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A study of herpes simplex virus proteins in human oral cancer using monoclonal antibodies



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# A study of herpes simplex virus proteins in human oral cancer using monoclonal antibodies

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# A STUDY OF HERPES SIMPLEX VIRUS PROTEINS IN HUMAN ORAL CANCER USING MONOCLONAL ANTIBODIES

## DEDICATION

This thesis is dedicated to my wife and my child whose love and support have made this all possible and is also dedicated to my parents and parents-in-law who encouraged and supported my study.

## ACKNOWLEDGEMENTS

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# A study of herpes simplex virus proteins in human oral cancer using monoclonal antibodies

## INTRODUCTION

In the United States, cancer is the second leading cause of death, next to heart disease. In 1983, there will be approximately 27,100 new cancer cases involving the lips, tongue, salivary glands, floor of the mouth, alveolar mucosa, buccal mucosa, and oropharynx (Silverberg 1983). These cancers of the head and neck will account for 3.2% of all estimated new cases of cancers. The mortality of these cancers is estimated at 9,150 cases, about 2% of all cancer-related deaths. In California, of 2600 new oral cancer cases, about 850 (33%) will die from the oral malignancy (Silverberg 1983). This indicates that the disease has a low incidence but a high degree of lethality. The importance of more detailed studies is based on the high degree of mortality, and the severe degree of dysfunction and mutilation in curable patients. The majority of oral cancers are squamous cell carcinomas, which account for more than 90% of all oral malignancies (Silverman 1981; Shafer et al. 1974; Binnie 1975) and have specific histopathologic characteristics.

Histopathologically, squamous cell carcinoma can be classified from grade I to grade IV, depending on the degree of epithelial differentiation (Shafer et al. 1974). In general, one or more of the following features may be recognized in this carcinoma: nuclear hyperchromatism, higher mitotic activity, loss of cell polarity, invasion of epithelium into connective tissue and metastatic potential (Shafer et al. 1974).

The etiology of oral squamous cell carcinoma is unknown. However, several factors contribute to an increased risk of developing cancer and are indicated as having

etiologic roles. Tobacco and alcohol consumption increase the risk of developing oral cancer (Binnie 1975; Shafer et al. 1974; Silverman 1981). Others, such as immunologic disturbance might be important in the etiology (Silverman 1981; Scully 1982). In recent years, the association between viruses and human cancers has been investigated. Many studies indicated that herpes simplex virus (HSV) might be associated with oral carcinoma (Silverman 1981; Shillitoe & Silverman 1979; Scully 1982), although the etiologic role of HSV in oral carcinoma is still unclear. In this study, an immunohistochemical technique-- avidin-biotin-peroxidase complex (ABC) staining -- and monoclonal antibodies against HSV infected cell proteins (ICPs) were applied in the search for the relationship between oral carcinoma and HSV.

## HSV-1 and oral carcinoma

Several reports have indicated that there is some relationship between recurrent labial herpetic infection and squamous cell carcinoma of the lip, which is the most common site of recurrent infection (Wyburn-Mason 1957; Kvasnicka 1965). However, there is no evidence that herpetic infection is a directly causative factor in the development of oral squamous carcinoma. Only one of 24 cases studied in our clinic revealed the viral cytopathic effect (CPE) on Vero cell culture (Shillitoe & Silverman 1979). An electronmicroscopic study also failed to detect any viral particles in the cytoplasm of the atypical cells from oral carcinoma (Shillitoe & Silverman 1979), although the cytoplasmic disorganization was present as in other reports (Chen & Harwick 1977). However, studies in animal models and cultured cells indicated that HSV-1 has transforming and oncogenic potential like HSV-2. The oncogenicity of HSV-1 in human oral squamous cell carcinoma was first suggested by the significant changes in cell-mediated and humoral immune responses to HSV-1 in patients bearing oral malignancies, including epithelial dysplasia.

Both humoral and cell-mediated immune (CMI) responses were significantly different in patients with oral cancer and precancer, especially in patients with epithelial atypia, when compared with matched control individuals. Concerning the association between oral cancer and HSV-1, the change of CMI response detected by lymphocyte transformation test to HSV-1 was related to the clinical severity of lesions (Lehner et al. 1973b). Both leukoplakia without dysplasia (keratosis and acacthosis) and carcinoma patients had significant depressed lymphocyte responses to HSV-1. Like patients with active herpetic infection (primary and recurrent), a significantly elevated CMI responses (both lymphocyte transformation test and macrophage migration inhibition test) to HSV-1 were detected in patients with dysplasia (Lehner et al. 1973a & 1973b; Shillitoe et al. 1976 & 1977; O'Reilly et al. 1977) in which a high incidence of malignant transformation had been established (Silverman et al. 1983). The lymphocyte responses to phytohaemagglutinin (PHA) in these patients were comparable with those to HSV-1, a change of CMI is consistent with that to HSV-1 (Lehner et al. 1973b). Clinically, patients with leukoplakia showing the sequential change of lymphocyte response to HSV-1 further developed into carcinoma in situ (Lehner et al. 1973b). Although defective CMI might have played an important role in the etiology and/or be a result of the lesion, the oncogenecity of the virus might have been activated by the change in the immune system. The results suggested that HSV-1 is a possible etiologic factor in oral carcinoma, and that the change of CMI might induce the oncogenic properties of herpes simplex virus to cause neoplastic transformation directly and with an elevated response to HSV-1 in dysplasia, since there is a high incidence of transformation from epithelial dysplasia to malignancy (Silverman et al. 1983).

Although CMI is always depressed in patients with malignancy, humoral immunity in these patients is more complicated. First, antibody production may be T-lymphocyte dependent, and therefore any defect of antibody response may be a result of defective cell-mediated immunity. Second, the functions of antibodies are distributed between the

different classes of immunoglobulin (Ig). Third, humoral immunity may play a role as blocking factors in the regulation of immunity (Scully 1982). Although the specific characteristics of different antibodies is unclear, the following evidence suggests a correlation between oral carcinoma and HSV-1.

Concerning humoral immunity in patients with oral cancer, it was reported that a significantly elevated level of both serum (Hughes 1971; Smith et al. 1976a & 1976b; Brown et al. 1974, 1975a & 1975b) and salivary (Brown et al. 1974, 1975a &1975b) immunoglobulin was found in these patients and was suggested that the stimulation of immune system was present in this population. Based on the Ig classes, there was no difference of serum IgG levels in patients and matched controls (Brown et al. 1975a). Also, the IgG level of saliva from parotid and submandibular glands in patients was not increased (Brown et al. 1975a), although the whole salivary IgG level was increased. A significant elevation of salivary IgA level existed in oral cancer patients. Salivary IgA in cured patients had returned to normal, but patients with recurrent cancer had elevated salivary IgA levels (Brown et al. 1974, 1975a & 1975b). However, the significance of the change of salivary IgA level in primary and recurrent patients may be due to the leakage of serum protein through a torn and damaged oral epithelium (Brown et al. 1975a). Unlike salivary IgA, serum IgA had a persistently elevated level in cured patients (Brown et al. 1975b).

Although the association between oral cancer and HSV-1 is still an area for research, an association between oral cancer, precancer and smoking is well established (Silverman 1981; Baric et al. 1982). There is also evidence for an association between smoking and antibody to HSV-1. Normal individuals who smoked had significantly higher titers to HSV-1 in their sera than those who did not smoke (Smith et al. 1976a & 1976b; Shillitoe et al. 1982).

There was no significant difference in serum antibody level of IgG antibody to HSV-1 among untreated and treated cancer patients, patients with recurrent herpes

labialis, and in control individuals (Shillitoe et al. 1983a). This is consistent with the results from Smith et al. (1976a & 1976b) in which serum IgG anti-HSVIA (HSV-induced antigen) antibody was not significantly different between patients with squamous cell carcinoma and patients with nonsquamous cell carcinoma and between normal individuals who were smokers and nonsmokers.

Patients with untreated oral cancer had serum antibody levels to HSV-1 similar to those of normal individuals who smoked. Also, patients with later stage tumors had higher antibody titers to HSV-1 than those with earlier stage tumors. In patients who were tumor free after treatment for oral cancer, higher antibody titers to HSV-1 were associated with longer survival times (Shillitoe et al. 1982). Smith et al. (1976a) also reported that patients with squamous cell carcinoma who were smoking heavily had significant higher serum IgA anti-HSVIA antibody than patients who were smoking lightly or were non-smokers. However, a recent study in our department (Shillitoe et al. 1983a) using enzyme-linked immunosorbent assay (ELISA) indicated that there was no significant differenc in serum IgA antibody to HSV-1 between patients with untreated and treated cancer, patients with recurrent herpetic labialis and control individuals.

Recent studies in our department (Shillitoe et al. 1983a & 1983b) indicated that a significantly elevated IgM antibody to HSV-1 antigens was present in patients with oral cancer, but with a similar level of IgG antibody to HSV-1, when compared with matched controls. Also the IgM antibody level was highest in patients with primary herpetic infection. The elevated IgM antibody from patients' sera was associated with both early and late HSV-1 antigens in infected HEp-2 cells (Shillitoe et al. 1983b). The reaction of serum IgM antibody to late antigen was significantly higher in oral cancer patients than in controls. Sera from patients with oral cancer also had higher IgM antibody to HSV-1 virion antigens. The elevated IgM antibody in sera from both patients with oral cancer and acute herpetic infection suggested that HSV-1 antigens might be present in tissue of oral cancer as well as in the lesion of acute infection to stimulate the immune system

and to induce IgM antibody formation rather than IgG antibody. This finding is consistent with that in patients with cervical carcinoma in whom a higher IgM antibody was detected to the specific HSV-2 AG-4 antigen (Aurelian et al. 1976).

Thus both IgM and IgA antibodies are elevated in patients with oral cancer. This parallels the changes in patients with cervical carcinoma. Also, the higher IgA antibody in patients with oral cancer is consistent with that in patients with nasopharyngeal carcinoma in whom a higher level of IgA antibody to EBV occurs, but not IgG antibody (Henle & Henle 1976). These results might indicate that tumorigenic HSV-1 antigens may stimulate the local antibody response in both groups of patients with primary and recurrent tumors and in smoking patients.

A higher percentage of adults has been infected by HSV-1 than by HSV-2, 50%-100% and 10-70%, respectively (Krause 1980), and most of HSV-1 infected adults have developed antibody to the virus before maturity. Thus attempting to use the seroepidemiologic method to quantitate the possible role of HSV-1 in human oral cancer would be more difficult, although a possible association of HSV-2 with cervical carcinoma has been demonstrated by analysis with this method. More advanced and precise techniques are extremely required for further study to connect HSV-1 and oral cancer.

## HSV-2 and cervical carcinoma

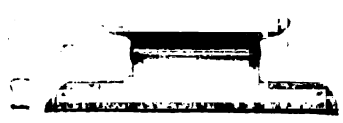
Although prior infection by a variety of organisms might be a cofactor of mild dysplasia of the uterine cervix, prior HSV-2 infection is more significantly related to severe dysplasia and carcinoma in situ of the cervix (Thomas & Rawls 1978). Other seroepidemiological surveys (Nahmias et al. 1970; Skinner et al. 1971 & 1977; Sprecher-

Goldberger et al. 1972; Adam et al. 1973; Kawama et al. 1974; Rawls et al. 1968; Kessler 1977) also provide evidence of this relationship between cervical carcinoma and HSV-2. Women with herpetic cervicitis have a significantly higher risk (from 4- to 16-fold) of developing carcinoma than those with a negative HSV-2 history (Nahmias et al. 1973; Naib et al. 1973). The prevalence of humoral (Nahmias et al.1973; Naib et al. 1973) and cell-mediated immune (CMI) responses (Smith et al. 1979; Thiry et al. 1977) to HSV-2 was significantly higher in cervical cancer patients than in matched control groups. Both humoral and CMI responses were HSV-2-specific, not specific to other viruses. Patients with other types of cancer, patients with cancer at other sites, and control individuals did not have a specific immune response to HSV-2, although antibodies to HSV-2 are normally present in 30-50% of the population.

A major early nonstructural protein, VP134 with a molecular weight of 134,000 contained ICP6 component (Courtney & Benyesh-Melnick 1974; Bone & Courtney 1974; Powell et al. 1975; Table 1), located in the cytoplasm of infected cells, was also found to correlate with cervical carcinoma. Patients with carcinoma have antibody against VP134 antigen when examined by radioimmune precipitation (Anzai et al. 1975). Quantitative assays by Melnick et al. (1976) showed that sera from patients with cervical cancer precipitated more VP134 than sera from patients with cancers of other types or from control women. However, antibody titers to VP134 were not significantly different before and after treatment.

With the particularly interesting and important HSV-2 viral protein AG-4, Aurelian et al. (1981) suggested that AG-4 is a cervical tumor antigen which fulfills the criteria for a marker of carcinogenicity. AG-4, immunologically identical to ICP-10 (Aurelian et al. 1980; Jariwalla et al. 1980; Strnad & Aurelian 1978), is an HSV-2-induced early protein with a molecular weight of 161,000 and has been purified from HSV-2 infected cells early in the reproductive cycle of the virus (Strnad & Aurelian 1978). This AG-4 viral protein is also expressed in cells transformed by the Bgl II-C fragment

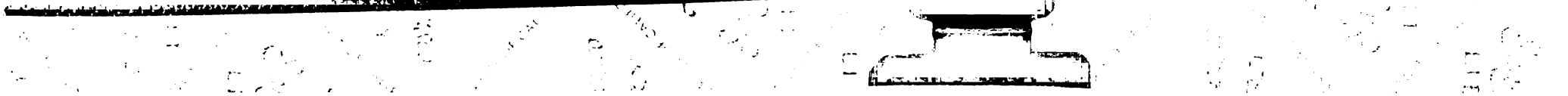
(Jariwalla et al. 1980), which is mapped between 0.43 and 0.58 of the HSV-2 DNA genome. The antibody to AG-4 is of the IgM class (Aurelian et al. 1976). Sera from patients with cervical carcinoma had a significantly higher frequency of antibody against AG-4 than sera from women without cancer or with other types of malignancy (Aurelian et al. 1976; Aurelian et al. 1973a & 1973b; Notter & Docherty 1976; Arsenakis et al. 1980; Heise et al. 1979; Kawana et al. 1976; Kawana et al. 1978). Unlike VP134 and HSV-TAA, AG-4 may have a favorable prognostic significance, since the serologic response to AG-4 followed the clinical history. Antibody titers to AG-4 reflected the active tumor growth of cervical tumor from dysplasia to invasive cancer. Antibody to AG-4 disappears after removal of the tumor and reappears with tumor recurrence (Aurelian et al. 1973a & 1973b; Aurelian et al. 1981). About 80-90% of patients with invasive carcinoma and only 10% of control women without tumor were AG-4 seropositive. About 35% of patients with dysplasia and 65% of patients with CIS were seropositive (Aurelian et al. 1973a & 1973b; Aurelian et al. 1981). Aurelian et al. (1981) reported a six-year follow-up study on patients with cervical carcinoma. Their microcomplement fixation assay of antisera to AG-4 in patients' sera gave significant evidence that AG-4 was a useful prognostic marker in cervical cancer. After therapy, 87 of 152 patients converted from being AG-4 seropositive to AG-4 seronegative, and none of these had recurrent disease. Twelve patients remained positive after therapy, of which 7 (66.7%) had recurrent disease and 3 died. Only 20 of 152 patients were seronegative initially (free of cervical anaplasia) and converted to seropositive after therapy, of which 9 had developed disease. Thirty-three patients were seronegative to AG-4 before and after therapy, of which 9 had developed mild to moderate dysplasia. In contrast to the cured patients with mild to moderate dysplasia in the seronegative group, patients with stage II and III invasive cancer might have a poor prognosis. This suggested that AG-4 may have a favorable prognostic significance (Aurelian et al. 1981; Aurelian et al. 1973b; Heise et al. 1979), based on the serologic conversion of AG-4 antibody.



Although three HSV-2-specific monoclonal antibodies, A6, H11 and E12, failed to react with cervical tumor cells or with transformed cells (McDougall et al. 1982), monospecific antisera to ICSP11/12 and antiserum to VP143 reacted positively with cervical tumor cells (McDougall et al. 1982). These two antisera also reacted with HSV-2-transformed cells (Lewis et al. 1982; McDougall et al. 1982; Flannery et al. 1977). Electrophoretic analysis of these two antisera suggested that ICSP11/12 and VP143 were immunologically identical, since they precipitated a single band with an estimated molecular weight of 118,000 (McDougall et al. 1982). The ICP8 protein, a DNA binding protein isolated from HSV-1 infected cells, was also identical to ICSP11/12 and VP143 (Powell et al. 1981) (Table 1). Patients with cervical carcinoma also contained anti-ICSP11/12 (VP143) antibody (Anzai et al. 1975). However, the significance of ICSP11/12 (VP143) can not be defined, since anti-ICSP11/12 antisera can crossreact with five different herpesvirus induced proteins (Yeo et al. 1981). In the study of McDougall et al. (1982), anti-ICSP11/12 and VP143 antisera were reactive with cytomegalovirus (CMV)-infected cells. But, the presence of ICSP11/12 (VP143) antigen in cervical tumor cells paralleled the finding of the RNA transcriptional sequences in those tissues by the in situ hybridization technique (McDougall et al. 1982). Another monospecific antiserum to ICSP34/35, which is a DNA-binding protein and is isolated from HSV-2-infected HEp-2 cells, also reacted with 38% of anaplastic cells from patients with cervical cancer (Dreesman et al. 1980).

Another HSV type-common antigen, AG-e, which is consistent with two viral proteins (ICP12 and ICP14) (Smith & Aurelian 1979), was expressed in anaplastic cells from patients with cervical cancer as well as infected cells from patients with herpetic cervicitis, but not in cells from normal tissues (Smith et al. 1980).

The in situ cytological hybridization technique was recently applied in studies of the relationship between HSV-2 and cervical carcinoma at the molecular level (McDougall et al. 1980 & 1982; Jones et al. 1979; Maitland et al. 1981). The detection of



viral RNA in cervical carcinoma (McDougall et al. 1980 & 1982; Maitland et al. 1981) by in situ hybridization was as positive as that in HSV-transformed cells (Collard et al. 1973; Frenkel et al. 1976a) and derived tumor sections (Copple & McDougall 1976). Attempts to find HSV-2 DNA sequences in human cervical tumor tissues failed (zur Hausen et al. 1974; Pagano 1975), except that in one cervical tumor 39% of the viral genome was found (Frenkel et al. 1972). About 37% (McDougall et al. 1980) to 67% (Maitland et al. 1981) of cervical tumor biopsies were positive for HSV-2-specific RNA, as shown by autoradiographic grains and the hybridization technique. In a very recent study using cloned subgenomic fragments of HSV-2 DNA cleaved by endonuclease as hybridization probes (McDougall et al. 1982), three regions of the viral genome located at map positions 0.07 to 0.40, 0.58 to 0.63 and 0.82 to 0.85 were detected as the RNA transcriptional sequences. This result paralleled to the finding of a positive reaction between ICSP11/12 (VP143) and cervical carcinoma. However, none of these sequences has been determined to be a tumor-specific gene.

Although AG-4 is specific and significant in cervical carcinoma, its location on the map position between 0.43 and 0.58 (Jariwalla et al. 1980) is different from that of three positive RNA transcriptional sequences found in an in situ hybridization study (McDougall et al. 1982). Further study to detail this specific association is necessary.

## Transforming and oncogenic potential of HSV

The observation of the biochemical changes in mouse L-cells after exposure to ultraviolet(UV)-irradiated HSV (Munyon et al. 1971) led to the study of the transforming and oncogenic potential of HSV. During the last decade, many investigators have worked on these specific characteristics of HSV. The wide application of UV-irradiated HSV had

successfully induced transformation in cells from primary cultures of hamster embryo fibroblasts (HEF) (Duff & Rapp 1971 & 1973; Duff et al. 1974; Kimura et al. 1975; Davis et al. 1974; Duff & Doller 1973; Macnab 1974; Rapp & Li 1975), rat embryo cells (RE) (Macnab 1974 & 1979), mouse cells (Munyon et al. 1971; Davis et al. 1974; Duff & Rapp 1975; Davidson et al. 1973; Garfinkel & McAuslan 1974; Munyon et al. 1972; Kurchak et al. 1977; Body & Orme 1975) and human cells (Davis et al. 1974; Kurchak et al. 1977). Photodynamic inactivation of HSV in the presence of neutral red (NR) (Rapp & Li 1975; Rapp et al. 1973; Li et al. 1975) was able to transform mammalian cells in culture. In addition, temperature sensitive (ts) mutants of both HSV-1 and HSV-2 were able to cause the biochemical transformation of cultured mammalian cells, included hamster and human embryo cells, after the primary cultured cells were infected at a nonpermissive temperature (Kimura et al. 1975; Takahashi & Yamanishi 1974; Darai & Munk 1973; Macnab 1975; Okazaki et al. 1981).

All three methods of inactivating the virus showed the transforming potential of HSV. They were able to transform cells either morphologically or biochemically or both. Basically, the following definitions were used to distinguish the difference between morphological and biochemical transformation. Biochemical transformation refers to the conversion of a thymidine kinase (TK)-negative cell to a TK-positive state following introduction and expression in the cell of the HSV TK gene. Morphological transformation refers to the alterations whereby cells express one or more phenotypic properties different from their "normal" counterparts which are associated with malignancy (Hamper 1981). Morphologically transformed cells induced by UV-irradiated HSV (Kimura et al. 1975; Macnab 1974, 1975 &1979; Rapp & Li 1975; Boyd & Orme 1975; Duff & Rapp 1971 & 1971a) and NR-treated HSV (Rapp et al. 1973) had the capacity in culture to form foci which lacked contact inhibition. HSV-1-transformed cells were of a predominantly epithelial morphology (Duff & Rapp 1973; Duff et al. 1974; Duff & Doller 1973; Rapp & Li 1975). HSV-2-transformed hamster cells exhibited a mixed morphology

consisting of fibroblasts, giant cells and primitive, undifferentiated cells, with the fibroblasts predominanting (Duff & Rapp 1971 & 1971a; Duff et al. 1974; Duff & Doller 1973; Rapp & Li 1975; Macnab 1975), but HSV-2-transformed rat cells were predominantly epithelial in morphology (Macnab 1975). Biochemically transformed cells did show thymidine kinase (TK) activity (Munyon et al. 1971; Davidson et al. 1973; Garfinkle & McAuslan 1974; Munyon et al. 1972; Macnab 1975; Macnab et al. 1980), whereas primary culture normal cells did not (Munyon et al. 1971; Davidson et al. 1973; Garfinkle & McAuslan 1974). The TK activity present in HSV-transformed cells is different from that in normal TK-positive cells (Kimura et al. 1975; Davis et al. 1974; Munyon et al. 1972) and the TK-minus mutants of HSV fail to cause the transformation of TK-negative L cells to TK-positive cells (Munyon et al. 1971), suggesting that the viral gene for TK is incorporated into the transformed cells. Unlike biochemically transformed cells, morphologically transformed cells did not show this specific TK characterization and their encoded genome for the transformation was different from biochemically transformed cells. Also, the loss of transforming capability in morphologically transformed cells after several passages did not happen to biochemically transformed cells (Davis & Kingsbury 1976). In addition, no infectious virus has been isolated from these HSV transformed cells (Duff & Rapp 1971 & 1973; Duff et al. 1974; Rapp & Li 1975; Kutinova et al. 1973; Boyd & Orme 1975; Rapp et al. 1973).

