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Identification of herpes simplex virus infection by immunoperoxidase and in situ hybridization methods

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Summary. Seven cases of visceral herpes simplex virus (HSV) infection were observed in five cases of hematopoietic disease and in one case each of a newborn baby and a pregnant woman. These seven cases were studied with an immunoperoxidase method and in situ hybridization. In HSV lesions of squamous epithelium, the immunoperoxidase method using rabbit anti-human HSV revealed positive staining, mainly in the nucleus but with some cytoplasmic staining. DNA in situ hybridization revealed stronger positive staining in the nucleus. In HSV hepatitis positive staining was seen in the nucleus and cytoplasm, both by immunoperoxidase and in situ hybridization methods. In the newborn baby, HSV lesions were observed in the brain only, with numerous positive astrocytes identified by the immunoperoxidase method and a few positive astrocyte nuclei by in situ hybridization. Cultured human fetal fibroblasts from the lung were infected with HSV. The immunoperoxidase method revealed diffuse positive staining in the nucleus and in the cytoplasm whereas in situ hybridization revealed fibrillar positive staining in the nucleus only. Thus, the immunoperoxidase method using rabbit anti-human HSV can detect the presence of HSV protein more sensitively than in situ hybridization, probably because of the greater quantity of HSV protein compared with HSV DNA in infected cells.

Key words: Herpes simplex virus – Immunoperoxidase method – In situ hybridization

Introduction

Despite recent progress in methods for isolating and identifying herpes simplex virus (HSV), many aspects of HSV infections are not fully understood (Rauls 1985). HSV is ubiquitous and contributes significantly to morbidity and mortality in different human populations (Rauls 1985). Disseminated HSV infection in adults is usually accompanied by stomatitis and as in infants, the outcome can be fatal (Gardner and McQuillen 1980). In adults, disseminated HSV infection is associated with compromised cellular immunity (Gardner and McQuillen 1980), where leukemia and lymphoma are the most common underlying disease processes (Buss and Scharyj 1979). Recently, there has been considerable progress in diagnosing HSV infection in tissue samples by the immunoperoxidase method using anti-HSV I and II and by in situ hybridization using a biotin-labelled DNA probe (Brigati et al. 1983). With seven cases of patients with visceral HSV infections, we have used both the immunoperoxidase method and in situ hybridization in order to compare these two methods. We have further tested these two methods with HSV-infected human diploid lung fibroblasts in tissue culture.

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Materials and methods

Among a total of seven cases, six were autopsies and one case was a biopsy specimen of the lung (Table 1). Without obvious immunosuppression, neonates (Nakamura et al. 1985; Whitley et al. 1980) and pregnant women (Goyete et al. 1974; Whitley et al. 1980; Young et al. 1976) represent the main target for disseminated HSV infection and one case each was included in this study (Table 1). The remaining five cases were all adults with hematopoietic diseases, including two cases of acute leukemia and one case each of multiple myeloma, aplastic anemia and idiopathic thrombocytopenic purpura (Table 1).

HSV infections were initially diagnosed by the presence of inclusions in routine hematoxylin and eosin (H&E) sections. These were further verified by an immunoperoxidase method with rabbit anti-human HIV type I and II (BioGenex, San Ramon, Calif.) (Balachandran et al. 1987) and DNA-DNA in situ hybridization using commercially available biotinylated human HSV-specific DNA probe (Buss and Scharyj 1979; Espy and Smith 1988; Langenberg et al. 1988). The hybridization cocktail (HSV Pathogene Kit, EP-875, Enzo, New York) contained a mixture of two HSV-DNA sequences of 3.0 and 8.0 kb of HSV I *Bam* HI restriction sites (Locker and Frenkel 1979) and 16.0 kb of HSV II *Bgl* II restriction site (Swain and Galloway 1983).

Table 1. Herpes simplex infection in immunosuppression

Case no.	Age/sex	Clinical Conditions	Distribution of herpes infection
1	5 days/M	Newborn	CNS
2	17/F	Pregnancy	Cervix, liver
3	42/M	ALL, bone marrow transplant[a]	Lungs (B) +CMV
4	44/M	Aplastic anemia[a]	CNS, tongue, esophagus, pancreas +CMV
5	66/M	Multiple myeloma[a]	Liver, adrenals
6	68/F	AML[a]	Esophagus
7	81/F	ITP[a]	Lungs +CMV

CNS, Central nervous system; B, biopsy; +CMV, concomitant CMV infection; ALL, acute lymphocytic leukemia; AML, acute myelocytic leukemia; ITP, idiopathic thrombocytopenic purpura
[a] Patients were treated with corticosteroid

