Carcinogenic Effect of a Dimethyl Sulphoxide Extract of Betel Nut on the Mucosa of the Hamster Buccal Pouch

THE high incidence of oral cancer in the Far East is generally attributed to the habitual use of betel nut "quids" which may contain, as well as betel nut, cured tobacco and various other organic and inorganic materials[1-4]. Attempts to extract from betel nut a material carcinogenic for oral tissues have not been successful[5,6], possibly because extracts were prepared with solvents in which the carcinogenic materials were not soluble. We have therefore extracted betel nut, alone or in combination with cured tobacco, with dimethyl sulphoxide (DMSO) and applied the extracts in this vehicle. Our choice of DMSO was based on the evidence that it is an excellent solvent with a low toxicity[7], that it has great powers of penetration[8] and that it enhances the absorption of various drugs through skin and mucous membranes without apparently affecting their pharmacological properties[9-12]. Furthermore, the use of DMSO as a vehicle decreases the latency period for the production of oral tumours in hamsters with dimethylbenzanthracene[13-14]. We have found that repeated, topical applications of DMSO extracts of betel nut to the mucosa of the buccal pouch of hamsters result in the development of leukoplakia and tumours. The incidence of tumours increases when the extract is made from a mixture of betel nut and cured tobacco, although extracts of tobacco alone cause leukoplakia but not tumours.

Extracts were prepared as follows. Betel nut (Banarsi Pan Shop, Jammu, India) was hand-ground in a porcelain mortar, and 30 g of the ground material mixed with 10 ml. of DMSO (Aldrich Chemical Co., Cedar Knolls, New Jersey) in a glass flask and kept in the cold (4°–5° C) for 48 h. The contents of the flask were then transferred to a porcelain mortar, hand-ground to a paste and thoroughly mixed with 10 ml. of DMSO. The mixture was stored at 4°–5° C in a glass flask for 24 h and strained through cheesecloth and stored at 4°–5° C for future use in a glass-stoppered, amber bottle. Extracts of a mixture of betel nut and tobacco were prepared in a similar fashion with 5 g of cured tobacco (Banarsi Pan Shop), ground by hand and added to the ground betel nut before the first mixing with DMSO. Extracts of tobacco alone, with 5 g of hand-ground tobacco serving as the starting material, were similarly prepared and stored.

Table 1 Occurrence of Leukoplakia and Tumours in Hamster Buccal Pouches after Chronic Topical Application of Dimethyl Sulphoxide (DMSO) Extracts of Betel Nut and of a Betel Nut and Tobacco Mixture

Treatment		No. of hamsters with leukoplakia	tumours
DMSO	(11)	0	0
Tobacco	(12)	8	0
Betel nut	(21)	19	8
Betel nut and tobacco	(21)	18	16

The number of hamsters is given in parentheses.

Male, golden Syrian hamsters (Dennen's Animal Industries, Gloucester, Massachusetts), approximately 9 weeks old, were housed in pairs in small mesh wire cages over a bedding of 'Pellicel'. Their diet consisted of laboratory chow and water given *ad libitum*.

Extracts and control solution (DMSO) were applied to the right buccal pouch mucosa with separate camel hair brushes three times a week for 21 weeks. Before each application, the bottles of extracts and control solution were allowed to reach room temperature. In the course of each application, the mucosa of the buccal pouch was examined for gross pathological alterations.

Table 1 shows that the application of extracts of betel nut alone resulted in the development of tumours in 38%, and of leukoplakia in 90%, of the hamsters receiving the extract. Application of the extract prepared from the mixture of betel nut and tobacco resulted in the development of tumours in 76% of the group. Extracts made from tobacco caused leukoplakia in 66% of the group, but no tumours were observed. Topical application of the control solution (100% DMSO) did not result in the development of leukoplakia or tumours. The cumulative incidence of leukoplakia and tumours during the 21 week experimental period is shown in Table 2. The data indicate an earlier appearance of tumours in the hamsters

