Contributions of molecular biology to diagnosis, pathogenesis and epidemiology of infectious diseases

Introduction

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Introductory remarks

Epidemiology is concerned with the cause, distribution and frequency of infectious diseases, as well as the distribution and transmission of infectious agents. Thus, diagnosis is the identification of an infectious agent in a host or in its products (urine, stool, saliva etc) and is intimately linked to epidemiology. The separation of the terms is in many cases artificial. The number of infectious agents is enormous. They range from viruses to multicellular eukaryotic organisms such as fungi and worms. Obviously, it is impossible to summarize the exciting new discoveries and developments concerning all infectious diseases in a reasonable space. Therefore, this multi-author review is restricted to the field of virology.

Viruses can neither be seen by the naked eye nor with the light microscope. Therefore, they are mysterious agents and were poorly understood for a long time. Since viruses do not possess synthesizing machinery they are obligatory parasites and multiply only in living cells. Virions – the extracellular infectious units – consist, after they have been released into the extracellular space, essentially of a program which is protected by protein molecules. The latter provide a few biological functions which allow a virus to penetrate a living cell. Thus, outside the living cell a virus only has an inactive potency.

In the viral kingdom, a bewildering variety of organizations of the programs is encountered. They may consist for instance of double-stranded or single-stranded deoxyribonucleic acid (DNA), or of double-stranded or single-stranded ribonucleic acid (RNA); they may be divided into split genomes, or contain partially double-stranded and partially single-stranded DNA. The single-stranded RNA may have a positive polarity serving as a messenger RNA or a negative polarity unable to serve as a messenger RNA. This flexibility of different program organizations provides the basis for the classification, and it explains the various multiplication strategies viruses use in the living cell.

The life-cycle of viruses can be formally divided into a number of phases. In a first phase, a virion is non-covalently bound to the cell surface if there are corresponding structures on both partners ('adsorption'). The virion or its program is then taken up, in eukaryotic cells either by fusion of the viral coat with the plasma membrane or by endocytosis ('penetration'). Within the cell, the program of the virion is released from the surrounding structures ('uncoating') to provide a template for biosynthesis of messenger RNAs or to act itself as a messenger RNA. The stage is now set to force the cell to synthesize all the macromolecules encoded in the viral program ('transcription', 'replication', biosynthesis of proteins, transport of these proteins). Finally, the virus-specific macromolecules form geometric structures ('morphopoiesis') and the progeny virions are released from the cell ('release').

The entire bacterial, plant and animal kingdoms are viral domains. Different viral families use different hosts and reservoirs, different mechanisms of transmission and survival. Most families span both vertebrate and invertebrate animals, and some viruses even infect animals as well as plants.

The symptomatology of the diseases results on the one hand from the interaction of the macroscopic host with the infecting virus population. Evidently, the organs or organ systems involved (liver, brain, etc.) reflect the pathways of distribution of the invading virus and the binding possibilities between cell types and viruses. On the other hand, the symptomatology of the disease reflects also the interaction at the cellular level. Thus, some general disease types may be discriminated such as a) the acute type with a transient production of many progeny viruses combined with eventual shedding into the bloodstream (e.g. yellow fever); b) the chronic-persistent type with a long-range production of low amounts of viruses (e.g. hepatitis); c) the latent type with recurrences (e.g. Herpes simplex or varicella zoster); and d) the slowly progressive type (e.g. certain brain diseases). One could add e) the integrative types, where viral genomes or parts of them are integrated into the host genome (e.g. papova viruses and retroviruses). However, (e) concerns rather the replication strategy and does not fit into the above scheme of general disease types.

Since viral replication encompasses multiple intracellular structures, including membranes (depending on the replication strategy of a given virus family) such as nuclear, endoplasmic, Golgi and cell surface membranes, viruses are also helpful tools in cell biology. Therefore a huge body of data concerning cell physiology as well as the pathophysiology of viral diseases has been accumulated[10].

Even though the virologist is confronted with seemingly endless variations, there is a common denominator unifying the whole viral kingdom: viruses consist of protein and nucleic acid. Proteins are antigens and accessible to a battery of serological assays. Nucleic acids are amenable to the methods of molecular biology. Thus, immunology and molecular biology provide the modern tools to study epidemiology, pathogenesis and diagnosis in the field of virology.

