Cell-Mediated Immune Status in Patients With Squamous Cell Carcinoma of the Oral Cavity

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Sixteen untreated patients with squamous cell carcinoma of the oral cavity were tested for *in vitro* immune status in comparison with the normal healthy donors. The parameters investigated were total leukocyte and lymphocyte counts, percentages and absolute counts of T- and B-cells in circulation, subsets of T-cells identified by the Fc receptors, phytohemagglutinin (PHA), and mixed lymphocyte culture (MLC) responses, natural killer (NK) and antibody-dependent cellular cytotoxicity (ADCC) activities, and circulating immune complexes (CICs). Eight of these patients were retested 3 to 6 months after surgery. The results showed that there was an increase in leukocyte and lymphocyte counts, an increase in the percentage and absolute number of B-lymphocytes, an increase in the percentage of T-gamma cells, suboptimal PHA and MLC responses, normal NK and ADCC activities, and increased levels of CICs in untreated oral cancer patients. In the postoperative stage, except for a reduction in leukocyte and lymphocyte counts, other abnormalities remained unchanged. The CICs in treated patients correlated with the tumor load in that in three patients showing recurrence, the CIC level remained elevated, whereas in patients without evidence of the disease the CIC level was either low or comparable to the upper normal limits.

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IN INDIA, a minimum of 40% of all cancers recorded annually are cancers of the oral cavity and pharynx, based on hospital registry.[1,2] This high incidence of cancers of the oral cavity has been attributed to the common habit of chewing betel leaf with areca nut, with or without tobacco.[2] Despite a predominance of oral cancers in India, there have been few investigations of the immune status of these patients.[3–5]

The current report deals with various immunologic parameters studied in patients with squamous cell carcinoma of the oral cavity before and after surgery in comparison with normal healthy controls. The parameters studied were absolute numbers of leukocytes and lymphocytes, absolute numbers and percentages of T- and B-lymphocytes and T-lymphocyte subsets, ability of T-lymphocytes to respond to mitogen phytohemagglutinin (PHA) and in mixed lymphocyte cultures (MLC), natural killer (NK) and antibody-dependent cellular cytotoxicity (ADCC) activities of peripheral blood lymphocytes, and levels of circulating immune complexes (CICs).

Materials and Methods

Patients and Controls

Sixteen patients diagnosed as having primary squamous cell carcinoma of the oral cavity, aged 29 to 60 years, were selected for the studies. The diagnosis was based on the clinical examination and histologic features of the biopsy material. In terms of the sites of the lesions, the majority of the patients under investigation had carcinoma of the buccal mucosa (seven) and carcinoma of the alveolous (five), whereas two patients had carcinoma of the tongue, and one patient each had carcinoma of the floor of the mouth and of the lip. The staging was done according to TNM classification, and patients with lesions in Stages T1 to T4, N0 or N1, and M0 and who were acceptable for surgery were studied. Eight of these patients were tested again after 3 to 6 months of surgery. At the time of testing, three showed recurrence of the disease, whereas five had no evidence of the disease (NED). Sixteen normal healthy donors aged 26 to 50 years belonging to the laboratory personnel were used as controls. A count of total leukocytes and

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lymphocytes per cubic millimeter of blood was done using routine methods.

Preparation of Lymphocytes

Peripheral blood mononuclear cells (MNC) were collected according to the method described by Boyum[6] and suspended in RPMI-1640 buffered with 25 mM Hepes, containing 4 mM glutamine and antibiotics (50 μg/ml streptomycin, 40μg/ml gentamicin, and 50 μg/ml Mycostatin [nystatin]), supplemented with either 10% heat-inactivated fetal calf serum (Difco, Detroit, MI) or 10% heat-inactivated human AB serum (complete medium, CMFCS or CMAB, respectively).

Total T- and B-Cell Population

The T-lymphocyte population was studied by its ability to form spontaneous rosettes with sheep erythrocytes (S-RBC) as described by Jondal *et al.*,[7] whereas the percentage of B-lymphocytes was assessed by the ability to form EAC-rosettes as described by Bianco *et al.*[8] Absolute numbers of the T- and B-cells were calculated from the total leukocyte and the total lymphocyte counts.

