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MICROCARRIERS IN ENZYME IMMUNOASSAY FOR THE DETECTION
OF HERPES SIMPLEX VIRUS ANTIGENS

A Thesis submitted to the
School of Graduate Studies
University of Ottawa

In Partial Fulfillment of
the Requirements for the Degree of
Master of Science,
Department of Microbiology and Immunology,
School of Medicine.

by

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UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

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ABSTRACT

The general objective of our investigations was to improve the detection of viral antigens in tube cultures by enzyme immunoassay (EIA) as applied to diagnostic virology. In the first phase of our work, we investigated the feasibility of growing HEp-2 and HFL cells on microcarriers (MC's) instead of monolayers. HEp-2 cells were first grown in spinner flasks (SF) and then transferred into stationary and rotating tube cultures. Study of the HEp-2 cell growth, pH, glucose utilization, CO_2 and O_2 metabolism over 7 days, indicated that cultures on MC in rotating tubes produced the best yield (50 cells/bead after 7 days of culture). Comparison of the HEp-2 cells grown on MC's and normal monolayers in tube cultures showed the highest yield produced by the stationary monolayer cultures (2.5×10^6 cells/tube), whereas the rotating MC cultures produced a significantly lower cell yield (1.4×10^6 cells/tube). With the HFL cells, the tube cultures were established by direct seeding of cells and MC's in stationary tube cultures only. Study of the growth curve, pH, glucose utilization, CO_2 and O_2 metabolism over 7 days showed a significant difference in cell yield at the end of 7 days when compared to HEp-2 cell yield. The HFL cells produced an average yield of approximately 30 cells/bead and the HEp-2 cells produced 15 cells/bead. However, comparison of the HFL cell yield on MC's with standard monolayer cultures set up under the same conditions showed no significant difference. The monolayer cultures produced an average yield of approximately 6×10^5 cells/tube and the HFL cells produced an average yield of

approximately 5×10^5 cells/tube.

The pH remained within reasonable limits (7.7-6.8) over the 7 days in all the HEP-2 or HFL cell cultures. The O_2 and CO_2 also decreased at a constant rate in all the cultures.

In the second phase of our study we investigated the use of the MC tube culture system in combination with enzyme immunofiltration (EIA-F) for the detection of HSV antigens. Using an HSV group monoclonal antibody, it was found to be as sensitive as direct IF. Multiplicities of infection ranging from 10^{-5} to 10^1 were tested at 8-96 hours post-infection. With very high infectious inocula HSV-1 positive EIA-F was obtained at the same time as the appearance of the cytopathic effect (CPE), while with HSV-2 it was within 10-24 hours of the appearance of CPE. With low inocula of both HSV-1 and HSV-2 specific identification was possible at 10-24 hours after the appearance of CPE.

In the third phase of our study we investigated the EIA-F and a commercial (Ortho Diagnostic Systems, Inc., U.S.A.) enzyme-linked immunosorbent assay (O-EIA) for the detection of HSV in direct clinical samples. A total of 166 clinical samples were tested and results were compared to those obtained in MRC-5 cell cultures. The sensitivity and specificity of the O-EIA were 54 and 83% respectively, compared to 16 and 93% respectively for EIA-F.

In the last phase of study, we applied the MC/EIA-F system to the detection of HSV-1 and HSV-2 cultures, by comparison with the detection of CPE. It was found that the system detected HSV antigens in 50% of the samples after 2 days and 100% of the samples after 3 days, and was almost as sensitive as the detection of CPE.

LIST OF ABBREVIATIONS

EIA - Enzyme immunoassay
MC - Microcarrier
SF - Spinner flask
IF - Immunofluorescence
EIA-F - Enzyme immunofiltration
O-EIA - ORTHO enzyme immunoassay
IP - Immunoperoxidase
ADC - Anchorage dependent cells
CPE - Cytopathic effect
DEAE - Diethylaminoethyl
EM - Electron microscopy
IEM - Immune electron microscopy
RIA - Radioimmunoassay
B-A - Biotin-Avidin system
ELISA - Enzyme linked immunosorbent assay
PBS - Phosphate buffered saline
FCS - Fetal calf serum
MOI - Multiplicity of infection

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INTRODUCTION

Despite the availability of herpes simplex virus (HSV) antigen detection tests such as direct and indirect immunofluorescence (IF), immunoperoxidase (IP), and enzyme immunoassay (EIA), and of DNA hybridization technology, isolation of HSV in cell culture still remains the "gold standard" and the most sensitive way to demonstrate the presence of this agent. However, IF, IP, and EIA are being increasingly applied for the rapid identification of HSV growing in cell cultures and are replacing more traditional methods such as microneutralization, for specific viral identification.

These procedures are costly in terms of culture material needed for antigen detection since they usually involve an entire tube culture cell monolayer to collect sufficient antigenic material for replicate testing, therefore preventing further observation or analysis by other immunochemical methods.

The concept of propagating anchorage-dependent cells (ADC) on the surface of small, solid particles suspended in the growth medium was first suggested and described by van Wezel in 1967. Since their original description, microcarrier (MC) cell cultures have been used extensively for the production of animal cells, and the culture of viruses and other cell products. In the MC system, ADC's are grown on beads made of either dextran, polyacrylamide, or polystyrene maintained in suspension by gentle stirring. Because of the higher cell surface to volume ratio, MC cultures produce higher cell yields than traditional monolayers at a lower cost. Growing cells on MC's makes repeated sampling possible

without disrupting the original culture, thus facilitating the harvesting of the small number of cells required for the identification of viral antigens.

The present studies were undertaken to develop a MC culturing system in tube cultures, and to use this system in conjunction with a modified EIA called enzyme immunofiltration (EIA-F) to overcome some of the limitations encountered in the identification of HSV infections in cell cultures.

Our studies proceeded in four phases:

a) the objective of phase I was to develop a MC tube culturing system which was convenient and simple for the growth of anchorage-dependent mammalian cells to allow sequential sampling without disrupting the original culture. This would facilitate the harvesting of the small number of cells required for the identification of HSV antigens by EIA-F. We studied the growth parameters of a continuous cell line (HEp-2) and of a diploid cell line (HFL) on MC's and monolayers in tube cultures. The two systems were compared over a 7 day period in order to determine if the MC system would produce higher cell yields and, more importantly, would allow for sequential sampling from one single tube culture.

b) the objective of phase II involved the use of the MC tube culture system developed in phase I, in combination with EIA-F for the detection of HSV antigens. The use of this MC/EIA-F system was examined in order to determine the sensitivity of the technique in the early detection of HSV antigens in comparison with direct IF and the detection of cytopathic effect (CPE).

Microcarrier tube cultures were infected with HSV-1 and HSV-2

strains using a wide range of multiplicities of infection (MOI; 10^{-5} to 10^1) in order to represent those encountered in diagnostic virology. The appearance of CPE, the detection of specific HSV-1 and HSV-2 by IF, and of specific EIA-F was determined at intervals.

c) in view of the good results obtained with the MC/EIA-F system in phase III we studied the sensitivity of the EIA-F assay when applied directly to clinical samples, in comparison to cell cultures. At the same time a commercially available EIA kit became available on the market and this kit was also evaluated in parallel with the EIA-F assay and compared to cell culture for the detection of HSV antigens.

d) the last phase of our studies involved the use of the MC/EIA-F system under field conditions to determine its sensitivity in comparison to cell culture in a clinical laboratory setting for the identification of HSV antigens.

REVIEW OF THE MICROCARRIER LITERATURE

1. INTRODUCTION

Mammalian cells propagated in vitro undergo several alternations during repeated culture transfers. As a result, the cell types obtained can be classified into three major groups, as proposed by Hayflick (Hayflick and Moorhead, 1961; Hayflick, 1965):

(1) primary cells - these are cultures started from cells or tissue taken directly from an organ before the first subculture.

(2) diploid cell line - these are cell lines in which, at least 75% of the cells have the same karyotype as the normal cells of the species from which the cells were originally obtained.

(3) established cell line - these cells have the potential to be subcultured indefinitely in vitro.

For the mass production of biologicals, it is preferable to use cells which grow as anchorage-independent cells, in homogenous suspension. However, these cells have a limited use in the production of biologicals because: a) transformed cells are considered to have a potential risk of carcinogenesis, b) propagation of viruses in suspended cultures causes, sometimes, rapid changes in viral markers, leading to a reduction in immunogenicity as compared to viruses propagated on anchorage-dependent cells (ADC). Thus, ADC are, today, the main substrates for the production of biologicals for human and veterinary use.

2. METHODS FOR LARGE SCALE PROPAGATION OF ADC

Traditionally ADC have been propagated as monolayers in glass or plastic flasks. The demand for large amounts of ADC led to the development of new equipment and systems for their propagation. In these systems, the surface growth area per unit volume of medium has been increased (Levine et al, 1979; Spier, 1980; Spier, 1982).

The two methods of choice for large-scale production of ADC are: a) the packed bed (Wohler et al, 1972; Whiteside, 1979; Merk, 1982) and b) the microcarrier culturing technique (van Wezel, 1967).

The packed bed is used for the large scale culturing of cells. The cells are grown on glass beads with a large total surface. Fresh medium is supplied automatically. The exchange of medium is controlled by monitoring the pH of the medium, and the cells are harvested in the amounts desired by trypsinization.

Our studies involved the use of microcarriers only and, therefore, this review is limited to the study of the different types of MC's available on the market today.

3. THE MC CULTURING SYSTEM: GENERAL DESCRIPTION

3.1 Concept

The concept of propagating ADC on the surface of small, solid particles suspended in the growth medium was first suggested and described by van Wezel in 1967 (van Wezel, 1967). The early studies based on diethylaminoethyl (DEAE)-Sephadex A-50 beads were only moderately successful, and for several years significant progress with this culture technique was limited both by the

toxicity of these MC's and the lack of matrices specifically designed for cell culture. These MC's were composed of cross-linked dextran charged with tertiary amine (DEAE) having an exchange capacity of 5.2 milliequivalent per gram (mEq/gm) of dry dextran.

Van Wezel (1976) reported that the DEAE-Sephadex A-50 MC's had toxic effects on cell growth. These effects were manifested by an initial destruction of cell inoculum, a long lag period, and a total loss of the culture at MC concentration greater than 2mg/mL.

Various approaches were employed to decrease the toxic effects and to improve MC's for the propagation of ADC. These approaches included: a) the addition of conditioned medium (Horng and McLimans, 1975) or medium ingredients (van Hemert et al, 1969) and an increase in the cell inoculum (Horng and McLimans, 1975); b) the treatment of DEAE-Sephadex A-50 MC's with serum (Spier and Whiteside, 1969), nitrocellulose (van Wezel, 1973), or carboxymethylcellulose (Levine et al, 1977a; Levine et al, 1977b); and c) the search for new anion exchange resins (van Wezel, 1973; Horng and McLimans, 1975; van Wezel, 1976; Levine, 1976). These approaches resulted in a partial improvement of MC's over the DEAE-Sephadex A-50. Levine et al (1977a) reported that the toxic effects of the DEAE-Sephadex A-50 could be dramatically reduced by lowering the exchange capacity of the MC to 2.0 mEq/gm dry dextran.

3.2 The Optimal MC

The requirements for an optimal MC can be summarized as follows (van Wezel, 1976; Hirtenstein et al, 1980; Nunc: Biosilon,

1981; Pharmacia Fine Chemicals, 1982):

- 1) a low positive charge: the lowest MC charge which allows cell adherence and growth with minimal adsorption of medium ingredients on the MC's.
- 2) the specific density of the MC should be between 1.03 and 1.10 in order to maintain the suspension of the MC's by slow agitation.
- 3) the MC dimensions should be at about 100-250um so that the MC will carry several hundred cells and still be easily suspended.
- 4) the MC size distribution should be as narrow as possible in order to achieve an even inoculum distribution.
- 5) there should be a smooth surface on the MC in order to allow good cell spreading.
- 6) the MC's should be transparent to allow microscopic observations of the cells on the MC's.
- 7) the MC's should be nontoxic for the cells and authorized for use in the production of biologicals for human use.
- 8) the MC's should be nonrigid in order to reduce possible damage to the cells due to MC collisions during stirring.
- 9) the MC's should be nonporous in order to minimize medium ingredients adsorption into the MC.

3.3 Commercial MC's

Since 1978 several companies have manufactured MC's suitable for propagation of ADC.

The most suitable MC's produced to date have been those based on a positively-charged cross-linked dextran matrix (Levine et al, 1979; Hirtenstein et al, 1980). Such MC's have been used for the culture of a wide variety of cells in culture systems ranging from

a few millilitres to several hundred litres (Levine et al, 1979; Hirtenstein and Clark, 1980; Meignier et al, 1980; van Wezel et al, 1980; Montagnon et al, 1981).

The presence of charged groups on these MC's was found to be essential for cell attachment and a specific degree of substitution of the MC matrix with DEAE groups was required for optimal cell growth (Whiteside et al, 1979; Hirtenstein et al, 1980). This optimum degree of substitution probably reflected a specific density of charges in the surface layer of the MC.

3.3.1. Tertiary Amino-Derived MC's

These MC's have a porous hydrophilic matrix, with tertiary amino-charged groups distributed throughout the whole matrix of the MC, thus leading to relatively high adsorption of medium components on the surface and inside the matrix of the MC. Cells having fibroblast morphology grow better on these MC's as compared with cells having epithelial morphology (Lewis and Volkers, 1979; Gebb et al, 1982; Pharmacia Fine Chemicals, 1982). The main disadvantage of these ionic charged MC's is that serum proteins adsorb strongly to the MC's (van Wezel, 1982; Pharmacia Fine Chemicals, 1982). This disadvantage led to the development of surface charged MC's.

3.3.2. Surface-charged MC's

These beads are derived with quaternary amines in order to prevent possible leakage of charged groups from the MC to the growth medium (Gebb et al, 1982). They also have a low total exchange capacity since the charged groups on the MC are distributed in a thin layer on the outer surface of the MC. This resulted in a decreased binding of the medium ingredients to the

MC's. These MC's have been recommended for use in propagation of cells having a pronounced fibroblast morphology (Gebb et al, 1982; Pharmacia Fine Chemicals, 1982).

3.3.3. Cytodex-3 MC

Collagen-coated surfaces have long been used in animal cell culture and have proved important for the growth of many types of epithelial and differentiated cells which are normally difficult to grow in culture (Reid and Rojkind, 1979; Bornstein and Sage, 1980). In addition, sensitive types of cells can be grown at much lower culture surfaces. This fact is potentially important for MC culture because it is often necessary to initiate cultures with only a small number of cells.

The process of attachment of cells to collagen surfaces is likely to involve steps in common with attachment to artificially charged surfaces. The glycoprotein fibronectin coming from serum is one of the main components involved in attachment of cells to both artificial substrates and collagen (Hook et al, 1977; Gold and Pearlstein, 1980; Grinnell et al, 1980). Fibronectin binds strongly to collagen and has an even higher affinity for denatured collagen (Engvall and Rouslahti, 1977; Engvall et al, 1978; Gold and Pearlstein, 1980). A denatured collagen coat on the MC's could be an important advantage when maximum recovery of cells from the MC's is required. The denatured collagen is susceptible to digestion by proteases. In preliminary experiments with Vero cells Levine et al (1979) found a recovery of cells greater than 95% with 95% viability from the coated MC's after treatment with standard trypsin procedures. Using the same procedure with Cytodex-1, the recovery of cells was 80-85%. The specific

protease, or collagenase, can provide a method for harvesting cells with maximum retention of membrane integrity. This factor is particularly important when harvesting cells which bind strongly to MC's (e.g. human kidney cells).

Cytodex-3 is the only commercial MC on which cell attachment is not a physical electrostatic or adsorptive phenomenon but a "natural" ligand type. Denatured collagen from porcine skin (M.W. 60,000-200,000) is cross-linked to the surface of Sephadex beads (Gebb et al, 1982; Pharmacia Fine Chemicals, 1982). The cross-linking of denatured type I collagen to the MC matrix avoids the problem of leakage of protein which is encountered with the standard techniques for coating cell culture surfaces. These MC's are recommended for propagation of cells having low plating efficiency or cells with a pronounced epithelial morphology (van Hemert et al, 1969; Pharmacia Fine Chemicals, 1982).

3.3.4. Polystyrene MC

These MC's have a hydrophobic nonporous matrix (tissue culture-treated polystyrene) with a low negative charge (Maroudas, 1976; Nunc:Biosilon, 1981). The main advantage of these MC's is their low-charged nonporous character, which results in negligible uptake of medium ingredients. They have, however, several drawbacks: (1) low rate of cell attachment, as compared to positively charged MC's. This is probably due to repulsion between the MC's and negatively charged cells. (2) cell damage is possible due to the rigidity of these MC's when there are collisions during stirring of these MC's. These MC's support epithelial cell growth better than cells having a fibroblast morphology.

3.3.5. DEAE-Cellulose MC

These MC's were produced as anion exchange resin mainly for liquid chromatography (Whatman, 1981). However, these products have been found to be useful as MC's for propagation of several ADC and for production of biologicals (Reuveny et al, 1980; Reuveny et al, 1982).

These MC's have several unique properties: (1) cylindrical shape, (2) low medium ingredient absorption to the MC's, (3) rate of cell attachment and spreading on these MC's is faster as compared to tertiary amino-derived MC's, (4) are cheaper in cost than all the current commercially available MC's. However, due to the elongated cylindrical form of these MC's the particles fully covered with cells tend to adhere to each other by forming cell aggregates.

The development of MC's with a low positive charge and collagen, coated carriers have contributed to a more general application of MC cultures. Cell attachment and growth on these types of MC's are more reproducible and the quality of the media is less critical

4. THE ADVANTAGE OF THE MC CULTURING TECHNIQUE

The technique has the following advantages over other ADC large-scale cultivation methods: (1) there is a high surface/volume ratio which can be varied by changing the MC concentration. This leads to high cell yields per unit volume. (2) the cell growth on the MC's can be easily monitored by microscopic observation. (3) there is more efficient use of growth medium as a result of the homogenous culture. (4) sampling

is easy and reproducible. (5) harvesting of cells and cell products is easy.

5. APPLICATION OF MC CULTURE IN CELL BIOLOGY

A wide range of cells can be cultured on various MC's. Anchorage-dependent and independent cells, transformed or normal cells and fibroblastic or epithelial-type cells can be propagated on the various MC's. Cells cultured on MC's can be used as a substrate for production of animal viruses. Poliomyelitis virus (van Wezel et al, 1980; Montagnon, 1981), Foot and Mouth Disease virus (Meignier et al, 1980), as well as other viruses have been propagated on Cytodex-1 MC cell cultures for industrial vaccine production. The conditions for optimal virus propagation in MC cell culture differ from those required for cell growth (Tyo and Wang, 1981).

Cell MC culture techniques were initially developed for obtaining large amounts of cells for production of viral vaccines or cell products. However, the new culturing techniques were found to be valuable for studies in cell biology. MC culture is a technique which has an enormous potential for the cultivation of animal cells and is currently used in a variety of situations where large amounts of cells, virus, or cell products are required.

6. SUMMARY

The ideal MC's for research purposes should be nonporous and uncharged. This is required in order to avoid non specific adsorption or penetration of various molecules (proteins,

hormones, vitamins, and nucleotides) into the MC's. The polystyrene MC's (nonporous) or the collagen-coated MC's (noncharged) are the most suitable MC's for this purpose.

Commercial companies and research groups still continue investigating and producing new MC's and this, therefore, emphasizes the fact that the available MC's are not optimized for use for all purposes. Each MC has its advantages and disadvantages and should be tested in the culturing system in which it is planned to be used. The selection of a specific MC depends on the cell type, the price of MC, adsorption characteristics of the MC, and the type and purity of the final product.

LITERATURE REVIEW OF HERPES SIMPLEX VIRUSES (HSV)

1. Structure and Composition

Herpes simplex viruses (HSV) are among the most common infectious agents of man. These viruses belong to the family Herpesviridae and are relatively large and complex, consisting of structural elements arranged in concentric layers (Furlong et al, 1972); a) a core consisting of a fibrillar spool on which the DNA is wrapped, b) an icosadeltahedral capsid containing 12 pentameric and 150 hexameric capsomeres, c) variable amounts of globular material asymmetrically arranged around the capsid and designated as the tegument, and d) a membrane or envelope surrounding the entire structure. The envelope, like any membrane, is less rigid and confers upon the complete virus particle a diameter varying from 150 to as much as 200nm (Becker et al, 1968; Kieff et al, 1971).

