XA9847616

Chapter 19

DNA TECHNOLOGY FOR DIAGNOSIS AND VACCINES FOR INFECTIOUS DISEASES

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Introduction

As far as the infectious diseases are concerned the outlook for the developing countries looks grim. In India, for instance, during last few years, there have been at least three killer epidemics - from encephalitis virus, leishmania and malarial parasite. Besides, leprosy is also endemic in certain parts of the country. These four infectious diseases have been chosen for discussion either because these are currently the major health problems or they have some interesting features making them attractive for research.

All the four diseases have a complex life history and some of the infectious agents have more than one host for multiplication. Secondly, these agents interact with the human system in a very intricate way. A useful generalization may be to bear in mind that on an average a viral genome may code for about a dozen to 300 genes, a bacterial genome for 2000 or so genes and a eukaryotic genome from 4000 genes and up. On the other hand, mutations may occur with a very high frequency, as for example, they do in AIDS virus and influenza virus - which creates new problems for diagnosis, vaccines and drug design.

Three or four general strategies are adopted for the control of infectious diseases. Early diagnosis, vaccination and chemotherapy. In the situations where there is transfer through mosquitoes or ticks from alternate hosts, control of the vector and of the infection in the alternate host are additional measures to be taken. This Chapter looks at the problems of disease control from the perspective of genetics, since molecular genetics now provides powerful tools in the form of radiolabelled DNA probes and clones of selected segments, useful for diagnosis as well as for vaccine design.

Molecular techniques in DNA diagnostics

DNA (Southern) blot technique is a very versatile one. In this technique, DNA is first digested with a restriction nuclease which may yield a number of fragments but of fixed size. These can be separated by agarose gel electrophoresis and blotted on to a membrane-filter. These are denatured and form the target sequence for a ^{32}P-labelled or S-labelled DNA probe. The probe or the detector DNA molecule is usually a segment of cloned DNA. Its hybridization with the target molecules indicates the presence of sequences homologous to the probe. Sensitivity of detection is so high that the DNA of a single hair or a small volume of blood, lysed and bound to filter membrane, can be used as the target for diagnostics.

DNA sequence analysis is accomplished either by the so-called dideoxy-nucleotide method or by chemical breakage induced at specific nucleotide sites. In the dideoxy method, a synthetic end-labelled oligonucleotide is used as a primer. Four different chain-extending reactions are set-up, each containing the identical primer and the template to be sequenced mixed with three deoxynucleotides mixed with one of the chain-terminating

dideoxynucleotides. When separated on polyacrylamide gels by electrophoresis, chain lengths are separated lengthwise differing in a single nucleotide length forming a kind of zigzag ladder from which the sequence can be read after autoradiography.

Japanese Encephalitis (JE)

It has a fairly wide distribution and is prevalent in Asia, southern USSR, much of China, Japan, Taiwan, Philippines and India. It has a mortality rate of 8%, neurological residual effect on 30% and persistent mental disturbance in 10% of the cases.

JE virus belongs to Flavivirus arthropod - borne virus group B. This group includes also the Dengue and Yellow Fever virus. These viruses can replicate both in invertebrate (mosquito/tick) and vertebrate (man) hosts. It is a RNA virus. The invertebrate host of the JE virus is generally a species of Culex mosquito. Man - mosquito - man cycle probably does not occur on any significant scale, and although swine are implicated as an alternate host, it has been proposed that the 'Basic Cycle' goes through birds in India. The inactivated Nakayama strain of the virus grown in mouse brain, is used as a vaccine of which only about two million doses are being produced in India. Inactivation with radiation of JE virus is being tried at present.

Leprosy

The prevalence worldwide of leprosy has been estimated to be between ten and twelve million cases, the disease being endemic in Asia, Africa and Latin America. The causative organism *Mycobacterium leprae* can develop resistance to the commonly used chemotherapeutic drug Dapsone, and thus a vaccine would be useful. Although both humoral and cellular immune responses to the infection occur, cellular immunity apparently determines the outcome of the infection. In lepromatous leprosy patients, there is a specific deficit in Cellular Immune Response (CIR) which then allows an unlimited replication and spread of the mycobacteria. Tuberculoid leprosy patients suffer from enhanced CIR resulting in the destruction of nerve endings.