T-Cell Subsets

T-cell subsets were assessed by their ability to form rosettes with erythrocytes coated with ox-erythrocyte-specific IgG (Tγ-rosettes) or IgM (Tμ-rosettes) as described earlier.[9]

Blastogenic Response to PHA and Allogeneic Cells

Triplicate cultures with 2×10^5 MNC/0.2 ml of CMAB per well of the microtest plate (Laxbro, India) were incubated with or without PHA (5 μg/ml, Burroughs Wellcome, UK) for 72 hours in humidified 5% CO_2 atmosphere at 37°C. Eighteen hours before harvesting, 0.5 μCi of ^{3}HTdR (specific activity 6–9 mCi/mM, BARC, Bombay, India) was added to each culture. The ^{3}HTdR incorporation by the cells of each well, harvested on Whatman No. 3 paper discs, was assessed on a Beckman LS 100 liquid scintillation counter. The mean counts per minute (CPM) in control cultures was subtracted from the CPM in PHA-treated cultures from each well to get the net CPM.

Allogeneic mixed lymphocyte cultures were performed in triplicates using round-bottom microtiter plates (Laxbro) with 2×10^5 responder cells and 2×10^5 cells treated with mitomycin C (MMC, 50 μg/6 $\times$ 10^6 cells/ml for 60 minutes at 37°C), in a total volume of 0.2 ml CMAB with additional 0.1 μM sodium pyruvate and 5×10^{-5} M 2-mercaptoethanol. In each experiment, lymphocytes from one oral cancer patient and one normal donor were stimulated with MMC-treated lymphocytes from each other. Controls consisted of responder cells alone and MMC-treated stimulator cells alone. Cultures were incubated for 144 hours in a humidified atmosphere of 5% CO_2 at 37°C, and 1 μCi ^{3}HTdR was added to each well for the last 18 hours of the culture. Cells were harvested and the ^{3}HTdR incorporation was measured as before. The results were expressed as the percent response of the oral cancer patients relative to the response of normal healthy volunteers. Initial experiments, in which responding lymphocytes from normal donors were stimulated with MMC-treated cells from one oral cancer patient and two normal allogeneic donors, confirmed that the stimulatory ability of lymphocytes from oral cancer patients was within the normal range.

Effector Cells for NK and ADCC Activity

Lymphocyte-rich MNCs suspended at 5×10^6 cells/ml in CMFCS were depleted of adherent cells by plastic adherence of the MNC. The nonadherent cells were cultural overnight in CMFCS (20% FCS) and used as effectors the next day.[10]

NK Cytotoxicity

The cultured human erythroleukemic cell line K562 was used as targets. One million viable K562 cells in growth phase were labeled by the addition of 100 μCi of sodium chromate ($Na_2{}^{51}CrO_4$, specific activity 1 mCi/ml, BARC), and incubated for 1 hour at 37°C, with occasional shaking.[10] The cells were then washed three times and resuspended at a concentration of 1×10^5/ml in CMFCS and checked for viability. Three effector: target ratios, which were 50:1, 25:1, and 12.5:1, were tested in triplicates in a 4-hour incubation assay. For maximum uptake, radioactivity incorporated in 1×10^4 target cells, a concentration used per replicate in the assay, was counted directly. The percentage of cytotoxicity for each replicate was calculated from the following formula to find out the mean percent cytotoxicity.

$$\%\ \text{cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum uptake} - \text{spontaneous release}} \times 100$$

The range of spontaneous release of label from 1×10^4 target cells at the end of 4 hours of incubation was 3% to 12%.

Antibody-Dependent Cellular Cytotoxicity

Chicken erythrocytes (CRBCs) were freshly collected and used as target cells.[11] Twenty million CRBCs were labeled with 100 μCi of ^{51}Cr as described earlier. After