The DNA's of HSV type 1 and HSV type 2 are linear double-stranded with a molecular weight of $85 \text{ to } 106 \times 10^6$.

The base composition of HSV-1 and HSV-2 is 67 and 69 guanine plus cytosine moles percent (Goodheart et al, 1968; Kieff et al, 1971).

Approximately 50 per cent of the DNA of HSV-1 and HSV-2 is transcribed during productive infection (Frenkel and Roizman, 1972; Roizman and Frenkel, 1973). Assuming that all the RNA transcripts specify proteins, it can be calculated that viral DNA codes the sequence of nearly 55,000 amino acids, that is 49 proteins, accounting for 75 per cent of total genetic information of the virus. Of this number, at least 27 are structural

proteins.

The newly assembled DNA-containing capsid accumulating in the nucleus consists of six proteins ranging in molecular weight from 25,000 to 155,000 (Gibson and Roizman, 1972). The proteins are packaged in layers, those in the outermost layer of the capsid are arranged in the form of an icosahedron containing 162 structural subunits or capsomeres (Wildy et al, 1960). Structurally, the capsomeres resemble hexagonal prisms with a hollow duct running parallel to the long axis. Just before envelopment, the capsid incorporates more proteins (the tegument), which remain tenaciously bound to it, even after the envelope is stripped with detergents (Gibson and Roizman, 1972).

The envelope forms a loose impermeable coat around the virus particle and morphologically resembles a trilaminar membrane. It contains lipids, polyamines and at least 12 glycoproteins (Gibson and Roizman, 1971; Heine et al, 1972). Analyses of the infectivity of the virion and of subviral particles (Roizman, 1969), indicate that the intact enveloped particle is most probably the epidemiologically important infectious unit. Although an intact envelope is not required for a particle to be infectious, capsids extracted from nuclei or obtained by stripping the envelope with detergents are not infectious. Even though the function of the envelope in conferring infectivity to the capsid is still unclear, it is likely that the envelope allows the virus to withstand physical stress and to adsorb to cells better than particles that are not enveloped. Since the glycoproteins determine the immunologic specificity of the virus (Roizman et al, 1973), considerable care must be taken in the selection of

reagents for virus identification and for sero-epidemiologic studies.

2. Immunological Specificity of HSV-1 and HSV-2

The relation between HSV-1 and HSV-2 has been extensively investigated. Although it is relatively easy to differentiate between the two viruses, it is difficult to differentiate clearly between antibodies to HSV-1 and HSV-2, particularly in serum specimens of persons who have antibodies to both virus types (Nahmias et al, 1970). Several types of measurements of the neutralization reaction clearly indicate that an antiserum prepared against HSV-1 neutralizes HSV-2 and vice versa (Plummer, 1973). The specificity of the reaction, defined as the ratio of antiserum titers against homologous and heterologous viruses, may vary from serum to serum (Savage et al, 1972). The antigenic sites reactive with neutralizing antibodies reside in the envelope of the virus. Membranes of infected cells carry the same glycoproteins as those present in the envelope of the virion.

The herpesvirion specifies at least 12 glycoproteins contained in the viral envelope. The question arises whether each of the glycoproteins share in common antigenic sites, or whether some glycoproteins carry antigenic sites unique for each site. Studies on the adsorption of glycoproteins to immunoadsorbent columns (Savage et al, 1972) indicate that antisera prepared in rabbits contain antibodies to all glycoproteins, moreover, all HSV-1 glycoproteins react with anti-HSV-2 serum and vice versa with the same average specificity as that measured in neutralizing tests. On the other hand, type specific antibodies, as assayed in

neutralization or membrane immunofluorescent tests, can be demonstrated by adsorption of serum prepared against one HSV type with cells infected with the other type (Nahmias et al, 1971a,b; Wildy, 1973).

Despite the differences in base composition between the DNA's of HSV-1 and HSV-2, there is at least 47 per cent homology, with 85% matching in base pairs (Kieff et al, 1972). The DNA-DNA hybridization studies suggest that the DNA's of HSV-1 and HSV-2 consist of two sets of sequences (Kieff et al, 1972). One set, which has been designated as the variable sequences, is unique for each virus. The other set, comprising the invariable sequences, is shared in common.

3. Clinicopathologic Aspects

The virus is recovered from the skin or mucocutaneous lesions, and, in rare instances, from blood, brain, spleen, lungs, peripheral leukocytes, and other sites. HSV-1 is associated with the majority of non-genital infections, while infections of genitals and neonates are caused most often by HSV-2. This association, however, is not absolute, either virus type may cause lesions anywhere on the body such as mouth and respiratory tract, lips, eyes, skin, urogenital tract, and nervous system.

The clinical course of an HSV infection can be of varying severity and duration. The incubation period for either primary HSV-1 or HSV-2 ranges from two to 20 days, with an average of around six days. The primary infection may be inapparent or it may be a clinically manifest course. The infection becomes latent. The term "primary" infection should be reserved to denote

the first exposure to either HSV-1 or HSV-2. Hence, a person can only have either a primary HSV-1 or a primary HSV-2 infection, and in such a case, antibody to only one of the two viruses would be demonstrable. Many primary infections are subclinical, partly as a result of being clinically inapparent to the affected person e.g. mouth (Hale et al, 1963), or uterine cervix (Yen et al, 1965; Josey et al, 1966; Josey et al, 1968). When clinically manifest, however, primary infections with HSV-1 or HSV-2 tend, in general, to be more severe with fever, more extensive lesions and local adenopathy, than in persons with prior antibodies to either or both HSV-1 or HSV-2 (Nahmias et al, 1970; Rawls et al, 1971). The latter type of patient might be one who has suffered from recurrent infections with one HSV type (e.g. HSV-1), and may then have acquired for the first time an exogenous infection with the heterologous type (e.g. HSV-2), which could then itself have caused clinically apparent or inapparent recurrences.

Infections due to HSV are among the most common of all virus infections, resulting in a wide variety of disease entities including keratitis, gingivostomatitis, whitlow, genital ulcers, encephalitis, and severe disseminated disease in newborns and immunocompromised hosts (Kibrick, 1980; Nahmias and Visintine, 1976; Whitley et al, 1980).

4. Epidemiology

The epidemiologic patterns of HSV infection became much more meaningful as a direct consequence of the demonstration of HSV-1 and HSV-2, which exhibit a large number of biological, biochemical and antigenic differences (Dowdle et al, 1967; Nahmias and

Dowdle, 1968; Plummer et al, 1970; Roizman et al, 1973; Scher et al, 1982).

Current serologic assays for detecting HSV-1, HSV-2 and dual (HSV-1 and HSV-2) antibodies (Rawls et al, 1969; Nahmias et al, 1970; Aurelian et al, 1970; Duenas et al, 1972) have pointed to the increasing acquisition of HSV antibodies with age, after transplacental antibodies had disappeared, usually within six months after birth. However, only 30 to 50 percent of adults of higher socioeconomic groups have been found to possess HSV antibodies, as compared to 80 to 100 percent of adults of lower socio-economic groups. The increased frequency of HSV antibodies in the lower socioeconomic populations appears to be due to a greater risk of becoming infected with both HSV-1 and HSV-2.

Genital HSV-2, and less commonly HSV-1 infections in pregnant women, have been found to be the major source of transmission of the virus to the newborn (Nahmias et al, 1969; Nahmias et al, 1971; Nahmias et al, 1972). Studies by Nahmias and Visintine (1976) have shown that the risk of transmission of infection is 50 percent when the baby is delivered by the vaginal route but can be decreased to 7 percent by Cesarean section (C-section). Pregnant women with a history of genital herpes have often been subjected to C-section because of the risk of neonatal HSV infection which may occur, even in the absence of obvious maternal herpetic lesions at the time of vaginal delivery (Whitley et al, 1980; Scher et al, 1982;). The majority of cases of neo-natal herpes are in fact acquired from asymptomatic mothers. These patients should be checked weekly for herpes infection after the 34th week of gestation (Committee on Obstetrics, 1980) to exclude the

possibility of asymptomatic genital excretion of HSV and so avoid unnecessary C-section, while reducing the risk of neonatal HSV infection. If the infection is transmitted to the newborn, the child should be cultured for HSV so that appropriate therapy can be used (e.g. Acyclovir, Ara-A).

The infant usually acquires the virus around the time of delivery if membranes have ruptured for more than six hours or on passage through the infected birth canal.

HSV-1 and HSV-2 appear to be transmitted by close personal contact e.g. kissing (Selling and Kibrick, 1964; Wheeler and Cabaniss, 1965; Porter and Baughman, 1965). The viruses can be spread by saliva, and this mode of spread may account for those outbreaks of HSV oral infections reported in closed populations, as well as those occurring in families (Hale et al, 1963). Viral spread via air droplets or via infected skin squames may also occur.

5. Diagnosis of HSV-1 and HSV-2 Infections

5.1. Clinical Diagnosis

Despite the availability of many laboratory aids, the diagnosis of HSV infections is still made in large part on clinical grounds. It so happens that most of the common forms of HSV infections, gingivostomatitis, cold sores, keratitis and dermatitis, can be recognized clinically with close, but not, 100 percent accuracy. Penile and vulvar herpes are not uncommonly mis-diagnosed as chancroid or other venereal diseases, and herpetic cervicitis or vaginitis is infrequently recognized clinically.

Certain HSV infections most often can only be diagnosed with laboratory aids. These include conjunctivitis, chorioretinitis, urethritis, prostatitis, cystitis, central nervous system involvement (encephalitis, meningitis, or myelitis), eczema herpeticum and severe forms of local or disseminated disease involving unusual sites. The findings in these conditions of concomitant skin, genital, mouth or lip lesions does not establish a herpetic cause, since HSV infection of these sites may be recurrences triggered by another process (e.g. bacterial meningitis).

5.2. Laboratory Diagnosis for HSV-1 and HSV-2

5.2.1. Benefits of Rapid HSV Viral Diagnosis

The benefits of rapid HSV diagnosis are numerous. Specific identification of the infection can prevent indiscriminate, expensive, and potentially harmful use of microbials. The diagnosis of the herpetic infections occurring in pregnancy plays an important role in patient care. Management of immunocompromised individuals exposed to herpesvirus infections, control of herpesvirus infections in recipients of organ transplants or neonates infected from their mothers are examples where rapid and specific viral diagnosis or determinations of immunity status are important. Rapid diagnosis of HSV can aid in controlling the spread and identification of sexually transmitted infections. The use of effective antiherpetic agents has emphasized the need for specific, as well as rapid identification of these viruses.

5.2.2. Detection of HSV in Tissue Culture

For over 50 years, the diagnosis of herpetic infection has been carried out by the morphologic examination of cells collected from infected sites, demonstrating the presence of multinucleated giant cells (Tzanck cells) and intranuclear inclusions (Scott and Tokumaru, 1964; Corey et al, 1983). This approach has been of particular value in the detection of asymptomatic herpetic cervicitis since the herpes-associated cells can be readily demonstrated by Papanicolaou smears obtained for routine cancer-screening purposes (Naib et al, 1973).

Although HSV culture is not as rapid as the direct viral examinations, culture is recognized to be the most sensitive technique available for the detection of HSV (Cho and Feng, 1978; Rawls, 1979; Moseley et al, 1981; Corey et al, 1982). This technique offers exquisite sensitivity since small amounts of virus in the specimen, which might be missed by direct examination or other assays, are amplified by replication in a susceptible host, thus positive result can be obtained with a single infectious virion. It is also highly specific, in that only the virus is amplified, thus sensitivity is increased without any loss of specificity. Direct examinations must, of necessity, be aimed at only a few selected agents, but viral cultivation is much broader in scope and has the potential for recovering a wide range of infectious agents. It can detect an unexpected agent depending on the host systems employed.

This technique, even though the most sensitive method for HSV virus detection, has a few drawbacks. It is often cumbersome and time consuming (Rawls, 1980), taking up to 7 days for the detection of small amounts of virus. In clinical situations, this

may be too long for the patient's management or the initiation of therapy. This technique only detects infectious virus, while immunoassay methods can detect HSV, whether viable or not. It also requires the use of appropriate culture systems in that some cell lines will support the growth of HSV better than others. Some immunoassays can also be used even when improper collection methods render the specimens inadequate for use in cell culture techniques.

5.2.3. Rapid and Specific Tests for HSV

Rapid diagnosis can be defined as specific viral identification within 24 hours or, at the very latest, within 48 hours of collection of the specimen. During the past 10 to 15 years much progress has been achieved in rapid viral diagnostic procedures. Much of this progress has been due to the better understanding of immunological and biochemical mechanisms and the significant discriminatory power of antibodies.

Ideally, rapid herpesvirus diagnostic techniques should be relatively simple procedures, providing results within 3 to 4 hours of specimen collection. In some instances this has been achieved. However, even techniques which shorten the number of days required for identification of HSV recovered in cell cultures or which permit more rapid serodiagnosis of infection or determinations of immunity status are also important goals of rapid HSV diagnosis.

A growing need for rapid, reliable laboratory diagnosis of HSV infections has taken place in recent years. This need has arisen from: a) the demonstration of the role of HSV in perinatal infections, b) the recognition of HSV as a sexually transmitted

disease, and c) the availability of effective antiviral chemotherapy for HSV infections.

The emphasis has been on techniques that permit herpesvirus detection directly in clinical specimens, since these avoid the need to cultivate the agents, and can detect herpesvirus in specimens in which the agent is no longer infectious.

All these problems have prompted the utilization of electron microscopy (EM) and assays based on immunological reactions for the rapid demonstration of herpesvirus antigens (Abelson et al, 1969). Utilization of antigen-antibody reactions for this approach to rapid virus detection is based on the fact that most viruses have definable antigens against which specific antibodies can be produced and labelled by fluorescent, radioactive or enzyme tags.

The past decade has seen the development of a variety of methods for the rapid detection of HSV antigens directly in clinical specimens for more rapid and specific identification or demonstration of viral antigens and antibodies. These progresses in rapid HSV diagnosis have made viral examinations much more practical and useful in clinical settings.

The currently used rapid viral diagnostic procedures fall into the following main categories:

- (1) Electron Microscopy (EM)
- (2) Immune Electron Microscopy (IEM)
- (3) Immunofluorescence (IF)
- (4) Enzyme Immunoassay (EIA)
- (5) Radioimmunoassay (RIA)

(6) Biotin-Avidin System (B-A)

(7) DNA Hybridization

5.2.3.1. Electron Microscopy (EM)

Electron microscopic examinations is expensive, and therefore, should be used selectively in HSV diagnosis (Lee et al, 1978; Almeida, 1980; Hammond et al, 1981; Field, 1982).

Although it requires an expensive instrument and highly trained personnel, and can handle only a small number of specimens, EM is particularly useful for rapid identification of HSV in specimens from vesicular lesions. However, EM cannot differentiate between members of the herpesviruses (e.g. cannot differentiate HSV-1 from HSV-2 or cytomegalovirus). Other drawbacks are that HSV must generally be present at a concentration of about 10^6 or more particles per milliliter, and the virus morphology must be preserved well enough to be readily distinguishable from debris.

The advantages of using EM for the identification of HSV is that it is relatively fast, and also that a single examination has the potential for detecting a wide range of viral agents, in contrast to immunologic methods which are limited by the narrow specificity of the antibody probes employed.

5.2.3.2. Immunologic Methods

Immunologic methods for the direct detection of viruses in clinical specimens are based on their interaction with specific viral antibodies, and then looking for evidence of an immunologic reaction between these antibodies and viral antigens in the

specimen. This approach permits detection and specific identification of the virus in a single step. Sensitivity for detection of viruses is greater than that of direct EM because the viral content of the specimen is amplified, either by aggregation or through the use of a label or "tag" on the antibodies used in the system.

I. Immune Electron Microscopy (IEM)

In IEM, the specimen is first reacted with viral antibodies before it is negatively stained and examined by EM. Antibody clumping of the viruses concentrates them, helping in their detection and in their specific identification (Kapikian et al, 1972; Feinstone et al, 1973).

Solid phase modifications of IEM have been developed in which virus is trapped by specific antibody coated grids (Derrick, 1972). Another modification consists of a "double antibody" technique in which viral (antibody coated grid traps virus and a second application of antibody coats and thus facilitates the viewing of the viral particles (Giraldo et al, 1982).

IEM is relatively simple to perform, however, interpretation of results requires considerable experience in distinguishing between spontaneous and antibody mediated aggregation of virus in the specimen (Kapikian et al, 1980).

II. Immunofluorescence (IF)

IF is one of the oldest methods used for the rapid identification of HSV (Berthiaume et al, 1981) and has been the most extensively used in rapid viral diagnosis (Emmons and Riggs,

1977; Gardner and McQuillin, 1980). This method is based on tagging antibody with a fluorochrome (fluorescein isothiocyanate) and demonstrating the antibody complexed with virus in the specimen by microscopic examination under ultraviolet illumination. HSV can be identified by either direct or indirect IF.

In the direct assay, the viral antibody is tagged with fluorescein, and the conjugate is applied to the material to be examined for the presence of virus. This approach requires labeling of specific antibody for each viral agent to be sought, and high-titered antisera are needed for conjugation. However, the method generally shows greater specificity than indirect IF and requires fewer manipulations and controls.

In the indirect IF, the unknown specimen is treated with known unlabeled viral antiserum, then labeled immune globulins directed against the animal species in which the viral antiserum was prepared. Indirect IF may be more sensitive than the direct method since the intermediate antiserum increases the surface area available for attachment of the labeled antibodies. A major advantage of the indirect method is the fact that it requires labeled antibodies only against immunoglobulins of certain animal species and not against individual viruses. However, since additional reagents are introduced in the reaction system, the indirect assay may be less specific than the direct method.

The success of IF staining for the detection of HSV depends to a large extent on the quality of the specimen; it must contain adequate numbers of virus-infected cells that are free from debris or contaminating organisms, that might produce nonspecific

fluorescence. In sampling of HSV vesicular lesions it is necessary to prepare smears of epithelial cells collected from the base of the lesion onto a swab or needle, vesicular fluids or crusts are not suitable for IF examinations.

An important advantage of IF staining, compared with viral isolation, is its ability to demonstrate HSV antigens in specimens taken late in the course of infection when infectious virus is no longer present either as a result of complexing with antibody or loss of infectivity (Fulton and Middleton, 1975; Schmidt et al, 1980; Berthiaume et al, 1981). The advantage of IF over enzyme immunoassays is that it is faster, but it is generally less sensitive.

The disadvantages of IF staining are the following: a) it is highly dependent upon the quality of the reagents used and requires a fluorescence microscope. Moreover, since there is no objective end point, diagnosis by IF microscopy requires experience and interpretation, b) technical problems, including the collection and preparation of a suitable specimen, autofluorescence, and nonspecific binding of antibody, and c) the inability to automate the process and thus to permit the evaluation of large numbers of specimens with high efficiency. In spite of these disadvantages, however, IF remains the most commonly rapid technique practised in diagnostic laboratories today.

III. Enzyme Immunoassays (EIA)

For EIA, antibody is labeled with an enzyme rather than with fluorescein, and the labeled antibodies bound to virus, or

virus-antibody complexes, are detected through the addition of a substrate upon which the enzyme acts to produce a colored product. In theory, EIA should be more sensitive than IF since the enzyme label can have a continuous action on the substrate, producing increasing amounts of reaction product, which amplifies the initial reaction at the site where virus is present in the specimen.

a) Immunoperoxidase Staining (IP) .

This technique uses horseradish peroxidase as the enzyme label and any one of several substrates which produce an insoluble reaction product (Kurstak et al, 1975; Benjamin, 1979).

IP staining has been applied to rapid diagnosis of HSV in the same manner as IF staining. The advantage of using IP staining over IF staining include the following: a) the potential for greater sensitivity due to amplification of the reaction by continuous action of the enzyme label on the substrate, b) the ability to use an ordinary light microscope, and c) the ability to make permanent preparations.

