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State of the art of new vaccine research and development

Immunization, Vaccines and Biologicals

World Health Organization

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Abbreviations and acronyms

AIDS	acquired immunodeficiency syndrome
ANRS	French National AIDS Research Agency
ARI	acute respiratory infection
BCG	Bacille Calmette–Guérin (vaccine)
BL	Burkitt's lymphoma
bp	base pair (ribo- or deoxyribo-nucleotide)
BV	Buruli ulcer
cAMP	cyclic adenosine monophosphate
CDC	Centers for Disease Control and Prevention (USA)
CFA	colonization factor antigen (*E. coli*)
CFR	case fatality rate
CRM	cross-reactive material (diphteria toxin)
CS	*E. coli* surface (antigen)
CSP	circumsporozoite protein
CT	cholera toxin
CTB	cholera toxin (subunit) B
CTL	cytotoxic T lymphocyte
CVD	Center for Vaccine Development (University of Maryland)
DHF	dengue haemorrhagic fever
DNA	deoxyribonucleic acid
DOTS	directly observed treatment, short course
DSS	dengue shock syndrome
DT	diphteria toxin
DV	dengue (fever) virus
DTH	delayed-type hypersensitivity
EBNA	Epstein-Barr (virus) nuclear antigen
EBV	Epstein-Barr virus
EDCTP	European Developing Countries Clinical Trials Partnership
EPI	Expanded Programme on Immunization (WHO)
ETEC	enterotoxigenic *Escherichia coli*
ERIG	equine rabies immunoglobulin
FDA	Food and Drug Administration (USA)
FIPV	feline infectious peritonitis virus

GAVI	Global Alliance for Vaccines and Immunization
GBS	Guillain-Barré syndrome
GMP	good manufacturing practice
GSK	Glaxo Smith Kline Biologicals
GST	glutathione S-transferase (Schistosomia)
HAV	hepatitis A virus
HBcAg	hepatitis B capsid antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCV	hepatitis C virus
HDCV	human diploid cell (rabies) vaccine
HEV	hepatitis E virus
Hib	*Haemophilus influenzae* type B
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPV	human papillomavirus
HRIG	human rabies immunoglobulin
HSV-1/-2	herpes simplex virus type 1/type 2
HuCV	human calicivirus
IAVI	International AIDS Vaccine Initiative
IFPMA	International Federation of Pharmaceutical Manufacturers
IS	intussusception
IVI	International Vaccine Institute (Seoul, Republic of Korea)
IVDU	intravenous drug user
JEV	Japanese encephalitis virus
kb	kilo-base (RNA or DNA)
kD	kilo-Dalton (molecular weight)
LPS	lipopolysaccharide
LRI	lower (tract) respiratory infection
LT	heat-labile toxin (*E. coli*)
MAP	multiple antigen peptide
MAPK	mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney (cell line)
MDR-TB	multidrug resistant tuberculosis
MHC	major histocompatibility complex
MIM	Multiple Initiative on Malaria
MRC	Medical Research Council (United Kingdom)
MVA	modified virus Ankara (vaccinia virus strain)
MVI	Malaria Vaccine Initiative (PATH, USA)
NIAID	National Institute for Allergy and Infectious Diseases
NIH	National Institutes of Health (USA)
NPC	nasopharyngeal cancer

NV	Norwalk virus
OMPC	outer membrane protein complex
ORF	open reading frame
PA	protective antigen
PATH	Program for Appropriate Technology in Health (USA)
PCECV	primary chick embryo cells (rabies) vaccine
PCR	polymerase chain reaction
PEP	post-exposure (rabies) prophylaxis
PFP	purified (paramyxovirus) F protein
PHKCV	primary hamster kidney cell (rabies) vaccine
PID	pelvic inflammatory disease
PIV (-1, -2, -3)	parainfluenza virus (type 1, 2, 3)
PS	polysaccharide (capsular)
R&D	research and development
RIG	rabies immunoglobunin G
RNA	ribonucleic acid
RRV	rhesus rotavirus
RSV	respiratory syncytial virus
RV	rotavirus
SARS	severe acute respiratory syndrome
SARS-CoV	SARS coronavirus
S/HIV	simian-human (chimeric) immunodeficiency virus
SIV	simian immunodeficiency virus
ST	heat-stable toxin (*E. coli*)
STD	sexually transmitted disease
SVDP	Schistosomiasis Vaccine Development Programme
TB	tuberculosis
TBEV	tick-borne encephalitis virus
TDR	(WHO) Tropical Disease Research (Programme)
TT	tetanus toxin/toxoid
UNAIDS	(Joint) United Nations (Programme) for AIDS
UNDP	United Nations Development Programme
UNICEF	United Nations Children's Fund
URI	upper respiratory infection
USAID	United States Agency for International Development
Vi	virulence (antigen)
VLP	virus-like particle
WHO	World Health Organization
WNV	West Nile virus
WRAIR	Walter Reed Army Research Institute (USA)
YF	yellow fever
YFV	yellow fever virus

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Preface

Infectious diseases are responsible for a third of all deaths worldwide, killing at least 15 million people a year. Of these, more than five million are children under five years of age. The health disparity between rich and poor countries results in average life spans of 77 and 52 years, respectively. Deaths attributable to infectious diseases contribute most to the disparity.

The most effective way to reduce disease and death from infectious diseases is to vaccinate susceptible populations. The impact of vaccination on the health of the world's population cannot be over stressed. With the exception of water sanitation, no other modality – not even antibiotics – has had such a major effect on mortality reduction and population growth. The campaign for the eradication of poliomyelitis, involving vaccination of more than 2 billion children, reduced the global incidence of polio by 99.9%, from 350 000 cases per year in 125 countries on 5 continents in 1998, to less than 800 cases in 6 countries on 2 continents in 2002; today there is hope to eradicate the disease within the next few years.

Today's technologies can provide adequate tools to detect, control and even prevent emerging infections. However, during the last decade, not only did malaria, tuberculosis (TB) and HIV* thrive, but there was actually a worsening of tuberculosis and malaria in the immunocompromised population infected by HIV. Worse, at least two million children still die each year from diseases that could have been prevented by already existing low-cost and effective vaccines. On top of this high death toll, millions more children are suffering disability and illness because they have not been properly immunized.

The global control of infectious diseases requires global and continuous collaboration, coordination of regulatory agencies and development of vaccines to prevent all diseases, as well as a sustainable economic system to support these initiatives.

Although highly effective vaccines are available against a number of pathogens, the world's poorest regions are still suffering a heavy toll of premature death and disability from infectious diseases for which vaccines either do not exist or need to be improved. For these diseases, it is of crucial importance that vaccine research and development be considered a priority. The present document represents an extensive analysis of the state of the art of vaccine research and development against infectious diseases of public health importance for which vaccines are still either non-existent or need substantial improvement.

* These three diseases alone contribute to half the global burden of infectious diseases.

1. Diarrhoeal diseases

Conservative estimates place the death toll from diarrhoeal diseases at four to six million deaths per year, with most deaths occurring in young children. In some developing countries, children have more than 12 episodes of diarrhoea per year and diarrhoeal diseases account for 15–34% of all deaths. The diversity of bacterial and viral infections that may cause diarrhoea complicates accurate surveillance and diagnosis, especially in developing countries with little or no access to modern laboratory procedures. The specific disease burden attributable to a particular infectious agent is especially complex, given the multiplicity of these agents and their serotypes, and depends largely on laboratory facilities. While, in the long term, access to clean water, better hygiene, adequate nutrition, and improvement of sanitary measures would certainly have the greatest impact on diarrhoeal diseases, immunizations against specific diseases are the best hope for the short and mid term. This is particularly true for viral diseases such as rotavirus, present in both high and low hygiene-level countries.

1.1 Caliciviruses

1.1.1. Disease burden

The role of caliciviruses as agents of gastroenteric diseases has long been unrecognized and under-appreciated because diagnostic tools were not commonly available or used. The application of new molecular diagnosis assays has shown that human caliciviruses (HuCVs) are significant contributors to diarrhoeal disease burden in children and adults. They appear to be the most common cause of gastroenteritis outbreaks in the United States, and a common cause of sporadic cases of acute gastroenteritis with vomiting, abdominal cramps, diarrhoea, headache and fever. The implicated vehicles of infection are contaminated water, shellfish, and food contaminated either at its source or by food handlers. Transmission also can occur by person-to-person contact or through contaminated objects.

HuCVs accounted for a 20% etiologic share of all episodes of acute gastroenteritis in prospectively followed children between two months and two years of age in Finland. In Japan, among 95 hospitalized children with acute gastroenteritis, rotavirus was detected in 47% of cases followed by HuCV in 18%. From a prospective population-based cohort study with a nested case–control conducted in the general Dutch population, it appeared that HuCVs were the second most common agent of severe diarrhoea in children, after rotavirus, and the most common cause of outbreaks of acute gastroenteritis, including those that are foodborne. The role of HuCV in developing countries remains to be established. However, in several southern and East African countries, most children have acquired serum antibodies to the

Norwalk agent very early in life; this suggests that these viruses may play a pre-eminent role in diarrhoeal diseases in children from developing countries. In Beijing, China, infants had a seroprevalence rate of 41% for HuCV at 7 months of age, with the figure increasing to 65% at 1 year, 85% at 3 years, and 100% at 8–9 years of age.

1.1.2. Virology

Human caliciviruses belong to the genera *Sapovirus* and *Norovirus* (previously referred to as the Norwalk family of viruses or Small Round Structured Viruses) in the family *Caliciviridae*. These are 27–35 nm nonenveloped icosahedral viruses whose genome is a single-stranded positive RNA molecule with the 5'part encoding nonstructural proteins, including the viral replicase, and the 3'part a unique 60 kD capsid protein. The virus does not grow in cell culture and its diagnosis remains limited to specialized laboratories capable of handling molecular tests.

1.1.3. Vaccine development

Norwalk virus (NV) capsid protein produced in a baculovirus system spontaneously assembled into virus-like particles (VLP) that were used as a test antigen to determine whether immune responses could be generated in volunteers who ingested transgenic potatoes. Healthy adult volunteers at the Center for Vaccine Development (CVD), University of Maryland, USA, received 2 or 3 doses of transgenic potato expressing the 60 kD NV capsid protein or 3 doses of wild-type potato. Most of the volunteers who ingested transgenic potatoes developed significant increases in the numbers of specific IgA antibody-secreting cells, 30% developed specific stool IgA and 20% specific serum IgG, but no increase in serum IgG titre was observed after the second dose.

NV VLPs administered by the intranasal or oral routes to mice induced a high serum antibody response as well as faecal IgA, which were enhanced when the heat-labile *E. coli* toxin (LT) or its nontoxic mutant R192G was coadministered with the VLPs. The safety and immunogenicity of NV VLPs are being evaluated with healthy human volunteers.

1.2. *Campylobacter*

1.2.1. Disease burden

Campylobacter jejuni ranks as the most common bacterial cause of diarrhoeas in many developed countries, with an estimated 1.5 million cases in the USA alone, but also represents the second cause of travellers' diarrhoea after enterotoxigenic *Escherichia coli* (ETEC). In developing countries, infection is nearly universal in early childhood. Perhaps of greater concern is its reported association with life-threatening cases of Guillain-Barré syndrome (GBS). *Campylobacters* are frequent commensals in the intestinal tract of animals – mostly birds – and, as such, are frequently implicated in food-borne diarrhoeal illness. Transmission generally occurs through consumption of contaminated water, raw milk or undercooked meats, especially poultry. Not washing one's hands and not cleaning kitchen utensils after carving a raw chicken carcass are major risk factors. Transmission also can occur through contaminated recreational waters. Estimates of infection are 400 million cases per year worldwide.

1.2.2. Vaccine development

Immunity to *Campylobacter* appears to be strain-specific and complex, and the antigens conferring immunity are not well understood. Uncertainty regarding the mechanism of GBS is another obstacle to *Campylobacter* vaccine development. A candidate vaccine consisting of heat- and formalin-killed whole bacteria combined with LT as a mucosal adjuvant has been developed by the Navy Medical Research Institute (USA) and shown to provide 87% protection against intestinal colonization in a small number of volunteers challenged post vaccination with a pathogenic *Campylobacter* strain. Other vaccine formulations are being tested, including a candidate multivalent vaccine expressing antigens from *Campylobacter, Shigellas* and ETEC *(see 1.6.3.)*.

1.3. Cholera

1.3.1. Disease burden

Cholera is an acutely dehydrating, watery diarrhoeal disease caused by intestinal infection with *Vibrio cholerae*. It probably has existed on the Indian subcontinent for thousands of years as can be judged from ancient manuscripts. Cholera was repeatedly one of the most dreaded pandemic diseases in history, being able to spread rapidly to large numbers of people, of whom a high proportion died. Before the advent of an effective rehydration therapy, cholera epidemics were associated with case–fatality rates exceeding 40% and led to tens of thousands of deaths. Cholera remains today an important disease in areas where population overcrowding and poor sanitation are common, such as in slums and refugee camps in developing countries. The year 1991 was marked by the entry of *V. cholerae* into Peru and other Andean countries, from which it has since spread throughout South and Central America. This was the first time cholera had invaded the Americas in more than 100 years.

In 2001, 58 countries officially notified WHO of a total of 184 311 cases and 2728 deaths but, due to considerable under-reporting, the true global figures are estimated as closer to 1 million cases. Estimates of global cholera-specific mortality are believed to be 100 000 to 130 000 deaths per year, with most of the deaths occurring in Asia and Africa. Case fatality rates (CFRs) vary greatly from country to country. For example, very low CFRs were recorded in South Africa (0.22%) whereas rates of up to 30% have been observed in other parts of Africa.

1.3.2. Bacteriology

Only enterotoxigenic *V. cholerae* serogroup O1 and new serogroup O139, which emerged in the 1990s in Bangladesh and India, are known to cause epidemics of cholera. Isolates of *V. cholerae* serogroup O1 are classified into two biotypes, El Tor and classical, on the basis of phenotypic characteristics. Currently, the El Tor biotype is responsible for virtually all the cholera cases throughout the world, and classical isolates have not been encountered since the mid-1990s in Bangladesh. Other serogroups of *V. cholerae* (O5, O37) can cause isolated cases of watery diarrhoea, but they do not cause epidemics. *V. cholerae* O1 can be classified into two serotypes, Inaba and Ogawa, based on serum agglutination. A possible third serotype, Hikojima, has been described, but is very rare. Immunity to *V. cholerae* infection is serogroup-specific.

Steps in the pathogenicity of cholera include colonization of the small intestinal mucosa and elaboration of the enterotoxin cholera toxin (CT), an 84 kD multimeric protein consisting of a central active A subunit bound to five surrounding B subunits. The B subunit is responsible for tight binding of CT to the GM1 ganglioside on the epithelial cell surface, whereas the A subunit is responsible for the toxic action of the toxin by causing hypersecretion of fluids and electrolytes through stimulation of the cell adenylate cyclase.

1.3.3. Vaccines

Although the whole-cell injectable vaccine is no longer recommended, it still is commercially available in some countries. This vaccine is made of killed *V. cholerae* strains of both Inaba and Ogawa serotypes. In controlled trials, vaccine efficacy was approximately 50% for about six months.

Currently, two new types of cholera vaccines are available; both are oral vaccines and have been shown to be safe, immunogenic and efficacious. The two vaccines have been licensed in some countries and are mainly used by travellers, but are now under consideration for use in public health. Several countries have already attempted to vaccinate their populations considered to be at high risk from cholera outbreaks (including Mayotte Island and Micronesia Islands).

The first vaccine (Dukoral, licensed by SBL Vaccin, Sweden) consists of four batches of heat- or formalin-killed whole-cell *V. cholerae* O1, representing both serotypes (Inaba and Ogawa) and both biotypes (classical and El Tor), and added with purified recombinant cholera toxin subunit B (CTB). The whole cell/recombinant B subunit (WC/rBS) vaccine – given orally with buffer to neutralize stomach acidity – was found, in field trials in Bangladesh and Peru, to confer 80–90% protection during 6 months in all age groups after administration of 2 doses 1–2 weeks apart. In Bangladesh, protection declined rapidly in young children after 6 months, but was still about 60% in older children and adults after three years. The vaccine was also successfully used for mass vaccination in a refugee camp in Uganda. Because LT cross-reacts with CT, the vaccine also provides short-term protection against ETEC, which is of added benefit for travellers.

A variant of the Dukoral vaccine containing no recombinant CTB-subunit has been produced and tested in Viet Nam. It is administered in two doses, one week apart. A field trial conducted in Nha-Trang, Viet Nam, showed an efficacy of 66% against *V. cholerae* El Tor after 8 months in all age groups tested. The vaccine has been licensed in Viet Nam and is currently being produced in Indonesia. A bivalent O1 and O139 whole-cell oral vaccine without CTB developed in Viet Nam was shown to be safe and immunogenic in both adults and children. This bivalent vaccine is currently the only existing vaccine against O139 serogroup infection.

The second type of oral vaccine consists of a live attenuated genetically modified *V. cholerae* O1 Inaba strain (CVD103-HgR), which has been engineered to produce CTB but not the A subunit of CT. The vaccine (Orochol, Berna Biotech, Switzerland) is given orally along with buffer to neutralize stomach acidity. The vaccine is available in two formulations, either a low dose formulation for developed countries or a 10-fold higher dose formulation for developing countries. A similarly modified *V. cholerae* O1 Ogawa (CVD111) also is available. Placebo-controlled trials in a number of South American and Asian countries have demonstrated the safety and

immunogenicity of a single dose of Orochol. The vaccine is currently licensed in several industrialized countries. Efficacy in adult volunteers in the USA was found to be about 80% against all diarrhoeas and 90% against severe diarrhoea, following challenge with *V. cholerae* O1 (of either El Tor or classical biotype) given 3 months after vaccination. However, in a subsequent large field trial performed in cholera-endemic Indonesia on 67 000 volunteers, the vaccine failed to demonstrate protection, although limited numbers of cholera cases were recorded. The reasons for such a discrepancy remain unknown.

Other candidate vaccines in development include:

- a live attenuated single-dose oral vaccine developed in Cuba, already tested in Phase I trials;
- a parenteral O-antigen-conjugated vaccine, in preclinical development at the Pasteur Institute in Paris;
- a naked DNA vaccine to be administered by intramuscular injection, in pre-clinical development at the Putra University in Malaysia and the Malaysia National Biotechnology Directorate; and
- a live oral recombinant vaccine strain already tested by Avant Immunotherapeutics (USA) and BioSidus S.A. (Argentina) in Phase II trials.

New attenuated strains of serogroup O139 for use as oral vaccines (CVD112 and Bengal 15) also are under development.

Since 1999, WHO recommends the use of killed oral WC/rBS vaccine as a tool to prevent cholera in populations at risk of a cholera epidemic. Such high-risk populations may include, but are not limited to, refugees and urban slum residents. In addition, in 2002 WHO recommended that demonstration projects with oral cholera vaccines be performed in populations at risk living in endemic settings.

1.4. Enterotoxigenic *Escherichia coli* (ETEC)

1.4.1. Disease burden

Disease caused by ETEC follows ingestion of contaminated food or water and is characterized by profuse watery diarrhoea lasting for several days. It may lead to dehydration and malnutrition in young children in developing countries. ETEC is the most frequently isolated enteropathogen in community-based studies of children aged less than 5 years in the developing world, and probably accounts for approximately 200 million diarrhoea episodes and 380 000 deaths annually. The peak incidence of ETEC diarrhoea in these settings occurs in the first two years of life, with a declining incidence with age thereafter. Surveillance of hospitalized cases of ETEC diarrhoea has however shown that almost half the cases occur in individuals over 10 years of age. In children, the tendency of ETEC to cause dehydrating diarrhoea is lower (approximately 5% of episodes) than that of rotavirus (approximately 36% of episodes). However, because the incidence of ETEC diarrhoea in children is considerably higher than that of rotavirus diarrhoea, the absolute number of dehydrating diarrhoea episodes due to ETEC is around 70% of that due to rotavirus. ETEC are also the number one cause of travellers'diarrhoea, affecting individuals from industrialized countries travelling to developing regions of the world.

1.4.2. Bacteriology

ETEC attach to specific receptors on the surface of enterocytes in the intestinal lumen by virtue of their hair-like fimbriae, which define strain-specific antigenicity. More than 20 types of fimbriae antigens, called *E. coli* surface antigens (CSs) or colonization factor antigens (CFAs) have been described. Antibodies targeted to fimbriae are protective but show tight serotype-specificity. Once attached to the intestinal epithelium, ETEC elaborate a heat-labile toxin (LT), which induces the watery diarrhoea. LT, like the CT to which it is highly homologous, is composed of an active A subunit surrounded by five attachment B subunits. Short-term protection against ETEC disease has been documented in individuals immunized with a CTB subunit vaccine *(see 1.3.3. above).*

1.4.3. Vaccine development

Natural history studies of ETEC infections in children in developing countries suggest that these infections are immunizing, as reflected by declining rates of ETEC diarrhoea with age, lower ratios of symptomatic to asymptomatic ETEC infections with increasing age, and the protective relationships between initial ETEC infections and subsequent infections that have similar toxin and/or colonization factor phenotypes. These data suggest that immunization against ETEC early in life may be an effective preventive strategy. Travellers from industrialized to developing country settings – including military troops on deployment – constitute another potential target population for vaccination against ETEC.

The oral killed WC/rBS cholera vaccine (Dukoral) was found to prevent 23% of all diarrhoea episodes and 52% of episodes due to ETEC in Finnish tourists visiting Morocco; this protection was reported, however, not to last more than a few months.

Several approaches have been pursued to develop specific ETEC vaccines including the use of purified colonization factors, of LT-only or LT-ST (heat-stable toxin) toxoid, or edible transgenic plants that express the cholera toxin B subunit (CTB).

The most successful approach, developed by investigators at the University of Göteborg (Sweden), is based on recombinant CTB combined with five strains of formalin-killed ETEC cells that collectively express the colonization factors of greatest epidemiological importance in developing countries. Phase II studies of a 2-dose regimen of this vaccine have been conducted in Bangladesh, Egypt, Israel, Nicaragua, the USA and Europe. These studies have found the vaccine to be safe and immunogenic, as manifested by induction of mucosal antibody responses to CTB and to the CFA components of the vaccine. A pilot efficacy trial of this vaccine in European tourists travelling to developing country destinations found the vaccine to confer about 80% protection against ST-ETEC diarrhoea (the only toxin phenotype detected in this study), although the small number of outcome events precluded statistically precise estimates of efficacy. Phase III trials of vaccine efficacy are ongoing in travellers from the USA to Latin America, European travellers to Kenya, Israeli military recruits, and Egyptian infants and young children.

The live vaccine approach is being pursued by investigators at the Center for Vaccine Development (CVD), University of Maryland (USA). Their strategy is to use live attenuated *Shigella* vectors for expression of ETEC fimbrial and LT antigens. Such constructs might thereby protect against both *Shigella* and ETEC. The same

approach is being followed by Microscience using their spi-VEC oral live attenuated typhoid vaccine as a vector for the delivery of ETEC antigens. Another live recombinant oral vaccine is developed in the USA to cover traveller's diarrhoeal diseases due to *Campylobacter, Shigella* and ETEC *(see 1.6.3.).*

Two nontoxinogenic ETEC strains have been attenuated by mutagenesis of the *aro*C and *omp*R genes or the *aro*C, *omp*C and *omp*F genes, respectively, to be used as candidate live, oral attenuated vaccines. The mutagenized strains were found to be well tolerated and immunogenic when fed to human volunteers.

Other approaches include colonization factor antigens encapsulated in biodegradable microspheres, developed by the CVD and tested in Phase I trials, and the expression of *E. coli* LTB in bananas, potatoes, tobacco and tomatoes. DNA immunizations are still at the preclinical development stage.

In addition, a mixture of fimbrial antigen CS6 and LT has been delivered to human volunteers using a new delivery technology, the transcutaneous immunization patch. An immune response to both antigens was elicited in about 50% of the volunteers. The presence of LT as an adjuvant was required for stimulation of responses to the CS6 antigen.

A recent international meeting at WHO recommended that further research is required in the field of ETEC vaccines and in elucidating the mechanisms of protection.

1.5. Rotavirus

1.5.1. Disease burden

Rotaviruses (RV) are the leading cause of severe diarrhoeal disease and dehydration in infants and young children in both developed and developing countries, with a distinct winter seasonality in temperate climates and year-round exposure in tropical countries. Virtually all children are infected by the time they reach two to three years of age. Most symptomatic episodes occur between 3 months and 2 years of age with a peak incidence between 7 and 15 months. Outbreaks in day-care centres and hospitals can spread rapidly among nonimmune children, presumably through person-to-person contacts, airborne droplets, or contact with contaminated toys. Prevalence of RV has been vastly underestimated until recently.

In the USA, RV gastroenteritis causes an estimated 70 000 or more hospitalizations a year, a half-million physician and clinic visits, and 20 to 40 deaths. Worldwide, RV is estimated to account for almost 40% of all cases of severe diarrhoea, which translates into 600 000 deaths each year, mostly in children under age 2. Up to 85% of these deaths occur in countries defined as "low-income". Thus, RV was detected in 60% of stool specimens from children hospitalized with severe diarrhoea in Viet Nam, 56% in Myanmar, 41% in China, and 29% in China, Hong Kong Special Administrative Region (Hong Kong SAR). From an epidemiological study conducted in Peru, RV was accountable for 64 000 clinic visits, 30 000 hospitalizations, and 1600 deaths per year. Because RV remains the most common cause of severe diarrhoea in children in all regions of the world, an RV vaccine would have universal application as part of childhood vaccination programmes.

1.5.2. Virology

Rotaviruses are 70 nm icosahedral viruses that belong to the family *Reoviridae*. The virus is composed of three successive protein shells, an outer and an inner capsids and an internal shell that encases the 11-segment double-stranded RNA genome. When mixed infections with distinct RV strains occur, the gene segments may reassort independently, producing progeny "reassortants" of mixed parentage, which is a source of important viral diversity. Two structural outer capsid proteins, VP7 (G protein) and VP4 (P protein) define the G and P serotypes of the virus, respectively. These are the major antigens involved in virus neutralization. Human RVs bearing G serotypes G1–G4 and G9 and P genotypes P[4], P[6] and P[8] are predominant worldwide. P[8]G1 is the globally predominant strain, followed by P[8]G3, P[4]G2, and P[8]G4. G9 strains have been emerging in the late 1990s and are now the predominant strain in some regions of the world.

Rotavirus surveillance system networks have been constituted with the collaboration of the Centers for Disease Control and Prevention in Atlanta (USA) and WHO to estimate the hospital-based disease burden of RV gastroenteritis in children less then five years of age, and to constantly update the frequency and characteristics of circulating strains. This aspect is of importance for the development of an RV vaccine and for studying the possible vaccine selective pressure leading to emergence of new strains in the vaccinated populations.

1.5.3. Vaccine development

The first RV vaccine to be tested in humans was the live bovine strain RIT4237 (P[1]G6). In two initial efficacy trials in Finland, the vaccine showed 50% and 58% protective efficacy, but little or no protection was observed when it was subsequently tested in the Gambia, Rwanda and South-Western USA. Another RV bovine strain, WC3 (P[5]G6) was evaluated as a live RV vaccine, induced virus neutralizing antibodies in 60–100% of infants but showed inconstant capacity to protect against disease. The third animal RV strain to be tested in infants was the simian RRV strain (P[3]G3) but, again, results of efficacy trials were variable, depending on the serotype of the prevailing RV strain in the setting of the trial.

In view of the inconsistency of these results, efforts were made to develop reassortant RV strains bearing a human RV gene for the VP7 protein together with the other 10 genes from a simian or a bovine RV strain. The first such vaccine was a tetravalent rhesus-human reassortant vaccine developed by the NIH, Bethesda, based on the rhesus RRV strain, which is a G3 strain that was mixed with three reassortant strains of G types 1, 2, and 4 from human RV in a RRV genome. The vaccine (RotaShield), which was introduced in 1998 on the market in the USA by Wyeth-Lederle Vaccines, was shown to provide 48–68% protection against any RV disease and 64–91% protection against severe disease in different studies. The vaccine was recommended for routine immunization of infants at two, four, and six months of age. Vaccination was however stopped less than a year later, and the vaccine discontinued for use, when several cases of intussusception (IS) following vaccine administration were reported. The vaccine already had been administered to 600 000 children in the USA. The risk of IS appeared to be highest during the period 3–7 days following the first and second immunizations, with relative risks of 37.27 and 3.8 for these 2 time periods, respectively. That risk, initially targeted at 1 in 2500 children immunized, has now been reassessed as 1 in 10 000, and could

be made as low as 1 in 40 000 if the vaccine were to be administered in the first 2 months of life. In any case, and quite unfortunately, the vaccine could not be evaluated in terms of risk–benefit for children in developing countries, as the ongoing trials in Africa (Ghana and South Africa) and Asia (Bangladesh and India) were stopped at the same time.

A biotech company called BIOVIRx Inc., has acquired the dossier and the master seed banks of the Rotashield vaccine from Wyeth and the NIH and is planning to manufacture the vaccine.

In China, a lamb-derived monovalent (P[12]G10) live-attenuated, 3-dose oral vaccine, developed by the Lanzhou Institute of Biomedical Products is licensed and used in the private sector. The vaccine is reported to induce neutralizing antibody responses in 60% of vaccinees but its efficacy is not known since it was not tested against placebo in a controlled Phase III trial.

Two RV vaccine candidates developed by multinational companies show good promise of efficacy and safety.

- First, a monovalent (P[8]G1), live-attenuated, 2-dose oral vaccine developed by Avant Immunotherapeutics from a human RV strain and licensed to GSK Biochemicals, who slightly modified the strain. The vaccine (Rotatrix) has been tested in 11 Latin America countries and Finland in Phase III trials on more than 63 000 children. Reports have indicated no increased attributable risk of IS in the high-risk period up to 30 days post any dose. The vaccine has already been licensed in the Dominican Republic and Mexico, and application for a license has been submitted in several other countries in Latin America and in Europe.
- Second, a pentavalent human-bovine (WC3) reassortant (G1, G2, G3, G4 and P[8]) live-attenuated, 3-dose oral vaccine, developed by Merck Research Co., Pennsylvania. This vaccine (RotaTeq) has been tested in a Phase III trial in Finland and the USA on more than 70 000 children who were carefully monitored for 2 weeks after each immunization for risks of IS. Results of the trial are expected in mid-2005 and application for a license to the Food and Drug Administration (USA) should follow shortly.