The sections on the poly-L-lysine (0.1% v/w) coated glass slides were deparaffinized and treated with freshly prepared protease solution (0.2 mg/ml, Sigma, St. Louis, Mo.) in 50 mM Tris-HCl buffer (pH 7.4) at room temperature for 15 min. The excess protease was removed and the tissue sections were washed twice in Tris-HCl buffer containing 2 mg/ml glycine to inhibit continuous digestion by protease. Then, a drop of biotin-labelled cocktail was placed on each section and covered with a coverglass. Both probe and cellular DNA were denatured together in a 95° C convection oven for 5 min. Hybridization was allowed to proceed at 37° C for 1 h. After the hybridization, the coverglass was removed and the sections were covered with post-hybridization wash solution and were rinsed with wash buffer. Sections were then subsequently treated with an avidin-horseradish peroxidase complex, wash buffer, Tris-HCl buffer containing diaminobenzidine tetrahydrochloride (DBA, Sigma) and hydrogen peroxide and were finally rinsed with distilled water. Another set of sections were treated with streptavidin-alkaline phosphatase complex (SA-5100, Vector, Burlingame, Calif.) (Mason and Sammons 1978) which was detected with an alkaline phosphatase substrate (SK-5100, Vector). Sections were lightly counterstained with hematoxylin and were coverslipped with Paramount. Diploid fibroblasts from the human fetal lung were cultured in flasks (15 ml) and were infected with the isolated HSV I virus. HSV infection was identified by distinctive cytopathic effects on fibroblasts (Ray et al. 1984). Cultured cells were washed with phosphate buffered saline, centrifuged and smears were made on poly-L-lysine coated glass slides, which were fixed with acetone and were used for routine H&E, immunoperoxidase staining and in situ hybridization. The latter was performed with a HSV-DNA probe without further digestion by protease. For transmission electron microscopy, cells were first spun down by centrifugation and were fixed in 2% glutaraldehyde in phosphate buffer (pH 7.4). For immunoelectron microscopy and in situ hybridization, cells were first spun down and were subsequently fixed in periodate-lysine-paraformaldehyde solution for 1 h (McLean and Nakane 1974). Cell blocks were washed with 10%, 15% and 20% sucrose solutions in PBS for 30 min each, then were frozen in solid carbon dioxide-acetone. Frozen sections (5–7 μm) were cut and placed on poly-L-lysine coated glass slides. Sections were air-dried and used for immunoelectron microscopy with rabbit anti-HSV I and II in the same way as in light microscopic immunohistochemistry. After processing with DBA and hydrogen peroxide, sections were washed and post-fixed with 1% osmium tetroxide for 20 min, then were subjected to the process of dehydration and flat embedding in Epon. For electron microscopic in situ hybridization, sections on the glass slides were treated with protease solution for 15 min at room temperature. The excess protease was removed and the tissues were washed twice in Tris-HCl buffer containing 2 mg/ml glycine.

The sections were then processed with hybridization cocktail, followed by staining with DBA and hydrogen peroxide, post-fixation with 1% osmium tetroxide and were processed in the same way as in immunoelectron microscopy.

Results

In a total of seven cases, five revealed HSV infection by routine H&E sections (Table 1, cases 2–6), whereas two (Table 1, cases 1 and 7) did not reveal obvious HSV inclusion bodies. All seven cases had visceral infections involving tongue, esophagus, liver, pancreas, lungs, adrenals, brain and uterine cervix (Table 1).

In herpetic lesions of the esophagus, tongue and cervix, the typical histopathology of intranuclear inclusions and ballooning of the cells were evident in the surface squamous cells at the edge of ulcer (Fig. 1). The intranuclear inclusions were positively stained by the immunoperoxidase method and in situ hybridization (Fig. 1). There was a more diffuse staining in the nucleus and cytoplasm by the immunoperoxidase method than by in situ hybridization. The latter revealed stronger staining in the nucleus only (Fig. 1). In the liver (cases 2 and 5), more than two-thirds of the cut surface revealed acute hemorrhagic necrosis of hepatocytes, surrounded by a small rim of viable periportal hepatocytes (Fig. 2). The margins of viable hepatocytes contained numerous eosinophilic intranuclear inclusions, which were variable in size, from small to slightly larger than normal hepatocytes. All were stained positive in the nucleus and cytoplasm with both immunoperoxidase and in situ hybridization (Fig. 2). The pulmonary lesions revealed diffuse necrosis. The adrenal lesions were in the deep cortex with occasional inclusions, similar to the hepatic lesion. The central nervous system of the infant (case 1) revealed massive necrosis of the cerebellar and cerebral hemispheres, dorsal ganglia and brain stem. There were chronic inflammatory infiltrates, gliosis, and calcification at the margin of necrosis where no definite intranuclear inclusions were observed in H&E sections. By the immunoperoxidase method, many astrocytes were positively stained, whereas by in situ hybridization only a few astrocyte nuclei were positively stained.

