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Demonstration of an Oral Cancer — Associated Antigen

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Introduction: Tumour-associated antigens have been demonstrated in experimental animal and human cancers (1,2,3,4). The neoantigens expressed by tumours may be released into the extracellular environment and hence, be found in free form and/or as antiten-antibody complexes (immune complexes) in body fluids or serum. The occurrence of tumour-associated antigens in tumour tissue, serum and in immune complexes has been demonstrated in experimental animal tumours and in a few human malignancies (1,5,6). Though elevated levels of circulating immune complexes have been observed in several types of human malignancy (1,6,7,8,9,10), attempts to demonstrate tumour-associated antigens in immune complexes have not met with much success. Identification and characterisation of tumour-associated antigens can lead to the assessment of their potential use as diagnostic and/or prognostic markers, and further, may provide insight into the host interactions with a developing tumour (1). Our earlier studies revealed elevated levels of circulating immune complexes in the serum of oral cancer patients (11,12). The present study was undertaken to verify the presence of any oral cancer-associated antigen in the tumour tissue and/or circulating immune complexes, and if present to isolate, purify and characterise the antigen(s). Results of our preliminary studies in this direction are presented in this paper.

Materials and Methods: Serum samples from patients with histopathologically proved squamous cell carcinoma of oral cavity were pooled. Circulating immune complexes (CIC) were isolated from the pooled serum by polyethylene glycol (PEG) precipitation method (13). Briefly, 2 ml of 3.5% PEG-6000 in 0.1 M borate buffer (pH 8.4) was added to 0.2 ml of serum, incubated at room temperature for 2 hr with occasional stirring and then centrifuged at 2500 xg for 30 min at 4°C. The pellet was washed 3 times with 3.3% ice-cold PEG solution and dissolved in a small quantity of 0.1 M phosphate buffer of pH 7.0. This preparation was purified for IgG - containing CIC by affinity chromatography on Protein A-Sepharose. The CIC dissolved in 0.1 M phosphate buffer was mixed with Protein A-

Sepharose CL-4B, equilibrated with the same buffer, for 2 hr at room temperature. The gel was then washed with the phosphate buffer till complete removal of unbound protein, and the bound protein was eluted with 0.1 M glycine-HCl buffer of pH 3.0 containing 0.5M NaCl. Three ml fractions were collected and protein monitored using a UV-spectrophotometer at 280 nm. The protein containing fractions were neutralised immediatelly with 1 M K_2HPO_4, pooled and dialysed against 0.01 M phosphate buffer of pH 7.2.

The CIC were dissociated by incubating in 0.01 M phosphate buffer (pH 7.2) containing 9 M urea for 3 hr at 37°C and then overnight at 4°C. After the incubation, it was loaded on to a DEAE-Sepharose CL-6B (Pharmacia) column of 35 x 1.5 cm size, equilibrated with 0.01 M phosphate buffer (pH 7.2) containing 9 M urea. Unbound protein was eluted with the same buffer. Two ml fractions were collected and protein content in the fractions was monitored at 280 nm in a UV-spectrophotometer. Bound protein was eluted with 0.01 M phosphate buffer containing 9 M urea and 0.5 M NaCl. Protein peak-forming fractions were identified, pooled and buffer transferred to 0.01 M phosphate buffer of pH 7.2 or PBS of pH 7.4. Undissociated CIC from the bound fractions from the column was removed by adsorbing with Protein A-Sepharose CL-4B.

Oral cancer biopsy specimens were collected, washed repeatedly with sterile normal saline to remove blood and stored at -85°C. Later, the specimens were pooled, minced in phosphate buffered saline (pH 7.4), homogenized, subjected to alternate freezing and thawing to rupture cells and homogenized again. The homogenate was centrifuged at 13,000 xg at 4°C for 30 minutes. The supernatent was collected and stored at -85°C until used. This prepation formed a crude oral cancer antigen (COCA). COCA was chromatographed on a Sephadex G-200 column (35 x 1.5 cm), equilibrated with PBS of pH 7.4. Flow rate was adjusted to 12 ml/hour. Two ml fractions were collected and protein monitored at 280 nm in a UV-spectrophotometer. Peaks were pooled and stored at -85°C. Immunologic activity of the different peaks was tested by immunodiffusion and/or ELISA.

Antibodies against CIC and COCA were raised in rabbits. Anti-COCA and Anti-CIC antisera were extensively adsorbed with normal tissue extracts and normal human serum constituents. For adsorption, these constituents immobilised on CNBr-Sepharose CL-4B (Pharmacia) were used. After the adsorption, IgG was isolated from the antiserum using Protein A-Sepharose or DEAE-Sepharose. Antibodies thus prepared were used in immunodiffusion and ELISA.

