Eur J Cancer Clin Oncol, Vol. 20, No. 7, pp. 891–897, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

Leucocyte Adherence Inhibition Assay (LAI) in Cancer of the Oral Cavity*

BALARAM PRABHA, KUMARY and DAMODARAN M. VASUDEVAN†
†*Research Division, Regional Cancer Centre, Trivandrum, Kerala—695 011, India*

Abstract—*Antitumor immunity to oral cancer was assessed in 46 patients suffering from squamous cell carcinoma of the oral cavity using a leucocyte adherence inhibition assay (LAI). The response shown by the leucocytes of oral cancer patients to the oral cancer antigen was compared to that shown by the leucocytes from 10 normal age- and sex-matched healthy controls and 43 patients suffering from other types of cancers. Seventy-six percent of the oral cancer patients showed a high degree of leucocyte adherence inhibition in the presence of the antigen. The specificity of this test was further assessed using combinations of the leucocytes from oral cancer patients with extracts from seven cancers occurring at various other sites and extracts of normal oral tissue. The test was highly specific and the leucocytes of oral cancer patients showed significant inhibition only in the presence of oral cancer extract. The inhibition was between 0 and 30% with most other cancer extracts except in the case of extract from cancer of the cervix, where 4/10 patients showed above 30% inhibition. Specific blocking of the LAI response was observed on addition of sera from oral cancer patients to leucocyte-antigen mixtures from oral cancer patients. This effect was not observed on addition of these sera to specific leucocyte-antigen mixtures from other cancer patients. These observations point towards the usefulness of this test in monitoring antitumor immunity in oral cancer patients.*

INTRODUCTION

THE LEUCOCYTE adherence inhibition assay (LAI), first developed by Halliday and Miller [1] and later modified by others [2–6], has produced promising results in the immunodiagnostic field of human cancers. This test is based on the observation that leucocytes from a sensitised person show reduced adherence to glass or plastic surfaces in the presence of the specific antigen. This has been shown to have good correlation with the status of cell-mediated immunity [5, 7–15]. The mechanism operating in this inhibition of adherence is not clear. Evidence indicates that the response obtained by the haemocytometer method is mediated through a lymphokine from T lymphocytes [16–19]. On the other hand, various observations using the tube LAI test point towards the possibility of monocytes being armed with cytophilic antibodies or soluble antigen-antibody complexes [20–23]. Dunn and Halliday [24] are of the opinion that the leucocyte adherence inhibition factor is produced by both T and B cells with the help of macrophages.

Various earlier studies have shown the specificity of this assay [7, 9, 14, 15, 25–27]. This test has also been used in studying the effect of serum factors on the cell-mediated immune response [1, 10, 28–31].

MATERIALS AND METHODS

Selection of patients

Forty-six patients with histologically proven squamous cell carcinoma of the oral cavity attending the clinics at the Regional Cancer Centre, Trivandrum were selected for the study on their first attendance before any type of therapy was given. Patients with other cancers included lymphomas (2), cancers of the skin (5), oesophagus (2), penis (2), colon (7) and breast (10), and squamous cell carcinomas of the larynx (5) and cervix (10). These were tested before the start

Accepted 6 January 1984.
*Supported by grants from the Indian Council of Medical Research, New Delhi and Foundation for Medical Research, Bombay.

of any therapy. Volunteers from the general population were used as normal controls.

Preparation of antigen

Tumor tissues of the oral cavity were collected from surgical specimens, the outer layer of adhering tissues removed, washed five times in media containing a high concentration of antibiotics, pooled and stored at −20°C. For preparation of the antigen the pooled tissues were minced and homogenised in PBS, pH 7.4 (1:4 w/v). Homogenate was centrifuged at 15,000 rev/min for 1 hr at 4°C and the supernatant stored in small aliquots at −20°C. The extracts from normal oral tissues collected from the oral cavity of post mortem cases immediately after death and the extracts from other cancers were made in the same manner as for the oral cancer tissues. The protein content of the extract was estimated using Lowry's method.

Preparation of leucocyte suspension

Leucocytes were separated from heparinised blood by the dextran sedimentation method of Skoog and Beck [32]. The red cells in the leucocyte-rich supernatant were lysed using 0.83% Tris-buffered ammonium chloride. Leucocytes thus collected were washed with Hank's balanced salt solution (HBSS) and suspended in media TC 199 containing 100 units of penicillin and 100 μg of streptomycin per ml and no serum.