The disadvantages include the need for a few more manipulations, and the fact that certain specimens will contain endogenous peroxidase activity that can cause nonspecific reactivity. However, methods are available for inactivating endogenous peroxidase (Pearson et al; 1979).

IP staining for direct examination of specimens has had the greatest use and success in detecting herpes simplex viral antigens in brain and lesion material (Benjamin and Ray, 1975; Benjamin, 1977; Moseley et al, 1981; Pearson et al, 1979). In a

comparative study, indirect IP staining was shown to be more sensitive than direct IF staining for detection of genital HSV infections (Moseley et al, 1981).

b) Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA technique as outlined by Engvall and Perlman (1972), is simple, and was initially used to quantitate antigens and subsequently antibody. The principles on which they were based were similar or even identical to those of radioimmunoassay (RIA). They therefore necessitated the use of a solid phase to separate the free antigen or antibody from the specific antigen-antibody complexes and were consequently termed heterogenous EIA's.

Various EIA's for the detection of viruses have been developed using substrates that produce soluble reaction products. This category of tests has sometimes been referred to as enzyme-linked immunosorbent assays (ELISA). The colored products can be quantitated by spectrophotometry or detected visually.

In the direct procedure, viral capture antibody is coated onto a solid phase such as polyvinyl or polystyrene microtiter cups or polystyrene beads. The test specimen is added, and virus is bound to the "capture" antibodies. After washing, the bound virus is detected through the addition of enzyme-labeled "detector" virus antibody, followed by substrate.

In the indirect procedure, the "detector" viral antibody is unlabeled, and it must be from animal species different from that of the "capture" antibody. This is followed by a labeled antibody directed against the species in which the detector antibody was

produced. As with IF staining, the indirect method may have greater sensitivity than the direct because of an increased surface area of reactants which bind the labeled antibody. It also has the advantage of requiring only anti-species-labeled immunoglobulins. However, the need to use viral antisera prepared in two different species may be a drawback. The direct procedure using fewer reagents may have greater specificity (Hammond et al, 1982; Rubenstein and Miller, 1982).

Advantages of these EIA assays are their sensitivity, accuracy and objectivity. Also a large number of specimens can conveniently be tested in a short time (Engvall and Perlman, 1972; Rosenthal et al, 1973; Walsh et al, 1979; Yolken, 1982; Goldstein et al, 1983). They can be performed on specimens in which viral infectivity has been lost or which do not contain intact infected cells. In contrast to RIA's, EIA assays use stable reagents and do not require radioisotopes. The sensitivity of some of these assays has been shown to be 1 to 10ng of viral protein. However, the sensitivity of EIA's, in terms of virus concentration required for identification, is approximately 10^4 to 10^5 plaque forming units/mL (Yolken, 1982).

The sensitivity, specificity, reproducibility, and precision of any EIA depends on its design and even more on the choice of solid phase, antibody, enzyme, enzyme measurement method, enzyme-antibody conjugate, and number of replicates. It should be stressed that, irrespective of the conjugate or solid phase used, EIA's always involve nonspecific adsorption to a degree depending on the area of the coated surface used in the assay. At present, this nonspecific adsorption constitutes one of the two main

factors limiting the accurate measurement by EIA of extremely small amounts of antigen. Nonspecific binding of viral antibodies to bacteria or bacterial products in the specimen may cause false-positive results (Yolken, 1982). Improper washing steps can result in nonspecific binding of reagents or in cross-contamination between specimens. Another remaining potential hazard of EIA procedures, however, involves the use of carcinogenic materials as substrates.

EIA's have been applied to the detection of HSV, and sensitivity compared with cell culture isolation has ranged from about 50 to 80%.

c) Radioimmunoassay (RIA)

Antibodies labeled with a radioisotope, usually ^{125}I , are used in the same way as enzyme-labeled antibodies, in either direct or indirect systems, to detect viral antigen in clinical materials (Forghani, 1979). Binding of detector antibodies is demonstrated by counting the radioactivity of the specimen in a gamma counter after completion of the reaction and demonstrating a significant increase in radioactivity over that of negative controls.

EIA assays applied to the direct detection of viral antigens in infected tissues have been troubled with difficulties due to high background activity caused by endogenous enzymes in the tissues. RIA avoids these problems and shows greater viral specificity in the examination of tissue specimens (Forghani, 1979). Overall, the RIA enjoys the same advantages that were mentioned for the EIA's. However, in contrast to EIA the

disadvantages are the following: a) the need for radioisotopes, b) the need for relatively expensive counting and monitoring devices, and c) the use of expensive reagents with short half lives, making it necessary to test large numbers of specimens in order to make efficient use of expensive assay kits.

d) Enzyme Immunofiltration (EIA-F)

This is a variant of the solid-phase assay already discussed. In this assay method, rather than virus-specific capture antibodies, filters are used for the nonspecific capture and immobilization of antigen. A specially designed filter manifold consisting of 96 individual filters in the wells of a modified microtiter plate rests upon a vacuum manifold. Each well has a tiny hole (0.5mm diameter) in its bottom. When an aliquot of a specimen is placed in the well and the vacuum is applied, whole cells, cell debris (greater than 1.5µm), and even cell-free virus are trapped on the filters. When a solution is added to the well with the vacuum off, surface tension keeps the fluid from draining through the tiny hole in the bottom, and the well thus serves as an incubation chamber. Application of the vacuum draws the fluid through the filter and out of the well in one or two seconds. All 96 filters can thus be rapidly washed in seconds by the addition of buffer to each well. The viral components remain immobilized during numerous incubations and washes. The immobilized viral components can then be detected and quantitated by any of a number of direct and indirect techniques (most commonly used is the indirect method) involving radiolabeled or enzyme-linked reagents.

This assay has been utilized for the detection and

quantitation of both antigens and antibodies (Cleveland et al, 1979; Redfield et al, 1981; Richman et al, 1981; Richman et al, 1982; Cleveland et al, 1982). The advantages to this assay compared to other EIA's is the fact that because the antigen capture is nonspecific, there is no need to prepare plates with specific capture antibodies or to incubate the specimen in the plate for capture of the desired antigen. It is also faster and more flexible than most other EIA's (Cleveland et al, 1982). The disadvantages are the same as with other EIA's already discussed.

e) Biotin-Avidin System (B-A)

The B-A system was originally described for immunochemical staining of tissues by Bayer and Wilchek in 1975. In this system, biotin-labeled antibody may be substituted for the usual detector antibody in sandwich-type EIA's. After washing, avidin coupled with an enzyme is added. Since avidin has four active binding sites, increased sensitivity can be achieved by using unlabeled avidin attached to the biotin-labeled antibody, which is then attached to the antigen. This, in turn, is detected by adding biotin labeled with an enzyme, followed by the enzyme substrate (Guesdon et al, 1979).

This system offers several advantages: a) avidin, a basic glycoprotein of approximately 68,000 molecular weight, has a high affinity for biotin (Green, 1963), b) each avidin molecule can bind 4 biotin molecules, c) biotin can be covalently coupled to antibody to a high specific activity without affecting the antigen binding capacity (Guesdon, 1979).

The B-A system has been used to enhance immunocytochemical

staining (Bayer and Wilchek, 1975) and IF (Heitzmann and Richards, 1974; Berman and Basch, 1980). Guesdon et al (1979) were the first to report the incorporation of the B-A system into ELISA techniques. In a B-A system, the biotinylated antibody is used for specific interaction with antigen in concert with avidin and biotinylated enzyme in that order. Alternatively, avidin can be conjugated directly to an enzyme and be used as the detection probe.

B-A system has been shown to afford more sensitivity than that observed in the standard ELISA (Kendall et al, 1983). This increased sensitivity may be attained as a result of several advantages inherent in the B-A system. Because avidin has 4 binding sites for biotin, theoretically it is possible to couple more biotinylated enzyme per avidin molecule than enzyme per antibody molecule without a concomitant loss of specific binding activity (Berman and Basch, 1980). Secondly, the high affinity of the bond between biotin and avidin insures that the B-A complex, once formed, is stable in later washes and incubations. In addition, the reaction is complete in a shorter time than is required for antigen-antibody interaction. Lastly, biotinylation of many proteins has been shown to have no effect on the protein's biological activities (Guesdon et al, 1979). This is in contrast to the glutaraldehyde procedures used to conjugate enzyme to immunoglobulins which are used as reagents in the standard ELISA techniques (Boorsma and Kalsbeek, 1975).

The B-A ELISA offers four distinct advantages over the more commonly used method of HSV isolation in tissue culture. First, the B-A ELISA is rapid, requiring only 4 hours to complete when

antibody-coated plates are available, whereas tissue culture isolation may take as long as 7 days. Second, accurate determinations can be obtained from contaminated specimens. Third, the B-A ELISA detects noninfectious as well as infectious virus. Fourth, the evaluation is objective rather than subjective.

This system is faced with the same disadvantages as other immunoassay systems already discussed.

IV. DNA Hybridization Technique

DNA hybridization has been used during the past 15 years to explore the structure and expression of cellular and viral genes. It is based on the fact that single-stranded nucleic acids can form stable duplexes if they encounter complementary sequences under appropriate conditions (Marmur and Doty, 1962).

If a clinical specimen is to be analyzed for the presence of viral nucleic acids, different techniques of nucleic acid hybridization may be applied. Regardless of the technique to be applied, DNA has to be extracted from the clinical specimen by conventional means, including lysis of cells, digestion of proteins with proteinase, and repeated extraction by phenol or phenol-chloroform mixtures and the DNA bound to nitrocellulose filters. Additionally, the viral DNA or RNA in question has to be available as a labeled hybridization probe. Cloning of viral DNA in a bacterial host provides almost unlimited quantities of the probe (Brandsma and Miller, 1980; Chou and Merigan, 1982; Scotto et al, 1982; Stalhandske and Pettersson, 1982; Weller et al, 1982; Flores et al, 1983; Redfield et al, 1983; Virtanen et al 1983).

The nucleic-acid probe can be labeled by any of a number of techniques. The most common is nick translation, in which DNAase and DNA polymerase are used to nick and repair an agent-specific DNA molecule and, in the process, to incorporate into it ^{32}P -labeled deoxynucleoside triphosphates (Rigby et al, 1977).

After hybridization of such a probe on filters containing DNA from the clinical specimen, the bound radiolabel is detected by autoradiography.

This technique has been used recently for the detection of HSV in clinical specimens by Richman et al (Richman et al, 1984). They found that the assay was 78 per cent as sensitive as viral culture with swabs of HSV-2 infected genital lesions and 90 per cent as sensitive as viral culture of HSV-1 infected eye lesions (Redfield et al, 1983; Richman et al, 1984). These results demonstrate the feasibility of using this approach as a rapid technique for HSV diagnosis. However, this method has its drawbacks: a) it requires some expertise and an advanced technology and is thus hardly applicable to diagnostic routine in laboratories, b) the sensitivity for HSV detection is equivalent to that of EIA's, c) the ^{32}P -labeled probes have short shelf lives and are usable for only 2 weeks, they entail problems with radiation exposure and disposal, d) they are expensive.

The advantages of this technique is the extreme sensitivity that is due to the high level of specific activity of the probes.

6. Serological Diagnosis

In first episodes of genital herpes, fourfold or greater increases in neutralizing or complement fixing antibodies between

acute and convalescent sera can be useful for documenting recent infection but serodiagnosis has little value for the diagnosis of recurrent genital herpes (Nahmias et al, 1970; Plummer, 1973; Smith et al, 1972). Because only 5 percent of patients with recurrent genital herpes show fourfold or greater rises in either anti-herpes simplex virus complement fixing or neutralizing antibody, viral isolation or antigen detection techniques are quicker and much more sensitive methods for confirming recurrent HSV infection. The measurement of serum antibodies to HSV has been useful in epidemiologic surveys evaluating the prevalence of past HSV infections.

7. Summary

From this review, it is apparent that there has been much interest and activity in the development of techniques for rapid HSV diagnosis which would allow successful intervention in the treatment of patients or their contacts or in the control of herpesvirus disease in the community. In the development of rapid HSV diagnosis the greatest emphasis has been on techniques that permit herpesvirus detection directly in clinical specimens, since these avoid the need to cultivate the herpesvirus and can detect them in specimens in which it is no longer infectious.

The use of immunoassays for more rapid identification of HSV isolated in the laboratory host systems and for the selective detection of viral antigens in inoculated cell cultures, possibly before the agents produce an observable effect, has been an important advance in herpes viral diagnosis.

However, despite the ease with which immunoassays are

performed, and the accurate and objective data they yield in as little as two hours, immunoassays are still too insensitive to be generally adopted. Ideally, a rapid HSV diagnostic technique would at least be as sensitive as tissue culture isolation, but all the available assays including DNA hybridization currently fall short of this goal. Isolation of HSV in culture is currently the most sensitive and accurate method of detecting HSV.



PLAN OF STUDY

Our studies were divided into 4 phases. In phase I, we studied and compared the growth characteristics and metabolic parameters of HEP-2 and HFL cells grown on microcarriers (Cytodex-3) with that of conventional monolayers in polystyrene tube cultures. In phase II we determined the sensitivity of the EIA-F assay for the early detection of HSV antigens in MC tube cultures over a wide range of multiplicities of infection by comparison with direct IF and the appearance of CPE. Phase III of our investigations involved the determination of EIA-F sensitivity for the direct detection of HSV antigens in clinical specimens by comparison with a commercially available EIA kit and cell cultures. Phase IV consisted of a field evaluation of the MC/EIA-F system developed in phase II for the detection of HSV antigens in MC tube cultures using wild HSV isolates by comparison with classical monolayers.

MATERIALS AND METHODS

1. Cells

The following anchorage-dependent cells (ADC) were used in our studies:

1.1 HEP-2 cells - this is a human epithelial cell line derived from a case of epidermoid carcinoma. A seed culture of these cells was originally received by us from the American Type Culture Collection (ATCC; Rockville, Md., U.S.A.) at passage number 353. As stock cultures the cells were routinely cultivated as monolayers in 75cm² plastic culture flasks (Flow Laboratories, Rockville, Md., U.S.A.) using Eagle's minimal essential medium (MEM) in Earle's base (Flow Labs.). The medium was supplemented with 10% virus- and mycoplasma-free fetal calf serum (FCS; Flow Labs.). Each monolayer was trypsinized using 2.0 mL of a mixture of 0.25% trypsin (Flow Labs.) and ethylenediamine-tetracetic acid (EDTA; 0.02%) in Ca²⁺- and Mg²⁺- free phosphate buffered saline (PBS). A split ratio of 1:4 was generally used for the passage of the cells.

1.2 HFL cells - this is a human diploid cell line, derived from the lungs of a 5cm long fetus. A seed culture of these cells was originally received by us from the Children's Hospital of Eastern Ontario, Regional Virology Laboratory (Ottawa, Ontario, Canada), at passage number 12. Stock cultures were cultivated as described for HEP-2 cells. Each monolayer was trypsinized and processes as for HEP-2 cells.

1.3 MRC-5 cells - this is a human diploid cell line, derived from an embryonic lung. A seed culture of these cells was originally received by us from the ATCC (Rockville, Md., U.S.A.) at passage number 18. Stock cultures were cultivated as described for HEP-2 cells. Each monolayer was trypsinized and processed as for HEP-2 cells.

2. MICROCARRIERS (MC)

All our studies were carried out using cytodex-3 MC's (Pharmacia Fine Chemicals, Uppsala, Sweden). The reasons for this choice were stated previously (see Microcarrier Literature Review).

Cytodex-3 MC's consist of a surface layer of denatured collagen covalently bound to a matrix of cross-linked dextran. The amount of denatured collagen bound to the MC matrix is approximately $60\text{ug}/\text{cm}^2$. The dry MC's, used at a concentration of $3\text{mg}/\text{mL}$, were added to a siliconized glass vial and were rehydrated in 15 to 30 mL of Ca^{2+} - and Mg^{2+} -free PBS for at least 3 hours at room temperature with occasional gentle agitation. The supernatant was decanted and the MC's were washed once with gentle agitation for a few minutes in fresh Ca^{2+} - and Mg^{2+} - free PBS. The PBS was discarded and replaced with fresh Ca^{2+} - and Mg^{2+} - free PBS and then the MC's were sterilized by autoclaving (115°C for 15 minutes at 15 p.s.i.). Prior to use, the sterilized MC's were allowed to settle, the supernatant was removed and the MC's washed in 5 to 10 mL of

prewarmed (37°C) culture medium. This wash reduced the dilution of the culture medium by PBS trapped between and within the MC's. The MC's were allowed to settle, the supernatant culture medium removed, and the MC's were resuspended in a small volume of culture medium and transferred into spinner flasks (SF; Bellco Glass, Inc.). The cytodex-3 MC's rehydrated in PBS gave a wet bead size of approximately 133 to 215µm diameter.

3. MC CULTURE IN BELLCO SF's

All glassware (pipets, cylinders, SF's, etc...) to be used in the procedures were siliconized in order to prevent the MC's from sticking to the glass. The siliconizing fluid used was 10% Prosil-28 (PCR Research Chemicals). A small volume of this agent was added to the clean culture vessels, all surfaces coming into contact with the MC's were wetted. Excess fluid was drained from the vessel and then the surfaces allowed to dry. All the glassware was washed thoroughly with distilled water and sterilized by autoclaving.

One hundred milliliter SF's (Bellco Glass, Inc.) were initiated by the addition of 3mg/mL of cytodex-3 MC concentration (this is equivalent to 1.2×10^6 MC's/SF) and 30 mL (1/3 final volume) of Eagle's MEM in Earle's base (Flow Labs.). The medium was supplemented with 10% virus- and mycoplasma-free FCS (Flow Labs.). The concentration of MC's used (3mg/mL) is usually the optimal concentration for general MC culture and results in the greatest proportion of cells attaching to MC's. Freshly trypsinized HEp-2 cells were inoculated at a density of 5 x

10^4 cells/mL. The SF's contained a suspended, magnetic-spin bar and the cultures were stirred for 2 minutes every 30 minutes interval at a speed of 30 r.p.m. After 6 hours of continuous stirring, the volume of the culture in the SF's was increased to its final volume of 100 mL with prewarmed (37°C) culture medium. The culture was equilibrated by the addition of an atmosphere of 95% air:5% CO_2 and the stirring was commenced at a speed of 60 r.p.m. (this speed was sufficient enough to keep the MC's in suspension), and incubated at 37°C on a magnetic stirrer (Bellco MC magnetic stirrer, Bellco Glass, Inc., Vineland, New Jersey, U.S.A.).

PHASE I

4 MC TUBE CULTURES

4.1 Established from SF's

After 48 hours of growth of the HEP-2 cells in the SF's, or when the MC's reached 25% confluency (equivalent to 5 to 10 HEP-2 cells/MC) the culture medium was replaced with Dulbecco's MEM in Earle's base (Flow Labs.). The medium was supplemented with 2% of virus- and mycoplasma-free FCS (Flow Labs.). The polystyrene tube cultures (Lux, Lab-Tek Division, Miles Laboratories, Inc., Napierville, Ill., U.S.A.) were prepared by taking 2 mL MC suspensions from the SF's and delivering it into the tube cultures (equivalent to approximately 10^5 cells/tube). Half of the tube cultures were incubated stationary, slanted at a 5° to 10° angle. The other half was incubated on a rotating drum (O.H. Johns Scientific, Lab-Line Instruments Inc., Melrose Park, Ill., U.S.A.) at a speed of 30 r.p.m. All the tube cultures were

incubated at 37°C.

The stationary and rotating MC tube cultures were assessed for cell yield, pH, glucose utilization, oxygen (O_2) and carbon dioxide (CO_2) on days 0, 1, 3, 5, and 7 of culture.

At the specified days, the MC's in the tube cultures were allowed to settle, the supernatant medium was removed and used for the measurement of pH, O_2 , CO_2 (ABL-2 Acid-Base Laboratory, Radiometer, Copenhagen, Denmark), and glucose concentration (Glucose System 1 Analyzer, Beckman Instruments, Inc., Fullerton, Ca., U.S.A.). The MC's in the cultures were washed briefly using 1.0 mL of a mixture of 0.25% (w/v) trypsin (Flow Labs.) and EDTA (0.02%) in Ca^{2+} - and Mg^{2+} - free PBS.