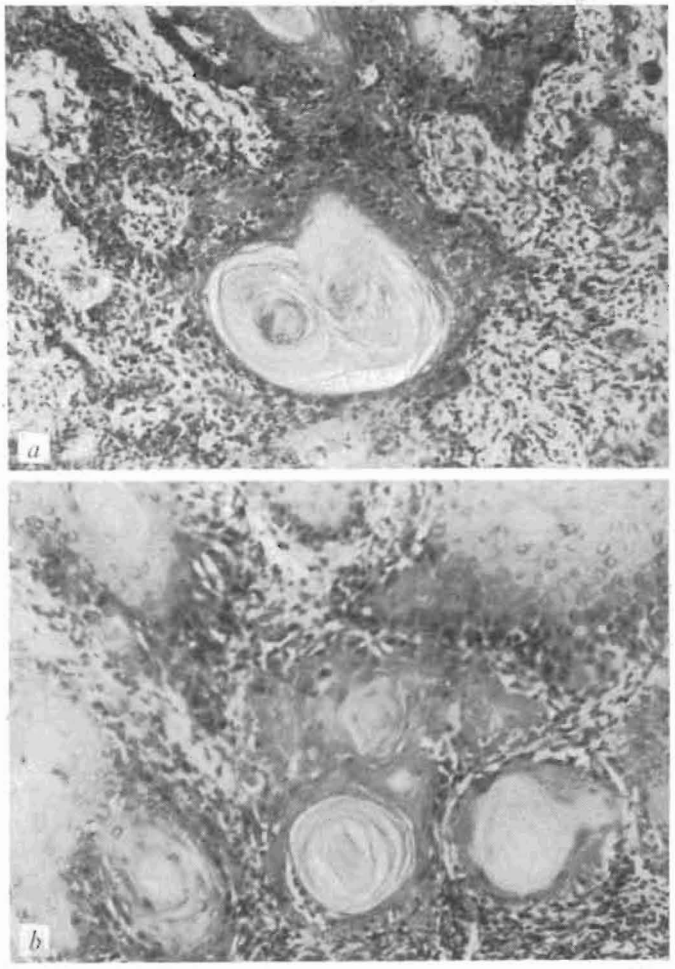

Fig. 1 Microscopic appearance of buccal pouch mucosa after chronic application of the DMSO extracts. *a*, Betel nut extract stained with haematoxylin and eosin (×15). Epithelial cells show loss of polarity. There is dyskeratosis with "pearl" formation. This tumour was classified as a squamous cell carcinoma, grade II. *b*, Betel nut and tobacco extract stained with haematoxylin and eosin (×15). Epithelial pearl formation is very marked. Cell polarity is lost. There is marked enlargement of cells and of their nuclei. Under higher power, numerous mitotic figures were clearly evident. This tumour was classified as a squamous cell carcinoma, grade III.

Table 2 Time Course of the Appearance of Leukoplakia and Tumours during the Application of Extracts of Betel Nut and of the Betel Nut and Tobacco Mixture

Time (weeks)	Leukoplakia		Tumours	
	Betel nut (%)	Betel nut and tobacco (%)	Betel nut (%)	Betel nut and tobacco %
1–6	0	10	0	0
7–9	19	24	0	10
10–12	38	38	14	33
13–15	52	57	24	57
16–18	76	76	38	76
19–21	90	86	38	76

receiving the extract made from the betel nut and tobacco mixture. In both groups, all tumours had developed by the end of 18 weeks. The incidence of leukoplakia, on the other hand, was still increasing at the end of the experimental period.

By gross observation, leukoplakia developed in the form of patches that had a bluish-white or milky-white appearance. Early in their development leukoplakia lesions were not indurated but later they developed a clearly elevated margin and became rough to palpation. Leukoplakia appeared before tumours, and tumours developed only in hamsters with leukoplakia. Grossly, the tumours were of the papillary type and had a nodular appearance. They seemed to grow more rapidly in hamsters that received the extract made from the betel nut and tobacco mixture. The extent of growth of the tumours also appeared to be greater in this group, and some of the tumours reached a very large size and bled quite easily when the camel hair brush was applied.

At autopsy, the hamsters were killed with ether and right and left buccal pouches were dissected, fixed in 10% neutral formalin, sectioned in paraffin and stained with haematoxylin and eosin. Submandibular lymph nodes were also removed for histological study. Microscopic examination revealed that the tumours were squamous cell carcinomas with a variable degree of malignancy (Fig. 1). Examination of the lymph nodes revealed no signs of metastases.