An important 65 kD antigenic protein of *M. leprae* has been characterized. It is somewhat similar to heat shock proteins. This protein contains an *M. leprae* B cell epitope and a number of T cell epitopes. A HLA-DR2 restricted, *M. leprae*-reactive T cell clone 2F 10 has been isolated from a human tuberculoid leprosy patient which seems to proliferate in an in vitro situation specifically in response to *M. leprae*. At low concentrations only Dharmendra lepromin or Armadillo- derived *M. leprae* induce a good proliferation response. Only *M. vaccae* at high concentration induces a fair response. None of the other 18 mycobacterium species tested including *M. tuberculosis* induced any response.

In order to characterize the epitopes involved in inducing this response, 65 kD protein of *M. leprae* produced from *E. coli* was tested. It gave a good response along with Armadillo-derived *M. leprae*. However, 65 kD protein from *M. bovis* BCG from *E. coli* gave no response. Sequence of the minimal peptide (the so-called peptide 17) for stimulation

of clone 2F, 10 has been determined and shown to be L Q A A P A L D K L. It is flanked by leucine at either end and omission of either leucine results in the loss of stimulatory activity. A specific monoclonal antibody III E9 also inhibits this activity. Similarities have been noted between peptide 17 and an HLA DR2 peptide. It is from the third hypervariable region of the DR2 B3 chain that stimulates proliferation of 2F10 although with a 100-fold lower efficiency. DR2 allele is apparently present only in about one third of most populations. Its role in pathogenesis of tuberculoid leprosy is suggestive.

Leishmaniasis

Leishmaniasis is caused by at least 14 different species or subspecies of leishmania. There are three types of leishmaniasis:

(a) simple cutaneous, which is often self-limiting,

(b) mucocutaneous, involving destruction of nasal tissue and

(c) visceral disease, a systemic infection which is fatal if not treated. The parasite is transmitted by a sandfly vector. Generally the diagnosis is done by direct examination of tissue biopsy or by a delayed type of hypersensitivity reaction (Montenegro test). Direct treatment requires that the strain is identified.

DNA probes are useful in that first of all it eliminates the need for culturing of the parasite. The major new world species of leishmania can be distinguished by DNA probes of total kinetoplast DNA (kDNA). Sample from the lesions can be directly applied to a nitrocellulose filter and processed further. In a comparison of tests (Montenegro, DNA hybridization, culture and histopathology), DNA hybridization test was more sensitive than either culturing or histopathology tests. It was positive in about 80% of the patients detected by Montenegro test. Montenegro test is an immunological test. So there is a possibility that it is positive even in patients with previous infection or cross-reacting antigen. On the other hand, kDNA probes used may not be appropriate for all patients. For the old world leishmania, even the species cannot be distinguished. Subcloning or some other constructs may help in distinguishing old world species and subspecies. Sandflies can be directly squashed on nitrocellulose filters and checked for the presence of leishmania by suitable DNA probes.

Malaria

Malaria parasite *Plasmodium falciparum* has a complex life cycle with stages in the female anopheles mosquito and in the liver and blood (erythrocytes) of the intermediate host (man). Symptoms are caused by asexual parasites and these proliferate in erythrocytes. Sporozoites released by mosquito in the human system enter hepatocytes and form schizonts

which release merozoites into the blood. Merozoites infect erythrocytes. Merozoites multiply in erythrocytes and rupture them. Some of these differentiate into male and female gametocytes. These are picked up by the mosquito and these develop into male and female gametes. Fertilization occurs and the zygotes develop into ookinetes which invade the gut where sporozoites form within oocyte. Sporozoites next migrate to the salivary gland of the mosquito and will infect humans during the next blood meal.

The detection of malaria is possible by microscopic examination of a blood smear. This is adequate for diagnosing acute malaria but is time-consuming and requires a trained technician when low numbers of parasites are present. ELISA is being developed but has the disadvantage that antibodies might be present even after disappearance of the malarial parasites.

DNA probes have been developed which detect the current infection in the blood. Even parasite density is measurable and the method is adaptable to field use. Blood sample obtained by a digital puncture can be directly applied to nitrocellulose filter.