Evidence of the transforming potential of HSV is supported by the following findings. HSV-specific antigens were present in the cytoplasm and on the cell surface of transformed cells, as detected by immunofluorescence (IF) (Flannery et al. 1977; Collard et al. 1973; Duff & Rapp 1971, 1971a & 1973; Duff & Doller 1973; Macnab 1974, 1975 & 1979; Rapp & Li 1975; Kutinova et al. 1973; Garfinkle & McAuslan 1974; Kurchak et al. 1977; Boyd & Orme 1975; Rapp et al. 1973; Li et al. 1975; Takahashi & Yamanishi 1974; Okazaki et al. 1981; Macnab et al. 1980; Davis & Kingsbury 1976; Kimura et al. 1979; Lausch et al. 1975; Kako et al. 1981; Buultjens & Macnab 1981). HSV-antigen has also

been found in the nucleus and cytoplasm of biochemically transformed cell by immunoperoxidase staining (Kurchak et al. 1977). Virus-neutralizing antibodies against HSV were found in sera from tumor-bearing animals which were inoculated by HSV-transformed cells (Duff & Rapp 1971, 1971a & 1973; Kimura et al. 1975; Duff & Doller 1973; Kutinova et al. 1973; Macnab 1979; Boyd & Orme 1975; Macnab et al. 1980), and these antibodies detected surface and cytoplasmic immunofluorescence on HSV-infected cells (Macnab 1979; Boyd & Orme 1975; Li et al. 1975; Macnab et al. 1980) and a surface reaction on the tumor cells (Li et al. 1975). Adsorption of anti-HSV serum with transformed cells reduced the neutralizing activity of antiserum (Lewis et al. 1982). Incubation of HSV-neutralizing antiserum in culture during the production of UV-HSV-transformed cells reduced the number of transformed-cell foci formed (Duff & Rapp 1975). Furthermore, the expression of virus-associated antigens in clonal cultures derived from single cells (Duff & Doller 1973) and the assay of cell-mediated cytotoxicity on transformed cells (Lausch et al. 1975; Sprecher-Goldberger et al. 1974) gave other evidence of the existence of viral information in transformed cells. Tumor cells recovered from these animals carried viral-specific antigens in their cytoplasm and on their cytoplasmic surface, just as their original transformed cells did (Duff & Rapp 1971 & 1973; Kimura et al. 1975; Macnab 1974 & 1979; Rapp & Li 1975; Boyd & Orme 1975; Li et al. 1975; Macnab et al. 1980; Buultjens & Macnab 1981) as demonstrated by IF. All of these results indicate that at least some portion of the HSV genome persists in the transformed cells, and supply circumstantial evidence of the transforming potential of HSV. However, expression of HSV-1 specific proteins seen in early passages of transformed cells is no longer detected after subsequent passage in vitro or after passage as tumor in animals in several studies (Minson et al. 1976; Frenkel et al. 1976a; Li et al. 1975; Davis & Kingsburg 1976). Unlike other DNA tumor virus induced transformed cells, in which the continued presence of the viral DNA is required, these HSV-1 induced morphologically transformed cells may only carry HSV-1 antigens for a limit number of

passages rather than were transformed by HSV-1. In contrast, HSV-1 induced biochemically transformed cells do express HSV gene indefinitely (Davis & Kingsbury 1976). Thus, there is controversy that morphologically transformed cells may carry HSV-antigens rather than are transformed by virus. A "hit and run" model had been proposed (Skinner 1976; Hampar et al. 1976; zur Hausen 1980; Schlehofer & zur Hausen 1982).

Many studies have identified the HSV type-specific and type-common antigens in transformed cells. Early and late antigens of infectious HSV-1 and HSV-2 have been shown in transformed rat cells by IF and prepared antisera against corresponding antigens (Macnab 1979; Macnab et al. 1980; Buultjens & Macnab 1981). Similarly, perinuclear and cytoplasmic reactions were recognized in these cells. An early, nonstructural HSV-2-induced polypeptide, VP143 with a molecular weight of 143,000 (Lewis et al. 1982) is immunologically identical to ICSP11/12 and ICP8 (McDougall et al. 1982; Powell et al. 1981), was detected in HSV-2-transformed and tumor-derived cell lines by hyperimmune antiserum against this specific polypeptide and IF (Lewis et al. 1982; Flannery et al. 1977). ICP8 is encoded within the sequences of transforming activity from the HSV-1 genome.

An HSV type-common virion antigen, CP-1 (Cohen et al. 1972), which resides on the 52,000 molecular weight glycoprotein is recognized as a precursor of an envelope glycoprotein (gDpp) with a molecular weight of 59,000 (Cohen et al. 1978). Both polypeptides are demonstrated to be related and are associated with one major glycoprotein (gD) of the virion envelope (Eisenberg et al. 1980; Cohn et al. 1978). Using antiserum against CP-1 and IF (Reed et al. 1975), both a UV-inactivated HSV-1 transformed cell line (14-012-8-1) and its tumor cell line (14-012-8-1,T-10) expressed reactive fluorescence on the cell membrane. Although antiserum to CP-1 was able to neutralize both HSV-1 and HSV-2, it was not expressed on UV-HSV-2 transformed-cell lines and NR-HSV-transformed cell lines.

Gupta and Rapp (1977) showed that at least ten polypeptides were present in the virion of HSV-1. Indirect evidence obtained by immunoprecipitation and SDS-PAGE techniques indicated that three HSV-1 surface virion polypeptides were detected in HSV-1-transformed hamster cell lines (14-012-8-1,T-10) (Gupta & Rapp 1977). Of these three polypeptides, two were also detected in HSV-2-transformed cell lines (333-8-9 and 333-2-29) as the HSV type-common virion polypeptides (Gupta et al. 1980). These two polypeptides were P7 and P8 with a molecular weight of 33,000 and 30,000, respectively (Gupta & Rapp 1977; Gupta et al. 1980). The P5 polypeptide with a molecular weight of 58,000 might be an HSV-1 type-specific virion polypeptide (Gupta & Rapp 1977). These three polypeptides belonged to surface glycoproteins (Gupta & Rapp 1977). These polypeptides expressed in transformed cells may also be virus specific, however, they have not yet been fully characterized and their associated glycoretein is not defined either.

Camacho and Spear (1978) also applied anti-VP7(B2) serum (AS-39), which recognized an isolated glycoprotein VP7(B2) (identical to gA/gB), and anti-gps serum (AS-18), which reacted with all major glycoproteins, to determine the viral antigens expressed in the cytoplasm of transformed cells by IF. Anti-VP7(B2) serum (AS-39) absorbed with transformed cells had reduced its neutralizing activity by about 90%. Both antisera were precipitated with proteins from radiolabelled extracts of transformed cells.

Recently, monospecific antisera to HSV-2 gA/gB and gX glycoproteins stained in HSV-2 transformed rat and derived tumor cell lines by immunofluorescence (Lewis et al. 1982).

Most of the transformed cells induced by the inactivated HSV-1 or HSV-2 or DNA fragment possess oncogenic potential. Subcutaneous injection of transformed cells into animals of the respective original species (Duff & Rapp 1971, 1971a & 1973; Kimura et al. 1975; Duff et al. 1974; Duff & Doller 1973; Rapp & Li 1975; Kutinova et al. 1973;

Macnab 1979; Li et al. 1975; Macnab et al. 1980; Buultjens & Macnab 1981) and into immunodefective mice (Buultjens & Macnab 1981) resulted in the tumor formation. These tumors were invasive and metastatic with characteristics of undifferentiated cells in their morphology (Duff & Rapp 1971, 1971a & 1973; Duff et al. 1974; Kimura et al. 1975; Duff & Doller 1973; Rapp & Li 1975; Macnab 1979; Li et al. 1975; Buultjens & Macnab 1981). Cells isolated from the induced tumor were reported to have higher oncogenic potential than the original transformed cells (Duff & Rapp 1971 & 1973; Duff et al. 1974; Kimura et al. 1975; Li et al. 1975).

It is interesting that HSV-1-transformed epitheloid cells implanted into hamsters resulted in the formation of carcinoma, and that isolated tumor cells were predominantly epitheloid in morphology after higher passage in culture (Duff & Rapp 1973; Duff et al. 1974; Duff & Doller 1973). In contrast, fibrosarcoma and fibroblastoid cells had the morphologic characteristics of HSV-2-induced tumor and transformed cells (Duff & Rapp 1973; Duff et al. 1974; Kimura et al. 1975; Duff & Doller 1973). Although there was no definite cell type in the induced tumors by HSV-1 and 2, the different genome in HSV-1 and 2 may contribute to the determination and maintenance of transformation.

More precise and sensitive techniques are also available in the search for the viral genome in transformed and derived tumor cells, and in the induction of transformed cells by part of viral genome. HSV-1 and HSV-2 DNA share their base sequences in part, and virus-specific RNA in each type of virus can cross-hybridize. On the basis of this information, Collard et al. (1973) applied the molecular hybridization technique and detected viral mRNA sequences in an HSV-2-transformed cell line (333-8-9), but not in adenovirus-transformed cells or non-infected HEp-2 cells. These 333-8-9 cells contained 10-30% of the virus sequences transcribed during productive infection. Part of the HSV-2 DNA sequence was also detected in UV-irradiated HSV-2-transformed cells and their derived tumor cell line by hybridization analysis (Frenkel et al. 1976a). Nucleic acid or DNA from transformed cells has been transferred to normal cells, and tests of these

normal cells by cell-mediated cytotoxicity further supported the persistence of the HSV-genome in the transformed cells (Sprecher-Goldberger et al. 1974).

Approximately 10% of HSV-1 or HSV-2 genome has been demonstrated in UV-irradiated HSV-1- or HSV-2-induced biochemically transformed cells, respectively, but not in UV-HSV-2 morphologically transformed cells (333-8-9 cell line) (Davis & Kingsbury 1976). Using restriction endonuclease cleaveage of viral DNA, Camacho and Spear (1978) demonstrated that as little as 15% of the HSV-1 genome was able to induce HEF to become transformed cells and its products were expressed in these cells. This particular portion of the HSV-1 genome, Xba I-F, was located between coordinates 0.30 and 0.45 on the physical map of the genome. A Bgl II-I fragment from HSV-1 DNA located between 0.311 and 0.415 and its encoded sequences, similar to the Xba I-F, has further been demonstrated as a transforming region in some transformed cells by the hybridization (Reyes et al. 1980). Most recently, HSV-1 Eco RI-F fragment at map position between 0.32 and 0.42 (Eizuru et al. 1983) which is colinear with the Xba I-F and Bgl II-I fragments has also been demonstrated as the most frequently transforming region in eight of ten 14-012 transformed cell lines. Concerning the HSV-2 strain 333, its Bgl II-N fragment with mapping between 0.582 and 0.628 was able to induce transformed cells with tumorigenicity (Reyes et al. 1980; Galloway & McDougall 1981). However , the encoded viral proteins could not be demonstrated in transformed cells (Reyes et al. 1980; Galloway & McDougall 1981). A study by Galloway et al. (1980) pointed out that the Bgl II-N fragment with mapping between 0.60 and 0.65 was present in 333-8-9 transformed cells. In addition, another fragment, Bgl II-G with mapping between 0.21 and 0.33, was detected in the transformed cells (Galloway et al. 1980). The Bgl II-I from HSV-1 and Bgl II-N from HSV-2 were defined as morphological transforming region I (mtr-I) and mtr-II, respectively (Reyes et al. 1980). These results strongly supported the concept that the HSV genome is involved in the transforming capability (Fig. 1).

Jariwalla et al. (1980) studied the transforming and tumorigenic capability of

HSV-2 strain S-1. The "CD" fragment of HSV-2 S-1 DNA cleaved by restriction endonuclease Bgl II-, Hpa I-, or Bgl II/Hpa I- was transferred into hamster embryo cells and induced morphological transformation. These transformed cells were oncogenic in newborn hamsters. A complement fixation assay provided evidence of the expression of viral protein ICP-10, a viral protein immunologically identical to the cervical-tumor-associated AG-4 antigen (Jariwalla et al. 1980; Strnad & Aurelian 1978), in transformed cells. This specific DNA fragment CD from HSV-2 S-1 exhibiting the transforming activity, was sequentially homologuous to the Bgl II/Hpa I fragment CD of DNA from HSV-2-333, and was mapped between coordinates 0.43 and 0.58 on the physical map of strain 333 DNA. The CD333 fragment was confirmed as oncogenic (Jariwalla et al. 1980) (Fig. 1).

The experiments of complementation of some HSV-2 ts mutants by rat transformed cells to form plaques also indicated the presence of HSV genetic information in these transformed cells (Macnab & Timbury 1976). This suggests that these particular RE 1-rat transformed cells express HSV information sufficient to complement at least three genes (Macnab & Timbury 1976). Similarly, recombination of transforming virus DNA from transformed rat cells with ts mutant DNA was successfully demonstrated by Park et al. (1980). These supplied more evidence that transformed cells contain HSV genes.

Taken together, all the evidence indicates that HSV-1 and HSV-2 have transforming and oncogenic potential, at least in some particular strains. In addition, gA/gB and ICP8 proteins were coded in the HSV-DNA fragments (Fig. 2) associated with the transforming potential (Fig. 1). Both proteins were also detected in transformed and tumor-derived cell lines (Lewis et al. 1982; Flannery et al. 1977; Camacho & Spear 1978). Although other proteins, such as P5, P7, P8, CP-1 (gD) and gX were also shown to have some associations with HSV oncogenicity, they were not yet fully characterized.

Herpes simplex virus infected cell polypeptides and monoclonal antibodies

Recently, many biochemical and immunologic studies have been done to search for the properties of HSV infected cell polypeptides (ICPs) in infected cells. ICPs were defined as virus-specific, since the synthesis of proteins coded for by the host cell rapidly declined after infection and the synthesis of viral polypeptides was increased after infection (Honess & Roizman 1973; Powell & Courtney 1975). Three groups of viral polypeptides were classified in the pathway of their synthesis. Basically, the synthesis of $\alpha$ , $\beta$ , $\gamma$ -polypeptides was coordinately regulated and sequentially ordered in by a cascade pathway (Honess & Roizman 1973, 1974 & 1975). $\alpha$-polypeptides (e.g. ICP4) are formed at the immediate-early stage after infection. The synthesis of $\beta$ - or early polypeptides (e.g. ICP6, ICP8) requires the presence of functional $\alpha$-polypeptides, and is in turn responsible for the following synthesis of $\gamma$ - or late polypeptides (e.g. ICP5, gB). In addition to the transition of synthesized polypeptides from $\alpha$ to $\beta$ to $\gamma$ group, the later polypeptides also regulate (inhibit) the synthesis of the earlier polypeptides. Evidence of the regulation of the synthesis of virus polypeptides has been based on studies of pulse-labeled amino acids and the application of specific chemicals, such as cycloheximide.

Studies indicate that $\alpha$ -polypeptides are synthesized immediately after infection, reach a maximal synthesis rate between 2- and 4- h postinfection, and decline thereafter (Honess & Roizman 1974). The onset of $\beta$ -polypeptides synthesis begins at 3 h postinfection, whereas $\alpha$ -polypeptides synthesis declines (Honess & Roizman 1974). The peak rates of synthesis of $\beta$-polypeptides is between 5 and 7 h postinfection and require the presence of functional $\alpha$-polypeptide (Honess & Roizman 1974 & 1975). $\gamma$ -polypeptides have an increasing rate of synthesis after 15 to 18 h postinfection and require the presence of functional $\beta$ -polypeptides (Honess & Roizman 1974). Also based on the temporal pattern of polypeptide synthesis, several classes of ICPs have been classified

(Honess & Roizman 1973). Proteins of class A (e.g. ICP5) are synthesized at gradually increasing rates thoughout infection; that is in infected cells ICP5 is made at progressively higher rates until at least 12 h postinfection; proteins of class C (e.g. ICP4, ICP6, ICP8) are synthesized at an increasing rates initially and thereafter their synthesis declines.

As to the approximately 50 proteins coded by HSV-1 and HSV-2, many of these proteins share type-common antigenic determinants that are cross-reactive with type-specific hyperimmune antisera, while others have type-specific antigenic determinants that are reactive with type-specific hyperimmune antisera only. Monospecific antisera were able to distinguish between the type-common and type-specific antigenic determinants (Heilman et al. 1978). Most recently, the successful production of monoclonal antibodies (Pereira et al. 1980; Killington et al. 1981; Balachandran et al. 1981; Showalter et al. 1981) following the hybridoma technique introduced by Kohler & Milstein (1975) were also employed to detect the specific determinants on individual polypeptides.

Monoclonal antibodies produced by hybridoma cells are able to recognize specific individual proteins (Showalter et al. 1981; Peterson et al. 1983) and glycoproteins (Balachandran et al. 1981 & 1982; Showalter et al. 1981; Peterson et al. 1983; Pereira et al. 1980, 1981 & 1982) as shown by radioimmunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (RIP-PAGE). Thus type-specific or type-common characteristics of monoclonal antibodies can be determined by this technique as well as the identification of specific proteins in infected cells by monoclonal antibodies and immunofluorescence assay. Similarly, immunofluorescent staining has been applied to demonstrate the existence of specific antigens in infected cells and to test the specificity of monoclonal antibodies (Pereira et al. 1980; Killington et al. 1981; Showalter et al. 1981). As hyperimmune antisera, only some monoclonal antibodies were able to neutralize HSV, further attesting their specificity (Pereira et al. 1980 & 1981;

Showalter et al. 1981). Furthermore, monoclonal antibodies may be useful as reagents to rapidly screen the different serologic type of virus (Peterson et al. 1983; Pereira et al. 1980 & 1982) instead of the hyperimmune antisera tests and restriction endonuclease analysis of viral DNA (Peterson et al. 1983).

More than one monoclonal antibody can be produced against the same viral protein (Pereira et al. 1980 & 1982; Showalter et al. 1981). Although some monoclonal antibodies did not neutralize infectivity, they did precipitate their respective polypeptides and did stain on infected cells by immunofluorescence (Pereira et al. 1980; Showalter et al. 1981). On the other hand, other antigenic domains on the same protein might function as an important part of virus infectivity. Also, by analysing the results from different tests of the different clones of antibodies which recognize the distinct antigenic sites on homologous polypeptide, it is possible to identify the specific strain or serotype of virus based on intratypic variation of antigenic determinants present on homologous protein (Pereira et al. 1982). Moreover, with specific biochemical analysis and monoclonal antibodies, it is possible to tell the specific amino acid feature for each polypeptide.

HSV-1 strain 14-012 has been demonstrated to be an oncogenic and transforming virus. Recently the hybridoma technique was introduced to form 52 monoclonal antibodies against 10 HSV proteins and glycoproteins (Showalter et al. 1981). Antibodies against four glycoproteins (gB, gC, gD and a 110,000 molecular weight protein) and six nonglycosylated proteins (ICP4, ICP5, ICP6, ICP8, ICP9, and a 68,000 molecular weight protein) were developed. Their characteristics and specificity were determined by RIP-PAGE, IF and neutralization assays as previously described (Showalter et al. 1981). In this study we included monoclonal antibodies against ICP4, ICP5, ICP6, ICP8 and gB, and used the ABC technique to localize the reactive pattern of different proteins in infected cells and to determine their specificity. ICP8 viral protein encoded by the HSV-1 genome was employed to study the relationship between HSV-1 and oral carcinoma, since a viral protein ICSP11/12 (VP143) was immunologically identical to ICP8 in the HSV-1 encoded

genome (Powell et al. 1981). ICSP11/12 (VP143) has been shown to have some association with cervical carcinoma (McDougall et al. 1982) and in situ HSV-transformed cells (McDougall et al. 1982; Flannery et al. 1977; Lewis et al. 1982). The HSV-glycoprotein gA/gB has been found on HSV-transformed cells and derived tumor cells (Lewis et al. 1982; Camacho & Spear 1978). Both ICP8 and gB encoded genomes are located in the Xba I-F fragment of HSV-1 with the physical map between 0.30 and 0.45 which has been shown to be a transforming fragment of the virus (Fig. 2). Other proteins such as P5, P7, P8, CP-1 and gX have been shown to have some associations with HSV transforming characteristics. However, they have not been fully characterized, and might be proteins now known by other names. Thus, in addition to the analysis of five monoclonal antibodies (anti-ICP4, ICP5, ICP6, ICP8 and gB) in HSV-infected HEp-2 cells, monoclonal antibodies were also employed in the frozen sections of squamous cell carcinomas by ABC technique to determine if these HSV proteins are present on the malignant cells. Sections of tissue from other conditions were included in this study as control groups. The results and their possible significance in the etiologic role of HSV in oral cancer are discussed.

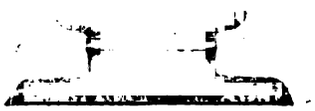
# MATERIALS and METHODS

## Cells and Viruses

HEp-2 and Vero cells were grown in M 199 medium containing 10% fetal calf serum. The virus strains used were HSV-1 strain 14-012 and HSV-2 strain 333 (obtained from Dr. F. Rapp, Hershey, PA). The virus was passaged in Vero cells at low multiplicity as previously described (Shillitoe et al. 1983a). Monolayers showing cytopathic effect of 80% were scraped into the culture medium and disrupted by ultrasonic vibration. Cell debris was pelleted by centrifugation at 1,000 x g for 15 minutes and discarded. The virus suspension was layered onto a 10-45% sucrose gradient and further centrifuged at 82,000 x g for one hour at 4° C. The virus band was then collected and diluted in phosphate-buffered saline (PBS) and precipitated at 100,000 x g. The virus particles were then resuspended in PBS by ultrasonic vibration. Virus infectivity was determined by plating the suspension onto Vero cells with a methylcellulose overlay, and was found to be $5x10^7$ plaque-forming units (PFU) per milliliter (ml).

## Infection of Cells

Monolayer cultures of HEp-2 cells in 75-$cm^2$ flasks trypsinized and the cells were transferred to microscope slides. Each 1 cm diameter area of the slides contained $2x10^5$ cells which were allowed to attach and then were infected by addition of virus at input multiplicities of 10-20 PFU/cell. After adsorption of virus for 1 hour, medium 199 containing 2% fetal calf serum was used to maintain the cells. The cultures were incubated at 37° C with 5% $CO_2$ for different time periods and then were fixed in 10% acetone for 10 minutes at 4° C. Cells fixed by acetone on slides were used directly for ABC staining or were stored at -70° C after drying in air.



