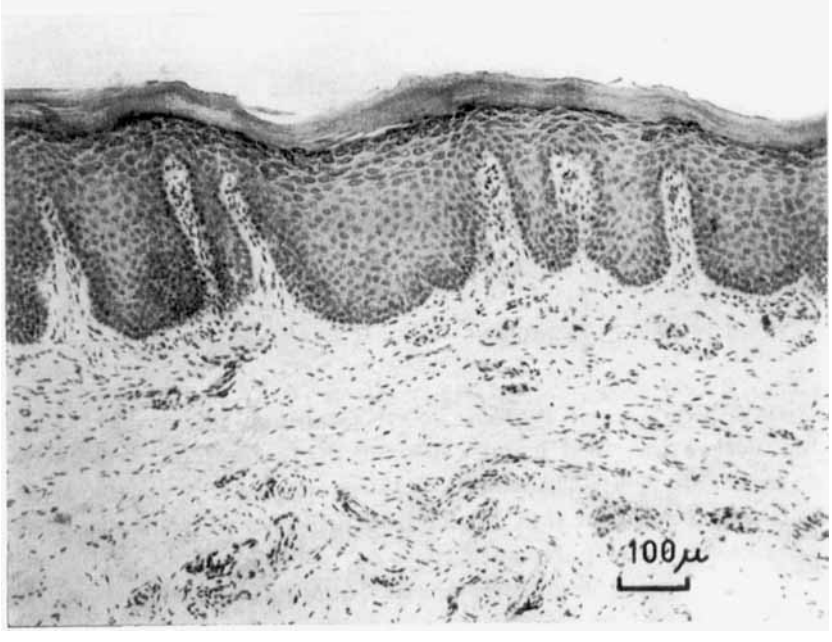

Fig. 5. Photomicrograph showing the histomorphology of a common type leukoplakia. Note orthokeratosis, acanthosis and slight subepithelial inflammation. Hematoxylin — eosin.

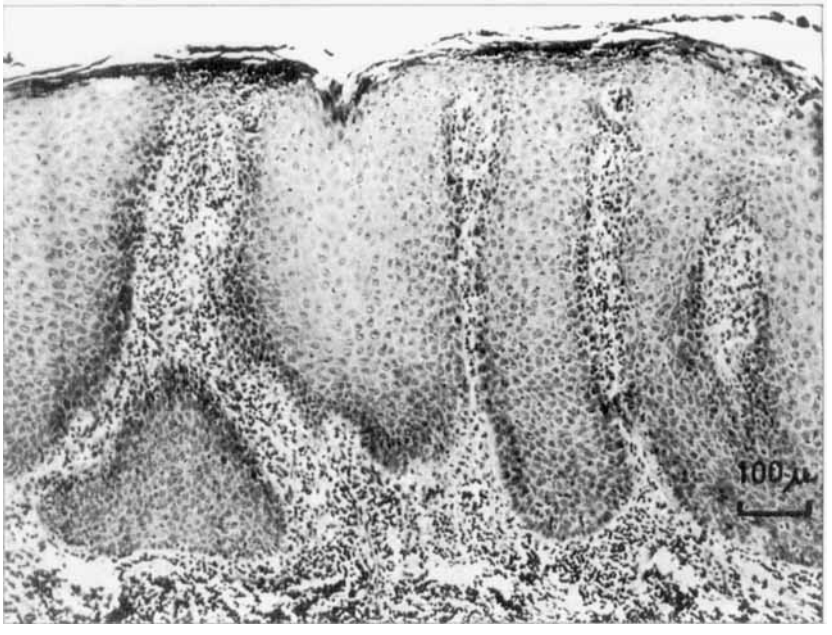

Fig. 6. Photomicrograph showing the histomorphology of a speckled leukoplakia. Note parakeratosis, epithelial hyperplasia, epithelial atrophy and heavy subepithelial inflammatory reaction. Hematoxylin — eosin.

