9. Demonstration of HSV-1 Antigen in Patients With Oral Cancer By Immunofluorescence And Immunoperoxidase Techniques

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Abbreviation used. PBS — Phosphate buffered Saline, HSV — Herpes Simplex Virus, Ab — Antibody

Studies on the both human and experimental animals suggest that viruses may play a role in the etiology of human cancers. Herpes Simplex Viruses are ubiquitous in the human population and are now implicated as the etiologic agents of a wide spectrum of diseases. They persist in the host in a latent state despite producing humoral antibody. Seroepidemiologic studies linking HSV and cervical cancer provide the most positive evidence that Herpes Simplex Viruses may be a human carcinogen (1-6). HSV-1 has been thought to produce lesions of the oral cavity [7-8]. The observations that the animals bearing tumours induced by HSV-transformed cells develop virus specific neutralizing antibodies supports the notion that HSV transformed cells may express virus antigens (9). The production of antibodies by these animals may be the result of host recognition of virus specific membrane antigens which are present in some HSV transformed lines. Oral cancer patients may have circulating antibody to tumour associated antigens derived from HSV-1 [10, 11]. In a previous study we have demonstrated high levels of antibody titres to HSV-1 antigen in oral cancer patients, compared to control individuals [8]. But the tumour specificity of such antigens is in doubt because independent efforts have failed to confirm this specificity [12-15]. In this context an attempt has been made to detect the presence of HSV-1 antigen in oral cancer cells using Immunofluorescence and immunoperoxidase techniques.

Cancer patients attending the clinics of the Regional Cancer Centre, Trivandrum were taken up for the study. Punch biopsies from clinically diagnosed and histopathologically proved cases of oral cancer were collected in Eagle's MEM. The tissues were washed with phosphate buffered saline to remove blood clots mucous etc, and cut into small pieces and minced gently to obtain single cell suspension. Cell dispersing enzymes were not used since they are found to alter the antigens [16, 17] as well as other characteristics of the cell surface. The cells were centrifuged and washed three times with Eagle's MEM containing 10% foetal calf serum. The supernatant was discarded, and the cell button was resuspended in a few drops of PBS. Smears were prepared, allowed to dry in air and immediately fixed in cold acetone for 10 minutes. The slides were preserved at-20°C till staining.

Normal oral tissues were obtained from surgical specimens and from persons undergoing plastic surgery for cleft lip. Specimens were also collected from oral cavity of the autopsies done immediately in the case of accidental deaths. Another group of control was constituted by the biopsy taken from the site other than the cancerous site of the cavity of the patients. All of them were processed as earlier and acetone fixed smears were kept at-20°C, until tested.

Virus Strains. Herpes Simplex Virus type-1, AC Strain, (kindly donated by Dr. Pradeep Seth, All India Institute of Medical Sciences, New Delhi) was grown in Vero Cells fed with Eagle's MEM containing 10% foetal calf serum. Culture bottles were incubated at 37°C for 24 to 36 hours. When they showed complete cytopathic effect, the cells were scraped off into the medium, centrifuged at 1000 x g for 15 minutes. One portion of the cells was purified as described by Shillitoe et al [18] resuspended in PBS and smears were

prepared as described earlier. The remaining cells were subjected to three cycles of freezing and thawing, finally suspending the cells in a small volume of maintenance medium. This was used as antigen for the preparation of antiherpes simplex virus-1 serum in rabbits [4]. Pre-immunisation serum was collected and preserved at-20°C. Non-infected Vero cells were prepared, dried, fixed in cold acetone and preserved at-20°C.

Absorption Procedures. The HSV-1 antiserum raised in rabbit was absorbed with non-infected Vero cells at 4°C overnight [19]. The rabbit HSV-1 antiserum was absorbed with homologues HSV-2 infected Vero cells by mixing equal volumes of the two and incubating at 36°C for 1 hour. The supernatant was used for the detection of HSV-1 antigens in non-infected Vero cells, HSV- 1 infected Vero cells, biopsy smears, cultured oral cancer cells and normal oral tissue cells.