Molecular biology and diagnosis

The goal of all the new developments in the field of virus diagnostics is an early, specific and sensitive diagnosis, if possible directly in the specimens obtained from the patient. In earlier approaches, researchers were forced either first to multiply viruses from patients in cell culture or to use sera from patients whose antibody titer against a given virus increased in the course of the disease. The specificity or sensitivity of these earlier tests was not high enough to allow direct diagnosis with patient specimens, and thus diagnosis was desperately late in many cases.

Today, molecular biology is the fastest growing area in medicine and provides more and more tools for rapid diagnosis.

The new approaches involve the application of recombinant DNA technology, protein biochemistry and monoclonal antibodies (MAB). Pure molecular species of DNA, RNA or protein can be prepared from cells from a mixture of either nucleic acids or proteins. I therefore expect that in the near future rapid diagnosis of microbial diseases directly in clinical specimens will gain more and more impact, based on the detection of a microorganism's proteins (antigens) by MAB's, or based on a microorganism's nucleic acids.

Diagnosis based on microorganism's nucleic acids (nucleic acid hybridization) [21–23, 40]

DNAs are thread-like double-stranded molecules, containing four types of base, adenine (A), cytosine (C), guanine (G) and thymine (T) which are covalently arranged in a 'species-specific' sequence within a strand. The bases of one strand bind specifically to the bases of the other strand according to the complementarity rule, forming hydrogen bonds between base pairs; G pairs with C and A with T. If a fragment of a known microbial single-stranded DNA is isolated and/or cloned in the laboratory by recombinant DNA technology, it may be either radiolabeled with ^{32}P or labeled with non-radioisotopic biotin and used as a probe to seek its complementary strand in a clinical specimen (DNA-DNA hybridization). The latter is 1) spotted on a solid support (e.g. nitrocellulose filters) and used immediately for hybridization or 2) is used first to infect a cell culture growing on the support. 3) If the specimen contains bacteria, they may be grown on the support for a limited time. 4) Also, preisolated nucleic acids can be spotted on (dot-assay) or transferred to solid supports (e.g. after electrophoresis; Southern blot for DNA, Northern blot for RNA). The goal of all these variations is the same; to release the unknown nucleic acid from the microorganism, if possible directly on to the support, to fix it to the support and to hybridize it with the known labeled probe. Identification of an unknown DNA is then based on a positive signal following autoradiography (the ^{32}P-labeled probe is bound to the unknown nucleic acid) or after an enzyme assay (the biotinylated probe is bound to the unknown nucleic acid and in turn binds a streptavidin-coupled enzyme). Different enzymes may be used; alkaline phosphatase may be mentioned as one example. If cell cultures on the solid support are infected, the cells themselves may be used to provide the unknown partner for the hybridization, be it RNA or DNA. This approach is called in situ hybridization, a technique which may be important in patients whose sera are negative for the suspected viruses, as for example in cases of hepatitis[4, 46]. Sometimes culturing cells is impossible, because tissue sections were initially fixed and (paraffin) embedded. Again, the nucleic acids in the infected cells can be used as hybridization partners[5]. An example is the detection of cytomegalovirus. The ^{32}P-probe technique will not be widely applied in clinical laboratories because of the short half-life and the bio-hazard of this isotope. A promising substitute will be biotin which is safe and stable. Biotin labeling has already been extensively used to detect in situ viral DNA or RNA in clinical specimens (cytomegalovirus[36], hepatitis B virus[35], Herpes simplex virus[5], adenovirus[5], Epstein-Barr virus[49]).
Recently, the in situ hybridization for detection of specific messenger RNAs in individual cells using isotopic and non-isotopic probes has been optimized[48].

Diagnosis based on MABs [13]