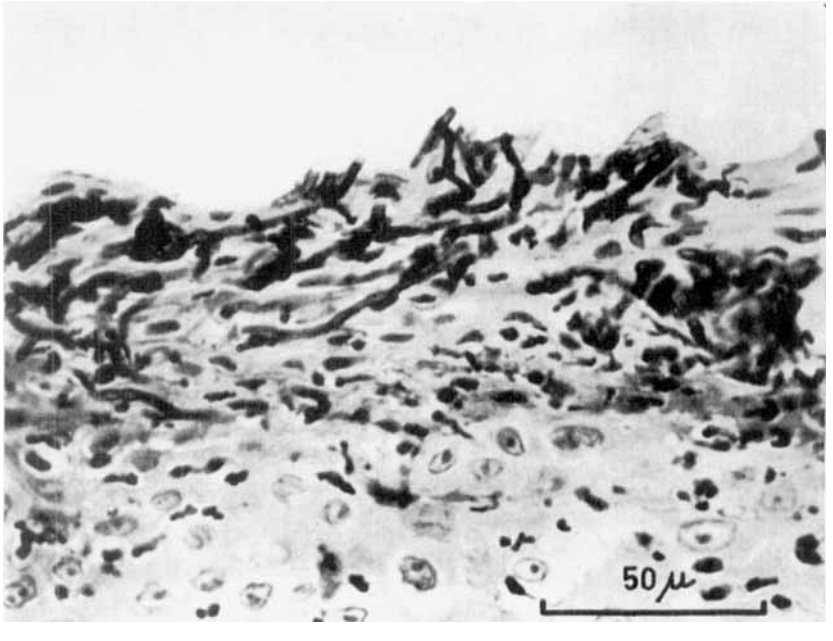

Fig. 7. Hyphal structures in the superficial layers of the epithelium in a section from a speckled leukoplakia. P. A. S.-hematoxylin.

Fig. 8 a.

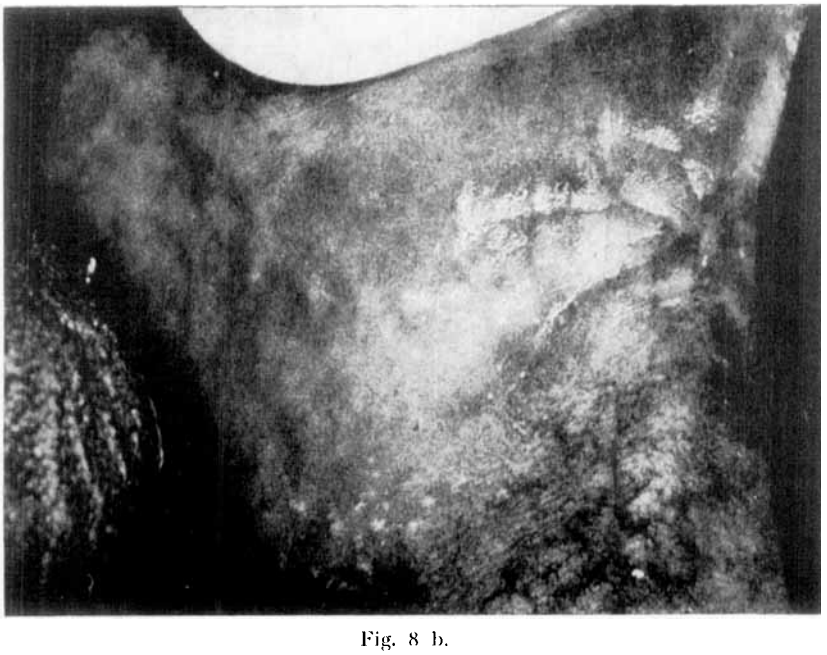

Fig. 8 b.

Fig. 8 a and b. Speckled leukoplakia before and after treatment with Nystatin. A change to common type leukoplakia is demonstrated. Note disappearance of erythematous areas.