Monoclonal antibodies

Five monoclonal antibodies to HSV-1 proteins ICP4, ICP5, ICP6, ICP8 and gB were used in this study. These antibodies were made by hybridomas as previously described (Showalter et al. 1981), and were kindly provided by Dr. M. Zweig. The properties of these monoclonal antibodies have been described in detail (Showalter et al. 1981). Briefly, anti-ICP4 antibody is type specific against an HSV-1 protein with a molecular weight of 175,000 as defined by RIP-PAGE and IF. Anti-ICP5 antibody is against the major capsid protein of MW 155,000 and cross-reacts with HSV-1 and 2. Anti-ICP6 is against a protein of MW 140,000 and cross-reacts with HSV-1 and 2. Anti-ICP8 antibody is specific for HSV-1 and is against the major DNA-binding protein with molecular weight of 132,000. The final monoclonal antibody is against gA/gB, a glycoprotein with molecular weight of 120,000 and is a type common antibody showing cell-membrane immunoflurorescent staining of infected cells. All monoclonal antibodies belong to $IgG_{2a}$ class, except that anti-ICP5 antibody is $IgG_1$ (Table 2).

All five monoclonal antibodies were used to stain on the infected cells by the ABC technique. Titration of antibodies for their specificity and staining patterns on infected cells was carried out from 1:20 to 1:32,000 for gA/gB and from 1:20 to 3,200 for other proteins. The optimal dilution of each antibody was employed on cells with different time periods of infection. For the study of tissue sections, dilutions of anti-ICP8 from 1:10 to 1:2,560 and of gA/gB from 1:40 to 1:2,560 were used. Normal mouse ascites fluid (Bethesda Research Laboratories; Gaithersburg, MD) was also used in the same dilutions.

In addition, two monoclonal antibodies against ICP8 were donated by Genetic Systems Corp. (Seattle, Washington) (thereafter named as G.S.#1 and G.S.#2) and were also used in several experiments for comparison with the 39S anti-ICP8 antibody (Table 2). These two antibodies have not been well characterized by the manufacturer.

Tissue Specimens

Specimens were obtained by biopsy from patients with oral lesions of leukoplakia or malignancy who attended the Oral Medicine Clinic of the School of Dentistry, University of California, San Francisco. All available specimens were chosen after the diagnosis was made. There were 5 specimens of gingiva with nonspecific inflammation (control group), 10 of leukoplakia, 11 of malignancy, and 6 of leukoplakia with atypia. Four of six leukoplakias with atypia were diagnosed clinically as proliferative verrucous hyperplasia or proliferative verrucous leukoplakia (PVL); Three of four PVL showed atypia and the other (R82-113) was carcinoma in situ. The other two atypia specimens were carcinoma in situ (R82-206) and dysplasia (R83-065). All of these biopsies were diagnosed by oral pathologists in the Division of Oral Pathology with regular hematoxylin and eosin stain.

Specimen preparation

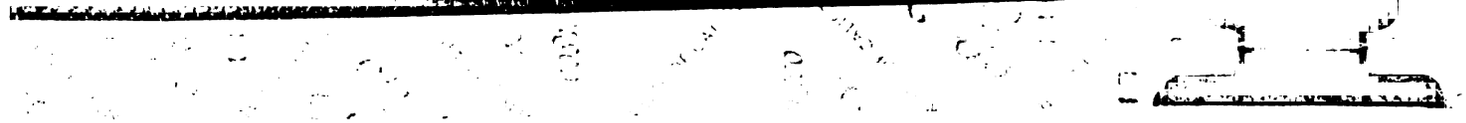
Half of the tissue from each biopsy specimen was fixed in 10% formalin, embedded in paraffin and cut for the regular hematoxyalin and eosin staining. This was done in the Division of Oral Pathology for the histopathological diagnosis. The other half of each specimen was quickly frozen in liquid nitrogen. Frozen specimens were mounted in optimal cutting temperature (O.C.T.) compound (Tissue-Tek Division, Miles Laboratories, Inc. Naperville, IL) and were cut with a Slee cryostat (South London Electrical Equipment Co. Ltd.) at -20° C to get sections with of 5 micron thickness. The sections were then mounted on formol-gelatin coated slides and fixed in acetone for 10 minutes at 4° C. These sections were stored at -70° C after fixing and air drying and were stained by the ABC technique within two weeks. Specimens were also cut and mounted on slides and fixed in ether/alcohol solution. These sections were later stained with hematoxylin and eosin.

Avidin-Biotin complex (ABC) immunoperoxidase technique (Fig.3)

The ABC method, introduced by Hsu et al. (1981), was used. The slides stored at -70° C were air dried for 10 minutes immediately before the ABC reaction. Briefly, ABC technique proceeded as follows. First, cells or sections were incubated with 3% normal horse serum (Cappel Laboratories; Cochranville, PA) for 20 minutes to block the possible nonspecific binding of the conjugated antibody (secondary antibody). Monoclonal antibody (primary antibody) was diluted in 0.5M Tris HCl-0.15M saline buffer and was then added to the appropriate areas on the slide for another 20 minutes. After washing out the nonreactive antibody in buffer for 10 minutes, a 1:200 dilution of horse anti-mouse IgG antibody (H & L chains) conjugated with biotin (Vector Laboratories; Burlingame, CA) was applied for 20 minutes to recognize the primary antibody binding to the targets on the slide. The excess, unbound conjugated antibody was then removed by another washing for 10 minutes. Avidin-biotin peroxidase complex (ABC) reagent (Vector Laboratories; Burlingame, CA), diluted 1:100 was added to bind with the biotin molecules conjugated on the antibody for 60 minutes. After another washing for 10 minutes, 0.01% hydrogen-peroxide mixed with 0.05% 3,3-diaminobenzidine tetrahydrochoride (DAB) (Litton Bionetics; Kensington, MD) solution was used to be oxidized by the peroxidase components of ABC reagent for 5 minutes. The DAB substrate formed an insoluble brown precipitate. Methyl green (4%) was followed as a counterstain and was reacted for 10 minutes (Figure 3).

Toluidine Blue stain

Prepared frozen sections were stained with 0.5% toluidine blue in distilled water for 1 minute to show mast cells. The stained slide was washed in running distilled water for 10 seconds and a coverslip was applied with glycerine jelly.



Double staining of mast cells

Prepared frozen sections were first stained with 0.5% toluidine blue in distilled water for 1 minute. The areas with metachromatic staining were then photographed. Stained sections were washed twice in Tris-buffer for 10 minutes to remove the stain on the tissue. After this, the ABC reaction was employed on the sections and they were rephotographed.

# RESULTS

## Specificity of monoclonal antibodies on HSV-infected cells

Titration of each monoclonal antibody was performed on cells infected with HSV for 24 hours, and on noninfected HEp-2 cells. End point titers of monoclonal antibodies were obtained as follows: greater than 1:3,200 for anti-ICP4, ICP5, ICP6 and ICP8 antibodies; greater than 1:32,000 for anti-gB antibody (Table 3). All monoclonal antibodies showed positive staining on infected cells, and no reaction was found on noninfected cells. The results from staining with monoclonal antibodies was consistent with the specificity of these monoclonal antibodies as previously determined by RIP-PAGE and IF (Table 2 and 3). Anti-ICP4 and anti-ICP8 were HSV-1 type-specific; anti-ICP5, anti-ICP6 and anti-gB antibodies were HSV type-common antibodies. The predominant staining pattern on 24 h infected cells for each antibody from ABC was identical to that with IF (Showalter et al. 1981). Each of ICP4, ICP5 and ICP8 appeared in the nucleus; both ICP6 and gB polypeptides appeared in the cytoplasm and gB glycoprotein also showed the cytoplasmic membrane staining. A dense nuclear staining of ICP4 was seen in infected cells, whereas ICP8 and ICP5 appeared as speckled staining.

Titration of G.S.#1 and G.S.#2 antibodies was performed under the same conditions. End point titers were 1:1,600 for both antibodies. Like 39S ICP8, a speckled nuclear staining was seen in infected cells. Unlike 39S ICP8, these two antibodies were type-common antibodies as detected by the ABC on HSV infected cells (Table 3).

## HSV-1 proteins in infected cells

Seven experiments were performed to study of the properties of the viral proteins ICP4, ICP8 and gB. Five of the seven experiments also included ICP5 and ICP6. The

dilution used of each antibody was 1:800 for ICP4 and ICP8; 1:400 for ICP6; 1:200 for ICP5; and 1:8,000 for gB. Three experiment also included G.S.#1 and G.S.#2 antibodies in which two only reacted on cells infected with virus at 24 h to compare their results with those of 39S ICP8 (Table, 5 & 6). The dilution used for G.S.#1 and G.S.#2 were 1:100 for both antibodies. Viruses and cells used in each experiment came from the same batch and were used under the same conditions.

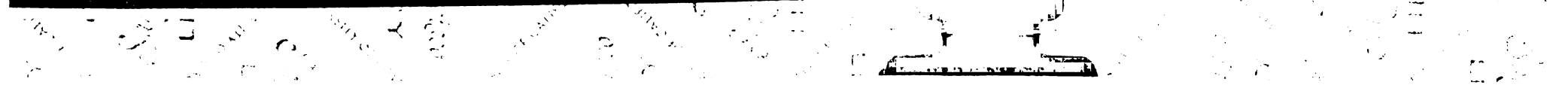
Immediate-early protein, ICP4: A 1:800 dilution of monoclonal antibody against the immediate-early protein ICP4 was employed to stain HEp-2 cells infected by HSV at different times postinfection: 1, 4, 8, 12, 24, 48 and 72 hours. At 1 h postinfection only the cytoplasm was stained by the monoclonal anti-ICP4 antibody. The accumulation of ICP4 in the cytoplasm increased with time postinfection, reaching a maximal intensity at 12 h postinfection. The intensity of staining of ICP4 in the cytoplasm then declined progressively until cell was lysed. Unlike the cytoplasm, the nucleus showed no obvious ICP4 protein immediately after infection until 4 h postinfection. The translocation and accumulation of ICP4 polypeptide in the nucleus of infected cells increased with time after infection until the cell was lysed by the virus. The nuclear ICP4 had a dense appearance (Table 4; Figure 4). No reaction was found in HSV-2 infected and noninfected cells when they were stained by anti-ICP4 antibody. Also the control mouse ascites fluid did not stain any cells infected with HSV-1 or HSV-2 at any time postinfection, or noninfected cells.

Early protein, ICP8: A 1:800 dilution of monoclonal anti-ICP8 antibody was employed in the study of the reactive pattern in infected cells at different time postinfection. ICP8, the major DNA-binding protein, was not seen at 1 h postinfection. The synthesis of ICP8 in the cytoplasm of infected cells was observed at 4 h postinfection in this study. However, the cytoplasmic ICP8 was not visible at later times postinfection (after 12 h

postinfection). The translocation of the cytoplasmic ICP8 into the nucleus was seen at 8 h postinfection. The nuclear staining of ICP8 reached the maximal intensity at 24 h postinfection. At 48 h postinfection a weaker staining of ICP8 was found in the nucleus and gradually disappeared after that time. At 72 h postinfection the nuclear ICP8 was not demonstrated any more, even in the few surviving cells (Table 4). Unlike 39S ICP8, however, the intensity of nuclear G.S.#1 and G.S.#2 ICP8 still present at 48 h and 72 h postinfection, although a decline of intensity was observed (Table 6). Unlike the dense nuclear staining of ICP4, ICP8 appeared as a speckled staining (Fig. 5). However, in several experiments we found that this DNA-binding protein, unlike other polypeptides except ICP5, demonstrated variable results in infected cells. The variety observed in our experiments occured under the same experimental procedure and conditions, except that the virus employed was from a different passage. All of these viruses come from the same original stock.

Variable results were obtained from three experiments in which the virus passages were the highest (Table 5 & 6). In these three experiments the nuclear ICP8 was difficult to observe but some cytoplasmic staining was seen after 12 h postinfection (Table 6). In two of these three experiments, G.S.#1 and G.S.#2 was also used on cells infected with HSV for 24 hours and they did show the nuclear staining but with a weaker intensity than the staining on cells infected with less passage of virus (Fig. 6). Variable results of ICP5 were also exsisted in two of these three experiments (Table 5 & 6). Other proteins (ICP4, ICP6 and gA/gB) studied in these experiments had the same results as those from other experiments.

Early protein, ICP6: A 1:400 dilution of anti-ICP6 antibody was used to study the reactive characteristics of the early protein, ICP6. An obvious cytoplasmic staining was demonstrated in infected cells. In both HSV-1 and 2 infected cells the positive staining appeared in the cytoplasm at 4 h postinfection and increased its intensity up to 12 h



postinfection. The amount of ICP6 protein seen in infected cells declined between 12 and 24 h postinfection. Another peak of staining was seen at 48 h postinfection. After 48 h postinfection the intensity of ICP6 fell again until the cells were lysed. There was no nuclear ICP6 present in infected cells before 8 h postinfection. Nuclear staining was seen after 8 h postinfection of HSV-1 and 2 infected cell becoming diffuse at 48 h postinfection, like that of gB glycoprotein in infected cell at 24 h postinfection. Like the cytoplasmic staining, the intensity of nuclear ICP6 decreased at 24 h postinfection and then reached a homogeneous and more defined staining at 48 h postinfection (Fig. 7).

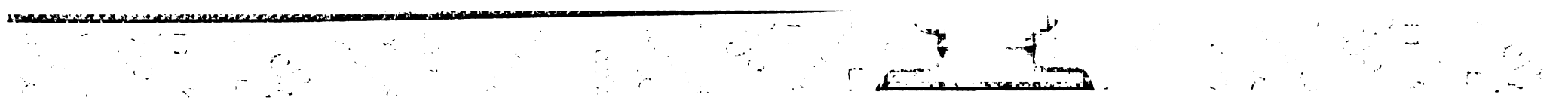
Late protein, gB: Monoclonal anti-gB antibody stained both HSV-1 and 2 infected cells. A 1:8,000 dilution of this antibody was used. The cytoplasmic staining was first seen at 4 h postinfection with a weak appearance. The intensity of the cytoplasmic staining increased and was obvious at 8 h postinfection, when cell membrane staining was also observed. Both the cytoplasmic and the cell membrane gB increased with time and reached the maximal amount at 24 h postinfection. Unlike other proteins, intensity of gB did not change after 24 h postinfection until cells were lysed at 72 h postinfection. A characteristic staining pattern of gB was also observed in which perinuclear staining was present in infected cells, particularly round cells, after infection. The perinuclear gB, like the cytoplasmic gB, also increased gradually postinfection. When the cells began to be lysed, the cell membrane staining became more obvious and the nuclear mophology could not be defined at this time, since the staining is diffused homogeneously in the cytoplasm and the nucleus. In contrast to ICP4 and ICP8, this glycoprotein was not easily observed in the nucleus. A weak, diffuse staining was first seen in the nucleus on infected cells at 12 h postinfection; and when the glycoprotein is accumulated in the cytoplasm later in the infective cycle, the diffuse staining spread out of the nucleus reaching a very homogeneous pattern when the cell was in the lytic stage (Table 4; Fig. 8).

Late protein, ICP5: Anti-ICP5 antibody against the major capsid protein ICP5 was used at a dilution of 1:200. This antibody cross-reacted with both HSV-1 and 2 infected cells. Both cytoplasmic and nuclear staining were present in HSV-infected cells with a predominantly nuclear staining at the late stage of infection. The cytoplasmic ICP5 first seen in infected cells at 4 h postinfection with a very weak staining. The intensity of the cytoplasmic staining gradually increased with time, becoming the strongest 12 h postinfection. The translocated ICP5 in the nucleus was present at 8 h postinfection; it accumulated gradually and reached its maximum at 24 h postinfection. Like ICP8 in the nucleus of infected cells, ICP5 showed the speckled staining in the nucleus of infected cells. At 24 h postinfection the cytoplasmic staining of ICP5 was weaker than that at 12 h postinfection. The decline in cytoplasmic ICP5 continued until the cells lysed. The nuclear ICP5 declined after 24 h postinfection in our study and smaller amounts of ICP5 were observed in the nucleus of infected cells at 48 h and 72 h postinfection and had a spot-like appearance (Table 4; Fig. 9). However, variant results were also obtaied in several experiments. Like ICP8, two experiments showed the intense cytoplasmic staining, but with only weak nuclear staining even though at 24 h after infection or later (Table 6).

HSV-1 proteins in tissues

All five monoclonal antibodies were used to look for HSV-1 antigens in human oral carcinoma and other tissues. All antibodies used in this study were serially diluted starting at 1:10. The same dilutions of control mouse ascites fluid were used as a control.

ICP8 and gB polypeptides in tissue sections: One specimen was reactive in epithelium



with anti-gB antibody, while no reaction was found with control ascites fluid. One case of leukoplakia (R82-162) was the only specimen to show positive staining in epithelium instead of in the connective tissue. This specimen was negative for both antibodies and control ascites fluid in the connective tissue and negative for anti-ICP8 antibody and control ascites fluid in epithelium. Anti-gB antibody had reacted with the the epithelium with cytoplasmic staining. The positive epithelial cells were scattered along the basement membrane, but not in those in other parts of the epithelium (Figure 10).

Some reactions appeared in the connective tissues of parts of specimens, both with control ascites fluid and monoclonal reagents, but at higher dilutions with the antibodies. The reactive stainings were present within the cytoplasm of cells with the appearance of mononuclear cells which were located in either the deep connective tissue or the tissue near the basement membrane or both (Fig. 11). A conservative definition of positive reaction was chosen for this study. A 4- fold higher dilution of antibody than control ascites fluid was said to be a positive result. An alternative method of defining positive and negative results was as follows: specimens stained by control ascites fluid greater than or equal to a dilution of 1:160 will be called nonspecific results for antibodies; specimens with dilution of antibody higher than 1:160 have a positive result. Both definitions result in a similar percentage of positive results, except atypia.

The following data is based on the first definition. Five of eleven (45%) of carcinoma specimens were positive for both ICP8 and gB in the connective tissue (Table 7). Three of six (50%) and one of six (17%) of atypia were positive for ICP8 and gB, respectively (Table 8). In the atypia group, there were four PVL in which three (75%) and one (25%) were positive with ICP8 and gB, respectively (Table 8). Three in ten (33%) and two of seven (29%) clinical leukoplakias were positive for ICP8 and gB, respectively (Table 9) (Fig. 12). For negative control, 5 specimens of gingiva did not react with antibodies, but one reacted with gB at 1:40 dilution. However, this gingiva specimen reacted with anti-gB antibody was also defined as negative when compared with the

reaction to ascites fluid.

The specimens of carcinoma positive for gB and ICP8 antigens were the same, although the intensity and dilution of positive results were different between these two antibodies (Table 7). The only one positive atypia or PVL specimen with anti-gB antibody was also reacted with anti-ICP8 antibody. However, a differently reactive pattern was present in leukoplakia specimens. Specimen R82-007 had a strong reaction for gB antigen, but was negative for ICP8 antigen. Three specimens of leukoplakia, R82-003, R82-073 and R82-008 lack enough tissue for the study of gB antigens. Two of these three specimens were ICP8 positive and the other was negative. Specimen R82-075 was the only one showing a positive result in the connective tissue for both of ICP8 and gB antigens (Table 9).

When the number of positive reaching by both definitions are compared, one different positive percentage is found in the atypia group. As defined by the first method, three of six and one of six are positive for ICP8 and gB antigens, respectively. However, by the second method, 2 of six have the reaction for both ICP8 and gB and the same two specimens are positive for both of these antigens.

ICP4, ICP5 and ICP6 proteins in tissue sections: Although the study of ICP4, ICP5 and ICP6 on the tissue sections was not complete due to the insufficient amount of each specimen, there was no positive reaction found in both the epithelium and the connective for these three antibodies in any specimen. The specimens with complete data included 5 gingiva for all three proteins; 2 leukoplakia, 4 atypia and 4 carcinoma for ICP4; 2 atypias and 5 carcinoma for ICP5; and 2 atypia and 4 carcinoma for ICP6. Other tissues with reaction for each protein were only at the lower dilution (1:40) and were much weaker than the reaction obtained from anti-ICP8 and gB antibodies (Table 7, 8 & 9).

Mast cells as the reactive components in the connective tissue:

Instead of the epithelium, we unexpectly found positive results in the connective tissue of studied specimens of carcinoma, atypia, PVL and leukoplakia without atypia, except for the R82-162 specimen which is a leukoplakia without atypia. In an attempt to recognize the reactive components in the connective tissue, we compared the results from the study of lymphocyte subpopulation by Dr. C. Migliorati in the same specimens with reactive staining in the connective tissue using other monoclonal antibodies, since our positive finding in the connective tissue had the appearance of mononuclear cells. Monoclonal antibodies OKT4 (helper T cells), OKT8 (suppressor T cells), OKT11 (T cells), OKT6 (Langerhans cells), OKM1 (macrophages), OKT7 (NK cells) and B1 (B cells) in the other study did not react with cells like those in this study. It seemed possible that they might be mast cells based on their size, morphology and their granular cytoplasm.

Toludine blue (0.5%) in distilled water was employed to demonstrate the morphology and distribution of mast cells (Fig. 13). Mast cells with reddish granular staining in the cytoplasm were easily recognized in the tissue sections. Serial sections from three specimens were employed for the reaction with toluidine blue, monoclonal anti-ICP8, and anti-gB antibodies. The result showed that the mast cells and the positive cells for either ICP8 antibody or gB antibody or both had the same distribution and location. Furthermore, we applied double staining for monoclonal antibody and mast cells on three specimens in the same sections as described in Materials and Methods. These results were photographed twice, one after toluidine blue staining and once after ABC reaction (Fig 14). The cells reacting positively with monoclonal antibodies were found to be mast cells by using both methods.

Both serial sections and double staining demonstrated that the cells which reacted with antibodies were mast cells. It is difficult to determine whether all mast cells react with antibody in positive specimens, since staining was weak, even when lower dilutions of antibody were used. However, not all mast cells were positively reactive with

antibodies (data not shown). The number of mast cells is also difficult to count due to the variation of size and shape of specimens and the amount of the connective tissue with or without invading tumor cells. Varying numbers of mast cells were observed among all specimens (data not shown). Furthermore, specimens with negative results were not due to the lack of mast cell in the tissue, even though in cases that the lowest dilutions (1:10 or 1:40) of antibodies do not show any positive reaction. The most obvious examples are the negative control gingiva in which many mast cells were present in the connective tissue but were non-reactive with antibodies.

## DISCUSSION

This study demonstrates that the ABC staining technique, using monoclonal antibodies, is a reliable method to identify specific proteins of HSV in infected cells, and to specify the type of virus. The specificity of the monoclonal antibodies used was consistent with that obtained from RIP-PAGE and IF (Showalter et al. 1981). Comparing the results from ABC and IF (Table 2 & 3), the dilution of each antibody that is able to recognize specific viral proteins for ABC was much higher than that for IF. At a very early stage 1 h postinfection, the optimal dilution of antibody for ICP4 was also able to detect the existence of viral proteins in infected cells (Table 4). This shows that the combination of ABC and monoclonal antibody provides a sensitive and useful method to detect viral proteins located in cultured infected cells. Furthermore, the application of anti-ICP8 and gB antibodies on the biopsy specimens also suggests that this is useful for the detection and localization of viral antigens in human tissues.