The MC's were allowed to settle and the trypsin was removed. One milliliter of the trypsin was added again to the MC's in the tube cultures and incubated for 15 minutes at 37°C. The tubes were agitated occasionally in order to facilitate trypsinization. The MC's were allowed to settle and the supernatant (containing the trypsinized cells off the MC's) was transferred into a clean sterile polystyrene tube culture (Lux, Lab-Tek Division). The MC's in the tube cultures were again washed with 2.0 mL of culture medium (to collect any remaining trypsinized cells), the MC's allowed to settle and the supernatant removed and pooled with the first supernatant. The trypsinized pool of cells were centrifuged, the supernatant medium was discarded and the pelleted cells were resuspended by adding 1.0 mL of Ca^{2+} - and Mg^{2+} - free PBS. The cells were counted on a hemacytometer by the trypan blue exclusion method (Patterson, 1979).

4.2 Established by direct seeding of cells and MC's

In this part of our investigations, we studied the growth of HFL and HEp-2 cells in MC tube cultures established by direct seeding of cells and MC's without prior growth in SF's.

To sterile polystyrene culture tubes (Lux, Lab-Tek Division) we added 2.0 mL of Eagle's MEM in Earle's base (Flow Labs.). The medium was supplemented with virus- and mycoplasma-free FCS (Flow Labs.). To each tube we then added 6mgm of freshly hydrated sterile cytodex-3 MC's. HFL and HEp-2 cells routinely cultured as monolayers in 75cm² plastic culture flasks (Flow Labs.) using Eagle's MEM in Earle's base (Flow Labs.) and supplemented with 10% FCS were trypsinized, and the cells used to seed the MC tube cultures at an inoculation density of 2×10^5 cells/tube. The MC tube cultures were again incubated stationary, slanted (5° to 10° angle) in a 37°C incubator.

4.3 Established by direct seeding of cells

Here we compared the growth of HFL cells growing on MC's in tube cultures (described above) and growing as normal monolayers in tube cultures. Tube cultures with HFL cells were set up as above except that the MC's were omitted from the tube cultures.

PHASE II

5.1 Constitution of an HSV-1 and HSV-2 Stock

Lesion swabs sent to the Regional Virology Laboratory, Children's Hospital of Eastern Ontario (C.H.F.O.), Ottawa, Canada, in transport medium (Virocult, Medical Wire and Equipment Co. Ltd., Corsham, Wilts, England) were expressed into 2.0 mL of inoculation medium (Hanks' Basic Salt Solution containing 0.2%

bovine serum albumin (BSA), HEPES (N'-2-Hydroxyethyl-piperazine N'-Ethanesulfonic Acid) 21.5mM, amphotericin B (8.6ug/mL), gentamycin sulphate (213ug/mL), and penicillin G (8.55×10^2 IU/mL). Tube cultures originally seeded with 2×10^5 MRC-5 cells and 2.0 mL of Eagle's MEM in Earle's base (Flow Labs.) and supplemented with 10% FCS, were inoculated with 0.1 mL of the inoculation medium containing the swab extract. When approximately one half of the cells in the tube cultures showed cytopathic effect (CPE), after 24 to 48 hours, the tube cultures were stored frozen at -20°C . At the time of use the tubes containing isolated strain 83C-2650 (HSV-1) and 83C-4420 (HSV-2) were thawed, the contents swirled on a vortex mixer and inoculated into 75cm^2 plastic culture flasks containing MRC-5 cells to produce stock virus at a concentration of 2.5×10^7 and 1×10^7 plaque forming units (PFU/mL) respectively.

The titration of the stocks was carried out by the plaque assay technique (Kaplan, 1957; Russell, 1962; Rapp, 1963). Vero cells in a 75cm^2 plastic culture flask (Flow Labs.) in Eagle's MEM in Earle's base (Flow Labs.), supplemented with 10% FCS, were obtained from the Regional Virology Lab. (C.H.E.O.). The monolayer was trypsinized using 2.0 mL of a mixture of 0.25% trypsin and EDTA (0.02%) in Ca^{2+} and Mg^{2+} -free PBS and 12-well plastic plates (Costar, Cambridge, Mass., U.S.A.), seeded at an inoculation density of 1.0×10^5 Vero cells/well. The plates were incubated in the presence of 5% CO_2 in a CO_2 incubator (Shel-Lab, Sheldon Manufacturing Inc., Portland, Oregon). The monolayers were generally ready for plaque assay within 48 hours of seeding. The medium was removed from the

wells, each well received 0.1 mL of the desired inoculum, the cultures were adsorbed at 37°C for 1 hour, and the inoculum dispersed every 15 minutes. Each of the control wells received 0.1 mL of PBS. The overlay medium consisted of MEM, 0.6% agarose type II (Sigma Chemical Company, St. Louis, Mo., U.S.A.), 5% FCS (non-inactivated; Flow Labs.), DEAE-Dextran (Sigma Chemical Co.), and glutamine (Sigma Chemical Co.). After 4 days incubation at 37°C, the cultures were fixed overnight in a 1:10 formalin (J.T. Baker Chemical Co.)-saline mixture. After the removal of the fixative and overlay medium, the monolayers were washed in tap water and then stained with a 1% aqueous solution of crystal violet.

5.2 Preparation of HSV Infected Cells for EIA-F

Plastic culture flasks (75cm²; Flow Labs.) were seeded with 3.0×10^6 MRC-5 cells using Eagle's MEM in Earle's base (Flow Labs.). The medium was supplemented with 10% FCS. The flasks (50 to 75% confluent) were infected with HSV-1 and HSV-2 at an MOI of 1. When all the cells showed CPE (24 hours), the plastic flasks were scraped with a rubber policeman. One uninfected control flask was also trypsinized to obtain a non-infected cell control. The non-infected and infected cell suspensions were centrifuged and resuspended in 10 mL of culture medium. The cell suspensions were used for the checkerboard titration of the group HSV monoclonal antibody (H3-30-1-B-3) produced at the Laboratory Centre for Disease Control (L.C.D.C.), Health and Welfare, Canada, and the antimouse peroxidase labeled conjugate (Zymed Inc., Burlingame, Ca., U.S.A.) to be used in the enzyme immunofiltration assay (EIA-F).

5.3 Titration of Group HSV Monoclonal Antibody

A group HSV monoclonal antibody (H3-30-1-B-3), produced at L.C.D.C., was used for our assay. This was mouse ascites fluid containing antibodies to the IgG₁ subclass, reacting with a 100,000 dalton protein. When tested by standard indirect IF with both HSV-1 and HSV-2 infected Vero cells, this monoclonal antibody had an optimal titer of 1:800 against both HSV-1 and HSV-2. To determine the optimal dilution of the monoclonal antibody to be used in our EIA-F assay, two fold dilutions of the group monoclonal antibody starting at 1:250 were tested against HSV-1 and HSV-2 infected MRC-5 cells and non-infected control cells. The antibody showed significant binding (specific absorbance of 0.139, and binding ratio greater than 34.75) up to a dilution of 1:256,000, with both HSV-1 and HSV-2 infected cells. The antibody was used at a final dilution of 1:32,000 in the EIA-F assay.

5.4 Titration of Enzyme Conjugated Reagents

Horseradish peroxidase labeled goat anti-mouse IgG, gamma chain specific (Zymed Labs.) purified by affinity chromatography was used in the EIA-F assay. To determine the optimal dilution to be used in the assay, two fold dilutions of the conjugate, starting at 1:250 were tested in a checkerboard titration against HSV-1 and HSV-2 infected MRC-5 cells and non-infected control cells. The conjugate showed the lowest background (absorbance at 0.010) and significant binding (absorbance at 0.139) between dilutions of 1:2000 and 1:4000, with both HSV-1 and HSV-2 infected cells. The conjugate was used at a final dilution of 1:3000 in the EIA-F assay.

5.5 Enzyme Immunofiltration Assay (EIA-F)

The procedure described by Cleveland et al (1979) was followed using the group HSV monoclonal antibody (L.C.D.C.) and peroxidase conjugate (Zymed Labs.) at dilutions of 1:32,000 and 1:3,000 respectively. The assay was performed in a 96-well microplate filter manifold (V&P Enterprises, San Diego, Ca., U.S.A.) containing a glass fiber disc and a drain hole in each well. The filters were conditioned for 10 minutes at 37°C using 0.1 mL fetal bovine serum buffer (FBS; PBS containing 10% FCS, 1% bovine serum albumin (BSA), 0.3% gelatin and 0.01% thimerosal) and the fluid removed by vacuum application. The next step consisted in the addition of antigen: a) in the case of the phase II experiments, frozen (-20°C) MC tube cultures were thawed, resuspended by vortexing and 50 uL of the suspension was added to 4 or 8 replicate wells, b) in phase III experiments 200 uL of preparation medium containing the swab extract was added to the wells, and c) in phase IV, 300 uL of MC suspension from infected tube cultures kept at 4°C was added to the wells. The fluid was removed by vacuum and the MC's containing the infected cells and other particulate materials were trapped on the filters. All the wells were washed 3 times with 0.3 mL of gelatin buffer (PBS containing 0.3% gelatin and 0.01% thimerosal). Fifty uL of the group monoclonal antibody at a final dilution of 1:32,000 was added to each well and the microplate incubated for 1 hour at 37°C. The fluid was removed by vacuum, and the filters washed 3 times with gelatin buffer. Then, 50 uL of the conjugate at a final dilution of 1:3,000 was added to each well and the microplate incubated for 30 minutes at 37°C. The fluid was again removed by vacuum and the filters washed 3 times

with gelatin buffer. The drain holes at the bottom of the plates were sealed with parafilm using a plate sealing device (V&P Enterprises), then 200 μ L of substrate buffer [20mg O-phenylenediamine (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and 6 μ L of 30% hydrogen peroxide in 30 mL of citric-acid-phosphate buffer (pH 5.0: 0.05M citric acid, 0.1M sodium phosphate)] were added to all the wells. The microplates were held in the dark for 30 minutes at room temperature (22° to 27° C), and then 100 μ L of 2M H_2SO_4 was added to each well. 100 μ L of the fluid from each well was transferred to a one-half area EIA plate (A/Z EIA plate, Costar, Cambridge, MA.). The absorbance was measured at 488nm with a Model MR 600 Microplate reader (Dynatech Laboratories, Alexandria, Va., U.S.A.).

5.6 Inoculation of MC Tube Cultures With HSV-1 and HSV-2

The growth of MRC-5 cells on the MC's in tube cultures was observed with an inverted microscope, and at 50 to 75% confluence, 12 replicate MC tube cultures were inoculated with HSV-1 and HSV-2 at different MOI's ranging from 10^{-5} to 10^1 . The MC's were sedimented by gravity with the tubes vertical, and the medium carefully removed. The inoculum (0.1 mL of HSV-1 and HSV-2) was added to the sedimented MC's in the 12 tube cultures and left to adsorb for 1 hour at 37° C. The cultures were washed twice with 0.01M PBS (pH 7.4) to remove any non-adsorbed virus and then added 2.0 mL of Dulbecco's MEM in Earle's base (Flow Labs.) supplemented with 2% FCS to each tube. The MC tube cultures were incubated stationary, slanted (5° to 10° angle) at 37° C. At 8, 18, 24, 48, 72, and 96 hours after viral

inoculation, the degree of CPE present on the MC's and the islands of cells growing on the wall of the tubes, was assessed. At each of the above times, one infected tube was processed for HSV-1 and HSV-2 direct IF and the other tube culture was frozen at -20°C to be assayed by EIA-F. Also, one replicate uninfected control MC tube culture was trypsinized and cell counts were obtained by the trypan blue exclusion technique in a hemacytometer (this was done to give us an indication of the number of cells/well we could expect in the EIA-F assay at the different time periods stated above). A second replicate uninfected control MC tube culture was frozen at -20°C for EIA-F.

5.7 Direct Immunofluorescence for HSV-1 and HSV-2 Antigens

The Micro-Trak (Syva Co., Palo Alto, Ca., U.S.A.) HSV-1 and HSV-2 Culture Confirmation Typing Test,⁴ which is a direct IF test was used for the detection of HSV antigen. This test utilizes fluorescein-labeled monoclonal antibodies; for HSV-1, a monoclonal antibody which reacts with the HSV-1 specific glycoprotein C complex is used, and for HSV-2, a monoclonal antibody that reacts with an HSV-2 specific 140,000 dalton protein is used. Each of these reagents contains fluorescein-labeled, purified murine monoclonal antibodies against HSV-1 or HSV-2 and an Evan's Blue counterstain in a protein-stabilized buffer solution (pH 7.4). At the different time periods one infected MC tube culture was trypsinized to obtain cell suspensions. Two 5mm wells on 8 well multitest slides (Flow Labs.) were filled with 0.03 mL of cell suspension, two wells per slide were filled with non-infected cells to serve as negative controls. The preparations were air dried and fixed in acetone for 20 minutes at room temperature.

The slides were allowed to air dry and then stained with the fluorescein-labeled HSV-1 or HSV-2 monoclonal antibodies by adding 0.03 mL of the reagent to the wells. The slides were incubated for 30 minutes at 37°C in a covered moist petri dish. The slides were rinsed gently in distilled water and the excess water was blotted. The slides were mounted and observed for fluorescence with a U.V. microscope at 40X (Leitz Laborluk K, Ernst Leitz Ltd., Midland, Ontario, Canada).

PHASE III

6.1 ORTHO Enzyme Immunoassay (O-EIA)

The ORTHO HSV antigen ELISA test system (ORTHO Diagnostic Systems, Inc., U.S.A.) is an EIA kit for the detection of HSV-1 and HSV-2 in clinical specimens. This test utilizes the double antibody, or "sandwich" technique. The system may be used to detect HSV antigen in direct specimen material or in inoculated cell cultures (culture amplified test). The clinical specimens used in our study were tested by the direct test only.

To the microwells coated with capture antibody (rabbit anti-HSV-1 and HSV-2) 200 uL of swab extracts was added (swabs emulsified in inoculation medium), and controls were added in duplicate. The following controls were included in each test: substrate blank well, two wells of ORTHO positive control, and 2 wells of negative control (inoculation medium used to emulsify the swabs in). The microwell plate was incubated at room temperature (22° to 27°C) for 2 hours, for the binding of HSV antigens present in the sample to the solid-phase capture antibody. The wells were washed with stock washing buffer (pH 7.4; wash buffer

concentrate, 0.05M PBS containing 0.05% Tween). The washing procedure consisted in first aspirating the sample solutions from all the microwells, filling the wells with washing buffer and allowing them to soak for exactly one minute. The wells were then aspirated dry. This fill-soak aspirate sequence was repeated 3 times. The immobilized antigen was then measured by adding 200 μ L of detector antibody (peroxidase-conjugated rabbit anti-HSV) to all the wells except the substrate blank well. The plates were incubated at room temperature for 2 hours. The wells were washed five times with diluted wash buffer as described above. Fresh substrate was prepared before use [10 mL substrate buffer (0.024M citrate-0.05M phosphate buffer, pH 5.1), 0.6% hydrogen peroxide (10 drops), substrate concentrate (10 drops; 4.5% O-phenylenediamine in solvent)]. The outside of the microwell bottoms were inspected for the presence of dried buffer salts or wash solution which might interfere with the spectrophotometric reading. The well bottoms were carefully cleaned with tissue paper, and 200 μ L of substrate solution [O-phenylenediamine (OPD) is used as the chromogenic substrate] was added. The plate was incubated for 30 minutes at room temperature. Fifty μ L of 2N hydrochloric acid was added to all the wells, and the intensity of the color produced in the wells, which is dependent on the amount of HSV antigen present was measured at 490nm with a Model MR 600 Microplate reader (Dynatech Laboratories, Alexandria, VA., U.S.A.).

6.2 Identification of HSV in Clinical Specimens

We obtained one hundred and sixty six routine swabs from the Regional Virology Laboratory (C.H.E.O., Ottawa, Canada). These

swabs were received in transport medium (Virocult, Medical Wire and Equipment Co., Ltd., Corsham, Wilts, England) and expressed into 2.0 mL of inoculation medium [Hanks' Basic Salt Solution containing 0.2% bovine serum albumin, HEPES (N'-2-Hydroxyethyl piperazine-N'-Ethanesulfonic Acid) 21.5mM, amphotericin B (8.6ug/mL), gentamycin sulphate (213ug/mL), and penicillin G (8.55×10^2 IU/mL)]. 100 uL of the swab extract was used to inoculate MRC-5 standard tube cultures and identified by direct IF (Micro-Trak kit, Syva Co.). The remainder of the swab extract was kept at 4°C and was subsequently used for HSV antigen detection by EIA-F and ORTHO EIA (200 uL/well of swab extract was used for each assay).

PHASE IV

7.1 Identification of HSV in Cultures of Clinical Samples

Routine unselected swabs of herpetic lesions sent to the Regional Virology Laboratory (C.H.E.O.) in transport medium (Virocult) were expressed into 2.0 mL of inoculation medium. Initially, positive HSV specimens were isolated by inoculating 0.1 mL of the swab extract into MRC-5 standard tube cultures and identified by direct IF (Micro-Trak, Syva Co.) when the cultures showed a +2 CPE. The positive swab extracts were kept at 4°C and subsequently 0.1 mL were inoculated into one MC tube culture growing MRC-5 cells (cells were 50 to 75% confluent on the MC's) without adsorption. The tube cultures were incubated stationary, slanted (5° to 10° angle) at 37°C. On days 1, 2, 3, and 4 after inoculation of the positive swab extracts, the degree of CPE was noted and 300 uL of the MC suspension was sampled.

Non-infected MC tube cultures, to be used as controls, were sampled on day 4 only. All the samples were kept at 4°C until assayed by EIA-F.

EXPRESSION OF RESULTS

8.1 EIA-F

An EIA-F result was considered positive if it met the three criteria defined by the original investigators of the method (Cleveland et, 1979): a) it yielded a specific absorbance value (test minus control) of greater than 0.05, b) the specific absorbance value was at least twice the standard deviation (S.D.) of both the test and the control absorbance values, and c) the binding ratio (B.R.) was greater than 1.4 (B.R.= the test absorbance divided by the absorbance value of the control).

8.2 ORTHO EIA

A positive direct test result using the ORTHO HSV antigen ELISA test system confirms the presence of HSV in the specimen. In order for the test to be considered positive, it must meet the following criteria: a) the two negative media control values must be within 0.04 optical density (O.D.) units, neither value can be 0.00 O.D. units, b) the negative media control mean (average of the two negative control values) must be less than 0.15 O.D. units, and c) the antigen standard mean must be greater than two times the cutoff value (negative media control mean value + 0.010). Any value greater than the cutoff value is considered positive for HSV antigen.