Several new vaccine approaches also are currently being pursued.

1) An alternative multivalent bovine-human reassortant vaccine has been developed by the National Institute of Allergy and Infectious Diseases (NIAID, NIH, Bethesda). Phase II data showed a good immune response and no adverse interference with concomitantly administered childhood vaccines. A non-exclusive license for production of the vaccine candidate is being negotiated with vaccine producers in Brazil, China and India.
2) A human neonatal P[6]G3 strain developed by Bishop and colleagues in Australia was found to be safe and well tolerated in a Phase I study. A small Phase II study with three doses of 10E5 pfu of the vaccine indicated relatively low immunogenicity as measured by serum IgA. However, the vaccine recipients who developed an immune response were protected against clinical disease in the following year. Further Phase II immunogenicity studies are planned with 10E7 pfu per dose.

3) Two naturally occurring human–bovine, neonate-derived strains (116E and I321) are under development in India in a consortium with partners in the USA, including the Children's Vaccine Program at PATH.

4) Rotavirus VLPs expressed in a baculovirus system and given as a killed injectable vaccine or an oral vaccine is an approach which is still in development. Efforts also are under way to produce subunit vaccines comprising a number of rotavirus structural proteins expressed in bacteria.

Future field efficacy trials will monitor IS in the vaccine recipients and placebo groups, as well as the serotypes and genotypes of viruses recovered from stools in both groups in order to test possible vaccine selection pressure. Several alternative vaccine approaches have been proposed to avoid IS. These include:

- non-oral vaccines, either vaccine-like particles administered intranasally or injectable inactivated vaccines;
- nasal administration of live vaccines; and
- administration of the vaccine in the neonatal period since natural IS is rare in infants less than two months old and bovine-derived vaccines have been found to be safe in this age group.

Another matter to watch carefully with the advent of the new RV vaccines will be that of cross-protection against the full range of RV strains, including serotype G9, which is becoming increasingly important across Asia, and G8, which is gaining prevalence across Africa.

1.6. Shigellosis

1.6.1. Disease burden

Shigellosis is endemic throughout the world where it is held responsible for some 165 million cases of severe dysentery with blood and mucus in the stools, the overwhelming majority of which occur in developing countries and involve children less than five years of age. More than one million people are estimated to die from *Shigella* infection each year. In addition, some 580 000 cases of shigellosis are reported among travellers and military personnel from industrialized countries. Since the late 1960s, pandemic waves of *Shigella* dysentery have hit sub-Saharan Africa, Central America and South and South-East Asia, often striking areas of political upheaval and natural disaster. During the 1994 genocide in Rwanda, approximately 20 000 Rwandan refugees who had fled into the North Kivu region of Zaire died in the first month alone from dysentery caused by a strain of *Shigella* that was resistant to all commonly used antibiotics. The combination of *Shigella* and HIV infections has deleterious consequences, due to compromised immunity in HIV-positive persons.

1.6.2. Bacteriology

Three major species of *Shigella* are responsible for bacillary dysentery: *S. sonnei, S. flexneri* and *S. dysenteriae*. *S. sonnei* is the causative agent of most shigellosis in industrialized countries where it accounts for 77% of cases (compared to 15% in developing countries), but it also seems to have become predominant in Thailand in recent years, a phenomenon possibly linked to the level of development of the country.

S. flexneri is endemic in developing countries (60%) and is the most frequently isolated species worldwide. *S. dysenteriae* (Sd1) is the cause of epidemic dysentery and can cause vicious outbreaks in confined populations, especially refugee camps. A major obstacle to the control of Sd1 is its resistance to antimicrobial drugs.

Shigella species are transmitted by ingestion of contaminated food or water, or by person-to-person contact, a most common source of transmission. The bacteria invade the colonic epithelium through M cells and then spread laterally from cell-to-cell. This invasive ability is due to several virulence factors encoded by a high molecular weight virulence plasmid. In addition, *S. dysenteriae* secretes the Shiga toxin, which inhibits protein synthesis in eukaryotic cells via inactivation of ribosomal RNA, leading to cell death.

1.6.3. Vaccine development

Candidate shigellosis vaccines currently in advanced development include both killed and live vaccines. The killed, subunit vaccine approach includes the following.

- Parenteral conjugate vaccines consisting of purified *S. dysenteriae* type one lipopolysaccharide (LPS) conjugated to tetanus toxoid, and *S. flexneri* and *S. sonnei* LPS conjugated to recombinant *Pseudomonas aeruginosa* exotoxin A. These vaccines, which are developed at the NIH, were shown to be 74% efficacious against disease when tested in field trials with Israeli military volunteers and demonstrated safety and immunogenicity in 4–7 year-old children.
- A parenteral nuclear protein/ribosomal vaccine approach, developed by the International Vaccine Institute (IVI) and the Walter Reed Army Institute of Research (WRAIR), still at a preclinical stage.
- A nasally administered proteosome vaccine consisting of *Shigella* LPS linked to micelles of the outer membrane protein of group B *Neisseria meningitidis.*
- In addition, Antex (USA) is developing both a *Shigella* inactivated whole cell vaccine and an oral traveller's diarrhoea vaccine (Activax) containing antigens from *Campylobacter, Shigella* and ETEC. These candidate vaccines should presently enter clinical testing.

Definite progress has been made with candidate live oral shigellosis vaccines, but the main problem remains the small margin that exists between under-attenuation responsible for excessive reactogenicity of the strains and over-attenuation leading to poor immunogenicity in human subjects. These approaches include the following.

- A live bivalent *S. flexneri* 2a and *S. sonnei* vaccine (FS) that was developed at the Lanzhou Institute of Biological Products. Large field studies in China have demonstrated 61–65% protection against *S. flexneri* 2a, 57–72% protection against *S. sonnei,* and 48–52% protective efficacy against heterologous *Shigella* species. However, the use of a 3-dose regimen with high doses of vaccine strain (>2x10E10 cfu) remains problematic. Further field studies of the FS vaccine in toddlers and infants may help define the public health application of this vaccine in China.

- A live, attenuated *S. flexneri* 2a strain (SC602), and an *S. dysenteriae* type 1 strain (SC599) carrying mutations in their *ics*A, *iuc*, *int* and *tox*A genes, that were developed at the Pasteur Institute, Paris. SC602 was tested in adult volunteers in the USA and in adults and children in Bangladesh in collaboration with WRAIR and IVI. A remarkable efficacy against challenge was observed in USA volunteers, but results of immunogenicity were disappointing in young infants in the field, due to lack of colonization of the gut, perhaps as a consequence of the presence of maternal antibodies from breastfeeding, or due to over-attenuation of the vaccine candidate for this population.
- Live attenuated *Shigella* vaccine candidates targeting *S. flexneri* types 2a, 3a and 6, *S. sonnei*, and *S. dysenteriae* type 1, which are in development at CVD, each carrying additional fimbriae genes from ETEC. A series of strains with progressive deletions of virulence genes (CVD1203, CVD 1204) was engineered, culminating in strain CVD1208S, which should presently enter Phase I clinical trials.
- An *S. sonnei* vaccine candidate (WRSS1) that has been developed with a single deletion mutation of the *Vir*G gene and tested in a Phase I study conducted at CVD, where the vaccine was found to be midly reactogenic at higher titres and elicited a significant immune response in the volunteers. Further development is planned.
- Streptomycin-dependent (SmD) vaccines have been developed and shown to be safe for *S. flexneri* 1, 2a, and 3a and *S. sonnei*. Large studies in the former Yugoslavia showed that the vaccines were protective in 82–100% of cases. However, side effects were observed and the development of the vaccine was not continued.

1.7. Typhoid fever

1.7.1. Disease burden

Typhoid fever is caused by *Salmonella typhi*, the typhoid bacillus. It is characterized by the sudden onset of sustained fever, severe headache, nausea, loss of appetite, constipation or sometimes diarrhoea. Severe forms have been described with mental dullness and meningitis. Case-fatality rates of 10% can be reduced to less than 1% with appropriate antibiotic therapy. However, strains resistant to chloramphenicol and other recommended antibiotics (ampicillin, cotrimoxazole and even ciprofloxacin) have become prevalent in several areas of the world. Paratyphoid fever can be caused by any of three serotypes of *S. paratyphi* A, B and C. It is similar in its symptoms to typhoid fever, but tends to be milder, with a lower fatality rate.

Typhoid fever remains a serious public health problem throughout the world, with an estimated 16–33 million cases and 500 000 to 600 000 deaths annually. In the last outbreak in the Democratic Republic of Congo, between 27 September 2004 and early January 2005, no less than 42 564 cases of typhoid fever were reported, including 214 deaths and 696 cases of peritonitis and intestinal perforations. In virtually all endemic areas, the incidence of typhoid fever is highest in children from 5–19 years old. The disease is almost exclusively transmitted by food and water contaminated by the faeces and urine of patients and carriers. Polluted water is the most common source of typhoid transmission. In addition, shellfish taken from

sewage-contaminated beds, vegetables fertilized with night-soil and eaten raw, contaminated milk and milk products have been shown to be a source of infection. Typhoid fever has been virtually eliminated in most areas of the industrialized world with the advent of proper sanitary facilities. Most cases in developed countries are imported from endemic countries. People can transmit the disease as long as the bacteria remain in their body; most people are infectious prior to and during the first week of convalescence, but 10% of untreated patients will discharge bacteria for up to 3 months. In addition, 2–5% of untreated patients will become permanent, lifelong carriers of the bacteria in their gall-bladder.

S. paratyphi is becoming predominant in some provinces in China and increasing numbers of cases are being reported from Pakistan.

1.7.2. Bacteriology

Taxonomy within the genus *Salmonella* has been the source of great confusion. The most recent classification, based on DNA sequences, has left only two species, *S. enteritica* and *S. bongori,* further subdivided into subspecies and serovars. To avoid confusion, *S. enteritica* serovar *Typhi* continues to be referred to as *S. typhi*. The bacteria is characterized by its flagellar antigen, H, its lipopolysaccharidic O antigen, and, in addition, its PS capsular Vi (for virulence) antigen, found at the surface of freshly isolated strains. The complete sequence of the 4,809 037-bp genome has been determined. In addition to the plasmid encoding antibiotic resistance, a virulence plasmid was found that shows homology with the virulence plasmid of *Yersinia pestis*.

Upon ingestion, typhoid bacilli rapidly penetrate the small intestinal mucosa by transcytosis through M cells and enterocytes, and are taken up by macrophages or diffuse into mesenteric lymph nodes. A primary bacteraemia follows and the pathogen rapidly attains intracellular haven throughout the reticuloendothelial system. This is followed by a sustained secondary bacteraemia asssociated with clinical illness. *S. typhi* also shows remarkable predilection for the gall-bladder, where infection tends to become chronic especially in individuals with a pathologic gall-bladder condition.

1.7.3. Vaccines

Heat-killed, phenol preserved whole-cell *S. typhi* were utilized as parenteral vaccines as far back as 1896 in England and Germany, and still are licensed today in many countries in spite of their high reactogenicity.

The attenuated *S. typhi* strain Ty21a was generated in Switzerland by chemical mutagenesis of wild-type strain Ty2 and developed as the first live oral typhoid fever vaccine. The strain is characterized as lacking both a functional galactose-epimerase (*gal*E) gene and the Vi antigen, although other mutations in the genome probably are responsible for the attenuated phenotype. The Ty21a vaccine is presented in the form of phtallate-coated gelatin capsules containing 2 to 6 x 10E9 cfu of Ty21a to be swallowed every other day for one week. It can be taken simultaneously with the attenuated CVD103-HgR *V. cholerae* vaccine. Ty21a is licensed in 56 countries in Africa, the Americas, Asia and Europe.

A subunit *S. typhi* polysaccharide (PS) vaccine was developed in the 1980s in the laboratory of John Robbins at the NIH and licensed to Sanofi-Pasteur. The vaccine is based on purified Vi antigen, a linear homopolymer of galacturonic acid that is purified from the bacteria by treatment with Cetavlon, the detergent used for the preparation of the meningococcal PS vaccine. The vaccine elicits serum anti-Vi antibodies in approximately 85–95% of adults and children above two years of age after a single parenteral injection of a 25 µg dose of purified PS. The lack of immunogenicity in younger children has prompted the development of a conjugate Vi vaccine using *Pseudomonas aeruginosa* exotoxin A as a carrier. The Vi vaccine has shown 72–77% efficacy in trials in Nepal and South Africa, and is now licensed in more than 92 countries in the five continents. The Vi conjugate vaccine has shown a 91.5% protection rate in a large-scale randomized, 2-dose controlled trial in 2–5 year-old children in Cambodia and Viet Nam, and a 88% protection rate at 16 months.

A head-to-head comparison of the Ty21a and Vi vaccines has been proposed by WHO in order to make future recommendations for countries severely affected by typhoid.

Among several other new attenuated *S. typhi* strains that could be used as live oral vaccines, three are currently at an advanced clinical stage of development.

1) The Ty800 live attenuated oral vaccine, a *pho*P/ *pho*Q deletion mutant of Ty2, has been shown to stimulate vigorous IgA and serum O antibody responses in Phase I trials (Avant Immunotherapeutics).

2) The CVD908-htrA live attenuated oral vaccine, an aroC/ *aro*D/ htrA deletion mutant, has successfully been tested in Phase II trials. Its Vi+ derivative, CVD909, which constitutively expresses the Vi antigen, has now completed Phase I testing (Acambis/Berna).

3) An attenuated *S. typhi* strain has been developed by Microscience (USA) as a live vector (spi-VEC) for oral vaccines.

2. Respiratory infections

Acute respiratory infections (ARI) continue to be the leading cause of acute illnesses worldwide. Whereas upper respiratory infections (URIs) are very common but seldom life-threatening, lower respiratory infections (LRIs) include more severe illnesses such as influenza, pneumonia, bronchitis and tuberculosis (TB) and are the leading contributor to the more than four million deaths caused each year by respiratory diseases. The populations at greatest risk for developing a fatal respiratory infection are the very young, the elderly, and the immunocompromised. A recent meta-analysis demonstrated that 70% of the children who died from ARI in 2000 were living in Africa and South-East Asia. In developing countries, most of the deaths caused by respiratory infection occur in children younger that 5 years of age and 30% of those are attributable to pneumonia, but precise etiological diagnosis is difficult and uncertain. One of the difficulties is that ARIs are often associated with other life-threatening diseases such as measles. In a study where 62% of reported childhood deaths had been attributed to ARI, the figure fell to 24% when measles-associated deaths were excluded. Better estimation of the burden of childhood pneumonia is needed and should be given high priority. The main etiological agents responsible for ARIs in children include *Streptococcus pneumoniae, Haemophilus influenzae* type b *(Hib),* respiratory syncytial virus (RSV), and Parainfluenza virus type 3 (PIV-3). In the elderly, influenza-related pneumonia remains a leading cause of infectious disease-related deaths. Human metapneumovirus, a member of the *Paramyxoviridae* family, also is a recognized cause of a large fraction of severe ARIs in infant, elderly and immunocompromised population. Finally, nosocomial or hospital-acquired pneumonia is a major infectious problem: pneumonia is the second most common type of all nosocomial infections, with an associated case fatality rate of 20–50%.

Viruses are a common cause of acute LRI in children worldwide. Available data suggest that dual infection with viruses and bacterial pathogens are more common in developing countries than in industrialized countries. In Pakistan, 26% of children infected with RSV also had *S. pneumoniae* or *H. influenzae* bacteraemia. In a study in Papua New Guinea, bacteria were isolated from blood culture or lung aspirate in two-thirds of children with viral ARI. Although the exact relationship between viral and bacterial infection in these cases has not been established, dual infection seems to increase the severity of the disease and to result in higher mortality.

2.1. Influenza

2.1.1. Disease burden

The burden of influenza in the USA is currently estimated to be 25–50 million cases per year, leading to 150 000 hospitalizations and 30 000–40 000 deaths. If these figures are extrapolated to the rest of the world, the average global burden of inter-pandemic influenza may be on the order of ~1 billion cases of flu, ~3–5 million cases of severe illness and 300 000–500 000 deaths annually. Epidemics and outbreaks of influenza occur in different seasonal patterns depending on the region in the world. In temperate climate zones, seasonal epidemics typically begin in the late fall and peak in mid- to late winter. In tropical zones, seasonal patterns appear to be less pronounced, with year-round isolation of virus. In developed countries, annual influenza epidemics infect about 10–20% of the population each season, and cause febrile illnesses that range in severity from mild to debilitating and can lead in some instances to hospitalization and even cause death. The latter mostly occur as a consequence of primitive fulminant influenza virus pneumonia or of secondary respiratory bacterial infections and are facilitated by underlying pulmonary or cardiopulmonary pathologies. The risk of developing serious complications is aggravated in the very young and in the elderly. Data collected in Michigan (USA) and in Japan indicate that the mass vaccination of school-aged children correlates with a reduced rate of respiratory illness in all age groups, suggesting that larger-scale immunization in childhood could favourably affect influenza epidemics.

The repetitive occurrence of yearly influenza epidemics is maintained through the ongoing process of "*antigenic drift*", which results from the accumulation of point mutations in the genes that encode the two viral surface proteins haemagglutinin (HA) and neuraminidase (NA), and leads to the constant emergence of new virus variants against which there is little or no pre-existing immunity in the population. At unpredictable intervals, due to the segmented nature of the influenza virus genome, these viruses also can acquire new genes from an avian or other animal influenza virus. This process is believed to occur most readily in pigs, as these animals can be infected by avian as well as human viruses. Co-infection in pigs can result in the emergence of a virus with a completely new glycoprotein subtype, which is referred to as an "*antigenic shift*" and, if the virus infects the human population and can efficiently spread from person-to-person, a worldwide epidemic known as a pandemic occurs. Three of these pandemics occurred in the last century (1918, 1957, and 1968). The most severe, in 1918, infected approximately 50% of the world's population, killing an impressive 20–50 million people, particularly those in the prime of their lives. This pandemic depressed population growth for the following ten years.

The last outbreak of influenza with high mortality and pandemic potential occurred in 1997, when a new influenza virus with an avian virus HA glycoprotein (H5N1) emerged in Hong Kong SAR, killing 6 of the 18 affected patients, mainly young adults. Fortunately, the virus was not able to spread from person-to-person and it was possible to stop the outbreak by massive culling of poultry. Another H5N1 strain was isolated in 2003–2004 in several countries in Asia, including Thailand and Viet Nam, killing altogether 31 of the 43 patients diagnosed with the virus. Again, from 24 December 2004 to 6 January 2005, six new human cases of H5N1 influenza were reported from Viet Nam, including five deaths.

These recent outbreaks have coincided with a major epizootic of avian flu in South-East Asia, due to a highly pathogenic H5N1 virus strain that not only kills domestic poultry (ducks excepted) but also wild birds such as geese, flamingos, and other species of aquatic birds. The virus also is pathogenic for ferrets, cats and tigers. Cats can be infected with H5N1 virus both by the respiratory route and by feeding on virus-infected birds. So far, no human-to-human transmission has been documented with certitude, but the fear is that the H5N1 virus could gain the capacity to spread into the human population through change in receptor-binding specificity by mutation or reassortment, leading to a new pandemic.

Other avian influenza viruses have occasionally caused a human outbreak, such as a H9N2 strain in 1999 in Hong Kong SAR, H7N7 virus in 2003 in the Netherlands, which caused 89 confirmed human cases with conjunctivitis and one death, and H7N2 and H7N3 in 2003–2004 in North America.

In the USA, the impact of a new pandemic, assuming it would be of a similar magnitude as the 1957 or the 1968 pandemics, and not like the 1918 pandemic, is projected to be 18–42 million outpatient visits, 314 000–734 000 hospitalizations and 89 000–207 000 deaths. Extrapolating these figures to the world population, a gross estimate of the impact of the next pandemic calls for 1–2 billion cases of flu, 5–12.5 million cases of severe illness, and 1.5–3.5 million deaths worldwide!

2.1.2. Virology

Influenza viruses are enveloped viruses with a segmented genome made of eight single-stranded negative RNA segments, most of which encode only one viral protein (HA, NA, M, NP etc.). Influenza viruses belong to the family *Orthomyxoviridae.* They are divided into three genera, Influenzavirus A, Influenzavirus B, and Influenzavirus C, based on antigenic differences in two of their structural proteins, the matrix protein (M) and the nucleoprotein (NP). Influenza A viruses are further divided into subtypes according to the antigenicity of their major envelope glycoproteins, HA and NA. Fifteen HA subtypes (H1 to H15) and nine NA subtypes (N1 to N9) have been identified so far. Only viruses of the H1N1, H1N2 and H3N2 subtypes are currently circulating in the human population.

Influenza A viruses also infect poultry, aquatic birds, pigs, horses, and sea mammals. Aquatic birds, in which the virus multiplies in the gut, usually have an asymptomatic infection and excrete the virus in their faeces. Aquatic birds serve as a natural virus reservoir and a potential source of new genes for pandemic influenza viruses. Swapping of genomic segments leading to the emergence of a new, reassortant progeny strain with a mixed genotype most readily occurs in pigs, as pigs have the complete set of sialylated receptors for avian, swine and human influenza virus strains.

HA is present at the surface of the flu virion in the form of a HA0 precursor which must undergo proteolytic cleavage to generate functional subunits HA1, which bears the receptor-binding site and neutralization epitopes, and HA2, which is responsible for the fusion of the viral envelope with the host-cell membrane. Classical avian virus strains have a HA0 trypsin-like cleavage site, hence their tropism for the gastrointestinal tract. In contrast, highly virulent avian strains such as the 2004 H5N1 strain from Thailand and Viet Nam have acquired through spontaneous mutations an ubiquitous furin-like cleavage site, which allows them to multiply in many tissues including the respiratory tract.

2.1.3. Vaccines

The currently available influenza vaccines are subvirion preparations made from inactivated, detergent-split influenza virus grown in the allantoic cavity of embryonated chicken eggs. These vaccines effectively prevent influenza-related illness and have a high benefit-to-cost ratio in terms of preventing hospitalizations and deaths, as shown in numerous studies on vaccination of the elderly and of individuals at high risk for severe outcomes of influenza. WHO estimates that there globally are about 1.2 billion people at high risk for severe influenza outcomes: 385 million elderly over 65 years of age, 140 million infants, and 700 million children and adults with an underlying chronic health problem. In addition, 24 million health-care workers also should be immunized to prevent them from spreading the disease to the high-risk population. At this time, the world's total vaccine production capacity is limited to about 900 million doses, which realistically does not suffice to cover the global high-risk population.

It is, therefore, quite evident that the global health infrastructure would not be able to handle the timely manufacture, distribution and delivery of a pandemic influenza vaccine which, in all likelihood, would have to be given as a two-dose regimen because people will not have had a previous exposure to the virus antigen. One solution to this problem would be to lower the quantity of antigen per dose and add an adjuvant to the vaccine, but this needs to be tested in clinical trials. Another solution would be to improve on current vaccine production technologies (egg-derived vaccines). Several pharmaceutical companies have embarked on projects for the development of cell-culture vaccines, as this could help overcome current vaccine production bottlenecks, limited availability of specific pathogen-free egg supply and time constraints. Furthermore, it would improve possibilities of scaling up vaccine production capacities in face of a pandemic.

It is now possible, using the techniques of reverse genetics, to first mutagenize the HA1/HA2 cleavage site of any potential pandemic virus strain such as the avian H5N1 strain, so as to attenuate its virulence, then to transfer its HA and NA genomic segments into an appropriate influenza A virus master strain such as the PR8 strain which has been adapted to grow on Vero cells, thus generating within a few weeks a reassortant virus with the antigenic specificity of the pandemic strain and the growth characteristics of the master strain, including adaptation to cell culture.

To assess dosage for the reverse-genetics vaccine against Viet Nam H5N1 virus, clinical trials are presently being conducted by the NIH using vaccine lots prepared by Chiron and by Sanofi-Pasteur. Japan is planning to organize trials in early 2005 and the European Union should eventually test a low dose pandemic H5N1 vaccine containing alum as an adjuvant. Intellectual property and liability issues also are major obstacles, not counting the fact that a reverse-genetics vaccine is considered a genetically modified organism and as such would need special clearance in Europe.

Another approach to influenza vaccines has been the development of cold-adapted (*ca*) virus strains which grow well in primary chick kidney cells and embryonated eggs at 25–33°C, have a reduced replication titre at 37°C, and show attenuated behavior in ferrets. Cold adaptation was found to be a reliable and efficient procedure for the derivation of live attenuated influenza virus vaccines for humans. A trivalent live cold-adapted vaccine (Flumist) has been developed for intra-nasal spray delivery by MedImmune and Wyeth. This vaccine was proven highly efficacious in Phase III

trials, showing a 92% overall protection rate over a 2-year study in children. The vaccine has been licensed in the USA for vaccination of persons from 5–49 years of age, in view of side effects in younger children (wheezing, nasal congestion) and absence of data in the elderly. The vaccine is safe, effective, and shows remarkable genetic stability, but it has to be kept at -18°C. A new, heat-stable version of the vaccine has recently been developed, and has shown remarkable efficacy in clinical trials in Asia and Europe, including young children. An application for European licensure is expected to be filed presently (MedImmune).

- Biodiem Limited (Australia) and Merck will be developing another live attenuated influenza vaccine, to be delivered by nasal spray.
- Still another cold-adapted live-attenuated virus vaccine grown in Madin-Darby canine kidney (MDCK) cells on microcarrier beads in serum-free medium is at an advanced preclinical development stage at the Vector Scientific Center in the Russian Federation.
- Berna Biotech is commercializing an influenza vaccine formulated in virosomes, with the surface spikes of the three currently circulating influenza strains inserted in the vesicle membrane of three corresponding virosome types. A nasal formulation of this vaccine was however recently withdrawn from the market, due to undesirable neurological side effects (Bell's palsy) linked to the presence of the *E. coli* labile toxin (LT) used as an adjuvant, most likely because of GM1 ganglioside binding of the B subunit of LT in neuronal tissues associated with the olfactory tract.
- Other formulations of inactivated influenza vaccine for mucosal delivery are in progress including immunostimulating complexes (ISCOMs).
- Protein Science is developing a subunit vaccine containing recombinant HA and NA proteins produced in a serum-free insect cell culture with a baculovirus vector. The vaccine has been successfully tested in a Phase II trial in 64–89 year-old volunteers in whom it induced good anti-HA antibody responses. A Phase III trial is pending.
- Yeda, an Israeli research and development (R&D) company, is developing a synthetic peptide influenza vaccine for nasal administration. The vaccine has shown protective efficacy in humanized mice and is planned to enter clinical trials in 2005.
- PowderJect (USA) is working on novel means of influenza vaccine delivery eliciting a higher antibody response than needle and syringe delivery.
- DNA vaccines for influenza are still at an early stage due to poor immunogenicity results of naked DNA in humans.
- Finally, a recombinant particulate vaccine has been engineered by genetically fusing copies of the influenza virus M2 protein to the hepatitis B core antigen (HBc). The (M2)-HBc fusion protein spontaneously assembled into virus-like particles (VLP) that provided complete protection against a potentially lethal influenza virus A challenge in mice. Similarly, a M2 peptide was conjugated with *Neisseria meningitidis* outer membrane protein complex (OMPC) and recently evaluated in animal models including monkeys. M2 is a highly conserved transmembrane protein in the virion. These approaches might thus serve as a basis for an universal influenza vaccine with broad spectrum of protective activity.

2.2. Parainfluenza viruses

2.2.1. Disease burden

Parainfluenza viruses cause a spectrum of respiratory illnesses, from URIs, 30–50% of which are complicated by otitis media, to LRIs, about 0.3% of which require hospitalization. Most children are infected by parainfluenza virus type 3 (PIV-3) by the age of two years and by parainfluenza virus types 1 and 2 (PIV-1 and -2) by the age of five years. PIV-3 infections are second only to RSV infections as a viral cause of serious ARI in young children. Pneumonia and bronchiolitis from PIV-3 infection occur primarily in the first six months of life, as is the case for RSV infection. Croup is the signature clinical manifestation of infection with parainfluenza viruses, especially PIV-1, and is the chief cause of hospitalization from parainfluenza infections in children two to six years of age. However, this syndrome is relatively less frequent in developing countries. The proportions of hospitalizations associated with PIV infection vary widely in hospital-based studies. Consequently, the annual estimated rates of hospitalization fall within a broad range: PIV-1 is estimated to account for 5800 to 28 900 annual hospitalizations in the USA, PIV-2 for 1800 to 15 600 hospitalizations, and PIV-3 for 8700 to 52 000 hospitalizations. Along with RSV, parainfluenza viruses are also leading causes of hospitalization in adults with community-acquired respiratory disease.

The seasonal patterns of PIV-1, -2, and -3 infections are curiously interactive. PIV-1 causes the largest, most defined outbreaks, marked by sharp biennial rises in cases of croup in the autumn of odd-numbered years. Outbreaks of infection with PIV-2, though more erratic, usually follow type 1 outbreaks. Outbreaks of PIV-3 infections occur yearly, mainly in spring and summer, and last longer than outbreaks of types 1 and 2. Although PIV-1 to PIV-3 have been described as a cause of LRI in developing countries, the disease burden has not been accurately quantified in these countries.

2.2.2. Virology

Parainfluenza viruses belong to the family *Paramyxoviridae*, subfamily *Paramyxovirinae*, itself subdivided into three genera: Paramyxovirus (PIV-1, PIV-3, and Sendai virus), Rubulavirus (PIV-2, PIV-4 and mumps virus) and Morbillivirus (measles virus). All are enveloped viruses with a negative strand, ~15 500 nucleotide-long nonsegmented RNA genome which encodes two envelope glycoproteins, the haemagglutinin-neuraminidase (HN), and the fusion protein (F, itself cleaved into F1 and F2 subunits), a matrix protein (M), a nucleocapsid protein (N) and several nonstructural proteins including the viral replicase (L).

2.2.3. Vaccines

Live, attenuated parainfluenza virus vaccines have been developed from both human and bovine strains in view of intra-nasal immunization. Candidate vaccines should be able to replicate and induce a protective immune response in young infants in the presence of maternally acquired antibody. Achieving an appropriate balance between attenuation and immunogenicity has however been a major obstacle to the development of these vaccines.