In 1975, Köhler and Milstein[27] developed a technique for producing large quantities of pure MABs with pre-defined specificity. Spleen cells of an immunized (e.g. with a viral protein or peptide as an antigen) animal are fused in vitro with myeloma cells, a B-lymphocyte cell line with malignant growth characteristics. In the hybrid cell, the spleen cell produces and sheds the antibody molecules; the spleen cell is immortalized by the myeloma cell. The hybrid cells secreting the appropriate antibody are then selected and propagated. In contrast to conventional polyclonal antibodies present in sera of immunized animals, MABs can be produced in unlimited amounts. In addition, they are highly specific reagents and do not vary from one batch to another. Some MABs may be less sensitive than polyclonal antibodies and others may be too specific because they bind only to some types of an organism but not others[17].
Immunocytochemistry[6] can then be used in tests for viral and bacterial diseases, but enzyme-linked immunosorbent assays[29] are preferable if large series have to be worked up daily.
The use of MABs can be combined with more elaborate procedures such as protein separation with electrophoresis and transfer of the separated proteins onto supports (Western blot). There are numerous viruses for which MAB tests have been developed. Tests for respiratory syncytial virus (RSV), Herpes simplex virus (HSV) and cytomegalovirus (CMV) are in an advanced stage. MABs were also used to detect RSV directly in nasopharyngeal aspirates[2, 24].
To prevent severe damage of the newborn, rapid diagnosis (within hours) of HSV infection of the cervix in pregnant women immediately before delivery will be of great importance[7].
In the case of CMV, which causes severe disease in immunocompromised patients, fast tests for diagnosis are also needed[19, 20, 36], since conventional cell cultures assays may take up to 6 weeks.

Molecular biology and pathogenesis

The last 20 years have brought great progress in the understanding of the biochemical mechanisms involved in viral pathogenesis. However, the same level of knowledge has not yet been achieved as in the field of intracellular replication. In order to propagate, viruses must not only find susceptible cells, but they must also surmount host defence mechanisms for a sufficient period of time. Therefore, host defense reactions may themselves contribute to pathogenesis.
Some viruses, such as respiratory viruses, replicate and provoke a disease at the site of entry. In contrast, other viruses, such as enteroviruses, enter the host and spread to other organs. Many host and viral factors interact to shape the outcome of infections at the microscopic and macroscopic host level. Some aspects will be reviewed here.

Entry into the host, binding to the cell surface and polarity phenomena

Most viruses enter their host through the mucosa of the respiratory and gastrointestinal tracts. In case of viruses producing a disease at the entry site (e.g. influenza, respiratory tract; rotavirus, gastrointestinal tract), the cells involved in the entry, the cells of the primary replication, and the cells of the target organ are identical. In case of viruses which distribute within the host, a mucosal barrier must first be penetrated (e.g. enteroviruses such as poliovirus: entry in the gastrointestinal tract, disease of the central nervous system; varicella virus: entry in the respiratory tract, disease of the skin). There are some fascinating aspects behind these facts.
a) Poliovirus, for example, is an unusually resistant virus and survives the milieu stresses of the gastrointestinal tract such as acid pH, detergent action of bile salts and proteolytic enzymes. Depending on the type, reoviruses may or may not be inactivated by chymotrypsin. The resistance in vitro of a given type towards this proteolytic enzyme correlates with its capacity to replicate in vivo[43]. Thus, a certain gene (the M2 gene) seems to be a determinant of reovirus virulence by

regulating viral survival at the mucosal barrier. b) Viruses penetrating the mucosal barrier may utilize specialized intestinal[56] or respiratory epithelial[56] cells, the M cells overlaying Peyer's patches, tonsils and bronchus-associated lymphoid tissue. These cells take up particulate materials and transport them to cells of the lymphoid tissue of the antiluminal surface. c) Polarity phenomena at the cellular level may explain why certain enveloped viruses such as influenza virus are shed into the lumen of the entry organ, thus provoking a local instead of a generalized infection. A model epithelial system in vitro is the Madin-Darby canine kidney cell line. When confluent, these cells form an epithelium-like sheet with circumferential tight junctions which divide the cells into two polar domains. An apical one corresponds to the luminal surface of the renal tubule, and a basolateral one corresponds to the laminar surface. Influenza and parainfluenza viruses mature only at the apical ('luminal') surface; vesicular stomatitis virus buds from the opposite surface[42]. This polarity of virus maturation may be a consequence of a directed integration of viral membrane proteins into the cell surface membrane, and this in turn seems to be due to a specific targeting in the Golgi complex[41]. In addition, enveloped animal viruses have an infection polarity topographically coinciding with their maturation polarity. In the case of the vesicular stomatitis virus, the infection polarity may reflect the receptor polarity[14]. In the case of influenza virus which contains neuraminidase, infection from the basolateral side may be impossible due to the presence of sialic acid-containing serum protein[15]. Other examples of polarity can be cited in addition, for example hepatitis A or B viruses. The B virus is shed into the bloodstream, whereas the very stable hepatitis A virus, a picorna virus[34], is secreted via bile and stool. The nature of viral binding sites on the cell surface – termed receptors – is in many cases undefined. Binding studies may reveal thousands of binding sites on permissive cells[12, 32]. Since even cell membrane-integrated viral proteins were shown to mediate adsorption of unrelated viruses[15], the term 'receptor' seems to be inappropriate, the more so since 'receptors' identified so far have their own physiological functions[9].

