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CD_2 Related Immunoregulation in Oral Cancer

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Sera from 93 patients with carcinoma of the buccal mucosa were analysed for its regulatory effect on CD_2 antigen expression using anti CD_2 monoclonal antibodies and sheep erythrocyte rosetting assay. The sera from 55.5% of the patients showed an inhibitory effect (blocker sera) while sera from 44.5% showed an enhancing effect (enhancer sera) on the CD_2 antigen expression. An interesting feature observed was the dominance of enhancer sera in the early stages of the cancer, well differentiated squamous cell carcinoma and verrucous carcinoma. The regulatory effect of the sera had no correlation to the concentration of circulating immune complexes (CIC), ferritin, and serum immunoglobulins on an individual basis. Taken as a whole, the concentration of CIC was higher in the enhancer sera while the concentration of IgG and IgM were higher in the blocker sera. The dominance of enhancer sera in groups with better prognosis in clinical practice suggest a correlation with the presence of serum enhancing factors to a good prognosis. The correlation of regulatory status to the prognosis of the patient is examined as the second phase of the study.
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INTRODUCTION

CANCER OF the oral cavity forms the major cancer among males and the third major cancer among females in Kerala, India. The age adjusted incidence rates in the Trivandrum District are probably the highest in the world [1]. Investigations carried out in this laboratory [2–4] and elsewhere [5–9] have demonstrated impairment of immune responses in these patients. Autologous serum exerts strong regulatory activity on these responses, the most reported being inhibitory [2, 4, 7, 9, 12]. During the course of our investigations, we observed a proportion of the cancer sera possessing an enhancing activity on the immune responses. Hence in this paper we have evaluated the regulatory effect of oral cancer sera on circulating T lymphocytes using expression of CD_2 surface antigen as the marker.

CD_2 antigen is one of the earliest T-cell markers expressed on thymocytes. A number of important functions have been ascribed to CD_2. CD_2 modulates cytotoxic T-cell mediated killing, helper T-cell proliferation, interleukin-2 (IL-2) receptor expression and IL-2 secretion [13–18]. CD_2 also appears to play a role in T-lymphocyte adhesion, signal transduction, differentiation and avidity with which a mature T-cell interacts with its antigen bearing cell [13, 18, 20]. CD_2 antigen takes part in the regulation of antigen independent pathway of activation [18, 22] and it appears that signals delivered via CD_2 are an integral part of antigen specific pathway. Although CD_2 and CD_3-T pathways are independent in most cell types, they are functionally linked in mature T-cells and regulate the quantitative expression of CD_4 and CD_8 surface antigens [23]. Perturbation of the CD_2 extra-cellular segments by certain combinations of anti-CD_2 monoclonal antibodies or other soluble factors, membrane derived molecules, etc. can activate T-lineage function. These CD_2 mediated activation events synergise with signals mediated through the TCR to augment T-cell responses [23, 24]. Brietmeyer has recently observed a down regulatory effect on engaging the CD_2 molecule [25]. Hence the observations of immunoregulations of CD_2 surface antigens by cancer serum could have direct implication on antitumour immunity and the prognosis of the patient.

PATIENTS AND METHODS

Study population

93 patients registering at the outpatient department of Regional Cancer Centre, with biopsy proven cancer of the buccal mucosa and 50 apparently healthy age matched controls were selected for the study. None of the patients had received any previous treatment for the malignancy. They were screened to rule out any symptoms of auto immune disorders or any immunosuppressive/stimulative therapy. The patients were staged into different stages using the TNM system of classification. They were also classified histologically into well differentiated squamous cell carcinoma (WDSCC), moderately differentiated squamous cell carcinoma (MDSCC),

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Poorly differentiated squamous cell carcinoma (PDSCC) and verrucous carcinoma.

Sample collection

Blood was collected by venepuncture. An aliquot of 5 ml anticoagulated blood was used for separation of lymphocytes on ficoll/hypaque gradient. Serum was separated from 8 ml clotted blood and stored at −20°C until it was used. Serum used for blocking assay was inactivated at 56°C for 30 min for complement destruction.

Quantitation and isolation of circulating immune complexes (CIC)

The CIC in serum was isolated using the method of Digeon *et al.* [26] and quantitated by Lowry *et al.*'s method [27] Briefly, serum (1 ml) was precipitated with equal volume of polyethylene glycol (PEG6000) in borate buffered saline (BBS pH 8.4). The precipitate was dissolved in 1 ml phosphate buffered saline (PBS) after two washes with 3.5% polyethylene glycol (PEG). The concentration of the CIC was adjusted to that of the original serum for use in the blocking assay.