The immediate-early protein ICP4 belongs to the α-polypeptides (Honess & Roizman 1974 & 1975; Courtney & Benyesh-Melnick 1974; Pereira et al. 1977; Fenwick & Roizman 1977). This protein was observed in the cytoplasm of infected cell at 1 h postinfection. As shown by the staining intensity, its synthesis increased and accumulated in the cytoplasm up to 8 h postinfection. Between 8 and 12 h postinfection, the level of ICP4 in the cytoplasm remains constant, whereas there is no detectable synthesis of ICP4 in infected cells at 9-10 h postinfection, according to Honess & Roizman (1975). After 12 h postinfection the cytoplasmic ICP4 declined. The translocation of ICP4 from the cytoplasm into the nucleus of infected cells was detected at 4 h postinfection, which is similar to the finding of others that the partially detected nuclear ICP4 translocated from the cytoplasm was present at 3 h postinfection (Pereira et al. 1977).

At least three forms of ICP4 -- a, b and c-- have been recognized based on their

electrophoretic mobility. Infected cell cytoplasm contains only ICP4a, which disappears entirely after its posttranslational modification, whereas ICP4b and ICP4c are the only two forms present in the nucleus and they are the modified products after the translocation of cytoplasmic ICP4a into the nucleus (Pereira et al. 1977; Fenwick & Roizman 1977). The monoclonal antibody against ICP4 employed in this study was able to recognize both the cytoplasmic and nuclear ICP4. This suggests that all three forms of ICP4 share at least one epitope which is recognized by this particular monoclonal antibody.

The first observation of the major DNA-binding protein, ICP8, was in the cytoplasm at 4 h postinfection. The time of maximal rate of synthesis of ICP8 is at 3 to 6 h postinfection (Knipe et al. 1982) or at 5 to 7 h postinfection (Honess & Roizman 1974) and the decrease in rate of synthesis of ICP8 normally occurs after 7 h postinfection (Honess & Roizman 1974). Our observation of the changes of the cytoplasmic ICP8 intensity between 4 and 12 h postinfection is consistent with previous reports. The translocation of ICP8 from the cytoplasm into the nucleus is seen at 8 h postinfection; it gradually increased in amount and reached its maximal level at 24 h postinfection. Previous reports (Knipe et al. 1979; Knipe & Spang 1982; Quinlan & Knipe 1983) also demonstrate that immediately after ICP8 is synthesized it is associated with the cytoplasmic framework; subsequently, it is transferred onto the nuclear framework. Late after ICP8 synthesis, it is translocated and bound to DNA, although its function in DNA replication is not clear. The results obtained from ABC and monoclonal antibody experiments demonstrated similar sequences of its synthesis and location, except that binding to DNA could not be demonstrated by this method. The predominant pattern of ICP8 was the nuclear staining at 24 h postinfection, whereas the cytoplasmic ICP8 was not seen.

Both the a and b forms of ICP8 are present in both the cytoplasm and nucleus of infected cells (Knipe et al. 1982; Knipe & Spang 1982). Although there is no

posttranslocational modification of ICP8, the fact that this monoclonal antibody recognized the antigenic determinant site of ICP8 protein suggests that both forms of ICP8 have the same precursor and that this antigenic determinant is maintained before and after translocation. Anti-ICP8 (39S) antibody failed to react with the nucleus of infected cells in some experiments, but monoclonal antibodies G.S.#1 and G.S.#2 against ICP8 had reactivity to infected cells in the same experiments. The staining of ICP8 by G.S.#1 and G.S.#2 was weaker than that seen in experiments with lower passage of virus (experiment G). This suggests that the antigenic determinant recognized by this 39S anti-ICP8 antibody may be changed in some virus stocks after several passages (Frenkel et al. 1975 & 1976; Bronson et al. 1973; Wagner et al. 1974), but the protein is not absent. However, we can not conclude, based on the absence of 39S ICP8 in nucleus of infected cells, that the virus is defective in DNA replication, since the G.S.#1 and G.S.#2 did show nuclear ICP8 but was weaker, and since the nuclear association of this DNA binding protein did not require viral DNA replication (Knipe & Spang 1982) Also, G.S.#1, G.S.#2 and 39S ICP8 recognized different antigenic determinant, since the observation of decrease and absence of 39S ICP8 in the nuclei of infected cells at 48 h and 72 h postinfection was not seen for G.S.#1 and G.S.#2 ICP8. At 48 h and 72 h postinfection G.S.#1 and G.S.#2 ICP8 still have very intense staining in the nuclei of infected cells, although 39S ICP8 decrease and was absent at those time, respectively.

Unlike other proteins recognized by monoclonal antibodies in this study, the disappearance of the cytoplasmic ICP8 suggests that the synthesis of ICP8 ceases between 12 and 24 h postinfection. This may explain the finding of a decline in nuclear ICP8 after 24 h postinfection, since the synthesis of ICP8 in the cytoplasm was completely ceased. Another explanation may be that the maximal amount of ICP8 present in the nucleus at 24 h postinfection reaches the limit of its requirement for viral DNA replication in individual host cell to produce the genome for virus transcription, and no further mRNA for ICP8 is transferred into the cytoplasm for further synthesis. This is

based on the suggestion of the association with viral DNA replication, the regulation of viral gene expression and ICP8 (Powell et al. 1981; Conley et al. 1981). However, the intense staining of G.S.#1 and G.S.#2 ICP8 in the nucleui of infected cells at 48 h and 72 h postinfection could not be explained by these ways.

Most of the β-polypeptide ICP6 is present in the cytoplasm (Pereira et al. 1977). Its synthesis was firstly observed at 4 h postinfection in the cytoplasm. Between 4 and 8 h postinfection there was large increase of ICP6 in the cytoplasm, reaching a maximum at 12 h postinfection. A second peak of ICP6 was present at 48 h postinfection. The results we obtained before 12 h postinfection are consistent with previous reports (Honess & Roizman 1974). However, the decrease of ICP6 after 12 h postinfection and its subsequent increase after 24 h postinfection has not been previously reported. ICP6 is found in both the cytoplasm and the nucleus after its maximal synthesis (Pereira et al. 1977). The ICP6 in the nucleus found after 12 h postinfection declined and increased after 24 h postinfection like that found in the cytoplasm. However, the diffuse pattern of nuclear ICP6 found at 48 h postinfection is more difficult to define. The significance of the change of ICP6 pattern observed at different time postinfection is not clear. It may relate to the two forms of ICP6 products in which part of them is modified and is not sensitively detected by this antibody at several time postinfection.

Although glycoproteins gA and gB can be differentiated on the basis of their electrophoretic mobilities in SDS polyacrymide gel (Spear 1976), both react with antisera prepared against each of the glycoproteins (Eberle & Courtney 1980b). Further study also indicates that gA and gB glycoproteins share antigenic determinant sites and at least portions of their primary structure (Eberle & Courtney 1980a & 1980b; Balachandran et al. 1982). Both gA and gB specified genes are mapped within the same region of the HSV genome (Ruyechan et al 1979). Thus, glycoproteins gA and gB either derive from a common precursor or arise from mRNA transcribed at least in part from a common set of DNA sequences (Pereira et al. 1981). The term "gA/gB" is frequently used to represent

both gA and gB glycoproteins (Eberle & Courtney 1980b).

Monoclonal anti-gB antibody used in this study (24S) is type-common for both HSV-1 and 2 as defined by IF and RIP-PAGE (Showalter et al. 1981) and confirmed by ABC staining. The predominant staining of gA/gB is in the cytoplasm and the cell membrane of infected cells (Showalter et al. 1981; Spear 1976; Eberle & Courtney 1980a). Glycoprotein gA/gB is first present in the cytoplasm at 4 h postinfection with a weak staining (Balachandran et al. 1982), but not on the cell membrane. The obvious cytoplasmic gA/gB is found at 8 h postinfection, where cell membrane involvement is also demonstrated (Spear et al. 1970; Norrild et al. 1980). Consistent with a previous report (Honess & Roizman 1974), we found that this $\gamma$ -polypeptide is synthesized gradually after 4 h postinfection and reaches the maximum at 24 h postinfection. The amount of gA/gB on the cell membrane and in the cytoplasm did not decline after its formation and was maintained until cell lysis. The accumulation of perinuclear gA/gB was also found especially in infected round cells (Figure 8). Diffuse nuclear staining of gA/gB was present after 12 h postinfection. Our results concering time sequence of synthesis and location in infected cells are consistent with other reports using antibody-dependent cell mediated cytotoxicity (ADCC) (Norrild et al. 1980) and study of isolated membrane fractions by acrylamide gel electrophoresis and electron microscopy (Spear et al. 1970).

The first sign of reactive staining of ICP5 in this study was present as weak staining in the cytoplasm at 4 h postinfection, and its amount gradually increased as the infective time increased, but declined slightly after 24 h postinfection. The translocation of the cytoplasmic ICP5 into the nucleus began after 4 h postinfection and was observed at 8 h postinfection. The nuclear ICP5 amount also gradually increased; a large increase of nuclear ICP5 happened after 12 h postinfection and reached a maximal intensity at 24 h postinfection. These results are consistent with previous reports as this is a $\gamma$-polypeptide (Honess & Roizman 1973). However, the nuclear amount of ICP5, like

the nuclear ICP8 protein, declined after 24 h postinfection and showed a weaker staining at 48 h postinfection. The decline in nuclear ICP5 continued until the cell was lysed. Unlike ICP8 protein, the cytoplasmic ICP5 is still present after the translocation of ICP5 occured, but with a weaker staining as the time increased to after 24 h postinfection.

Although we sugested that the changes of the DNA-binding protein ICP8 is not due to the defective virus in its DNA replication, several reports demonstrated that the virus (strain KOS and F) with inhibited DNA synthesis after treatment with cytosine arabinoside (Powell et al. 1975) or phosphonoacetate (Knipe & Spang 1982; Conley et al. 1981) and the DNA defective viruses (ts mutant) (Bone & Courtney 1974; Courtney & Benyesh-Melnick 1974; Conley et et. 1981) revealed a decrease in the accumulation of late ($\gamma$) polypeptides, including ICP5. Combining our results of the decrease in the intensity and change of the staining patterns of ICP8 and ICP5, we may suggest that ICP8 may function in DNA replication (Knipe & Spang 1982) and the alteration of its existence or synthesis in infected cells may result in the inhibition of DNA replication at least some components of DNA. The inhibited parts of DNA replication may further affect the synthesis and/or nuclear transport of ICP5 (Knipe & Spang 1982; Conley et al. 1981), since the cytoplasmic 39S ICP8 and ICP5 were present in these experiments in which nuclear 39S ICP8 and ICP5 were absent, and in which G.S.#1 and G.S.#2 ICP8 were weakly present in the nuclei of infected cells.

Such passage induced mutations might involve only a single ICP8 antigenic epitope. This would result in the alteration of cascade regulation and further affect the synthesis of ICP5. Alternatively, different ICP8 antigenic epitopes might exist with a domain recognized by G.S.#1 and G.S.#2 which is less altered in the passage induced mutation. Whether the mutation of an ICP8 antigenic epitope results in defective infectivity or not can not be concluded in this study, although the viral cytopathic effect had been observed in all experiments. Finally, the changes of reactive pattern of ICP8 and ICP5 observed in several experiments with different virus passages may also be

associated with the changes of ICP4 and ICP6 proteins with an increase of their synthesis (Conley et al. 1981; Powell et al. 1975; Courtney & Benyesh-Melnick 1974) in these experiments which had variant results of ICP5 and ICP8. However, the ABC staining method is not able to quantitatively detect these changes.

A fragment of the HSV-1 DNA genome located between 0.30 and 0.45 of the physical map is associated with the HSV-1 morphologically transformed cells (Camacho & Spear 1978; Eizurz et al. 1983). Both ICP8 and gB polypeptides are encoded in this region of the HSV-DNA genome (Ruyechan et al. 1979; Morse et al. 1979; Roizman 1979; Halliburton 1980) (Fig.2). These two polypeptides, indeed, have been shown in and on transformed cells (Lewis et al. 1982), and ICP8 polypeptide is also associated with cervical carcinoma (McDougall et al. 1982). However, there is no documented evidence of the existence of HSV-1 antigens in oral carcinoma, although HSV-1 may be suggested as being involved in the etiology of oral carcinoma based on the immunological investigation of patients with oral carcinoma in relation with HSV-1. This study, using monoclonal antibodies against HSV-1 proteins and ABC, is attempting to approach this question.

An unexpected result in the tissue study was that all but one of the positive staining was present in the connective tissue and not in the epithelium. The positive components in the connective tissue that reacted with either anti-ICP8 or anti-gB antibody or both were recognized as mast cells by studies of serial sections and double staining on the same section from three specimens. The possible significance of the reactive mast cells in oral cancer and pre-cancer specimens will be discussed below. However, one possibility is that the reaction of mast cells with either one or both of antibodies may have been a nonspecific reaction, since these cells contain Fc-receptors of $IgG_a$ on their surface (Halper & Hetzger 1976; Konig & Ishizaka 1976; Moller & Konig

1980). Both anti-ICP8 and anti-gB antibodies belong to $IgG_{2a}$ (Table 2). Although further study using the Fab fragment of antibodies to detect the specificity between mast cells and antibodies has not been done, several observations suggest their specific reaction with antibodies rather than nonspecific binding through Fc receptors. Positive specimens for ICP8 and gB did not react positively with two other $IgG_{2a}$ monoclonal antibodies. Although ICP4, ICP5 and ICP6 stained in the connective tissue of some specimens, they were only present in the lower dilution same as that in ascites fluid. These results of ICP4, ICP5 and ICP6 were negative by the criteria used result. Not all specimens showed positive result. Mast cells were present in sections stained by toluidine blue, even though the control gingiva specimens were entirely negative for both antibodies. Furthermore, the staining components of the positive specimens were present within mast cells with some granular appearance, but not on the cell membrane of mast cells. Thus, mast cells containing HSV antigens may express their specificity and significance.

Although our results were unexpected in that the viral antigens were present in the connective tissue instead of in epithelium, at least one specimen of leukoplakia did show the viral antigen recognized by anti-gB as antibody present in the epithelial cells along the basement membrane rather than in the connective tissue. The only positive specimen (R82-162) showing the presence of viral gA/gB antigen in the epithelium was clinically and histologically diagosed as leukoplakia. This leukoplakia specimen did not show any reactivity to antibodies in the connective tissue, although it does have mast cells present in the studied section. Other specimens with positive results had reactions with mast cells in the connective tissue. By combining the information obtained here with the "hit and run" hypothesis (reviewed by Galloway & McDougall 1983), we may suggest:

1) There is some relationship among oral cancer, precancer and HSV-1.

2) HSV-1 induced oral cancer may began at a very early stage as precancer or

leukoplakia; and the HSV-related precancer results from the introduction of a part of the viral genes which is related to the transforming capability of HSV-1.

3) Viral protein encoded by the genome associated with the transforming capability present in transformed cells at the very early stage disappears when cells divide and move into the upper layers of epithelium, since only one precancer specimens was detected to have gA/gB antigen in the basal cell layer but not in the other cell layers, and no cancer specimens had viral antigen.

4) Part of the viral information is still functioning and present in cells but is undetectable by current technique and material.

5) The viral antigens released from transformed epithelial cells into the connective tissue is picked up or endocytosed by mast cells.

6) Viral antigens stimulate the mast cell and cause the secretion of mediators that are able to destroy several components of the basement membrane thereby enhancing the invasion and/or metastasis of the induced tumor or transformed cells or act directly on epithelial cells and cause malignant behavior.

Despite the negative result of HSV antigens in epithelium, mast cells reaction with HSV-antibodies may provide a basis for understanding a relationship between HSV-1, oral cancer, precancer and PVL. Forty-five percent of the carcinoma specimens , 75% of the PVL, 50% of the atypia, 30% of the leukoplakia and none of the control gingiva stained with antibody to ICP8. Forty-five percent of the carcinoma, 25% of the PVL, 16% of the atpia, 29% of the leukoplakia and none of control gingiva stained for gB. These data demonstrate the association between precancer, cancer and HSV-1. The risk of malignant transformation from clinical hyperkeratosis and verrucous hyperkeratosis is high (Silverman et al. 1983). In their study, forty-five of two hundred and fifty-seven (17%) patients with oral leukoplakia subsequently developed squamous carcinoma in the hyperkeratotic epithelial site and 8 of 45 (18%) patients with malignant transformation

originally had epithelial dysplasia. The role of HSV-1 in leukoplakia, atypia and PVL may result in further transformation and may explain the high risk for malignant transformation in these three groups of lesions. Seventy-five percent of the atypia had the ICP8 protein in the connective tissue. Possibly HSV-1 may be involved in the etiology of the malignant transformation of PVL (Silverman et al. 1983).

Basic on the "hit and run" model (Skinner 1976; Hampar et al. 1976; zur Hausen 1980; Schlehofer & zur Hausen 1982; Galloway & McDougall 1983), the expression of the HSV-1 transforming genome and its coded protein may occur relatively rapidly after cells are transformed. Viral antigens in precancer or cancer must be detected at the right moment, when they are present. If testing for these viral antigens in the tissue in situ is delayed, false negative findings in epithelium can occur. Thus, the time of the examination for HSV-1 antigens in the biopsied specimens may be crucial. This may explain why only one specimen of precancer showed positive HSV gB antigen in the epithelial cells.

The elevated serum antibody titer to HSV-1 in patients with untreated oral cancer (Shillitoe et al. 1982), especially IgM antibody (Shillitoe et al. 1983b), suggests that HSV-1 antigens continuously stimulate antibody production. At least three different antigens of HSV-1 are associated with the elevated serum antibody of patients with oral cancer and acute herpetic infection (Shillitoe et al. 1983b). Two of the three HSV-1 antigens recognized the IgM antibody are late proteins. Both ICP8 and gB proteins are assumed to be related with these recognized proteins, since both of their encoded genomes possess transforming capability (Figure 1 & 2), and since the maximal levels of these proteins are detected in infected cells at the same time postinfection at which these antigens are recognized by serum IgM antibody (Shillitoe et al. 1983b). The presence of both ICP8 and gB antigens in mast cells in some patients with oral cancer, precancer and PVL, but not in the negative control gingiva, suggests this may be the source of viral antigens which continuously re-stimulate the antibody formation. However, the unexpected finding of

viral antigens expressed on mast cells may suggest the stimulation is originating from the connective tissue rather than from the malignant cells. It is possible that viral antigens other than ICP8 and gB, such as gD (Reed et al. 1975), P7 and P8 (Gupta & Rapp 1977), are also responsible for the changes in humoral immune response. Humoral immunity may also be stimulated by transformed epithelial cells which express ICP8 and gB during and after the transition from the epithelial area into the connective tissue. However, further detailed studies are necessary to provide the exact model.

HLA-DR positive cells, Langerhans cells, are able to present antigens and induce proliferative T cell responses to HSV-Ag (Braathen et al. 1980). Some studies (Streilein et al. 1982; Hoefsmit et al. 1982) indicate the possible movement of Langerhans cells between epithelium and the connective tissue. Thus Langerhans cells may take up and present HSV-1 antigens be released from transformed cells during growth. This mechanism may explain the presence of ICP8 and gB antigens in the connective tissue and in mast cells.

Elevated serum IgE immunoglobulin has been reported in patients with oral cancers (Scully et al. 1982). Specific anti-HSV-1 and 2 IgE antibodies were also reported in animal model after challenge by virus (Day et al. 1976; Ida et al. 1983). Conceivably presentation of HSV-1 antigens in patients with oral cancer and precancers <u>in situ</u> may result in the production of specific IgE antibody against these viral antigens (Bahna et al. 1978). Further interaction between anti-HSV IgE and mast cells in localized area where the HSV-1 antigens are present in cells may result in the enhancement of the release of mediators (Ida et al. 1983), especially protease which is able to degrade the type IV collagen in the basement membrane. This model may be significance in our finding of the existance of HSV-1 antigens in mast cells of oral cancer and precancer specimens and may give a possible route of the tumor invasion.

HSV-1 antigen presenting cells from epithelium migrate into the connective tissue and stimulate plasma cells to induce of antibody formation, including IgE anti-HSV

antibody (Day et al. 1976). The binding of IgE antibody with mast cells further stimulates the release of mediators from mast cells through the activation by HSV-1 antigens. The activated action of HSV-1 antigens bound on IgE antibody may be the result of the expression of antigens on mast cells found in this study.

Although the meaning of mast cells presenting the HSV-1 antigens is not clear, mast cells do contain many chemical mediators and enzymes which are important in the host defensive mechanisms. It is not known whether HSV-1 antigens present in mast cells stimulate them to secrete the chemical mediators or enzymes or not. The assumption of the stimulation response of mast cells to HSV-1 antigens may suggest that enzymes secreted from these cells are significant, especially protease in its relationship to tumor invasion. Sage et al. (1979) demonstrated the specific function of mast cell protease in the degradation of type IV collagen, which is the major component of the basement membrane. A higher level of mast cell protease has been found in parasite-stimulated mast cells (Woobury & Miller 1982). The distribution of mast cells (Carranza & Cabrini 1955) (Fig. 13) and protease (Seppa 1978) found in the connective tissue just next to the basement membrane may play a significant role in breaking down this particular protein, if these HSV-1 antigens are able to stimulate mast cells.

The failure to demonstrate HSV-1 genome coded products in oral malignant cells may suggest the following model of the unrestricted growth of oral cancer. The insertion of the HSV-1 genome into nonpermissive cells results in the induction of cellular DNA synthesis in which an adaptive mechanism is acquired to overcome the limitation of cellular DNA synthesis. However, either based on the "hit and run" mechanism or the genome being defective in late functions (zur Hausen 1980), the initiation of cellular DNA synthesis continues without the presentation of inserted genomes and result in unrestricted growth of affected cells. The rapid disappearance of effectors (HSV-1 genomes) fail to represent their products on affected cells, which will then escape the host defense mechanisms developed at the early stages in response to the expression of

effectors. The integration of HSV-1 DNA into the host cell genome or the modification of HSV-1 DNA may be the function permitting escape from host defense system (zur Hausen 1980). Although humoral immune responses to HSV-1 exist in patients with oral cancer, the loss of a target on tumor cells results in the failure of their protective function.

Higher titers of anti-HSV-1 antibody are present in patient with later stages (III-IV) of oral cancer than in patients with tumors in earlier stages (Shillitoe et al. 1982). The presentation of HSV-1 antigens in the connective tissue rather than in tumor cells may support the model described above, although it is not so easy to explain the change of the humoral immune response. The higher antibody response to HSV-1 is found in patients with late stage tumors who have worse survival rates.

If this model is followed by HSV-1 in the induction of tumor formation, we must face the dangerous fact that the immune response will not be able to protect the host against the HSV-1 induced tumor. The early diagnosis, detection and treatment of precancerous lesions caused by HSV-1 is very important in clinical work.