Two attenuated strains have been studied: a) a *ts* human PIV-3 strain, *cp*45, which was selected after 45 passages of the virus in African green monkey cells at low temperature; and b) bovine PIV-3, which is closely related antigenically to human PIV-3, can protect monkeys against challenge with human PIV-3, and replicates poorly in humans. Both *cp*45 and bovine PIV-3 have been evaluated in Phase I/II trials in RSV seropositive and seronegative children and in young infants. Both candidates were found to be over-attenuated in seropositive children, but immunogenic in seronegative children and infants, although the magnitude of the anti-HN response was lower in children who received the bovine PIV-3 vaccine.

This prompted the engineering of chimeric bovine/human PIV-3 candidate vaccines that contain the human PIV-3 F and HN genes and internal genes from bovine PIV-3. One of such chimeric viruses, hPIV-3-Nb, is a human PIV-3 with the human nucleocapsid (N) gene replaced by its bovine counterpart. The virus was found to be attenuated and protective in nonhuman primates, and is at Phase I clinical trial stage.

Chimeric bovine PIV-3 expressing the F and HN proteins of human PIV-3 have been used as a backbone into which the F, or F and G ORFs of RSV A or RSV B were inserted to provide a bivalent candidate vaccine against RSV and PIV-3 infections in young infants *(see 2.3.3.)*.

A few attempts also have been made at developing PIV-1 vaccines. Sendai virus, a murine PIV-1, was found to protect African green monkeys against human PIV-1 challenge but does not seem to be sufficiently attenuated to be used as a Jennerian vaccine in human infants. NIAID has produced attenuated chimeric viruses that contain PIV-3 *cp*45 internal genes with the F and HN genes from either PIV-1 or PIV-2 but experiments in hamsters have not been conclusive.

In addition, Berna Biotech is developing a virosomal formulation of a PIV-3 vaccine.

2.3. Respiratory syncytial virus (RSV)

2.3.1. Disease burden

RSV is the single most important cause of severe LRIs in infants and young children. RSV disease spectrum includes a wide array of respiratory symptoms, from rhinitis and otitis media to pneumonia and bronchiolitis, the latter two diseases being associated with substantial morbidity and mortality. Humans are the only known reservoir for RSV. Spread of the virus from contaminated nasal secretions occurs via large respiratory droplets, so close contact with an infected individual or contaminated surface is required for transmission. RSV can persist for several hours on toys or other objects, which explains the high rate of nosocomial RSV infections, particularly in paediatric wards.

The global annual infection and mortality figures for RSV are estimated to be 64 million and 160 000 respectively. In temperate climates, RSV is well documented as a cause of yearly winter epidemics of acute LRI, including bronchiolitis and pneumonia. In the USA nearly all children, by two years of age, have been infected with RSV, is estimated to be responsible for 18 000 to 75 000 hospitalizations and 90 to 1900 deaths annually. The incidence rate of RSV-associated LRI in otherwise healthy children was calculated as 37 per 1000 child-year in the first two years of

life (45 per 1000 child-year in infants less than 6 months old) and the risk of hospitalization as 6 per 1000 child-years (11 per 1000 child-years in the first six months of life). Incidence is higher in children with cardio-pulmonary disease and in those born prematurely, who constitute almost half of RSV-related hospital admissions in the USA. Children who experience a more severe LRI caused by RSV later have an increased incidence of childhood asthma. These studies serve as a basis for anticipating widespread use of RSV vaccines in industrialized countries, where the costs of caring for patients with severe LRI and their sequelae are substantial. RSV also is increasingly recognized as a important cause of morbidity from influenza-like illness in the elderly.

Few population-based estimates of the incidence of RSV disease in developing countries are available, although existing data clearly indicate that, there also, the virus accounts for a high proportion of LRIs in children. Studies in Brazil, Colombia and Thailand show that RSV causes 20–30% of LRI cases in children from 1–4 years of age, a proportion similar to that in industrialized countries. In addition to accurate incidence rates, other important data for developing countries are lacking, such as the severity and case–fatality rates for RSV infection at the community level and the median age of first infection. Preliminary data from community-based studies suggest that the median age of first infection may vary between communities. This information is important for vaccination programme planners, when considering the optimal schedule for vaccination. For example, maternal immunization against RSV would be a desirable strategy to adopt if rates of infection during the first two months of life were found to be high.

Another confusing aspect of the epidemiology of RSV infection that may have an impact on vaccine use is the seasonality of the disease. In Europe and North America, RSV disease occurs as well-defined seasonal outbreaks during the winter and spring months. Studies in developing countries with temperate climates, such as Argentina and Pakistan, have shown a similar seasonal pattern. On the other hand, studies in tropical countries often have reported an increase in RSV in the rainy season but this has not been a constant finding. Indeed, marked differences in the seasonal occurrence of RSV disease have been reported from geographically contiguous regions, e.g. Mozambique and South Africa, or Bangladesh and India. Cultural and behavioral patterns in the community might affect the acquisition and spread of RSV infection. A clear understanding of the local epidemiology of the disease will be critical for the implementation of a successful vaccine development and introduction programme.

2.3.2. Virology

RSV belongs to the family *Paramyxoviridae*, subfamily *Pneumovirinae*, genus Pneumovirus. The genome of RSV is a 15 222 nucleotide-long, single-stranded, negative-sense RNA molecule whose tight association with the viral N protein forms a nucleocapsid wrapped inside the viral envelope. The latter contains virally encoded F, G and SH glycoproteins. The F and G glycoproteins are the only two components that induce RSV neutralizing antibody and therefore are of prime importance for vaccine development. The sequence of the F protein, which is responsible for fusion of the virus envelope with the target cell membrane, is highly conserved among RSV isolates. In contrast, that of the G protein, which is responsible for virus attachment, is relatively variable; two groups of RSV strains have been described, the A and B groups, based on differences in the antigenicity of the G glycoprotein. Current efforts are directed towards the development of a vaccine that will incorporate strains in both groups, or will be directed against the F protein.

2.3.3. Vaccines

Development of vaccines to prevent RSV infection have been complicated by the fact that host immune responses appear to play a role in the pathogenesis of the disease. Early studies in the 1960s showed that children vaccinated with a formalin-inactivated RSV vaccine suffered from more severe disease on subsequent exposure to the virus as compared to unvaccinated controls. These early trials resulted in the hospitalization of 80% of vaccinees and two deaths. The enhanced severity of disease has been reproduced in animal models and is thought to result from inadequate levels of serum-neutralizing antibodies, lack of local immunity, and excessive induction of a type 2 helper T-cell-like (Th2) immune response with pulmonary eosinophilia and increased production of IL-4 and IL-5 cytokines.

In addition, naturally acquired immunity to RSV is neither complete nor durable and recurrent infections occur frequently. In a study performed in Houston, Texas, it was found that 83% of the children who acquired RSV infection during their first year of life were reinfected during their second year, and 46% were reinfected during their third year. At least two thirds of these children also were infected with PIV-3 in their first two years of life. Older children and adults, however, usually are protected against RSV-related LRIs, suggesting that protection against severe disease develops after primary infection.

Passive immunization in the form of RSV-neutralizing immune globulin or humanized monoclonal antibodies given prophylactically has been shown to prevent RSV infection in newborns with underlying cardiopulmonary disease, particularly small, premature infants. This demonstrates that humoral antibody plays a major role in protection against disease. In general, secretory IgAs and serum antibodies appear to protect against infection of the upper and lower respiratory tracts, respectively, while T-cell immunity targeted to internal viral proteins appears to terminate viral infections.

Although live attenuated vaccines seem preferable for immunization of naive infants than inactivated or subunit vaccines, the latter may be useful for immunization of the elderly and high-risk children, as well as for maternal immunization. Candidate vaccines based on purified F protein (PFP-1, -2 and -3) have been found safe and immunogenic in healthy adults and in children over 12 months of age, with or without underlying pulmonary disease, as well as in elderly subjects and in pregnant women. A Phase I study of PFP-2 was conducted in 35 women in the 30th to 40th week of pregnancy; the vaccine was well tolerated and induced RSV anti-F antibody titres that were persistently fourfold higher in newborns to vaccinated mothers than to those who had received a placebo. No increase in the frequency or morbidity of respiratory disease was observed in infants from vaccinated mothers. Maternal immunization using a PFP subunit vaccine would be an interesting strategy to protect infants younger than six months of age who respond poorly to vaccination.

The efficacy of a subunit PFP-3 vaccine was tested in a Phase III trial on 298 children 1 to 12 years of age with cystic fibrosis. The vaccine was well tolerated and induced a four-fold increase in RSV neutralizing antibody titres, but this was not associated with significant protection against LRI episodes as compared to placebo recipients.

A subunit vaccine consisting of co-purified F, G, and M proteins from RSV A has been tested in healthy adult volunteers in the presence of either alum or polyphosphazene (PCPP) as an adjuvant. Neutralizing antibody responses to RSV A and RSV B were detected in 76–93% of the vaccinees, but titres waned after one year, suggesting that annual immunization with this vaccine will be necessary.

A subunit approach also was investigated using the conserved central domain of the G protein of an RSV-A strain, whose sequence is relatively conserved among groups A and B viruses. A recombinant vaccine candidate, BBG2Na (Pierre Fabre), was developed by fusing this domain (G2Na) to the albumin-binding region (BB) of streptococcal protein G. The candidate vaccine elicited a protective immune response in animals, but was moderately immunogenic in adult human volunteers and its clinical development was interrupted due to the appearance of unexpected side effects (purpura) in a few immunized volunteers.

Another RSV candidate vaccine is a synthetic peptide of the conserved region of the G protein administered intranasally, either alone or in combination with cholera toxin. Protection was conferred to mice even without the cholera toxin.

Live, attenuated RSV vaccines that could be delivered to the respiratory mucosa through intranasal immunization have been in development for more than a decade, based on temperature-sensitive (*ts*), cold-adapted (*ca*) or cold-passaged (*cp*) mutant strains of the virus. Difficulties for such a vaccine arise from over- or under-attenuation of the virus and limited genetic stability. Most attenuated live RSV strains tested in humans to date caused mild to moderate congestion in the upper respiratory tract of infants one to two months old and, therefore, were considered as insufficiently attenuated for early infancy. Recombinant RSV vaccines with deletion mutations in nonessential genes (SH, NS1 or NS2), and both *cp* and *ts* mutations in essential genes, are currently being evaluated.

Recombinant DNA technology also has provided the possibility of engineering a chimeric virus containing the genes of human PIV-3 surface glycoproteins F and NH, together with those of RSV glycoproteins F and G, in a bovine PIV-3 genetic background. A first candidate vaccine was found to be attenuated and to induce an immune response to both human PIV-3 and RSV in rhesus monkeys and should presently enter clinical trials. Similarly, a bovine PIV-3 genome was engineered to express human PIV-3 F and HN proteins and either native or soluble RSV protein F. Resulting recombinants induced RSV neutralizing antibodies and protective immunity against RSV challenge in African Green monkeys. These b/h PIV3/RSV F vaccines will presently be tested for safety and efficacy in human clinivcal trials as bivalent vaccines to protect infants from both RSV and PIV-3 infection and disease.

Finally, a combination of a live-attenuated vaccine with a subunit vaccine also is being considered for protecting adults against RSV illness, although a preliminary test of this strategy in healthy young and elderly adults was inconclusive.

2.4. Severe acute respiratory syndrome (SARS)

2.4.1. Disease burden

Severe acute respiratory syndrome (SARS) is a severe respiratory illness caused by a newly identified virus, the SARS coronavirus (SARS-CoV). The disease emerged in southern China in late 2002 and spread in the spring of 2003 to some 30 countries within Asia, Europe and North America. The epidemic finally came to a stop in July 2003 through strict implementation of quarantine and isolation procedures and international collaboration under the coordination of WHO. SARS is characterized by fever, headache, cough and dyspnea, and rapidly progresses to respiratory distress syndrome in more than 20% of the patients, who then necessitate prolonged hospitalization, intensive care and mechanical ventilation. According to WHO, 8,437 cases had been identified worldwide as of July 2003 and 813 patients had died, a 9.6% mortality rate. Only sporadic mini-outbreaks have been reported since then in China, Singapore and China (Province of Taiwan), two of which were linked to laboratory contaminations.

Inapparent, nonpneumonic infections also seem to be quite common, as judged from seroprevalence in healthy populations of blood donors or medical personnel in Hong Kong SAR. Transmission is thought to mostly occur by respiratory droplets. Several instances of nosocomial infection have been reported and health-care workers are at a high risk of infection. Although there is evidence that SARS-CoV emerged from a nonhuman source, no animal reservoir has yet been identified with certainty. Masked palm civet cats and raccoon dogs have been found to be carriers of the virus, and Chinese wild-animal traders show high seroprevalence figures, especially civet cats traders. SARS-CoV also has been recovered from rats but there is no evidence that it is naturally transmitted among that species. The virus has been found to multiply asymptomatically in mice and cats and is pathogenic for some monkeys and ferrets. In spite of the limited extension and relatively rapid control of the epidemic by national Authorities and WHO, the highly contagious nature of the disease and its high fatality rate have prompted the search for a vaccine.

2.4.2. Virology

SARS-CoV belongs to a newly identified group in the family *Coronaviridae*, which are enveloped viruses whose envelope is characterized by crown-like proteinic spikes. Its RNA genome is an exceptionally long 29 727 nucleotides single-stranded positive RNA molecule which encodes 23 different proteins, including the replicase molecule (1a and 1b), spike protein (S), envelope protein (E), membrane protein (M), and nucleocapsid protein (N). Among other members of the *Coronaviridae* family are human coronaviruses HCoV-229E and HCoV-OC43 (agents of the common cold), the feline infectious peritonitis virus (FIPV), the avian infectious bronchitis virus (IBV) and the pig transmissible gastroenteritis virus (TGEV). The S protein of these viruses is known to be responsible for the induction of virus neutralizing antibodies.

2.4.3. Vaccine development

The S protein of SARS-CoV contains the viral receptor binding site and neutralization epitopes in its N-terminal half and a fusion domain together with other neutralization epitopes in its C-terminal half. The spike protein therefore is a prime target for the generation of neutralizing antibodies against SARS-CoV and the development of protective humoral immunity. A human neutralizing monoclonal antibody targeted to the S protein was found to block the attachment of the virus to its receptor and provided remarkable protection in a mouse model of SARS-CoV infection, paving the way for its eventual use in passive serotherapy to provide immediate protection against infection for contacts and medical personnel.

Less than one year after SARS first appeared, half a dozen candidate vaccines already were in development. At this time, a number of candidate vaccines are on track:

- several whole inactivated vaccine preparations, one of which already was tested in Phase I clinical trials in China;
- a live modified virus Ankara (MVA) recombinant vaccine and a live bovine PIV-3 recombinant vaccine, both expressing the SARS-CoV S protein;
- a live recombinant nonreplicative adenovirus vaccine expressing the S, M and N proteins;
- DNA vaccines expressing either the N or the S proteins; and
- several subunit vaccines made of recombinant SARS virus proteins;
- in addition, coexpression of SARS-CoV S, M, and N proteins in human 293 renal epithelial cells in culture resulted in the production of SARS-CoV VLPs that will eventually be developed into a particulate recombinant vaccine.

All these vaccines face uncertainties, however, not the least of which is the lack of a reliable animal model in which to test them. Another uncertainty is the possibility of immune enhancement, a phenomenon which was observed when studying vaccination of cats against the feline coronavirus, FIPV: animals vaccinated with a whole inactivated virus vaccine showed accelerated disease and death after exposure to wild-type virus. The fact that ferrets vaccinated with an experimental MVA/SARS-CoV recombinant vaccine suffered from increased liver inflammation upon SARS virus challenge casts a cautionary note that immunization with some SARS vaccines might worsen the disease rather than prevent it.

2.5. *Streptococcus pneumoniae*

2.5.1. Disease burden

Based on available data, *S. pneumoniae* is estimated to kill annually close to one million children under five years of age worldwide, especially in developing countries where pneumococcus is one of the most important bacterial pathogens of early infancy.

In developed countries, virtually every child becomes a nasopharyngeal carrier of *S. pneumoniae* during the first year of life. Many go on to develop one or more episodes of otitis media, whereas a smaller number develop more serious invasive pneumococcal infections. Bacteraemic pneumonia is a common form of invasive

pneumococcal disease, the next most common being pneumococcal meningitis, with or without bacteraemia. *S. pneumoniae* is the leading cause of nonepidemic childhood meningitis in Africa and other regions of the developing world. In the USA, most cases of invasive pneumococcal disease are characterized by febrile bacteraemia without specific localization. Less severe but more frequent forms of pneumococcal disease include middle-ear infection, sinusitis or recurrent bronchitis. Thus, in the USA alone, seven million cases of otitis media are attributed to pneumococci each year. Although all age groups may be affected, the highest rate of pneumococcal disease occurs in young children and in the elderly population. In addition, persons suffering from a wide range of chronic conditions and immune deficiencies are at increased risk. In Europe and the USA, pneumococcal pneumonia accounts for at least 30% of all cases of community-acquired pneumonia admitted to the hospital, with a reported annual incidence of 5500 to 9200 per 100 000 persons 65 years of age or older, and a case fatality rate of 10–30%. *S. pneumoniae* is an under-appreciated cause of nosocomial pneumonia in hospital wards, intensive care units, as well as in nursing homes and long-term care institutions.

2.5.2. Bacteriology

S. pneumoniae is a Gram-positive encapsulated diplococcus. Based on differences in the composition of the polysaccharide (PS) capsule, 90 serotypes have been identified. This capsule is an essential virulence factor. The majority of pneumococcal disease in infants is associated with a small number of these serotypes, which may vary by region. Current data suggest that the 11 most common serotypes cause at least 75% of invasive disease in all regions. Several other virulence factors have been described, including pneumolysin which leads to pore formation and osmotic lysis of epithelial cells, autolysin, and pneumococcal surface protein A (PspA), which interferes with phagocytosis and immune function in the host. Pneumococci are transmitted by direct contact with respiratory secretions from patients and healthy carriers. Although transient nasopharyngeal colonization rather than disease is the normal outcome of exposure to pneumococci, bacterial spread to the sinuses or the middle ear, or bacteraemia following penetration of the mucosal layer, may occur in persons susceptible to the involved serotype. Pneumococcal resistance to essential anti-microbials such as penicillins, cephalosporins and macrolides is a serious and rapidly increasing problem worldwide.

2.5.3. Vaccines

Protective immunity is conferred by type-specific, anticapsular antibodies, although the serological correlates of immunity are poorly defined. Antibodies to pneumococcal surface proteins (PspA) have been demonstrated to confer protection in animal models but the role of these antibodies in humans is yet to be determined.

Currently licensed vaccines are polyvalent PS vaccines containing per dose 25 µg of purified capsular PS from each of the 23 serotypes of *S. pneumoniae* that together account for most cases (90%) of serious pneumococcal disease in western industrialized countries. Relatively good antibody responses (60–70%) are elicited in most healthy adults within 2–3 weeks following a single intramuscular or subcutaneous immunization. The immune response is however mediocre in children less than two years of age and in immunocompromised individuals (HIV/AIDS). Furthermore, PS vaccines do not induce immunological memory which is required for subsequent booster responses. The polyvalent PS vaccine is recommended for

healthy people over 65 years of age, particularly those living in institutions. Randomized controlled trials in healthy elderly people in industrialized countries have, however, failed to show a beneficial effect of the vaccine, so that recommendation for its use in the elderly is based on data from observational studies showing a significant protective effect against invasive (bacteraemic) pneumococcal disease, but not pneumonia.

Following the vaccination of pregnant women with PS vaccines, anti-PS antibodies are transferred both via the placenta and in the breast milk, but formal demonstration that maternal vaccination actually protects newborn infants against pneumococcal disease is still lacking.

Over the past 15 years, several vaccine manufacturers have developed pneumococcal conjugate vaccines in which a number of *S. pneumoniae* PS are covalently coupled to a protein carrier. Conjugate vaccines elicit higher antibody levels and a more efficient immune response in infants, young children, and immunodeficient persons than the PS vaccines, as well as a significant immunological memory resulting in a booster antibody response on subsequent exposure to the antigen. Moreover, these vaccines suppress nasopharyngeal carriage of the pathogen and reduce bacterial transmission in the community through herd immunity, which adds considerable value to their implementation. Conjugate vaccines immunization followed by PS vaccine boosting might provide a foundation for lifelong protection against pneumococcal disease.

Introduction of the conjugate vaccine in early 2000 in the USA resulted in dramatic decline in the rates of invasive pneumococcal disease, with reductions also seen in unvaccinated individuals as a result of herd immunity. In a double-blind Phase III study of the 7-valent vaccine, Prevnar (Wyeth), conducted at northern California Kaiser Permanente medical centres on 37,868 infants, 40 cases of invasive *S. pneumoniae* disease were seen in the study population, 39 of which were in the control group, representing a 97% vaccine efficacy. The vaccine was found to be 100% efficacious in the few low birth-weight and preterm infants included in the study. Post-licensure follow-up studies conducted in the same setting have shown an 87% reduction in invasive pneumococcal diseases caused by vaccine serotypes in children less than one year of age, and a 62% reduction in children less than five years of age, with no difference between a two-dose or a three-dose immunization regimen. The vaccine also elicited moderate protection against otitis caused by vaccine serotypes. However, the decrease in cases of vaccine-type otitis media was offset by an increase in those due to non-vaccine-types of *S. pneumoniae* and by *H. influenzae*, a phenomenon referred to as "replacement disease". This phenomenon also has recently been observed for invasive pneumococcal disease, although the increase in non-vaccine types was small relative to the decrease in vaccine-type invasive disease caused by vaccination.

The currently licensed 7-valent vaccine, Prevnar, does not contain some of the serotypes that cause severe disease in developing countries, notably serotypes 1 and 5. New conjugate vaccines that provide more optimal serotype coverage in these countries are in clinical development, including a 9-valent Wyeth vaccine, and an 11-valent GSK and Sanofi-Pasteur vaccines. The protein carrier used by Wyeth is CRM197, a genetically detoxified mutant of diphtheria toxin, whereas that used by GSK is the *H. influenzae* protein D. Merck is using the outer membrane protein

complex (OMPC) from *N. meningitidis*. The 9-valent vaccine has been tested in South Africa with remarkable efficacy results in children less than two years of age, including HIV-positive infants. In addition, an unexpected benefit of vaccination was the decrease of symptomatic pneumonia cases associated with a viral infection, whether influenza virus or one of the paramyxoviruses. The vaccine is now being tested in the Gambia. Sanofi-Pasteur 11-valent vaccine is undergoing an efficacy trial in the Philippines, but it is not clear at this time whether all these conjugate candidate vaccines will be taken to licensure.

Newer vaccine approaches are being developed in order to provide protective immunity against a larger number of *S. pneumoniae* serotypes, and to circumvent the complexity of manufacture of conjugate vaccines. Several pneumococcal proteins, including pneumolysin, PspA, pneumococcal surface adhesin (PsaA), neuraminidase, and autolysin are at an early clinical stage development. PiaA and PiuA, two newly identified lipoprotein components of *S. pneumoniae* iron uptake ABC transporters, elicit protective immunity against invasive pneumococcal disease in mice through induction of opsonophagocytosis-promoting antibodies.

Through screening with human convalescent sera of a *S. pneumoniae* genomic expression library, Shire Biologicals, Canada (now ID BioMedical) has identified what appear to be remarkably conserved bacterial surface proteins (BVH-3 and BVH-11) able to induce protective anti-pneumococcal antibodies in the mouse model. A recombinant 100 kD hybrid protein, BVH3/11V, was engineered by fusion of parts of the two genes and expressed with high yields in *E. coli*. The fusion protein has successfully been tested in Phase I dose ranging clinical trials in toddlers and elderly volunteers. A 2-dose immunization regimen was able to induce a 50-fold increase in anti-*S. pneumoniae* antibody levels. Phase II clinical studies in infants and elderly persons have been initiated. This vaccine should be serotype-independent as the BVH3 and BVH11 antigens are common to all 90 serotypes of *S. pneumoniae*.

2.6. Tuberculosis

2.6.1. Disease burden

An estimated one third of humanity (approximately two billion people) is infected with tuberculosis (TB). Amongst those carrying the pathogen, around 8 million persons come down with clinical disease every year; and out of these, about 1.6 million die, not counting tuberculosis-related deaths in TB-HIV co-infected individuals. Over 1.5 million new TB cases per year occur in sub-Saharan Africa, nearly three million in South-East Asia and over a quarter of a million in Eastern Europe. In 1993, WHO declared tuberculosis a global emergency, reflecting the magnitude of the concern about the TB epidemic. It is estimated that between 2000 and 2020, nearly one billion people will be newly infected, 200 million will get sick, and 35 million will die from TB – if control measures are not significantly improved.

TB is a poverty-related disease: it has long been recognized that war, malnutrition, population displacement and crowded living and working conditions favour the spread of TB among humans, whereas periods of improvement in societal conditions and hygiene favour its rapid decline. TB is highly contagious. Left untreated, each patient with active TB will infect on average between 10 and 15 people every year.

Transmission is common in families, schools, hospitals and prisons. The high contagiousness is related to the production of small particle droplets when a patient coughs, and to the low dose of bacilli needed to infect a person. Substantial and successful progress has been made worldwide to contain the TB pandemic, especially through the WHO-recommended strategy for its detection and treatment (directly observed treatment short course, DOTS). However, these efforts are antagonized by the emergence of multidrug-resistant TB (MDR-TB) strains, the favourable environment created by HIV infection, and the frequent lack of modern tools to diagnose, treat and prevent the disease.

MDR-TB is defined as the disease due to TB bacilli resistant to at least isoniazid and rifampicin, the two most powerful anti-TB drugs. MDR-TB is rising at alarming rates in some countries, especially in the Newly Independent States of the former Soviet Union, and threatens global TB control efforts. From a public health perspective, poorly supervised or incomplete treatment of TB is worse than no treatment at all. TB also is the leading cause of death among people who are HIV-positive, accounting for about 25% of AIDS deaths worldwide. In sub-Saharan Africa, HIV was the single most important factor determining the increased incidence of TB in the last 10 years: in some regions, up to 75% of new active TB cases are in HIV-infected people. Paradoxically, TB seems to be severely aggravated in these dually infected patients when active antiretroviral therapy is initiated. Another factor that helps the spread of TB is global movement of people. In many industrialized countries, about one half of TB cases occur in foreign-born or migrant populations. Untreated TB spreads quickly in crowded refugee camps and shelters. It is estimated that as many as 50% of the world's refugees may be infected with TB.

But the major bottleneck for higher success rates in controlling TB is the fact that currently only about 40% of all sputum-positive TB are detected. Thus, the majority of TB cases remain untreated or are treated only at a very late and highly infectious stage, causing enormous individual hardship as well as creating a public health time bomb. For all these reasons, plus the relative ineffectiveness of the current BCG vaccine, the development of improved TB vaccines has become a necessity for adequate control and elimination of tuberculosis.

2.6.2. Bacteriology

Mycobacterium tuberculosis, the agent of human TB, was discovered in 1882 by Robert Koch and for a long time called after his name (the Koch bacillus). All members of the Mycobacterium genus share the property of acid-fastness (Ziehl-Neelsen staining), due to their mycolic acid-rich cell wall structure. They include *M. tuberculosis*, *M. africanum*, and *M. ulcerans*, which are primary human pathogens, *M. bovis,* the agent of TB in cattle and other animals, which also can cause disease in humans, and a great many nontuberculous or environmental species, some of which can be pathogenic in humans such as those belonging to the *M. avium-intracellulare* complex.

TB bacilli usually multiply first in the lung alveoli and alveolar ducts and in draining lymph nodes. They also multiply in the macrophages that were attracted from the bloodstream and killed, progressively creating a primary tubercle. Delayed cutaneous hypersensitivity develops and together with other cellular immune reactions, leads to the caseous necrosis of the primary complex. Bacilli eventually spread to

many parts of the body such as liver, spleen, meninges, bones, kidneys and lymph nodes, where they can either be a source of overtly disseminated TB or, more commonly, remain dormant. Occasional decline in cell-mediated immunity leads to reactivation TB, most frequently seen in adults as a pulmonary disease with infiltration or cavity in the apex of the lung. This is the most infectious form of TB.

CD4+ T-cells play a major role in containment of infection; progressive TB is usually associated with a Th2 T-cell response, whereas a pure Th1 response mediates protection. The tuberculin skin test has long been used as evidence of TB infection or as a sign of adequate response to BCG vaccination, although no clear relationship between delayed-type hypersensitivity and protective immunity could be established. A number of antigens found in *M. tuberculosis*, including Ag85, MPT64, ESAT-6 and CFP10, have been identified which may play a major role in cellular immunity and the induction of a protective IFN-γ response.

2.6.3. Vaccines

By culturing a *M. bovis* isolate from a cow for a period of 13 years and a total of 231 passages, Calmette, a physician, and Guérin, a veterinarian, created an attenuated variant of *M. bovis*, Bacille Calmette–Guérin (BCG). In 1921 BCG was first tested in infants as an oral vaccine. New methods of administration were later introduced, such as intradermal, multiple puncture, and scarification. Since 1974, BCG vaccination has been included in the WHO Expanded Programme on Immunization (EPI), resulting in more than four billion doses injected worldwide (approximately 100 million immunizations in children each year). As recently shown by sequencing, the original BCG strain lost the RD1 region of the *M. tuberculosis* genome in the course of the selection process. Major BCG vaccine strains in use today differ even further from the original BCG strain and from each other, with "stronger" strains (Pasteur 1173 P2, Danish 1331) being more reactogenic and, presumably, more immunogenic, than "weaker" strains (Glaxo 1077, Tokyo 172).

No other widely used vaccine is as controversial as BCG. Its effects in large randomized, controlled, and case–control studies, have been widely disparate, from excellent protection against TB to no protection. Most studies have demonstrated that BCG vaccines afford a higher degree of protection against severe forms of TB, such as meningitis and disseminated TB, than against moderate forms of the disease. The efficacy of neonatal BCG vaccination also wanes with age, dropping in one study from 82% in children less than 15 years of age to 67% in the 15–24-year-old group, and to 20% only in persons over 25 years of age. Studies that evaluated meningitis or miliary TB demonstrated that BCG can provide good protection against these serious forms of TB in young children, with reported efficacy ranges from 46–100%. In contrast, efficacy against pulmonary TB, which is more prevalent in adolescents and adults, has ranged from 0–80%.