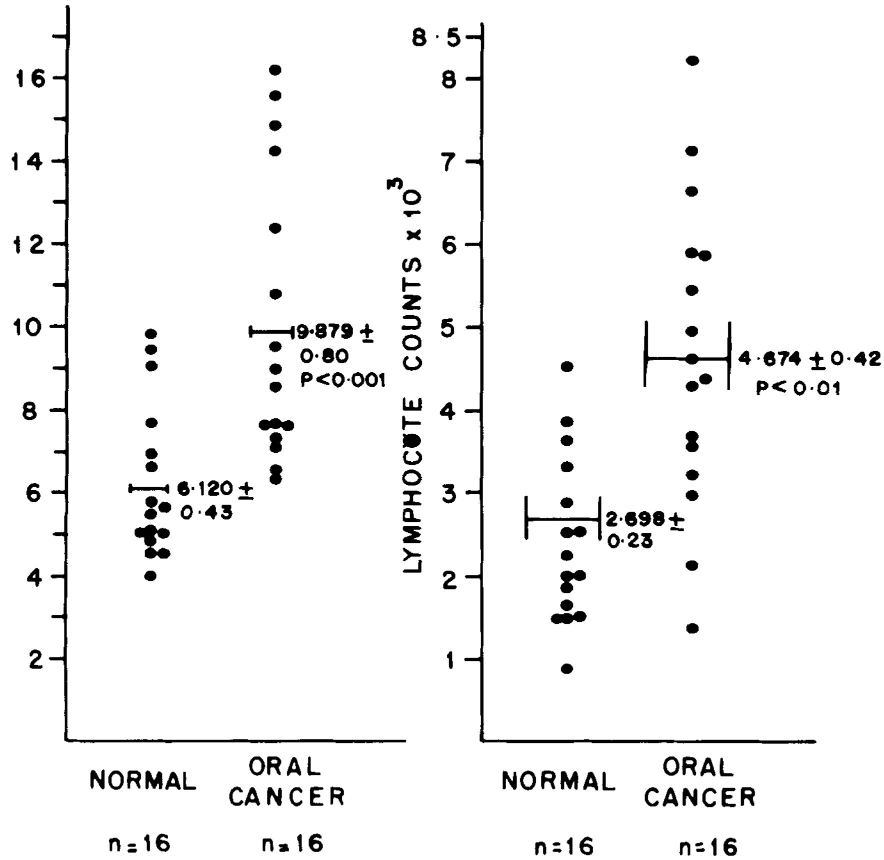

FIG. 1. Leukocyte and lymphocyte counts of untreated oral cancer patients and normal individuals.

labeling and washing, CRBCs were resuspended at a concentration of 1×10^6 cells/ml. In 1×10^5 target cells, 100 μl of 1:1000 dilution of rabbit anti-CRBC serum and 1×10^6 effector cells were added. Test controls did not contain the antisera. Incubation, harvesting, and calculation of specific chromium release was carried out in the same way as that of NK cytotoxicity.

Circulating Immune Complexes

Circulating immune complexes were assessed by a fluid phase radioiodinated C1q binding assay as described earlier.[12] Heat-aggregated human gamma globulin (AHG) at various concentrations was used as positive control, and the World Health Organization (WHO) supplied AHG standards (kindly given by Dr. U. Nydegger) (WHO, Immunology Research and Training Centre, Geneva) that were used for provisory reference curve. Results were expressed as percent C1q binding activity (C1q-BA) as well as μg/ml AHG equivalent.

Results

Leukocyte and Lymphocyte Counts

The total leukocyte and lymphocyte counts from 16 untreated oral cancer patients are shown in Figure 1, in comparison with those of 16 healthy controls. It was observed that oral cancer patients showed a significant increase in the leukocyte count (9879 ± 800) and lymphocyte count (4674 ± 420) in the peripheral blood, compared with normal donors (6120 ± 430 and 2698 ± 230, respectively).

Lymphocyte Subpopulations

Figure 2 shows the percentage of T- and B-lymphocytes in the peripheral blood of untreated oral cancer patients. A significant elevation in the percentage of B-lymphocytes was shown by oral cancer patients (39 ± 2.6) compared with controls (30 ± 1.6), whereas the percentage of T-lymphocytes appeared to be equivalent to that of normal controls (52 ± 2.1 and 50 ± 2.4, respectively). Since the total lymphocyte count of oral cancer patients was increased, the absolute values of total B-lymphocytes in patients showed further increase (1784 ± 204) in comparison with healthy donors (709 ± 77, $P < 0.001$). Similarly, there was a significant difference in the absolute number of T-lymphocytes in oral cancer patients (2432 ± 240) when compared with controls (1165 ± 99, $P < 0.001$) because of differences in total lymphocyte count in circulation.