Assay

The haemocytometer method of Halliday and Miller [1] was used with some modifications. The results in the modified method was comparable to the original and had the advantage that large numbers of samples could be handled at a time. Briefly, the siliconised tubes containing the different test combinations [5×10^6 leucocytes + 1.25 mg of extract in a total volume of 0.5 ml of media containing 0.05 ml of the required serum (Table 1) per tube] were incubated at 37°C for 30 min. This was done to allow the formation of the hypothetical mediator before the cells spread out. Following this, 0.1 ml of the admixture was taken on to measured areas (7 × 20 mm) on clean cover slips and incubated in a moist chamber for 90 min at 37°C. The cover slips were gently removed and washed by dipping in saline six times. They were dried, fixed in methanol for 5 min and then mounted onto the haemocytometer on a drop of dilute Leishman's stain. For counting, the adherent cells in the outer four squares of the haemocytometer were counted and the number for the whole area of spreading calculated. The percentage adherence was calculated as the percentage of the number of adherent cells in relation to the total number layered. The tests were carried out in duplicate and the mean values were used for computation of the results. The percentage inhibition of leucocyte adherence was calculated using the modified form of the formula used by Holt *et al.* [25]:

$$\% \text{ LAI} = 100 - \frac{\text{mean No. of adherent cells in the presence of antigen}}{\text{mean No. of adherent cells in the absence of antigen}} \times 100.$$

Statistical analysis was done using Student's *t* test.

The assay was carried out using the combinations presented in Table 1. All the tumor extracts have not been tested in every patient. The numbers of oral cancer patients on whom the extract from a particular tumor was tested are given in Fig. 2.

In the preliminary experiments 250 μg protein of oral cancer extract per million cells gave the optimum results (Fig. 1). The same protein concentrations of other tumor extracts were used

Table 1. Serum blocking activity in patients with oral cancer

Source of leucocytes	Source of extract	Source of inactivated serum	% LAI ± S.D.	Blocking activity No. tested	Blocking activity No. positive
Normal controls (10)	normal oral tissue	ox serum	8.4 ± 2.6		
	oral cancer tissue	pooled oral cancer sera	7.6 ± 3.0	10	0
Oral cancer (46)	oral cancer tissue	ox sera	40.5 ± 15.5		
	oral cancer tissue	autologous serum	25.4 ± 12.2	46	36
	other cancers	ox serum	15.0 ± 12.5		
	normal oral tissue	ox serum	14.4 ± 6.34		
Other cancers* (43)	respective tumors	ox sera	52.8 ± 16.2		
	respective tumors	pooled oral cancer sera	45.2 ± 20.4	36	3
	oral cancer tissue	ox sera	12.8 ± 7.1		

The numbers in parentheses indicate the number of individuals in each group.
*Other cancers: lymphomas, and cancers of the skin, breast, cervix, oesophagus, larynx, penis and colon.

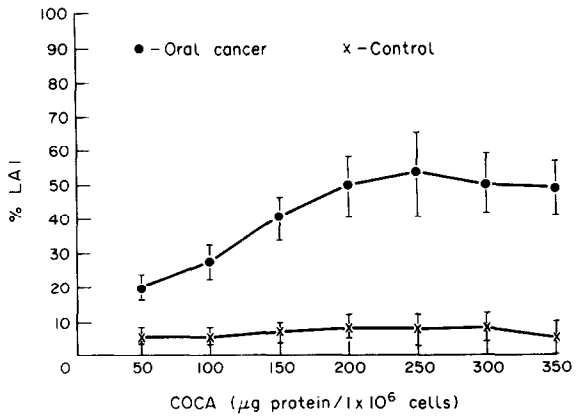

Fig. 1. The dose-response curve of leucocytes from oral cancer patients (O) and normal healthy controls (X) to crude oral cancer antigen (COCA).

for the tests. The results shown on addition of 10% inactivated ox serum or 10% inactivated normal human AB serum were comparable. Therefore only the results of the tests done in the presence of ox serum are presented here.

The blocking effect of sera from oral cancer patients was assessed by comparing the adherence inhibition in the presence of the respective extracts and normal ox serum with that in the presence of sera from oral cancer patients.