RESULTS

Phase I

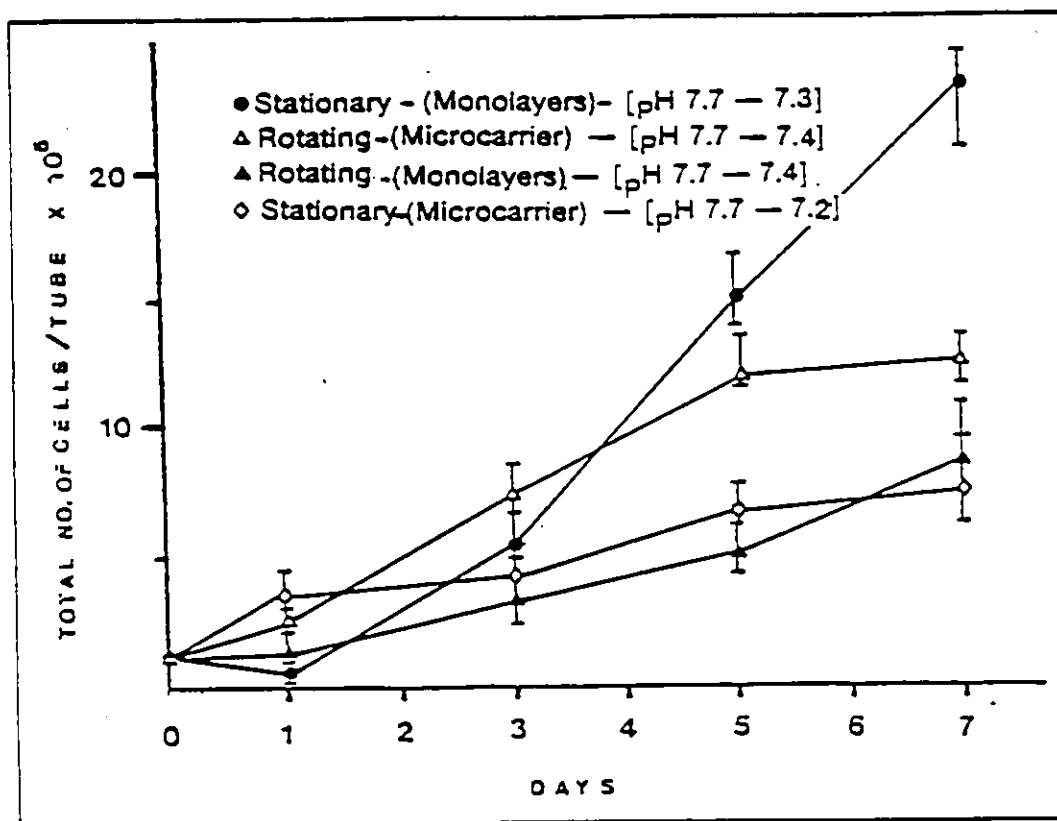
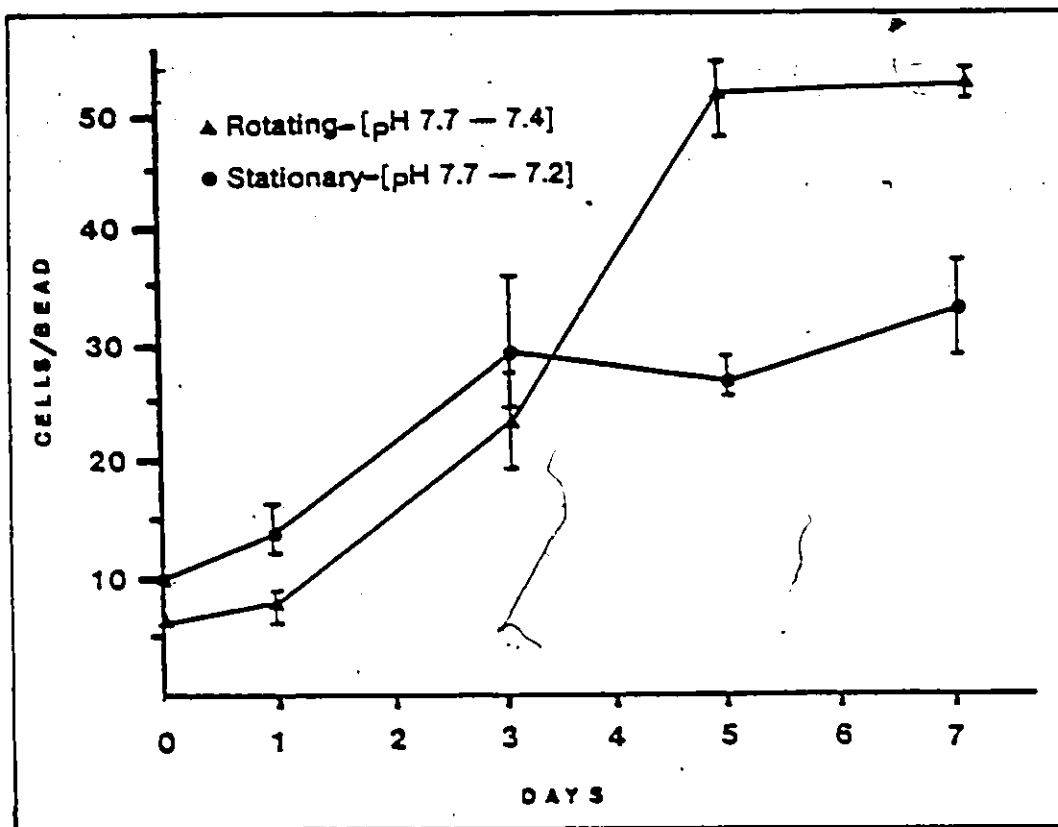
1. MC and Monolayer Tube Cultures

The data for the HEP-2 cell yield of partially grown MC's in stationary and rotating tube cultures which were established from SF's are presented in Fig. 1. Cell counts (4 replicates) were determined on days 0, 1, 3, 5, and 7 and expressed as the number of cells/bead (there are approximately 2.4×10^2 MC/tube) in the tube cultures. At day 0, the tubes were seeded with approximately the same number of cells/bead, the rotating tubes contained 6 cells/bead while the stationary tubes contained 10 cells/bead. Over the first 3 days of culture there appeared to be no significant difference in the number of cells/bead in both culture conditions. However, at the end of the 7 days, a significant difference was noted in the cell yield in the stationary and rotating cultures. The rotating cultures produced an average cell yield of approximately 50 cells/bead (equivalent to 10^6 cells/tube), and the stationary cultures produced an average cell yield of approximately 30 cells/bead (equivalent to 6×10^5 cells/tube). The pH values noted over the 7 day period in both culture conditions remained within normal limits (Ceccarini and Eagle, 1971a,b; Ceccarini, 1975), the values ranging from 7.7 on day 0, to 7.2 at the end of the 7 days of culture.

Comparison between the HEP-2 cell growth curves in MC tube cultures established from SF's and normal monolayer cultures in both stationary and rotating cultures are presented in Fig. 2. Cell counts (4 replicates) were determined on days 0, 1, 3, 5 and

Figures 1 and 2

1. Number of HEP-2 cells ($\pm$ S.D.) per microcarrier in stationary and rotating tube cultures over a 7 day period.
2. Comparison of the growth curves of HEP-2 cells ($\pm$ S.D.) grown on microcarriers and in monolayers, in stationary and rotating tube cultures, over a 7 day period.



7 and expressed as the total number of cells/tube. All the tubes were seeded with approximately 2×10^5 cells/tube on day 0. As can be seen, there appeared to be no significant difference between the different culture systems studied over the first 5 days. However, after 7 days, a significant difference was noted between the stationary monolayer cultures and the rotating MC cultures. The stationary monolayer cultures produced a final cell yield of approximately 2.5×10^6 cells/tube, while the rotating MC cultures produced a final yield of only 1.4×10^6 cells/tube. There was no significant difference seen in the yields between the rotating monolayer cultures and the stationary MC cultures at the end of 7 days. The pH in all the cultures remained within normal limits, ranging from 7.7 on day 0, to 7.2 at the end of the 7 days.

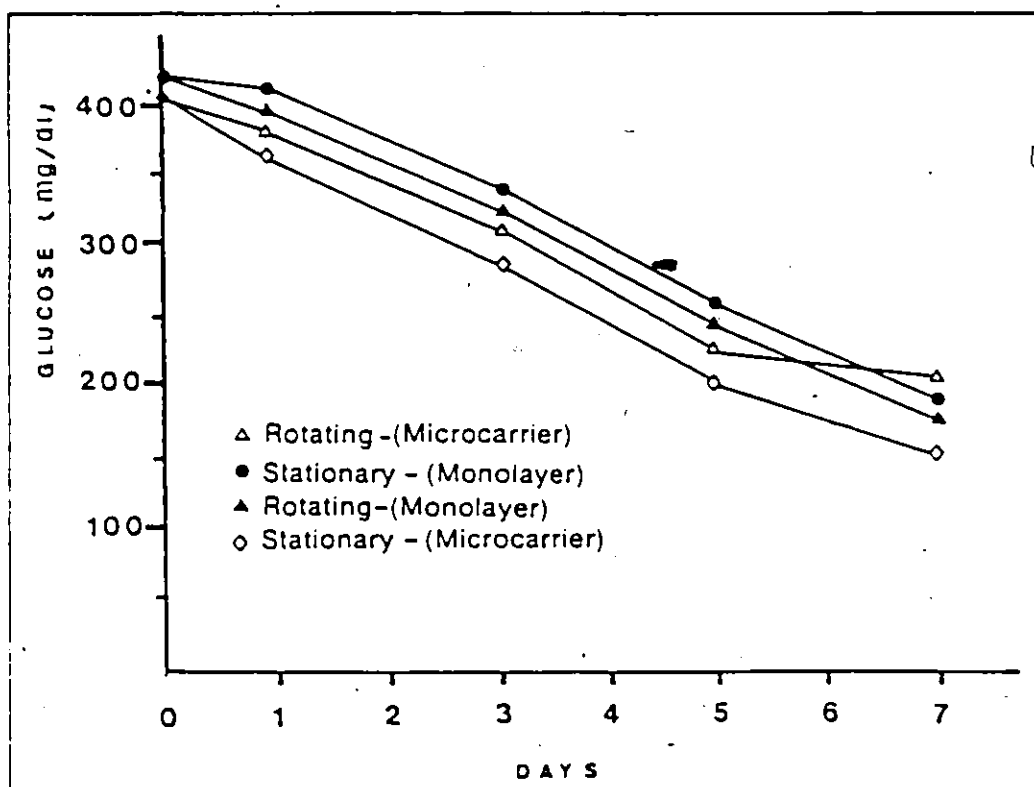
The different rates of glucose utilization are presented in Fig. 3. It shows that all the culture systems studied in this experiment appeared to metabolize glucose at an equal rate, and that the differences noted in the growth curves were not the result of glucose starvation. The glucose decreased from approximately 400 mg/dl on day 0, to approximately 200 mg/dl on day 7. Similarly the O_2 (Fig. 4) and CO_2 (Fig. 5) metabolism appeared to proceed at a similar rate in all the cultures studied.

2. Direct Seeding of MC Tube Cultures

In an attempt to simplify culture procedures for MC, HFL and HEP-2 cells were seeded directly in tube cultures containing freshly hydrated MC's. The data for the cell yields obtained in the stationary MC tube cultures are presented in Fig. 6. Cell

Figure 3

Glucose concentration (mg/dl) in stationary and rotating HEp-2 cell cultures grown on microcarriers or as monolayers over a 7 day period.



Figures 4 and 5

4. Oxygen concentration (%) in stationary or rotating HEP-2 cell cultures grown on microcarriers or as monolayers over a 7 day period.
5. Carbon dioxide concentration (%) in stationary and rotating HEP-2 cell cultures grown on microcarriers or as monolayers over a 7 day period.

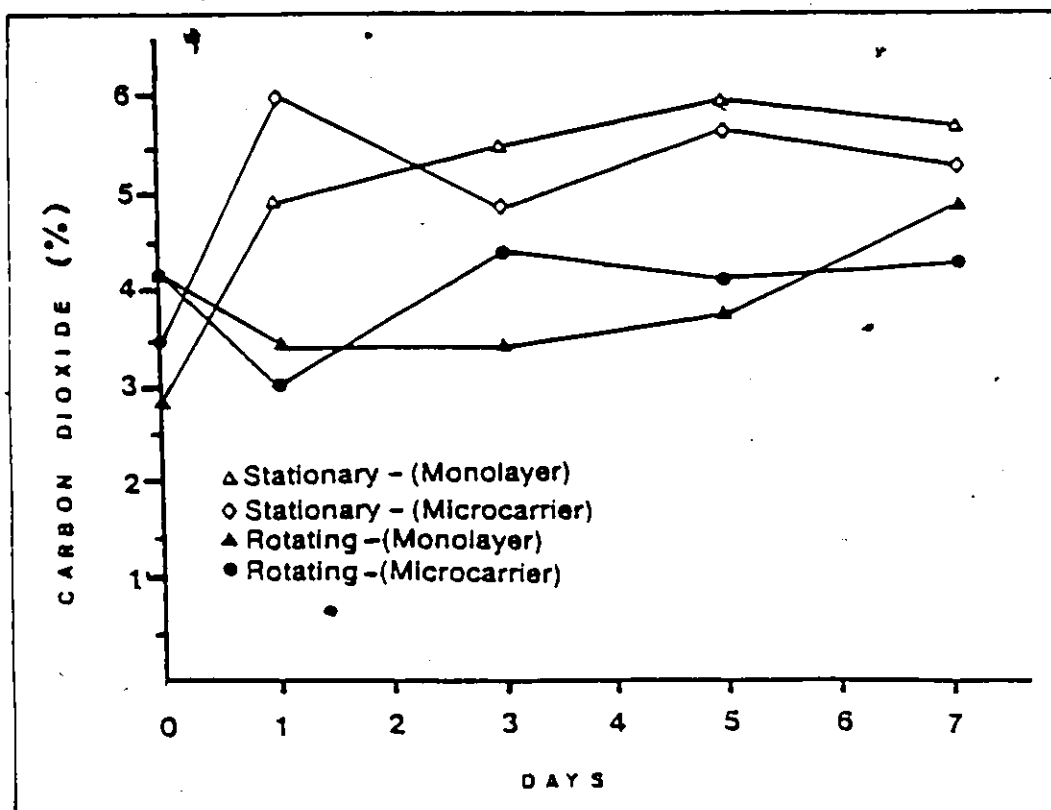
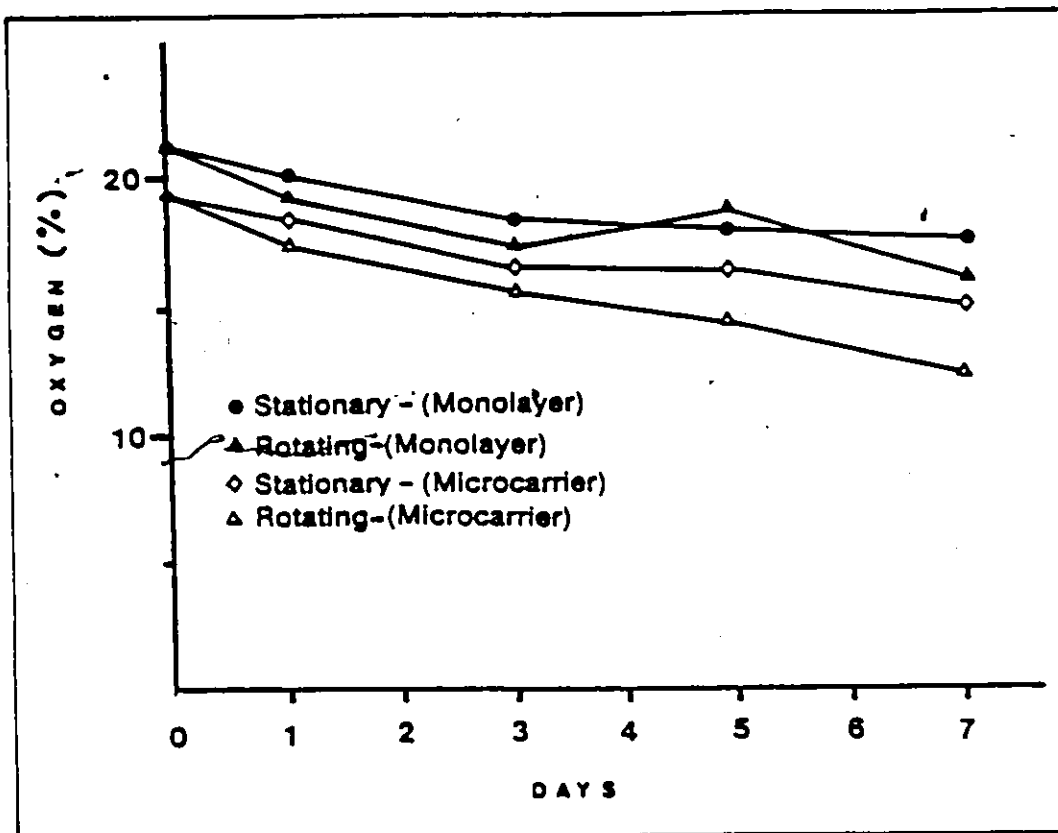
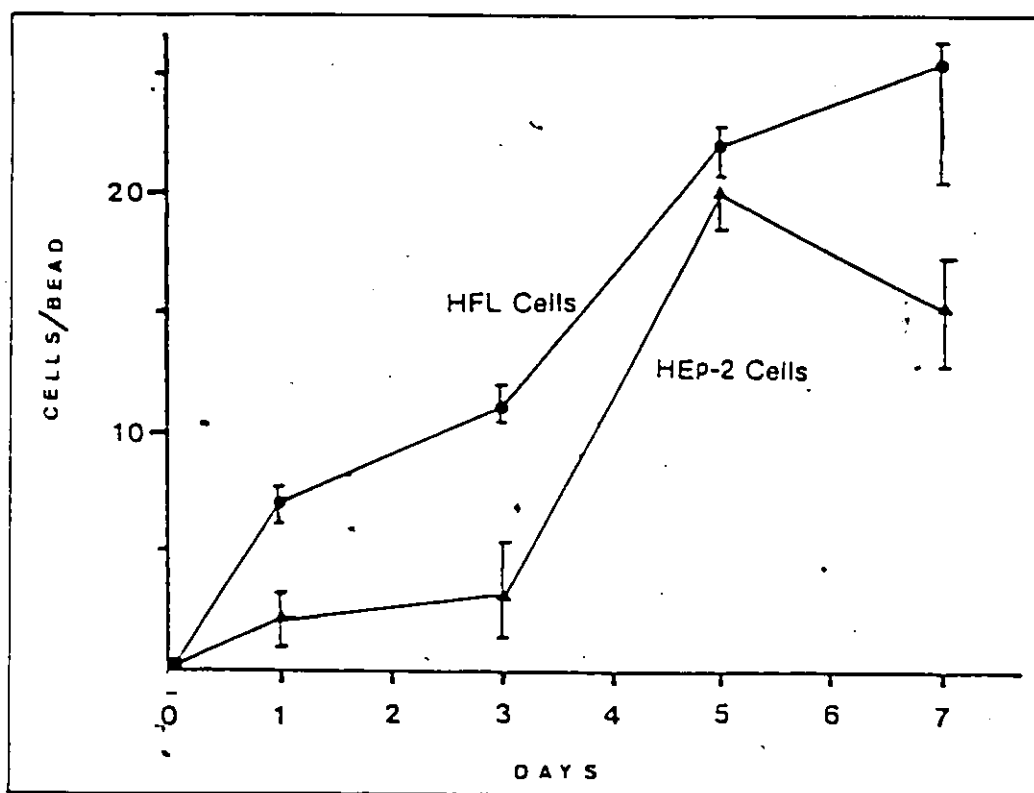


Figure 6

Number of HFL ($\pm$ S.D.) and HEp-2 cells ($\pm$ S.D.) per microcarrier in stationary cultures over a 7 day period.