Quantitation of serum immunoglobulins and ferritin

Quantitation of serum immunoglobulins was carried out with the help of commercially available single radial immunodiffusion plates. Serum ferritin quantitation was done by enzyme linked immunosorbent assay (ELISA) technique using Tandem-E-Ferritin kits (Hybritech, Europe).

Evaluation of immunoregulatory effect

Lymphocytes isolated by F/H gradient was incubated with either 10% patient/normal individual serum/10% normal AB serum or 10% CIC (concentration equivalent to that in the serum) for 30 min at 37°C. A part of the lymphocytes was washed and further incubated with anti-CD_2 monoclonal antibody (Dakopatts, Denmark). The CD_2 cells were counted using a Leitz UV microscope. The other part was used for erythrocyte rosetting assay. Erythrocyte rosetting assay was carried out by incubating the lymphocytes with washed sheep erythrocytes as routinely evaluated. Sheep erythrocytes were also used after incubation for 1 h with patient's serum to evaluate the effect of serum on erythrocyte surface markers such as LFA. The serum immunoregulatory effect was evaluated using the equation.

$$\%\ \text{Blocking or enhancing} = 1 - \frac{\text{Presence of autologous serum/CIC}}{\text{Erythrocyte rosettes } (CD_2+) \text{ in presence of normal AB serum.}} \times 100.$$

Based on the result, the individuals were classified into blockers (+ve blocking) and non-blockers or enhancers (−ve blocking).

Statistical analysis

The percentage of blocker and enhancer sera were tested for its significance using 'Proportion test' and 'χ^2' test [28]. Students t-test was used for comparison of degree of immunoregulation and serum values with normal controls.

Table 1. Pretherapeutic immunoregulatory effect of autologous serum on CD_2 expression and correlation with stage and histology of disease

Description (No.)	Blockers No. (%)	Enhancers No. (%)	*P* value
Normal control (50)	15 (30)	35 (70)	
Oral cancer patients (93)	52 (55.5)	41 (44.5)	<0.01
Stage I (10)	4 (40)	6 (60)	N.S.
Stage II (35)	20 (55.5)	15 (44.5)	<0.05
Stage III (24)	13 (54.2)	11 (45.8)	<0.05
Stage IV (24)	15 (62.5)	9 (37.5)	<0.01
WDSCC (53)	30 (56.6)	23 (43.4)	<0.05
MDSCC (19)	9 (47.4)	10 (52.6)	N.S.
PDSCC (14)	10 (71.4)	4 (28.6)	<0.01
Verrucous carcinoma (7)	3 (42.8)	4 (57.2)	N.S.

WDSCC = well differentiated squamous cell carcinoma.
MDSCC = moderately differentiated squamous cell carcinoma.
PDSCC = poorly differentiated squamous cell carcinoma.
Comparisons have been made with normal controls.

RESULTS

The results of this study show the expression of CD_2 antigen to be perturbed by autologous serum of cancer patients. Incubation of sheep erythrocytes with cancer serum prior to incubation with lymphocytes had no effect on rosette formation. The immunoregulatory effect was not destroyed by heating to 56°C. Based on the regulatory effect, the patients could be classified into blockers and enhancers. Inhibition of CD_2 antigen expression was exhibited by 55.5% of the cancer sera while 44.5% showed an enhancing activity. This was in contrast to 30% of the blockers and 70% of the enhancers in the normal controls (Table 1). The number of patients showing blocking/enhancing was significantly different from normal controls except in the stage I group of patients, moderately differentiated squamous cell carcinoma and verrucous carcinoma group (Table 1). The degree of blocking in cancer patients ranged from 1.75 to 87% with a mean value of $25.60 \pm 21.68\%$. In the controls, however, the range was much narrower ranging from 1.0 to 30% with a mean value of $8.6 \pm 7.4\%$. On the enhancement side, the range fell between 8 and 130% with the mean value of $30.31 \pm 30.04\%$ in the patients group in contrast to a range of 1–70% in the controls (mean value 13.5 ± 12.2). Both the enhancing activity in the enhancers and blocking activity in the blockers were significantly higher in the oral cancer patients when compared to normal controls (*P* value <0.001).

Correlation with disease

There was a dominance of sera with enhancing activity in the early stages of cancer paralleled by that of blocker sera in the advanced stages of the cancer (Table 1).

On correlation with the histology of the tumour, sera from PDSCC group exhibited a higher degree of blocking (71.7%, Table 1). Incidentally, of the 14 patients belonging to this group, 8 had stage IV disease. In case of verrucous carcinoma, the enhancers outnumbered the blockers. It was of importance that the blocking activity exhibited by the blockers among the verrucous carcinoma were of a very low degree (Table 1).

