B: Cell Culture

- Cell culture as a technique began with whole tissues or organs then progressed to utilization of cells.

- The advent of antibiotics made it possible to utilize cells and culture them and avoid the problem of contamination.

- In 1949, the breakthrough was made. Enders, Weller, and Rubin propagated polioviruses in primary human cell cultures marking the beginning of the “golden age of virology”.

- The utilization of this technique in virus isolation allowed the production of the first man-made vaccine for polioviruses in 1955, which resulted in the control of poliomyelitis. Poliovirus used to spread in epidemics causing tens of thousands of paralytic illnesses among children and sometimes death.
  - Poliomyelitis still takes place in certain developing countries and some few developed ones, but it has been proved under control in most of the world. And we are about to eradicate poliovirus.
  - The first polio vaccine was heat (or formalin) - inactivated, then a live attenuated (weakened) vaccine was produced early in the 1960’s, and it was used with the inactivated vaccine, they are still in use together (each has its own indications.)

- With the availability of this technique, widespread viruses were isolated from individuals who were not suffering from overt (obvious) clinical manifestations, indicating that subclinical infections are commonly caused by viruses. That’s another piece of evidence that most viral infections are subclinical or asymptomatic. Cell cultures are of different types depending on the origin of these cells, the ability of the cells to grow in the lab and the periods of time taken by each type.
Types of Cell Cultures:

1. **Primary cell cultures**
2. **Secondary cell cultures**
3. **Continuous cell cultures**: which are referred to as “cell lines” because a line can be established from these cells.

1) **Primary cell cultures**:

- Examples: Human Embryonic Kidney (HEK), Guinea Pig Embryo (GPE), Chick Embryo (CE), Primary Monkey Kidney (PMK).
- Epithelial cells in the origin.
- They are derived from fresh fetal tissues, whether human or animal fetal tissues.
- They can be cultured and grown in the lab for a few generations (2-3) generations or passages. This means that if we want to prepare a primary culture we can take cells from a culture and subculture them and from that (latter) subculture we can take cells and then subculture them again and so on. The maximum number is 3 generations, after which the cells won’t grow, and they will become no longer useful for virus isolation.
- These cells are very expensive because they can not be propagated for a large number of generations and they are obtained from fetuses (which aren’t easy to obtain); so for human tissues; they are obtained after cases of abortion, and for animal tissues they should be grown in the lab.

2) **Secondary cell cultures**: (Diploid)

Examples: Human Diploid Fibroblasts (HDF), Human Newborn Foreskin (HNF) which is obtained in cases of circumcision where foreskin can be obtained and prepared for virus isolation, Medical Research Council (MRC-5), Winstar Institute (WI –38).

- They are fibroblasts in origin.
- They are obtained from human or animal tissues.
Those cells differ from primary cell cultures in the fact that they can propagate for a relatively large number of passages (40-50) passages or subcultures so more number of source.

They are less expensive if compared to primary cell cultures because if you have a stock of cells you can divide them to a number of groups and put each in a vial, then you can subculture each vial for (40-50) passages (which produces a larger number.)

They retain the original diploid chromosome number throughout their propagation in vitro.

3) Continuous cell lines:

Cells (malignant in nature) of a single type capable of indefinite propagation in vitro (immortal).

They represent cancer cells (monoclonal proliferation of one cell type.)

Decreased ability to support viral replication after 100-120 passages. The fact that these cell can grow indefinitely does not mean that they are suitable for virus isolation indefinitely. It was observed that after 100-120 passages, these cells start to deteriorate in quality and they are no longer suitable for virus isolation. This is because they undergo changes that may be confused with cytopathic effects (CPE) produced by viruses. And this may lead to false positive results due to the decay of these malignant cells (After the 100-120th passage of the malignant cells, they start producing CPE similar to the ones produced by viruses, which may cause confusion in diagnosing and isolating viral infections.)

They are cheap; you can use one vial to prepare a large number of these cells.

Examples: Human Epidermoid cancer of the larynx (HEp-2), lung Adenocarcinoma (A - 549), Henrietta Lacks cervical carcinoma (HeLa (human)), Vero (monkeys), Madin Darby Canine kidney (MDCk (dogs) the most sensitive cell line for the isolation of the
influenza virus), L929, 3T3 (mice), Baby Hamster Kidney (BHK-21 (hamster)), and Rabbit Kidney (RK –13 (rabbit)).

