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In Vitro Bromodeoxyuridine Labelling of Squamous Cell Carcinomas of the Oral Cavity

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Abstract—*Samples of 33 primary and previously untreated squamous cell carcinomas of the oral cavity were labelled by* in vitro *bromodeoxyuridine (BURD) incubation. Positive cells were visualized by indirect immunofluorescence antibody labelling. The cells were examined by fluorescence microscopy and the labelling index (LI) was calculated. The values ranged from 0 to 23.2% with a median of 2.6%. The LIs of T3 tumors were significantly higher than those of T1 and T2 carcinomas. The LIs of primary tumors showing cervical lymph node metastases at the time of biopsy also had significantly higher values than tumors without evidence of lymph node involvement. The BURD technique seems to represent a promising tool for studying the clinical implications of the proliferative behavior of human tumors.*

INTRODUCTION

ANTIBODY LABELLING of bromodeoxyuridine (BURD), which is incorporated into DNA during replication, has greatly facilitated the analysis of cellular proliferation. The most powerful attributes of this technique are its extreme sensitivity as well as the ease and rapidity of sample processing [1].

Recently, this method has been introduced into the study of cytokinetics even in solid human tumors. BURD labelling by either *in vivo* or *in vitro* application could be demonstrated in tumors of the stomach [2, 3], brain [4, 5], breast [6], bladder [7], endometrium [8], and kidney [9, 10]. The goal of the present study was to analyze tumor cell proliferation of a homogeneous collective of squamous cell carcinomas of the oral cavity by *in vitro* BURD labelling and to evaluate the prognostic implications of the results.

MATERIALS AND METHODS

Thirty-three patients with squamous cell carcinoma of the oral cavity were investigated including tumors of the floor of the mouth, tongue, lips, jaws, and palate.

Samples were taken by incision biopsies or cut from surgically removed material and homogenized immediately by thorough mincing with surgical knives. The suspension was passed through a 50 μm nylon mesh and the cells were spun down.

For *in vitro* BURD labelling 5 ml minimum essential medium (MEM) suspended with 10% calf serum and 1% gentamycin (Biochrom, Berlin, F.R.G.) were added to the vital cells immediately after sample preparation. Bromodeoxyuridine/deoxycytidine (Sigma, Deisenhofen, F.R.G.) stock solution, 15 mM each, was added to a final concentration of 15 μM. After 60 min incubation at 37°C the cells were fixed (70% ethanol) and stored at −20°C.

After removal of the ethanol the cells were incubated for 20 min in 1.5 N HCl at room temperature. The denaturation was stopped by washing twice with PBS (pH 7.0). 5 μl anti-BURD monoclonal antibody (Partec, Arlesheim, Switzerland) and 100 μl PBS were added directly to the pellet and incubated for 1 h at 37°C. After washing with PBS 5 μl FITC (fluorescein isothiocyanate) conjugated goat anti-mouse monoclonal antibody (Dianova, Hamburg, F.R.G.) and 100 μl PBS were added to the pellet. The cells were incubated for 30 min at room temperature.

The samples were examined using a fluorescence microscope. The LI was calculated by counting a total of at least 500 nuclei (LI > 5%). If less than 25 positive cells were scored, counting was continued until 25 labelled nuclei could be detected (5% < LI < 1%). The cell count was interrupted at a maximum of 2500 even if less than 25 positive cells were scored (LI < 1%).

RESULTS

The *in vitro* BURD labelling indices were determined from 33 patients with primary and previously

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untreated squamous cell carcinoma of the oral cavity. The LIs varied in a wide range (Table 1) from one specimen showing only single labelled cells in the entire sample (0.0%) up to 23.2% (median 2.6%).

Taking into account the mode of scoring, LIs lower than 1% may show a certain statistical inaccuracy. The 95% confidence interval of the 0.5% value, for example, ranges from 0.22 to 0.78%. However, since these LIs nevertheless are much lower than the median, the significance of the median test especially, by which differences of the frequency distribution of values higher or lower than the median are analyzed (Fisher's exact probability test) is not influenced significantly.

No significant differences were found with regard to tumor localization (data not shown) and histological grading (Table 1). However, T3 tumors and carcinomas with metastatic cervical lymph nodes at the date of biopsy show significantly higher LIs compared with T1/T2 tumors and tumors without lymph node involvement, respectively (Table 1, Fig. 1).

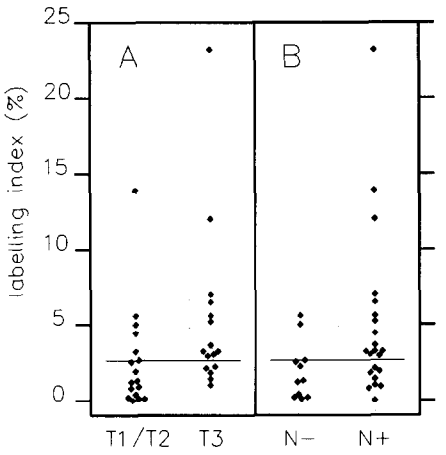

Fig. 1. BURD labelling indices of squamous cell carcinomas of the oral cavity classified with regard to tumor size (A) and lymph node involvement (B). The median value of all patients is indicated by a horizontal line.