Antigen/antibody activity of different preparations was checked by immunodiffusion technique and ELISA. Immunodiffusion was performed following the method reported by Catty and Raykundalia (14). Samples were added into wells punched in different combinations into 1% agarose gel in barbitone buffer of pH 8.6, carried on a microscope slide. The slides were incubated at 37°C for 24 to 48 hrs and examined for

precipitation lines after washing in normal saline or after staining with Amido Black.

ELISA was done following the method outlined by Catty and Raykundalia (15). Wells of ELISA plates were coated with antigen dissolved in 0.05M carbonate-bicarbonate buffer of pH 9.6 at 4°C overnight. After washing 3 times with phosphate buffered saline (pH 7.4) containing 0.05% Tween 20 (PBST), any unoccupied binding sites in the wells were blocked with 1% BSA by incubating at 37°C for 90 min to 2 hours. Antibody, dissolved in PBST-BSA, was added into the wells after washing off the blocking solution with PBST, and incubated at 37°C for 45 min to an hour. The wells were then washed 5 times with PBST, loaded with the second antibody (anti-human IgG or anti-rabbit IgG) labelled with horse raddish peroxidase (1 : 1000 dilution in PBST-BSA) and incubated at 37°C for 45 minutes. After washing the wells with PBST for 5 times, substrate solution (citrate [0.15 M, pH 5.0] buffer of pH 4.5 containing 0.05% orthophenylene diamine and 0.003% H_2O_2) was added and incubated at room temperature for 20 to 30 min for colour development. The reaction was stopped with 2.5 M HCl. Antigen and antibody negative controls were routinely incorporated in every test. BSA solution in carbonate-bicarbonate buffer (100 μg/ml) was used for coating antigen negative wells, and PBST with 1% BSA was used as antibody negative solution.

Results and Discussion: Anti-CIC antibodies, after adsorption with normal human serum constituents, gave a single precipitation line with CIC from oral cancer patients' serum samples (OCIC) in immunodiffusion studies and failed to react with the CIC from normal serum (NIC), demonstrating the presence of a specific antigen in OCIC. When tested with ELISA, the antibody gave positive reaction with patient serum and OCIC, but not with normal serum and NIC.

Ion-exchange chromatography of 9 M urea treated OCIC on DEAE-Sepharose yielded 2 protein peaks. The 1st peak, eluted with 0.01 M phosphate buffer containing 9 M urea, reacted with antihuman IgG antibodies in immunodiffusion. The second peak, eluted with the phosphate buffer containing 9 M urea and 0.5 M NaCl, reacted with the anti-CIC antibody in immunodiffusion and ELISA, revealing the presence of the OCIC-associated antigen in this peak. This peak also contained undissociated CIC, which were removed by adsorption with Protein A-Sepharose. After the adsorption, its reactivity with anti-CIC antibodies was retained, but that with anti-human IgG antibody was lost. ELISA results showed that the 1st peak contained the IgG antibodies from the dissociated OCIC and the 2nd peak contained the antigen.

In immunodiffusion, the anti-CIC antibody reacted with COCA, and this precipitation line was continuous with that of OCIC. This indicated the presence of a common antigen, the tumour-associated antigen, both in OCIC and COCA. The anti-CIC antibody did not react with the PBS extract of normal oral tissue in immunodiffusion or ELISA.

Anti-COCA antibody, after adsorption with normal tissue extracts, reacted immunologically with COCA, OCIC and the second peak of OCIC

from DEAE-Sepharose. The antibody did not react with NCIC and normal tissue extracts.

COCA was chromatographed on a Sephadex G-200 column and resolved into 4 protein peaks (PK): PK I - fractions (fr) 15 to 27; PK II - fr 32 to 38; PK III - fr 48 to 62 and PK IV - fr 70 to 76. The PK I reacted immunologically with anti-human IgG and IgG antibodies and also with anti-CIC and Anti-COCA antibodies, raising the possibility of the presence of immune complexes in this peak. The PK II reacted with antihuman IgG antibodies. The PK IV reacted with anti-CIC and anti-COCA antibodies, revealing the presence of the tumour-associated antigen in the peak.

In ELISA, the PK IV of COCA and the second peak of OCIC from DEAE, after adsorbing with protein A-sepharose and normal serum constituents, gave positive reaction with serum samples from oral cancer patients. No reaction was observed when tested with normal serum samples. These results suggested the presence of specific circulating antibodies to the tumour associated antigen in the serum of oral cancer patients.

The results of our preliminary studies reported here provided evidence for the occurrence of an oral cancer-associated antigen(s) in the cancer tissue and in CIC. Further work is underway to purify and characterise the antigen(s).

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