2



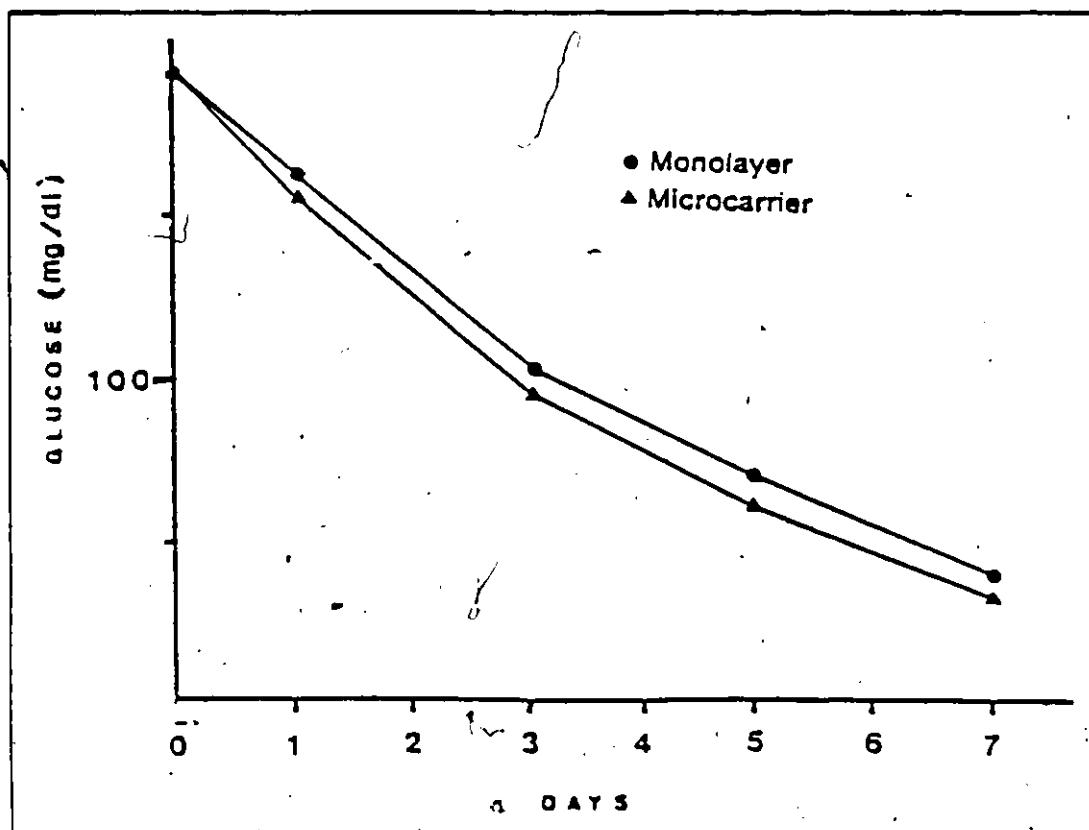
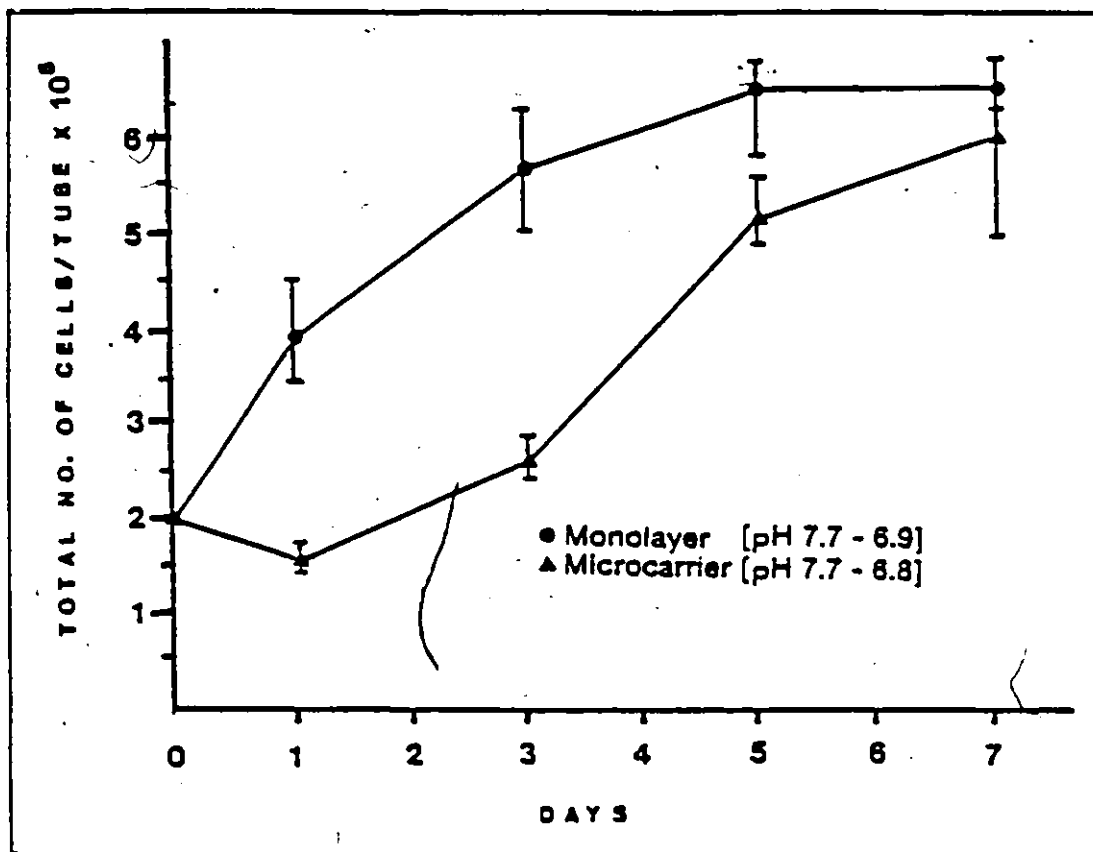
counts (10 replicates) were determined on days 0, 1, 3, 5 and 7 and expressed as the number of cells/bead. The tube cultures were seeded with approximately the same cell inoculum (2×10^5 cells/tube), and a significant difference in the cell yield of HFL and HEp-2 cells was noted at the end of 7 days. The HFL cells produced a yield of approximately 30 cells/bead while the HEp-2 cells produced a yield of only 15 cells/bead. Furthermore, the HEp-2 cell yield per MC dropped significantly between day 5 and day 7.

Because the HFL cells seeded directly with MC's in tube cultures appeared to grow well, we compared the growth of HFL cells grown on MC's and in normal monolayer cultures. As can be seen in Fig. 7, cell counts (10 replicates) were expressed as the total number of cells/tube and determined for 7 days (on days 0, 1, 3, 5 and 7). For the first 5 days of culture, there appeared to be a significant difference in the cell yields in the 2 systems studied. But, at the end of the 7 days, this was no more the case. The monolayer cultures produced a yield of approximately 6×10^5 cells/tube and the MC cultures produced a yield of approximately 5×10^5 cells/tube. The pH in both systems remained within reasonable levels, ranging from 7.7 on day 0 to 6.8 on day 7.

As can be seen in Fig. 8, the glucose utilization decreased at a similar rate in both systems, from approximately 200 mg/dl to approximately 25 mg/dl over 7 days. In as far as the O_2 (Fig. 9) and CO_2 (Fig. 10) concentrations are concerned, there was virtually no difference between the 2 systems.

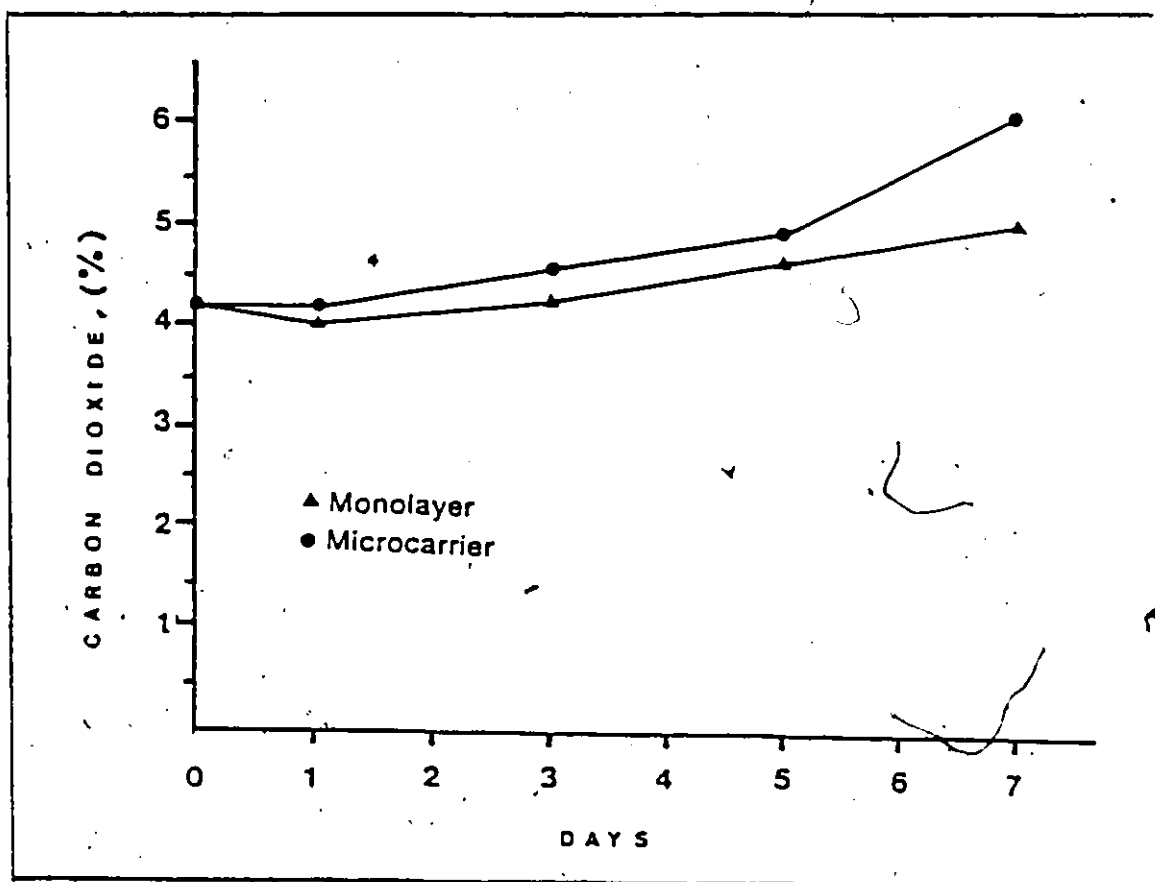
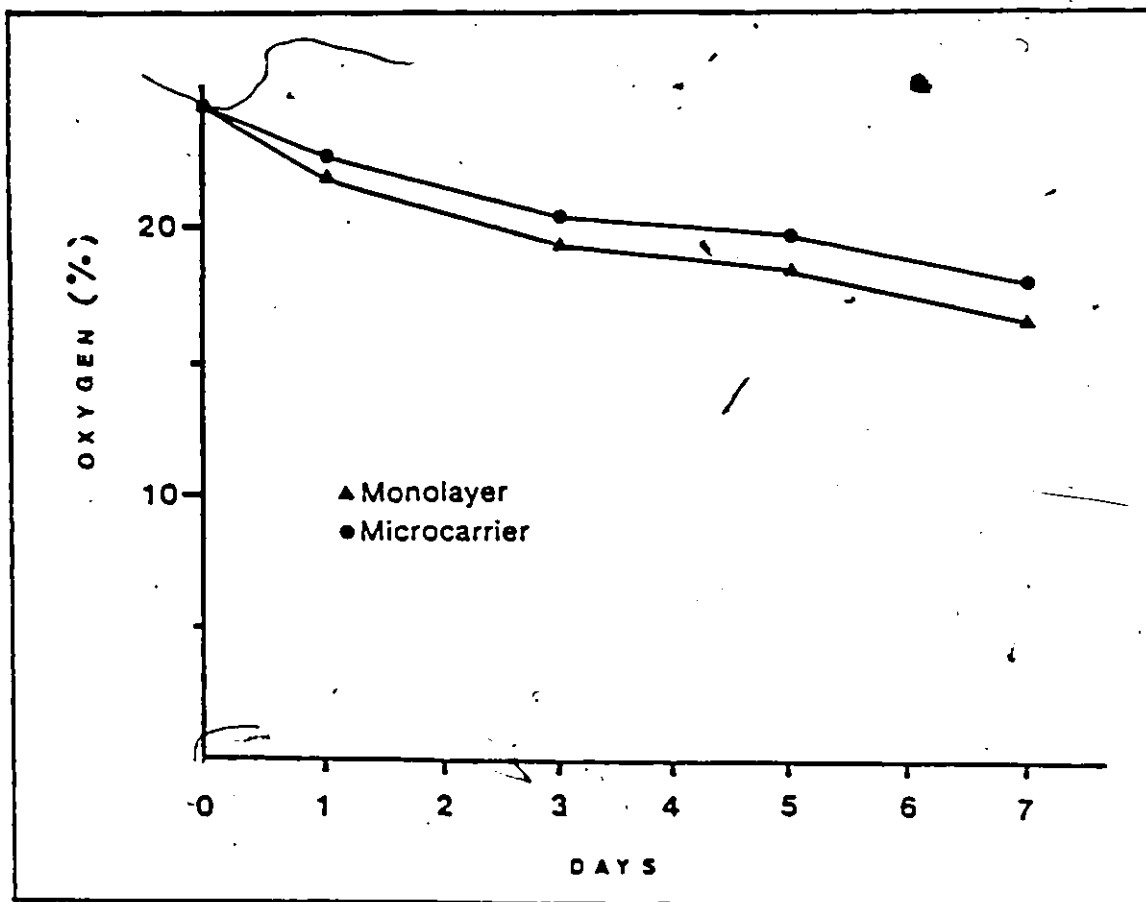
Figures 7 and 8

7. Comparison of the growth curves of HFL cells ($\pm$ S.D.) grown on microcarriers and monolayers in stationary cultures over a 7 day period.
8. Glucose concentration (mg/dl) in stationary cultures of HFL cells grown on microcarriers or as monolayers over a 7 day period.



Figures 9 and 10

9. Oxygen concentration (%) in stationary cultures of HFL cells grown on microcarriers or as monolayers over a 7 day period.
10. Carbon dioxide concentration (%) in stationary cultures of HFL cells grown on microcarriers or as monolayers over a 7 day period.



Phase II

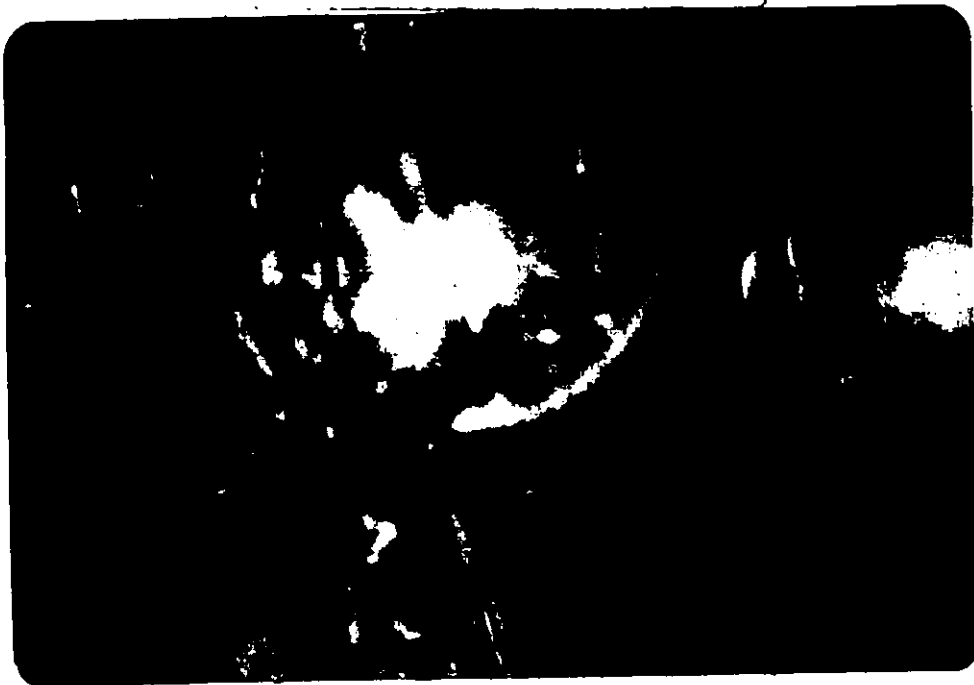
3. Checkerboard Titration of the Group HSV Monoclonal Antibody and Conjugate

In order to determine the optimal dilution of antibody and conjugate to be used in the EIA-F assay, a checkerboard titration was set up using the group HSV monoclonal antibody (diluted from 1:250 up to 1:256,000) against the conjugate (horseradish peroxidase; diluted from 1:250 up to 1:8000). A maximum amount of antigen (greater than 12,500 cells/well) in the presence of HSV-1 or HSV-2 infected MRC-5 cells was used. The data presented in Fig. 11 indicate that the chosen antibody dilution was at the lower end of the curve (at dilution 1:32,000) at a conjugate dilution between 1:2000 and 1:4000. At this dilution the background levels were negligible. The conjugate to be used was determined to be between 1:2000 to 1:4000, since at these dilutions the background absorbance was negligible. The conjugate was used at a dilution of 1:3000, which was in agreement with the one suggested by the manufacturers.

4. Detection of HSV-1 Antigen at Low Multiplicity of Infection (MOI)

MC tube cultures containing approximately 0.7×10^5 MRC-5 cells/tube were inoculated with $10^{0.7}$, 10^1 , 10^2 , and 10^3 plaque forming units (PFU) of HSV-1 resulting in a range of MOI's from 10^{-5} to 10^{-2} . The data for the development of CPE, the appearance of specific HSV-1 immunofluorescence, and specific EIA-F absorbance as a function of time and increasing inoculum size are presented in Table 1. The appearance of CPE was usually followed by that of specific HSV-1 IF 24 hours later. The

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Photograph showing the growth of HEp-2 cells on Cytodex-3 microcarriers in tube cultures (40x).

Figure 11

Checkerboard titration of group HSV monoclonal antibody versus horseradish peroxidase conjugate [doubling dilution of antibody starting at 1:250 (1 on the graph) to 1:256,000 (11 on the graph), and doubling dilution of conjugate starting at 1:250 to 1:8000].

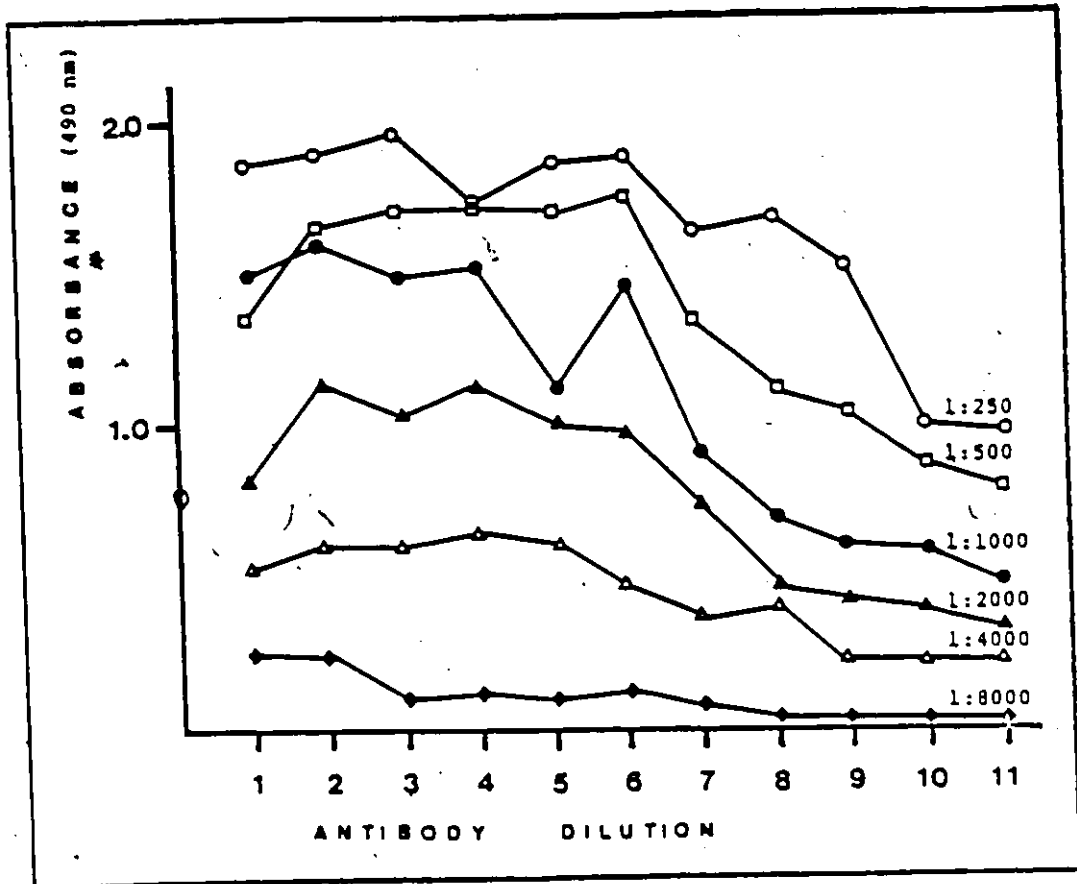


TABLE 1

Development of CPE, specific HSV-1 fluorescence and specific absorbance by enzyme immunofiltration in MRC-5 microcarrier (MC) tube cultures inoculated at low multiplicity of infection (MOI) with HSV-1.