Efficacy of BCG vaccination also appears to vary with geographic latitude – the farther from the equator, the more efficacious the vaccine. Presumably, exposure to nonpathogenic mycobacteria, which is more intense in warm climates, induces a degree of protective immunity in exposed populations, masking potential protection from BCG.

Vaccination with BCG still remains the standard for TB prevention in most countries because of its efficacy in preventing life-threatening forms of TB in infants and young children, and also because it is the only vaccine available, is inexpensive, and requires only one encounter with the baby.

A spectrum of innovative new approaches have been applied to TB vaccine development during the last decade and, as a consequence, several new TB candidate vaccines now are in clinical trials or at late stages of preclinical development. Some approaches have relied on strengthening the immunogenicity and/or persistence of genetically modified BCG strains, such as those described below.

- A recombinant BCG vaccine (BCG30) that was engineered at University of California at Los Angeles (USA) to express the 30 kD major secretory protein Ag85B, and is in Phase I trial in the USA.
- A BCG::RD1 recombinant, in which the RD1 segment of the *M. tuberculosis* genome has been reintroduced, resulting in the expression of ESAT-6 and Ag85A proteins. This new BCG strain, developed at the Pasteur Institute, Paris, showed increased persistence and improved protection against challenge with virulent *M. tuberculosis* in animal models.
- Another improved BCG, rBCG: Ä*ure*C-*Hly*, was engineered at the Max Planck Institute for Infection Biology in Berlin (Germany) to express listeriolysin O, which increases MHC class I presentation, and its urease gene was deleted in order to prevent neutralization of the acidic pH in phagosomes. This recombinant BCG was found to be devoid of pathogenicity for SCID mice and provided greatly improved protection against aerosol TB in the mouse model. The vaccine is expected to enter clinical trial in the course of 2005.
- Other live-attenuated candidate TB vaccines include a *Pho*P mutant of *M. tuberculosis*, developed at the Pasteur Institute in Paris, and double auxotrophic mutants of *M. tuberculosis* developed at the Albert Einstein College of Medicine in New York. These vaccines have been shown to be safe in animals but their evaluation in humans is met with technical and psychological barriers.

Due to safety concerns, in particular in immunocompromised persons, as well as to technical challenges regarding manufacture and reproducibility, live mycobacteria vaccines are not the product of choice of most vaccine manufacturers. Many new TB vaccines approaches are therefore focused on recombinant subunit vaccines, DNA vaccines, or attenuated Salmonella vector- or virus vector-based vaccines that express antigens such as 85A (Ag85A), HSP65, the R8307 protein, a 36kD proline-rich mycobacterial antigen, or the 19kD and 45kD proteins.

The first of the genuinely new candidates, a recombinant MVA construct carrying the secretory Ag85A, has completed Phase I safety evaluation in humans in the United Kingdom (UK) without major adverse events and is now being evaluated in the Gambia.

A live, nonreplicative adenovirus expressing Ag85A is developed by the Aeras Global TB Vaccine Foundation (USA) and Crucell NV (Netherlands).

Several non-living TB vaccine candidates also have entered or will soon be entering human clinical trials, including two recombinant protein subunit vaccines, one based on an Mtb32/Mtb39 fusion protein, developed by Corixa Inc. (USA) and GSK, and the other based on an ESAT-6/Ag85B fusion protein, developed by the Statens Institute in Copenhagen. In addition, a multi-epitope polypeptide, as well as nonproteinic antigens such as mycolic acids and carbohydrate moieties, are being developed as candidate antigens.

Evaluation of many of these new candidate vaccines is planned, at least initially, in prime-boost regimens with BCG as the priming agent. Indeed, the MVA-Ag85A recombinant was found to induce significantly stronger cellular immune responses in BCG-primed than in BCG-naive individuals, even if the immunization was as long as 38 years after the priming: at 24 weeks after immunization, the levels of IFN-γ secreting T-cells were 5–30 times greater in the BCG-vaccinated than in the BCG-naïve individuals. Likewise, a BCG prime followed by an Mtb72 fusion protein boost in combination with GSK adjuvant formulation gave remarkable protection results against TB challenge in mice and monkeys, and should enter clinical trials soon.

In the absence of a valid surrogate of vaccine-induced protection and in order to avoid long duration and/or enormous cohort sizes, the first Phase III efficacy trials of new TB vaccines are likely to be performed in high-risk populations such as household contacts of TB patients and health-care workers. New TB vaccine developers will have to face important ethical issues, such as withholding preventive isoniazide treatment of individuals at high risk, and withholding BCG vaccination during clinical trials, including in populations where HIV infection is prevalent.

As observed for HIV/AIDS vaccines, human clinical studies will, from now on, act as the principal driving force for the development of new TB vaccines. Testing of such a wide variety of vaccine types using different immunization strategies directed against a sole pathogen is unique in the history of vaccine development. It will make the valid comparison of clinical data most challenging. Therefore, it will be all the more important that this effort be tightly coordinated to provide maximal comparability and transparency. WHO is working with all stake holders in the field to standardize key parameters such as trial entry criteria, endpoints, immunoassays, etc. The main players in this area include, among others, the US National Institute for Allergy and Infectious Diseases (NIAID), the Aeras Global TB Vaccine Foundation, supported by the Bill and Melinda Gates Foundation, a network of European researchers supported by the European Commission, and pharmaceutical manufacturers including GlaxoSmithKline (GSK) and IDRI-Corixa.

3. Bacterial infections

A variety of infectious diseases caused by pathogenic bacteria have been regrouped in this chapter and are presented below in alphabetical order: *Helicobacter pylori*, *Neisseria meningitidis*, *Mycobacterium ulcerans, Staphylococcus aureus*, and Group A and Group B *Streptococci*. Other bacterial infections have been classified on type of disease, route of transmission or animal reservoir.

3.1. *Helicobacter pylori*

3.1.1. Disease burden

The isolation of *Helicobacter pylori* from the human gastric mucosa in 1982 and the demonstration of its involvement in gastritis, peptic ulcer disease and gastric adenocarcinomas have radically changed our perception of these diseases. *H. pylori* is a small, spiral, gram-negative bacillus that appears to inhabit the mucous layer overlying the gastric epithelial cells in humans. It produces a potent urease, which, by producing ammonia, may help to neutralize gastric acid, but the mechanism by which the bacteria produces gastric inflammation is not clear as it does not invade the mucosa. Development of atrophy and metaplasia of the gastric mucosa are strongly associated with *H. pylori* infection. Oxidative and nitrosative stress in combination with inflammation plays an important role in gastric carcinogenesis.

H. pylori has an estimated prevalence of about half the world's population, possibly reaching up to 70% in developing countries and 20–30% in industrialized countries. Although infected individuals often have histological evidence of gastritis, the vast majority of infections are asymptomatic. Infections seem to be more common with age but, in the tropics, they often occur before the age of 10 years, especially in high-density populations with low socioeconomical status. Transmission is from person-to-person, presumably oral-oral and/or faecal-oral. In the absence of treatment, infection is potentially lifelong. Treatment is based on the use of a proton-pump inhibitor and antibiotics (metronidazole and clarithromycin).

3.1.2. Vaccine development

H. pylori has been shown to be heterogeneous at the genomic level with a high variability in some genes. The feasibility of preventive vaccination has been proven in animal models (mice, dogs) using whole cell vaccines as well as subunit vaccines comprising selected antigens such as VacA, CagA, NAP, hsp, urease or catalase. One of the difficulties met in vaccine studies is the absence of correlates of protection; another is to develop a vaccine that will be efficacious at the mucosal level. In humans, several Phase I studies have been conducted using:

- recombinant attenuated *Salmonellas* expressing *H. pylori* urease, that showed mediocre immunogenicity by the oral route;
- an oral whole-cell vaccine adjuvanted with wild-type LT, that was discontinued because of excessive side effects;
- purified urease co-administered with LT, also put on hold;
- a recombinant VacA, CagA and NAP vaccine in alum that proved to be safe and strongly immunogenic. The companies which were involved are Antex, Acambis and Chiron in the USA and the Commonwealth Serum Labs in Australia.

A prophylactic vaccine would be cost-effective in preventing gastric cancer and duodenal ulcer.

3.2. *Neisseria meningitidis*

Bacterial meningitis remains a serious threat to global health, accounting for an estimated annual 170 000 deaths worldwide. Even with antimicrobial therapy and the availability of sophisticated intensive care, case fatality rates remain at 5–10% in industrialized countries, and are even higher in the developing world. Between 10–20% of survivors develop permanent sequelae such as epilepsy, mental retardation or sensorineural deafness. Three species, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitidis*, are responsible for most cases of bacterial meningitis occurring beyond the neonatal period. Since the introduction of *H. influenzae* type b (Hib) conjugate vaccines, *N. meningitidis* and *S. pneumoniae* have become the commonest causes of bacterial meningitis in the world. *N. meningitidis* moreover is the only bacterium capable of generating epidemics of meningitis.

3.2.1. Disease burden

N. meningitidis is a common inhabitant of the mucosal membranes of the human nasopharynx, where it usually lies as a harmless commensal. Up to 5–10% of a population may be asymptomatic carriers in nonepidemic settings. Most cases are acquired by person-to-person contact through aerosol droplets or contacts with respiratory secretions from the asymptomatic carriers. A small minority of those who become infected will develop an acute inflammation of the meninges, the membranes covering the brain and the spinal cord. The disease mainly affects infants and young children, but also can readily be found in older children and young adults.

Meningococcal disease is a global problem that occurs in all countries. Group A meningococci are characterized by their propensity to cause large-scale epidemics in developing countries but they rarely cause disease in North America or in Europe. They are the major cause of both epidemic and endemic meningococcal disease in Africa, with the highest burden of disease occurring in a sub-Saharan area from Senegal to Ethiopia that is referred to as "the meningitis belt". Meningococcus group A epidemics occur there in irregular cycles every 5 to 12 years, last for 2 to 3 years, peaking in March–April at the end of the dry season, and dying out during the intervening rainy seasons. Extensive population travel, such as for the Hadj pilgrimage in Saudi Arabia, facilitates the circulation of virulent strains from country to country. Three quarters of the cases occur in individuals less than 15 years of age.

The size of these epidemics can be enormous. During the 1996 epidemic in sub-Saharan Africa, around 200 000 cases were reported with 20 000 deaths. Attack rates in epidemic years can be as high as 1000 per 100 000 population, with a case fatality rate between 10% and 15%. Incidence remains relatively high in non epidemic years. Thus, in Niger, the average annual incidence of meningitis during 11 inter-epidemic years in the 1980s and early 1990s was 30/100 000, a 10-fold higher rate than in the UK. Since 2000, the potential emergence of group W135 as the cause of outbreaks (Saudi Arabia, 2000; Burkina Faso, 2003) has added complexity to the epidemiological situation in the region. Group A epidemics also have occurred in other parts of Africa and in Asia (China, Mongolia and Nepal).

Serogroup B meningococcus is the most important cause of endemic meningitis in industrialized countries, accounting for 30–40% of cases in North America and for 30–80% in Europe, with most of the remaining cases caused by group C strains. The proportion of group B strains is especially high in Denmark, Germany, the Netherlands and Norway, while increasing proportions of group C strains have been reported from the Czech Republic, Greece, Ireland, Slovakia, Spain and the UK. In all countries, the incidence of group B and C disease is highest in winter in infants less than one year-old. In the USA, an estimated 3000 cases occur per year, with a case fatality rate of 12%, involving serogroups B, C, and Y in roughly equal proportions. Case fatality is higher for infants less than one year of age and for the elderly. Group B epidemics have occurred in some middle-income countries of the Americas (Brazil, Chile, Colombia and Cuba), but are rare in Africa or in other areas of the developing world. Since 1991, New Zealand has experienced an epidemic of group B meningococcal disease with incidence rates of up to 10 times the background incidence, especially in Pacific Islanders and Maori populations, progressively increasing from 53 cases per year (1.6 per 100 000 population) in 1990 to a peak of 613 (16.9/100 000) in 1997, and consistently remaining high thereafter, with an average of 500 cases per year (about 14 per 100 000 population). In contrast with serogroup A and C epidemics, which usually resolve in one to three years, serogroup B epidemics begin slowly but may persist for 5 to 10 years or longer, as seen in Cuba, Norway, areas of Chile and currently in New Zealand. Serogroup B global incidence has been estimated between 20 000 and 80 000 cases per year, accounting for 2000–8000 deaths annually.

3.2.2. Bacteriology

N. meningitidis is a gram-negative encapsulated diplococcus. At least 13 different serogroups have been defined on the basis of the immunochemistry of the capsular PS, but serogroups A, B, C, Y, and W135 account for almost all cases of disease. Meningococci are further classified into serotypes and subtypes on the basis of the immunologic reactivity of their PorA and PorB outer membrane proteins, respectively. Approaches such as multilocus enzyme electrophoresis, now replaced by multilocus sequence typing, have been used to monitor the global epidemiology of meningococcal disease. The complete nucleotide sequence of the genome of isolates from serogroups A, B and C has been determined. Many *N. meningitidis* strains have reduced susceptibility to penicillins, but high levels of resistance are rarely found. Still, the antibiotic of choice for treatment of meningococcal meningitis in outbreaks in developing countries is oily chloramphenicol.

3.2.3. Vaccines

3.2.3.1. Groups A, C, Y, W

The first successful capsular polysaccharide (PS) vaccines against *N. meningitidis* groups A and C were developed 30 years ago in response to meningitis epidemics among military recruits in the USA and were widely tested in Africa, Latin America and Europe. They proved to be safe and effective in preventing group C disease in military recruits in the USA, and in controlling group A epidemics during mass campaigns in Africa. Multivalent PS vaccines against groups A and C, or A, C, Y and W-135, are licensed (GSK and Sanofi-Pasteur) and available worldwide, but not always affordable for developing countries. During the epidemic season in the African meningitis belt, vaccine from an international stockpile is made available to countries through the International Coordinating Group on Vaccine Provision for Epidemic Meningitis (ICG) set up in 1997 by WHO. Since 2003, the W-135 serogroup of *N. meningitidis* has emerged as a potential epidemic strain in countries of the meningitis belt, prompting the development of a trivalent PS A/C/W135 vaccine (GSK). However, most PS vaccines are poorly immunogenic in young infants and children less than two years old, fail to induce immunological memory and do not provide protection for more than 3–5 years.

Experience with Hib and pneumococcal conjugate vaccines has shown that the immunogenicity of PS can be greatly improved by chemical conjugation to a protein carrier. The resulting PS-protein conjugate vaccines are safe, immunogenic in young infants and induce long-term immune memory. Immunization also decreases nasopharyngeal carriage and transmission of the pathogen (herd immunity).

Meningococcal group C conjugate vaccines were developed by Chiron and Wyeth, using a genetically detoxified diphtheria toxoid (CRM197) as the protein carrier, and by Baxter, using tetanus toxoid as the carrier. These vaccines were introduced in November 1999 into the UK as an addition to routine infant immunization at two, three and four months of age. The vaccine had a tremendous impact on the incidence of the disease, resulting in a more than 90% decrease in the number of deaths and clinical cases, and a 66% decrease in asymptomatic carriage. Interestingly, the vaccine also decreased by 70% the number of cases in nonvaccinated people, a substantial benefit due to herd immunity. This vaccine is now used in routine three-dose infant immunization schedule in the UK, although there is evidence that one dose could be sufficiently protective in infants and young children to allow postponement of the two booster doses to an older age. Also, the possibility of using PS vaccines for booster immunization has been entertained.

Among other conjugate meningitis vaccines under development is a tetravalent vaccine incorporating PS from groups A, C, Y and W-135. Wyeth is currently testing in Phase III trials a conjugate 9 valent *S. pneumoniae*/Meningococcus C vaccine. No major technical problems are anticipated with these vaccines, which should become available in the USA and Europe within a few years. An interesting initiative has been launched in June 2001 by the Meningitis Vaccine Project (MVP) that resulted in a partnership between the WHO and PATH to develop a meningococcus group A conjugate vaccine at an affordable price for the developing world. MVP has identified a European manufacturer of clinical-grade group A PS, a developing country manufacturer of high quality tetanus toxoid to be used as the carrier, and a public biological research centre in the USA which provided expertise in the design of

conjugate vaccines. The Serum Institute of India is in charge of the conjugation and of filling, lyophilization and packaging of the vaccine which they expect to produce at the rate of 25 million doses per year, at a cost of less than US$0.5 per dose in ten-dose vials. Phase I clinical trials of the vaccine are planned to start by early 2005 in India and to be followed by a dose escalating Phase II trial in late 2005 in Africa. MVP plans to use the monovalent A conjugate vaccine as a single-dose in mass vaccination campaigns in persons 1–29 years of age, and in a 2-dose schedule (14 weeks and 9 months) in routine childhood immunization.

3.2.3.2. Group B

In contrast with group A and C meningitis, development of vaccines against group B meningococcal infections remains problematic because: a) unlike other meningococcal capsular PS, group B capsular PS is poorly immunogenic; and b) it cross-reacts with sialylated proteins in human tissues, including the neural cellular adhesion molecule involved in cell-to-cell adhesion. An attempt was made at developing a conjugated vaccine using group B capsular PS in which the N-acetyl groups of the sialic acid residues had been replaced with N-propionyl groups. The vaccine was tested in a Phase I trial on 17 adult volunteers and found to be safe, but the antibodies induced were devoid of functional activity (Sanofi-Pasteur). Consequently, vaccine research against serogroup B meningococcus has mostly focused on cell-surface protein antigens contained in outer-membrane vesicles (OMV).

One of the outer meningococcal membrane proteins (OMP), PorA, was identified as a major inducer of, and target for, serum bactericidal antibodies. This protein is expressed by almost all meningococci. However, there is a large number of PorA proteins with different antigenic specificities so that eliciting an immune response against one PorA antigen does not confer protection against strains with heterologous PorA antigens. OMV vaccines are thus strain-specific vaccines: they can be used against clonal disease outbreaks but not for prevention of sporadic disease caused by diverse strains. The two best studied OMV vaccines were produced in response to national outbreaks in Norway and Cuba, respectively. Both these vaccines have been used for epidemic control in their respective countries and, in the case of the Cuban vaccine, in Brazil and Chile. The PorA vaccines were found to be 50–80% effective, but they did not protect young children and the immune response was of short duration.

In an effort to develop a vaccine against the New Zealand (NZ) group B strain, the RIVM in the Netherlands has used recombinant technology to produce both a monovalent NZ PorA vaccine and a hexavalent vaccine containing six PorA proteins, including that of the NZ strain. The performance of the NZ PorA antigen in the hexavalent formulation administered as a three dose series was only modest in infants, but it was shown to stimulate a satisfactory immune response after a fourth dose in toddlers. This vaccine has now been tested in Phase II trials (GSK).

A PorA vaccine developed by Chiron in partnership with the New Zealand Ministry of Health and the University of Auckland, MeNZB, was successfully tested in Phase I and a series of successive Phase II trials in school children, then toddlers and infants. A three dose regimen of immunization was found to elicit bactericidal antibodies in 70% of children 6–24 months of age as well as in 8–12 year-old children. The vaccine elicited a 90% response in teenagers. Strain-specific anti-OMP immune

responses also were generated in infants. Sequential, nationwide introduction of the vaccine in the population under 20 years of age is currently ongoing with intensified Phase III/IV monitoring.

The successful development of a broad specificity Group B vaccine may come in the end as a consequence of the sequencing of the meningococcal genome, which opened new possibilities of identifying antigens to be included in candidate vaccines. Of the 600 potential meningococcus antigens whose sequence was recorded, 350 were expressed as recombinant proteins in *E.coli*, purified, and tested for the induction of bactericidal activity in the mouse. This approach, which has been dubbed "reverse vacccinology", has led to the discovery of 28 novel proteins with immunological potential for the development of new meningococcal vaccines. Novel surface-antigens which appear to be well conserved among *N. menigitidis* isolates are currently entering clinical evaluation as candidate vaccines (Chiron, Microscience).

Global elimination of bacterial meningitis may well be an achievable target when potent and affordable vaccines against meningococcus ACYW and B become available within the next decade.

3.3. *Mycobacterium ulcerans* (Buruli ulcer)

3.3.1. Disease burden

Buruli ulcer (BU), also known as tropical ulcer, is an emerging necrotic skin disease caused by *Mycobacterium ulcerans*. *M. ulcerans* is the third most important mycobacterial pathogen of man after *M. tuberculosis* and *M. leprae.* It is found in water-dwelling insects, snails and fish. *M. ulcerans* also has been described in native Australian animals including the koala, possums and the long-footed potoroo. Transmission to humans is by an unknown mechanism, presumably as a consequence of local trauma, although recent evidence suggests that aquatic insects may be the natural reservoir and their bite may transmit the disease to humans. BU presents as a chronic, essentially painless skin ulcer with whitish or yellowish base that usually starts as small nodules which subsequently ulcerate. Most lesions are located on the extremities and occur among children living near wetlands and rain forests in rural tropical environments. Bones and joints may be affected and cases of osteomyelitis have been reported with increasing frequency. Although most ulcers eventually heal, poorly managed patients eventually present with scars, oedema, and local deformities including disabling contractures. *M. ulcerans* secretes a polyketide macrolide toxin, mycolactone, a virulence factor which has both necrotising and immunosuppressive properties. Other toxins and virulence determinants such as phospholipase C may also play a role. BU is associated with severe illness and permanent disabilities in >25% of patients. Currently, the only treatment for lesion of *M. ulcerans* infection is wide surgical resection. Unfortunately, many patients do not present until there is extensive and disfiguring ulceration.

BU is currently endemic in West Africa. It is also reported from the Americas, Asia, Australia and Papua New Guinea. Environmental changes that promote flooding, such as deforestation, dam construction and irrigation systems, often are associated with outbreaks of BU. The global burden of BU is, however, unknown. A report from Ghana estimated a national prevalence of 20.7/100 000 in 1999.

In some West African communities, BU was reported to have replaced TB and leprosy as the most prevalent mycobacterial disease, affecting up to 22% of the population.

The rising incidence of the disease, its predilection for poor rural communities, the cost of complex surgical treatment, the lost productivity during illness, and the reduced fitness after recovery combine to make BU a major economic burden in West Africa.

3.3.2. Vaccines

Two large randomized controlled trials of BCG vaccination for the prevention of BU were conducted in Uganda during the late 1960s. The overall protective efficacy of BCG against *M. ulcerans* infection was 47%, but the effect was short lived, ranging from six months in one study to one year in the other. BCG was shown to be protective in a mouse model of *M. ulcerans* using a small challenge inoculum, but protection was overcome when using larger challenge doses.

Taken together, these results strongly suggest that a new vaccine is needed for the prevention of BU. To our knowledge, no private investment is devoted to the research on BU vaccines at present, and most BU research activities are coordinated by the Global Buruli Ulcer Initiative.

3.4. *Staphylococcus aureus*

3.4.1. Disease burden

Staphylococcus aureus is responsible for many serious community- and nosocomially-acquired infections, being the most frequently isolated bacterial pathogen from patients with hospital-acquired infections, especially immunocompromised patients with implants or prostheses. Asymptomatic *S. aureus* colonization occurs intermittently in children and adults, most commonly in the anterior nasal vestibule, but occasionally on the skin, hair, nails, axillae, perineum, and vagina. Invasive infections of the skin occur in previously healthy individuals, ranging from impetigo to abscess formation, cellulitis and lymphadenitis. Ocular infections include conjunctivitis and endophtalmitis. *S. aureus* is a frequent cause of endocarditis with possible complications of pericarditis, respiratory tract infections, osteomyelitis and septic arthritis. Most often, *S. aureus* infections are associated with medical insertion of foreign metal, plastic or Gore-Tex devices such as those used for haemodialysis, venous catheterization, or artificial prostheses. *S. aureus* also is the cause of a number of toxinoses, including toxic shock syndrome (TSS), food poisoning, scalded skin syndrome and necrotizing pneumonia. Before the introduction of antimicrobials in the 1940s, the mortality rate of *S. aureus* invasive infection was about 90%. The initial success of antibiotherapy was rapidly countered by the successive emergence of penicillin-resistant, then methicillin-resistant *S. aureus* (MRSA) strains and, since 2002, by that of vancomycin-resistant strains. The development of antibiotic resistance in *S. aureus* is a strong incentive that spurs vaccine development.

The virulence of *S. aureus* is due to a combination of virulence factors that include several exotoxins – such as a) α-hemolysin, which lyses erythrocytes, necrotizes skin, and causes the release of cytokines that may produce shock; b) toxins A and B, which cause the sloughing of skin that characterizes the scalded skin syndrome; and c) the toxic shock syndrome toxin-1 (TSST-1) which is responsible for most TSS cases, especially those associated with menses – and enterotoxins that cause

vomiting and diarrhoeas when ingested and are responsible for food poisoning. Enterotoxins and TSST-1 have superantigen activity which results in a massive release of cytokines that is responsible for the clinical picture of TSS. Case fatality rates in some S. *aureus* infections today still can reach 30%.

3.4.2. Vaccines

Substantial controversy exists as to whether *S. aureus* infections may be prevented by a vaccination approach and, if so, which patients should be targeted for vaccination. A killed whole-cell vaccine, which displayed no protection per se in a rabbit endocarditis model, was combined with an α-hemolysin toxoid and tested in patients undergoing continuous ambulatory peritoneal dialysis; no protection could be observed against peritonitis, catheter-associated infection or asymptomatic carriage.

Attention therefore shifted to the *S. aureus* capsular PS. Eight types of PS have been identified, with serotypes 5 and 8 representing 85–95% of infections. Types 5 and 8 capsular PS were purified and conjugated to a *Pseudomonas aeruginosa* detoxified exotoxin A carrier. The resulting vaccine (StaphVAX, Nabi Biopharmaceutical) provided 75% protection in mice against *S. aureus* challenge and transfer of the mouse IgG provided 75% passive protection. The vaccine was then tested on human volunteers. In a Phase III clinical trial on 1850 end-stage renal disease (ESRD) patients in haemodialysis, a 60% efficacy was observed for up to 10 months following vaccination but the figure dropped to 26% at 1 year, perhaps due to the decline in antibody levels. A second Phase III trial was started on 3600 ESRD patients on haemodialysis who were vaccinated twice, eight months apart, with results expected by the end of 2005.

Anti-*S. aureus* IgGs (Altastaph) derived from donors vaccinated with StaphVAX are being evaluated at the same time in a Phase II trial in neonates.

3.5. Group A *Streptococcus*

3.5.1. Disease burden

Group A streptococci (hemolytic *Streptococcus pyogenes*) cause a broad spectrum of diseases, ranging from simple and uncomplicated pharyngitis and skin infection to life-threatening invasive illness that includes pneumonia, bacteraemia, necrotizing fasciitis, streptococcal toxic shock syndrome (TSS), and nonsuppurative sequelae such as acute rheumatic fever and glomerulonephritis. Streptococcal pharyngitis continues to be one of the most common childhood illnesses throughout the world. The incidence of rheumatic fever has declined in industrialized countries since the 1950s and now has an annual incidence of around 0.5 cases per 100 000 children of school age. In contrast, it remains an endemic disease in developing countries, with annual incidence rates ranging from 100 to 200 cases per 100 000 school-aged children. It also is a major cause of cardiovascular mortality. Australia's aboriginal population suffers the highest incidence worldwide. Group A streptococci, *S. pneumoniae* and *Staphylococcus aureus* are important causes of severe infection in young children in the Papua New Guinea highlands. It has recently been estimated that there currently are more than 18 million cases of severe group A streptococcal disease such as rheumatic heart disease in the world, with more than 500 000 deaths each year. Prospective, longitudinal studies are clearly needed to better understand the epidemiology of streptococcal infections in developing countries and implement more effective public health prevention programmes.

3.5.2. Vaccines

Immunity to group A streptococci is mediated by antibodies against the M protein, a coiled-coil α-helical bacterial surface protein. Vaccine development faces substantial obstacles. Firstly, opsonizing antibodies directed against the M protein are serotype-specific and there are more than 100 identified serotypes. Secondly, immunological cross-reactivity has been demonstrated between epitopes in the M protein and several human tissues, including heart, kidney and cartilage. Although the pathogenesis of rheumatic fever is not yet understood, increasing evidence indicates the existence of an auto-immune process. And, thirdly, because humans are the only hosts for group A streptococci, no relevant animal model is available.

To develop a suitable vaccine candidate, non-cross-reactive, serotype-specific epitopes were selected and linked to a conserved epitope in the C-terminal half of the protein, J14, which is shared by about 70% of isolates. Such prototype vaccine constructs have demonstrated excellent immunogenicity and protection in mice and tolerance in human volunteers, including a hexavalent, a heptavalent and a 24-valent M protein vaccines. ID Biomedical Corp is currently evaluating a 26-valent peptide vaccine in clinical trials in Canada.

In parallel, attempts at developing vaccines based on other bacterial proteins, such as the SCPA peptidase, or the fibronectin-binding protein I, or on toxoids made from the *S. pyogenes* exotoxins SPE A and SPE C that are involved in TSS and scarlet fever, are now in progress.

Efficacy trials of group A streptococcal vaccines are expected to be difficult and long, and will require large sample sizes, especially if efficacy endpoints are clinical endpoints. WHO is currently involved in a process to develop standard protocols for the clinical evaluation of group A streptococcal vaccines.

3.6. Group B *Streptococcus*

3.6.1. Disease burden

Group B streptococci are one of the most important infectious causes of neonatal morbidity and mortality. Women vaginally or rectally colonized with Group B streptococci during pregnancy are at increased risk of transmitting the bacteria to their newborn infant during labour and delivery. Pregnancy-associated streptococcal infection can result in maternal sepsis. It also is the leading cause of chorioamniotitis and one of several infections now thought to enhance the risk of preterm rupture of membranes. In the newborn, early onset of the disease is recognized as pneumonia and bacteraemia within the first seven days of life, whereas late onset disease primarily occurs in the form of meningitis between 7 and 90 days of age. Vaginal group B streptococcal colonization has been reported to occur in about 12–27% of women in North Africa, India, the Middle East, Pakistan, Saudi Arabia and the USA. Surprisingly, a WHO collaborative study on serious infections in young infants conducted in four developing countries showed that Group B streptococci were found in only 2 of 167 blood culture isolates and 1 of 40 CSF isolates, whereas Group A streptococci were recovered from 29/167 blood isolates and 3/40 CSF isolates. These data may have been biased by the fact that infants who develop streptococcal sepsis on the day of birth will usually not survive. It might

also be that Group B streptococcal-related morbidity in developing counties often manifests itself through miscarriage or preterm delivery, in which case infants may not survive to develop confirmed sepsis.