In the tissue culture study we found, within 3 days of HSV infection, that smear preparation of cultured human fetal fibroblasts revealed scattered, numerous cells with large nuclei and ballooning degeneration (Fig. 3). The immunoperoxidase method revealed relatively diffuse positive staining of nuclei and of some cytoplasm both in large and normal-sized cells (Fig. 3). In situ hybridization clearly demonstrated positive nuclear staining of a somewhat caterpillar or fibrillar appearance and occasional granular staining in the cytoplasm (Fig. 3).

Electron micrographs revealed aggregates of cross-sections and occasional fibrillar tangential sections of viral particles in the nucleus, whereas intracytoplasmic viral particles depicted central cores with single or double envelopes (Fig. 4). The immunoperoxidase method

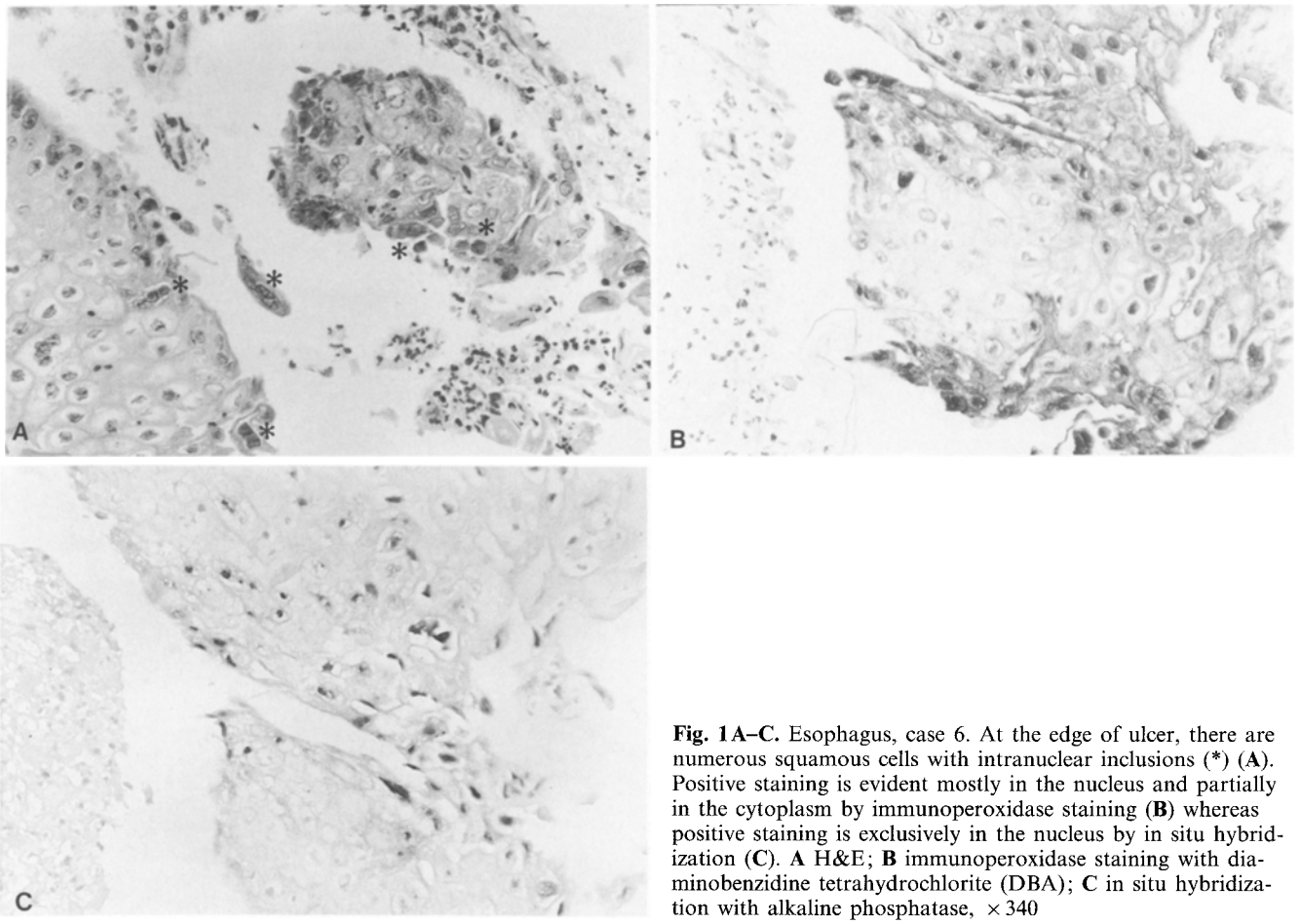


Fig. 1A–C. Esophagus, case 6. At the edge of ulcer, there are numerous squamous cells with intranuclear inclusions (*) (**A**). Positive staining is evident mostly in the nucleus and partially in the cytoplasm by immunoperoxidase staining (**B**) whereas positive staining is exclusively in the nucleus by in situ hybridization (**C**). **A** H&E; **B** immunoperoxidase staining with diaminobenzidine tetrahydrochlorite (DBA); **C** in situ hybridization with alkaline phosphatase, × 340