These cells are obtained from different animals and each cell is suitable for certain type of virus and, in practice lab one of each of these cell type is used for isolation of virus and that’s why the physician should indicate to lab the virus that’s suspected so that the lab used the suitable cell line so usually for virus we use primary, fibroblast and continuous cell types.

➢ these cell lines are incubated together and they are followed up for the development of cpe which is the characteristic of different viruses so we have different characteristic or different CPE for different viruses that’s why in isolation of viruses the lab should be more than one cell type, two or three are used one primary, one secondary one continuously cell type

Techniques used to prepare cell cultures:
These cell line are prepared in culture continuous that could be:

- **Flasks:** which contain one flat surface.
- **Roller tubes:** which are tubes with a concave side and a flat side, they are put on drums and they rotate so that continuous supply with nutrients takes place.
- **Microtiter trays:** which are trays with multiple wells in them.
- **Shell vials:** (cover slips) are small vials that contain square or round cover slips like the ones used for light microsopical examination of slides. The covers are used as substrates to grow cells on it. And shell vials undergo centrifugation (we’ll come to this in a moment).

the cell are prepared from either cell line that are growing or tissues that are obtained from animals embryo (fresh tissues) with fetal or embryonic or fibroblast like newborn skin.

where the tissue is cut, minced then treated with trypsin and seeded in the surface and the cell culture will be grown and it is recommended usually to inoculate virus before the formation of the complete
monolayer so that viruses will infect cells during the formation of monolayer and changes that are formed as consequence of viral condensation in these cells are detected early before the deterioration of the monolayer and there are certain viruses like cmb and adeno viruses that require 2 to 4 week to grow caused evident (changes in the cell culture) so to avoid the problem of confusion with deterioration with the normal deterioration of cell, the culture is inoculated with clinical specimen before the formation of the monolayer and then changes can be detected before the deterioration of the cell culture (the normal tissue of the cell culture), and viruses can be identified by the changes that are in the form of the cytopathic effect (that can be either cell lysis or fusion of adjacent cells).

- **Identification** is based on:
  - **CPE**: cytopathic effect that develop, they are character
  - **Electron Microscopic examination**: certain viruses have characteristic morphology that enables their diagnosis.
  - **Immunologic methods**: including immunofluorescence (IF); by staining cells with a fluorescent dye, hemagglutination (HA), Hemagglutination inhibition (HA inhibition), Hemadsorption or hemadsorption inhibition, complement fixation (CF) and neutralization.

“CPE (1)”

*The right image: changes including rounding of infected cells in the monolayer (noticed mainly in the focus or the center) and detachment of the cells from the substrate when compared to normal uninfected cells far from the center.
*The left image: this is an advanced stage of infection when there is lysis of the monolayer.

“CPE (2)”

Syncitia formation can occur as shown in both images. It could be caused by RSV or measles viruses, they are known to cause fusion of adjacent cells with the formation of polykaryons or multinucleated giant cells.
**“Hemadsorption”**
If the monolayer was infected with a hemadsorbing virus, the addition of erythrocytes will lead to their attachment (adsorption) to the monolayer. Washing would not remove red blood cells if the cell culture was infected by the virus (because of the binding of erythrocytes to hemagglutinin), if not, the RBCs would be washed away.

Hemadsorption: the adherence of red cells to other cells, particles or surfaces. Hemadsorption and hemadsorption inhibition are used for the assay of some viruses and their specific antibodies, e.g. paramyxovirus.

**Slide 26:**
The first column (A+D): these are normal cells (fibroblasts). The second column: Epithelial cells are infected. Image (C): after infection of the cell, viral replication takes place and the monolayer starts to deteriorate until the whole layer is lysed.

**Primary changes can indicate a certain virus, if one virus was suspected, we can use immunological methods to confirm the diagnosis of viral infection.**

Although cell culture is the golden standard for diagnostic virology, it has some problems. Major problems with cell culture are:

1. **The long period of incubation required for results (up to 4 weeks).** Certain viruses like cytomegalovirus and adenoviruses may require 4 weeks to grow, by which time the cell culture cells will deteriorate.