Invasive group B streptococcus disease has also been frequently reported in adults with diabetes, neurological impairment, breast cancer and cirrhosis. Its manifestations include soft tissue infections, bone and joint infections and pneumonia or, more rarely, endocarditis and meningitis. Adults over 65 years of age are at the highest risk of dying from invasive group B disease.

3.6.2. Vaccines

Early onset of Group B disease in neonates can be prevented by the use of intrapartum chemoprophylaxis, as was done in South Africa. Active immunization of mothers during the third trimester of pregnancy to elicit an antibody response and passively protect the newborns represents an attractive alternative strategy. Streptococcal capsular polysaccharides (PS) have been found to elicit serotype-specific protective immunity, but showed low immunogenicity if not conjugated to a protein carrier. Conjugated PS vaccines have been developed using as a protein carrier either the tetanus toxoid (TT) or a recombinant cholera toxin B subunit (CTB) administered intranasally to increase the mucosal antibody response, or group B streptococcal surface antigens such as the C5a peptidase or the C protein. Most of the resulting formulations have been tested in mice and a few in non-pregnant women. A bivalent Ia and Ib PS-TT conjugate vaccine was well-tolerated in women and elicited a dose-dependent antibody response that correlated with in vitro opsonophagocytosis. A type III PS-TT conjugate vaccine administered to third-trimester pregnant women was well tolerated and induced PS-specific antibodies that were efficiently transported to the infant and could be detected through two months of life. Microscience (USA) and Intercell (UK) are developing vaccines based on novel surface protein candidates.

A major difficulty in developing Group B streptococcal vaccines is the existence of a multiplicity of serotypes with different geographical distributions. A vaccine suitable for Asian or European populations may not be suitable for African populations. Another difficulty, similar to that encountered with Group A streptococcal vaccines, is the implementation of efficacy trials. A Phase III evaluation of candidate vaccines in women before pregnancy will require large sample sizes and take a long time. Administration of the vaccine to pregnant women may be difficult because of fear of risks of birth defects and subsequent liability.

4. Parasitic diseases

Parasitic diseases caused by helminths and protozoa are major causes of human disease and misery in most countries of the tropics. They plague billions of people and kill millions annually, and inflict debilitating injuries such as blindness and disfiguration on additional millions. WHO estimates that one person in every four harbors parasitic worms. Attempts to develop vaccines against these pathogens have been hampered by the difficulty to cultivate them in vitro, the complexity of their multicellular organization and/or multistage development, added to their impressive antigenic variability. Although remarkable progress has been made in the last decade in the cloning and expression of protective antigens from a large number of parasites, the prospect of using these antigens for the development of preventive vaccines has been met with little enthusiasm from industrial vaccine manufacturers, due to general scepticism as to the capacity for defined antigens to elicit sterilizing immunity against complex organisms, especially metazoan organisms. Definite scientific and technical advances have nevertheless been made in recent years in the field, including the complete sequencing of the genome of *Plasmodium falciparum*, and quite a number of groups are now supporting research on vaccine development against parasitic diseases. Significant progress has been made over the past five years in the development of vaccines against malaria and leishmaniasis. Vaccine development efforts for Chagas' disease (American trypanosomiasis) have been curtailed because of successful efforts at vector control, whereas vaccine development for African sleeping sickness (African trypanosiomiasis) still is hampered by the phenomenon of antigenic variability.

4.1. Amoebiasis

Amoebiasis is due to invasion of the intestinal wall by the protozoan parasite *Entemoeba histolytica*. Amoebic colitis results from ulcerating mucosal lesions caused by the release of parasite-derived hyaluronidases and proteases. Hepatic infection occurs as a consequence of entry of the parasite into the afferent bloodstream. The disease is prevalent throughout the developing nations of the tropics, at times reaching a prevalence of 50% of the general population and is estimated to cause more than 100 000 deaths per year.

Evidence from a cohort of Bangladeshi children suggests that mucosal IgA directed against the major amoebic adherence molecule, a 170 kD lectin, correlates with resistance to reinfection with *E. histolytica.* Gerbils immunized with this lectin antigen were reported to show significant decrease of liver abscesses following parasite challenge, suggesting that a subunit vaccine might elicit protective immunity.

4.2. Hookworm disease

4.2.1. Disease burden

Human hookworm infection is a soil-transmitted helminth infection caused by the nematode parasites *Necator americanus* and *Ancylostoma duodenale*. It is a leading cause of anaemia and protein malnutrition, afflicting an estimated 740 million people in the developing nations of the tropics. The largest numbers of cases occur in impoverished rural areas of sub-Saharan Africa, Latin America, South-East Asia and China. *N. americanus* is the most common hookworm worldwide, while *A. duodenale* is more geographically restricted.

Hookworm transmission occurs by skin contact with infective third-stage larvae (L3) that have the ability to penetrate through the skin, frequently entering the body through the hands, feet, arms, or legs. *A. duodenale* L3 also can be ingested. L3s migrate through the body and enter the lungs from which they are expelled by cough and swallowed into the intestine where they first moult twice to become adults. Adult hookworms are approximately one-centimeter-long parasites that cause host injury by attaching to the mucosa and submucosa of the small intestine and producing intestinal blood loss. The presence of between 40 and 160 adult hookworms in the human intestine results in blood loss sufficient to cause anaemia and malnutrition. The term "hookworm disease" refers primarily to the iron-deficiency anaemia with reduced host haemoglobin, serum ferritin, and protoporphyrin that results from moderate and heavy infections and is in direct correlation with the number of parasites (as measured by quantitative egg counts). In children, chronic hookworm infection has been shown to impair physical and intellectual development, reduce school performance and attendance, and adversely affect future productivity and wage-earning potential.

Unlike other soil-transmitted helminth infections, such as ascariasis and trichuriasis, in which the highest intensity infections occur primarily in school-aged children, high intensity hookworm infections also frequently occur in adult populations. This is an important health threat to adolescent girls, women of reproductive age, and to outcomes in pregnancy. Up to 44 million pregnant women are estimated to be infected with hookworm. In pregnant women, anaemia resulting from hookworm disease results in several adverse outcomes for both the mother and her infant, including low birth weight, impaired milk production, and increased risk of death for both the mother and the child.

Efforts to control hookworm infection include the sanitary disposal of faeces and educational campaigns about the proper use of latrines. At this time, the most cost-effective way to control hookworm infection has been through population-wide treatment with either albendazole or mebendazole. A resolution adopted at the 2001 World Health Assembly advocates the anthelminthic treatment of 75% of all at-risk school-aged children by 2010. In time this would become the largest health programme ever attempted. However, both children and adults usually become reinfected within a few months after deparasitation, which implies repeated and frequent use of the drugs, and there is concern that heavy and exclusive reliance on albendazole and mebendazole might lead to drug resistance. Therefore, a safe and cost-effective vaccine would provide an important new tool for the control of hookworm infection.

4.2.2. Vaccines

The feasibility of developing a human anti-hookworm vaccine is based on the previous success of using live, irradiated L3s as a vaccine for canine hookworm infection. The Human Hookworm Vaccine Initiative (HHVI), a programme of the Sabin Vaccine Institute, together with the George Washington University (USA), the Oswaldo Cruz Foundation (FIOCRUZ, Brazil), the Chinese Institute of Parasitic Diseases, the Queensland Institute of Medical Research (Australia), and the London School of Hygiene and Tropical Medicine (UK), has identified, isolated, cloned, and expressed the major L3 antigens, and then tested them as recombinant vaccines. The leading candidate, the *Ancylostoma*-secreted protein (ASP), was selected because it can be recognized in a subset of individuals who have low intensity hookworm infection, and is partially protective in laboratory hamsters and dogs against challenge with *A. ceylanicum* and *A. caninum*, respectively. With support from the Bill and Melinda Gates Foundation, as well as additional support from the NIAID, NIH, and the March of Dimes Birth Defects Foundation, the HHVI has completed manufacture of the *Na*-ASP-2 hookworm vaccine. A Phase I dose-escalating trial of the vaccine is tentatively planned to take place in the USA in early 2005. Further planning is in progress for a Phase IIb trial to determine the vaccine's ability to protect against high intensity hookworm infection in Brazil. It is anticipated that industrial-scale manufacture of the vaccine will take place in Brazil.

Additional studies are in progress to develop a second antigen from adult hookworms. Candidates of choice are the haemoglobin-degrading proteases found to line the brush border membrane of the hookworm gastrointestinal tract. These have been expressed in eukaryotic expression systems such as yeasts or baculovirus, to keep their native conformation intact for better immunogenicity. Work is in progress to combine them with ASP in a multivalent vaccine.

4.3. Leishmaniasis

4.3.1. Disease burden

Leishmaniasis is caused by several species of flagellated protozoan parasites found in many areas of the world, particularly in Africa, Latin America, South and Central Asia, the Mediterranean basin and the Middle East. In its more severe forms, the disease can cause serious disfigurement as well as death. WHO estimates the worldwide prevalence to be approximately 12 million cases, with annual mortality of about 60 000. The size of the population at risk is about 350 million. Transmission is most often zoonotic: the parasites (*Leishmania*) are transmitted from a wild-animal reservoir (small rodents, dogs) by the bite of the female phlebotomine sandfly. It also can be anthroponotic, the parasite being transmitted by the sanfly from an infected human host. Several forms of the disease exist: cutaneous (CL), mucocutaneous (MCL) and visceral (VL, also called "kala-azar"), which, after treatment, is often followed by a dermal manifestation known as "post-kala-azar" dermal leishmaniasis (PKDL). CL and MCL in Central and South America are caused by members of the *L. mexicana* and *L. braziliensis* species, whereas CL in South and Central Asia and the Middle East is caused by *L. tropica* and *L. major*: The majority of MCL cases occur in Bolivia, Brazil and Peru, and 90% of CL cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria. VL ("kala-azar"), the most lethal form of the disease, is caused by *L. donovani* in Bangladesh, Chinnda, Iia, Nepal and Sudan by *L. infantum* in North Africa and southern Europe, and by *L. chagasi* in Latin America.

For many years, the public health impact of leishmaniasis has been grossly underestimated, as a substantial number of cases were never recorded. About 1.5–2 million new cases are estimated to occur annually, but only 600 000 are officially declared. In addition, deadly epidemics of VL periodically flare up but go mostly unnoticed in spite of case–fatality rates as high as 10% or more. In the 1990s Sudan suffered a crisis with an excess mortality of 100 000 deaths among people at risk. The expansion of leishmaniases and the alarming rise in the number of cases is related to environmental changes such as deforestation, building of dams, new irrigation schemes and migration of non-immune people to endemic areas, and resulted in significant delay in the implementation of development programmes in the Amazons and the tropical regions of the Andean countries, Morocco and Saudi Arabia.

More recently, as a result of epidemiological changes, a sharp increase in the overlapping of HIV infection and visceral leishmaniasis has been observed, especially in intravenous drug users in South-Western Europe. The situation may soon worsen in Africa and Asia where the prevalence and detection of HIV and *Leishmania* co-infections still are probably largely underestimated.

The first line drugs for treatment of leishmaniasis are antimonials, which remain expensive, require repeated injections, and are associated with important side effects. Drug resistance also is becoming common in certain areas (i.e. Bihar, India). Miltefosin, a recently developed drug which is active orally against VL, has not yet been widely used. Vector and reservoir controls may be useful under certain conditions but are not applicable in every epidemiological setting and require infrastructure and vigilance beyond the capability of many endemic countries. Vaccination, therefore, remains the best hope for control of all forms of the disease.

4.3.2. Vaccines

There is as yet no effective vaccine for prevention of any form of leishmaniasis. A first generation vaccine was prepared using whole killed parasites combined or not with BCG. The combination of autoclaved *L. major* promastigotes with BCG as adjuvant was tested in Iran against CL and in Sudan against VL. A limited efficacy was noted in converters to positive skin reaction to leishmania antigen (leishmanin) and unexpectedly in boys. Similar observations had been made earlier in Brazil using killed promastigotes without BCG. Alum precipitated autoclaved *L. major* promastigotes plus BCG have demonstrated safety and substantial immunogenicity in Phase I studies in Sudan. Additional trials are under way to test new formulations with IL-12. It is of note that treatments combining administration of antimonials and first generation leishmania vaccines in patients suffering from "post-kala-azar" dermal leishmaniasis (PKDL) have shown benefit to the patients, suggesting that even suboptimal leishmaniasis vaccines could have a role in the therapeutic setting.

Various subunit recombinant candidate vaccines also have been tested in mice and provided some degree of protection against infection. These vaccines were based on:

- recombinant surface antigen gp63, a glycoprotein with protease activity;
- lipophosphoglycan, a surface glycoconjugate;
- a 46 kD promastigote antigen derived from *L. amazonensis*;
- or the *Leishmania*-activated C kinase (LACK), among others.

Protection against *L. major* infection in mice was provided by DNA constructs encoding a number of *Leishmania* antigens, including gp63 and LACK.

It has been demonstrated in experimental animal models that a dominant Th1 lymphocyte response (IL-2, IFN-γ) is associated with self-limited disease, whereas a dominant Th2 response (IL-4, IL-5) is linked to progressive disease. Addition of Th1-driving adjuvants such as IL-12 or oligodeoxynucelotides (CpG) to leishmanial antigens (TSA, LeIF, LmSTI-1) resulted in complete protection of susceptible mice against progressive disease, whereas no protection was observed in the absence of adjuvant. The Bill and Melinda Gates Foundation has funded the development of a chimeric vaccine made of these three recombinant leishmanial antigens (LeIF, LmSTI-1 and TSA) in the form of a fusion protein combined with monophosphoryl lipid A in squalene oil as adjuvant. Phase I trials of this vaccine in healthy volunteers in the USA and initial efficacy testing as a therapeutic vaccine in patients in Latin America suggest the safety and immunogenicity of the vaccine.

Recent evidence indicates that a 15 kD protein antigen derived from the salivary glands of the sandfly vector also could be protective in mice when given as a vaccine.

Generally, recovery from CL leads to protection against future infections. For centuries, in some of the hyper-endemic areas of the Middle East, the pus of an active lesion was used to inoculate young children to protect them against future lesions on the exposed parts of the body, especially the face. *L. major* promastigotes grown in culture under good manufacturing practice (GMP) guidelines, rather than the exsudates from active lesions, have been used for inoculation as a live vaccine. The practice is known as leishmanization. Genetically manipulated parasites with attenuated virulence or high sensitivity to chemotherapy might represent the ideal form of a live vaccine.

4.4. Malaria

4.4.1. Disease burden

Malaria is by far the world's most important tropical parasitic disease, killing more people than any other communicable disease except AIDS and tuberculosis. Worldwide prevalence of the disease is in the order of 350–500 million clinical cases each year, with an estimated annual death toll of over 1.1 million deaths. The vast majority of deaths occur among children under five years of age, especially in remote rural areas with poor access to health services. One century ago, malaria was endemic across every continent except Antarctica. Control programmes based on the use of insecticides led to its elimination from Australia, Europe and the USA by the 1950s, but the disease still remains endemic in some 100 countries in Africa, the Americas, the Eastern Mediterranean Region, the South-East Asia Region, and the Western Pacific Region. These countries are inhabited by more than 2.4 billion people – 40% of the world's population.

Human malaria is caused by four species of the protozoan parasite *Plasmodium*. The disease thrives where the environment supports one of the 50 species of *Anopheles* mosquitoes that serve as the vector for transmission. Transmission of malaria is affected by climate and geography, and often coincides with the rainy season. Global warming and other climatic events such as "El Niño" also play their

role in increasing the risk of disease, presumably because the associated weather disturbances influence vector-breeding sites. Quantitative leaps in malaria incidence coincident with ENSO (El Niño/southern Oscillation) events have been recorded in Africa, South America and Asia (in Pakistan and Sri Lanka). The disease has now spread to highland areas of Africa. A change in risk of malaria also can be the unintended result of economic activity or agricultural policy that changes the use of land, such as the creation of dams, new irrigation schemes, commercial tree cropping and deforestation. Urban malaria is increasing due to unplanned development around large cities, particularly in Africa and South Asia.

Symptoms associated with malaria include high fever, malaise, headache, myalgia, nausea and vomiting. Bouts recur every 48–72 hours. Severe disease cases occur more frequently with *P. falciparum* because of its ability to adhere to capillary walls. Acute renal failure, cerebral malaria and pulmonary oedema occur most commonly in populations that are immunonaïve, such as young children and travellers. Fatally afflicted children often die less than 72 hours after developing symptoms. In those children who survive, malaria also drains vital strength, impairing their physical and intellectual development. Malarial sickness is one of the principal reasons for poor school attendance. Other high-risk groups are women during pregnancy, non-immune travellers, refugees, displaced persons and labourers entering endemic areas. Malaria causes severe anaemia, a major factor contributing to maternal deaths in pregnant women. Pregnant mothers who have malaria and are HIV-positive also are more likely to transmit HIV to their newborn.

Malaria therefore exacts an enormous toll in lives, in medical costs, and in days of labour lost. For the individual, costs include the price of treatment and prevention, and lost income. In rural areas, the rainy season is often a time of intense agricultural activity, when poor families earn most of their annual income. Malaria can make these families even poorer, hitting young adults especially hard: a single bout of the disease costs an estimated equivalent of 10 working days. The estimated costs of malaria, in terms of strains on the health systems, are enormous: in endemic countries, as many as 3 out of 10 hospital beds are occupied by victims of the disease. The direct and indirect cost of malaria in sub-Saharan Africa is estimated to be 1–5% of the gross domestic product (GDP).

The battle to control malaria is being fought by efforts to implement improved diagnosis and chemotherapy, as well as integrated vector control through the use of insecticide-treated bednets and residual house spraying. International efforts to combat malaria are unprecedented, including among others a Global Malaria Control Strategy coordinated by the WHO, involving three UN agencies (UNDP, UNICEF and WHO) and, together with the World Bank and the Multilateral Initiative on Malaria (MIM), regroups a number of institutions wishing to promote malaria research in Africa. The UNDP/World Bank/WHO Special Programme on Tropical Diseases (WHO/TDR) has joined the initiative, establishing a task force to address the needs of endemic countries and to fund activities related to strengthening research capacities on malaria. However, emergence as well as resurgence of malaria continues to be evident worldwide, much of it due to drug-resistant parasites and insecticide-resistant vectors. Therefore, the development of a safe, effective and affordable malaria vaccine is a critical global public-health priority.

4.4.2. Parasitology

The agents of human malaria are four species of *Plasmodium* protozoa: *P. falciparum, P. vivax, P. ovale* and *P. malariae*. All are transmitted by *Anopheles* mosquitoes. *P. falciparum* causes the greatest number of deaths, whereas *P. vivax* has the greatest geographic distribution. All strains of *Plasmodium* have a complex life cycle that begins when a female mosquito injects sporozoites into the human host when taking a blood meal. The sporozoites enter the bloodstream and within less than 30 minutes migrate to the liver and invade hepatocytes. Sporozoites mature in the liver where they give rise to tens of thousands of merozoites over a period of 6–16 days. Merozoites erupt into the bloodstream and invade erythrocytes where they multiply and mature over a period of 24–72 hours. Infected red blood cells (RBC) then lyse and liberate the merozoites which immediately proceed to invade new RBCs to repeat the cycle. The classical signs of malaria, acute febrile episodes and rigors that occur every 48 to 72 hours, coincide with the synchronized lysis of infected RBCs releasing the newly matured merozoites. Some of the merozoites eventually develop into sexual-stage gametocytes, which can sexually combine and develop into new sporozoites when taken up by an anopheline mosquito, thus reinitiating the cycle.

The complete nucleotide sequences of both the *Plasmodium* parasite and of the *Anopheles gambiae* vector have been determined.

4.4.3. Vaccines

The major malaria vaccine funding agencies are the NIH in the USA, the Wellcome Trust in the UK, the European Union – either directly through the European and Developing Countries Clinical Trials Partnership (EDCTP) or through the European Malaria Vaccine Initiative (EMVI) – USAID, the Malaria Vaccine Initiative (MVI) of PATH and the Bill and Melinda Gates Foundation. In addition, the African Malaria Network Trust (AMANET) was recently established to build African capacity to plan and coordinate malaria vaccine trials in Africa.

Several lines of evidence suggest that a prophylactic malaria vaccine for humans is feasible. Firstly, immunization with irradiated sporozoites was shown to confer 90% protection against experimental infection following laboratory-bred, sporozoite-infected mosquito bites in naïve human volunteers. Secondly, naturally acquired immunity progressively builds up during the first two decades of life in people living in malaria-endemic countries. This immunity primarily impacts the severity of clinical disease, and appears to be linked to continuous antigenic stimulation, waning rapidly when exposure ceases. Thirdly, protection has been elicited by passive transfer of hyperimmune immunoglobulins from malaria-immune adults into malaria-naïve human volunteers.

However, key obstacles to the development of a vaccine include the lack of immune correlates of protection, the lack of reliable and predictive animal models, and the developmental and antigenic diversity and variability of the parasite. Much work has been done to determine which protective antigens or epitopes should be used in the construction of recombinant or synthetic malaria vaccines. The *Plasmodium* genome is A-T rich, unlike most of the microbial organisms or animal cells used to express recombinant antigens, and it also shows quite different codon usage. Enhanced expression of recombinant *Plasmodium* antigens therefore requires the use of synthetic genes reconstructed with optimized codons best suited for the expression system used for production.

The traditional approach to develop malaria vaccines has been the targeting of the different stages of parasite development (pre-erythrocytic, asexual and sexual stages).

Pre-erythrocytic vaccine strategies aim to generate an antibody response that will neutralize sporozoites and prevent them from invading the hepatocyte, and/or to elicit a cell-mediated immune response that will inhibit intra-hepatic parasites. This type of vaccine would be ideal for travellers because it would prevent the advent of clinical disease.

Asexual blood-stage (erythrocytic) vaccine strategies aim to elicit antibodies that will inactivate merozoites and/or target malarial antigens expressed on the RBC surface, thus inducing antibody-dependent cellular cytotoxicity and complement lysis; they also are meant to elicit T-cell responses that will inhibit the development of the parasite in RBCs. This type of vaccine would mostly serve as a disease-reduction vaccine in endemic countries by decreasing the exponential multiplication of merozoites.

Vaccines targeting the sexual stage of the parasite do not aim to prevent illness or infection in the vaccinated individual, but to prevent or decrease transmission of the parasite to new hosts.

Other novel approaches being currently taken include the development of combination multicomponent vaccines, a commercial irradiated sporozoite vaccine, and an anti-parasite toxin vaccine. This type of potential anti-disease vaccine would target parasite toxins contributing to the disease, such as the glycosylphosphatidyl inositol (GPI) anchor.

4.4.3.1. Pre-erythrocytic vaccines

These vaccines aim to protect against malaria infection and ideally should provide sterilizing immunity for non-immune individuals.

- The most advanced and well-documented pre-erythrocytic (liver-stage) vaccine candidate is derived from the circumsporozoite protein (CSP) that is found at the surface of the sporozoite and of the infected hepatocyte. This candidate vaccine, RTS,S/AS02, developed by GSK and the WRAIR, comprises the antigenic C-terminus (amino acids 207–395) of the CSP from *P. falciparum* fused to the hepatitis B surface antigen and expressed in the form of VLPs in *Saccharomyces cerevisiae*. Initial Phase I clinical trials of RTS,S formulated with GSK AS02 adjuvant (containing MPL, QS21 and an oil-in-water emulsion) showed protection against malaria challenge in six out of seven volunteers. A dose-range Phase I/II study showed levels of efficacy from 30% (single dose) to 55% (3 doses), with an overall protective efficacy of 41% among 41 vaccinees. However, protective efficacy was not long-lived. Further trials in the Gambia demonstrated a 70% protection efficacy against infection for the first 9 weeks, with efficacy waning rapidly thereafter. Volunteers who received a fourth dose of the vaccine the following year, prior to the onset of the malaria season, again exhibited a 47% protection over a 9-week follow-up period. Recent field testing on 2022 children in Mozambique showed that efficacy of three doses of RTS,S/AS02 at preventing a first malaria attack in 1–4 year-old children was about 30%, with a 37% decrease in blood parasitemia frequency at six months, and a 58% overall decrease of severe

disease incidence (the figure was 77% in children between ages 1 and 2). Other clinical studies are under way, including one aimed at combining RTS,S with the blood-stage antigen MSP-1 *(see below).*

- Another CSP-based candidate vaccine includes a 102-amino acid synthetic peptide representing the C-terminus of the CSP antigen formulated with Montanide ISA 720. The formulation, which is developed by Dictagen Inc., in collaboration with the University of Lausanne (Switzerland), was shown to be safe in human volunteers and to elicit both an antibody and a cellular immune response including secretion of IFN-γ. The vaccine has now undergone further Phase I studies comparing doses and adjuvants, and is currently undergoing Phase IIa clinical trial at the University of Nijmegen (Netherlands).
- An alternative approach to synthetic vaccines was to incorporate several copies of a protective CSP epitope into a multiple antigenic peptide (MAP). As expected, the resulting vaccine was found to elicit an immune response only in the volunteers with cognate HLA haplotypes. In an effort to by pass MHC restriction, the peptide was linked to a "universal" T-cell epitope. This construct was shown to elicit robust immune responses in humans with diverse genetic background. The B-cell and T-cell epitopes from the MAP trials have been incorporated into a recombinant VLP based on hepatitis B core particles. This CSP-HBc particle vaccine, known as ICC-1132, is being developed by Apovia in the USA with the help of the MVI, but results of a Phase II study have been disappointing.
- Other vaccines based on the CSP antigen include live recombinant vaccines that use MVA, Adenovirus, Sindbis virus, or a cold-adapted attenuated influenza virus strain as a vector. Prime-boost combinations of some of these vaccines with the RTS,S/AS02 vaccine are in progress.
- The United States Department of Defense, in collaboration with Vical Inc., has developed candidate DNA vaccines for malaria (the Multi-Stage DNA Operation, MuStDO), including a liver-stage DNA vaccine that encodes the CSP of *P. falciparum*. This DNA vaccine was tested as a "proof-of-concept" in a Phase I study carried out by the United States Navy Malaria Program. The vaccine elicited cell-mediated immune responses but only modest antibody responses. No serious adverse events were recorded, but the vaccine did not elicit protection against experimental challenge.
- A multiple-antigen DNA vaccine, MuStDO-5, has been designed to encode five different liver-stage antigens: CSP, liver stage antigens 1 and 3 (LSA-1 and -3), exported protein 1 (EXP1), and the sporozoite surface protein 2 (SSP2, also known as thrombospondin-related adhesive protein, TRAP). Various studies conducted in endemic areas have linked LSA-1 and LSA-3 with protective immunity. MuStDo-5 is manufactured as a combination of five separate plasmids. The vaccine, administered with GM-CSF DNA as an adjuvant, was safe and well tolerated in mice and rabbits, but showed only weak immunogenicity in primates. In addition, competition between the plasmids was observed, due to immunodominance of one of the antigens.
- The Oxford University Malaria Vaccine Clinical Trials Group conducted studies of a DNA, a fowlpoxvirus (FPV), and an MVA-based vaccine expressing TRAP fused to a polyepitopic construct, demonstrating strong correlation between the induction of IFN-γ-secreting CD4+ and CD8+ T-cell responses and protection against malaria in a mouse model. The DNA and MVA candidate

vaccines were combined in a prime-boost immunization trial in human volunteers in the Gambia. No protection was observed against occurrence of disease, but malaria-related mortality was reduced. A similar recombinant FPV/MVA prime-boost immunization regimen is currently being tested in Kenya.

- Additional antigens that have been targeted for pre-erythrocytic vaccine development include the liver stage antigens 1 and 3 (LSA-1 and -3), the sporozoite and liver stage antigen (SALSA) and the sporozoite threonine and asparagine rich protein (STARP). LSA-3, a highly conserved pre-erythrocytic antigen, has been demonstrated to induce protective immunity against *P. falciparum* sporozoite challenge in chimpanzees and Aotus monkeys. LSA-3 formulated with AS02 and a LSA-3 lipopeptide candidate are about to enter Phase I trials.
- A glutamate-rich protein (GLURP) long synthetic peptide vaccine developed by the Staten Serum Institut in Denmark in collaboration with EMVI is in early clinical studies in European adult volunteers.
- The SPf66 vaccine candidate that had been developed in Colombia was a synthetic multiepitope, multistage peptide vaccine mixed with alum as an adjuvant. The vaccine was tested in several Phase III field trials involving thousands of volunteers, but its reported efficacy was too low to warrant further development, although one may suspect that the vaccine might have fared better with more potent adjuvants.

4.4.3.2. Asexual blood-stage vaccines

These vaccines are aimed to primarily protect against severe malaria disease, and not against infection.

- The most advanced asexual blood stage vaccines are based on the use of merozoite surface protein 1 (MSP-1), which is part of a complex involved in red blood cell invasion, MSP-2, MSP-3, the apical membrane antigen 1 (AMA-1), a type 1 integral membrane protein and the glutamate-rich protein (GLURP). Antibodies to MSP-1 have been shown to block parasite invasion of red blood cells in vitro. AMA-1 is a natural target of protective responses in vivo. Both AMA-1 and MSP-1 have their 3-D conformation stabilized by intramolecular disulphide bonds which are critical for optimal immunogenicity of the molecule. MSP-1 contains two cysteine-rich epidermal growth factor (EGF)-like domains that generate protective antibodies and are conserved across all species of *Plasmodium*.

Current work has concentrated on either the entire MSP-1 molecule, its 42 kD C-terminal moiety, or a further-processed 19 kD fragment. These were expressed either as such or as part of fusion molecules using baculovirus, *E. coli*, or yeast (*Saccharomyces* or *Pichia*). Recombinant MSP-1 42 kD and19 kD fragments have been shown to protect both mice and Aotus monkeys against lethal parasite challenge, but a Phase I trial of the 19 kD fragment carried out at Baylor University (USA) demonstrated that the vaccine was poorly immunogenic and had unacceptable side effects.