RESULTS

Immunoreactivity of leucocytes from oral cancer patients

The inhibition of adherence of peripheral blood leucocytes from oral cancer patients was tested in the presence of extracts from oral cancer tissue, normal oral tissue and a panel of other tissues such as cancers of larynx, breast, oesophagus, penis, colon, cervix and skin. The results indicate an increased inhibition of adherence of leucocytes from oral cancer patients in the presence of oral cancer tissue extract (40.50 ± 15.50) when compared to the response in the presence of extracts from other cancers (15.0 ± 12.5) or normal oral tissue (14.4 ± 6.34) (Table 1, Fig. 2). The difference was statistically significant ($P < 0.001$). The responses of these leucocytes to the extracts of cancer of the cervix was noteworthy in that they showed a higher inhibition (4–45%) of leucocyte adherence than the responses to other tumor extracts (0–31%) or normal tissue extract (8–25%) (Fig. 2).

The specificity of this test was further ascertained by testing the response of leucocytes from other cancers, such as those of the skin, lymphoma, cervix, breast, oesophagus, larynx, penis and colon to the extract of oral cancer. The response of leucocytes from normal, apparently healthy individuals were taken as the control values. The percentage inhibition of adherence in both the other cancer groups and the control group were in the range 0–24%, with mean values of 12.8 ± 7.1 and 8.4 ± 2.6 respectively (Table 1).

From the above observations the upper limit of non-specific leucocyte adherence inhibition could be taken as 30%, and this was taken as the cut-off point. Any response greater than 30% was considered to be a specific positive response to the extract. On this basis, oral cancer patients showed 76% (35/46) positive response with oral cancer extract and only 12.5% (5/33) positive response with extracts from other tumors. In the latter, four positive cases were seen in response to cervical cancer extracts and one in response to extract of colon cancer (Fig. 2). On the other hand, leucocytes from the patients having other cancers or normal controls showed negative responses (<30%) to oral cancer extract.

The LAI response was further compared in the different stages of the disease. The results indicate

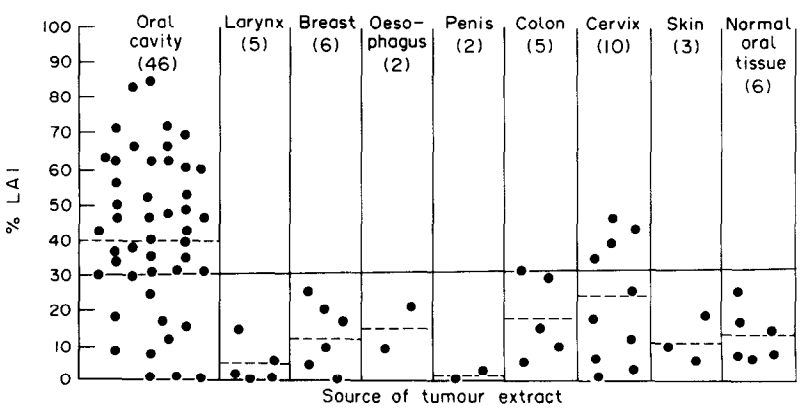

Fig. 2. LAI immunoreactivity of leucocytes from oral cancer patients to various tumor extracts. The numbers in parentheses indicate the number of patients tested. – – – – Mean value in each group; —— cut-off point for assessment of positivity.