# CONCLUSIONS

Recently developed monoclonal antibodies and ABC technique were used in this study to demonstrate different viral proteins in cells infected with HSV-1 at different time periods and to look for the existence of viral proteins in biopsied tissues of oral cancer and precancer. The combination of monoclonal antibodies and the ABC had been proven to be a useful and reliable method in the detection of viral proteins in infected cells after fixation with acetone and freezing at -70°C. Five different HSV-1 proteins were studied. The immediate-early protein ICP 4 was detected at a very early stage in the cytoplasm and later in both the cytoplasm and nucleus of infected cells with a predominant nuclear staining. This protein existed in infected cells during the infectious cycle of HSV-1 until cells were lysed. The major DNA-binding protein ICP8 was seen as nuclear staining predominantly in infected cells at a late stage. However, at an early stage after infection the cytoplasmic staining of ICP8 was also detectable and the translocated nuclear ICP8 was seen at 8 h postinfection. Later after infection (after 24 h) the intensity of nuclear ICP8 declined until the cells were lysed. Also, more than one antigenic determinant of ICP8 was suggested. The early protein ICP6 was also a DNA-binding protein. Its predominant staining was present in the cytoplasm of infected cells during the reproductive cycle, although a diffuse nuclear staining was observed at the late stage (24 h postinfection) of infection. The late protein gB is a major virion glycoprotein. It was present in the cytoplasm of infected cells and with a particular cell membrane staining after its synthesis. The late protein ICP5 is a major capsid protein. Its existence was observed in the cytoplasm of infected cells first and then the translocated nuclear ICP5 was the predominant staining in infected cells. However, like ICP8, inconsistent results were observed for ICP5 in several experiments and suggested the possibility of the change of the structure of ICP8 of HSV-1 after several passages.

The study of HSV-1 proteins in tissue sections indicated the association between oral cancer, precancer and HSV-1. Five of eleven (45%) of the carcinoma specimens, three of six (50%) of atypia, three of four (75%) of PVL and three of ten (30%) of leukoplakia without atypia were positive in the connective tissue for ICP8. Five of eleven (50%) of the carcinoma specimens, one of six (17%) of atypia, one of four (25%) of PVL and two of seven (29%) of leukoplakia without atypia were positive for gA/gB. Both ICP 8 and gA/gB are encoded in the DNA genome between 0.30 and 0.45 map units which is associated with the oncogenicity of HSV-1. Also, both proteins have been demonstrated in the HSV-transformed cells and cervical carcinoma in other studies. None of five control gingiva tissues was positive with these two proteins. No specimen was positive for other proteins ICP4, ICP5 and ICP6. The higher percentage of HSV-1 protein present in atypia group may explain the higher risk of malignant transformation of patients with atypia, especially PVL.

Both serial sections and double staining on the same section with toluidine blue and the ABC indicated that the positively stained cells were mast cells. The results suggested that the presence of HSV-1 proteins in the connective tissue may continuously re-stimulate the immune system and result in the elevation of immune responses in patients with oral cancer and precancer.

Only one specimen of leukoplakia without atypia showed the presence of gA/gB protein in the basal layer of epithelial cells but not in the connective tissue also suggested the association among the oncogenicity of HSV-1, oral malignancy and the "hit and run" mechanism which was previously described for HSV.

The possible model of HSV-1 which functions as an initiator or an effector in the induction of tumor formation was discussed, and the significance of HSV-1 proteins in mast cells in malignant change and metastasis was also considered.

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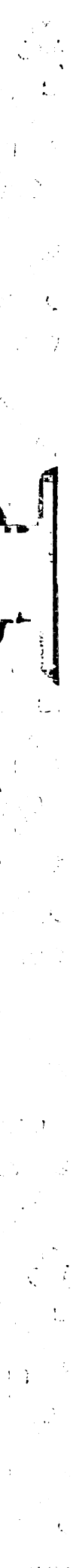

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## FIGURE LEGENDS

Figure 1. HSV-DNA fragment associated with transforming potential.

Figure 2. HSV-1 proteins with transforming potential coded in Xba I-F (0.30-0.45) and Eco RI-F (0.32-0.42) fragments.

Figure 3. Avidin-Biotin-peroxidase complex technique.

Figure 4. Immediate early protein ICP4 in HEp-2 cells infected with HSV-1 at different time periods. (a) At 8 h postinfection, high intensity of cytoplasmic and nuclear translocated ICP4 is observed. (b) The predominant nuclear ICP4 is seen at 24 h postinfection as a dense appearance. Unlike ICP8, the visible cytoplasmic ICP4 is still present. (c) Strong intensity of staining of ICP4 is still present at 48 h postinfection.

Figure 5. ICP8 in HEp-2 cells infected with HSV-1 at different time periods. (a) The translocation of cytoplasmic ICP8 into nucleus is seen at 8 h after infcetion. (b) The predominant staining of ICP8 at 24h after infection is a speckled nuclear stain; (c) The intensity of nuclear ICP8 declines at 48 h postinfection. The cytoplasmic ICP8 is not easily seen after 24 h infection.

Figure 6. The change of intensity of staining and of the reactive pattern of ICP8 on cells infected with different passage of the viruses. (a) Very weak staining of 39S ICP8 on cells infected with higher passage of HSV-1 at 24 h. (b) and (c) The detectable G.S.#2 ICP8 on cells infected with higher passage (b) and lower passages (c) of HSV-1 at 24 h. A weaker nuclear staining is seen on (b) than (c).

Figure 7. Early protein ICP6 in HEp-2 cells infected with HSV-1 at different time periods. (a) Obvious cytoplasmic ICP6 is seen at 8 h after infection; Stronger cytoplasmic ICP8 is present at 12 h (b) than at 24 h (c) after infection; (d) Increased cytoplasmic ICP6 at 48 h after infection which is associated with a diffuse staining in nucleus.

Figure 8. Late protein gA/gB in HEp-2 cells infected with HSV-1 at different time periods. (a) Cytoplasmic and cell membrane staining is present at 12 h after infection and is associated with a low intensity of diffuse nuclear staing; stronger cell membrane, cytoplasmic and perinuclear staining is seen at 24 h (b) and 48 h (c) postinfection and with a diffuse nuclear staining.

Figure 9. Late protein ICP5 in HEp-2 cells infected with HSV-1 at different time periods. (a) Cytoplasmic and translocated nuclear ICP5 is observed at 12 h postinfection. (b) The predominant nuclear ICP5 observed at 24 h after infcetion is a speckled staining. (c) Weaker staining of ICP5 is present at 48 h after infection with a spot-like appearance.

Figure 10. Tissue number R82-162 (leukoplakia without atypia) shows the staining in the epithelial cells scattered along the basement membrane. (a) low magnification (100x) and (b) higher magnification (250x).

Figure 11. ICP8 and gB proteins stained in tissue sections. (a) Stained cells for ICP8 in the connective tissue invaded by the malignant epithelium. The arrows indicate that the cells are large in size and stained in the cytoplasm of cells. (b) Positive cells in the deep connective tissue of leukoplakia and (c) cells near the basement membrane in the tissue of leukoplakia.

Figure 12. The results of ICP8 and gB proteins in the tissue sections are present in four different groups.

Figure 13. Cells with reddish stain from toluidine blue in tissue (metachromatic staining) are recognized as mast cells. (a) low magnification (100x) and (b) higher magnification (250X).

Figure 14. Double staining of section of toluidine blue and ABC. (a) Tissue stained by toludine blue first and (b) Stain by the ABC for ICP8 protein. Arrows indicated that same cells are recognized as mast cells by toluidine blue and later stained with anti-ICP8 antibody.

Figure 1, HSV-DNA fragment associated with transforming potential

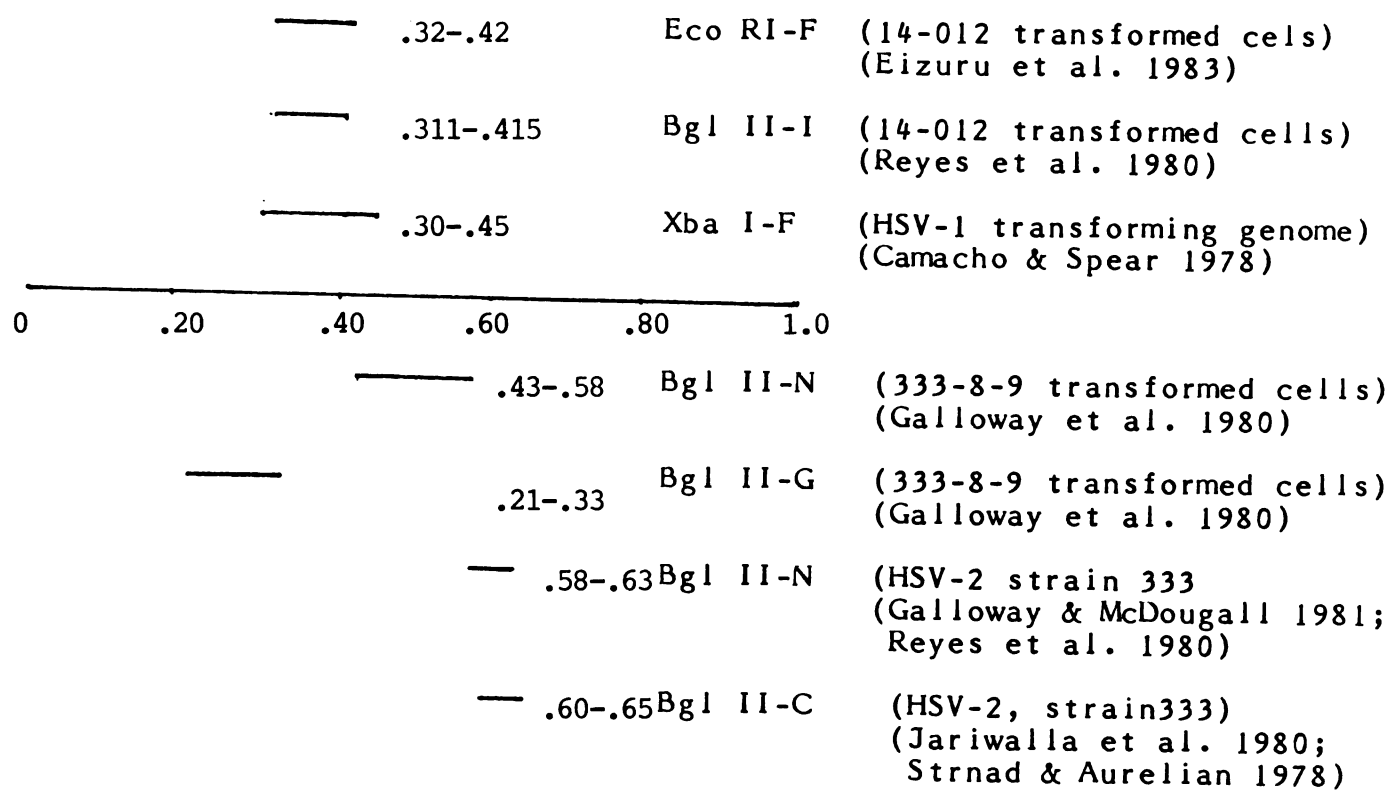
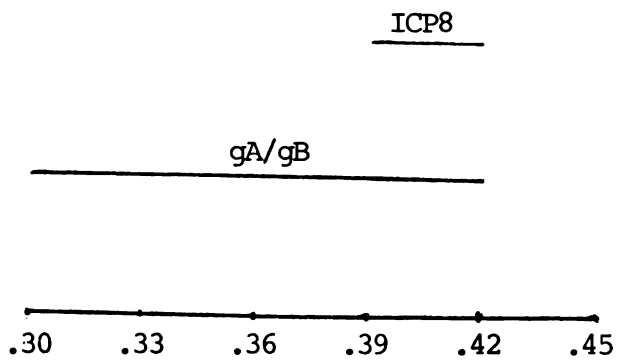


Figure 2, HSV-1 proteins with transforming potential coded in Xba I-F (0.30-0.45) and Eco RI-F (0.32-0.42) fragments




From: Roizman 1979; Halliburton 1980;
Ruyechan et al. 1979 & Morse et al. 1979.

Figure 3, AVIDIN-BIOTIN-PEROXIDASE COMPLEX TECHNIQUE

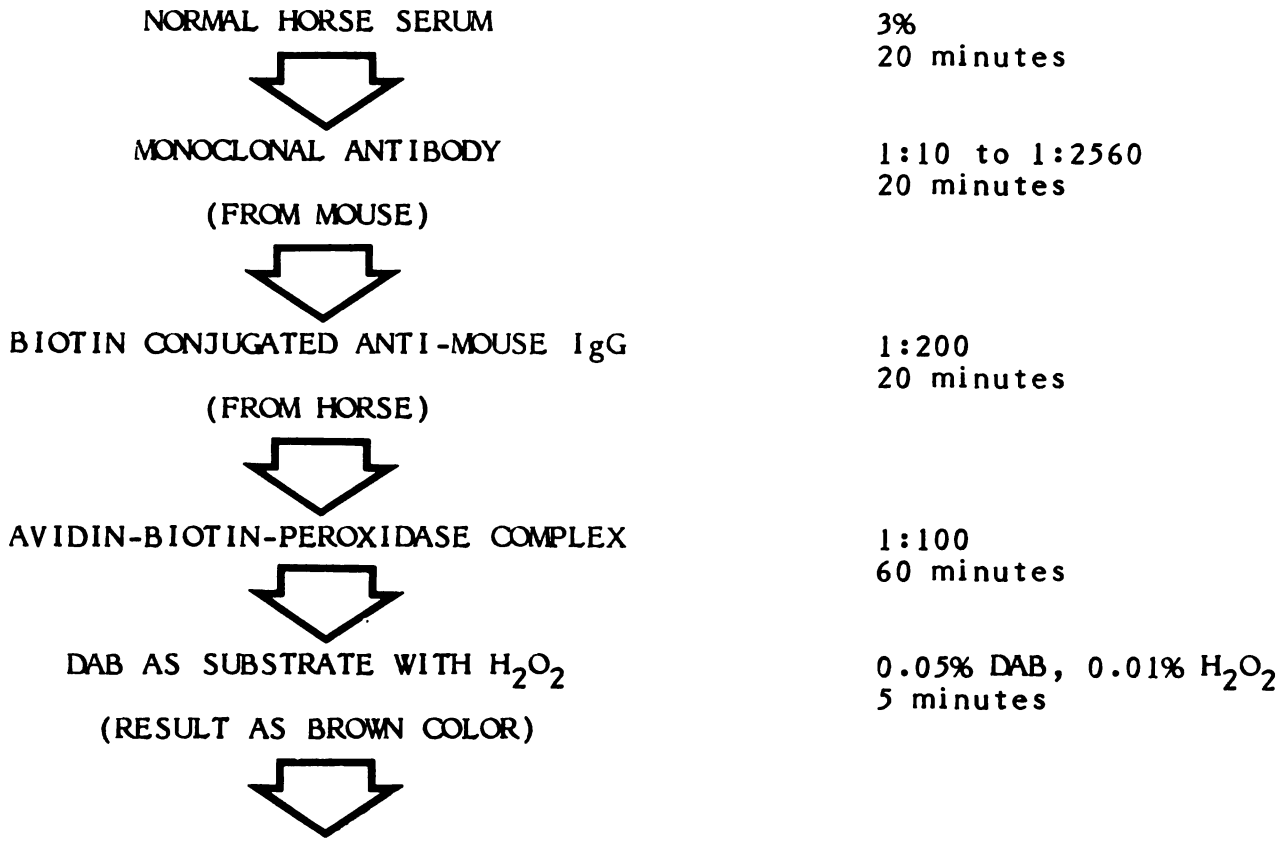
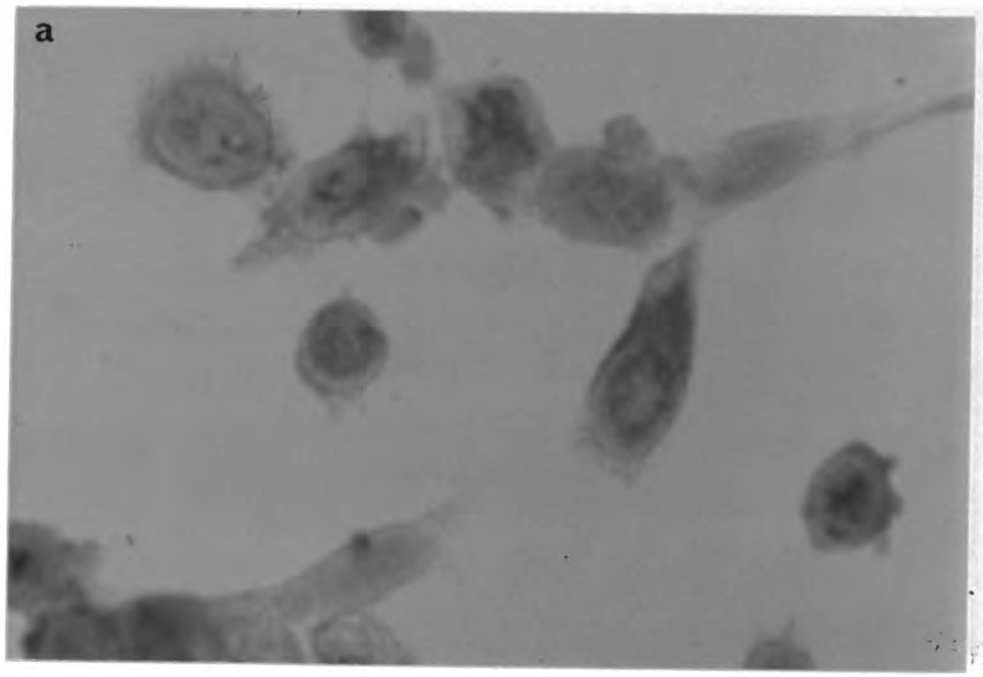
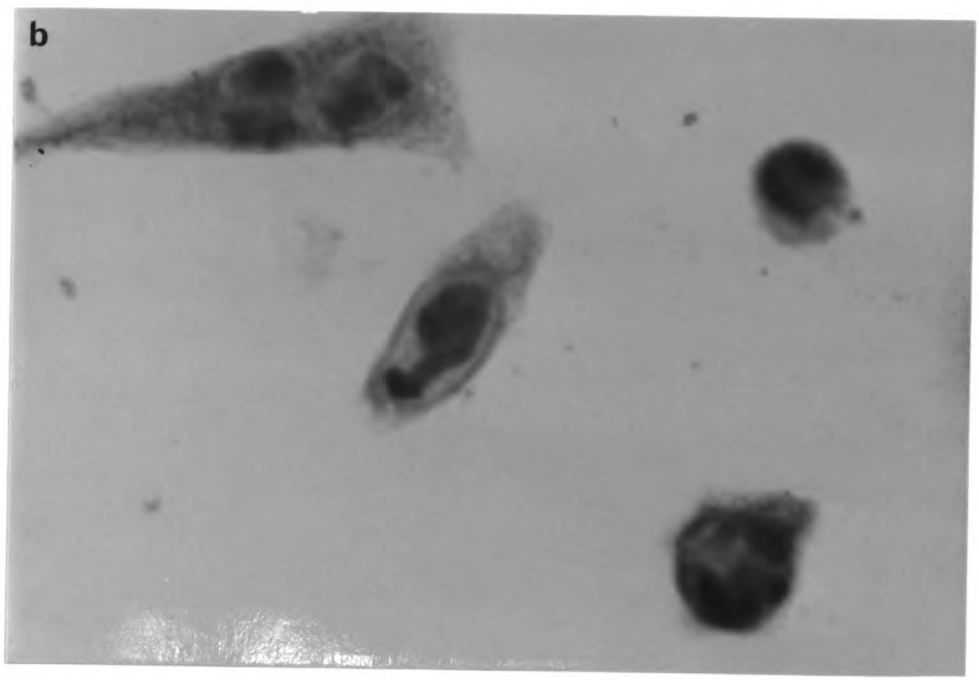


Figure 4



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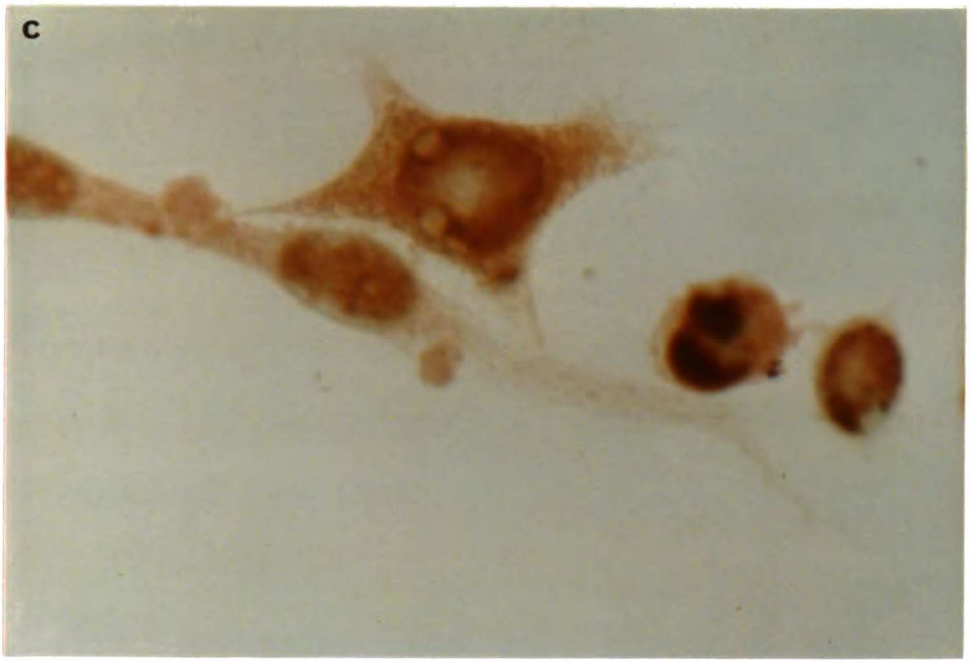

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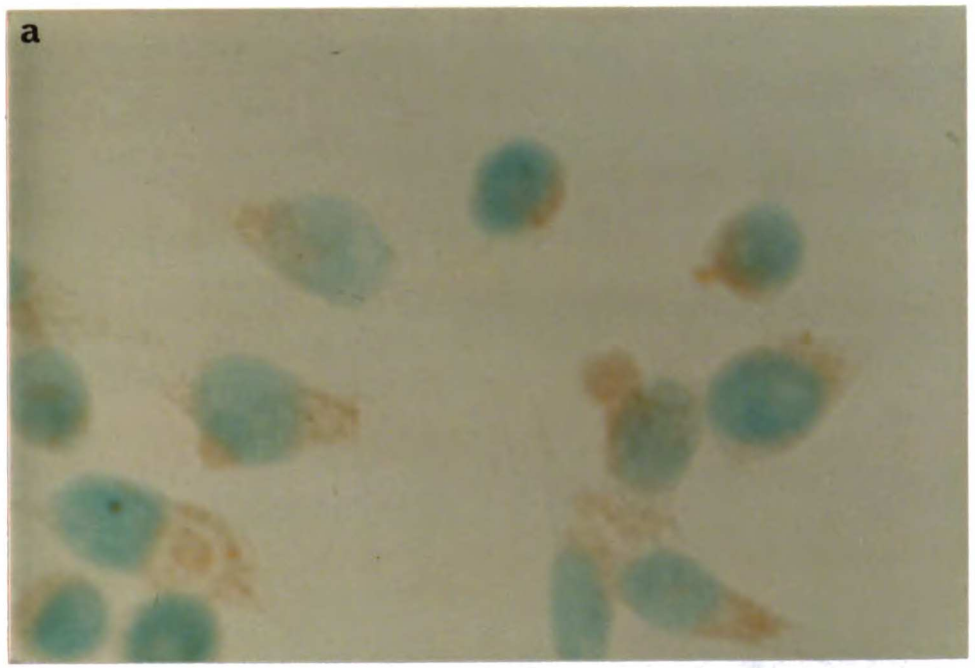