- The MSP-1 42 kD fragment formulated in AS02 adjuvant by GSK in collaboration with WRAIR and the MVI was found to be safe and very immunogenic in human volunteers in Kenya, Mali and the USA. The vaccine is presently entering Phase II trial in Kenya.
- Another vaccine studied in collaboration between WRAIR and GSK is based on the AMA-1 protein formulated in AS02. The vaccine was studied in a Phase I study in the USA and should now proceed to Phase I studies in Kenya and Mali.
- A MSP-1/AMA-1 fusion antigen was produced in Shanghaï using *Pichia pastoris* and Montanide ISA 720 adjuvant and showed good immunogenicity in rabbits and non-human primates. WHO sponsored and coordinated the first clinical testing of this vaccine (PfCP 2.9) in collaboration with the Second Military Medical University in Shanghai. A Phase I trial occurred in China and showed the vaccine to be safe and immunogenic. Plans are under way for further clinical development.
- MSP-3 is being developed both as a long synthetic peptide by the Pasteur Institute and EMVI and as a recombinant protein by the Pasteur Institute. The vaccine construct contains B and T-cell epitopes that were selected based on their targeting by cytophilic antibodies that interact with monocytes in the antibody-dependent cellular inhibition (ADCI) assay. AMANET, in collaboration with EMVI, sponsored and coordinated a recently completed Phase I study of the vaccine in Burkina Faso, where the vaccine was shown to be safe. Vaccine-induced antibodies demonstrated ADCI activity in vitro and in vivo in a new mouse model of *P. falciparum* malaria.
- Another long synthetic peptide vaccine, developed by the Staten Serum Institute in Denmark in collaboration with EMVI, is based on the glutamate-rich protein (GLURP). GLURP formulated in alum and Montanide ISA 720 has been tested in a Phase Ia clinical trial and is planned for further clinical development.
- Furthest along the vaccine development pathway of blood-stage malaria vaccine candidates is the "Combination B" vaccine, which results from a collaborative effort by the Papua New Guinea Institute for Medical Research along with the Australian Cooperative Research Center for Vaccine Technology in Queensland, The Walter and Eliza Hall Research Institute and the Swiss Tropical Institute. This vaccine combines MSP-1 and MSP-2 with *P. falciparum* ring-stage infected erythrocyte surface antigen (RESA) in a Montanide adjuvant formulation. Recent Phase I/IIb trials in 120 5–9 year-old children in Papua New Guinea showed a 62% reduction in parasite density in vaccinees. Analysis of the genotype of breakthrough parasites showed a significant increase in the opposite dimorphic form of MSP-2. A new version of the vaccine is being developed using both variants of MSP-2 in order to target both genotypes.
- Additional merozoite surface antigens under development as vaccine candidates include MSP-4, -5, -8 and -9. These molecules contain one or more of the hallmark EGF-like domains present in MSP-1. MSP-5 is of particular interest because it lacks the sequence variation between different isolates of *P. falciparum* from different geographical locations that is typically seen with most of the MSPs.

- The erythrocyte-binding antigen (EBA-175) and its paralog in *P. vivax*, the Duffy binding antigen (DBA), are two other antigens currently developed as recombinant vaccine candidates expressed either in *E. coli* or in *Pichia pastoris* or as a DNA prime-boost vaccine.

Plasmodium species do not carry out N- or O-linked glycosylation. It has been observed that glycosylation occurring in some expression systems may mask the immunogenicity of protective epitopes. Thus, a recombinant version of AMA-1 in which the glycosylation sites had been mutagenized was shown to elicit protective immunity in Aotus monkeys whereas the glycosylated version did not.

Despite encouraging progress, the lack of immune correlates of protection and that of predictive animal models, together with the polymorphism and strain variability of many asexual stage antigens constitute major challenges to the development effort of asexual stage vaccines. In contrast with pre-erythrocytic vaccine candidates, asexual stage vaccine candidates also lack a human artificial challenge model and have to rely on natural challenge in field trials to provide proof-of-concept. Their development is therefore slower and necessitates major commitment, intensive collaboration as well as high-level coordination supported by adequate funding.

4.4.3.3. Transmission-blocking vaccines

These vaccines are aimed to induce antibodies against the sexual stage antigens in order to prevent the development of infectious sporozoites in the salivary glands of the *Anopheles* mosquitoes. The leading candidate vaccines contain the *P. falciparum* surface protein antigens Pfs25 and Pfs28 or their *P. vivax* homologues Pvs25 and Pvs28. These vaccines are currently being developed at the NIH as recombinant yeast-secreted proteins (*S. cerevisiae*). Initial human Phase I trials have been conducted for Pfs25 and should follow soon for Pvs25. Other sexual stage-specific antigens that are being developed as transmission-blocking vaccines are Pfs48/45 and Pfs230.

4.4.3.4. Other approaches

Various groups such as the US CDC, NMRC and WRAIR are developing multi-antigens, multi-stage vaccine concepts. Advantages of a combination vaccine include the potential to address the problem of antigenic variation, that of inducing immunity in genetically heterogeneous populations and that of possibible immune escape of the parasite. However, the risk of interference between components of the vaccine and increased reactogenicity of the formulation must be taken into account.

The concept of attenuation and parasite challenge to elicit immunity also is explored. An attempt to develop a commercial attenuated sporozoite vaccine has been undertaken by Sanaria Inc., with the support from the Bill and Melinda Gates Foundation and the NIH.

Finally, the glycosylphosphatidylinositol (GPI) anchor, which tethers several of the *Plasmodium* antigens to the membrane, has been shown to be highly toxic in mouse models. An anti-toxic vaccine is currently being developed as a carbohydrate anti-GPI vaccine. A proof-of-principle study testing synthetic GPI as a vaccine in rodent models of malaria showed that the candidate vaccine was immunogenic and protected the animals from significant malaria pathology and mortality. Whether further development of malaria toxin neutralization as a vaccine strategy will continue is not however certain.

4.5. Schistosomiasis

4.5.1. Disease burden

Schistosomiasis, also known as bilharziasis, is second only to malaria in public health importance. It is estimated that 200 million people worldwide are infected with the snail-transmitted, water-borne parasitic helminth, and that 20 000 deaths are associated with the severe consequences of infection, including bladder cancer or renal failure (*Schistosoma haematobium*) and liver fibrosis and portal hypertension (*S. mansoni*). In sub-Saharan Africa where schistosomiasis constitutes an important public health problem, a survey in 2000 of disease-specific mortality reported that 70 million individuals out of 682 million had experienced haematuria and 32 million dysuria associated with *S. haematobium* infection. It was estimated that 18 million suffered bladder wall pathology and 10 million hydronephrosis. Infection with *S. mansoni* was estimated to cause diarrhoea in 0.78 million individuals, blood in stool in 4.4 million and hepatomegaly in 8.5 million. Using the very limited data available, mortality rates due to non-functioning kidney (from *S. haematobium*) and haematemesis (from *S. mansoni*) have been estimated at 150 000 and 130 000 per year, respectively. Although these are global estimates of the schistosomiasis disease burden, the public health impact of schistosomiasis in the field has been poorly evaluated and is still subject to controversy. Apart from a few situations where schistosomiasis is or was recognized as an obvious public health problem, as in Brazil, China, Egypt, the Philippines, northern Senegal and Uganda, the disease is often not a priority for health authorities. Moreover, the lack of a simple clinical case definition does not enable rapid identification of the disease by health personnel.

High rates of schistosomiasis occur near bodies of fresh water. Environmental changes linked to water resource development, population movements and population growth have led to the spread of the disease to previously low or non-endemic areas, particularly in sub-Saharan Africa. The building of the Diama dam on the Senegal River for example introduced intestinal schistosomiasis into both Mauritania and Senegal. Refugee movements and population displacements in the Horn of Africa introduced intestinal schistosomiasis to Somalia and to Djibouti. In contrast, successful schistosomiasis control has been achieved in several countries in Asia, the Americas, North Africa and the Middle East. Schistosomiasis has been eradicated from Japan and some of the islands in the Lesser Antilles. Four national control programmes (Brazil, China, Egypt, and the Philippines) have demonstrated that concerted control efforts together with economic development can decrease morbidity to low levels. Chemotherapy was central to these successes. The current drug of choice, praziquantel, reverses pathology in as little as six months after treatment in *S. haematobium* infections. The cost of praziquantel has decreased significantly over the past 20 years. Nevertheless, large scale use of praziquantel can impose a heavy burden on health systems. In addition, concerns remain over the potential threat of the emergence of praziquantel-resistant parasites.

4.5.2. Parasitology

The three major species of schistosomes, *S. mansoni*, *S. haematobium*, and the *S. japonicum* complex (including *S. japonicum* and *S. mekongi*) are distinguished by their snail vectors, location within the host vasculature, and egg morphology. *S. haematobium* is found in the Middle East and Africa, including the islands of Madagascar and Mauritius. Intestinal schistosomiasis due to *S. mansoni*, is now found in the Arabian peninsula, most African countries north of the equator (Egypt, Libya, Sudan, Somalia, Mali, Senegal, Mauritania), as well as in Brazil, some Caribbean islands, Suriname and Venezuela. *S. japonicum* is endemic in China where bovines are the main reservoir, as well as in Indonesia and the Philippines (with dogs and pigs as reservoir). *S. mekongi* is mostly found in Cambodia and Laos, along the Mekong River.

Asexual reproduction of the parasites occurs in fresh-water snails that release in the water large numbers of free-swimming larval schistosomes known as *cercariae.* The cercariae are attracted to the human skin through which they penetrate then lose their tail upon entry to become *schistosomulae* which migrate through the blood stream and the lung of the host until they reach the liver. Schistosomules differentiate in the liver into male and female schistosomes that migrate through the portal vasculature to settle in the mesenteric or bladder venules where they lay eggs. The latter exit from the body in faeces or urine and hatch in fresh water, giving birth to *miracidia* that swim via the action of their cilia until they find a suitable snail host in which they will give rise to thousands of progeny.

Most of the morbidity associated with Schistosomiasis occurs when eggs remain trapped in the intestinal or bladder wall or in the liver, eliciting the formation of granulomas and fibrosis. In the liver, fibrosis leads to portal hypertension and splenomegaly. The most severe forms of the disease are due to *S. japonicum.*

4.5.3. Vaccines

The administration of radiation-attenuated cercariae to laboratory animals provided protection against experimental *S. mansoni* infection by blocking the migration of the parasite out of the lung. IFN-γ and Th1 cellular immune responses appear to play a key role in this process.

Great attention has been paid to the use of antigens from schistosomules, with disappointing protection results so far. Somewhat better results have been obtained with antigens that are shared between schistosomules and schistosomes, such as the 63 kD parasite myosin, the 97 kD paramyosin, the 28 kD triose phosphate isomerase (TPI), a 23 kD integral membrane protein (Sm23), and the 26 and 28 kD glutathione-S-transferases (GSTs). In recent Phase I and II clinical trials, the 28 kD *S. haematobium* GST (Sh28GST) developed by Institut Pasteur de Lille (France), was safe and showed good immunogenicity in human volunteers in France, Niger and Senegal.

The Schistosomiasis Vaccine Development Programme (SVDP), based in Egypt and supported by USAID, has focused on two *S. mansoni* antigens: paramyosin and a synthetic peptide construct containing multiple antigen epitopes (MAP) from the triose phosphate isomerase (Bachem Company, Los Angeles, USA).

Another candidate vaccine, which is developed by FIOCRUZ (Rio de Janeiro, Brazil), is based on the use of Sm14, a 14 kD fatty acid-binding *S. mansoni* protein with cross-reactivity with *Fasciola hepatica*. In mice, Sm14 provided a 67% protection against challenge with *S. mansoni* cercariae and full protection against *F. hepatica* metacercariae.

None of the above candidate vaccines has, however, been able so far to provide more than a partial reduction in challenge-derived worm burdens relative to non-immunized controls. It is hoped that better success can be achieved using cocktails of recombinant antigens.

Another approach to vaccination against schistosomiasis has been to target the fecundity of the female schistosome in order to diminish egg excretion. Success with this approach has been reported in mice and large animal reservoir hosts, including pigs and water buffaloes, using *S. japonicum* 26 kD GST and paramyosin. The suggestion was made, and the hope entertained, that vaccination of the reservoir host might be sufficient to reduce *S. japonicum* transmission to humans.

5. Sexually transmitted diseases

Syphilis, gonorrhoea and chancroid are generally considered as the main sexually transmitted diseases (STDs), but a wide variety of pathogens also are s exually transmissible, including Herpes simplex virus type 2 (HSV-2), *Chlamydia trachomatis*, human papillomaviruses (HPV), human T lymphotropic virus type 1 (HTLV-1), human immunodeficiency virus (HIV), and hepatitis B virus (HBV). Women, particularly adolescents, are disproportionately vulnerable to STDs, many of which remain asymptomatic, favouring spread of infection. The risk of acquiring an STD is highest in urban areas, in low socioeconomic groups, in the young, and in association with illicit drug use and prostitution. In the USA, as an example, rates of gonorrhoea and syphilis are greater for African Americans and Hispanics than in the non-Hispanic white American population, and African American and Hispanic women suffer a greater share of severe complications of these diseases, such as pelvic inflammatory disease (PID) caused by bacterial infections, and cervical cancer caused by HPV infection.

Apart from the HIV epidemic, STDs cause significant morbidity and contribute greatly to increasing health-care costs. Several common STDs adversely affect pregnancy, causing spontaneous abortions, stillbirths, preterm delivery and postpartum endometritis. Neonatal infections include gonococcal conjunctivitis which may lead to blindness, chlamydial pneumonia which may lead to chronic respiratory disease, and herpes encephalitis. Genital infection with HPV is causally associated with cervical cancer, the most common cause of cancer-related death in women throughout the world. For this reason, HPV will be discussed in Chapter 8 *(see 8.3.)*.

A consensus has emerged that the prevention and control of STDs require a global initiative, the success of which will largely depend on the development of safe and effective vaccines. Currently, except for hepatitis B infection, no such vaccines exist.

5.1. *Chlamydia trachomatis*

5.1.1. Disease burden

More cases of STD are caused by *Chlamydia trachomatis* than by any other bacterial pathogen, making *C. trachomatis* infections an enormous public health problem throughout the world. In both men and women, silent, asymptomatic infection is common. The bacterium is transmitted from one partner to another by sexual intercourse. In men, *C. trachomatis* is the commonest cause of non-gonococcal (non-specific) urethritis. Conjunctivitis (that does not progress to blindness) and joint inflammation may occur. Men with asymptomatic infection serve as carriers of the disease, spreading the infection while only rarely suffering long-term health problems. Women, in contrast, are at high risk of severe complications of infection.

Acute infection with *Chlamydia* can result in acute salpingitis and PID, whose long-term consequences include chronic pain, ectopic pregnancy and infertility. Contamination of the hands with genital discharge may lead to a conjunctival infection following contact with the eyes. Babies born to mothers with infection of their genital tract frequently present with chlamydial eye infection within a week of birth (chlamydial "*ophthalmia neonatorum*"), and may subsequently develop pneumonia. Various studies have estimated that there are four to five million new cases of chlamydial infection each year in the USA alone. Among urban adolescent females, the incidence rate can be as high as 30%. The annual costs of treating and caring for patients with PID might be as high as US$10 billion.

Worldwide, the most important disease caused by *C. trachomatis* is trachoma that affects the inner upper eyelid and cornea and is one of the commonest infectious causes of blindness. The disease starts as an inflammatory infection of the eyelid and evolves to trachomatous trichiasis (at least one eyelash rubbing on the eyeball, or loss of interned eyelashes) and blindness due to corneal opacity. In some parts of the developing world, over 90% of the population is infected. Despite long-standing control efforts, it is estimated that more than 500 million people still are at high risk of infection, over 140 million persons are infected and about 6 million are blind in Africa, the Middle East, Central and South-East Asia, and countries in Latin America. The disease is particularly prevalent and severe in rural populations living in poor and arid areas of the world where people have limited access to water and personal hygiene is difficult. Visual loss from trachoma often starts in middle life and is 2–3 times more common in women. It is therefore a major cause of disability in affected communities, attacking the economically important middle-aged female population. Trachoma is a communicable disease of families, with repeated reinfection occurring among family members.

5.1.2. Bacteriology

C. trachomatis is a small obligate intracellular bacterium found in two forms: the elementary body (EB) and the reticulate body (RB). The infectious form is the EB which lies outside cells. After attachment, EBs penetrate into their host cells where they reorganize into metabolically active and replicative RBs that accumulate by division in a large cytoplasmic inclusion. RBs then reorganize into infectious and spore-like EBs, which are released by host cell lysis or extrusion. The genus *Chlamydia* includes three species: *C. trachomatis*, an exclusively human pathogen; *C. psittaci*, which infects a variety of animals and can cause pneumonia or psittacosis in humans; and *C. pneumoniae,* a relatively common cause of LRIs in humans. Based on the type of disease produced, *C. trachomatis* has been divided into biovars, including: the lymphogranuloma (LGV) biovar, associated with *lymphogranuloma venereum*, an inguinal lymphadenopathy; and the trachoma biovar, associated with human conjunctival or urogenital columnar epithelium infections. *C. trachomatis* is further divided into serotypes (or serovars), some of which produce almost exclusively ocular trachoma in endemic countries, whereas others are associated with both ocular trachoma and genital-tract infections. The whole nucleotide sequence of the genome of *C. trachomatis* (serovar D) has been determined. *Chlamydiae* are sensitive to a number of antibiotics including erythromycin and tetracyclins. Chemotherapeutic intervention thus consists of topical (tetracyclin) or systemic (azithromycin) treatment with antibiotics. Other interventions consist of surgery of the eyelid. Global elimination of trachoma as a disease of public health importance has been targeted by WHO for 2020.

5.1.3. Vaccines

A safe vaccine administered prior to adolescence that is effective through childbearing age would have a significant impact on the spread of the disease. The lack of a suitable animal model and the difficulties in genetic manipulation of the bacterium have hampered progress in the field.

Antex Biologics has developed a subunit vaccine candidate (TRACVAX) which has been tested in a randomized Phase I trial designed to assess the safety and immunogenicity of the candidate vaccine.

Identification of potential vaccine antigens is today an active area of research which is greatly helped by the availability of the complete *C. trachomatis* genome sequence, allowing for the identification and testing of candidate proteins based on their similarity to proteins important in protective immunity against other bacterial pathogens.

5.2. Gonorrhoea

5.2.1. Disease burden

Gonorrhoea is caused by *Neisseria gonorrhoeae*. It usually is characterized by purulent genital discharge, urethritis and dysuria. Infection also can be asymptomatic, especially in women. Asymptomatic carriers are more likely to transmit the disease than people with overt infections. Similarly, anorectal and pharyngeal infections, which are not uncommon in women and men who have sex with men, frequently remain asymptomatic but constitute a potential source of transmission. Global estimated incidence of gonorrhoea is 62 million infected people annually. Complications of the disease include epididymitis in men and PID in women, with subsequent risk of infertility and ectopic pregnancy. In about 1% of cases, the gonococcus becomes invasive and a bacteraemia develops, leading to disseminated gonococcal infection characterized by skin rash and asymmetrical septic polyarthritis. The most common manifestation of gonorrhoea in the newborn is purulent conjunctival infection (gonococcal *ophtalmia neonatorum*), which constitutes a medical emergency because blindness may rapidly ensue. The incidence of the disease has been greatly reduced by routine prophylactic administration of 1% silver nitrate eyedrops.

5.2.2. Bacteriology

N. gonorrhoeae is a gram-negative diplococcus. Specific serological reactions serve to distinguish gonococci from other species of *Neisseria* and permit serogrouping of gonococcal strains. The gonococcal liposaccharide (LPS) consists of branched oligosaccharide chains whose antigenic heterogeneity constitutes the basis of interstrain differences. The bacterial envelope is traversed by long pili constituted of repeated peptide subunits (pilin) that are characterized by both antigenic and phase variations. Antigenic variations result from chromosomal rearrangements altering the expression of any one of several silent pilin genes. Phase variation (*pi* + to *pil*-) occurs when the rearrangement involves a defective pilin gene. The predominant protein in the gonococcal outer membrane is termed protein I. This protein, which exists in two allelic forms, PIA and PIB, forms anion-selective transmembrane channels through the outer membrane and thus functions as a porin (POR protein). It is possible to divide gonococci into at least 24 PIA serovars and 32 PIB serovars on the basis of antigenic determinants on protein I. The complete nucleotide sequence of *N. gonorrhoeae* has been determined.

The life cycle of the bacterium was studied using a variety of cell culture systems. These studies have shown that the bacterium not only adheres to the epithelial cells but also penetrates and transits across the epithelial layer and exits into the subepithelial space where the symptoms of the disease are actually elicited.

5.2.3. Vaccines

The lack of a suitable animal model and the considerable antigenic variability of the bacterium have hampered the development of a vaccine for gonorrhoeal disease. Attachment of gonococci to mucosal cells is mediated in part by the pili, and it was found that rabbit antibody to pili reduces attachment of the bacteria to mammalian cells. Pilin was therefore chosen as the most likely vaccine candidate and tested for efficacy in military recruits and in volunteers challenged urethrally. This approach was met with some success, but protection was strain-limited, due the high rates of antigenic variation of pili. Porin also was studied as a vaccine antigen but the induced anti-porin antibodies were not bactericidal.

Identification of potential vaccine antigens will hopefully be helped by the availability of the complete genome sequence, allowing the search of candidate proteins with similarity to proteins important in immunity to other bacterial pathogens.

5.3. Herpes simplex type 2

5.3.1. Disease burden

Herpes simplex virus type 2 (HSV-2) is the cause of genital herpes. The hallmark of herpesvirus infections is the establishment of a lifelong, latent infection that can reactivate to cause one or more rounds of disease. Latent HSV-2 infection occurs primarily in neurons found in the sacral root ganglia. The clinical spectrum of HSV-2 includes primary infection, characterized by the appearance of vesicles on the vulva or the penis that soon break to leave shallow, painful ulcerating lesions. The ulcers heal in 2–3 weeks, although healing may be very slow in immunocompromised patients. Primary infection is then followed by recurrent episodes of clinical disease (4–5 per year). The proportion of symptomatic infections is estimated to be between 13% and 37%, and probably higher in HIV positive individuals. Subclinical infection may be associated with infectious viral shedding. The virus is transmitted in genital secretions. Transmission of HSV-2 to newborns at the time of delivery may lead to devastating systemic infection with encephalitis. The risk of neonatal herpes fortunately is low among HIV-negative pregnant women living in industrialized countries (less than 3%), but few data are available on neonatal herpes in developing countries.

Genital herpes is one of the most common ulcerating diseases of the genital mucosa. It is estimated that in the USA, for example, from 40 to 60 million people are HSV-2-infected, with an incidence of 1–2 million infections and 600 000–800 000 clinical cases per year. Prevalence in the 30–40 year-old population is about 30%. Overall prevalence is higher in women compared with men, especially among the young. The same independent factors of HSV-2 infection were identified in both genders: older age, higher lifetime number of sexual partners, positive HIV serology and positive syphilis serology. Prevalence in developing countries can vary from 2–74% according to the country, age, gender, or urban versus rural areas. Rates up to 40% have been reported among women 15–19 years of age in rural Costa Rica,

Kenya (Kisumu) and Mexico (Mexico-City). A study conducted on truck drivers in Bangladesh showed a high prevalence of HSV-2 (25.8%), compared to syphilis (5.7%), gonorrhoea (2.1%), and chlamydia (0.8%).

There is now ample evidence that HSV-2 infection is a major cofactor of HIV infection. In developed countries, where acquisition of HSV-1 in childhood has decreased, HSV-2 seroprevalence has increased, suggesting a possible protective effect of HSV-1 against HSV-2 acquisition. Although HSV-1 does not actually seem to modify the risk of HSV-2 acquisition, it appears to increase the proportion of asymptomatic seroconverters.

5.3.2. Virology

HSV-2, together with HSV-1 and the varicella-zoster virus (chickenpox), belongs to the subfamily *Alphaherpesvirinae* in the family *Herpesviridae*. These are large, complex enveloped viruses with an outer lipid envelope studded with at least 10 viral glycoproteins, an intermediate tegument layer comprising at least 15 viral proteins, and an icosahedral nucleocapsid containing the double-stranded DNA genome. The genome is organized into a 126-kb long and a 26-kb short region of double-stranded DNA bracketed by inverted repeat sequences that readily allow isomerization or recombination of the two regions. The genome comprises some 84 open reading frames. These have been divided into immediate-early genes, whose transcription depends on a virally-encoded activating protein, VP16, and which encode the viral a proteins; the early genes, which are turned on by the a proteins and whose products (β proteins) are involved in DNA replication; and the late genes, the products of which (γ proteins) are virion structural proteins and proteins needed for virus particle assembly and egress. Some of the viral envelope glycoproteins (gD) are antigenically related to those of HSV-1, whereas most are type-specific (particularly gG1 and gG2). Numerous viral gene products, which are dispensable for virus growth in vitro, can be considered as virulence genes that are involved in preventing apoptosis in the infected host cell, blocking the induction of interferons, or downregulating the presentation of viral antigens in the context of class I histocompatibility antigens (HLAs).

When the latent state is established in the neural ganglia, transcription is severely restricted such that a single transcript is produced from the latency-associated transcript (LAT) promoter, and only a few viral proteins are made. At intervals, changes in neuronal physiology induced by trauma, hormones, stress or immune suppression, render the neurones permissive to virus replication, resulting in full transcription of the genome and a burst of progeny virions.

5.3.3. Vaccines

The prospect for developing a vaccine against HSV-2 that could provide sterilizing immunity is thought to be unrealistic. The goals of the vaccines under development are rather to prevent the establishment of latent infection by blocking access of the virus to sensory ganglia, to reduce the severity of the symptoms, and/or to reduce the frequency of recurrences. The correlates of protective immunity against HSV-2 are not entirely understood. Passive maternal antibody seems important in preventing infection of the newborn and CD4+ Th1 T-cells appear to be crucial to the immune response. IFN-γ secretion and CD8+ CTL may also play a major role, particularly in the prevention of recurrences.

HSV-2 subunit vaccines were developed based on the use of viral envelope glycoproteins.

- A two-component gB2 and gD2 recombinant glycoproteins subunit vaccine formulated in MF59 adjuvant was developed by Chiron. The 2-component vaccine induced high antibody titres and showed 26% efficacy in women for a period of six months but protection did not persist and male volunteers were not protected.
- GSK developed a single component gD2 vaccine formulated in AS04 adjuvant (alum + monophosphoryl lipid A). The gD2 vaccine induced good Th1 immunity in mice, including high IFN-γ secretion, and provided good protection against vaginal HSV-2 challenge in female guinea pigs. The vaccine was tested in two large, double-blind, controlled Phase III trials on volunteers with a partner with genital herpes disease. In the first study, 847 subjects were selected as seronegative for both HSV-1 and HSV-2, whereas in the second study the 2491 selected subjects were selected only on the basis of HSV-2 seronegativity. The vaccine was 73% efficacious against genital herpes ***disease*** in doubly seronegative women. Trends towards protection against ***infection*** were also observed, but the figures were not statistically significant (less than 48% efficacy). Most unexpectedly, however, the vaccine was not effective in women previously seropositive for HSV-1 and in men, regardless of their HSV seropositivity status. This suggests that HSV-1 immunity is protective against HSV-2, but no satisfactory explanation is available of why subunit vaccines seem to provide only gender-specific protection. Further Phase III efficacy trials of the gD2 vaccine (Herpevac) are in progress in collaboration with the NIH, involving about 7500 persons from 18 to 30 years of age, double HSV-1/HSV-2 seronegative women. A vaccine that protects women could be expected to decrease the rate of neonatal HSV infection and have an impact on the epidemic spread of genital herpes. Lack of efficacy of vaccines in HSV-1 infected individuals would however render the vaccine of little use in developing countries, where HSV-1 infection is ubiquitous.
- A novel, live attenuated HSV-2 candidate vaccine has been developed by Xenova/GSK using a replication-impaired virus mutant that lack the gene of the essential glycoprotein gH (ICP8 gene mutation) as a disabled infectious single cycle (DISC) virus vaccine. The vaccine was tested in Phase II trials in the USA as a therapeutic vaccine in HSV-2 seropositive symptomatic patients. It was well tolerated and induced neutralizing antibodies and CTL in 83% of the vaccinees, but no difference in time to recurrence and no difference in virus shedding were observed as compared with controls. The development of the DISC vaccine has been refocused towards its use as a prophylactic vaccine.
- Another live, replication-impaired vaccine is currently under development by Avant Immunotherapeutics. Other viral mutants that are defective for replication and impaired for establishment of latency, such as mutant dl5–29, are at a preclinical stage of development.
- A live attenuated vaccine based on a replication-competent ICP10 mutant of HSV-2 developed by AuRix is in Phase II clinical study.

DNA vaccine formulations have shown incomplete efficacy in animal models. Similarly, whole inactivated virus vaccines did not show efficacy and their development has been stopped.

5.4. HIV/AIDS

5.4.1. Disease burden

The acquired immunodeficiency syndrome (AIDS) emerged in the human population in the summer of 1981. There now is convincing evidence that its agent, the human immunodeficiency virus (HIV), probably crossed the simian-human species barrier before the middle of the 19th century. At the end of 2004, the number of adults and children living with HIV/AIDS was estimated by WHO/UNAIDS to have reached 39 million worldwide. An estimated 4.8 million people (including 600 000 children less than 15 years of age) becomes infected each year, 95% of whom live in developing countries, and an annual 2.9 million people die of the disease. Today, HIV/AIDS is the leading cause of death in sub-Saharan Africa and the fourth biggest killer in the world. The number of HIV infections is equally distributed between men and women, but infection rates in young women in today's Africa are close to three times higher than those among young men, reflecting the degree to which gender inequities are driving the epidemic, as many women in developing countries lack socio-economic independence, education and access to health information and services, and have difficulty avoiding exposure to the virus.