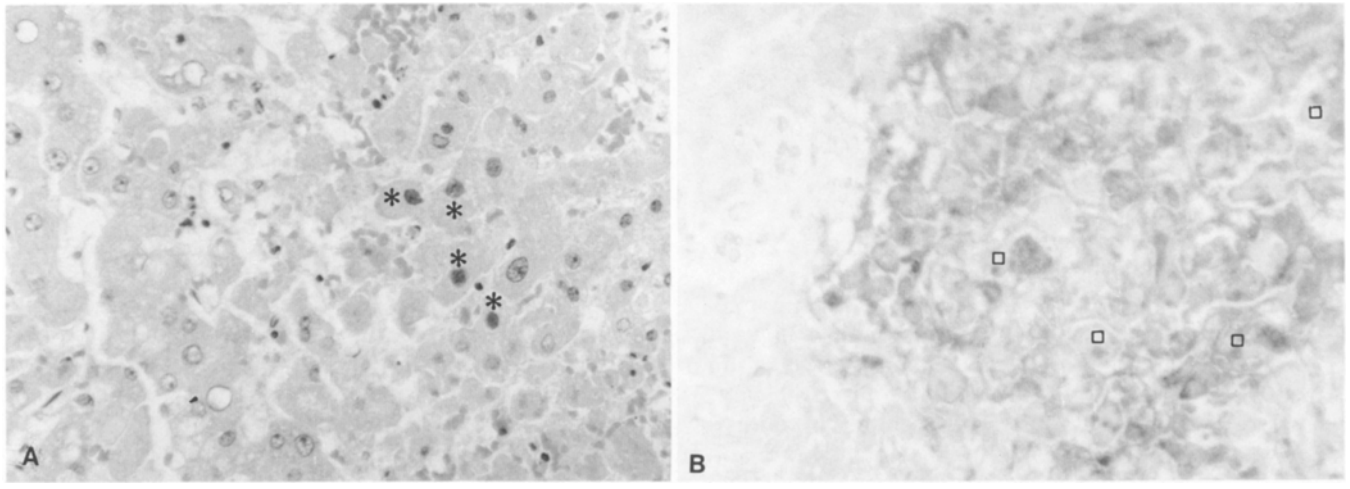


Fig. 2A, B. Liver, case 2. The majority of hepatocytes are necrotic, some of which contain eosinophilic intranuclear inclusions (*) (**A**). Necrotic hepatocytes are positively stained in both nucleus (□) and cytoplasm by in situ hybridization (**B**). The viable hepatocytes are in the left one-fifth. **A** H&E; **B** in situ hybridization with alkaline phosphatase, × 340

revealed positive staining in the nucleus and central core of some intranuclear and most of the intracytoplasmic viral particles (Fig. 5). In situ hybridization showed positive staining in the nucleus and central cores of intracytoplasmic viral particles, whereas practically all extracytoplasmic viral central cores were strongly positive Fig. 6).

Discussion

Among 56 reported autopsy cases of adult non-genital HSV infection, only 7 (12.5%) were clinically suspected, whereas 40 cases (71.4%) presented with no clinical signs or symptoms of HSV infection (Niedt et al. 1985). The most common underlying disease process had been a