Cultured Tumour Cells. The tumour cells obtained from biopsies of the oral cancer patients were inoculated into tissue culture bottles containing minimum essential medium with 20% foetal calf serum. Period of culturing of the different specimens ranged from two to twenty weeks. Slides were prepared with cultured cells from 10 specimens (which showed positivity in fresh acetone fixed smears), dried and acetone fixed.

Staining Procedure. In every series of tests conducted, known positive and negative control smears using HSV-1 infected and non-infected Vero cells were included.

Immunofluorescence. The smears were covered with anti HSV-1 serum and incubated at 37°C for 30 minutes, washed with PBS, dried, and incubated with FITC conjugated immunoglobulin. The slides were again washed with PBS, dried in air and mounted with 1: 1 PBS glycerol mountant. The smears were examined immediately under a Leitz orthoplan fluorescent microscope and were graded as 1+, 2+ and 3+depending upon the degree of fluorescence.

Immunoperoxidase. The method of Handlers et al [20] was employed. In short, the endogenous peroxidase activity was blocked by 0. 3% H_2O_2 washed with PBS and then overlaid with normal murine serum, to reduce nonspecific staining. These slides were then incubated with Horse raddish peroxidase conjugated rabbit antihuman HSV type 1 for 30 minutes and washed with PBS. This is then treated with a solution of Diaminobenzidine in PBS containing few drops of H_2O_2. After washing the cells were counterstained with Mayer's haematoxylin, dried mounted and evaluated under light microscope.

Staining with HSV-I Absorbed with Homologous Virus. Anti HSV-1 rabbit serum was mixed with an equal amount of HSV-1 specific antigen (HSV-1 infected Vero cells) and incubated at 36°C for 1 hour. The mixture was centrifuged and supernatant used for staining smears from the patients with oral cancer.

Specific HSV-1 infected Vero cells, which served as positive controls, showed good fluorescence and intense peroxidase staining whereas in the case of non-infected vero cells, it was completely negative. Among the twenty normal control specimens, 30% showed fluorescence while 20% showed positive peroxidase staining. Out of the ten biopsies taken from the other site of the oral cavity of the oral cancer patients, four showed positive FITC labelling and three showed positive peroxidase staining (Table-1). Brilliant fluorescence was shown by 135 specimens (77%) and positive peroxidase labelling was shown by 124 specimens (71%).

Acetone fixed smears of cells from fresh positive biopsies and cells cultured from the same patients showed approximately the same percentage of positivity and degree of fluorescence (Table-III). Specific anti HSV-1 serum stained larger number of cells compared to the heterologus anti-HSV-2 serum. No positivity was seen when both absorbed homologous and heterologous serum were used with uninfected Vero cells. The percentage of cells showing fluorescence/ staining varied from patient to patient.

In 20 smears showing strong positive fluoresence, instead of anti-HSV serum, PBS was used for the first incubation, followed by FITC Labelled or peroxidase anti-immunoglobulin, three showed fluorescence/

staining (Table-IV). In this preparation, the number of cells showing fluorescence/staining was less. The exact percentage of fluoresence/staining shown by HSV-1 antibody was calculated by substracting the percentage of cells showing fluorescence with PBS-FITC conjugated immunoglobulin from anti-HSV-1 and FITC conjugated immunoglobulin. Ten smears from patients with breast cancer and leukoplakia showed no fluorescence/staining. FITC conjugated preinoculated serum also showed negative results.