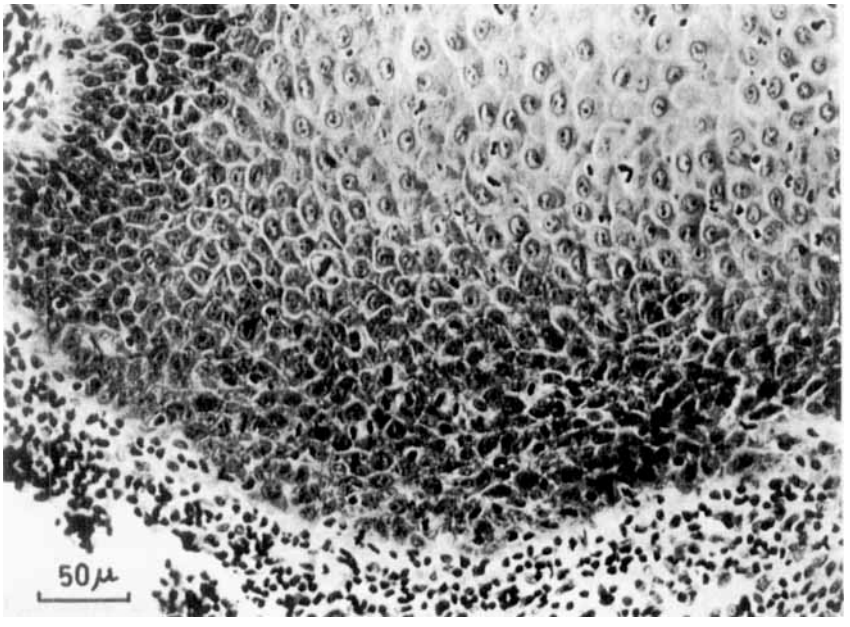

Fig. 9. Photomicrograph from the same section as seen in Fig. 6, showing increased mitotic activity in the basal layers of the epithelium. Hematoxylin — eosin.

Histopathology

Eleven of the speckled leukoplakias and 13 of the common type were biopsied. 7μm sections were stained with hematoxylin-eosin as well as P.A.S.

Examination of the speckled leukoplakias disclosed the following characteristics, which were seen in all sections: hyperparakeratosis, acanthosis, alternating epithelial hyperplasia and atrophy, superficial, intraepithelial leucocyte-infiltration and moderate or heavy subepithelial, chronic inflammatory reaction. P.A.S.-stained sections revealed hyphal structures in the uppermost parakeratotic layers of the epithelium. Epithelial atypia, malignant features or frank carcinoma were not seen.

The common type leukoplakias showed hyperorthokeratosis or hyperparakeratosis or both, acanthosis, slight or moderate chronic inflammation in the connective tissue, mild hyperplasia and in two cases a few superficially located hyphae, only one of these

accompanied by intraepithelial leucocyte-infiltration. None of these sections showed epithelial atypia or carcinoma. Illustrations 5, 6 and 7 demonstrate the above mentioned findings.

The mitotic activity in the basal cell layers of the epithelium, however, seems to be considerably higher in the speckled than in the common type, but this problem has not been studied in detail. See Fig. 9.

Following the *antimycotic treatment,* biopsies were taken from 4 of the 13 cases of speckled leukoplakia previously biopsied.

Histological examination revealed the following changes in comparison with the microscopical findings prior to treatment:

The hyphal structures and leucocyte-infiltration in the superficial epithelial layers had disappeared. The hyperparakeratosis had changed completely or partly to hyperorthokeratosis with a distinct granular cell layer. The epithelial atrophy had diminished and in 3 of 4 cases the chronic, subepithelial inflammation showed a regressive tendency. Increased mitotic activity as illustrated in Fig. 9 was not seen in these 4 post-treatment biopsies.

Treatment

In an attempt to clarify the possible influence of a mycotic infection on the clinical appearance of the speckled leukoplakia, treatment was undertaken, using Nystatin (MYCOSTATIN SQUIBB) tablets*), each containing 500,000 units. One tablet was allowed to dissolve in the mouth three times a day for 14 days or in a few instances for 4 weeks.

At the end of the treatment the speckled leukoplakias in 15 patients (22 lesions) had changed to common type leukoplakias. All symptoms had disappeared, and posttreatment-smears and -cultivation were negative in all instances. Figures 8 a and b show a lesion before and after treatment. Side effects from administration of the drug were not seen, but the majority of the patients complained of bad taste of the tablets.

One patient received no treatment, because the leukoplakia in this case spontaneously had changed to a common type with negative smear but still with positive yeast-cultivation.

*) The tablets were kindly provided by BIOFARMA A/S, Copenhagen, Denmark.