Viruses such as hepatitis B and many of the arthropod-borne toga- and bunyaviruses are inoculated directly into the bloodstream. Here, the virions are immediately distributed to their target organs. Again, polarity phenomena may play a role. The representative of the encephalitic togaviruses, Semliki Forest virus, infects through the basolateral surface[15].

Endocytosis and cell-cell fusion 'from within'

Many cell-bound virus types are ingested by endocytosis, that is through coated pits and endosomes. Apparently, even the most complex virions which are enveloped by a lipid bilayer, studded with virus-coded proteins (such as parainfluenza-, influenza-, rhabdo- and togaviruses) are taken up as a whole. Endosomes carry proton pumps[54] which acidify their contents; thereby viral envelope proteins may undergo a conformational change which triggers the fusion of the viral envelope with the endosomal membrane[50]. In consequence of this, the inner part of the virion – the nucleocapsid or core –, containing the genomic nucleic acid is ejected into the cytoplasm.

The viral proteins involved in fusion are called fusion (F) proteins (e.g. Sendai virus: the envelope glycoprotein F[44]; influenza virus: the hemagglutinin, an envelope glycoprotein[25, 30]). In these cases, a proteolytic cleavage is required during maturation to render the virion fusogenic and infective. Staphylococcus aureus strains secrete a protease which performs the cleavage activation of influenza hemagglutinin[52a]. Cultured cell lines which lack a trypsin-like enzymatic activity to perform this cleavage undergo a single cycle infection. Having been infected with a competent, fusogenic virus, these cells produce noninfectious, nonfusogenic progeny virus incapable of a next round of infection. These virions can be rendered infectious by trypsin treatment in vitro[30]. Sendai virus with uncleaved F does not replicate in the lungs of mice unless previously activated by trypsin[52]. Influenza A viruses with noncleaved hemagglutinin are not even internalized after adsorption[45]. Probably the cleavage results in fusogenicity by creating and exposing a hydrophobic N-terminus of the fusogenic protein which then interacts with a bilayer[1, 18]. It must be noted that the fusogenic behavior is not observed solely during infection, either at the plasma membrane (fusion from without, FFWO) or in endosomes, but also during maturation. This is explained by the fact that in infected cells newly synthesized viral envelope proteins are transported by vesicles in a fashion similar to host cell surface proteins from the endoplasmic reticulum to the Golgi apparatus. These are finally integrated as transmembrane proteins into the cell surface membrane. In most cases, the organization of such viral transmembrane proteins (envelope proteins) is relatively simple and corresponds to the arrangement in the released mature virion as for example in case of Semliki virus[16]: a short, C-terminal domain in the cytoplasmic compartment is followed by a hydrophobic transbilayer stretch and a long N-terminal, glycosylated, sequence is localized on the extracellular face. Therefore, (pH-dependent) conformational changes can be artificially introduced and result in cell-cell fusion in an infected monolayer cell culture[26, 37]. This is called fusion from within (FFWI)[3] and corresponds to the well-known giant cell or syncytium formation which is one of the classical forms of cytopathic effects (CPE) in cell culture or in vivo[11]. We therefore consider endosomal fusion during infection to be a very similar phenomenon to FFWI during maturation at a topographically different place of the cell.

Cellular injury

Viral virulence is expressed in many cases in the destruction of infected cells in the target tissues. The specific basis for this deleterious effect is not well understood. Most viruses undergoing a lytic replication cycle reduce or eliminate host-cell DNA, RNA and protein biosynthesis.