A *Plasmodium falciparum* genomic DNA library has been constructed which was screened by colony hybridization using nick translated *P. falciparum* DNA. From one thousand colonies, seven were selected for their hybridization intensity. DNA of these was digested, electrophoresed and transferred to nitrocellulose. Filters were probed either with human DNA or *P. falciparum* DNA. pPF14 showed intense hybridization suggesting the presence of highly repeated DNA sequences. *P. falciparum*, *P. vivax* and *P. cynomolgi* DNAs were all probed with pPF14. Of these, only *P. falciparum* hybridization was positive, indicating specificity of the probe. The detection level was down to 10 pg which is equivalent of about 100 malarial parasites. It is estimated that whereas one microscopist can read 60 slides per day, with DNA hybridization, almost 1000 samples can be examined per day.

Making of malarial vaccines has been greatly facilitated by the development of technology for culturing of *P. falciparum*. Antigens involved in protective immunity could be identified, and the immunity is both species- and stage-specific. Several ideas for making vaccines have been tried, three of which are discussed. The first one is a vaccine prepared by synthesis of a peptide consisting of four amino acids Asp - Ala - Asp - Pro (NANP) repeated three times. It is a dominant epitope of *P. falciparum* and monoclonal antibodies to sporozoite react with it.

$(NANP)_3$ sequence is repeated 37 times in circumsporozoite (CS) protein of Brazilian *P. falciparum* but only 23 times in Thai *P. falciparum*. This vaccine is being tried in humans in Africa.

Yet, another potential vaccine has been prepared by fusing the immuno - dominant repetitive epitope of CS protein to the pre-S2 region of the surface antigen of hepatitis B virus. Such hybrid proteins assembled into particles induce a high-titre antibody response.

Finally, a model oral vaccine has been prepared and shown to induce cellular immunity in mice. A *Salmonella typhimurium* WR 4017 avirulent strain with impaired ability to multiply in macrophages is transformed with plasmid pMGB2 which consists of a plasmid with *P. berghei* CS protein expressed constitutively. Female BALB/c mice 6-8 weeks old were immunized by the subcutaneous (10^4 bacteria) or oral (10^9 - 10^{10} bacteria) route. Only subcutaneous treatment induced low levels of antibodies at four weeks but not at five weeks. Mice that were given bacteria orally made no antibodies. Mice challenged between four to nine weeks with sporozoites (apparently) induced cell immunity. Thus, this technique is also important for T-cell epitope identification.

Comments

Life cycle, DNA diagnostics and potential vaccines has been reviewed for four infectious diseases. These are Japanese Encephalitis, leprosy, leishmania and malaria. They are quite common in many developing countries and need the attention of researchers and health authorities. There is very little or no characterization of pathogenic strains that cause encephalitis. Vaccines can be effective only when these are specific to the particular strain. Thus, initial effort on a large-scale should be directed towards identification and characterization of pathogenic strains.

Leishmania is currently raging in North India. Although, the new world species can be distinguished by kinetoplast DNA (kDNA) probes, this is difficult for the old world species. Probably a set of subclones of kinetoplast DNA or RFLP (Restriction Fragment Length Polymorphism) analysis of genomic DNA may be of help.

Research on malaria is quite advanced. Specific probes like pPF14 are already available and perhaps that identify drug resistance strains probes can also be made. The malaria parasite's epitopes consisting of repetitive amino acid sequence are intriguing. Their number can vary as has been shown for Thai and Brazilian strains. The significance of this is not clear nor is the mechanism of generation understood.

With regard to malaria vaccines, perhaps the induction of cell - mediated immunity may turn out to be more efficacious. Although there are two other vaccines, viz. synthetic and as fused protein, their usefulness remains to be demonstrated. The mouse model system has the added advantage that some antigen genes may be introduced to make transgenic mice and their expression seen.

Leprosy is endemic in quite a few developing countries. Recent researches have provided new insight that cell-mediated immunity may be more important. Specificity of peptide 17 (or a ten amino acid epitope of it) of 65 kD protein in inducing proliferation of T-cell 2F10 cell line derived from tuberculoid leprosy patients suggests that for diagnosis, DNA sequence corresponding to it may provide a useful probe. Because ten other mycobacterium species failed to stimulate the proliferation of 2F10 cells, it may be assumed that it will also be a distinguishing probe. The similarity of this epitope to HLA-DR2 B3

hypervariable region suggests that this may have a role in the pathogenesis of tuberculoid leprosy. Possibilities for further sophisticated research are abundant both in malaria and in leprosy.

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