DISCUSSION

There are several studies demonstrating analyses of cellular proliferation in collectives of different kinds of solid tumors using the BURD technique [11, 12]. Only a limited number of studies have been based upon the analysis of a considerable number of cases comprising comparable kinds of tumors [3–10]. A common characteristic of these studies seems to be the great variability of the individual labelling indices found even in the same histological type of cancer. A study of 27 tumors of the kidney shows LIs varying from 0.0 to 52% [10]. A comparable range of 4.0–41.4% was found in 24 gastric tumors [3]. A more narrow distribution could be detected in 12 breast tumors varying from 0.8 to 13.8% [6] as well as in two studies on brain tumors showing LIs in 18 patients from <1 to 12.7% [5] and in 22 patients from 0.9 to 13.8%, respectively [4]. The values determined in 33 oral carcinomas demonstrated in the present study obviously fit well with these results (Fig. 1).

The representative nature of the biopsy remains a key point for the validity of the method. However, in order to decrease the risk resulting from spatial heterogeneity attention was paid so that the samples were taken from the tumor periphery. Furthermore, the statistical significance of the median probability test applied to evaluate the data distribution is not much influenced by small deviations of the values.

It is self-evident that the great variety of the LIs does not necessarily express comparable differences in the cytokinetic characteristics of the tumors since this factor represents only one out of several parameters which determine actual cellular proliferation of malignant tissues. Unfortunately, the determination of important factors like growth rate, cell cycle duration or cell loss rate directly at the tumor site is widely restricted by the *in vitro* BURD method. However, it has turned out that the LI is correlated with the growth fraction [13] and consequently represents a functional cytokinetic parameter, which reflects proliferative activity at least to a certain extent. Hence, the clinical relevance of the

Table 1. Statistical analysis of the bromodeoxyuridine labelling indices of oral carcinomas classified by size, lymph node involvement and histological grading

Factor	*n*	Range	Median	*U*-test	Median test (Fisher's exact)
All patients	33	0.0–23.2	2.6	—	—
T1/T2	17	0.0–13.9	1.3	$P = 0.008$	$P = 0.038$
T3	16	1.0–23.2	3.2		
N−	11	0.1–5.6	1.3	$P = 0.021$	$P = 0.027$
N+	22	0.0–23.2	3.2		
G1	8	0.1–13.9	2.9		
G2	16	0.1–12.0	2.7	n.s.	n.s.
G3	6	0.0–23.2	3.1		

BURD LI should be demonstrable by comparison with other clinical parameters commonly accepted as influencing prognosis.

In fact, significant differences could be demonstrated comparing the BURD LIs of 16 benign meningiomas ranging from 0.9 to 3.9% (median 2.0%) and six malignant gliomas showing a distribution from 3.8 to 7.6% (median 6.3%) [4].

Also, in the present study on squamous cell carcinomas of the oral cavity, it could be shown that there are significant differences of the LI distribution wih regard to tumor size as well as to lymph node involvement (Fig. 1). Carcinomas with high LIs are obviously found more frequently in advanced as well as in metastatic tumor stages. However, since T3 carcinomas of the oral cavity usually show lymph node involvement, these factors might be connected. No correlation between the degree of histological differentiation, which is also a factor of great prognostic importance in oral carcinomas, and the specific LI could be found.

Comparable results have been reported in a study giving BURD LIs of 24 gastric carcinomas [3]. High values could be found to be significantly more frequent in advanced stages as well as in tumors showing lymph node metastasis than in early stages without lymph node involvement.

In spite of these promising results indicating a certain prognostic relevance of the BURD LI, the broad overlap of values of different clinical subgroups greatly restricts the clinical validity of this parameter with regard to the individual patient. However, tumor size as well as lymph node involvement have turned out to represent two of the most important prognostic parameters influencing the outcome of the disease in oral carcinoma [14]. Since the purpose of identifying prognostic covariates is the definition of risk groups, *in vitro* BURD labelling seems to represent an appropriate approach in addition to this histopathological and clinical characterization of the tumor, since high labelling seems to be correlated with poorer prognosis and vice versa. It is obvious that more detailed studies with higher numbers of homogeneous malignancies are indispensable to evaluate the prognostic implications of the BURD LI with better accuracy. However, since the application of the BURD method is characterized by its ease and rapidity and, furthermore, advanced equipment is not needed, this technique represents a promising tool for routine application in studying the cytokinetic behavior of solid human tumors and its clinical implications.

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