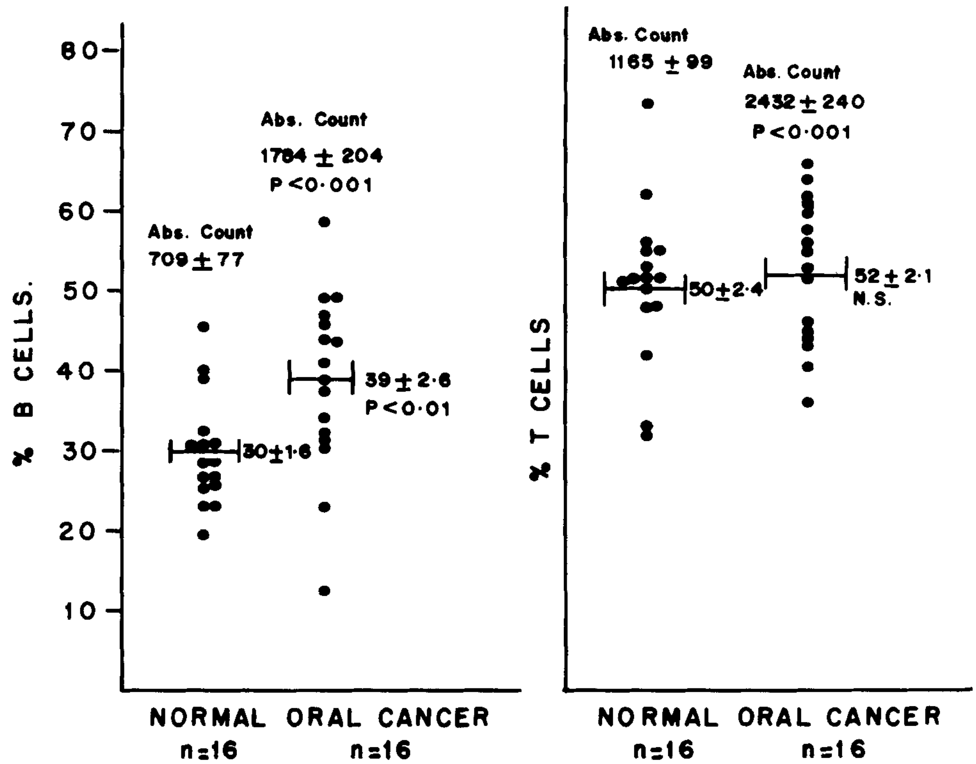

FIG. 2. Percent B- and T-cells and absolute counts from the peripheral blood of untreated oral cancer patients and normal individuals.

Subsets of T-Lymphocytes

The percentage of Tγ- and Tμ-cells, representing suppressor and helper populations, respectively, from untreated oral cancer patients and controls are shown in Figure 3. It appears that there was no significant difference in the percentage of T-cell subsets in oral cancer patients (Tγ, 25 ± 3.8; Tμ, 39 ± 4.3) when com-

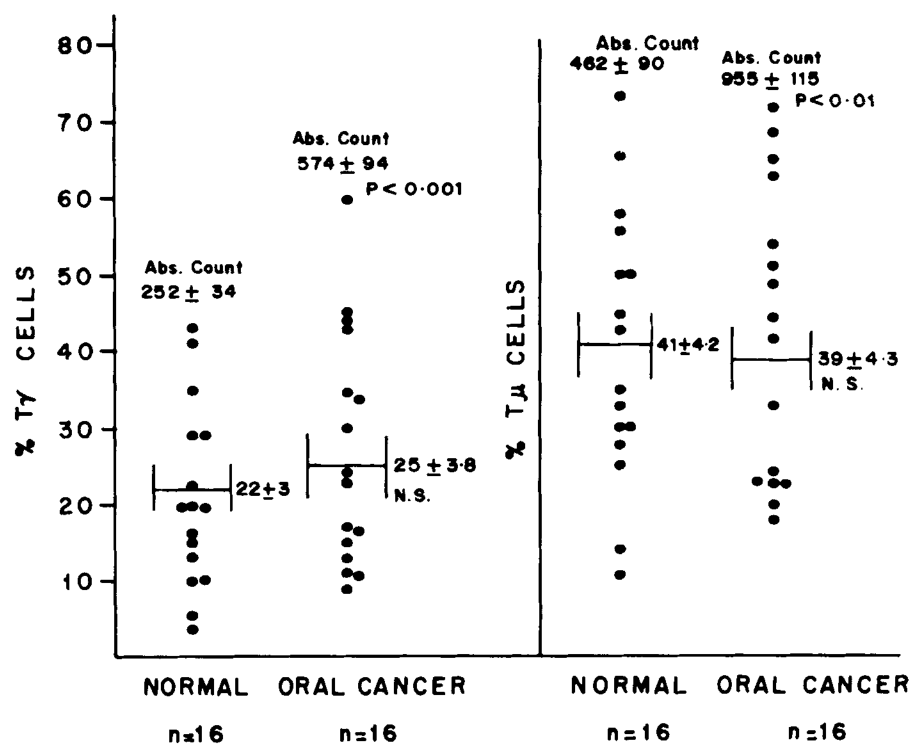

FIG. 3. Percent Tγ- and Tμ-cells and absolute counts in the peripheral blood of oral cancer patients and normal individuals.