Vesicular stomatitis virus rapidly shuts off cellular RNA synthesis at the level of nuclear transcription[55]. This effect may be brought about by an action of the glycoprotein of the infecting virions[33]. Similar effects have been reported for mumps viral glycoproteins[57]. At least one factor required for specific transcription by RNA-polymerase II is deficient in extracts from poliovirus-infected cells[8]. Vaccinia virus, on the other hand, induces a rapid degradation of cellular messenger (m)RNA. This in turn leads to inhibition of cellular protein synthesis and its diversion to exclusive production of viral proteins[39]. Many cytocidal RNA viruses (polio, mengo, reo, Newcastle disease virus, vesicular stomatitis virus, Sindbis virus) cause an inhibition of host DNA synthesis. The mechanism is obscure; in some cases, hydrolytic enzymes of precursor deoxynucleotides may become active[28]. Several viruses block the synthesis of host proteins. In the case of Semliki Forest virus protein biosynthesis is slowly depressed, until finally only viral proteins are formed. A candidate for the inhibition of host cell protein synthesis is the viral core protein[51].

In contrast, polio infection results in a rather fast shutoff of host protein biosynthesis. The cap binding protein complex which together with the m^7 Gppp N(m) cap structure at the 5'-end of mRNA facilitates the attachment of 40 S ribosomal subunits to host mRNA during initiation of translation is apparently inactivated[53]. This factor is not needed for polio-

virus mRNA translation since this RNA is not capped. Inactivation of the cap binding protein therefore results in a preferential translation of polio mRNA.

On the other hand, influenza virus uses cap structures and adjacent oligonucleotides present on host mRNAs as primers for viral mRNA synthesis; they are transferred in the cell nucleus to influenza RNA by a virus-specific enzyme[38]. Inhibition of host-cell protein biosynthesis may be due to this mechanism. In adenovirus-infected cells, the fiber protein appears to be responsible for the shutoff of host-cell macromolecular synthesis[31]. Inhibition of protein synthesis may explain several additional observations, for example the disruption of the cytoskeleton or parts of it as in reovirus infection[47]. Thus, there is circumstantial evidence that CPE (other than FFWI) are connected with virus-induced effects on host-cell metabolism. However, the reaction chains leading to CPE and finally cell death are not known. It is also not known whether there are common reactions or whether each virus has its own strategy to overpower and finally kill the host cell.

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Molecular biological methods in the diagnosis of viral disease

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Summary. Molecular biology allowed considerable improvements in diagnostic procedures by production of new and more specific sonds for the detection of traces of viruses, both on the nucleic acid and protein levels, and by determining the immune response of the host to specific antigens. Improvements in sensitivity and improved correlation to the stage of viral disease are already evident from several applications and strongly suggest a broad application of these approaches.
Key words. Nucleic acid hybridization; synthetic peptides; computer analysis; antigen selection; recombinant antigens; rapid diagnosis.

Introduction

Development of virus-specific therapy and the need for fast differential diagnosis for intensive medical care challenge quick diagnosis which should also allow conclusions for the stage of disease.

Classic methods of viral disease diagnosis are based on the cultivation of agents, direct demonstration of viral antigens, e.g., by immunofluorescence, and the detection of specific antibodies. Many of the procedures currently in use have the disadvantage that the reading of results requires well-trained personnel and disease-related advice is often not given or comes too late. This is particularly true for chronic neoplastic or reactivated acute virus-related diseases. Additional problems arise, e.g. form Hepatitis B, through long transitions from antigen or antibody excesses in connection with a complete exclusion of remaining infectiosity.

Detection of genetic material of viruses by nucleic acid hybridization

Considerably influenced by the findings of tumor virology, i.e., that viruses persist in cells and can change these without

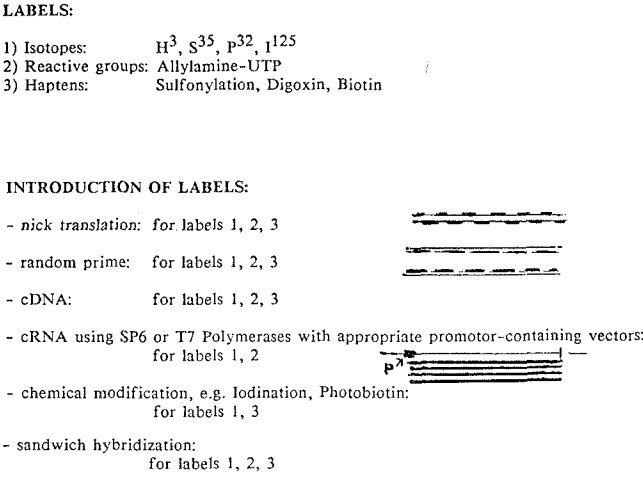

Figure 1. Introduction of label in nucleic acids for use as hybridization probe[1, 7, 8, 13, 14, 16, 19, 23, 24].