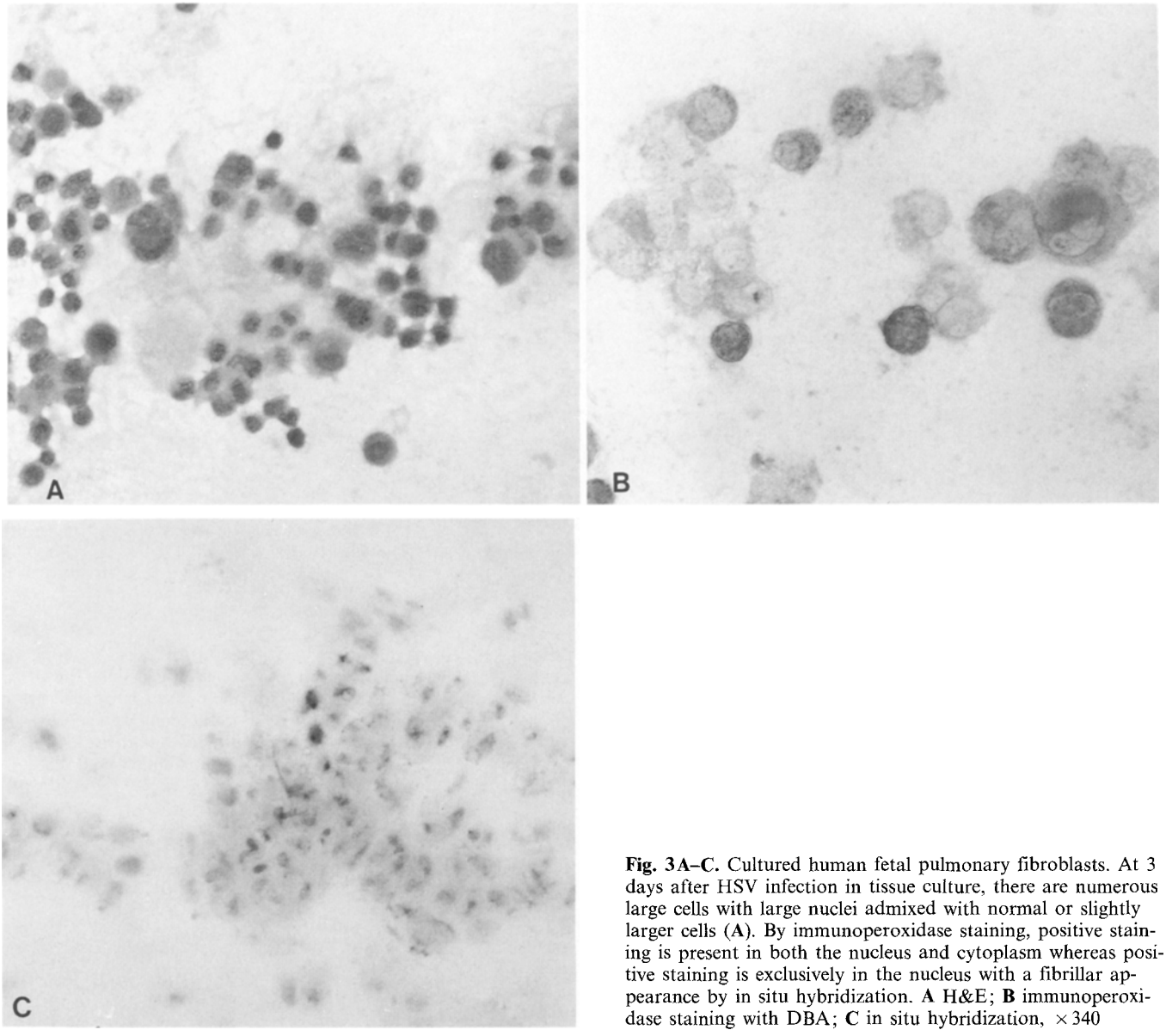
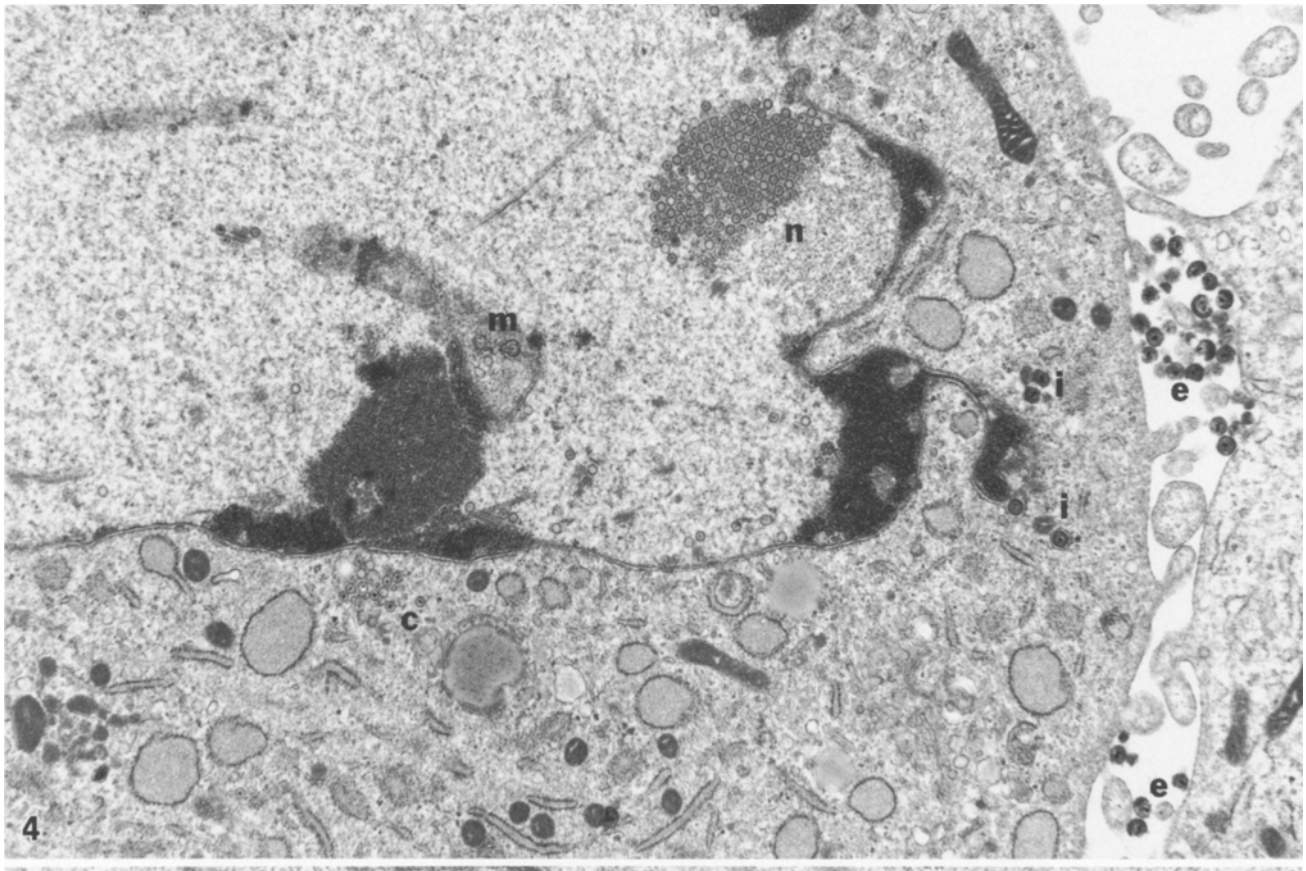