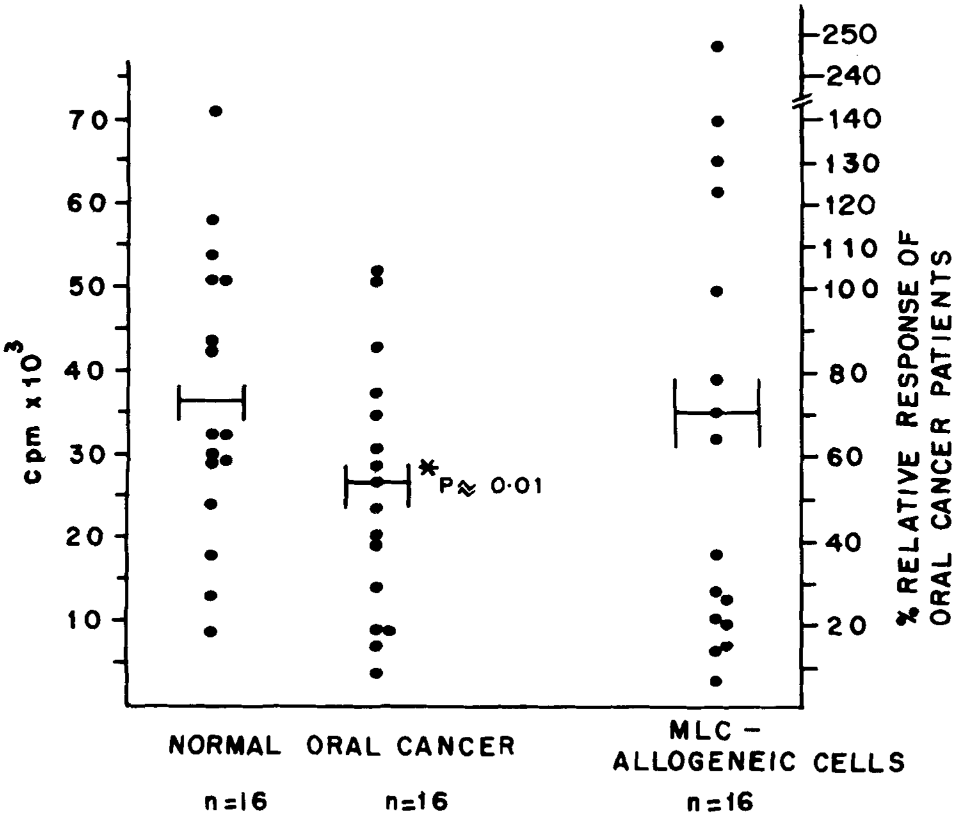

FIG. 4. Response of untreated cancer patients and normal individuals to PHA and allogeneic cells. *Response to PHA statistically analyzed using Wilcoxon's matched-pairs signed-ranks test.

pared with normal controls (Tγ, 22 ± 3.0; Tμ, 41 ± 4.2). However, the absolute numbers of regulatory T-cells in circulation again showed variation. The total number of Tγ-cells in circulation in cancer patients (574 ± 94) was significantly greater than in controls (252 ± 34, $P < 0.001$). Also, the total number of Tμ-cells in the peripheral blood of oral cancer patients (955 ± 115) showed significant elevation above that of normal controls (462 ± 90, $P < 0.01$). Since both subsets showed elevation, the Tμ:Tγ ratios were not significantly altered.

TABLE 1. NK Cytotoxicity and ADCC of Peripheral Blood Lymphocytes From Untreated Oral Cancer Patients and Normal Individuals

	Normal donor (n = 16) ($\bar{X}$ ± SE)	Oral cancer patients (n = 16) ($\bar{X}$ ± SE)
NK activity % specific cytotoxicity at E:T*		
50:1	39.0 ± 4.0	34.0 ± 4.4 NS
25:1	25.0 ± 3.0	23.7 ± 3.5 NS
12.5:1	15.3 ± 3.4	14.0 ± 2.5 NS
ADCC % specific cytotoxicity at E:T†		
10:1	49.8 ± 3.9	49.2 ± 4.4 NS

* Targets: K562.
† Targets: chicken erythrocytes.
NK: natural killer cell; ADCC: antibody-dependent cellular cytotoxicity; SE: standard error; E:T: effector:target ratio; NS: not significant.

Blastogenic Response to PHA and Allogeneic Cells

Figure 4 illustrates the ^{3}HTdR incorporation of lymphocytes from oral cancer patients and normal donors after stimulation with PHA and allogeneic cells. The PHA responsiveness of lymphocytes from controls and patients, conducted simultaneously, was evaluated statistically using Wilcoxon's matched-pairs signed-rank test, whereas the ability of lymphocytes from cancer patients to respond to alloantigens was expressed in terms of percent relative response in relation to response of normal lymphocytes stimulated simultaneously. As can be seen from Figure 4, lymphocytes from oral cancer patients showed reduced response to PHA. Also, 11 of 16 (70%) patients showed reduced values of percent relative response in MLC, the response varying between 7% and 79% of that of normal lymphocytes.