The tendency of recurrence is not evaluated, the observation time being too short.

DISCUSSION

When studied by cultivation on agar-plates the occurrence of yeasts in the oral cavities of 48 patients with oral leukoplakia is found not significantly different from the occurrence in 45 controls without pathologic conditions of the oral mucosae (56 per cent and 47 per cent, respectively). The latter figure corresponds quite well with those of other investigators (*Fox & Ainsworth* 1958, *Stenderup & Pedersen* 1962), whereas the 56 per cent figure can not be supported by any previous figures, due to lack of investigations on this special subject.

It is remarkable that all yeast-like fungi, isolated from the speckled leukoplakias, proved to be Candida albicans. In comparison with the controls, relatively many types, different from Candida albicans, were found in the common type leukoplakias.

In the smears from the speckled leukoplakias, Candida albicans is found exclusively as hyphae (pseudo-hyphae), while the phase of blastospore or chlamydospore is not seen. Several authors, thus *Gresham & Burns* (1960), *Gresham & Whittle* (1961), *Kozinn & Taschdjian* (1962), consider Candida albicans a parasite in the mycelial phase, but a saprophyte in the blastospore phase. This is in agreement with the common finding, that Candida albicans in case of generalised candidiasis is found in the hyphal phase in the tissues. However, in the present investigation single hyphae are found in smears from 4 of 45 controls. It therefore seems reasonable to assume that development of pathologic conditions also is dependent on the number of hyphae present.

The histological picture of the speckled leukoplakia, described in the present investigation, corresponds well with the findings of *Pindborg et alii,* except that epithelial atypia or carcinoma is not seen.

In addition to the histological details, as described by *Pindborg et alii,* the present material reveals leucocyte-infiltration and hyphal structures in the most superficial epithelial layers.

The massive invasion of Candida albicans in the speckled leu-

koplakias can not be readily explained. No relation to antibiotic or steroid therapy can be demonstrated in the present series.

A parallel between increased mitotic activity and hyperparakeratosis in leukoplakias was demonstrated by *Renstrup* (1963). This corresponds well with the present findings, where a decrease in mitotic activity, following treatment, parallels the change of parakeratosis to orthokeratosis.

The clinical, microscopical and therapeutical findings indicate a strong, superimposed Candida albicans infection in the speckled leukoplakia, an infection which perhaps increases or in certain instances alone is responsible for the inflammatory reaction found in the oral mucosa. This in connection with the proven keratolytic properties of the fungus (*Kapica & Blank* 1957) may account for the characteristic pattern of the speckled leukoplakia.

As mentioned previously, *Pindborg et alii* found a high frequency of epithelial atypia and invasive carcinoma in the speckled leukoplakias, but made no comment on this interesting point. One of the characteristic histological features of the speckled leukoplakia is a heavy, subepithelial inflammation, and in this connection it seems worth while to consider the role of inflammation in carcinogenesis as done by *Menkin* (1960). This author isolated a nucleo-peptide from inflammatory exudate and demonstrated that injected subcutaneously it caused epithelial hyperplasia and hyperkeratosis. It was further shown, that in experimental carcinogenesis produced by dimethyl-benzanthracene, a strong acceleration of tumour development was seen, when at the same time the nucleo-peptide was injected. *Menkin* named the substance "the growth factor" and stated, that "it behaves as a cocarcinogen, thus pointing to a definite role of inflammation in carcinogenesis via the growth-promoting factor of exudates".

Renstrup (1962) called attention to the possible influence of inflammation on experimental, chemically induced carcinogenesis in the hamster cheek pouch. The chemical agent alone caused carcinoma in 10 weeks, whereas carcinogen plus local, mechanical irritation, resulting in heavy inflammatory reaction, developed malignancy in only 4 weeks.

Gandini & Gartler (1964) demonstrated a mitogenic effect of extracts from whole frozen yeast (Saccharomyces cerevisiae) on leucocytes in vitro.

In view of these results, it should be conceivable to correlate the higher frequency of epithelial atypia and carcinoma in the speckled leukoplakias with the heavy inflammatory response, due to superimposed Candida albicans infection.