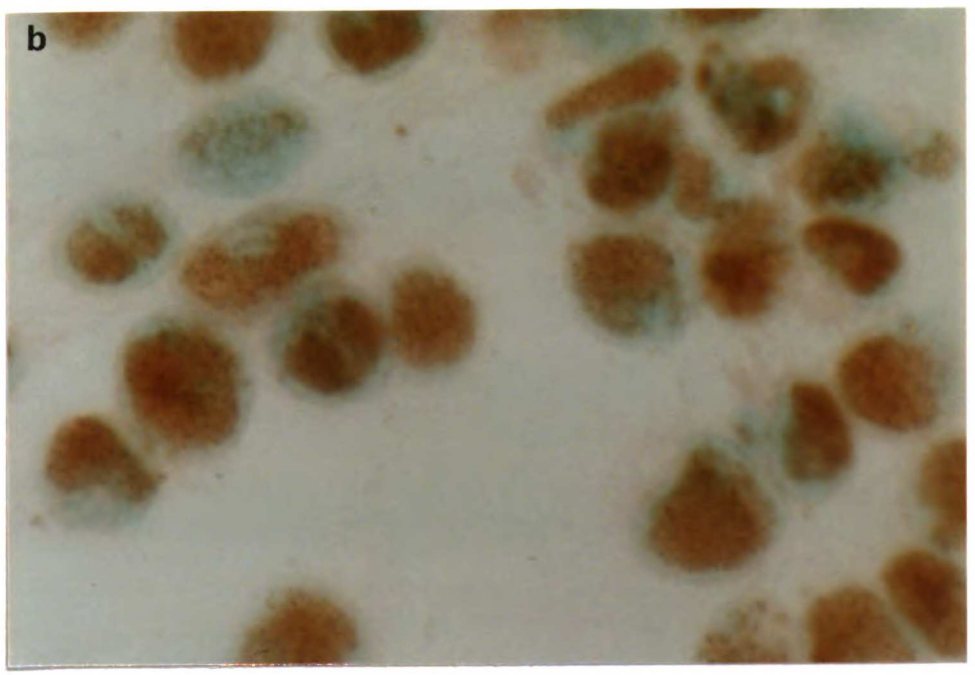

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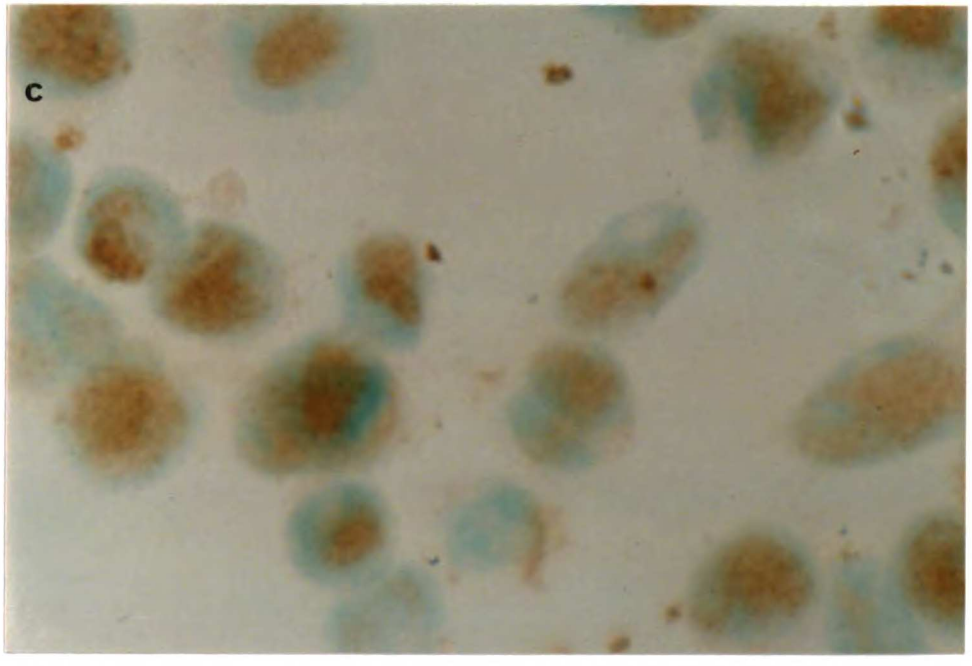

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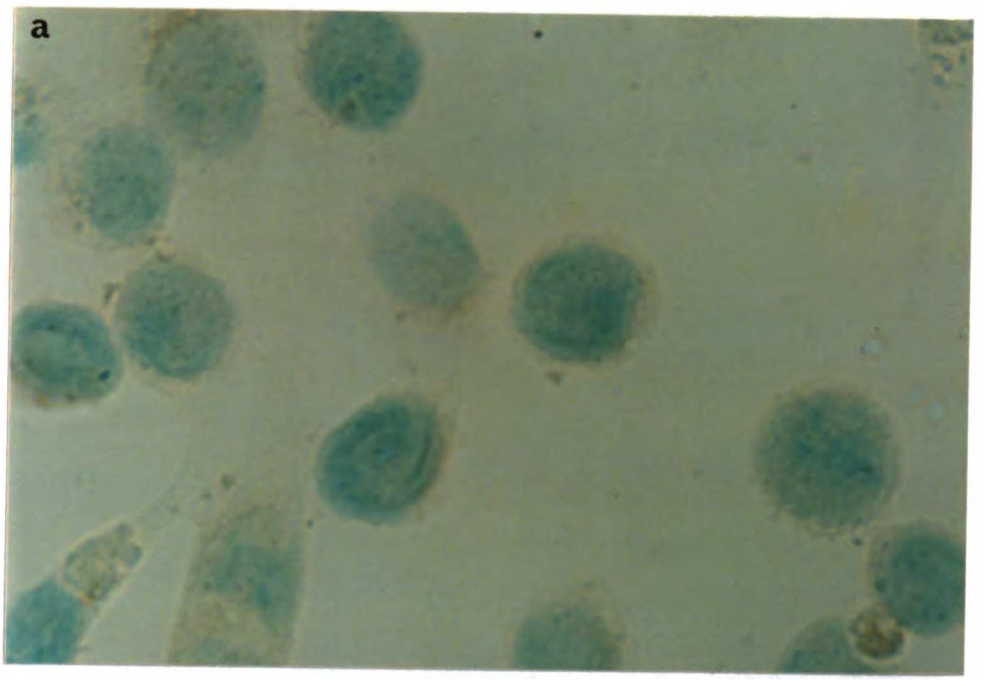


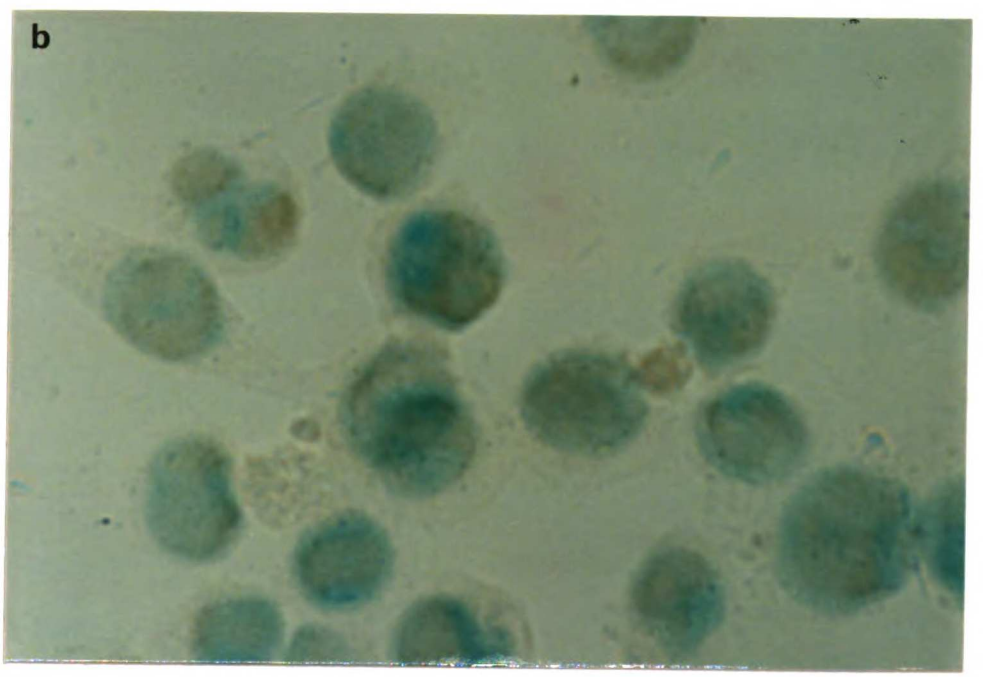

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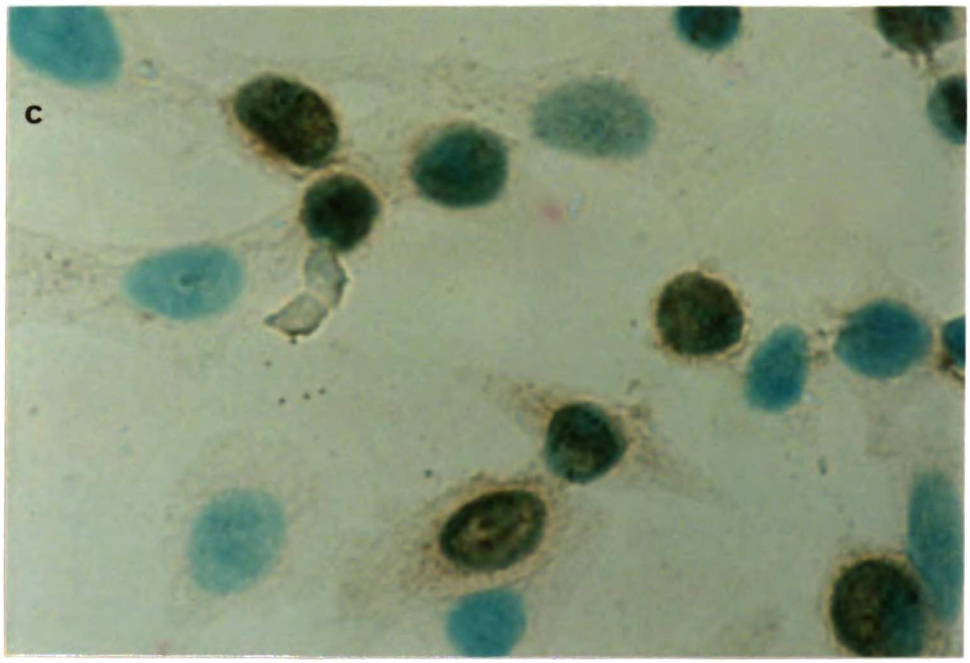

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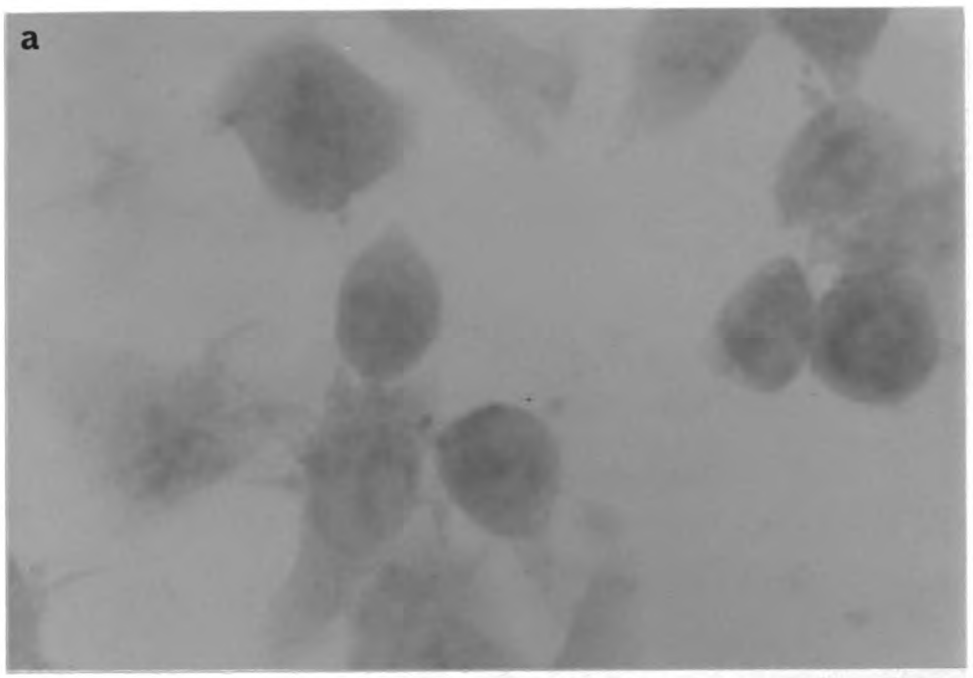


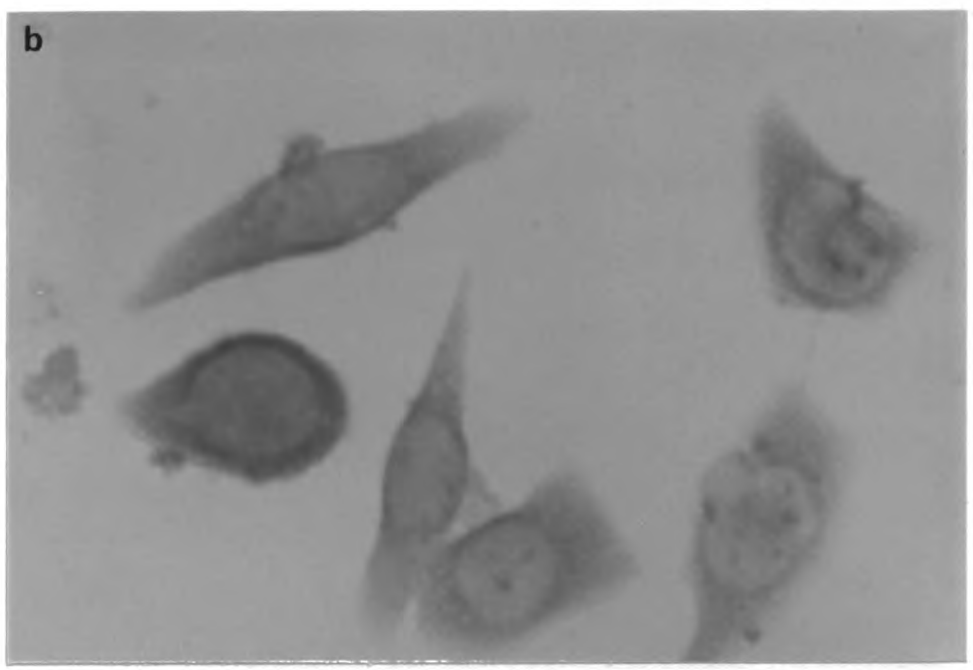

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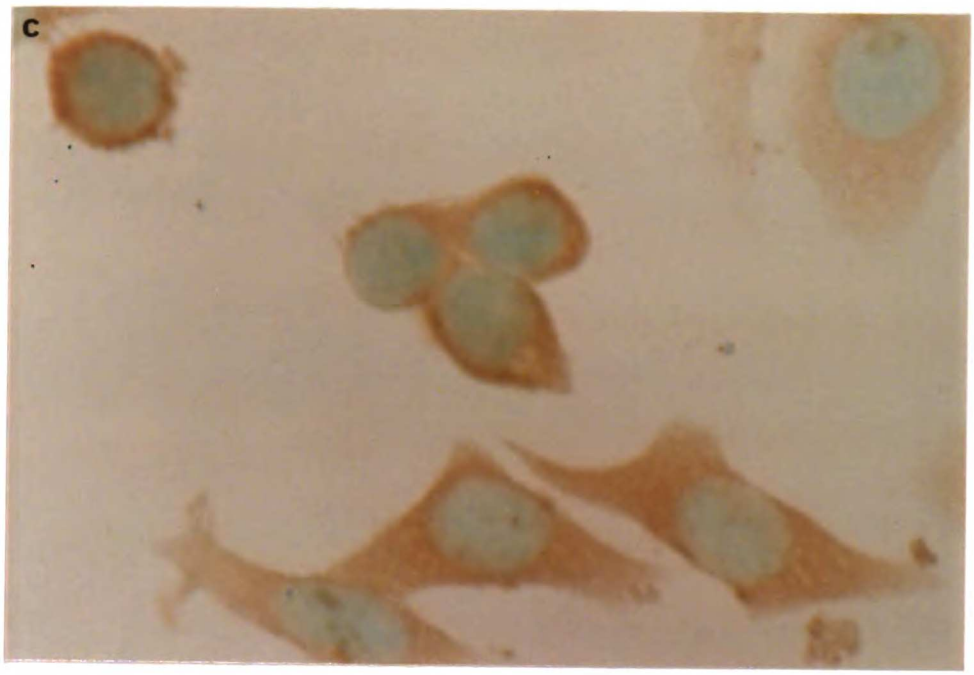


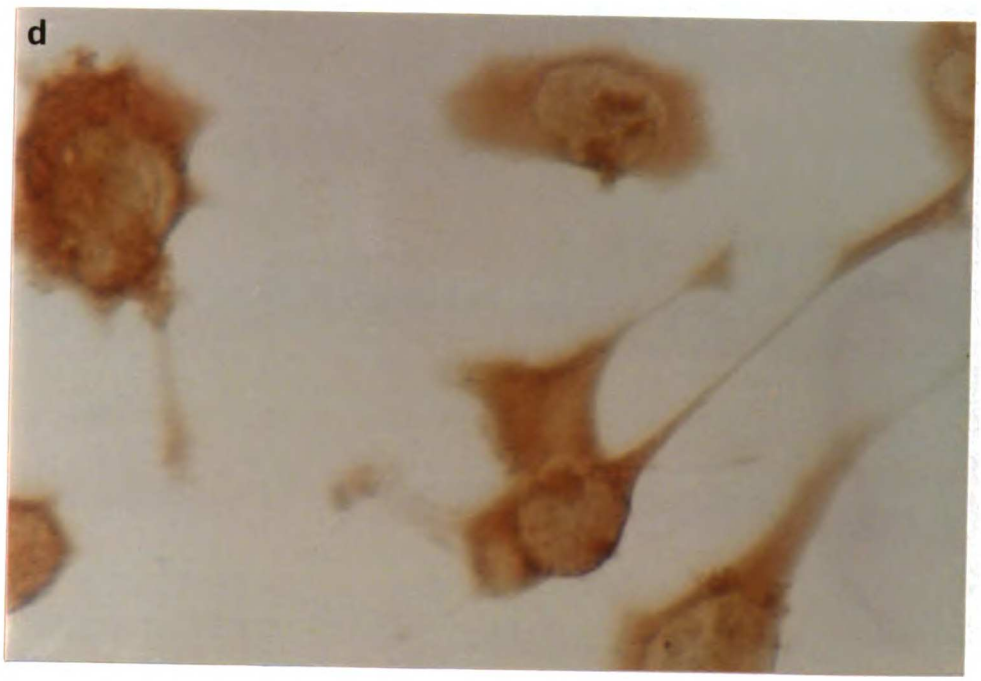

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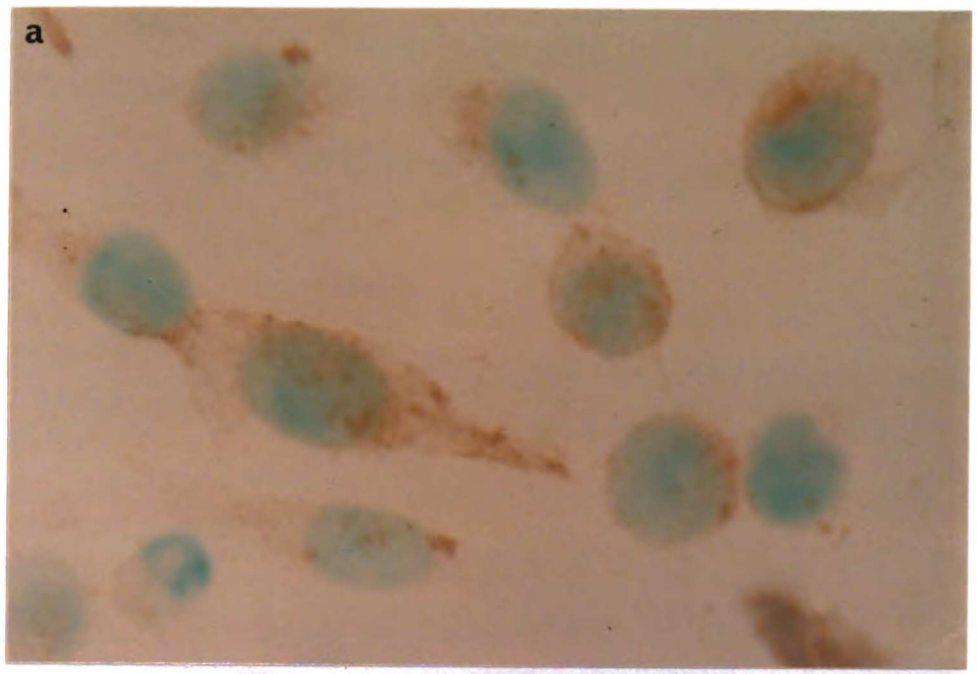