NK and Antibody-Dependent Cellular Cytotoxicity

Peripheral blood lymphocytes from 16 oral cancer patients and 16 normal donors were tested for NK and ADCC activities, as summarized in Table 1. It was seen

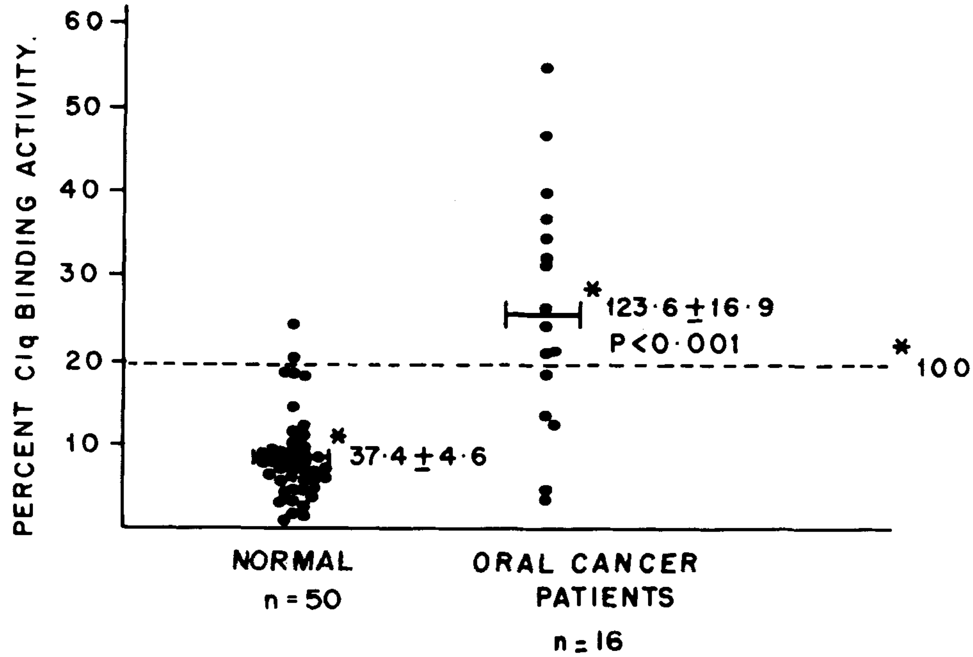

FIG. 5. CICs assessed by ^{125}I-C1q binding in sera of untreated oral cancer patients. Results expressed in percent C1q-BA and (*) μg/ml AHG equivalent.

that the untreated oral cancer patients had NK and ADCC activities equivalent to those of normal individuals.

Circulating Immune Complexes

The C1q-BA of sera from 16 oral cancer patients and normal donors is summarized in Figure 5. A large number of normal sera were tested for C1q-BA to get the mean percent binding activity and to establish upper limits, since the normal Indian population shows considerable variation in CICs. Sixteen controls used in the current study have been included in these data. As compared with sera from 50 normal healthy individuals who showed percent C1q-BA to the level of 8.8 ± 0.82, the group mean values of serum C1q-BA was significantly increased in the oral cancer patient (25.4 ± 3.6, $P < 0.001$). The upper limit of controls (mean + 2 standard deviations [SD]) was established at 19.66% for the 50 control sera, as shown in the scattergram (Fig. 5). Within this group, C1q-BA was not detected in 6 of 50 (12%) sera samples, and 2 of 50 (4%) tests showed values greater than the upper limit. Elevated C1q-BA was observed in 11 of 16 (68.7%) oral cancer patients.

With several batches of C1q, the variation in the C1q-BA measured on an internal standard was relatively limited. The interassay coefficient of variation was 8%, at 34% C1q-BA.

Comparison of Immune Parameters Before and After Surgery

Table 2 gives the comparative data on the same immune parameters studied on eight patients 3 to 6 months after surgery. With each blood sample of the follow-up patient, a blood sample from one normal healthy donor was tested as before. As indicated in the table, three patients showed recurrence of the disease at the time of retesting.