2. **Often very poor sensitivity, sensitivity depends to a large extent on the condition of the specimen.** In cases of screening of viruses, we need a very sensitive technique that can detect all infections. Sensitivity of cell culture is low; which means that you can not detect every viral infection present in the cell culture. It has a high specificity to viruses but a low sensitivity.

3. **Susceptible to bacterial contamination.** In this case, a combination of antibiotics and antifungal agents are used to prevent contamination of cell culture. However, sometimes we are faced with resistant strains of bacteria that may contaminate the cell culture, and therefore damage or destroy it.
3. Susceptible to toxic substances which may be present in the specimen.

4. Many viruses will not grow in cell culture e.g. Hepatitis B, Diarrhoeal viruses (eg: rotaviruses which cause diarrhea), parvovirus (which causes infections of immature erythrocytes) and papillomaviruses. And there is not, yet, a cell culture system that can support the growth of these viruses.

- The problems of low sensitivity of viral isolation in cell cultures and the long period of time required for such isolation can be overcome by the application of Rapid Culture Techniques.

**C: Rapid Culture Techniques**

- These rapid techniques include what’s known as the “Shell Vial” technique; which is the most widely used rapid diagnostic technique that utilizes cell culture.

- The cell sheet is grown on individual cover slips in a plastic bottle. Following inoculation, the bottle then is spun at a low speed for one hour (to speed up the adsorption of the virus) and then incubated for 2 to 4 days. The cover slip is then taken out and examined for the presence of CMV (cytomegalovirus) early antigens by immunofluorescence.

  **Explanation**: In the shell vial technique, a monolayer of cells is grown on a cover slip which is added to a vial, then the clinical specimen is inoculated. This is followed by centifugation of the cell vial for about one hour.

- This centrifugation is believed to enhance the attachment of the virus to the cell culture (and thus viral isolation) by applying force; which decreases the time required for attachment. And this can reduce the time required to diagnose certain viruses from weeks to 2-4 days (period of incubation). This technique is used to demonstrate the presence of viral antigens like CMV in the monolayer.
Rapid Methods:

1. **Microscopy (Light, EM (Electron Microscopy), IEM (Immune Electron Microscopy)).**
   has different in magnification .
   LM: we can not detect the virus because the largest (poxvirus) is not in the range of power LM .
   
   (EM ,IEM ): can demonstrate the shape,size and the arrangement of the virus and can be used to establish diagnoses like ( adenovirus , Rotavirus ) that don't grow well in cell culture .
   
   IEM : very specific because we use antibody , and antibody bind to specific into the virus
   so other than the shape and arrangement which can be judged upon by EM , we have addition of specify of the reactant to the factor or the technique use , so it is most specific in making diagnoses .

2. **Antigen detection (IF, Agglutination, EIA (Enzyme ImmunoAssay)).** All of them are specific and are rabid methid of antigen detection .

3. **Serological methods** are used in most of the cases to confirm an infection that has taken place because most antibodies of acute infections are not produced early in the process of the disease, they are produced later. So if we demonstrate antibodies by complement fixation (CF), Immunoflouresence (IF),

RadiolImmonoAssay (RIA), Enzyme ImmunoAssay EIA or other methods, this can furnish the past diagnosis of viral infection. This applies for most viruses except for few ones where the incubation period is very long or the infection is chronic (like hepatitis B virus and HIV).In this case, we can demonstrate antibodies with the presence of the virus.
**4. Molecular Methods:** like *In situ hybridization* and *PCR* can detect viral genomes utilizing molecular methods which are very specific and very sensitive. They are widely utilized for viral isolation nowadays.

*In situ hybridization (ISH):* is a type of hybridization that uses a labeled complementary DNA or RNA strand (i.e., probe) to localize a specific DNA or RNA sequence in a portion or section of tissue.

**Electron Microscopy:**

- 10^6 virus particles per ml required for visualization, 50,000 - 60,000 magnification normally used.

- It is useful especially in the cases where viruses do not grow in cell cultures. It is the only method that can be used sometimes to document the presence of the virus as a consequence of examining of clinical specimens with high magnification.