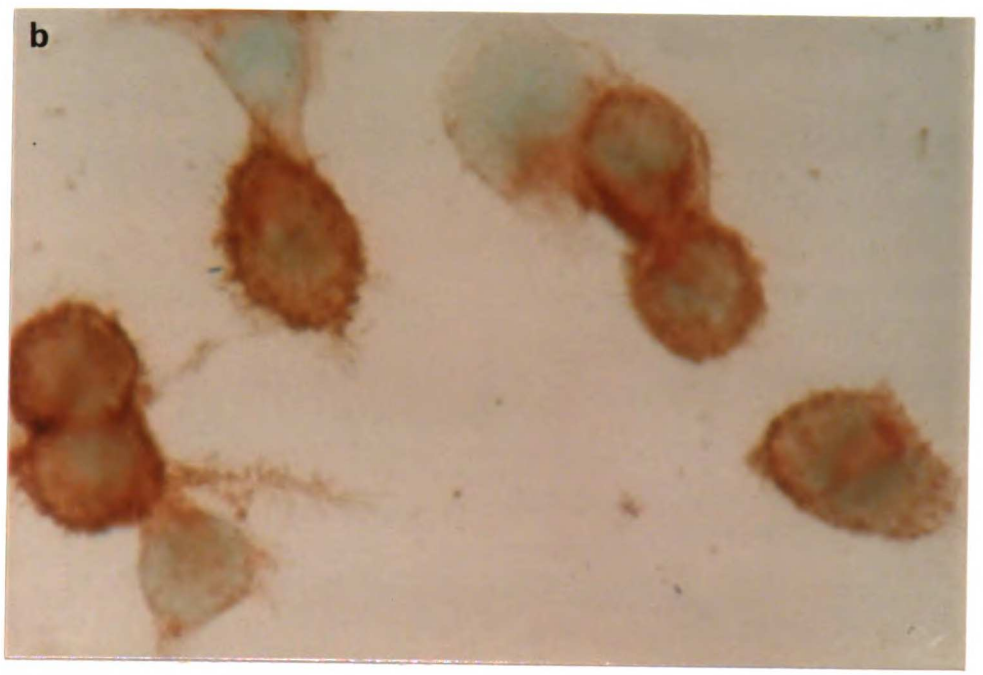

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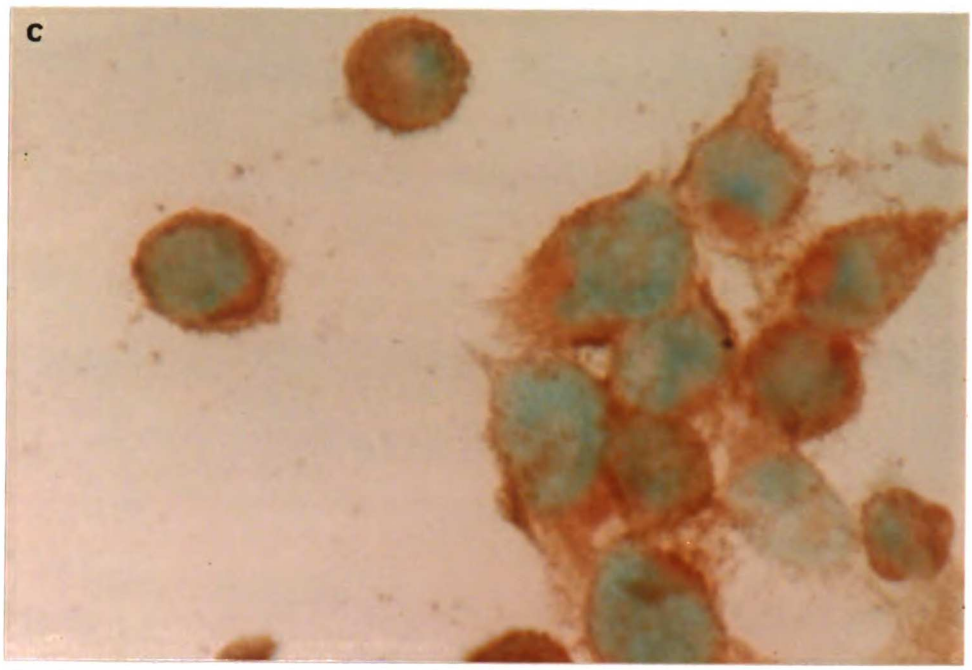

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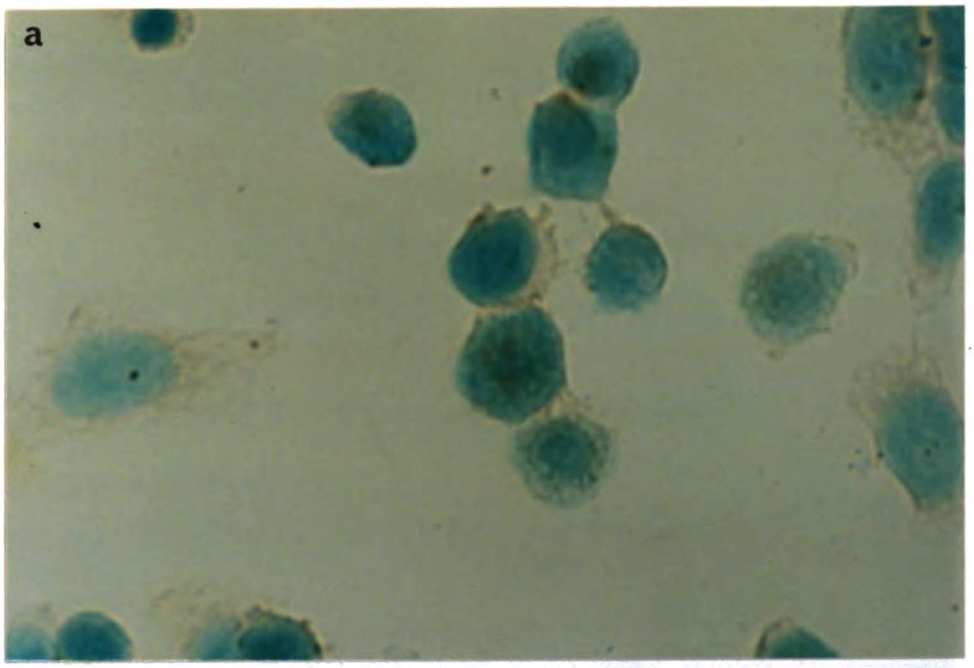


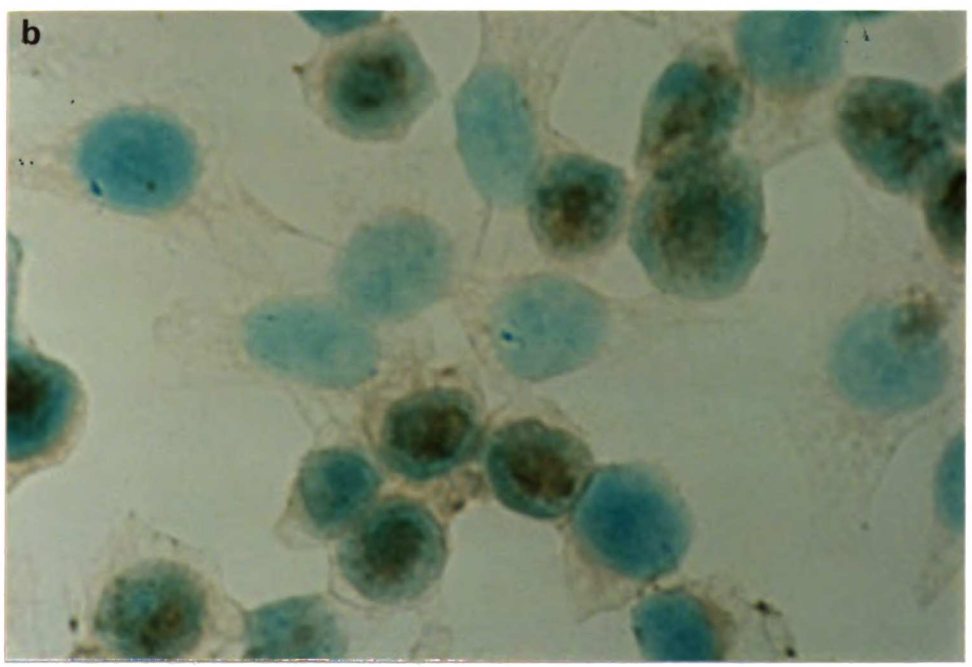

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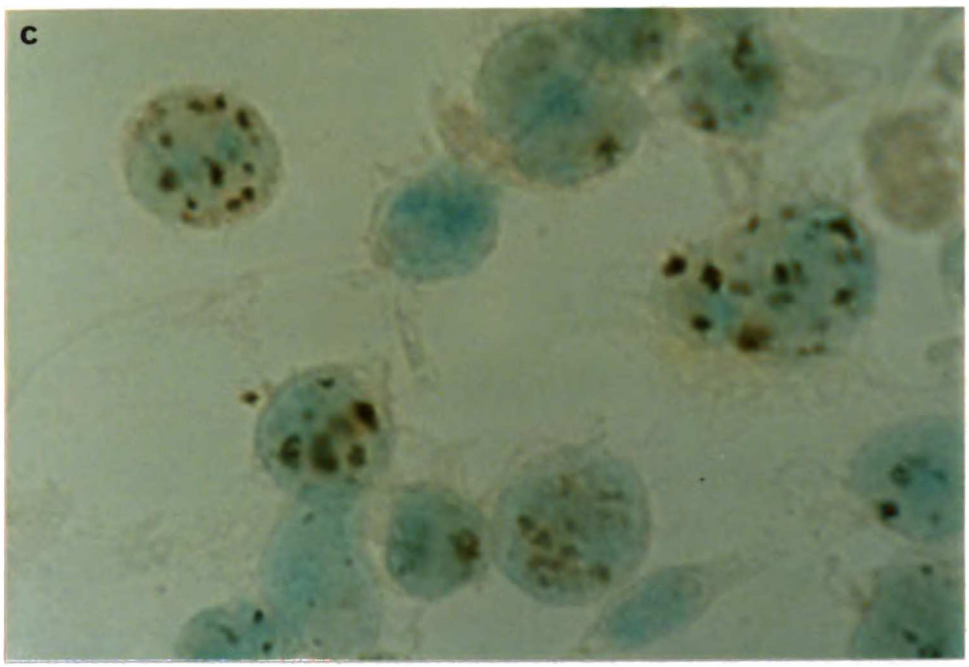

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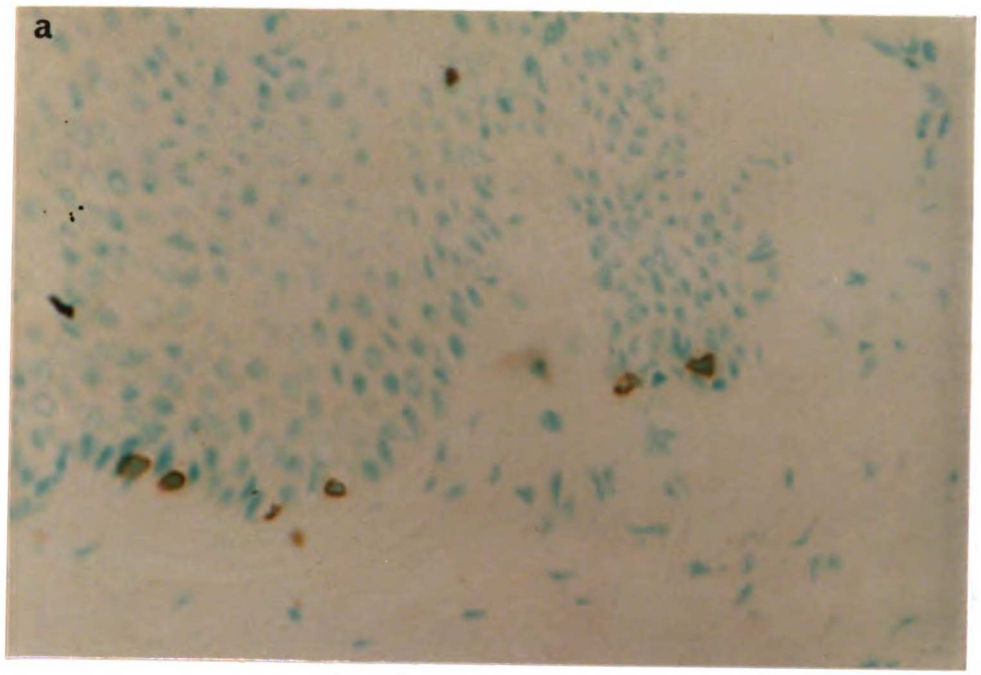


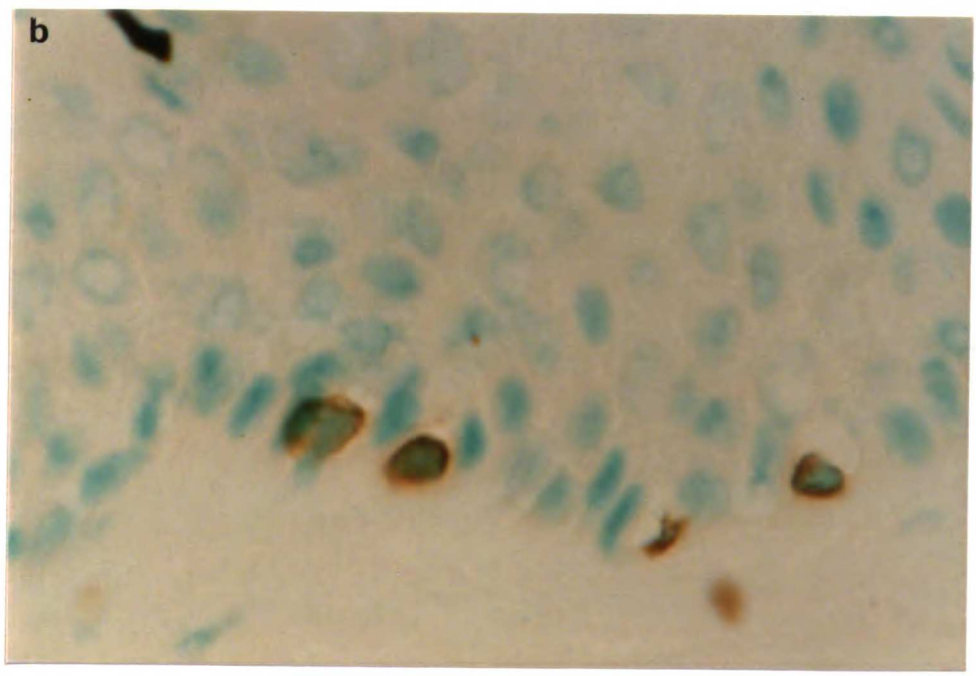

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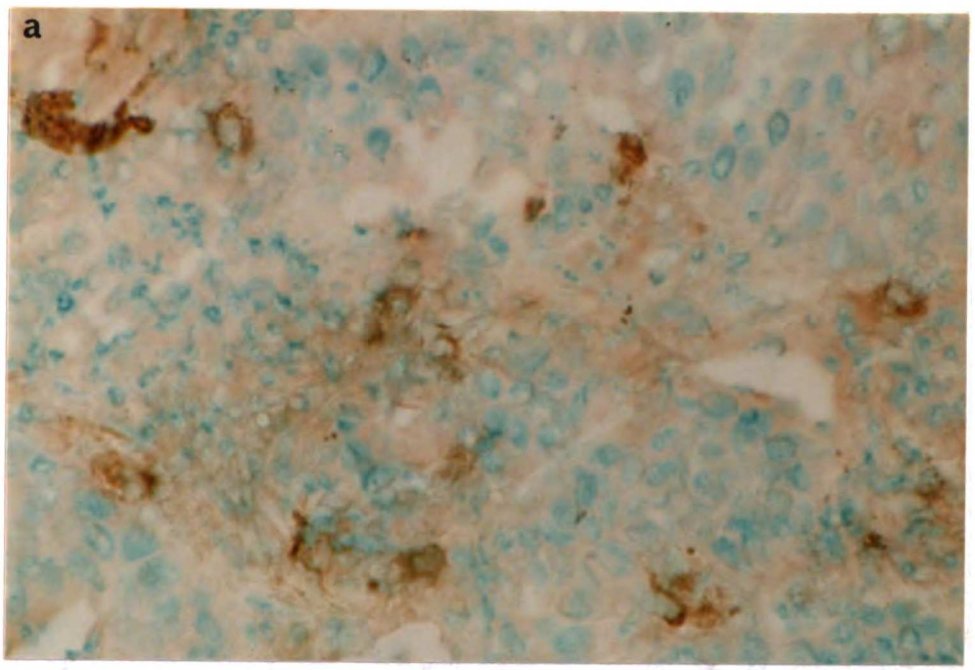


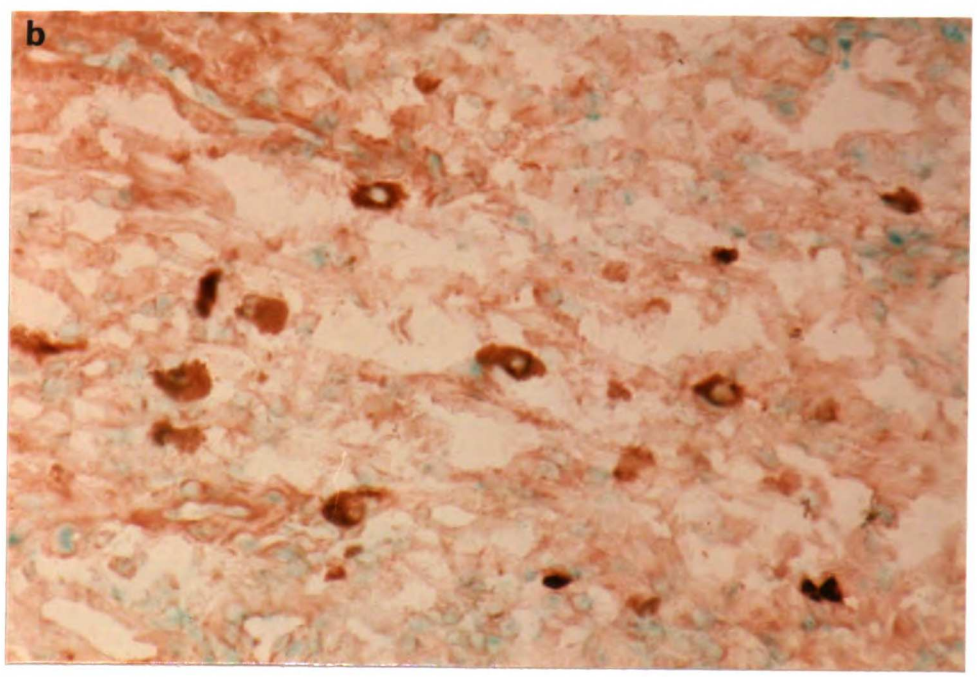

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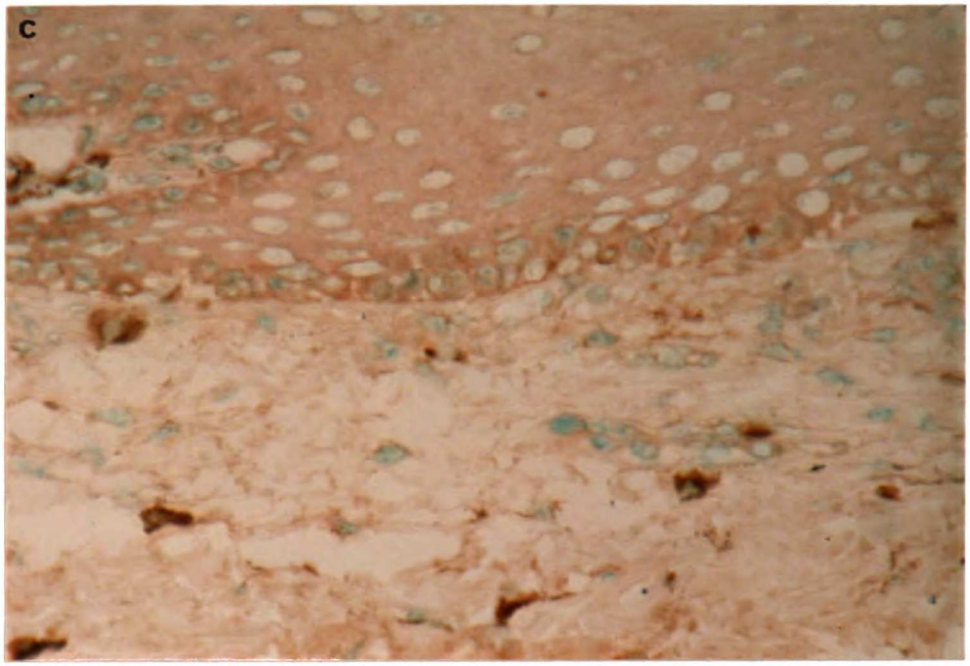

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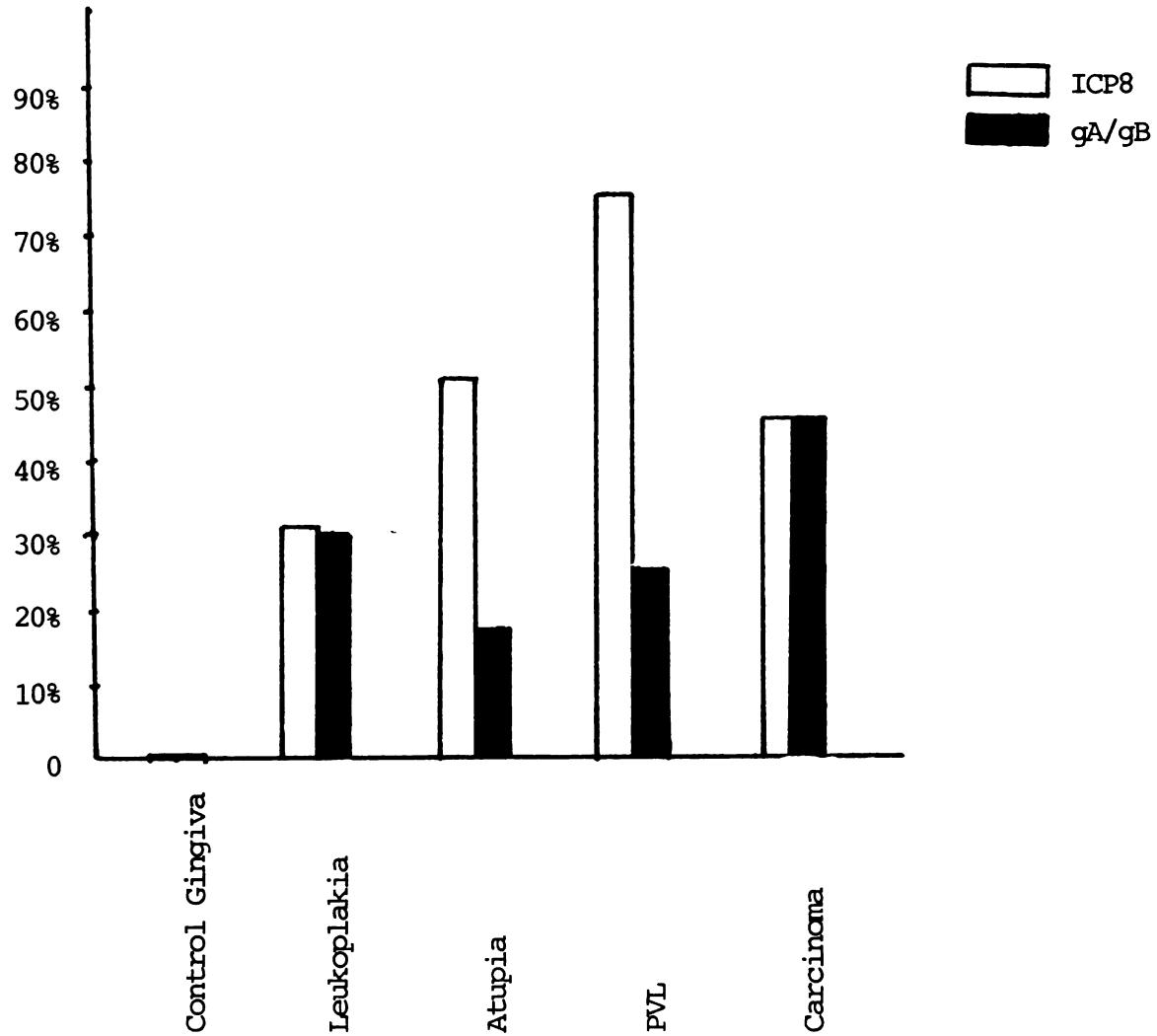
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80%
70%
60%
50%
40%
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10%
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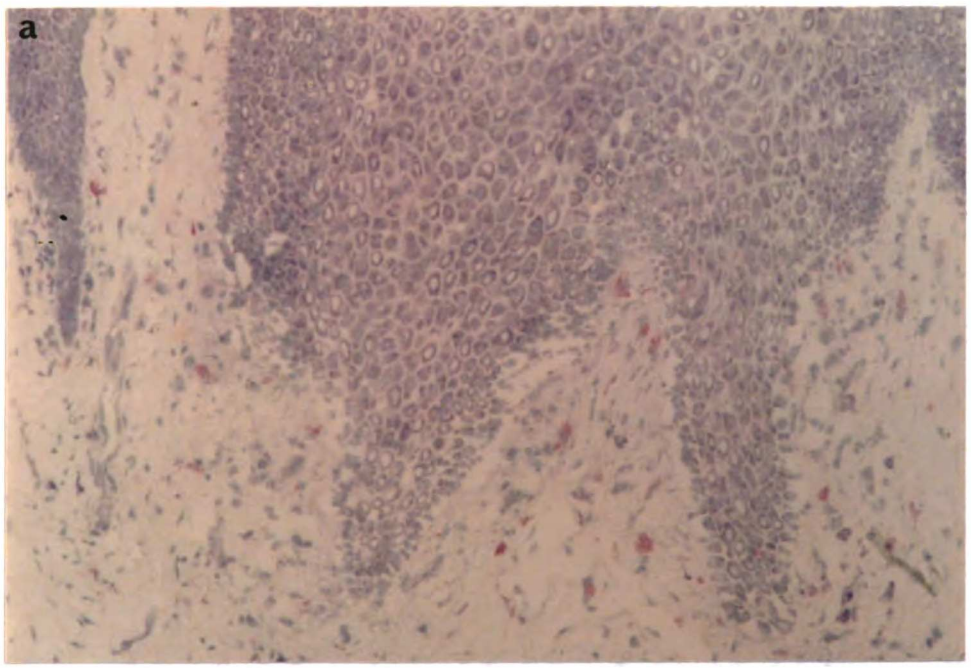
Control Gingiva
Leukoplakia
Atupia
PVL
Carcinoma