Table 2. The effect of autologous serum on the LAI test

Clinical staging	Patient No.	Mean % adherence of total cells layered			% LAI (OS)	% LAI (AS)	% blocking
		No Ag*	Ag + OS†	Ag + As‡			
	1	85.0	29.70	55.3	65.0	35.0	30.0
	2	76.5	65.0	68.8	15.0	15.1	0.0
T_1	3	60.3	33.5	36.4	44.5	39.7	4.8
	4	67.3	25.7	34.8	61.7	48.1	13.6
	5	42.8	26.7	33.7	37.5	21.3	16.2
	6	54.8	37.4	37.0	31.3	32.1	0.0
Mean		64.4 ± 13.9	36.3 ± 13.4	44.3 ± 13.2	42.6 ± 17.3	31.9 ± 11.0	
	7	91.0	28.7	83.0	68.5	7.7	60.8
	8	25.0	22.5	22.3	9.8	10.8	0.0
	9	47.5	29.9	39.4	37.0	17.1	19.9
	10	52.2	17.2	52.2	67.0	7.0	60.0
	11	48.0	32.5	32.1	32.3	33.1	0.0
	12	60.3	38.5	51.7	42.0	14.3	27.7
	13	82.5	28.9	78.3	65.0	5.1	59.9
T_2	14	73.7	43.4	56.7	41.1	23.1	18.0
	15	55.0	42.5	42.3	22.7	23.1	0.0
	16	77.9	35.8	59.2	54.0	24.1	29.9
	17	48.3	23.2	38.6	51.9	20.9	31.1
	18	48.0	18.7	37.9	61.1	21.0	41.1
	19	65.2	34.4	47.6	47.3	27.0	20.3
	20	52.8	32.7	45.9	38.0	13.1	24.9
Mean		59.1 ± 16.8	30.6 ± 7.9	49.1 ± 16.0	46.2 ± 18.9	17.7 ± 18.0	
	21	84.0	16.0	74.8	81.0	11.0	70.0
	22	77.5	35.5	47.9	54.2	38.2	16.0
	23	62.5	39.6	58.1	36.7	7.0	29.7
	24	49.0	24.5	49.0	50.0	0.0	50.0
	25	67.8	20.3	54.2	70.1	20.1	50.0
	26	65.1	54.4	54.6	16.4	16.8	0.0
	27	34.3	35.1	33.9	0.0	1.2	0.0
	28	37.0	25.1	36.2	32.1	2.2	29.9
	29	58.2	30.0	53.0	48.5	9.0	39.5
T_3	30	71.5	73.0	72.0	0.0	0.0	0.0
	31	49.0	14.9	34.3	69.5	30.0	39.5
	32	80.5	56.4	60.6	30.0	24.7	5.3
	33	38.0	34.3	34.2	9.8	10.0	0.0
	34	49.0	34.3	46.5	30.1	5.0	25.1
	35	67.5	40.5	58.7	40.0	13.0	27.0
	36	69.0	22.2	61.4	67.8	11.0	56.8
	37	74.0	44.8	66.6	39.5	10.0	29.5
	38	43.0	16.8	33.5	62.0	22.1	39.9
Mean		59.8 ± 15.3	33.8 ± 15.1	51.6 ± 12.9	41 ± 24.5	12.9 ± 10.4	
	39	51.7	11.5	47.7	77.7	7.7	70.0
	40	80.3	59.4	64.0	26.0	20.0	6.0
	41	73.0	24.8	61.3	66.0	16.0	50.0
	42	46.0	30.4	46.9	34.3	0.0	34.3
T_4	43	70.2	42.1	63.2	40.0	10.0	4.8
	44	30.0	28.4	29.8	5.5	0.7	4.8
	45	70.2	42.1	63.2	40.0	10.0	30.0
	46	47.0	49.2	48.4	0.0	0.0	0.0
Mean		56.1 ± 15.7	36.5 ± 14.5	51.3 ± 10.7	32.3 ± 26.5	7.25 ± 7.14	

*Ag, antigen.
†OS, 10% ox serum.
‡AS, 10% autologous serum.

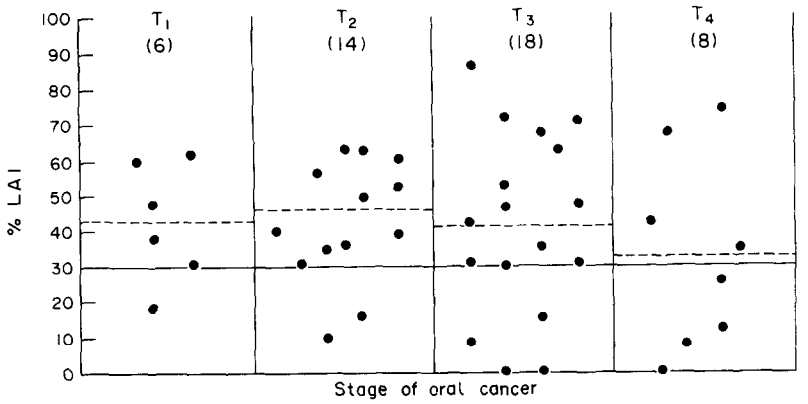

Fig. 3. LAI immunoreactivity of leucocytes from oral cancer patients of different stages of the disease to oral cancer extract. - - - - - Mean value in each group; —— cut-off point for positivity.

an impairment of the response in the T_4 stage (32.3 ± 26.48) compared with the response in the T_1 (42.66 ± 17.88), T_2 (46.28 ± 18.95) and T_3 (41 ± 24.57) stages of the disease. The percentage of patients showing an LAI response of <30% were 16.6 (T_1), 14 (T_2) and 22% (T_3), while it was 50% in the T_4 stage (Table 2, Fig. 3).