The major deviations in the immune parameters of preoperative oral cancer patients were increased leukocyte and total lymphocyte counts, increased levels of B-lymphocytes and Tγ-cells, and decreased PHA and MLC responses. It appears that in most of the cases there was a postoperative reduction in total leukocyte and lymphocyte counts. However, the B-cell proportions showed recovery only in three cases, whereas recovery was seen in percentage and absolute number of Tγ-cells only in one patient. The same patient showed improvement in PHA response after surgery; however, the relative MLC response was still low after surgery (Patient AR12204). In fact, none of the follow-up patients tested for MLC showed improvement of reactivity in comparison with simultaneously studied controls. The low NK activity in Patient AR4807 was boosted considerably after treatment, whereas that of Patient AR12204 was reduced. The CICs appeared to be either reduced or maintained at the upper limit of normal values (*i.e.*, 19.66) postoperatively in NED patients, whereas they were still increased in three patients with recurrence. In fact, Patient AR5233, who had shown a low C1q-BA value preoperatively, shwoed increased CICs and recurrence when tested again. This appeared to be the only parameter that distinguished patients with recurrent from those with nonrecurrent disease. The data from Table 2 thus show that there was no appreciable recovery

TABLE 2. Comparison Between Immune Parameters of Oral Cancer Patients Before and After Surgery

Patients		Total leukocytes × 10^3	Total lympho	T		B		Tμ		Tγ		PHA (cpm)	MLC (RRN)*	NK (50:1)	ADCC (10:1)	CICs
				%	Abs	%	Abs	%	Abs	%	Abs					
AR4807 (Rec)	Pre	16.2	7128	66	4705	44	3136	22.6	1063	9.3	438	52061	27.5	5.5	33.1	31.4
	Post	5.25	3255	58	1888	43	1400	48.6	918	37.8	714	4850	35.5	73.8	73.3	39.3
AR6598 (Rec)	Pre	10.8	5940	45	2637	32	1900	32.9	879	10.5	281	9441	ND	25	48.9	39.7
	Post	4.95	3366	25	842	10	337	53.5	1088	40.8	769	6038	ND	25.9	49.4	38.2
AR5233 (Rec)	Pre	6.6	1386	58	804	46	638	23	183	59.6	479	26779	ND	40	79	4.8
	Post	7.5	4950	40	1980	26	1287	68	1652	54.5	1322	10451	ND	50	ND	68.6
AR7686 (NED)	Pre	7.6	4864	52	2529	47	2286	57	1444	42.9	1085	4677	15.6	35.8	48.9	31.9
	Post	5.0	2850	72	2052	64	1824	48.7	999	37.1	761	2244	51.0	25.7	21.4	15.2
	Pre	6.35	3620	44	1593	13	471	72	1147	34.5	550	7536	71.0	19.0	35.3	5.3
AR10014 (NED)	Post	3.7	1887	51	962	21	396	63.6	612	48.4	466	5055	69.6	29.0	38.0	20.4
	Pre	7.65	2984	39	1168	29	865	22.5	262	7.5	87	20457	ND	ND	ND	36.9
AR9380 (NED)	Post	4.65	1256	49	615	49	615	58.4	359	53.8	531	12978	ND	ND	ND	21.3
AR2235 (NED)	Pre	8.55	2137	41	876	23	492	23	202	16	145	23737	ND	14.5	14.7	13.0
	Post	7.45	4396	69	3033	52	2286	26.5	788	40	1212	9132	50.9	25	19.3	18.2
AR12204 (NED)	Pre	14.25	5843	40	2337	38	2220	63.2	1477	33.9	792	19462	ND	16.5	32.2	12.5
	Post	2.65	1484	68	1009	20	297	68.5	692	20.3	202	45495	12.4	0.5	57.6	12.2

* Percent of relative response in relation to normal lymphocytes assayed simultaneously.
Pre: presurgery; Post: postsurgery; Lympho: lymphocytes; Abs: absolute number; NK: natural killer cells; PHA: phytohemagglutinins; MLC: mixed leukocyte culture; ADCC: antibody-dependent cellular cytotoxicity; CICs: circulating immune complexes; RRN: relative response in relation to normal; Rec: recurrence; NED: no evidence of disease; ND: not done.

of abnormalities when oral cancer patients were retested after surgery.