Figure 13



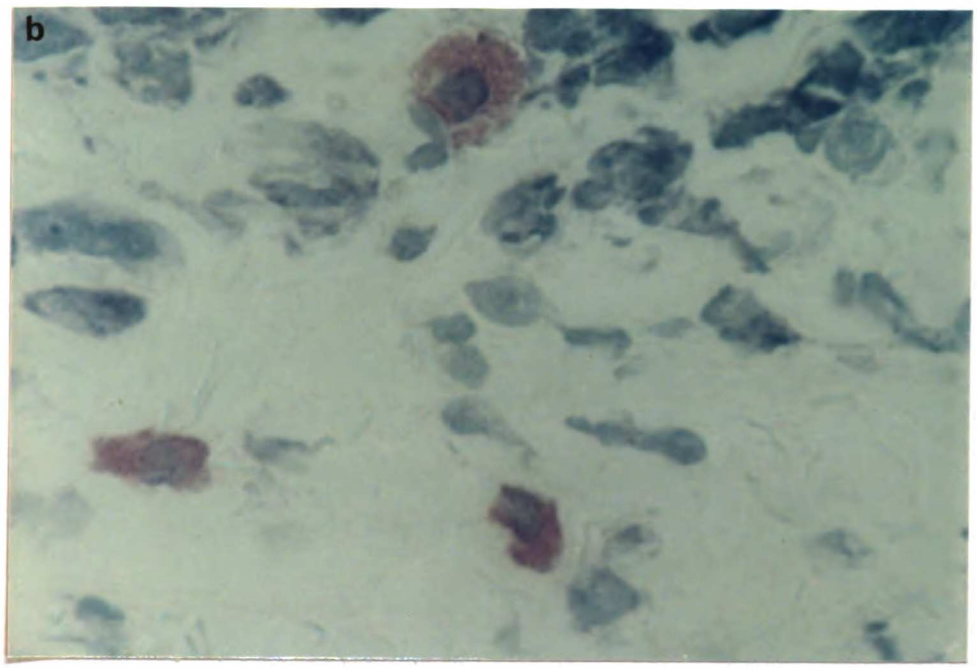
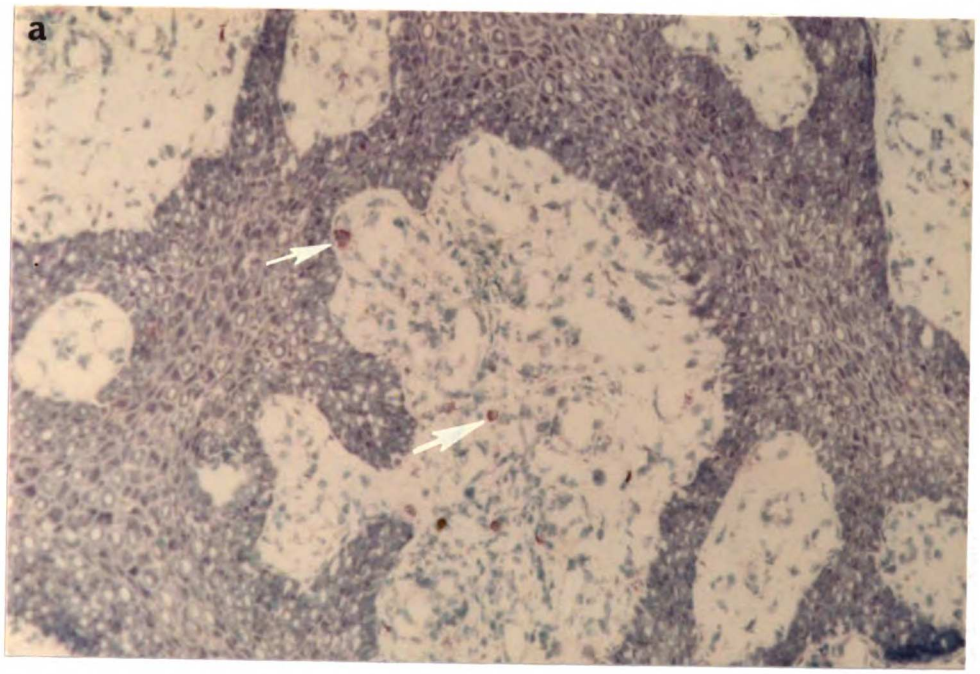



Figure 14



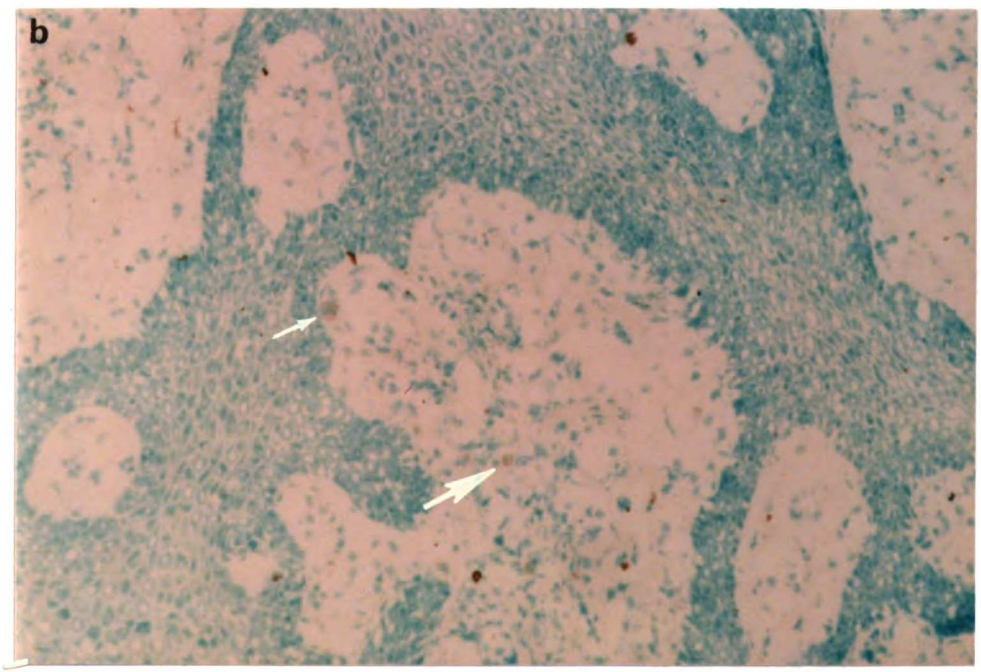



Table 1, Characteristics of HSV proteins in this study

| Viral proteins (MW) | Class | Structural | Characteristics | Other nomenclatures | References |
|---|---|---|---|---|---|
| ICP4 (175,000) | α | +/-* | Immediate-early protein; Minor virion protein; DNA-binding protein. | VP175; VP4; ICSP5-8. | (1). |
| ICP5 (155,000) | γ | + | Major structure protein; Major capsid protein. | VP154; VP5. | (2). |
| ICP6 (140,000) | β | +/-** | Minor structure protein; DNA-binding protein. | VP134; VP6. | (3). |
| ICP8 (132,000) | β | - | Major DNA-binding protein; Function in DNA-replication? | ICSP11/12; VP130; VP143. | (4). |
| gB (120,000) | γ | + | Major virion glycoprotein; Function in virion penetration. | VP7; VP7.5; VP7($B_2$). | (5). |

+: Structural; -: Nonstructural.

*: Studies marked by * defined ICP4 as nonstructural protein; **: Studies marked by ** defined ICP6 as nonstructural protein.

(1): Honess & Roziman 1973, 1974 & 1975; Courtney & Benyesh-Melnick 1974*; Powell & Purifoy 1976*; Powell et al. 1975*; Bone & Courtney 1974*.

(2): Honess & Roizman 1973, 1974 & 1975; Knipe & Spang 1982. Bone & Courtney 1974; Powell et al. 1975; Courtney & Benyesh-Melnick 1974.

(3): Honess & Roizman 1973, 1974 & 1975; Wilcox et al. 1980. Bone & Courtney 1974**; Powell et al. 1975**; Courtney & Benyesh-Melnick 1974**.

(4): Honess & Roizman 1973, 1974 & 1975; Knipe & Spang 1982; Knipe et al. 1982; Lewis et al. 1982; McDougall et al. 1982; Powell & Purifoy 1976; Powell et al. 1981.

(5): Camacho & Spear 1978; Ruyechan et al. 1979; Sarmiento & Spear 1979; Spear 1976.

Table 2, Properties of monoclonal antibodies to ICP4 (58S), ICP8 (39S), ICP6 (48S), ICP5 (56S) and gB (24S)

| Monoclonal antibodies | Immunoglobulin classes | RIP-PAGE (Acetone fixed) (HSV-1/HSV-2) | FA HSV-1 | FA HSV-2 |
|---|---|---|---|---|
| ICP4 (58S) | $IgG_{2a}$ | +/- | 320* | <20 |
| ICP5 (56S) | $IgG_1$ | +/+ | 640 | 640 |
| ICP6 (48S) | $IgG_{2a}$ | +/+ | 320 | 320 |
| ICP8 (39S) | $IgG_{2a}$ | +/- | 640 | <20 |
| gB (24S) | $IgG_{2a}$ | +/+ | 2,560 | 2,560 |

From Showalter et al. 1981
RIP-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of radioimmunoprecipitates.
FA: Immunofluorescence assay.
*: End point titer.

Table 3, Results of monoclonal antibody titers by ABC

| Monoclonal Antibodies | ABC HSV-1 | ABC HSV-2 | Optimal dilution for ABC |
|---|---|---|---|
| ICP4 (58S) | > 3,200* | <20 | 800 |
| ICP5 (56S) | > 3,200 | > 3,200 | 200 |
| ICP6 (48S) | > 3,200 | > 3,200 | 400 |
| ICP8 (39S) | > 3,200 | <20 | 800 |
| gB (24S) | > 32,000 | > 32,000 | 8,000 |
| G.S.#1 | 1,600 | 1,600 | 200 |
| G.S.#2 | 1,600 | 1,600 | 200 |

ABC: Avidin-Biotin peroxidase Complex (ABC) technique.
*: End point titer.

Table 4, Reactive patterns of monoclonal antibodies on HSV-1 infected HEp-2 cells at different times postinfection

| Time | ICP4 (58S) | | ICP8 (39S) | | ICP6 (48S) | | ICP5 (56S) | | gB (24S) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Postinfection | C | N[a] | C | N[b] | C | N[c] | C | N[b] | C | PN | CM | N[d] |
| 1 hr | ++ | - | - | - | - | - | - | - | - | - | - | - |
| 4 hrs | +++ | + | ++ | - | ++ | - | +/++ | - | + | + | + | - |
| 8 hrs | ++++ | ++ | +++ | ++ | ++ | + | +++ | +/++ | ++ | ++ | ++ | ++ |
| 12 hrs | ++++ | ++++ | ++ | +++ | ++++ | ++ | ++ | ++ | +++ | ++ | ++ | + D |
| 24 hrs | +++ | +++++ | +/- | ++++ | +++ | + D | ++ | ++++ | +++++ | +++++ | ++++ | +++ D |
| 48 hrs | + | +++++ | +/- | +++ | +++++ | +++ D | +/- | +++ | +++++ | +++++ | ++++ | +++ D |
| 72 hrs | + | ++++ | +/- | +/- | ++ | + D | +/- | ++ | ++++ | ++++ | ++++ | ++ D |

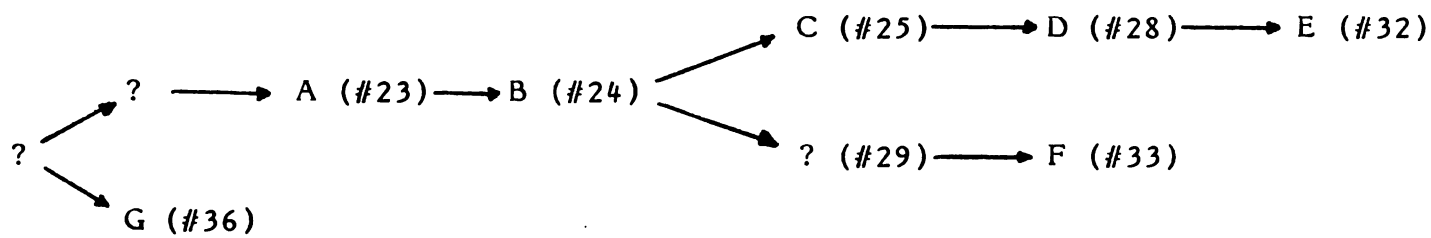
C: Cytoplasm; N: Nucleus; PN: Perinucleus; CM: Cell membrane; D: Diffuse.

a: Dense nuclear staining; b: Speckled nuclear staining; c: Diffuse staining; d: Diffuse nuclear staining found in most cells after 24 h postinfection.

Table 5, Different results of staining patterns of ICP8 and ICP5 on HSV-1 infected cells

| Experiment | Virus passage | Inconsistent results of viral proteins[a] 39S ICP8 | G.S.#1 ICP8 | G.S.#2 ICP8 | ICP5 |
|---|---|---|---|---|---|
| A | 3 | - | ? | ? | ? |
| B | 4 | - | ? | ? | - |
| C | 5 | - | ? | ? | - |
| D | 6 | + | ? | ? | ? |
| E | 7 | + | +* | +* | + |
| F | 6 | + | +* | +* | + |
| G | 2 | - | - | - | - |

Passage history of viruses used for each experiment:




+: Inconsistent results of staining patterns on infection cells.
-: No change of staining patterns on infected cell.
?: Not studied.
*: Only on cells infected at 24 hours.
#: Number of batch virus used.
a: Results from experiments E and G are listed in table 6.

Table 6. ICP5 and G.S.#1, G.S.#2 and 39S ICP8 proteins stained in cells infected with HSV-1 at different time postinfection

| Time of Infection | ICP8 | | | | | | | | | | | | ICP5 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Exp. E | | | | | | Exp. G | | | | | | Exp. E | | Exp. G | |
| | 39S | | G.S.#1 | | G.S.#2 | | 39S | | G.S.#1 | | G.S.#2 | | | | | |
| | C | N | C | N | C | N | C | N | C | N | C | N | C | N | C | N |
| 1 | - | - | | | | | - | - | - | - | - | - | - | - | - | - |
| 4 | - | - | | | | | ++ | - | ++ | - | ++ | - | + | - | +/++ | - |
| 8 | - | - | | | | | +++ | ++ | +++ | +++ | +++ | +++ | + | - | +++ | +/++ |
| 12 | ++ | - | | | | | ++ | +++ | + | ++++ | + | ++++ | +++ | - | ++ | ++ |
| 24 | ++ | - | +/- | ++ | +/- | ++ | +/- | ++++ | +/- | +++++ | +/- | +++++ | +++ | - | ++ | +++ |
| 48 | ++ | - | | | | | +/- | +++ | +/- | ++++ | +/- | ++++ | +++ | - | +/- | +++ |
| 72 | - | - | | | | | +/- | ++ | +/- | +++ | +/- | +++ | + | - | +/- | ++ |

Table 7, Staining of sections of carcinoma with monoclonal antibodies to proteins of HSV-1

| Specimen Accession Number | NMAF | ICP8 protein | | gB protein | | ICP4 protein | | ICP5 protein | | ICP6 protein | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| R80-010 | 1:20 | - | <1:10 | - | 1:40 | - | <1:10 | - | <1:10 | - | <1:10 |
| R80-011 | 1:20 | +++ | 1:1280 | + | 1:320 | - | 1:20 | - | 1:20 | - | 1:20 |
| R82-010 | <1:10 | ++ | 1:320 | + | 1:160 | ? | >1:10 | ? | --- | ? | >1:20 |
| R82-066 | 1:40 | >+ | >1:640 | >++ | >1:1280 | ? | >1:80 | ? | >1:20 | ? | >1:40 |
| R82-072 | <1:10 | - | 1:80 | - | 1:40 | - | 1:80 | - | <1:10 | - | <1:10 |
| R82-089 | 1:10 | >++ | >1:320 | + | 1:160 | ? | >1:10 | ? | >1:20 | ? | >1:20 |
| R82-093 | <1:10 | - | <1:10 | - | 1:40 | - | <1:10 | - | <1:10 | - | <1:10 |
| R82-108 | <1:10 | ++ | 1:320 | +++ | 1:640 | ? | >1:20 | ? | >1:20 | ? | >1:20 |
| R83-075 | 1:40 | - | <1:10 | - | <1:40 | ? | --- | ? | --- | ? | --- |
| R83-088 | 1:40 | - | 1:40 | - | 1:40 | ? | --- | ? | --- | ? | --- |
| R83-063 | 1:160 | - | 1:80 | - | 1:80 | ? | --- | ? | --- | ? | --- |
| Positive/ Total | | 5/11 (45%) | | 5/11 (45%) | | 0/4 (0%) | | 0/5 (0%) | | 0/4 (0%) | |

-: Defined negative result for NMAF; and anti-ICP8 and anti-gB antiibodies when compared with NMAF.
+: 4-fold higher dilution of antibodies (anti-ICP8 and anti-gB) than that of NMAF.
++: 5-fold higher dilution of antibodies than that of NMAF.
+++: 6-fold higher dilution of antibodies than that of NMAF.
?: Titration not done due to lack of tissue.

Table 8, Staining of sections of atypia and PVL with monoclonal antibodies to proteins of HSV-1

| Specimen Accession Number | NMAF | ICP8 protein | | gB protein | | ICP4 protein | | ICP5 protein | | ICP6 protein | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| R81-092* | 1:80 | ++ | 1:2560 | - | 1:640 | ? | >1:10 | ? | >1:10 | ? | >1:10 |
| R82-065* | 1:10 | + | 1:160 | + | 1:160 | ? | --- | ? | --- | ? | --- |
| R82-113** | 1:160 | + | 1:1280 | - | 1:320 | - | 1:40 | ? | >1:10 | ? | >1:10 |
| R83-083* | 1:40 | - | 1:80 | - | 1:80 | ? | --- | ? | --- | ? | --- |
| R82-206+ | <1:10 | - | <1:10 | - | <1:40 | - | <1:10 | - | <1:10 | - | <1:10 |
| R83-065++ | <1:10 | - | <1:10 | - | <1:40 | - | <1:10 | - | <1:10 | - | <1:10 |
| Positive/ Total | | | 3/6 (50%) | | 1/6 (17%) | | 0/4 (0%) | | 0/2 (0%) | | 0/2 (0%) |
| Positive/ PVL | | | 3/4 (75%) | | 1/4 (25%) | | 0/1 (0%) | | ? | | ? |

*: PVL; **: PVL with the feature of carcinoma in situ; +: Carcinoma in situ; ++: Dysplasia.

Table 9, Staining of sections of leukoplakia without atypia with monoclonal antibodies to proteins of HSV-1

| Specimen Accession Number | NMAF | ICP8 protein | | gB protein | | ICP4 protein | | ICP5 protein | | ICP6 protein | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| R79-649 | 1:640 | - | 1:2560 | - | 1:2560 | ? | >1:80 | - | >1:10,<1:80 | ? | >1:80 |
| R81-089 | 1:80 | - | 1:320 | - | 1:160 | ? | >1:10 | ? | >1:10 | ? | >1:10 |
| R83-003 | 1:20 | >+ | >1:320 | ? | >1:40 | - | 1:80 | ? | >1:10 | ? | >1:10 |
| R82-007 | 1:10 | - | 1:80 | ++++ | 1:2560 | ? | >1:10 | ? | >1:10 | ? | >1:10 |
| R82-008 | 1:20 | - | 1:40 | ? | --- | ? | --- | ? | --- | ? | --- |
| R82-009 | 1:320 | - | 1:320 | - | 1:160 | ? | >1:10 | ? | >1:10 | ? | >1:10 |
| R82-073 | <1:10 | >++ | >1:320 | ? | >1:40 | - | 1:80 | ? | >1:10 | ? | >1:10 |
| R82-075 | 1:10 | >+ | >1:160 | >+ | >1:160 | ? | >1:10 | ? | >1:10 | ? | >1:10 |
| R82-162 | <1:10 | - | <1:10 | - | <1:40 | ? | --- | ? | --- | ? | --- |
| R82-216 | 1:160 | - | 1:160 | - | 1:160 | ? | >1:10 | ? | >1:10 | ? | >1:10 |
| Positive/ Total | | 3/10 (30%) | | 2/7 (29%) | | 0/2 (0%) | | 0/1 (0%) | | ? ? | |