HSV-1 inoculum in PTU (MOI)											
Hours after HSV-1 inoculation	No. of cells in control MC samples	Absorbance values ^a in control MC samples $\pm$ SD	$10^{-7} (10^{-5})$			$10^{-4} (10^{-4})$			$10^{-2} (10^{-3})$		
			CPE	IF	Test absorbance value ^a $\pm$ SD	CPE	IF	Test absorbance value ^a $\pm$ SD	CPE	IV	Test absorbance value ^a $\pm$ SD
8	4.1×10^3	0.008 ± 0.004	-	neg.	0.017 ± 0.010	-	neg.	0.017 ± 0.010	-	neg.	0.017 ± 0.010
16	7.4×10^3	0.013 ± 0.010	-	neg.	0.018 ± 0.010	-	neg.	0.013 ± 0.010	-	neg.	0.025 ± 0.010
24	15.3×10^3	0.023 ± 0.010	-	neg.	0.011 ± 0.010	-	neg.	0.019 ± 0.010	-	neg.	0.022 ± 0.010
48	9.8×10^3	0.017 ± 0.010	-	neg.	0.012 ± 0.010	-	neg.	0.020 ± 0.020	+	neg.	0.115 ± 0.040^b
72	23.0×10^3	0.018 ± 0.010	+	neg.	0.207 ± 0.180	+	pos.	0.345 ± 0.134^b	+	pos.	0.378 ± 0.150^b
96	22.5×10^3	0.006 ± 0.004	+	pos.	0.238 ± 0.230	+	pos.	0.325 ± 0.100^b	+	pos.	0.311 ± 0.130^b
											0.327 ± 0.120^b

^a Mean absorbance, at 488 nm, of 8 replicate wells.

^b Met the criteria for a positive enzyme immunofiltration test.

CPE was first detected at an MOI of 10^{-2} at 24 hours and last at an MOI of 10^{-5} at 72 hours. A positive EIA-F specific absorbance was first detected at 48 hours at an MOI of 10^{-3} and 10^{-2} and at 72 hours at an MOI of 10^{-4} . At an MOI of 10^{-5} , the test absorbance values at 96 hours did not meet the criteria for a positive EIA-F test although CPE and IF were positive. At an MOI of 10^{-4} , EIA-F specific absorbance and direct IF were positive simultaneously with the CPE at 72 hours. The EIA-F preceded IF by 24 hours at an MOI of 10^{-3} and IF preceded EIA-F at an MOI of 10^{-5} . The CPE preceded IF by 24 hours at an MOI of 10^{-2} , 10^{-3} , and 10^{-5} .

The corresponding non-infected MC samples, which were used to obtain the control absorbances at each time period, contained 4.1×10^3 cells at 8 hours and increased to 22.5×10^3 cells at 96 hours. The slight variations in the control cell counts were probably due to tube-to-tube and sampling variations.

5. Detection of HSV-2 Antigen at Low MOI

The same procedure for infecting the MC tube cultures at the different MOI's was followed as with the HSV-1 experiment above. As can be seen, the data in Table 2 show that CPE and IF were positive simultaneously. The CPE was first detected at an MOI of 10^{-2} at 72 hours and last at an MOI of 10^{-4} at 96 hours. Positive EIA-F was first detected at 72 hours at an MOI of 10^{-3} and 10^{-2} , and at 96 hours at an MOI of 10^{-4} . The CPE, direct IF, and EIA-F were all negative at an MOI of 10^{-5} , however, at 96 hours, the CPE, IF, and EIA-F were all positive simultaneously. In contrast to the HSV-1 experiment, there was a correlation between the appearance of CPE, direct IF, and EIA-F,

TABLE 2

Development of CPE, specific HSV-2 fluorescence and specific absorbance by enzyme immunofiltration in MRC-5 microcarrier (MC) tube cultures inoculated at low multiplicity of infection (MOI) with HSV-2.

Hours after HSV-1 inoculation	No. of cells in control MC samples	Absorbance values ^a in control MC samples $\pm$ SD	HSV-2 inoculum in PFU (MOI)											
			$10^{0.7} (10^{-5})$			$10^1 (10^{-4})$			$10^2 (10^{-3})$			$10^3 (10^{-2})$		
			CPE	IF	absorbance value ^a $\pm$ SD	CPE	IF	absorbance value ^a $\pm$ SD	CPE	IF	absorbance value ^a $\pm$ SD	CPE	IF	absorbance value ^a $\pm$ SD
8	4.1 $\times 10^3$	0.018 $\pm$ 0.014	-	neg.	0.017 $\pm$ 0.001	-	neg.	0.014 $\pm$ 0.011	-	neg.	0.012 $\pm$ 0.001	-	neg.	0.011 $\pm$ 0.008
18	9.2 $\times 10^3$	0.013 $\pm$ 0.010	-	neg.	0.044 $\pm$ 0.056	-	neg.	0.006 $\pm$ 0.007	-	neg.	0.013 $\pm$ 0.011	-	neg.	0.010 $\pm$ 0.007
24	15.4 $\times 10^3$	0.018 $\pm$ 0.010	-	neg.	0.012 $\pm$ 0.004	-	neg.	0.015 $\pm$ 0.001	-	neg.	0.028 $\pm$ 0.006	-	neg.	0.007 $\pm$ 0.004
48	22.6 $\times 10^3$	0.017 $\pm$ 0.010	-	neg.	0.007 $\pm$ 0.004	-	neg.	0.008 $\pm$ 0.001	-	neg.	0.013 $\pm$ 0.003	-	neg.	0.025 $\pm$ 0.005
72	24.5 $\times 10^3$	0.007 $\pm$ 0.005	-	neg.	0.029 $\pm$ 0.006	-	neg.	0.020 $\pm$ 0.000	+	pos.	0.087 $\pm$ 0.038	+	pos.	0.165 $\pm$ 0.006
96	21.2 $\times 10^3$	0.018 $\pm$ 0.010	-	neg.	0.008 $\pm$ 0.003	+	pos.	0.139 ^b $\pm$ 0.005	+	pos.	0.261 ^b $\pm$ 0.078	+	pos.	0.219 ^b $\pm$ 0.026

^a Mean absorbance, at 488 nm, of 8 replicate wells.

^b Met the criteria for a positive enzyme immunofiltration test.

except at an MOI of 10^{-3} at 72 hours, where the CPE and IF were positive simultaneously while the EIA-F was negative.

The corresponding non-infected MC samples ranged from 4×10^3 cells/tube at 8 hours and increased to 21×10^3 cells/tube at 96 hours. Here again, the slight variations in the control cell counts were probably due to tube-to-tube and sampling variations.

6. Detection of HSV-1 Antigen at High MOI

To determine if the EIA-F could detect HSV-1 antigen earlier than CPE at higher inocula, MC tube cultures containing approximately 4.6×10^5 cells/tube were inoculated with 10^4 , 10^5 , 10^6 , and 10^7 PFU of HSV-1, resulting in a range of MOI's from 10^{-2} to 10. The data for the development of CPE, the appearance of HSV-1 IF and specific EIA-F absorbance as a function of time and increasing inoculum size are presented in Table 3. At an MOI of 10^{-2} , 10^{-1} , and 1, the specific EIA-F absorbance appeared at 48, 24, and 18 hours respectively which was 24 to 48 hours after the appearance of CPE and at the same time as the specific HSV-1 IF. At an MOI of 10, the CPE and the specific EIA-F were positive at 8 hours after infection followed by IF 10 hours later. The specific EIA-F at an MOI of 10^{-2} appeared at 48 hours, which was at the same time as in the previous experiment, despite the difference in the number of cells sampled.

7. Detection of HSV-2 Antigen at High MOI

The same procedure as above was followed for infecting the MC tube cultures at the different PFU with the HSV-2 strain. Table 4 shows that at an MOI of 10^{-1} , 1, and 10 the specific EIA-F

TABLE 3

Development of CPE, appearance of specific HSV-1 fluorescence and specific absorbance by enzyme immunofiltration in MC-5 microcarrier (MC) tube cultures inoculated at high multiplicity of infection (MOI) with HSV-1.

Hours after inoculation	No. of cells in control MC samples	Absorbance values ^a in control MC samples $\pm$ SD	HSV-1 inoculum in PFU (MOI)											
			$10^4 (10^{-2})$			$10^5 (10^{-1})$			$10^6 (10^0)$			$10^7 (10^1)$		
			CPE	IF	Test absorbance value ^a $\pm$ SD	CPE	IF	Test absorbance value ^a $\pm$ SD	CPE	IF	Test absorbance value ^a $\pm$ SD	CPE	IF	Test absorbance value ^a $\pm$ SD
8	23.0×10^3	0.020 ± 0.010	-	neg.	0.011 ± 0.010	-	neg.	0.007 ± 0.004	+	neg.	0.014 ± 0.010	+	neg.	0.110 ± 0.050^b
18	25.0×10^3	0.014 ± 0.010	-	neg.	0.008 ± 0.010	+	neg.	0.039 ± 0.020	+	pos.	0.200 ± 0.030^b	+	pos.	0.314 ± 0.010^b
24	35.0×10^3	0.018 ± 0.003	+	neg.	0.049 ± 0.010	+	pos.	0.196 ± 0.030^b	+	pos.	0.290 ± 0.020^b	+	pos.	0.252 ± 0.020^b
48	42.5×10^3	0.019 ± 0.010	+	pos.	0.298 ± 0.020^b	+	pos.	0.333 ± 0.090^b	+	pos.	0.284 ± 0.020^b	+	pos.	0.296 ± 0.010^b

^a Mean absorbance, at 488 nm, of 4 replicate wells.

^b Met the criteria for a positive enzyme immunofiltration test.

TABLE 4

Development of CPE, appearance of specific HSV-2 fluorescence and specific absorbance by enzyme immunofiltration in MRC-5 microcarrier (MC) tube cultures inoculated at high multiplicity of infection (MOI) with HSV-2

Hours after inoculation	No. of cells in control MC samples	Absorbance values ^a in control MC samples	HSV-2 inoculum in PFU (MOI)											
			$10^4 (10^{-2})$			$10^3 (10^{-1})$			$10^2 (10^0)$			$10^1 (10^1)$		
			CPE IF	Test absorbance value ^a $\pm$ SD	CPE	IF	Test absorbance value ^a $\pm$ SD	CPE	IF	Test absorbance value ^a $\pm$ SD	CPE	IF	Test absorbance value ^a $\pm$ SD	CPE
8	21.2×10^3	0.021 ± 0.010	-	neg. 0.017 $\pm$ 0.004	-	neg.	0.010 $\pm$ 0.002	-	neg.	0.013 $\pm$ 0.001	-	neg.	0.070 $\pm$ 0.008 ^b	
18	27.4×10^3	0.018 ± 0.010	-	neg. 0.029 0.023	+	neg.	0.007 0.004	+	pos.	0.048 0.011 ^b	+	pos.	0.131 0.028 ^b	
26	32.6×10^3	0.019 ± 0.010	$\pm$	neg. 0.029 0.026	+	pos.	0.022 0.007	+	pos.	0.107 0.035 ^b	+	pos.	0.197 0.013 ^b	
48	39.1×10^3	0.014 ± 0.014	+	pos. 0.028 0.021	+	pos.	0.117 0.002 ^b	+	pos.	0.206 0.023 ^b	+	pos.	0.202 0.001 ^b	

^aMean absorbance, at 488 nm, of 4 replicate wells.

^bMet the criteria for a positive enzyme immunofiltration test.

absorbance appeared at 48, 18, and 8 hours respectively, which was 48 hours after the appearance of the CPE and at the same time as the specific HSV-2 IF. At an MOI of 10, the CPE was doubtful but specific EIA-F absorbance was positive 8 hours after infection, followed by IF 10 hours later. At an MOI of 10^{-2} at 48 hours, the test absorbance values did not meet the criteria for a positive EIA-F test although the CPE and direct IF were positive.

Phase III

8. Direct Identification of HSV Clinical Samples by EIA-F and O-EIA

In view of the good results obtained with EIA-F for the detection of HSV in cultures, we examined the specificity and sensitivity of the EIA-F test and of the ORTHO^R ELISA test system for the direct detection of HSV antigens in skin samples. This was done in parallel with cell cultures. The data for the EIA-F test results versus MRC-5 cell culture for the HSV diagnosis in clinical samples are presented in Table 5. We examined a total of 166 specimens of which 92 were from genital lesions, 19 from other skin lesions, and 55 were from unknown sites. Of the 44 positive clinical specimens in cell culture only 7 of these were found to be positive by EIA-F, i.e. a sensitivity of only 16%. The false positivity rate was 53% and the false negative rate was 25%, with a specificity of 93%.

As can be seen from the data presented in Table 5, where the O-EIA was compared in parallel with cell culture, of the 44 positive clinical samples by cell culture, 24 were found to be positive by O-EIA, i.e. a sensitivity of 54%. The false positive

TABLE 5
Enzyme Immunofiltration and ORTHO^R ELISA Test
System Versus Cell Culture (MRC-5) for HSV Diagnosis

ELISA test	CELL CULTURE		TOTAL	% SENSITIVITY	% SPECIFICITY	% FALSE	
	Positive	Negative				Positive	Negative
EIA-F	7	8	15	16	93	53	25
	37	114	151				
• TOTAL	44	122	166				
O-EIA	24	20	40	54	83	45	16
	20	102	122				
TOTAL	44	122	166				

rate was 45% and the false negative rate was 16% with a lower specificity (83%) than EIA-F assay.

Phase IV

9. Identification of HSV in Cultures of Clinical Samples

The data on the appearance of CPE and the development of specific absorbance in MC tube cultures inoculated with 13 HSV positive clinical samples are presented in Table 6. As can be seen, in the MC tube cultures, the CPE appeared as early as in the standard monolayer cultures (usually 2 days after inoculation). A positive EIA-F test was detected on day 2 in half of the cultures, while on one occasion, it was detected after only one day, which was at the same time as the CPE. On day 3, all the MC tube cultures were positive by EIA-F irrespective of the HSV type, while increased absorbance values were reached on day 4. As a whole, absorbance values obtained in this study were found to be higher than in the HSV-1 and HSV-2 model experiments (refer to phase II study). This may have been due to the larger sample size used in this study and also a high infectivity in clinical specimens of genital origin.

TABLE 6

Development of CPE and appearance of specific HSV absorbance by enzyme immunofiltration in MRC-3 standard and microcarrier (MC) tube cultures inoculated with HSV positive clinical samples

HSV type and location	Type of culture and day on which CPE detected		Specific absorbance value ^d in MC cultures on day			
	standard	MC	1	2	3	4
1 ^a	3	2	-0.008	0.040	0.353 ^c	0.422 ^c
1 ^a	2	2	-0.002	0.032	0.309 ^c	0.473 ^c
1 ^b	3	2	-0.007	0.086	0.390 ^c	0.402 ^c
1 ^b	1	1	0.092 ^c	0.386 ^c	0.616 ^c	0.608 ^c
1 ^b	4	2	0.010	0.033	0.388 ^c	0.483 ^c
1 ^b	2	3	0.022	0.009	0.105 ^c	0.411 ^c
2 ^b	2	2	0.000	0.095 ^c	0.507 ^c	0.540 ^c
2 ^b	1	2	-0.001	0.237 ^c	0.525 ^c	0.422 ^c
2 ^b	2	2	0.019	0.360 ^c	0.677 ^c	0.492 ^c
2 ^b	1	2	0.014	0.025	0.412 ^c	0.589 ^c
2 ^b	1	2	0.015	0.091 ^c	0.322 ^c	0.367 ^c
2 ^b	2	2	0.017	0.127 ^c	0.437 ^c	0.489 ^c
2 ^b	1	2	0.013	0.002	0.073 ^c	0.355 ^c

^aMouth

^bGenital

^cMet criteria i and iii for a positive enzyme immunofiltration test. Control cultures mean absorbance values on day 4 was 0.010 ± 0.015.

^dTest absorbance minus control absorbance in 300 µl samples.

DISCUSSION

The general objective of our study was to develop convenient procedures for MC cultures applicable to virological diagnosis and modern immunoassay. These procedures should allow sequential sampling of the original culture, thus facilitating the harvesting of the small number of cells required for the identification of viral antigens by a modified EIA called the enzyme immunofiltration assay (EIA-F; Cleveland et al, 1979). Subsequently, the good results obtained with the EIA-F with the MC cultures led us to investigate its use for the direct identification of herpes simplex viral antigens in clinical specimens.

In phase I of our studies, the specific objective was to develop a MC culturing system in tube cultures and compare this system to the conventional system of growing cells as monolayers. We investigated the growth parameters of the MC and monolayer tube culture systems using HEp-2 and HFL cell lines, since these cell lines are routinely utilized in diagnostic laboratories and will also support the growth of HSV.

The HEp-2 cells which were first established in spinner flasks using Cytodex-3 MC's, were used to seed polystyrene tube cultures and, subsequently, the growth parameters monitored on days 0, 1, 3, 5 and 7. However, with the HFL cells, because of the fact that they are notoriously difficult to grow in large volume spinner flasks, the studies were carried out in tube cultures containing freshly hydrated MC's right from the start. In order to achieve the full potential of any cell culture system, various critical parameters must be considered, such as pH

(Ceccarini and Eagle, 1971a,b; Ceccarini, 1975; Paul, 1975), glucose (Fulton and Winnick, 1953; Eagle, 1955; Jones and Bonting, 1956; Paul, 1975), and of O_2 (Cooper et al, 1958; Brosemer and Rutter, 1961; Pace et al, 1962; Paul, 1975; Taylor et al, 1978), and CO_2 partial pressures (Paul, 1975).

In the investigations of the HEP-2 cells growing in stationary and rotating MC tube cultures, we noted a significant difference in the number of cells/MC over the 7 days: the rotating cultures, producing the highest yield. However, when we compared the rotating MC tube cultures to standard monolayer cultures, the latter produced the highest cell yield. In the MC cell culture system, collisions between cells and MC are important to achieve the full potential of the system (van Wezel, 1973; van Wezel, 1976; Levine et al, 1977; Levine et al, 1979; Pharmacia Fine Chemicals, 1980). This is suggested by our findings, since, the MC tube cultures produced a higher cell yield when rolled. However, when we compared the monolayer system to the MC system, it was noted from the findings that in monolayer systems the HEP-2 cells like to grow in a stationary phase and do not like to be rolled, contrary to the MC system. There are also other reasons for the lower cell yields obtained with the MC system which will be discussed later in the text.

The HFL cells growing on MC tube cultures and in monolayers were studied but in this case only stationary tube cultures were set up. The comparison of the cell yields of the HEP-2 and HFL cells in stationary MC tube cultures showed a significant difference in cell yield: the HFL cells produced the highest yield. This finding was surprising, since, as mentioned above,

diploid cell lines are difficult to grow on MC's. This can be accounted for by the fact that the HFL cells have fiber-like extensions (pseudopodia) and as a result wrap around the collagen coated Cytodex-3 MC's much more avidly than HEp-2 cells. This strong attachment was noted in the greater difficulty experienced in trypsinizing the HFL cells from the MC by comparison with the HEp-2 cells. In contrast to the HEp-2 cells, experiments comparing the MC system to the monolayer system, the HFL cells grown on MC's or monolayers in tube cultures resulted in no significant difference in the two systems by the end of the 7 days of culture.

The pH in all the cultures studied was monitored very carefully, since pH is known to be a critical parameter, for a high cell yield. It has been shown (Ceccarini and Eagle, 1971a,b; Ceccarini, 1975; Paul, 1975) that insufficient buffering capacity and/or pH control will inevitably lead to a decrease in pH during culture and result in the deterioration of growth and/or detachment of cells from MC's or other culture surfaces used. The pH values throughout our studies with either the HEp-2 or HFL cells during the 7 day culture period, remained within reasonable limits (7.7-6.8). These findings further suggest that the added surface provided by the MC's in the tube cultures does not affect the pH control and that sampling from these cultures is a possible for at least 7 days, without the cells detaching or deteriorating in the tube cultures.