Fig. 3A–C. Cultured human fetal pulmonary fibroblasts. At 3 days after HSV infection in tissue culture, there are numerous large cells with large nuclei admixed with normal or slightly larger cells (**A**). By immunoperoxidase staining, positive staining is present in both the nucleus and cytoplasm whereas positive staining is exclusively in the nucleus with a fibrillar appearance by in situ hybridization. **A** H&E; **B** immunoperoxidase staining with DBA; **C** in situ hybridization, ×340

malignant disease, especially lymphoma and leukemia, representing two-thirds of cases. The remaining one-third were associated with a variety of non-malignant conditions, including kidney transplantation (Berglin et al. 1982; Walker et al. 1981). Thus, all adult cases were associated with compromised cellular immunity. The development of HSV hepatitis is relatively rare and often fatal when complicated by hepatic and adrenal necrosis and disseminated intravascular coagulopathy (Goyette et al. 1979; Lee and Fortuny 1972). The most common HSV organ involvement is the esophagus in 50 out of 56 cases (89%), in which 41 of these cases there was no other organ involvement (Espy and Smith 1988). In our study, we have two cases of esophageal involvement: one case (case 6) was in the esophagus only and the other (case 4) was a generalized HSV infection, including the esophagus. Case 3 was diagnosed clinically as HSV infection by lung biopsy and was subsequently treated with acyclovir, resulting in a complete recovery. Acyclovir therapy has been the proven and effective treatment for HSV encephalitis and disseminated HSV infection and this potent drug of low toxicity is used in the therapy of HSV type I and II and varicella zoster virus. On occasions its use may be life saving (Cox et al. 1986; Jeffries 1986; Whitley et al. 1980). Many clinically evident HSV infections have been effectively treated with acyclovir; this may have contributed to the reduction in visceral HSV infection at autopsy in recent years.

The staining characteristics of HSV were different using the immunoperoxidase method with rabbit anti-human HSV and the DNA-DNA in situ hybridization method. In cultured fibroblasts infected with HSV, the immunoperoxidase method showed diffuse nuclear and cytoplasmic staining whereas in situ hybridization showed fibrillar positive staining exclusively in the nucleus with some granular staining in the cytoplasm (Fig. 3). The rabbit anti-human HSV was generated against HSV