Specificity of fluorescence and peroxidase staining was confirmed by making the highly positive smears to react with known HSV-1 antibody negative serum, specific anti HSV-2 antibody and anti HSV-1 serum absorbed with HSV-1 infected Vero cells. Even though 30% of the smears showed positivity with anti-HSV-2 antibody, fluorescence and peroxidase staining was very much reduced in the other two sets of experiments both in the number of positive specimens and degree of fluorescence/staining. In 20 of the positive cases when anti HSV-1 serum absorbed with HSV-1 infected Vero cells were used, the fluorescence/staining were completely negative in majority of the cases. In the remaining, the intensity of fluorescence/staining were also reduced (Table-IV).

Comparing the degree of fluorescence with clinical staging (TNM classification of the UICC) of the disease, only one case in T_1 state showed 1+, whereas most of the specimens in T_2, T_3 & T_4 stages showed 2+ to 3+. This cannot be taken into account as there is only one case in T_1 stage (Table-V). In eight leukoplakia cases studied, no HSV antigen was detected.

The association of HSV-2 antigen with carcinoma of the uterine cervix has been well documented [4-6]. An increase in the antibody titres to HSV-1 antigen in oral cancer patients compared to age matched normal controls were reported earlier [7, 8]. The increased incidence of HSV antibodies in oral and cervical cancers were postulated to be due to recurrent herpetic infections which can produce higher anti-body titres [21]. But the tumour specificity of such antigens is not yet confirmed [12-15]. Immunofluorescence studies have shown that cells infected with HSV develop new antigens which alter the antigenic specificity of the host cell membranes [18]. Virus induced virus specific antigens appear on HSV infected BHK cells [22]. During infection structural components of the virus are synthesised and become incorporated into membranes of infected cell surfaces [19, 23]. Hence in this study an increased incidence of HSV antigen in the oral cancer cells is demonstrated by immunofluorescence and immunoperoxidase techniques.

In this study we have shown that cancer cells from patients with histologically proved carcinoma of the oral cavity contain antigens related to those found in cells infected with HSV-1. The presence of HSV-1 associated antigen was shown by 77% of cases by Immunofluorescence and 71% by Immunoperoxidase. The fluorescence was reduced with the use of HSV-1 antiserum absorbed with HSV-1 infected cells, in a good percentage of highly positive smears. It is possible that all or some of these cells are infected with HSV-1, perhaps abortively, or It may be due to the result of exposure to HSV antigens from malignant cells expressing HSV antigens [24].

The fluorescence shown by infiltrating inflammatory cells may be due to the presence of the Fc receptors on the immunologically active lymphocytes and plasma cells. Positivity seen in the case of smears incubated with PBS instead of HSV-1 antiserum and FITC labelled immunoglobulin can be attributed to the presence of heterophilic antibody in conjugated antiserum preparation. Similarly HSV-2 antiserum also gave 30% positivity due to the presence of common antigens between HSV type 1 and type-2.

Aurelian et al [25] found the presence of HSV-2 antigen in 86% of the exfoliated cells in cervical cancer. But Royston and Aurelian [26] could not get positive results in biopsy materials taken from such patients, leading to the theory of latency. Our previous finding of higher percentage of HSV-1 antibodies in oral cancer patients [8] and the present finding of the increased incidence of HSV-1 antigen in oral cancer cells further supports the possible correlation of HSV-1 with oral cancer either alone or with other biological factors.

ACKNOWLEDGEMENT

The financial assistance given by the Indian Council of Medical Research is gratefully acknowledged.

Table-I

PRESENCE OF HSV-1 ANTIGEN IN NORMAL CONTROLS

Study groups	Total number tested	Immuno -fluore-scence	Immunoperoxidase
Surgery specimen	5	0 (Nil)	0 (Nil)
Autopsy	5	2 (40%)	1 (20%)
Scrapings from apparently normal Buccal Mucosa of the patient	10	4 (40%)	3 (30%)
Total	20	6 (30%)	4 (20%)

Table-II

PRESENCE OF HSV-1 ANTIGEN IN ORAL CANCER PATIENTS

Study groups	Total number tested	Immunofluorescence	Immunoperoxidase
Oral cancer patients	175	135 (77%)	124 (71%)
Controls	20	6 (30%)	4 (20%)