Discussion

Impairment of cell-mediated immunity measured *in vitro* has been reported in patients with squamous cell carcinoma of the head and neck region.[13-18] These studies involve measurement of percentage and absolute numbers of lymphocyte populations and/or their mitogen and alloantigen responses.[13,17-20] Cancer of the oral cavity is a very predominant type of cancer in India.[1,2] Some studies on tumor-associated antigen responses of oral cancer patients[3] and their ability to respond to recall antigens *in vivo* and PHA *in vitro*[4] have been reported by us before. It is now known that interactions of regulatory and effector cells are the major contributory factors for the normal immune functions. While assessing the immune status, it is therefore necessary to study these cellular components simultaneously. In this article, we report a number of *in vitro* parameters of immune status simultaneously in patients with cancer of the oral cavity in comparison with normal healthy donors. We also attempted to determine if the deviations from the normal responses are normalized after surgical therapy and their relationship to the recurrence of the disease in a small group of follow-up patients.

Several workers have reported decreased percentage and total T-lymphocyte counts in oral cancer patients,[19,20] whereas our data show that there was a significant increase in total leukocyte and lymphocyte counts in untreated oral cancer patients. Although the percentages of T-lymphocytes in our studies were comparable to those of normal donors, their absolute numbers were elevated because of the increased absolute lymphocyte numbers. Similar discrepancies in the total numbers and percentages of lymphocyte populations have been reported before.[13,17] It appears that the percentages of T-lymphocytes in oral cancer patients as well as in controls reported by us are lower than those normally reported. This could be because we have not used neuraminidase-treated SRBCs for rosetting, which is known to improve the rosette formation. However, in studies conducted by Bier *et al.*,[19] similar low numbers of T-lymphocytes are reported in controls (53%) and in oral cancer patients (39%). Also, we observed a major imbalance in the percentage and absolute numbers of B-lymphocytes in oral cancer patients in contrast with the decreased values reported before.[21,22] Since we used the EAC-rosette technique for enumeration of B-cells, it is possible that we may have counted some monocytes along with the B-cells, both in controls and in oral cancer patients. However, the increase is higher than that accountable by monocyte contamination.

For the analysis of subsets of T-lymphocytes we have used IgG FcR and IgM FcR as markers. Recently, the use of monoclonal antibodies for the assessment of T-lymphocyte phenotyes has been more popular. However, as shown by Ballieux and Heijnen,[23] both of these methods detect overlapping populations. For example, approximately 40% of the Tγ-cells express OKT8 marker, whereas approximately 40% of the OKT8 positive cells form E(0X)-IgG rosettes. Since Tγ- and Tμ-cells have been shown to conduct suppressor and helper functions, respectively,[23] we have used these markers to identify subsets of T-lymphocytes. Our observations on the subsets of T-lymphocytes indicate the normal percentages of the regulatory cells in oral cancer patients; however, the absolute number of cells in the helper as well as suppressor population were increased. Decreased lymphocyte proliferative response to PHA[14,15,18,22] as well as MLC[13] in oral cancer patients has been reported by others, although no attempt has been made to correlate these reduced functions with an imbalance in the regulatory cells in circulation. Our studies showed that since there was an increase in absolute counts of both regulatory cells, the reduced T-cell function may not be attributable to the imbalance in Tμ:Tγ ratio. We are currently investigating whether generation and/or response to interleukins could contribute to the deficient T-cell function in oral cancer.

Another discrepancy noted by us in the T-cell subset studies was that in some instances the addition of Tγ- and Tμ-cell percentages exceeded 100 (Table 2). This could be a result of occasional expression of dual receptors, as suggested by Pichler and Broder.[24]

It appears from our studies that the NK and ADCC activities of oral cancer patients are within the normal range. These effector mechanisms have not been studied in oral cancer patients, although increased cytotoxicity of patients' lymphocytes to squamous cell carcinoma cell lines has been reported.[25]

We have also reported here increased CICs in untreated oral cancer patients, which is in accordance with the observations reported by Scully *et al.*[26] Whether the increased number of B-lymphocytes represents a hyperactive humoral responsiveness, perhaps leading to an increased number of CICs, is difficult to assess. On the other hand, an increased number of CICs might reflect the tumor burden of the patient to a certain degree, which has been reported repeatedly,[12] and which is also evident from the observation that patients with nonrecurrent diseases postoperatively showed fewer CICs in circulation.

Thus, the major deviations in immune parameters in oral cancer patients appear to be increased leukocyte and lymphocyte counts, increased B- and Tγ-cells, decreased PHA and MLC responses, and increased levels of CICs. Individual comparisons between the immune parameters pre- and postoperatively indicate that the major abnormalities in regulatory cells and T-cell functions still persisted postsurgery and they did not correlate with the load of tumor in the patients. Bier *et al.*[19] have also reported that there is no correlation between the clinical course of the disease and nonspecific immune reactivity in patients with head and neck cancer. The only parameter that apparently correlated with the tumor burden was the level of CICs.

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