DMSO can solubilize so many compounds that it is not possible to make a reasonable speculation about the identity of the active carcinogenic materials in betel nut. It seems likely that they are fairly potent, for they were effective in producing pathological lesions in spite of the simplicity of the method used to prepare the extracts.

It has been suggested that lime salts in the betel quid eaten in some countries are the carcinogen[6]; our results do not support this suggestion, for lime salts were not added to our extracts. Nor, according to our results, is the tobacco in the quid the source of the carcinogenic materials[1,5]. They suggest, however, that the tobacco contains materials which, although not themselves carcinogenic, can enhance the carcinogenic actions of substances present in betel nut. But it is possible that a carcinogenic action of the tobacco extract could have been demonstrated in a longer experiment or if a more potent extract had been utilized.

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Epidermal Antigens in Cutaneous Dysplasia and Neoplasia

THE loss of tissue-specific antigens, which has been considered to be important in neoplastic behaviour[1-3], occurs in both experimental and human tumours, including squamous cell carcinomas of human[4,5] and murine[6] origin. Techniques used to demonstrate antigen deletion have involved heterologous anti-tissue sera or naturally occurring organ-specific autoantibodies. Among the latter, the discovery of anti-skin activity in the sera of human subjects with the skin diseases pemphigus vulgaris[7] and bullous pemphigoid[8] has provided reagents for the demonstration of antigenic change in experimental skin dysplasia and neoplasia.

We have assessed antigenic patterns in the epidermal elements by the indirect immunofluorescence method[9] using (*a*) pemphigus vulgaris serum, which stains specifically the intercellular areas and cell membranes[7,10] of stratified squamous epithelium, particularly of the stratum spinosum, and (*b*) bullous pemphigoid serum, which reacts specifically with the basement membrane zone[8] of stratified squamous epithelium. Prevention of immunofluorescent staining by serum absorptions with human epidermis, but not other tissues, confirmed the specificity of the reactions studied.

Dysplastic and neoplastic lesions were induced in the skin of hairless mice by five applications of a 0.5% solution of 3-methylcholanthrene in benzene at 14 day intervals[11] or by daily irradiation for 1 min with ultraviolet light of wavelength 2800–3200 Å. The histology of the various skin lesions which developed was correlated with the pattern of epidermal antigens. Epidermal hyperplasia developed in 1–2 months, dysplastic changes in 2–5 months, and squamous cell carcinomas from 4 months onwards. Both ultraviolet light and 3-methylcholanthrene induced similar skin lesions, with the same antigenic patterns, although the changes occurred earlier with 3-methylcholanthrene.

In simple hyperplastic lesions, antigen deletion was not observed—the intercellular and cell membrane immunofluorescence was uniform throughout the entire thickness of the epidermis, which varied from four to eight cells, and the underlying basement membrane zone stained evenly throughout its length. In dysplasias, characterized by focal areas of three to six irregular layers of heaped up cells in the basal region of the epidermis, a loss of intercellular and cell membrane antigenicity was detected by immunofluorescence (Fig. 1). Conventional histological examination showed that the morphology of the antigen-negative areas was that of premalignancy. The cells were pleomorphic, had frequently lost their polarity and their squamous maturation was poor; a clear perinuclear halo zone was often seen in the cytoplasm and the nuclei were hyperchromatic. The fluorescent staining of the adjacent basement membrane zone, however, remained essentially normal.

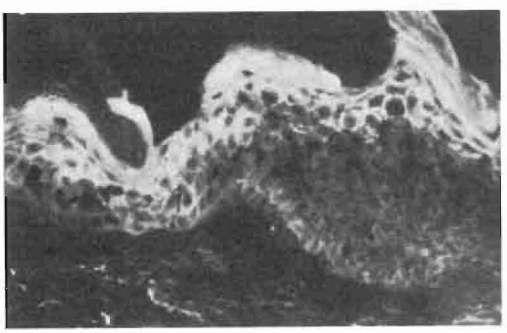

Fig. 1 Immunofluorescent staining by pemphigus serum of dysplastic hairless mouse epidermis. Staining is absent in dysplastic epidermis in contrast to bright fluorescence in adjacent areas. (× 200.)