Correlation with serum proteins

All the serum proteins measured CIC, ferritin, IgG, IgA and IgM showed significant increase in oral cancer patients

Table 2. Levels of serum proteins in oral cancer

	Ferritin ng/ml	CIC† μg/ml	IgG mg/dl	IgA mg/dl	IgM mg/dl
Oral cancer	110±72*	1257*±620	2014±486 N.S.	286±58*	176±47 N.S.
Normal controls	71±25	612±112	1921±375	196±41	167±33

N.S. = Not significant. *$P<0.001$. †Circulating immune complexes.

Table 3. Serum ferritin levels in different histological phenotypes

Histology	WDSCC	MDSCC	PDSCC	VC	Normal control
Ferritin levels (ng/ml)	135.0867±76.6034	126.8157±36.81	140.895±44.9790	95.64±42.52	59.7565±17.7574

(Table 2). The ferritin levels showed almost a 3-fold increase in oral cancer patients. Comparison of the levels in blocker and enhancer groups did not yield any significant difference. There was no correlation of this protein with the severity of the disease or the histological type of the cancer. However, the levels were much lower in case of verrucous carcinoma (Table 3). The degree of blocking or enhancement in individual patient was not related to the concentration of this protein in the serum.

Circulating immune complexes, a widely reported immunosupressant was found in significantly raised levels in oral cancer patients. Comparison between the blocker and enhancer groups showed marked increase of these in the latter. No individual correlation of CIC levels with degree of immunoregulation could be noticed. Immune complexes isolated from the sera exhibited inhibition in about 73.7% patients. Even in those CIC samples where no blocking was noticed, the degree of enhancement was less than 15%.

All the three immunoglobulins studied were increased significantly in oral cancer patients. On comparison of blockers and enhancers, levels of IgG and IgM were significantly increased in the blocker group. All the same, no individual correlation could be obtained between the degree of immunoregulation and concentration of the immunoglobulins.

DISCUSSION

The results of the study clearly demonstrate differential regulatory effect of cancer serum on expression of CD_2 antigens on lymphocytes. Inhibition of erythrocyte rosetting by serum has been reported in solid tumours [2–12]. A number of reports detail a proportion of the cancer sera which do not possess an immunosuppressive activity [4, 7, 9–12]. It is not clear from these reports whether the observation is that of a zero effect or an enhancing activity also. In a recent study, Kitaoka *et al.* reported the detection of an enhancing factor in adult T-cell leukaemia which is homologous to thioredoxin of *E. coli* which acted as a systemic immunopotentiator [29]. The serum factors bring about their effect by influencing the T-cell surface antigens and not the erythrocyte surface as prior incubation of erythrocytes with cancer serum did not affect the immunoregulatory effect. The mechanism responsible for this phenomenon could be either due to binding of the serum factor/s to the CD_2 antigen or in its vicinity thus altering the characteristics of the antigen or by expression of a defective CD_2 antigen [30]. Circulating immune complexes, circulating tumour products and non-specific factors such as viral proteins, serum proteins such as ferritin, acute phase proteins, etc. are some of the immunosuppressive factors reported. In this study, isolated CICs showed immunosuppression *in vitro* in most cases. In some cases, however, no inhibition was noticed. Preformed CIC of high antigen antibody ratio is found to be immunostimulatory. It is not clear whether it has any bearing on a non-specific T-cell activation pathway such as the CD_2 pathway. The lack of correlation of the immunoregulatory activity with the concentration of the CIC could also mean either, that only a few molecules of the CIC are necessary to bring about the effect or that the immunosuppressive effect of CIC is negated by other serum factor(s) which either stimulates the production of CD_2 antigens or act as "unblocking" factors. Presence of blocking antibodies have been documented in cancer patients and the increased levels of IgG and IgM in blockers probably point towards the presence of blocking antibodies, which could be interfering with the T-cell surface antigen. We have also noticed such a phenomenon of serum immunoregulation in corneal transplant patients where presence of enhancing sera was associated with graft rejection (data not shown) showing that the serum exerts a nonspecific immunoregulatory effect on the anti-transplant/tumour immune response.

In conclusion, it is evident that autologous serum exerts strong immunoregulatory effects at the T-cell surface, thereby affecting the overall immune responses. The observation that the proportion of patients with enhancer sera was predominant in the early stages of the cancer and in verrucous carcinoma—groups with good response to therapy—suggest its correlation to good prognosis. The long term effects and potential application can, however, be evaluated only on a long term follow-up which is being carried out as the second phase of the study.

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