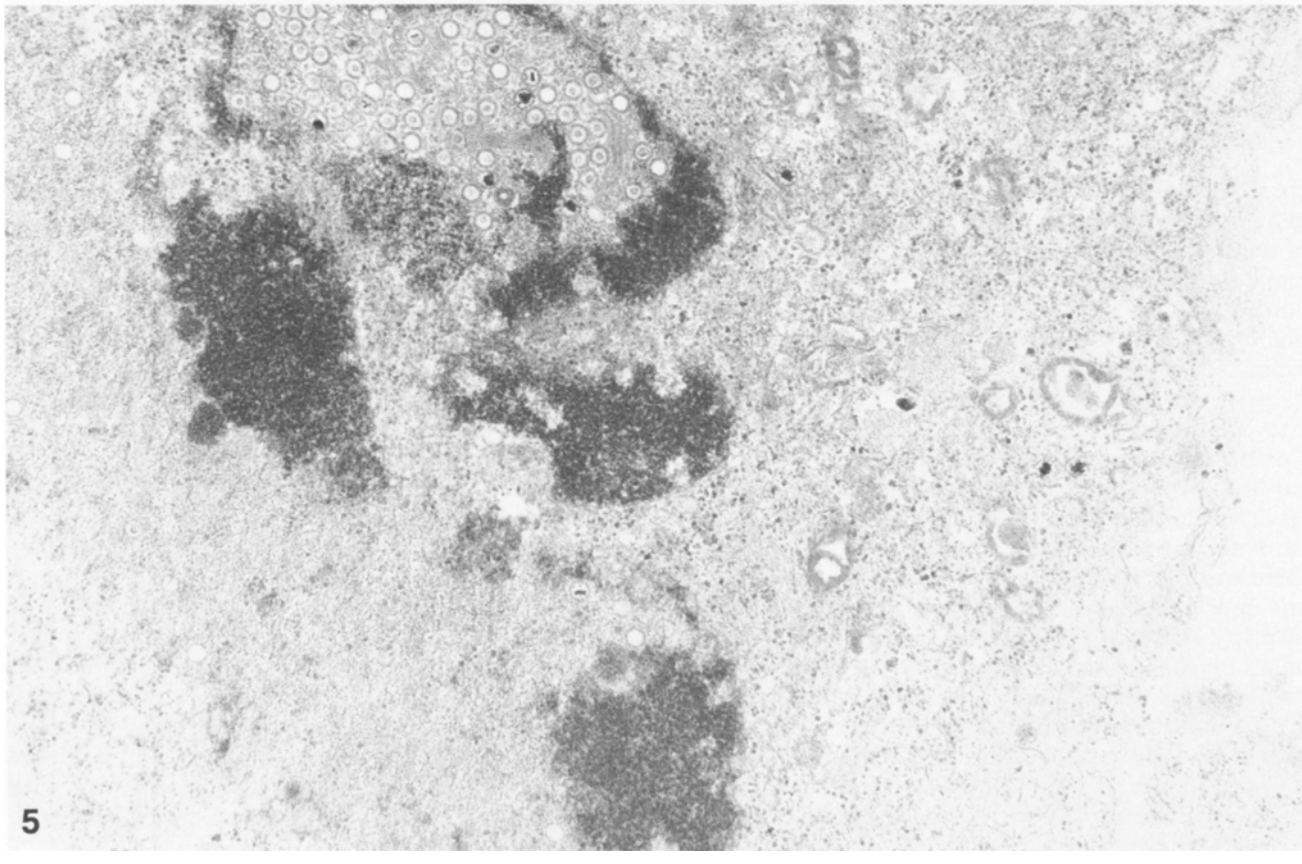


Fig. 4. Cultured human fetal pulmonary fibroblasts. All intranuclear (*n*) and a few intracytoplasmic (*c*) viral particles are enveloped with single membrane whereas all extracellular (*e*) and the majority of intracytoplasmic (*i*) viral particles are enveloped with two membranes. The processing of the double membrane is observed at nuclear membrane (*m*). Central cores are apparent in all viral particles. ×15750

Fig. 5. Cultured human fetal pulmonary fibroblasts, immunoperoxidase staining. Positive staining is observed in the nucleus and central cores of intranuclear and intracytoplasmic viral particles. ×31250