Sub-Saharan Africa remains the hardest-hit region in the world, with at least 25 million infected people, accounting for 70% of the people living with HIV/AIDS and 77% of AIDS deaths worldwide. The overwhelming majority of HIV transmission in the region stems from sexual behavior. In some African countries, overall prevalence in the adult population can be greater than 10%, with figures reaching up to 38.8% in some areas. Among the most severely hit countries are South Africa, with more than 5.6 million infected people, together with Bostwana, Mozambique, Tanzania and Zimbabwe. Highest infection rates are found among commercial sex workers, truck drivers and seasonal migrant workers. Sub-Saharan Africa also is home to an estimated 500 000 infants who contracted HIV each year before the onset of prevention of vertical transmission by use of antiretroviral drugs: transmission from mother-to-child can occur *in utero*, at birth or as a result of breastfeeding. In addition, sub-Saharan Africa faces numerous wars and civil conflicts, producing large numbers of refugees who are at heightened risk of contracting HIV. A remarkable success story in the fight against AIDS was undertaken in Uganda, which was facing a severe HIV epidemic in the mid 1980s. Through voluntary HIV counselling, expanded treatment of STDs, awareness campaigns and community mobilization encouraging delayed initiation of sexual activity, monogamy and use of condoms, the level of infection declined significantly since 1992 – from nearly 30% to 11.2% in prenatal settings in Kampala and from 13% to 5.9% in clinics outside major urban areas.

The estimated number of people living today with HIV in Asia and the Pacific Region is more than seven million, but the accuracy of the figure is questionable, in view of the fast pace at which the epidemic is expanding. It has been projected that the region will contribute 40% of all new infections by the end of the decade, with China reaching 10 million infected persons, from an official 800 000 today, and India 20–25 million, from 5.1 million today. Increasing sex trade, use of illicit drugs, and rates of sexually transmitted infections contribute to an increased vulnerability in the region. Injection drug use and heterosexual intercourse are the primary modes of transmission, although improper blood donation practices in China

and unsafe injection practices in health-care settings in India and surrounding countries have resulted in hundreds of thousands of infections. Substantial transmission also occurs in men who have sex with men, with prevalence rates of 14–20% reported in male homosexual communities in Cambodia, India and Thailand. Gender inequities play a major role in the epidemic as young girls are frequently steered toward sex work by their families.

The estimated number of adults and children living with HIV in Latin America and the Caribbean at the end of 2003 was two million. While in some countries HIV infections remain concentrated mainly in men who have sex with men and injecting drug users, others are experiencing increasing rates of heterosexual transmission.

The Eastern European countries continue to experience one of the sharpest increases in the number of new HIV infections, most of which occur among injecting drug users. The number of people with HIV/AIDS in the region is estimated to be 1.3 million.

In industrialized countries, highly active antiretroviral treatment (HAART) has considerably reduced disease progression to AIDS and transformed HIV/AIDS from a deadly disease to a somewhat manageable chronic disease. However, successes in treatment and care are not being matched by progress in prevention. Each year, some 75 000 individuals become infected with HIV in industrialized countries, where an estimated 1.6 million people are living with HIV/AIDS (1 million in North America alone), and where new evidence of rising HIV infection rates is emerging, particularly in marginalized communities.

5.4.2. Virology

The human immunodeficiency virus (HIV), together with the simian, the feline, and the bovine immunodeficiency viruses (SIV, FIV, and BIV, respectively), the Visna virus of sheep, the caprine arthritis-encephalitis virus (CAEV) and the equine infectious anaemia virus (EIAV), belongs to the genus Lentivirus in the family *Retroviridae*. These enveloped RNA viruses produce characteristically slow, progressive infections. Their replication depends on the presence of an active reverse transcriptase responsible for the transformation of the RNA genome into a DNA copy that integrates into the host cell chromosome in the form of a provirus. The provirus is eventually transcribed into a set of mRNAs that encode the viral proteins and into progeny genomic RNA. The genome of HIV is a single-stranded positive sense RNA molecule, 9.5 kb in length, which encodes the typical retrovirus proteins Gag (further cleaved into Matrix, Capsid and Nucleocapsid proteins), Pol (itself cleaved into Protease, Reverse Transcriptase and Integrase) and Env (a 160 kD glycoprotein eventually cleaved into a gp120 external subunit and a gp41 transmembrane subunit that form together trimeric spikes on the surface of the virion). In addition, the genome encodes a variety of nonstructural proteins, such as regulatory proteins Tat and Rev and accessory proteins Nef, Vif, Vpr and Vpu. The gp120 subunit binds the CD4 receptor and CCR-5 or CXCR-4 co-receptors on the surface of target cells, whereas gp41, which anchors the spikes in the viral envelope and maintains their trimeric organization, plays a major role in fusion of the virus and cell membranes. Neutralizing human monoclonal antibodies have allowed the identification of several neutralization epitopes on gp120 that overlap the receptor or co-receptor binding sites, but they appear to be little accessible to the cognate

antibodies due to hindrance by the many glycosylation motifs on the molecule as well as by the presence of hypervariable loops that act as antigenic decoys. Fusion-blocking antibodies also have been described, with corresponding epitopes located at the base of the gp41 ectodomain. Contrary to laboratory-adapted virus strains ("X4" strains) against which protection in chimpanzees could readily be obtained with neutralizing antibodies, field virus isolates ("R5" virus strains) have turned out to be extremely difficult to neutralize, casting doubt on the feasibility of a vaccine to elicit protection against infection by induction of neutralizing antibodies alone.

Two types of HIV have been described: HIV-1 and HIV-2, the latter being less virulent and geographically limited to West Africa. HIV-1 is phylogenetically close to SIVcpz, a commensal virus in chimpanzees, whereas HIV-2 is closely related to SIVmac, the agent of simian AIDS, and to SIVsm, a commensal virus in sooty mangabey monkeys. HIV-1 is further divided into three groups, M, N, and O. The vast majority of the HIV-1 strains responsible for the global pandemic belong to group M. These have further been classified in 10 subtypes, also known as clades, which have been designated by letters from A to K. HIV-1 subtype B predominates in industrialized countries as well as in Latin America and the Caribbean. Subtypes A and D are more common in Central Africa. Subtype C accounts for the majority of infections in southern Africa, parts of Eastern Africa and India. Interclade recombinant strains are relatively common and have been designated "circulating recombinant forms" (CRF). Major CRFs are CRF_AG, prevalent in western Africa, CRF_AE, which predominates in south-eastern Asia, and CRF_BC, prevalent in China. Amino acid sequence of the viral envelope glycoprotein shows 25–35% divergence between clades and up to 20% divergence within any given clade, which constitutes a formidable challenge to vaccine development.

5.4.3. Vaccines

The development of a safe and effective vaccine is hampered by the high genetic variability of HIV, the lack of knowledge of immune correlates of protection, the absence of relevant and predictive animal models, and the complexity of the implementation of efficacy trials, especially in developing countries. The first Phase I trial of an HIV vaccine was conducted in the USA in 1987. Since then, more than 30 candidate vaccines have been tested in over 80 Phase I/II clinical trials, involving more than 10 000 healthy human volunteers. Two Phase III trials have been carried to completion and a third one is in progress. Most of the effort to develop and evaluate HIV vaccines is borne by the NIH, CDC and WRAIR in the USA and by ANRS in France, with strong help from the International AIDS Vaccine Initiative (IAVI) in New York, the European Union, initiatives in WHO and UNAIDS, and the recent commitment of the Bill and Melinda Gates Foundation for a Global Enterprise. The HIV Vaccine Trial Network (HVTN) established by NIAID in 2000, with 25 clinical sites in four continents, represents a major resource for clinical HIV vaccine research. The European Union has created the European and Developing Countries Clinical Trials Partnership (EDCTP) with the aim of helping developing countries build up their capacity in testing the efficacy of new drugs, microbicides, and vaccines.

In the absence of identified correlates of immune protection, multiple vaccine concepts are being explored in parallel.

5.4.3.1. Live attenuated vaccines

The observation that *nef*-deleted mutants of SIV could elicit protection against challenge with pathogenic SIV in rhesus macaques served as a model in favour of a live attenuated HIV vaccine approach. The SIV Ä*nef* mutants, however, establish a lifelong, persistent low grade infection that does not protect the vaccinated monkeys against superinfection with wild-type virus, although the animals seem to be protected against subsequent disease. In addition, the attenuated virus still may cause AIDS when administered orally to infant monkeys. Additional deletions or mutations can further attenuate the virus but at the expense of its protective efficacy. Mostly because of safety concerns, this approach was therefore not pursued.

5.4.3.2. Subunit vaccines

A subunit HIV vaccine was developed based on monomeric gp120 added with alum (VaxGen). The vaccine was tested in two double-blind, controlled Phase III efficacy trials, one on 5000 volunteers at risk (mostly men who had sex with men) in the USA, with sites in Canada and in the Netherlands, using a mixture of two subtype B gp120s as the immunogen, the other on 2500 volunteers in Thailand (mostly drug users), using a mixture of a subtype E (CRF_AE) and a subtype B gp120s. None of these studies showed a statistically significant reduction of HIV infection in the vaccinees in spite of biannual booster immunizations. A reduction of the number of HIV infections was observed in certain ethnic subgroups in the first study, correlating with a higher level of anti-gp120 antibody, but the numbers were too small to provide statistical confidence. The same subtype E/B gp120 vaccine is planned to be used for booster immunizations in a prime-boost Phase III trial which was launched in late 2003 in Thailand in collaboration between the Ministry of Health of Thailand, WRAIR, Sanofi-Pasteur and VaxGen, and uses for priming a recombinant canarypox virus (ALVAC) that expresses CRF_AE gp120 and subtype B Gag, Pol and Nef antigens. The trial will enrol 16 000 heterosexual volunteers and is expected to last four years.

Other approaches aimed at eliciting HIV neutralizing antibodies are at an early clinical stage. These include the use of:

- trimeric gp140 molecules (gp120 + the ectodomain of gp41) with a deletion of the hypervariable V2 loop in order to expose the neutralization epitopes overlapping the CD4-binding site;
- oligomeric gp140 molecules covalently coupled to synthetic mimics of the CD4 receptor that should expose neutralization epitopes overlapping the coreceptor (CCR5 or CXCR4)-binding site;
- gp120/gp41 trimers internally stabilized by disulfide bond formation (SOS proteins) which should elicit both neutralizing and fusion-blocking antibodies.

Induction of fusion-blocking antibodies by immunization with recombinant oligomeric gp41 molecules is a promising new approach that still is at an early preclinical stage of development.

5.4.3.3. Live recombinant vaccines

Rather than attempting to elicit a neutralizing antibody response, recent HIV vaccine approaches have aimed to elicit a T-cell response, especially a CD8+ CTL response, whose role in control of virus load and evolution of disease has been well documented in the monkey model. In addition to perforin-based cellular cytotoxicity, CD8+ T-cell secrete antiviral cytokines (IFN-γ), still unidentified antiviral factors (CAF) and virus entry-blocking β-chemokines that have been correlated with protection against SIV infection in the monkey model, as well as associated to asymptomatic HIV-1 infection in humans and slower disease protection in HIV-2-infected patients. Vaccines that stimulate the T-cell arm of the immune response are however not expected to protect against infection, but rather to control its course and reduce viral loads, thus preventing or at least delaying the occurrence of symptoms. Reduction of viral loads in vaccinated but HIV-infected individuals also would hopefully result in lowering the probability of virus transmission to their partners.

Several prime-boost strategies involving priming with a DNA vaccine followed by boosting with a live recombinant vector-based vaccine have been tested in monkeys against challenge with a lethal dose of simian–human immunodeficiency virus (SHIV) that causes AIDS-like illness in the animals. These strategies resulted in reduction in virus load and provided protection against disease and death in the vaccinated animals. The same approach was however less successful in protection against SIV challenge.

A number of these vaccines also have been tested in Phase I/II trials in humans, including plasmid DNA and poxvirus vectors (MVA, fowlpox or canarypox viruses) expressing a variety of HIV antigens, such as Gag, Env, Pol and Nef.

Replication-defective adenovirus type 5 (Ad5) represents another promising vector: a recombinant Ad5-*gag/pol/nef* vaccine has entered Phase II trials on some 1200 men and 400 women at high risk who will be followed for 3 years after 3 immunizations at 0, 4 and 26 weeks (Merck).

The list of other vectors is long. It includes, among others:

- BCG (NIH Japan)
- *Salmonella* (IAVI/Institute for Human Virology, University of Maryland)
- Venezuelian equine encephalitis virus (VEEV; Alphavax)
- adenovirus-associated virus (AAV; IAVI/ Targeted Genetics)
- Sendai virus (NIH Japan)
- vesicular stomatitis virus (VSV; Yale University/Wyeth)
- Newcastle Disease Virus (NDV; Mount Sinai, New York, and Kyoto University, Japan)
- measles virus (Pasteur Institute/GSK).

The immunogenicity of the DNA and poxvirus vector vaccines in humans has usually been relatively weak, with generally less than 35% of the vaccinees scoring positive at any time point, as determined by IFN-γ ELISPOT assays. This was emphasized by the very disappointing results of a recent DNA prime-MVA boost Phase I study in Kenya which showed that the promising immunogenicity data obtained in monkeys with clade A HIV DNA and MVA constructs could not be repeated in human volunteers.

The best results so far, in terms of the percentage of human responders, level of T-cell responses and duration of immune responses have been obtained with the Ad5 vector. However, Ad5-based recombinant vaccines are confronted with the problem of a frequent pre-existing anti-vector immunity in the human population, especially in developing countries, which dampens the immune response to the HIV transgene. This has prompted the development of less prevalent human adenovirus serotypes (Ad35, Ad11, or Ad24) as nonreplicative vectors. Like Ad5, these vectors readily multiply to large yields in PRC-6 cells in fermenters. Nonreplicative chimpanzee adenovirus vectors (AdC68, AdC6 and AdC7) also are being developed. Combinations of different vectors in mixed modality prime-boost regimens will likely be developed in the future.

In rhesus monkeys, responses arising from an Ad5 priming-poxvirus (MVA or ALVAC) boosting regimen were significantly greater than those elicited by homologous regimens with the individual vectors, but this was not observed in human volunteers (Merck and Sanofi-Pasteur).

5.4.3.4. Other vaccinal approaches

Induction of persistent HIV Gag-specific CD8+ CTL responses was attempted in a Phase I trial involving immunization with a fusion protein comprising the HIV p24Gag protein and detoxified *Bacillus anthracis* lethal factor *(see 7.1.2.)* to target the antigen to antigen presenting-cells (Avant Therapeutics and WRAIR).

Multi-epitopic combinations of peptides, fusion proteins and long lipopeptides also are at an early stage of clinical development, either alone or in prime-boost combinations with live vector-based recombinant vaccines. Lipopeptides whose sequence corresponds to that of CTL epitopes-rich regions in the Gag and Nef viral proteins are in Phase II trials in the USA and in France (NIAID/ANRS).

Finally, a number of candidate vaccines that target nonstructural viral proteins such as Tat, Rev, Vif and Nef are being developed using viral vectors such as MVA or Ad5 (bioMérieux/Transgene), fowlpoxvirus (AVC), DNA (Vical, Istituto superiore dei Sanita/Parexel) recombinant proteins (FIT Biotech, Institute for Human Virology) or fusion proteins (GSK), or polyepitopic peptides (Wyeth/Duke University, Epimmune). Some of these candidate vaccines will be tested as therapeutic vaccines in patients as a complement to antiretroviral therapy. Tat has been shown to act as a viral toxin and to promote apoptosis of uninfected bystander T-cells and secretion of Th2 cytokines.

The population-wide effects of partially effective vaccines that do not prevent infection but only can reduce viral loads are largely unknown. Mathematical models predict that the factor with the greatest impact on reducing infections and deaths will be the degree of virus load reduction. A 90% reduction in viral load, which is a reasonable expectation with current candidate vaccines under development, would significantly reduce HIV mortality within 20 years after introduction of the vaccine.

The development of a safe, effective, and affordable HIV vaccine remains a formidable scientific and public health challenge at the dawn of this century.

6. Vector-borne viral infections

6.1. Dengue fever

6.1.1. Disease burden

Dengue fever, a usually mild albeit debilitating viral fever (breakbone fever), is prevalent throughout the tropics, where the urban-dwelling mosquito *Aedes aegypti* is a major vector. A related mosquito, *Aedes albopictus*, also can act as a vector. The dengue viruses, of which four serotypes are known (DV-1, -2, -3, and -4), are the most widespread arthropod-borne viruses (arboviruses). They also are the only known arboviruses that have fully adapted to the human host and lost the need of an enzootic cycle for maintenance. During the 20th century, the distribution and density of *Aedes aegypti* expanded dramatically in tropical areas, beginning in large cities then spreading to the countryside. This was followed by global circulation of the four DV serotypes. Because there is no cross protection between different serotypes, a population could experience a dengue-1 epidemic on one year, followed by a dengue-2 epidemic on the next year. Most primary infections cause a debilitating, but nonfatal, form of illness. Some patients, particularly children, experience a more severe and occasionally fatal form of the disease, called dengue haemorrhagic fever (DHF), the most severe form of which is referred to as dengue shock syndrome (DSS). The presence of antibodies to one serotype of DV is believed to facilitate the occurrence of DHF/DSS in certain individuals through immune-enhancement when infected by a second serotype. It is estimated that from 50–100 million cases of dengue fever, 500 000 cases of DHF/ DSS and more than 20 000 deaths occur each year.

Dengue today is present on most continents, and it is estimated than 2.5 billion people (including 1 billion children) are exposed to infection. Although the virus may circulate in endemic cycles, it periodically causes acute, widespread epidemics, such as in 1987 when 175 000 cases with more than 1000 deaths were reported from Thailand, or in 1996 when Brazil reported 180 000 cases, or again in 1998 when 1.3 million cases of dengue fever and DHF and over 3 500 deaths were reported from Latin America, the South-East Asia Region and the Western Pacific Region. More recently (2004), an outbreak was reported from Indonesia with more than 650 deaths. The burden of severe disease remains proportionately much greater in Asian and Pacific countries although it considerably increased in recent years in the Americas. Unplanned urbanization, lack of mosquito control, population growth in urban centres of tropical countries, and increased movement of viruses in infected humans through modern transportation have all contributed to the marked increase in epidemic activity.

6.1.2. Virology

DVs are members of the genus Flavivirus in the family *Flaviviridae*, which includes more than 70 related but distinct viruses, many of which are mosquito-borne, such as the yellow fever (YF) virus. Flaviviruses are enveloped, 40–50 nm-diameter viruses with an icosahedral capsid that contains the single-stranded, positive sense RNA genome. DV envelope surface projections are composed of dimers of the viral envelope (E) glycoprotein and of the membrane (M) protein, itself derived by furine-mediated cleavage from a prM precursor. The only other protein constituent in the virion is the capsid (C) protein. The E glycoprotein is responsible for virion attachment to receptor and fusion of the virus envelope with the target cell membrane and bears the virus neutralization epitopes. On native virions, the elongated three-domain molecule lies tangentially to the virus envelope in a head-to-tail homodimeric conformation. Upon penetration of the virion into the target cell endosome, E dimers are converted to stable target cell membrane-inserted homotrimers that reorient themselves vertically to promote virus-cell fusion.

The 10.5 kb-long genomic RNA is a monocistronic mRNA which is translated into a precursor polyprotein from which the individual viral proteins eventually derive by cleavage, starting with the C, prM and E proteins followed by nonstructural proteins NS1 to NS5. NS3 is a protease and a helicase, whereas NS5 is the RNA polymerase in charge of viral RNA replication. In addition to the E glycoprotein, only one other viral protein, NS1, has been associated with a role in protective immunity. This glycoprotein is not present on the virion, but is found on the surface of infected cells. Immunization with NS1 has been shown to elicit protective immunity in animal models. NS3 harbors the largest number of T-cell epitopes.

6.1.3. Vaccines

As there is no cross-protection between the four serologically distinct DV types (DV-1 to DV-4), and because of the possibility of immune enhancement by monotypic antibody leading to DHF with subsequent natural infections, the control of dengue will be possible only after an efficient tetravalent vaccine has been developed. Progress in vaccine development has been slow, mainly because these viruses grow poorly in cell culture and there is no reliable animal model for DHF.

Today, the most favoured strategy is to develop a live vaccine. This was achieved by Mahidol University, Bangkok, in collaboration with Sanofi-Pasteur, and by the United States Army (WRAIR) working with GSK. Attenuation was obtained by repeated passage of wild-type strains of DVs in cell culture, using primary dog kidney then green monkey or fetal rhesus monkey cell cultures. The difficulty in this approach has been to find the correct balance between insufficient attenuation and over-attenuation of the candidate vaccine strains, as criteria of virus attenuation in vitro, such as small plaque phenotype and temperature-sensitive growth, do not appear to be predictive of attenuation in vivo. In addition, whereas monovalent attenuated vaccine lots showed good immunogenicity, their combination into a tetravalent vaccine initially generated disappointing immunogenicity results, due to a phenomenon of interference between strains, the highest antibody titres being against DV-3.

- Recent clinical lots of the live attenuated vaccine prepared by Sanofi-Pasteur and tested in collaboration with WRAIR in a Phase I/II study on 78 children in Bangkok, showed that a two-dose schedule elicited 85% neutralizing antibody responses to DV-1, 78–DV-2, 100% to DV-3 and 76% to DV-4. A booster immunization one year later was followed by 100% responses to all four serotypes. Five year follow-up data have shown some asymptomatic infections and two breakthrough infections in spite of neutralizing antibodies. No cases of DHF/DSS were observed.
- A similar approach was chosen by WRAIR and GSK to develop a live, attenuated tetravalent vaccine. A number of various formulations had to be tested to find one able to elicit a balanced immune response. In pilot studies in humans, 3 doses of the most promising vaccine combination induced 90% neutralizing antibody responses to DV-1, and 60% to DV-2 and DV-3, but only 25% to DV-4. A small scale Phase I study in children 6–9 years of age has recently been conducted in Thailand, resulting in acceptable immunogenicity and reactogenicity profiles.
- Several research groups are successfully exploring an infectious clone technology for the development of a dengue vaccine. The ChimeriVax system, originally developed to construct a Japanese encephalitis vaccine *(see 6.2.3.)*, has been applied to DVs by Acambis in the USA. Chimeric yellow fever–dengue viruses (ChimeriVax-DV) were prepared using a recombinant infectious cDNA clone of the YF 17D vaccine strain as a backbone, and substituting the prM and E coding sequences by those of each of the DVs in turn. Each of the recombinant plasmids was then transcribed into chimeric infectious RNA molecules and the corresponding virus was generated by transfection in Vero cells. The chimeric viruses were shown to be attenuated in mice and monkeys, including after interacerebral injection, and showed 92% efficacy at protecting cynomolgous monkeys from homologous DV challenge. They showed no ability to grow in mosquitoes after oral feeding on blood-virus mixtures. A monovalent ChimeriVax-DV-2 (CVD2) vaccine formulation was evaluated on 56 human volunteers in a Phase I clinical trial in the USA, resulting in 100% neutralizing antibody responses to DV-2. A Phase I trial of the tetravalent combination has been carried out in collaboration with Sanofi-Pasteur, the results of which should be made available presently.
- A different strategy has been used by the NIH, which developed a DV-4 mutant with a 30 nucleotide deletion in the 3' non-coding region of the genome as a genetic background for the construction of chimeric viruses. Viruses with deletion mutations are genetically stable and less likely to revert to wild type than point mutation mutants. Phase I clinical trials of the DV-4 deletion mutant were carried out in adult humans showing good safety and immunogenicity profile of the vaccine. The attenuated virus was then used as the backbone for the construction of chimeric viruses with serotype 1, 2 and 3 envelope glycoproteins. Alternatively, the deletion mutation was introduced into DV-1, -2 and -3 as a means of attenuation. Monovalent candidate vaccines are in Phase I evaluation, and the evaluation of tetravalent formulations is being planned.

- A similar work is ongoing at the US CDC using an attenuated DV-2 vaccine mutant (strain 16681, PDK-53) as a backbone to construct chimeric attenuated viruses by swapping its prM and E coding sequences with those from DV-1, -3 and -4 serotypes. The tetravalent DV-2, DV-2/1, -2/3 and -2/4 mixture elicited neutralizing antibodies in mice against all four dengue serotypes at titres similar to those elicited by monovalent immunizations, showing the absence of viral interference in this setting.
- Finally, the FDA has started developing attenuated DV strains by site-directed mutagenesis of the 3' terminal stem and loop structure. These mutant candidates still are at a preclinical stage of development.

Other approaches for the development of dengue vaccines include DNA vaccines, inactivated and subunit vaccines, and recombinant vaccinia virus (MVA) vectors. Most advanced are efforts by Hawaii Biotech to develop a subunit, tetravalent vaccine using a mixture of the aminoterminal 80% of the E protein from the four DV serotypes and the nonstructural NS1 protein of DV-2 as immunogens in a proprietary adjuvant. Tetravalent formulations of the vaccine are being tested in non-human primates.

Efforts at developing DV vaccines are helped by the Pediatric Dengue Vaccine Initiative funded by the Bill and Melinda Gates Foundation.

6.2. Japanese encephalitis

6.2.1. Disease burden

Japanese encephalitis (JE) is a mosquito-borne, zoonotic arbovirus infection, in which pigs play the role of amplifying host in rural areas. The virus exists in a transmission cycle between mosquitoes and pigs and/or water birds. Humans, who become infected only incidentally when bitten by an infected mosquito, are a dead-end host. The disease is endemic with seasonal distribution in parts of China, the Russian Federation's south-east, and South and South-East Asia. Annual incidence ranges from 6 to 10 cases per 100 000 inhabitants within heavily endemic areas such as Thailand and Viet Nam. JE is a leading cause of severe central nervous system infection in Asia and Australia, where 30 000–50 000 cases are reported annually. Of these cases, about 30–35% are fatal, and 50% result in permanent neuropsychiatric sequelae. JE also poses risks to travellers and to military personnel deployed overseas. Large outbreaks of JE, often involving adults, have been reported on the Indian subcontinent, and the disease is currently considered hyperendemic in northern India and southern Nepal as well as in parts of central and southern India. The spread of JE in new areas has been correlated with agricultural development and intensive rice cultivation supported by irrigation programmes.

Most JE infections are asymptomatic. Clinical disease varies from a nonspecific febrile illness, which may include cough, nausea, vomiting, diarrhoea and photophobia, to a severe disease with meningoencephalitis, aseptic meningitis or a polio-like flaccid paralysis. About 30% of survivors have persistent motor deficits and about 20% have severe cognitive and language impairment.

6.2.2. Virology

JE virus (JEV) is a member of the genus Flavivirus in the family *Flaviviridae,* together with YFV and DV *(see above)*. JEV belongs to the same serological group as West Nile virus (WNV) *(see below)*; Kunjin virus in Australia and Papua New Guinea; the Murray Valley encephalitis virus (MVEV) also in Australia, Papua New Guinea and western Indonesia, and the St. Louis encephalitis virus (SLEV) in North and South America. All these viruses are transmitted by *Culex* mosquitoes (*Cx. tritaeniorhynchus)*. Wild birds and pigs play a major role in the enzootic cycle of JEV, which replicates in both species and in the mosquito. Humans occasionally may be bitten by an infected mosquito but are dead-end hosts and contribute little to the spread of the natural infection.

The 10 976 nucleotide-long JEV single-stranded RNA genome encodes, as for other flaviviruses, structural proteins C (capsid), prM (precursor to membrane protein M) and E (envelope), and nonstructural proteins NS1–NS5 which are involved in genome replication and viral protein processing. The 53 kD surface glycoprotein E is responsible for the viral attachment to cellular receptors, specific membrane fusion, and elicitation of virus-neutralizing, haemagglutination-inhibiting and anti-fusion antibodies. Four major genotypes of JEV exist, which show different geographic distribution, but all belong to the same serotype.

6.2.3. Vaccines

JE control programmes include mosquito control (spraying of pesticides, impregnated mosquito nets), pig control (segregation, slaughtering, and vaccination) and human vaccination. Several vaccines are now available and others are under development.

- A purified formalin-inactivated JE vaccine made from either the Nakayama strain or the Beijing strain of JEV propagated in mouse-brain tissue (Biken and Kaketsuken) has been successfully used to reduce the incidence of JE in China (Province of Taiwan), Japan, the Republic of Korea, Thailand and Viet Nam. Immunogenicity studies in areas devoid of endemic transmission have indicated that three doses of the vaccine are required to provide adequate level of antibody. Since 1988, this vaccine has gradually been introduced into the EPI in Thailand and administered with the fourth dose of DTP at 18 months.
- Another formalin-inactivated JE vaccine is prepared in China from the P3 strain of JEV propagated in primary Syrian hamster kidney-cell cultures. This strain is more immunogenic and confers greater protection in mice than the Nakayama strain. This used to be the most widely used JE vaccine worldwide.
- Other inactivated JE vaccines have been developed using better standardized cell substrates, including Vero cells. Vero cell-derived inactivated JE vaccines have been developed in China where the vaccine is now licensed, as well as by Biken and Chemo-Sero Therapeutic Research Institute in Japan.
- Now being the most widely used JE vaccine in China, the live, attenuated JEV strain SA 14-14-2 was obtained after 11 passages in weanling mice followed by 100 passages in primary hamster kidney cells at the National Institute for Control of Pharmaceutical and Biological Products (NICPBP) in Beijing in the early 1970s. This strain was shown to be safe and immunogenic in mice,

pigs, horses and humans. Expanded field trials in southern China involving more than 200 000 children confirmed the strain safety and yielded efficacies of 88–96% over 5 years. The SA 14-14-2 strain also elicits seroconversion rates of 99–100% in nonimmune subjects. The live attenuated SA 14-14-2 vaccine is produced on primary hamster kidney cells, lyophilized, and administered subcutaneously to children at one year of age and again at two years in annual spring campaigns. In a case–control study in Nepal, an efficacy of 99.3% was reported after a single dose of the vaccine. Currently, more than 60 million doses are distributed annually in southern and western China, and the vaccine is exported to Nepal and the Republic of Korea. Efforts are ongoing to produce the vaccine to GMP standards.