Studies intended to throw light on the pathogenicity of Candida albicans are in progress.

SUMMARY

With the purpose of investigating the possible role of yeasts in the development of the so-called speckled leukoplakia, showing erythematous areas between white patches, 48 patients with a total of 64 oral leukoplakias were examined qualitatively by cultivation and quantitatively by smear-technique for occurrence of yeast-like fungi.

On cultivation, fungi were isolated from all cases of speckled leukoplakia, and laboratory identification constantly showed Candida albicans.

Microscopic examination of smears showed a clear distinction between speckled and common type leukoplakias, in that the former revealed colonies of hyphae, the latter only single or no yeast elements.

Clinically, treatment with Nystatin in all cases changed the speckled type to the common type with a homogenous, whitish, slightly wrinkled surface.

Histologically, the hyperparakeratosis of the speckled leukoplakias changed to orthokeratosis with a decrease in subepithelial inflammatory reaction. Regression of epithelial atrophy and mitotic activity in the basal cell layers was noted.

These clinical, microscopic and therapeutic findings indicate a strong, superimposed Candida albicans infection in the speckled leukoplakias, which may explain the typical clinical pattern.

Further studies are in progress in order to throw light on the pathogenesis of Candida albicans-induced epithelial changes.

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RÉSUMÉ

MYCOSE DANS LA LEUCOPLASIE BUCCALE

Dans le but d'étudier le rôle éventuel des levures dans le développement de la leucoplasie dite "mouchetée", présentant des zones érythémateuses entre des plaques blanches, 48 patients ayant en tout 64 leucoplasies buccales ont fait l'objet d'examens qualitatifs par culture, et d'examen quantitatifs par la méthode des frottis, pour la recherche de champignons levuriformes.

A la culture, des champignons ont été isolés dans tous les cas de leucoplasie "mouchetée", et l'identification au laboratoire a chaque fois mis en évidence Candida albicans.

L'examen microscopique de frottis montrait clairement une différence entre les leucoplasies "mouchetées" et celles du type ordinaire, les premières révélant des colonies de filaments mycéliens, les autres ne présentant que des éléments isolés de levure ou aucun élément.

Du point de vue clinique, le traitement à la Nystatine a dans tous les cas provoqué une transformation du type des lésions, passant du type "moucheté" au type ordinaire, avec surface homogène, blanchâtre, légèrement ridée.

Du point de vue histologique, l'hyperparakératose des leucoplasies "mouchetées" s'est transformée en orthokératose, avec diminution de la réaction inflammatoire sous-épithéliale. Une régression de l'atrophie épithéliale et de l'activité mitotique dans les couches de cellules basales a été notée.

Ces résultats cliniques, microscopiques et thérapeutiques indiquent la superposition d'une forte infection à Candida albicans dans les leucoplasies "mouchetées", ce qui peut expliquer l'aspect typique du comportement clinique.

Des études complémentaires sont en cours dans le but d'ap-

porter des éclaircissements sur la pathogénèse des altérations épithéliales provoquées par Candida albicans.

ZUSAMMENFASSUNG

MYKOTISCHE INFEKTION IN ORALE LEUKOPLAKIEN

Zu dem Zweck, die eventuelle Bedeutung der Pilze in der Entwicklung der sogenannten Fleck-Leukoplakie, die als erythematöse Gebiete zwischen weissen Flecken erscheint, darzutun, wurden 48 Patienten mit im ganzen 64 oralen Leukoplakien auf das Vorkommen von Gärungspilzen hin untersucht, teils qualitativ durch Zucht und teils quantitativ durch Ausstrichtechnik.

Bei der Zucht wurden die Pilze von allen Fällen der Fleck-Leukoplakie ausgeschieden, und eine genauere Typenbestimmung ergab in allen Fällen Candida Albicans.

Eine mikroskopische Untersuchung der Ausstrichpräparate ergab eine deutliche Sonderung zwischen der fleckigen und der gewöhnlichen Leukoplakie, indem erstere Hyphenkolonien aufwies, während bei letzterer nur vereinzelte oder auch gar keine Pilzelemente zu beobachten waren.