Table-III

COMPARISON OF THE PRESENCE OF HSV-1 ANTIGEN IN ACETONE FIXED BIOPSY CELLS CULTURED CELLS AND LIVE CELLS

Study groups	Total number tested	Immunofluorescence	Immunoperoxidase
Biopsy cells	10	10 (100%)	9 (90%)
Cultured cells	10	9 (90%)	8 (80%)
Live cells	10	1 (10%)	1 (10%)

Table-IV

COMPARISON OF FLUORESCENCE/STAINING IN SMEARS WITH DIFFERENT ABSORPTION PROCEDURES

Method	Total number studied	HSV-1 Ab Negative serum	Specific anti HSV-2 Ab	PBS	HSV-1 serum absorbed with HSV-1 infected Vero Cells
Immunofluorescence	20	20 (100%)	6 (30%)	3 (15%)	6 (30%)
Immunoperoxidase	20	19 (95%)	6 (30%)	3 (15%)	5 (25%)

Table-V

DEGREE OF FLUORESCENCE WITH RESPECT TO THE STAGE OF THE DISEASE

Stage	Total number tested	Degree of fluorescence			
		-ve	i+	2+	3+
T_1	4	2	1	1	—
T_2	71	20	1	32	18
T_3	72	14	4	28	26
T_4	28	4	1	16	7
Total	175	40	7	77	51

REFERENCES

1. Naib, Z. M., Nahmias, A. J., Josey, W. E: Histopathology of cervical Herpes Simplex infection-Cancer. **19**, 1026-1031 (1966).

2. Josey, W. E., Nahmias, A. T., Naib, Z. M: Genital Herpes simplex infection-present knowledge and possible relationship to cervical cancer. Am. J. Obstet. Gynaec. **101**, 718-729 (1968).

3. Sprecher Goldberger, S., Thiry, L., Cattoor, J. P., Hoobe, R., Pestain, J: Herpes virus type 2 infection and Carcinoma of the cervix, Lancet. **2**, 266 (1970).

4. Rawls, W. E., Adama, E., Melnick, J. L: An analysis of Seroepidemiological studies of Herpes Virus type 2 and carcinomas of the cervix. Cancer Research, **33**, 1477-1482 (1973).

5. Seth, P: Association of Herpes Simplex Virus type 2 with squamous cell carcinoma of the uterine cervix. Ind. J. Microbiology **20,** 167-173 (1980).

6. Lakshmi, S., Pradeep, S., Walter, A., et al: Herpes Simplex Virus antibody in South Indian patients with carcinoma of cervix. Ind. J. Med. Res. **79,** 13-18 (1984.)

7. Shillitoe, E. J., Greenspan, D., Greenspan, J. S., Hansen, L. S., Silverman, S: Neutralising antibody to Herpes Simplex Virus type 1 in patients with oral cancer. Cancer. **49,** 2314-2320 (1982).

8. Kumari, T. V. Shanmugham, J., Prabha, B., Vasudevan, D. M: Prevalence of antibodies against Herpes simplex and Adeno virus in oral and cervical cancer patients-a preliminary report. Ind. J. Med. Res. **75,** 590-592 (1982).

9. Rapp, F: Herpes Viruses and Cancer. Adv. Cancer Res.**19,** 265-302 (1974).

10. Sabin, A. B., Tarro, G: Herpes Simplex and Herpes genitalis Viruses in etiology of some human cancer. Proc. Natl. Acad, Sci. US, **70,** 3225-3229 (1973).

11. Hollinshead, A. C., Lee, O., Cretein, P. B., Tarpley, J. L., Rawls, W. E., Adam, E: Antibodies to Herpes virus nonvirion antigens in squamous carcinomas. Science, **182,** 713-715 (1973).

12. Jan Vaage: Influence of tumour antigen on maintenance viruses depression of tumour specific immunity. Cancer Res. **33,** 493-503 (1973).

