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EVALUATION OF THREE ANTIGEN NON-SPECIFIC METHODS FOR DETECTING CIRCULATING IMMUNE COMPLEXES IN ORAL CANCER

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ABSTRACT

Circulating immune complexes levels in sera of oral cancer patients were compared with normal sera by three different antigen non-specific methods, namely polyethylene glycol (PEG)-3.5% precipitation assay, PEG turbidity assay and PEG-complement consumption assay. By the first two methods the CIC levels were found to be significant ($p<0.05$) whereas by the third method it was highly significant ($p<0.001$). Further, the number of positivity was also high in the PEG-CC assay (84%) in comparison to the 3.5% PEG assay (44%) and PEG-turbidity assay (40%). Thus PEG-CC method is more sensitive of the three methods used for CIC assay.

INTRODUCTION

Antigen-antibody complexes are known to be involved in the pathogenesis of a variety of diseases. Circulating immune complexes (CIC) are common features in neoplasia and a strong correlation has been reported between their levels and the progress of cancer (1, 2). The antigen part of these CIC, however is not known. As a result, specific test for detecting human cancer at an early stage is still not available. In recent years, a large number of tests for detecting circulating immune complexes, each based on different physicochemical and biological properties have become available (3). These are all antigen non-specific assays and they serve two functions namely, the detection and also the isolation of complexes for identification of the antigen(s) and antibody. Once the antigen has been isolated and characterised from these complexes, the ideal antigen specific CIC assay may be developed.

In this paper, we evaluated three antigen non-specific methods for detecting immune complexes. All the three, involve the insolubilization of the immune complexes by polyethylene glycol (PEG) (mol. wt. 6000) at different concentrations.

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MATERIALS AND METHODS

Sera from 25 oral cancer patients, histologically diagnosed as squamous cell carcinoma, were compared with 25 sera samples from healthy blood donors in all the three analyses. The study was carried out before anti-cancer therapy. Five ml of blood was collected and allowed to clot at room temperature for 2 to 4 hours. Sera were collected after centrifugation at 1,100 g and stored at -70°C without thawing until further use.

a) *PEG 3.5% precipitation assay:* The precipitation was done by PEG as reported by Fust *et al* (4) with slight modification. After thawing, 0.2 ml of the serum in each sample was diluted with 4.8 ml of borate buffered saline pH 8.4 and an equal volume of cold 7% PEG dissolved in borate buffer pH 8.4 was added to make the final concentration of PEG, 3.5%. The mixture was kept overnight at 4°C and centrifuged at 3200 g for 30 minutes at 4°C. The precipitate was washed twice with 3 ml of cold 3.5% PEG, and centrifuged again at 3200 g at 4°C for 20 minutes. The precipitate was redissolved in 2 ml of phosphate buffered saline, pH 7.4 and the total protein was estimated at 280 nm in a spectrophotometer.

b) *PEG turbidity assay:* In this method the increase in turbidity due to insolubilization of immune complex by PEG is measured at 450 nm, as reported by Haskova *et al* (5). One hundred μl of serum in each sample was mixed with either 900 μl of 4.166% PEG in 0.1 M borate buffer pH 8.4 or 900 μl borate buffer alone and incubated at room temperature for 120 minutes. Final dilution of serum was 1 : 10 and PEG final concentration was 3.75%. Tubes were then mixed and read at 450 nm. in a spectrophotometer. The OD 450 of the serum in buffer was subtracted from the OD 450 of the serum in PEG to get the $\triangle$ OD 450 value. Individual patient results were expressed as $\triangle$ OD $\times 10^3$.