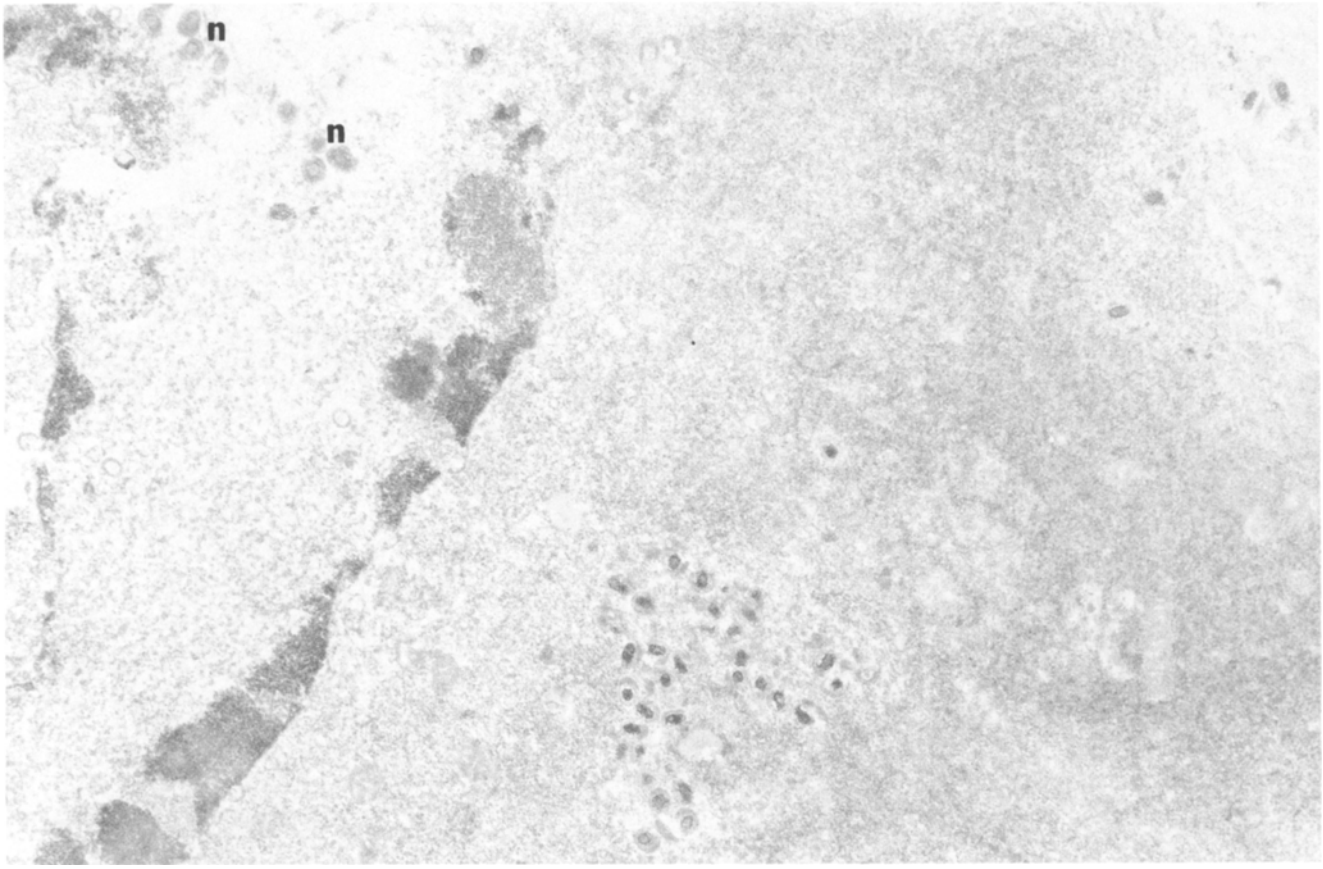


Fig. 6. Cultured human fetal pulmonary fibroblasts, in situ hybridization. Positive staining is observed in the intranuclear viral particles (*n*) and central cores of intracellular viral particles. × 40000

protein which immunoreacts with both nuclear and cytoplasmic viral proteins. The DNA probe represents a mixed sequence of type I and II HSV of relatively small molecular weights which facilitates the DNA probe penetration into the nucleus and cytoplasm. The positive staining of the DNA probe was distinctively fibrillar and corresponded very well to electron microscopic locations of viral particles (Fig. 4). Thus, in situ hybridization appears to be more specific for viral DNA whereas immunoperoxidase staining, which immunoreacts with both nuclear and cytoplasmic viral proteins, is more sensitive but less specific than DNA-DNA in situ hybridization. In case 1, there was very little viral DNA in the nucleus but there was more immunoreactive viral protein in the nucleus and cytoplasm using immunoperoxidase staining. Thus, it appears that the quantity of viral DNA is not as much as that of viral protein in the HSV-infected cells and this fact may have contributed in part to the different staining characteristics between immunoperoxidase and in situ hybridization methods. These staining characteristics are in contrast with what is found with cytomegalovirus, in which a DNA probe is both more specific and sensitive than an immunoperoxidase method (Keh and Gerber 1988; Masih et al. 1988; Myerson et al. 1984). It appears that sensitivity and specificity depend on each antibody and DNA probe for each virus.

HSV is composed of a double-stranded DNA core measuring 30–40 nm in diameter, a protein capsid of about 100 nm, and an envelope acquired when passing the nuclear membrane, measuring 120–130 nm (Gardner and McQuillen 1980). As observed in Fig. 4, intranuclear viral particles are surrounded by a single envelope and extracellular viral particles at the cell surface are surrounded by two envelopes. The majority of intracytoplasmic viral particles consist of two envelopes but some intracytoplasmic particles with a single envelope are also observed (Fig. 4). At least some viral particles are processed through the Golgi apparatus to be equipped with a second envelope, although the majority are acquired at the nuclear membrane (Gardner and McQuillen 1980). Recently, human herpesvirus-6 (HHV-6) has been implicated as a promoter of human immunodeficiency virus 1 (HIV-1) infection (Horvat et al. 1989; Lusso et al. 1989). HHV-6 is a new herpesvirus, antigenetically distinct from other human herpesviruses and has been recently isolated from the peripheral blood lymphocytes of patients with AIDS (Lopez et al. 1988; Tedder et al. 1987) and from patients with lymphoma and leukemia (Salahuddin et al. 1986). We may well see more evidence of HSV infection including HHV-6 in the patients with compromised cellular immunity especially in those with AIDS.

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