Effect of serum factors on LAI

The effect of serum from oral cancer patients on the LAI response of leucocytes from various cancers to the respective tumor extracts was assessed. To assess the blocking activity in oral cancer patients, serum from the same patient was used, while those sera showing a positive blocking activity in oral cancer patients were pooled to assess their effect on the LAI response of leucocytes from other tumors to their respective extracts. The results are presented in Table 1. Almost all the patients (33/35) showing a positive LAI response to the antigen had blocking factors in the autologous serum. However, three patients in the T_4 stage not showing positive LAI had low degrees of blocking factors in the serum. On the other hand, one patient from T_1 and another from T_2 who showed positive LAI responses did not show any blocking activity in their serum. The serum from oral cancer patients, however, showed a blocking effect on the LAI response of leucocytes from other cancers only in 3/36 cases (8.6%) to the respective tumor extracts (Table 1). There was no blocking effect on the response of leucocytes from normal individuals to the extract of normal oral tissue or cancer tissue (Table 1).

The blocking effect was seen in the sera from all stages of the cancer in 4/6 (5–30%) in T_1, 11/14 (15–60%) in T_2, 14/18 (5–70%) in T_3 and 7/8 (5–70%) in T_4.

DISCUSSION

The results of this study indicate that LAI can also be used as a simple, qualitative and technically feasible method for detecting specific antitumor immunity in oral cancer. This is based on the finding that 76% of the patients with histologically defined squamous cell carcinoma of the oral cavity responded strongly to the pooled extract of oral cancer tissue with an inhibition of >30% while no controls (normal controls and other cancers) showed a similar degree of inhibition (Table 1, Fig. 2). Conversely, patients with oral cancer showed no positive response (<30% LAI) to a panel of extracts from other cancers except four patients who showed positive response to the extract of the cancer of the cervix and one patient to the extract of colon (Fig. 2). One of the four patients positive to the cervix cancer extract had a total hysterectomy 3 years ago for reasons unknown to the patient. These observations point towards the high specificity of this assay. The tumor-type specificity of this assay has been noticed by various workers in various other instances [7, 9, 14, 15, 25–27].

The LAI test appeared to be very sensitive and showed a strong response to the specific extract even in the T_1 stage of the disease. The response in the precancerous stage is under investigation in order to ascertain the earliest stage at which a positive LAI response can be observed. This response, however, appeared to be impaired in the T_4 stage as seen from the increased percentage of patients showing a negative LAI response. Some patients in the T_3 and T_4 stages failed to show any reactivity to the extract (Fig. 3). This finding is in agreement with that of the results in lung cancer found by Thomson *et al.* [33] and in contrast to the findings of Kotlar *et al.* [8].

According to Thomson *et al.* [22, 34], the presence of LAI activity indicated a limited tumor load, whereas absence of an LAI response signified a large tumor burden, leading to shedding of excess of tumour antigens into circulation which coats the LAI reactive cells thus abrogating their response [22, 35]. Various other

reports have shown the absence of any such correlation between the LAI response and the stage of the disease [12, 16, 36].

Specific serum blocking factors were observed in about 77% of the patients. Their presence was seen in all stages of the disease. The tumor-type specificity observed in the leucocyte reactivity was also observed in the case of blocking activity. The serum from oral cancer patients blocked the LAI response of leucocytes only from patients with oral cancer (Table 1). In normal subjects and patients with unrelated tumors the blocking activity of the oral cancer serum was rarely found (Table 1).

The results presented here, obtained from limited material, indicate that the LAI assay can detect antitumor immunity in patients with oral cancer. This assay is simple and reproducible and might augment the present methods of detection of antitumor immunity. An early detection of this response in the precancerous stage will be of immense help in treating the cancer successfully.

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