c) *PEG precipitation - complement consumption assay:* This assay was done as reported by Harkiss and Brown (6) with modifications. It is based on the isolation of CIC through PEG precipitation and quantitation of the precipitated and redissolved CIC by their ability to activate the complement cascade. To 300 μl of serum, 50 μl of borate buffer pH 8.4 and 50 μl of 0.2 M EDTA were added and mixed gently. An aliquot of 100 μl of 12.5% PEG was added and the tubes were vortexed and then kept at 4°C for 90 minutes. They were centrifuged at 1700 g for 10 minutes at 4°C, the supernatants discarded and the pellets were washed with 1 ml of 2.5% PEG at 1700 g for 15 minutes at 4°C. Once again, the supernatant was discarded and the pellet was dissolved in 30 μl of warm complement fixing diluent (veronal buffered saline) by vortexing. To this is added 15 μl of pooled normal human serum (fresh) as a source of complement and incubated at 37°C for 30 minutes and then on ice. Each sample was then made upto 750 μl with warm complement fixing diluent and then 250 μl- of warm 0.2% sensitized sheep RBC was added to each tube and further incu

bated at 37°C for 15 minutes. The optical density of each sample was then noted at 545 nm. A 100% hemolysis control containing 30 μl complement fixing diluent plus 15 μl of pooled normal human serum and 0% hemolysis control containing 45 μl complement fixing diluent and no normal human serum were also included in the study. The results were expressed as the percentage of complement consumption (% CC).

RESULTS

Table 1 shows the means and standard errors of titres obtained in the three assays from 25 oral cancer patients and 25 normal healthy donors. Significantly high titres were found in all the three methods in comparison to the control group. However, highly significant difference ($P<0.001$) was obtained in the PEG precipitation complement consumption assay whereas in the other two assays it was significant to the extent of $P<0.05$.

Similarly, the incidence of positive results in the two groups were compared. Using the 95th percentile (mean $\pm$ 2 S.D.) of the CIC levels in normal subjects as the cut-off limit, as many as 84% were considered positive in the PEG-CC assay, 44% in the 3.5% PEG assay and 40% in the PEG turbidity assay. All the three assays was able to discriminate between healthy persons and persons with oral cancer but the highest incidence of positivity was found in the PEG-CC assay. One control sample in the 3.5% PEG assay and one control in the PEG-CC assay were positive but it was not from the same patient.

DISCUSSION

The antigen non-specific methods used for detection of CIC in serum are numerous. Each method depends on one or the other limited characteristics of the complexes and the correlation between the results obtained by different assays is often very poor (3, 4). The PEG precipitation method serves as a simple and rapid method for the isolation of immune complexes in large amounts, from which the antigen and antibody can be dissociated, purified and characterized.

Out of the three methods employed only PEG-CC assay could discriminate between the normal controls and oral cancer patients in a better way. Euler *et al* (7) showed that 70% Hodgkin's disease cases had increased levels of precipitable immune complexes as compared to normals by the 3.5% PEG assay, whereas we obtained only 44% positivity by the same method in oral cancer patients. By the PEG-turbidity assay, Rayner *et al* (8) and Chhajlani *et al* (9) demonstrated highly significant values of CIC in different types of cancers in comparison to the normals. But, by the same method we got only 40% positivity and significance high upto $p<0.05$.

TABLE 1. *Mean values, standard errors and incidence of positive results in the different immune complex assays in sera of oral cancer patients and healthy blood donors*

Immune complex assay	PEG-3.5% assay (OD 280 nm) mg%			PEG-turbidity assay (OD 450 nm)			PEG-CC assay (OD 545 nm)		
	Mean ± S.E.	No: +ve	% +ve	Mean ± S.E.	No: +ve	% +ve	Mean ± S.E.	No: +ve	% +ve
Oral cancer patients n=25	278.74 ± 42.6	11	44	322.32 ± 35.96	10	40	59.45 ± 4.12	21	84
Healthy controls n=25	147.56 ± 11.58	1	4	192.69 ± 18.53	0	0	11.9 ± 2.77	1	4
	$P<0.05$			$P<0.05$			$P<0.001$		

n=number of samples

However by the C.C. method, our results were comparable to Brandslund *et al* (10) who reported high concentration of CIC in more than 90% of patients with active rheumatoid arthritis. It seems that a whole spectrum of immune complexes with different properties circulate in the blood of patients with various types of cancer. These complexes may differ from each other in size, in composition or in the nature of antigen and antibody components. In our study, the PEG-CC method was found to be the most sensitive out of the three methods used.

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