Durch Behandlung mit Nystatin änderten sich alle Fälle der Fleck-Leukoplakie in den gewöhnlichen Typus mit einer homogenen, weisslichen, leicht runzeligen Oberfläche.

Histologisch liess sich eine Änderung der Hyperparakeratosis der Fleck-Leukoplakie in Orthokeratosis mit einer Verminderung der subepithelialen Entzündungsreaktion beobachten. Ferner war eine Regression der epithelialen Atrophie und der mitotischen Aktivität in den basalen Zellenschichten zu bemerken.

Diese klinischen, mikroskopischen und therapeutischen Ergebnisse lassen auf eine kräftige, superimposierte Candida Albicans-Infektion in der Fleck-Leukoplakie schliessen, was deren typisches klinisches Bild erklären mag.

Weitere Studien zur Erhellung epithelialer Änderungen, durch Candida Albicans verursacht, sind vorgesehen.

REFERENCES

Cawson, R. A., 1963: Denture sore mouth and angular cheilitis. Brit. dent. J. *115:* 441—449.

Cooke, B., 1964: Leukoplakia buccalis. Ann. roy. Coll. Surg. Eng. *34:* 370—383.

Fox, E. C. & G. C. Ainsworth, 1958: A contribution to the mycology of the mouth. Brit. med. J. No. *5100:* 826—828.

Gandini, E. & S. M. Gartler, 1964: A yeast mitogenic factor active on human peripheral leucocytes in culture. Nature (Lond.) *203:* 898—899.

Giunchi, G., 1958: Candidosis following antibiotic therapy. Sci. med. ital. *6:* 580—628.

Gresham, G. A. & M. Burns, 1960: Tissue invasion by Candida. In: Progress in the Biological Sciences in Relation to Dermatology. Ed. *A. Rock.* Cambridge: Cambridge Univ. Press pp. 174—183.

Gresham, G. A. & C. H. Whittle, 1961: Studies of the invasive, mycelial form of Candida albicans. Sabouraudia *1:* 30—33.

Hobæk, A., 1946—47: Leukoplakia oris. Acta odont. Scand. *7:* 61—91.

Hultin, M., 1954: Candidos och antibiotica. Svensk tandläk.-T. *47:* 441—451.

Kapica, L. & F. Blank, 1957: Growth of Candida albicans on keratin as sole source of nitrogen. Dermatologica (Basel) *115:* 81—105.

Kozinn, P. J. & C. L. Taschdjian, 1962: Enteric candidiasis: Diagnosis and clinical considerations. Pediatrics *30:* 71—85.

Lehner, T., 1964: Chronic candidiasis. Brit. dent. J. *116:* 539—545.

McCarthy, F. P., 1936: Etiology, pathology and treatment of Leucoplakia buccalis. Arch. Derm. Syph. (Chic.) *34:* 612—623.

Menkin, V., 1960: Role of inflammation in carcinogenesis. Brit. med. J. No. *5186:* 1585—1594.

Pindborg, J. J., G. Renstrup, H. E. Poulsen & S. Silverman, Jr., 1963: Studies in oral leukoplakias. Clinical and histologic signs of malignancy. Acta odont. Scand. *21:* 407—414.

Prinz, H., 1928: Leukoplakia oris — a clinical study. Dent. Cosmos *70:* 663—672 & 801—811.

Renstrup, G., 1958: Leukoplakia of the oral cavity. A clinical and histopathological study. Acta odont. Scand. *16:* 99—111.

—»— 1963: Studies in oral leukoplakias. Mitotic activity in oral leukoplakias. Acta odont. Scand. *21:* 333—340.

Renstrup, G., J. B. Smulow & I. Glickmann, 1962: Virkningen af kronisk mekanisk irritation på kemisk fremkaldt cancer i kindposen på hamstre. Tandlægebladet *66:* 661—676.

Shafer, W. G. & C. A. Waldron, 1961: A clinical and histopathologic study of oral leukoplakia. Surg. Gynec. Obstet. *112:* 411—420.

Sovadina, M., 1955: Ad Leukoplacia oris. Cs. Stomat. *55:* 116—120.

Stenderup, A. & G. T. Federsen, 1962: Yeasts of human origin. Acta path. microbiol. Scand. *54:* 462—472.