13. Sabin, A. B: Herpes simplex virus non-virion antigens and their implication in certain human cancers-unconfirmed. Proc. Natl. Acad. Sci. US., **71,** 3248-3252 (1974).

14. Notter, M. F. D., Docherty, J. J: Comparative diagnostic aspects of herpes simplex virus tumour associated antigens. JNCI, **57,** 483-488 (1976).

15. Shillitoe, E. J., Tarro, G., Lehner, T: Cell mediated immunity to herpes simplex virus type 1 and 2 antigens in leukoplakia and carcinoma in man. Oncology, **33,** 192-195 (1976).

16. Hanschka, T. S., Mitchell, J. T., Neiderpuren, D. J: A reliable frozen tissue bank-viability and stability of 72 neoplastic and normal cell types after prolonged storage at -78°C. Cancer Res. **19,** 643-653 (1959).

17. Kahn J., Smith, A. M. J., Robinson, D. M: A cautionary note on the use of pronase in Tissue Culture Exptl cell Res. **40,** 445-446 (1965).

18. Shillitoe, E. J., Greenspan, D., Greenspan, J. S., Silverman, Jr. S: Antibody to early and late antigens of Herpes Simplex type 1 in patients with oral Cancer. Cancer. **54,** 266-273 (1984).

19. Riozman, B., Spear, P. G: Herpes virus antigens on Cell membranes detected by centrifugation of membrane anti-body complexes. Science. **171,** 298-300 (1971).

20. Handlers, J. P., Melrose, R. J., Abrams, A. M., Taylor, G. R: Immunoperoxidase technique in diagnosis of oral pemplugus vulgeris. An alternative method to Immunofluorescence. Oral surgery oral medicine oral pathology. **54,** 207-212 (1982 .

21. Aurelian, L., Strandberg, J. D., Melendez, L. V., Johnson, L. A: Herpes virus type 2 isolated from cervical tumour cells grown in tissue culture. Science. **174,** 704-706 (1971).

22. Gedder, L., Skinner, G. R. B: Differentiation between type 1 and type 2 strains of herpes simplex virus by an indirect immunofluorescent technique. J. Gen. Virol. **12,** 179-192 (1971).

23. Ben-Porat, T., Shimono, H., Kaplan, A. S: Synthesis of proteins in cells infected with herpes virus-2. Flow of structural viral proteins from cytoplasm to nucleus. Virology. **37,** 56-61 (1969).

24. Heise, E. R., Kucera, L. S., Raben, M., Homesley, H: Serological response patterns to herpes virus type 2 early and late antigens in cervical carcinoma patients. Cancer Res. **39,** 4022 (1979).

25. Aurelian, L., Strandberg, J. D., Davis, H. J: Herpes virus type 2 antigens absent from biopsied cervical tumour cell. A model consistent with latency. Proc. Soc. exp. Biol. Med. **140,** 404-408 (1972).