- Viruses may be detected in the following specimens:
  
  a. **Feces:** Rotavirus, Adenovirus, Noroviruse, Astrovirus, and Calicivirus. Which are all causative agents of diarrhea. Diagnosis could be made within hours in such cases.

  b. **Vesicle Fluid:** HSV (Herpes Simplex Virus) and VZV (Varicella Zoster Virus) we can obtain fluids from the vesicles, or skin lesions that develop, then examine them for the presence of viruses.

  c. **Skin scrapings:** like in the case of Papillomavirus, orf, and Molluscum Contagiosum virus. And these don't grow in cell culture and that's why EM are used.

“Electronmicrographs”:

These are examples of viruses that can be demonstrated by electron microscopy. Adenovirus (left), Rotavirus (right).

**Molecular Methods:**
Methods based on the detection of viral genome are widely utilized nowadays. With the development of the hybridization techniques and the Polymerase Chain Reactions (PCR), these techniques have become more popular and sometimes they are the only techniques used. It is often said that molecular methods is the future direction of viral diagnosis.

However, in practice, although the use of these methods is indeed increasing, the role played by molecular methods in a routine diagnostic virus laboratory is still small compared to conventional methods.

These methods can not be used for every situation.

It is certain though that the role of molecular methods will increase rapidly in the near future.

The PCR relies on the amplification of small sequences or small amounts of DNA present; so if we have one molecule of DNA or RNA we can amplify it to millions, and these can be detected by electrophoresis.

Serological Methods:

Rely on the demonstration of antibodies (IgG or IgM antibodies) thorough viruses.

Titer: *The dilution of a serum containing a specific antibody at which the solution retains the minimum level of activity needed to neutralize or precipitate an antigen.

* A dilution of a substance with an exact known amount of fluid. For example, one part of serum diluted with four parts of saline is a titer of 1:4.

Criteria for diagnosing - Primary Infection:

- 4 fold or more increase in titer of IgG or total antibody between acute and convalescent sera (serum after recovery); meaning that two specimens are needed. One at the time of illness, and the other must be obtained 2 or 3 weeks later.
** Remember: many infections stay for a short time, and their antibodies are produced after the infection is gone. This is why measuring the number of antibodies produced in the recovery phase is a reliable evidence proving that the individual was infected.

- Serial dilution can be prepared by addition of certain volumes of the clinical specimen to a diluent (which is saline). Now the 4 fold policy is that the titer should increase at least by a 4 fold difference and not less than that; because a difference less than 4 fold could be due to reading errors (examiner errors). So if the clinical specimen titer was 20, we need to demonstrate a titer of 80 in the convalescent specimen; which means that antibody concentration increased during this while.

  o **Presence of IgM:** If IgM is present its usually the result of a primary infection because in secondary infections IgG is produced.

  o **Seroconversion:** is when the serum converts from negative to positive. If an individual was negative to HIV (for example) and suddenly he becomes positive, this represents seroconversion.

  o **A single high titer of IgG (or total antibody) - very unreliable.**

riteria for diagnosing- Reinfection

  o **4 fold or more increase in titer of IgG or total antibody between acute and convalescent sera**

  o **Absence or slight increase in IgM .**
Typical Serological Profile After Acute Infection

* There are two curves the dotted > IgM concentration, the continuous line > IgG concentration.

* The first exposure to the virus happens in the first week, the second exposure (reinfection) happens in the 5th week.
1) The infection starts with a production of IgM in small amounts, which last for a short while and then the levels of IgG increase.
2) In the case of reinfection (reexposure to the same virus) IgG increases. The time period required for the increase of IgG is less and small amounts of IgM are produced but IgG is produced at higher concentrations. That's why IgG is the most important in serological diagnosis of viral infections.

Usefulness of Serological Results

- How useful a serological result is dependent on the individual virus.

- For viruses such as rubella and hepatitis A, the onset of clinical symptoms coincide with the development of antibodies.

- The detection of IgM or rising titers of IgG in the serum of the patient would indicate active disease. Some viruses are monotypic (of a single type; no subtypes) so the infected individuals will develop immunity for life and they will not be reinfected by the virus. This shows
that the demonstration of these antibodies is a very sensitive and specific marker of infection.

- Many viruses often produce clinical disease before the appearance of antibodies such as respiratory and diarrheal viruses.

- So in this case, any serological diagnosis would be retrospective (indicative of past infections) and therefore will not be that useful.

- There are also viruses which produce clinical disease months or years after seroconversion e.g. HIV and rabies. So serological diagnosis is very important in these cases.