The other parameters which we investigated and which have also been shown to be critical for successful cell culture techniques are O_2 , CO_2 , and glucose. The gas mixture

which was used for the tube cultures and which is standard in most cell culture systems (compositions based on the gas composition of alveolar spaces) was 95% air:5% CO_2 . Excess O_2 is detrimental to the growth of cells in culture, and the reported optimal tension for O_2 is usually less than 10% (Cooper et al, 1958; Brosemer and Rutler, 1961; Pace et al, 1962; Paul, 1975; Taylor et al, 1978). Our data indicate that the O_2 tension with both the HEP-2 and HFL cells remained constant between 10-20% during the culture period. This is greater than the optimal O_2 tension reported above, but the cell growth we observed was excellent, with very little cell detachment, either from the MC's or the monolayers. Similarly, the CO_2 was often less than the routine 5% throughout the culture period without any effect on pH or cell growth.

The glucose concentrations in all the tube cultures (MC or monolayers) studied was seen to decrease at an equal rate over the 7 days of culture. The glucose at the end of 7 days, was not completely exhausted in either system studied.

The MC tube culturing system makes available a larger surface area, and one would expect therefore a greater cell yield. However, the expected higher cell yields were not obtained with the MC system, by comparison with the standard monolayer culture system. These findings can be attributed to several problems encountered in the use of the MC culturing technique: a) the loss of many MC's due to the attachment to the tube surface and their incorporation into the monolayer, b) the presence of beads without cells attached to them, c) variable degrees of cell confluence on the MC's, and d) clumping of MC's and cells, resulting in many

bare MC's. These problems contributed to the lower cell yields obtained, especially with the HEp-2 cell experiments, where the cell yields obtained with the MC system were significantly lower than the standard monolayer system. As a result of the above problems encountered with the MC system, all of the cell counts obtained with the MC system are only minimal values, accounting for the significantly lower cell yield obtained by comparison with the monolayer system. Despite these drawbacks, the MC system has its advantages for the purpose this system was originally designed for.

Our primary objective in developing this system was to obtain cultures, allowing for repeated sampling, without having to destroy the monolayer from one tube cultures. Several of the methods used for the identification of viral antigens in cell cultures, such as EIA or IF, require that the cell monolayer be first stripped off the glass or plastic before being processed. This is a costly procedure, the tube itself still representing the major cost of the culture.

Even if maximum cell yields were not obtained, enough cells/MC (whether HEp-2 or HFL cells) were obtained to be used in the EIA-F assay. Since each tube contains 2 mL of the MC suspensions and using 50 to 300 μ L per sample, we calculated that we could expect to sample up to 40 times, therefore allowing us to sample for up to a period of 10 days before the cell supply is exhausted, using only one single tube culture. The results of the growth parameters after 7 days in the MC tube culture system, suggests that this is indeed a possibility, and that the cultures can be kept for longer periods, with the addition of fresh medium

to the tube cultures. Because the sampling can be achieved with this MC system, the need for several replicates for EIA-F or IF can therefore be met. Another consideration in the use of this MC system is that we observed that cell transfer did take place between the MC's and the wall of the tube cultures resulting in the formation of cell islands. It was found that these cell islands made it easier to detect CPE than on the MC's, where spherical aberration was encountered. The system is also very economical, the addition of MC's costs only 6 cents per tube culture, with very little extra labor.

The above findings led us to investigate the use of the EIA-F assay in conjunction with the MC tube culture system for the identification of HSV, in order to overcome some of the limitations encountered when using standard monolayer cultures.

In these studies, HSV was used as a model, since, this virus has a brief replicative cycle (Rawls, 1979), is a frequent isolate in a clinical laboratory, and has been shown to replicate and grow readily in a number of primary and established cell culture systems (Cho and Feng, 1978).

Specimens submitted for HSV diagnosis show a range of infective virus from 10^1 to 10^7 PFU/specimen. This range is dependent upon: a) whether the lesion is a primary or recurrent one, b) the stage of the skin lesion, c) the use of antiviral drugs, d) delays in transit of the specimen to the laboratory, and e) the type of transport medium employed for the specimens. Most of the diagnostic laboratories today use standard monolayer tube cultures to identify the infecting agent in clinical specimens. One or two cultures are inoculated per specimen. The sequential

use of modern immunological confirmatory tests such as IF or EIA; is not practical, since the amount of viral antigen needed for these assays usually requires trypsinization of the whole tube culture monolayer, to obtain enough antigen to identify the agent. It thus eliminates the culture from further observation. Furthermore, in solid phase EIA, samples should be examined in duplicates, to decrease the inherent coefficient of variation of the assay. As a result, the continuous observation of the cultures for CPE becomes impossible.

The MC tube culture system (MRC-5 cells were used instead of HFL cells) we used in conjunction with EIA-F in the HSV-1 and HSV-2 model experiments, overcomes most of the problems encountered in the identification of HSV in standard monolayer cultures. This system was found to identify inocula as small as 10 PFU of HSV 72 hours after inoculation. This was as early as the appearance of both CPE and HSV-1 direct IF. The EIA-F required only 50 μ L of MC suspension for identification, thus leaving the tube intact for further observation or subsequent immunological tests. Multiple specimens harvested over several days can be accommodated in one single assay from one single tube culture. Also, tests can be performed on specimens in which viral infectivity has been lost or which do not contain intact infected cells. The technique is therefore applicable when poor collection methods render specimens inadequate for use in cell culture. The assay was carried out rapidly, in less than 3 hours, and, by using the group monoclonal antibody specific for HSV, conferred absolute specificity to the assay and allowed the results to be quantitated in an automated spectrophotometer.

With higher inocula, such as 10^6 PFU, with both HSV-1 and HSV-2, it was found that the interval between inoculation of the culture and a positive result could be reduced to 18 hours. With an inoculum of 10^7 PFU (HSV-1), both EIA-F and CPE were positive 8 hours after inoculation. With HSV-2 at the same inoculum it was noted that the CPE was doubtful while the EIA-F was positive. These results may indicate the detection by EIA-F of unadsorbed input virus, which was not entirely eliminated by the two washings which followed inoculation of the MC tube cultures. The positive CPE present at that time does not therefore indicate viral replication, since a cytotoxic effect can be separated from productive infection (Darlington and Granoff, 1973) and the complete HSV replicative cycle is 12-18 hours (Rawls, 1979). It is unlikely that the higher number of cells sampled for the EIA-F tests carried out at an MOI of 10^{-2} to 10^1 were the cause of the earlier detection of a positive absorbance value. At an MOI of 10^{-2} , in two different experiments, with a significantly different number of cells present in the MC samples, the EIA-F tests became positive at the same time (48 hours). This suggests that beyond a minimal number of infected cells, EIA-F was not critically affected by the number of cells present in the sample. This is supported by the fact that the higher number of cells sampled in both HSV-1 and HSV-2 model experiments, carried out at an MOI of 10^{-2} to 10^1 did not affect the absorbance and variance values of the non-infected cell controls at the different sampling times. The number of cells present in those samples which were positive in the EIA-F assay were similar to that of other investigators, using

a different monoclonal antibody (Cleveland et al, 1982).

When the MC/EIA-F system was compared with CPE at low inocula, the only instance where CPE showed an advantage was at the 5 PFU inoculum level with HSV-1, but with HSV-2, the CPE and EIA-F were both negative even at 96 hours. When the EIA-F was compared with direct IF there were two 24 hour discrepancies with HSV-1, one to the advantage of EIA and the other to the advantage of IF. With HSV-2 there was only a 24 hour discrepancy to the advantage of IF. At high inoculum levels (10^4 - 10^6 PFU), CPE appeared to have a 24 hour advantage over the MC/EIA-F system or direct IF with HSV-1, however, with HSV-2, there appeared to be a 48 hour advantage of CPE over the MC/EIA-F system at 10^5 PFU.

The titration of the group monoclonal antibody was determined in the presence of a maximum amount of antigen, using approximately 12,500 infected cells/well. This was to ensure that small amounts of antigen present in the model experiments or in clinical samples would be detected. From the results it is evident that the antibody can be used at a lower dilution than the one that was chosen (1:32,000), but that will increase the risk of unacceptable high background levels. The conjugate, however, could only be used at dilutions between 1:2000 to 1:4000. Otherwise, the background levels would reach unacceptable levels. In the results of the model experiments using HSV-1 and HSV-2, there are discrepancies when using the MC/EIA-F system which suggest that the group monoclonal antibody may have a higher affinity for HSV-1 antigen or that a significantly higher number of cells were sampled in the HSV-1 experiment, therefore resulting

in these discrepancies.

The MC/EIA-F system was not as sensitive as cell culture, however, in general, it was found to be more sensitive than direct IF in these model studies. On theoretical grounds, the EIA should be more sensitive than IF staining. The enzyme label can have a continuous action on the substrate, producing increasing amounts of reaction product, which will therefore amplify the initial reaction at the site where virus is present in the specimen (Walsh et al, 1979; Yolken, 1982; Goldstein et al, 1983).

With the increasing number of adult genital HSV infections and consequently increased neonatal HSV infections transmitted from vaginal deliveries in the presence of active viral shedding, the need for rapid and accurate diagnosis of genital HSV infection has become very important. In view of the good results obtained with the HSV-1 and HSV-2 model studies using the MC/EIA-F system, the next phase of our study was directed at the detection of HSV antigen in direct clinical specimens using the EIA-F assay. We compared these results in terms of sensitivity and specificity to a commercially available EIA kit, as well as to the presence of CPE in cell cultures.

As was suggested by our findings in the phase II, culture was the most sensitive technique for the detection of HSV and confirms the results obtained by Cho and Feng (1978). However, this method can be slow depending on the level of viral concentration in the specimen. Another limitation of the method is that only infectious virus is detected in culture, so that stringent specimen transport conditions are necessary to avoid loss of infectivity. Detection of HSV antigen in the absence of virus

isolation is not uncommon (Moseley et al, 1981; Vestergaard and Jensen, 1981; Goldstein et al, 1983). Various phenomena account for this: a) the incomplete expression of the viral genome or the production of defective viral particles during the course of infection, b) the inactivation of virus by neutralizing antibodies, and c) the loss of viral infectivity due to inappropriate handling before culture. Those limitations are critical when it comes to the prevention of neonatal herpes.

The current dogma for the prevention of neonatal herpes calls for a C-section if HSV is present at the time of delivery, whether or not herpetic lesions are present. In fact, it is recognized that the majority of cases of neonatal herpes are acquired from asymptomatic mothers. However, the morbidity associated with C-sections is much greater than that of neonatal herpes and there is presently great concern at the increasing rate and costs of C-sections in this country and elsewhere. It is therefore essential that in a pregnant mother, no C-section should be carried out on the basis of a false positive HSV diagnosis. It is in this perspective that we examined the sensitivity and specificity of the EIA-F and O-EIA tests for the direct detection of HSV antigens in skin samples in parallel with cell culture. Both EIA direct detection systems for HSV in clinical samples were found to be not only insensitive but also to produce a significant proportion of false positive results.

Results in the phase III study with the clinical specimens indicate that the ORTHO-EIA (O-EIA) and EIA-F assays cannot replace culture for HSV detection. The O-EIA was found to detect 54% of HSV positive specimens, and the EIA-F was found to detect

only 16% of the HSV positive specimens by tissue culture. This direct antigen detection rate with the O-EIA, is in keeping with earlier reports by other investigators (Warford et al, 1984). The discrepancies between viral isolation and the two EIA's could be a result of the quality and handling of the specimens, as well as the stage of the lesions. There is also some doubt about the stability of the temperature (4°C) at which our specimens were stored before testing. In addition, they may have contained very few cells, as the CPE in the cell culture with most of our specimens took an average of 5 days to develop, suggesting that a small amount of virus was present. The low sensitivity of the O-EIA and EIA-F can also be attributed to several other causes: a) a majority of the specimens submitted were from asymptomatic patients, and b) the volume of the transport medium used in our study (2.0 mL) and antibiotics diluted the concentration of virus on the swab specimen. Reports by Morgan and Smith (1984) have shown that the detection of HSV antigen by ELISA is influenced by the amount of serum free medium (SF) used for specimen extraction. They reported a decrease of 15% in HSV antigen detection when using 3.0 mL SF medium as opposed to 2.3 mL of medium. More perplexing than the lack of sensitivity of these two EIA's in detecting HSV antigen in the clinical samples, was the number of false-positive results obtained. The EIA-F and O-EIA had a false positive rate of 53% and 45% respectively. These findings might have been due to the non-specific binding of viral antibodies to bacteria or other bacterial products in the samples, improper washings between steps, or the presence of noninfectious HSV antigen.

It is unlikely that in our series all the false-positive results were due to inactivation in transport. In the EIA-F test, the presence of endogenous peroxidase in the samples was controlled for by using wells containing the sample and the addition of substrate only, which would demonstrate the presence of peroxidase in the sample. However, in the O-EIA assay, the test does not control for the presence of endogenous peroxidase.

The results obtained with both of these assays suggest that they cannot replace cultures for HSV detection but are a reliable alternative to conventional viral isolation in cell cultures since 50% of the specimens can be detected in a matter of hours.

The last phase of our study was concerned with the use of the MC/EIA-F system for the detection of HSV-1 and HSV-2 in cultured clinical specimens to determine the sensitivity of the system by comparison with the detection of CPE. Using 300 uL samples of MC's, the EIA-F test became positive at the same time as the CPE, in approximately 50% of the cases, 2 days after inoculation. All the specimens were positive by EIA-F one day later. This 24 hour delay in the identification of 50% of the specimens is in fact illusory, since the detection of CPE is not a definitive result but a presumptive finding requiring a further confirmatory test.

The results of all our studies suggest that the MC/EIA-F system, IF, or other EIA's cannot replace cell culture for HSV detection but is a reliable alternative to conventional viral isolation in cell cultures.

CONCLUSION

The 2 objectives of our study were, (1) the development of a microcarrier culturing system in tube cultures to allow sequential sampling, (2) the determination of the sensitivity and of the specificity of the enzyme immunofiltration assay in combination with the microcarrier tube culture system for the detection of HSV antigens, by comparison with direct IF and the detection of CPE.

Experiments with HEp-2 and HFL cells on microcarriers in tube cultures over 7 days demonstrated that, although maximum cell yields were not obtained, nevertheless the cell yield (number of cells/bead) with both cell types was more than adequate for use in the EIA-F assay. Sequential sampling was possible, allowing for several replicate specimens for EIA-F or IF to be obtained from a single tube culture, without destroying the monolayer. As a result, this microcarrier system was very economical by comparison with standard tube monolayer cultures.

Experiments with the EIA-F in combination with the microcarrier tube culture system demonstrated that this system was very specific and almost as sensitive as cell culture, and more sensitive than direct IF for the detection of HSV antigens. The capability of this system to detect HSV antigens almost as rapidly as the appearance of CPE in cell cultures and the possibility of sequential sampling from one single infected tube culture makes it useful because: a) a large number of specimens can be tested in about 3 hours time, b) the diagnosis of HSV is performed by a quantitative method instead of relying on the detection of CPE by trained personnel, c) since sequential sampling is possible, specimens can be tested for infectivity everyday from the same

tube culture, d) the system is very economical, e) tests can be performed on specimens in which viral infectivity has been lost or which do not contain intact infected cells.

By contrast, the EIA-F was not efficient for the direct detection of HSV antigens in clinical samples. The EIA-F as well as commercial EIA kits were found to be insensitive and to produce a high percentage of false positive reactions. The use of these EIA assays for clinical specimens from pregnant females or for other clinical conditions where rapid diagnosis is needed are therefore not very reliable. As a result cell culture for HSV detection still remains the method of choice.

APPENDIX I

ENZYME IMMUNOFILTRATION ASSAY

Assay Reagents and Conditions

1. 10X concentrated PBS (0.1M)
 - a. 1 liter distilled H_2O
 - b. 2.0 g KH_2PO_4
 - c. 2.0 g KCl
 - d. 80.0 g NaCl
 - e. 21.6 g $Na_2HPO_4 \times 7H_2O$ or 11.44 g Na_2HPO_4

Mix in order given, place in 500 mL bottles and autoclave.

2. Gelatin buffer (0.3% gelatin in 0.01M PBS)
 - a. 430 mL of distilled H_2O
 - b. 50 mL of 10X PBS
 - c. 15 mL of a 10% aqueous swine skin type 1 gelatin stock solution warmed to $37^\circ C$. The stock solution must be prepared by autoclaving at $121^\circ C$ for 15 min.
 - d. 5 mL of a 1% thimerosal stock solution
 - e. Adjust pH to 7.4 with 1.0 N NaOH.

3. BSA buffer (1% BSA added to gelatin buffer)
 - a. Gelatin buffer plus enough powdered bovine serum albumin fraction V to make it 1% BSA
 - b. Adjust pH to 7.4 with 1.0 N NaOH.

4. FBS buffer (fetal bovine serum added to BSA buffer)
 - a. BSA buffer plus enough fetal bovine serum to make it 10% FBS.
5. Storage conditions for the reagents:
 - a. Room temperature - 10X PBS, 10% gelatin, 1% thimerosal, and 1.0 N NaOH
 - b. 4°C - Gelatin buffer,
 - c. -20°C - BSA buffer, FBS buffer
6. All reagents are warmed to 37°C just prior to use.

Substrate Buffers and Conditions

Horseradish peroxidase

Citrate Buffer:

Make 0.1 M citric acid solution by adding 9.6 g citric acid (anhydrous) to 500 mL distilled H₂O.

Make 0.2 M Na₂HPO₄ solution by adding 26.87g Na₂HPO₄ x 7H₂O to 500 mL distilled H₂O.

Add 486 mL of 0.1 M citric acid to 500 mL of 0.2 M Na₂HPO₄, pH mixture to 5.0, with NaOH or HCl, autoclave, store at room temperature.

Substrate:

10X substrate solution, add 240 mg of O-Phenylenediamine (OPD) to 60 mL of citrate buffer, add 3 mL to 1 dram vials and freeze at -20°C . When ready to use add contents of 1 vial to 27 mL of citrate buffer then add 6 μL of 30% hydrogen peroxide to each 30 mL of citrate buffer containing substrate, mix, use immediately. This is enough to do one plate. Make a fresh batch of frozen OPD concentrate each month.

ORTHO ENZYME IMMUNOASSAY

1. Capture antibody - 8x12 microwell strips (total of 96 wells) coated with a mixture of HSV-1 and HSV-2 antibodies (rabbit) and blocked with protein stabilizer.
2. Antigen standard - 1x2.6 mL of HSV-1 antigen in 0.05% phosphate buffered saline (PBS) containing 0.1% sodium azide and 1% carrier protein.
3. Detector antibody - 1x21 mL of peroxidase-conjugated HSV-1 and HSV-2 antibodies (rabbit) in 0.05 M PBS containing 10% carrier protein and 0.04% preservatives and stabilizers.
4. Substrate buffer - 1x28 mL of 0.024 M citrate - 0.05 M phosphate buffer, pH 5.1.
5. Hydrogen peroxide - 1x1.5 mL of 0.6% H_2O_2 in distilled water.
6. Substrate Concentrate - 1x1.5 mL of 4.5% o-phenylenediamine (OPD) in solvent.

7. Wash buffer concentrate - 1x50 mL of a 20X concentrate of 0.05 M PBS containing 0.05% Tween.

Preparation of reagents:

- a. Prepare a diluted wash buffer stock solution (pH 7.4) by adding the contents of the wash buffer concentrate bottle to 950 mL of distilled water.
- b. Prepare substrate solution immediately before use. From the total number of microwells to be used, determine the volume of substrate solution that will be needed (200 μ L per well). Allowing for a small excess, round off to the next mL and pipette the appropriate number of mL of substrate buffer. Add one drop per mL of hydrogen peroxide to the buffer and mix. Then add substrate concentrate, one drop per mL, and mix again.
Substrate solution must be used within 15 minutes.

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