Fig. 1

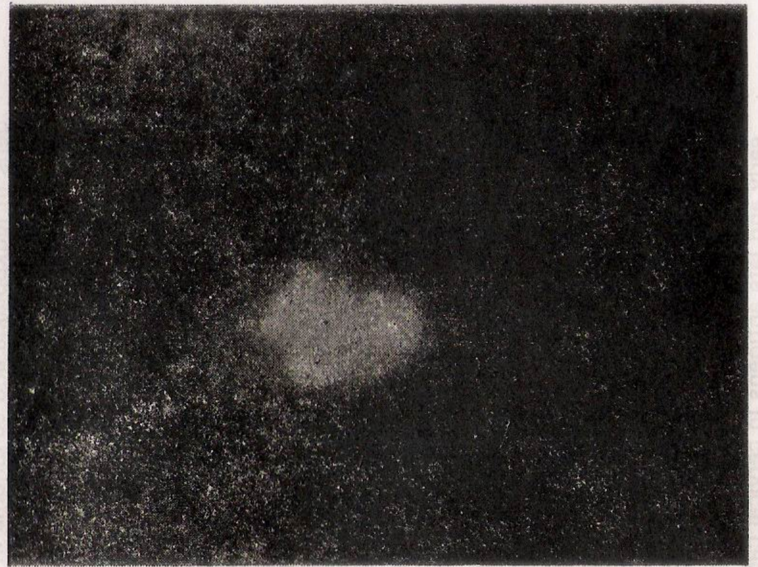

Fig. 2

Fluorescence shown by acetone fixed cells from biopsies of carcinoma of the oral cavity upon incubation with specific anti HSV-1 antibody and FITC conjugated goat anti rabbit antibody. Both cytoplasmic and nuclear fluorescence is seen (Low power).

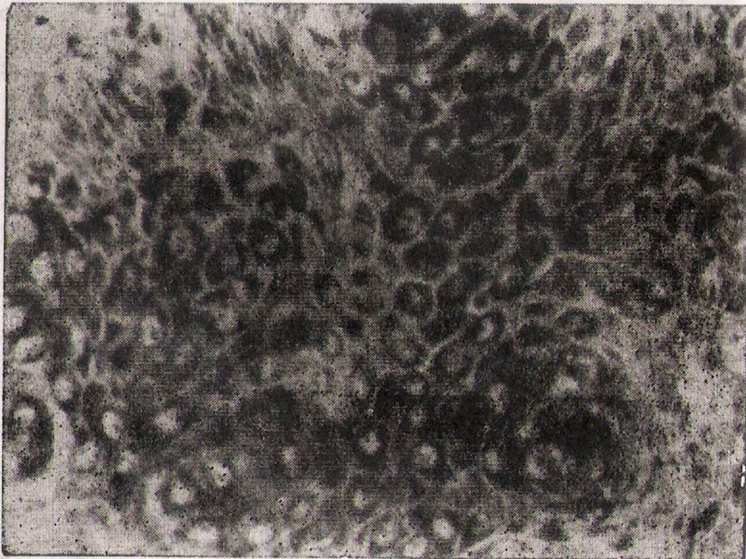

Fig. 3

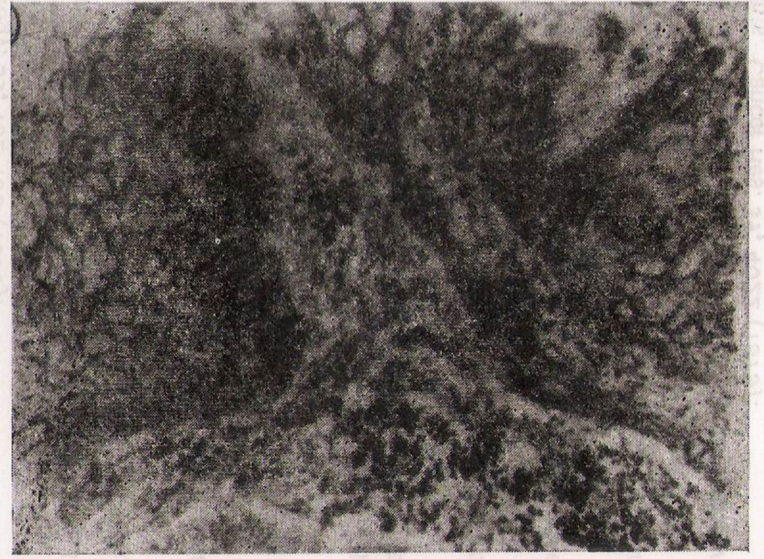

Fig. 4

Dewaxed paraffin sections of carcinoma of the oral cavity tissues incubated with horse raddish peroxidase conjugated rabbit anti-human HSV-1 stained with diaminobenzidene containing 0.02% H_2O_2 and counterstained with haematoxylin.