- In the case of these viruses, the mere presence of antibody is sufficient to make a definitive diagnosis.

Problems with Serology

- Long period of time required for diagnosis for paired acute and convalescent sera. In the case of corona viruses (which cause SARS “Severe Acute Respiratory Syndrome” that caused epidemics years ago) antibodies were not produced in the infected individuals before 21 days; one had to wait until 3 weeks to establish diagnosis of SARS. Therefor no public health measurements would be possible depending on serological data which took too long.

- Mild local infections such as HSV genitalis (which cause cutaneous lesions: HSV1: causes orofacial lesions, HSV2: causes genital lesions) may not produce a detectable humoral immune response. In such infections, antibodies are either not produced or are produced in very low concentrations; so they are not detectable. These minor localised infections may not be associated with seroconversion that usually happens due to the production of antibodies to viruses.

- Extensive antigenic cross-reactivity between related viruses e.g. HSV and VZV, Japanese B encephalitis, Dengue and pox
viruses **may lead to false positive results.** Some individuals with autoimmune diseases produce nonspecific antiviral antibodies, and that’s why in such cases where cross reactivity is extensive, serological diagnosis is unreliable.

- **Immunocompromised patients often give a reduced or absent humoral immune response.** They do not produce antibodies in sufficient amounts to be detected by serological diagnosis due to the immune suppressive state; HIV patients may not respond specifically to viral infections by production of antibodies.

- **Patients with infectious mononucleosis and those with connective tissue diseases such as SLE may react nonspecifically giving a false positive result.**

- **Patients given blood or blood products may give a false positive result due to the transfer of antibody;** passive transfer of antibodies. Blood that is donated may contain antibodies for certain viruses to which the blood donor was exposed to, so these antibodies will be transferred from the blood of the donor to the patient. If the patient develops an infection and serological diagnosis is performed, it may give false positive diagnosis of a viral infection due to the presence of antibodies in the serum of the individual.

**CSF antibodies**

- Antibodies are mainly present in the serum, but they are also present and can be produced intrathecally in the brain; particularly in the CSF (CerebroSpinal Fluid).

- **Used mainly for the diagnosis of** infections of the CNS like herpes simplex and VZV encephalitis or other types of meningitis.

- **CSF normally contains little or no antibodies.** The source of these antibodies is the serum, they cross the blood-brain barrier in very small amounts.
The following equation utilizes an indicator system like Albumin titer serum in CSF over serum (by dividing albumin in CSF concentration over the albumin in serum concentration), normal level is usually less than 1/100.

\[
\frac{\text{CSF antibody titer}}{\text{Serum antibody titer}} > \frac{1}{100}
\]
is indicative of meningitis

Presence of antibodies (more than 1/100) suggests meningitis or meningoencephalitis.

Diagnosis depends on the presence of an intact blood-brain barrier. If the result of the previous equation was more than 1%, this would indicate a non-intact blood-brain barrier, because an intact blood-brain barrier will prevent the leakage of antibodies into the CSF. However, if the blood-brain barrier was inflamed, its permeability increases and antibodies leak to the CSF.

In Summary :-

Diagnosis of viral infections can be done by:

1) **Visualize the virus; by EM like (adenovirus)** which is insensitive and laborious.

2) **Culture the virus;** viruses need living cells (animal, egg, culture) time consuming, a large proportion of clinically relevant viruses cannot be grown in vitro like HBV.

3) Detect antibodies against the virus, **indirect approach, dependent on host immunity.** It is usually a retrospective diagnosis of viruses.

4) **Find the viral nucleic acid (molecular technique), universally applicable, revolutionary technical developments** that allowed accurate, very specific and very sensitive diagnosis of viral infection.
That’s why a shift from all other techniques to molecular technique is taking place worldwide.

** Now, in different labs protocols are available for:

- Respiratory viruses (or respiratory pathogens in general); they have a multiplex PCR methodology, which uses probes to many viruses and bacteria that cause respiratory diseases.

- GI protocols; for viruses or bacteria that cause GI infections.

- CNS protocols.

Whenever infection is suspected, multiplex PCR is applied and we will be able to detect any of these agents and confirm diagnosis, that’s why molecular techniques have revolutionarised diagnosis of infectious diseases in general including viruses.

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