- The SA 14-14-2 strain also has been adapted to growth on primary dog-kidney cells and on Vero cells at WRAIR for use as an inactivated vaccine. Phase I and II studies of an inactivated SA 14-14-2 strain propagated in Vero cells have been carried out, showing excellent safety and immunogenicity profiles (Intercell, Vienna). A Phase III trial of the vaccine is scheduled to start in 2005.
- Live attenuated recombinant JE vaccine candidates based on poxvirus vectors (NYVAC, ALVAC) expressing the prM, E, NS1 and NS2A proteins have been tested in monkeys and in humans, but their development was stopped.
- A most promising genetic approach has been the construction of a chimeric live-attenuated vaccine that comprises the prM and E coding sequences of the JEV SA 14-14-2 strain inserted in Phase into the 17D YFV strain genome. The resulting virus can be cultivated on Vero cells, has proved to be highly immunogenic in rhesus monkeys and to protect against intracerebral and intranasal challenges with wild-type JEV. The prototype vaccine, ChimeriVax-JE (Acambis), has been tested successfully in 99 adults in the USA, showing good safety and immunogenicity; 94% of the vaccinees developed JEV-neutralizing antibodies after a single dose. There was no interference with chimeric vaccination by prior immunity to YF, but a slight interference was noted in persons given YF 17D 30 days after ChimeriVax-JE. The vaccine is under Phase II clinical trials with promising early results. A first paediatric evaluation of the vaccine is scheduled for 2005 in Thailand.
- Other attempts at developing new JE vaccines have focused on DNA vaccines. A single intramuscular immunization of DNA carrying the prM and E coding sequences from JEV or WNV protected mice and horses from virus challenge. The use of multivalent combination DNA vaccines designed to immunize against multiple flaviviruses is an interesting area of development, although the immunogenicity of DNA vaccines in humans first needs to be greatly improved.

6.3. Tick-borne encephalitis

6.3.1. Disease burden

The endemic area for tick-borne encephalitis (TBE) spreads from Alsace-Lorraine in the west to Vladivostok and north-eastern regions of China in the east, and from Scandanavia to Italy, Greece and Crimea in the south. TBE also is endemic in North Japan, where the virus has repeatedly been isolated from blood samples of sentinel dogs, ticks, and rodent spleens. TBE is a serious acute central nervous system infection which may result in death or long-term neurological sequelae in 35–58% of patients. The fatality rate associated with clinical infection is 0.5–20%. The proportion of cases involving subclinical infection varies between 70% and 98%. Symptomatic infection occurs in all age groups.

The agent is the TBE virus (TBEV), a flavivirus that is transmitted to humans by the bite of a tick. Eight species of ticks have been identified that can transmit TBEV. The chief vectors are *Ixodes ricinus* in Europe and the western part of the Russian Federation, and *Ixodes persulcatus* in the eastern part of the Russian Federation. Occasionally, transmission also can occur through consumption of raw milk from an infected cow, goat or sheep. There are two subtypes of TBEV: eastern and western that show slight differences in the structure of the viral proteins. In the past, diseases caused by the two variants were referred to as "Russian spring/summer encephalitis" and "central European encephalitis", respectively. The virus subtype largely determines the clinical course of the disease. The eastern variant has proven to be more virulent and to more often lead to severe illness. Transmission of the disease is seasonal and occurs in spring and summer, particularly in rural areas, where two seasonal peaks of the disease are typically seen, one in June–July and the other in September–October, corresponding to two waves of feeding of tick larvae and nymphae. A rise in incidence of TBE has been observed in recent decades in some regions – presumably linked to global warming – as milder winters lead to proliferation of rodent populations (voles and field mice) which are the main hosts and reservoirs of the virus and, as a consequence, also of ticks.

6.3.2. Vaccines

At least four formalin-inactivated TBE vaccines are available, made of virus grown on chick embryo fibroblasts, inactivated by formaldehyde and purified by continuous flow-density centrifugation. These vaccines are administered along a two-dose schedule followed by a booster immunization at one year and recommended booster injections every 3–5 years. They are produced in Austria (Baxter Vaccine, previously Immuno), Germany (Chiron, previously Beringwerke) and the Russian Federation (Institute of Poliomyelitis and Viral Encephalitidis and Virion Company). Active surveillance in Austria has demonstrated a dramatic decline in the incidence of TBE in vaccinated groups, with a reported vaccine efficacy of 95%. The main reported side effect with currently available vaccines is postvaccination fever and allergic reactions in children. A series of improvements to the available vaccines were introduced to reduce their reactogenicity. In Germany, the vaccine is widely used to immunize children in high-risk areas. The Russian vaccine induces high seroconversion rates and is believed to be highly effective.

So far, attempts to develop a live attenuated TBE vaccine have been unsuccessful, although promising preliminary results have been obtained in a mouse model using deletion mutants of the 3' noncoding region of the TBEV genome combined with a mutation (Thr 310) in the putative receptor-binding site in the E glycoprotein. The development of a live attenuated vaccine would provide major advantages with respect to generating long-lasting immunity without the need of frequent booster injections.

Other approaches to develop TBE vaccines are based on the use of DNA or RNA vaccines.

6.4. West Nile virus

6.4.1. Disease burden

The identification of West Nile virus (WNV) in the suburbs of New York City in the summer of 1999 marked the first detection of the mosquito-borne virus in the western hemisphere. During the following years the virus extended its range throughout most of the eastern parts of the North American continent, and then spread to the southern and western parts of the USA and to parts of Mexico. It is feared that expansion of the virus range in the direction of Central and South America is presently going to occur. WNV, a member of the Japanese encephalitis virus complex in the family *Flaviviridae,* is now one of the most widely distributed flaviviruses worldwide. Its geographic range includes Africa, North America, West Asia, Australia, Europe and the Middle East, where both sporadic cases and major outbreaks of encephalitis with fatalities have been reported.

The virus infects birds, horses and other equines, but has also been recovered from cats, dogs, skunks, rabbits, squirrels, raptors, camels and alligators! In North America the virus has been detected in dead birds of at least 140 different species, particularly crows and jays which serve as sentinel species. In contrast, no bird mortality from WNV infection has been reported in Europe. WNV has a complex life-cycle involving birds as primary hosts and *Culex* mosquitoes as primary vectors. The virus is amplified during the period of adult mosquito blood-feeding by continuous transmission between mosquito vectors and birds. Humans, horses and other mammals are usually "dead-end" hosts, because they do not produce sufficient viremia to contribute to the transmission cycle.

Most human WN infections are asymptomatic. In about 20% of cases, however, febrile influenza-like illness (West Nile fever) is observed with fever, headache, body aches, myalgia, and anorexia. A maculopapular rash occurs in about half the persons with WN fever. Ocular pain, pharyngitis, nausea, vomiting, diarrhoea and abdominal pain can also occur. In less than 1% of cases, neuroinvasive forms are reported, with meningitis and/or encephalitis leading to paralysis, and coma resulting in death. Advanced age is the most important predictor of death. Mortality among patients with neuroinvasive disease is about 10%. Among the survivors, long-term cognitive and neurologic impairment may occur. The neuropathologic lesions are similar to those of Japanese encephalitis, with diffused central nervous system inflammation and neuronal degeneration. The virus is also found in the spleen, liver, lymph nodes and lungs of infected individuals. WNV infection may also cause an acute flaccid paralysis syndrome, which results from an anterior horn cell process in the spinal cord suggestive of poliomyelitis. Long-term improvement is variable, but complete recovery is uncommon.

During the period 1999–2000, 142 cases of neuroinvasive WNV disease of the central nervous system (including 18 fatalitites), and seven cases of uncomplicated WN fever were reported in the USA. These figures have been increasing ever since. Between April and October 2004, no less than 2230 human cases were reported from 40 states and the District of Colombia (DC), 73 of which were fatal. During the same period, 196 presumptive WN viremic blood donors were identified, three of whom later came down with a neuroinvasive illness and 46 with WN fever. At the same time, 5416 dead corvids and 1316 other dead birds with confirmed WN infection were reported from 45 states and New York City. The relative severity of the WNV epidemic in the USA has been attributed to the novelty of its introduction on the continent, the pathogenicity of the introduced virus strain for wild birds, but also to the fact that American *Culex pipiens* mosquitoes seem to more readily bite birds and then humans than European *Cx. pipiens*, which feed overwhelmingly on birds and ignore humans.

An investigation conducted by the US CDC, the Food and Drug Administration (FDA), the American Red Cross, and State Health Departments in Georgia and Florida has confirmed transmission of WNV through organ transplantation from a single donor to four organ recipients. Transmission through transfused blood, transplacental transmission and transmission among haemodialysis patients also have been reported.

6.4.2. Vaccines

Vaccination against JE or dengue is unlikely to prevent WNV infection but might protect against disease, as judged by the report that, in animal models, immunization with heterologous flavivirus vaccines reduced the severity of subsequent WNV infection.

Specific WNV vaccines under development include the following.

- A candidate live attenuated chimeric WN vaccine that uses an infectious dengue virus type 4 (DV-4) cDNA clone as a backbone. The chimera was constructed by replacing the genes for the structural prM and E proteins of DV-4 by the corresponding genes from WNV strain NY99. The WN/DV-4 chimera was highly attenuated, as compared with the WNV parent, and provided complete protection against lethal WNV challenge in mice.
- A live-attenuated chimeric vaccine (ChimeriVax-WN), which was developed by Acambis using the attenuated yellow fever 17D strain as a live vector, as done for DV and JEV vaccines *(see above)*. Attempts were first made to engineer attenuation mutations in the prM-E coding sequences from WNV. Engineering three point mutations (L107F, A316V and K440R) in the WNV ORF rendered the virus attenuated for mice and monkeys including by the intracerebral route. The mutagenized prM-E cassette from the attenuated WNV was then inserted in place of the corresponding sequence in the 17D strain, thus generating an YF/WN chimera with the three attenuation mutations. The chimeric virus induced high anti-WN neutralizing antibody titres in mice and monkeys and protected 12 of 12 monkeys against intracerebral challenge with WNV. A small Phase I clinical trial in 15 subjects showed the chimera to be well tolerated in humans. A Phase II study is planned to start soon.

- A subunit WN vaccine formulated in proprietary adjuvant by Hawaii Biotech, which has successfully been tested in preclinical challenge studies.
- Other technologies include a naked DNA vaccine which showed protective efficacy in horses, mice and birds, and attenuated WNV variants that have lost the neuroinvasive characteristics of the parental virus through serial passages in mosquito cells and neutralization escape from WNV-specific monoclonal antibody.

A Phase I/II randomized, placebo-controlled trial is in progress to assess the safety and efficacy of intravenous immune globulin with high titres of anti-WNV antibody in patients at high risk for progression to encephalitis and/or myelitis.

West Nile encephalitis also has emerged as a significant problem in veterinary medicine. One major veterinary manufacturer (Fort Dodge) has developed a formalin-inactivated vaccine which was given conditional approval for use in horses, but showed little efficacy in avian species.

7. Zoonotic infections

Zoonotic infections (animal infections transmitted to man) are responsible for a large and growing proportion of the mortality and morbidity throughout the world. Diseases such as Japanese encephalitis, yellow fever, West Nile fever, haemorragic fevers and leishmaniasis affect untold numbers of people in the world's poorest countries. Rabies remains a public health problem in the same countries. Other zoonotic infections include SARS and hepatitis E. Many zoonoses are promoted by human behavior, such as bush-meat hunting (EBOLA, and probably HIV), the sale of live wild animals (SARS), intense farming and sale of different animal species next to each other (avian influenza), or deforestation and building of dams (leishmaniosis, Rift Valley fever). Finally, the threat of using zoonotic pathogens as bioterrorism agents, as demonstrated in the case of anthrax, has prompted the development of new vaccines against anthrax, plague and EBOLA fever, among others. This chapter will only deal with anthrax, plague, hepatitis E and rabies as most other zoonotic infections have been inserted in preceding chapters.

7.1. Anthrax

7.1.1. Disease burden

Anthrax, a zoonotic disease caused by *Bacillus anthracis*, has three forms: cutaneous, inhalational and gastrointestinal, with mortality rates if untreated of 20%, 100% and 25–75%, respectively. The cutaneous form is the most usual in humans. Natural human cases primarily are associated with infections in sheep, goats or cattle, and/or with exposure to contaminated animal products that include wool, goat hair, hides or carcasses. Pasture contamination is the source of most animal cases in endemic countries. Natural disease in humans is not a major public-health problem in the world today, although occasional epidemics do occur. The largest epidemic in modern times occurred between 1979 and 1985 in Zimbabwe, with approximately 10 000 human cases reported; the source of infection was infected cattle. An unusual epidemic occurred in the Russian Federation (Sverdlovsk) in 1979 after an accidental release of spores in an aerosol from a military microbiology facility, resulting in at least 77 human cases and 66 deaths from inhalation.

In view of the infectiousness of *B. anthracis* spores and the high mortality of inhalation anthrax, the primary concern with anthrax in modern times is its use as a biological weapon. The admission by Irak that it produced weapons containing anthrax spores which it was prepared to launch during the 1991 Persian Gulf War and the intentional use of *B. anthracis* as a bioterrorist weapon in the autumn of 2001 in the USA prompted the development of improved anthrax vaccines. The anthrax terror attacks in the autumn of 2001 in the USA (the District of Columbia, Connecticut, Florida,

New Jersey and New York) resulted in 11 confirmed inhalational cases, and 7 confirmed and 4 suspected cutaneous cases of anthrax from exposure to contaminated mail, and led to 60-day prophylactic antibiotic treatment in more than 10 000 persons.

7.1.2. Bacteriology

B. anthracis is a gram-positive bacillus which can sporulate in response to oxygen. Spores survive for decades in the soil, and eventually are ingested by cattle when grazing on contaminated land. The spores are phagocytised by macrophages in which they germinate, giving rise to bacilli whose virulence is due to two plasmids, pXO1 which carries the three toxin genes *cya* (oedema factor), *lef* (lethal factor) and *pag*A (protective antigen), and pXO2, which carries the gene for polyglutamate capsular filaments that play a major role as an invasiveness factor. Bacteria lacking plasmid pXO2 are attenuated for animals and have been used as a live attenuated vaccine. Lethal toxin (LT) is formed by the binding of the lethal factor (LF) to receptor-bound protective antigen (PA). LF is a Zn+ metalloprotease that cleaves cellular mitogen-activated protein kinase kinases (MAPK kinases) and prevents the induction of a number of anti-apoptotic genes in activated macrophages, thus causing macrophage death together with release of TNF-α. Oedema toxin (ET) similarly is formed by the binding of the oedema factor (EF) to the protective antigen (PA). EF is an adenylate cyclase that promotes tissue oedema by increasing the intracellular concentration of cyclic AMP (cAMP) and also acts on macrophages. LT and ET thus contribute to the ability of *B. anthracis* to evade host innate immune responses by deregulating proinflammatory cytokines, inducing apoptosis in activated macrophages, inhibiting phagocytosis and suppressing the respiratory burst in polymorphonuclear cells.

7.1.3. Vaccines

An attenuated, unencapsulated strain, the Sterne strain, was developed in the 1930s as a live anthrax vaccine that still is in use worldwide for domesticated animals.

A live human vaccine consisting of a suspension of live spores from another unencapsulated strain, SST-1, has been developed in the Russian Federation. The vaccine is administered by skin scarification at 0 and 21 days, followed by yearly boosters.

A similar vaccine, made of live spores from still another avirulent strain, A16R, has been developed by the Langzhou Institute of Biological Products in China and is given by scarification as a single dose to be followed by a booster dose 6–12 months later.

The human anthrax vaccine licensed in the USA is made from cell-free filtrates of bacterial cultures of an unencapsulated strain of *B. anthracis* adsorbed to aluminium hydroxide and treated with formaldehyde (AVA/Biothrax, BioPort). Its main constituent is the PA antigen. The vaccine is administered in six doses at 0, 2, and four weeks followed by booster immunizations at 6, 12 and 18 months. Subsequent boosters are given annually. A similar vaccine was developed in the UK and is administered in 4 doses at 0, 3 and 6 weeks followed by a booster dose at 7.5 months and subsequent yearly booster injections.

The often important reactogenicity of these different vaccines has prompted the development of purified recombinant PA subunit vaccines adjuvanted with Aluminium hydroxide (Biosector, Denmark), which were found to provide high-level and lasting protection against aerosol challenge with multiple *B. anthracis* isolates in rabbits and rhesus monkeys.

Live recombinant vaccines using *B. subtilis*, *Salmonella* or vaccinia virus vectors, as well as naked DNA vaccines expressing the PA antigen, still are at an early clinical trial stage.

Finally, the demonstration that spore components offer additional protection in small animal models has led to the development of a dual component vaccine made of recombinant PA added with formaldehyde-inactivated spores. This vaccine, which is in development at the Pasteur Institute, Paris, should enter a dose-escalating Phase I trial in 2005.

7.2. Hepatitis E

7.2.1. Disease burden

Hepatitis E virus (HEV) was first identified in India in 1955, and has since been recognized as the principal cause of acute hepatitis in young adults throughout much of Asia, the Middle East, and northern and western Africa. Viral hepatitis is a disease that has been recognized as a clinical entity since antiquity. Five viruses are known to cause hepatitis in humans. They have been designated hepatitis A (HAV), B (HBV), C (HCV), D (Delta) and E (HEV). Other viruses such as CMV, EBV, HSV, dengue virus, Rift Valley fever virus and adenoviruses also can cause severe hepatitis. HAV infects more than 80% of the population of many developing countries by late adolescence, and also is common in industrialized countries, where outbreaks occur at daycare centres, nursing homes and restaurants where inappropriate food-handling might occur. It still accounts for approximately 55% of acute hepatitis cases in the USA, in spite of the availability of two formalin-killed licensed vaccines for adults and children over 2 years of age (Merck and GSK).

Hepatitis E, like hepatitis A, is transmitted by the faecal-oral route but, in contrast with HAV, less than 10% of children under 10 years of age have antibodies to HEV. In Nepal, where the disease is endemic, 75% of infections occur in people aged 15–34 years. The virus can cause large water-borne epidemics as well as sporadic cases. Most outbreaks have occurred following monsoon rains, heavy flooding, faecal contamination of well water, or massive surges of untreated sewage into city water treatment plants. The zoonotic origin of HEV is suspected, as monkeys, rats, cattle, sheep, goats, ducks and especially pigs are susceptible to infection with HEV. Recent evidence suggests that there is a low prevalence of HEV in several industrialized countries, for example Italy and Spain, where HEV may cause sporadic illness or unapparent infections. The low amount of intact HEV particles present in patient stools accounts for the generally lower secondary attack rate of person-to-person transmission of hepatitis E (2%) as compared with hepatitis A (10–20%). There is no evidence for sexual transmission or for transmission by blood transfusion. Vertical transmission of HEV from mothers to their infants has been reported.

In most cases, HEV infection remains asymptomatic. When symptomatic, the disease is characterized by jaundice, like hepatitis A: the two diseases are virtually indistinguishable and show similar pre-icteric and icteric phases. HEV is the first cause of hospitalization for jaundice in Nepal. The disease is self limited and most patients recover completely without complications or sequelae. Viremia is thought to last between 14 and 28 days. Both IgG and IgM antibody responses are detected soon after infection, with peak antibody titres at 2–4 weeks. No chronic or carrier state has been demonstrated after HEV infection. A low mortality rate (0.5–4%) is associated with HEV infection, with the dramatic exception of third-trimester pregnant women who can develop fulminant hepatitis with a case fatality rate of 10–42%. An Ethiopian study found that 35% of HEV-infected hospitalized pregnant women had premature delivery.

7.2.2. Virology

HEV is a 27–34 nm non-enveloped icosahedral virus with a single-stranded, positive-sense RNA genome. The virus had wrongly been classified into the family *Caliciviridae*, but now is classified as a separate, unclassified virus. Although at least four major genotypes can be distinguished, the virus seems to exist as a single serotype. The 7.5 kb RNA comprises three overlapping ORFs: ORF1 which codes for proteins involved in viral genome replication and viral protein processing, ORF2 which codes for one or more capsid proteins, and ORF3 for a small nonstructural phosphoprotein. The ORF2 protein carries the neutralization epitopes.

7.2.3. Vaccines

Although HEV does not replicate well in cell culture, animal models have been developed (cynomolgus macaques, chimpanzee, rat, tamarin monkey). Cynomolgus monkeys were successfully protected against challenge by passive immunization with convalescent serum or active immunization with an ORF2-based vaccine. At present, no commercially available HEV vaccines exist. However, several studies for the development of an effective vaccine against hepatitis E are in progress.

- A 56 kD recombinant ORF2 protein produced in insect cells infected with recombinant baculovirus and adjuvanted with alum was used to vaccinate rhesus monkeys against different strains of hepatitis E. The ORF2 protein spontaneously assembles into VLPs, whose oral administration to monkeys induced serum IgG, IgA and IgM anti-HEV response. The vaccine did not provide protection against infection, but protected the monkeys from the symptoms of disease. The 56 kD antigen was found to be safe and immunogenic in Phase I trials conducted by WRAIR and GSK in the USA then in Nepal when given as a three-dose regimen at 0, 1 and 6 months. The vaccine has recently undergone Phase II/III efficacy trials in Nepal; results of the trial are pending.
- The direct intramuscular injection of purified plasmid DNA containing the full-length ORF2 of HEV was shown to elicit a prolonged humoral immune response in 80% and 100% of two separate groups of vaccinated mice, respectively.
- Recently, swine HEV was found to be immunologically cross-reactive with human HEV and might thus prove useful as an attenuated “Jennerian” vaccine for immunization against human hepatitis E.

7.3. Leptospirosis

Leptospirosis is a ubiquitous zoonosis caused by one of the pathogenic spirochetes of the family *Leptospiraceae*. Humans are infected from carrier animals, primarily feral and peri-domestic rodents, especially rats, and domestic farm animals (dogs, pigs and cattle). Transmission occurs from occupational or recreational immersion in contaminated water or by direct occupational contact with carrier animals. Seasonal rises in incidence and epidemics occur in wet spring and summer seasons and at harvest time, when the population of rodents is highest. Epidemics also follow natural disasters, such as floods and earthquakes, which drive rats out of sewers. The disease, which is not communicable, is usually a mild but incapacitating febrile illness with frequent transient impairment of renal function, followed by spontaneous recovery within 6 to 12 weeks. More severe cases with aseptic meningitis, uveitis, and liver, kidney or heart failure presumably occur in developing countries, but reliable statistics on the incidence of the disease are lacking.

A killed vaccine is available for humans in Asia (China, Japan and Viet Nam).

7.4. Plague

7.4.1. Disease burden

Although epidemics of urban plague have dramatically waned, plague still is a significant public health problem, especially in Africa, Asia, and South America. Worldwide, there are 1000 to 2000 cases of plague reported each year, with a fatality rate between 5% and 15%. Historically, the first major epidemic of plague was recorded in China in 224 BC. Plague in Europe came in long-lasting pandemic waves. The first documented pandemic, the Justinian plague, killed several million people in the Byzantine Empire during the 6th to 8th centuries. The second pandemic, the "Black Death", caused some 25 million deaths (more than 30% of the European population) starting in the mid 14th century and culminating with the Great Plague of London in 1665. The third pandemic started in China in the middle of the 19th century and caused 10 million deaths in India alone.

Plague is a zoonosis. Plague bacteria persist in the environment as the result of a stable and constant rodent-flea infection cycle, causing a fatal disease in murines and sciurides. However, when the rodent population is reduced as a consequence of the disease or of rodent control measures, humans as well as other warm-blooded mammals serve as alternative hosts.

The bubonic form of the disease typically is transmitted from rodents through the bites of infected fleas, usually the rat flea, *Xenopsylla cheopsis*. It is characterized by the development of swollen and excruciatingly painful lymph nodes called *buboes* that often are located in the inguinal region (*Bubo* in Greek means groin) and may be associated with gastrointestinal symptoms and diarrhoea. Severe peripheral tissue necrosis and gangrene are one of the manifestations of the disease, reminiscent of the medieval epithet "Black Death". Septicemic plague is an acute toxic illness with elevated fever, malaise and gastrointestinal disturbances which often leads to systemic inflammatory response syndrome such as disseminated bleeding, respiratory distress syndrome, shock and organ failure, with a 20–40% case–fatality rate.

In some instances, bacteraemic spread of plague bacilli to the lungs leads to the development of the pulmonary form of the disease, which can then be transmitted from person-to-person by airborne respiratory droplets and results in most feared epidemics of pneumonic plague associated with a case–fatality rate of 95–100% if untreated.

Recently plague has attracted a considerable attention because of its possible use as an agent of biological warfare or bioterrorism.

7.4.2. Bacteriology

The etiologic agent of plague is *Yersinia pestis*, a gram-negative bacterium that belongs to the family *Enterobacteriaceae*. *Y. pestis* has a number of virulence genes that appear to be primarily located on three plasmids; the low calcium response (Lcr) plasmid which encodes a number of *Yersinia* outer membrane peptides (Yops), the Pesticin plasmid which encodes a surface-bound plasminogen activator with potent fibrinolytic activity, and the Tox plasmid which carries the *caf* operon that regulates the synthesis of the F1 capsular antigen, a 17 kD polypeptide. Among known virulence determinants are the pH6 antigen, a fibrillar adhesin induced by low pH conditions, as well as the F1 capsular antigen and a number of Yops such as YopE, YopH, and the V antigen, a secreted protein that down-regulates the production of IFN-y and TNF-α by the host.

7.4.3. Vaccines

Killed whole-cell plague vaccines have been in use since their initial development by Haffkine (1897), but only the Commonwealth Serum Laboratories in Australia are still manufacturing a heat-killed *Y. pestis* vaccine.

A live, attenuated vaccine was developed in the former Soviet Union using an attenuated strain of *Y. pestis* (EV76) but this vaccine is no longer available.

At this time, subunit vaccines are being developed that include recombinant F1 antigen and V antigen. The combination of the two antigens together provided better protection against high-dose challenge in mice, including inhalation challenge, than each component alone. The F1+V subunit vaccine is now in advanced clinical development. A similar vaccine is based on an F1-V fusion protein produced in recombinant *E. coli* and adjuvanted with alhydrogel (DynPort Vaccine). The vaccine provided 80% protection against aerosol challenge in nonhuman primates and is now in clinical trials.

A candidate DNA vaccine expressing a secreted form of the V antigen that provided protection against lethal intranasal challenge in mice also is in development at the University of Massachussetts Medical School (USA).

7.5. Rabies

7.5.1. Disease burden

The first written description of rabies can be found in the *Babylon Codex*, 23 centuries BC. The disease thrived from ancient times to the end of the 19th century when, in 1885, Louis Pasteur succeeded in the first prevention of human rabies by postexposure vaccination. One hundred and twenty years later, this zoonotic viral disease still continues to plague humankind, especially in developing countries in Africa, South and South-East Asia and, to a lesser extent, Latin America. According to WHO, more than three billion people are at risk for rabies in over 85 countries and territories worldwide, and about 50 000–60 000 human deaths from rabies are estimated to occur annually, even though effective vaccines for postexposure prophylaxis (PEP) are available and over 10 million individuals actually receive rabies PEP each year. The true disease burden of rabies is largely under-estimated, especially in Africa. For example, in Tanzania, incidence of human rabies mortality was estimated to be about 1500 deaths per year (4.9 deaths/100 000 persons) when calculated from active surveillance data on bite incidence, but national statistics reported only 193 deaths per year (0.62/100 000).

In Central and Eastern Europe and North America, most human rabies cases are associated with contacts with wild carnivore species (e.g. foxes, raccoons, dogs, skunks and coyotes), which are the main reservoir of rabies virus, or with bats whose role has been considerably increasing in recent years. In contrast, in canine rabies-endemic regions with large stray-dog populations, the overwhelming majority (95–98%) of cases of human rabies occurs following dog bites. Control of disease in these settings often is hampered by cultural, social and economic realities (Buddhist and Hindu ethics restrain culling of the canine population; India and Thailand have prohibited the killing of stray dogs by municipalities). Reduction of the stray-dog population by capture and euthanasia is ineffective in the long term and there is today no convenient tool for dog reproduction control. In addition, vaccines for human PEP too often are not available or not affordable. All these factors are responsible for the high disease burden in these countries.

The risk of rabies to travellers depends on the country of destination, exposure to animals (especially dogs, but also cats, skunks, raccoons, and bats), conditions of travel and length of stay. Long-stay travellers and expatriates may have an incidence of animal bites equivalent to that in the local population, which can be as high as 100–200 suspect bites per 100 000 person-years.

Animal bites introduce rabies virus into tissue and muscle rich with nerve endings by which the virus can invade the peripheral nervous system, reach the neural ganglion and travel through the spinal cord to the brain. This process usually requires weeks or months, depending upon the distance from the bite site to the brain. Replication of the virus in the brain causes hydrophobia, hallucinations and aggressive behavior, eventually followed by paralysis, coma and death. Meanwhile the virus can spread to salivary glands, from where it will eventually be transmitted to a new host, but also to the skin, cornea, nasal and intestinal mucosa and other organs, including the kidneys.

In rare occasions, the virus was shown to be transmitted by aerosols in caves populated by rabies-infected bats. Cases of transmission by organ transplant (cornea as well as solid organs) also have been reported.

7.5.2. Virology

Rabies virus is an enveloped bullet shape virus, which belongs to the genus Lyssavirus in the family *Rhabdoviridae*. The negative sense RNA genome encodes a small leader sequence followed by the N (nucleocapsid), P, M (membrane), G (envelope glycoprotein) and L (replicase) proteins which are translated from five capped and polyadenylated monocistronic mRNAs, each encoding one of the five viral proteins. Seven virus genotypes have been described, including several bat lyssaviruses (Lagos bat virus, genotype 2, Central Africa Mokola virus, genotype 3, South Africa Duvenhage virus, genotype 4, European bat lyssaviruses, genotypes 5 and 6, and Australian bat lyssavirus, genotype 7). All these viruses, except Lagos bat, can be pathogenic for humans. The G glycoprotein, which forms spikes at the surface of the virion, is responsible for attachment to viral receptors and bears the neutralization epitopes. There seems to be little cross protection between genotypes 1 (rabies virus) and genotypes 2 (Lagos bat) and 3 (Mokola virus).

7.5.3. Vaccines

Several effective inactivated rabies vaccines are currently available worldwide. Nervous tissue-derived vaccines are prepared from rabid sheep, goat (Semple vaccines in Asia) or suckling mouse brains (Fuenzalida Palacios vaccine in South America) by phenol-inactivation. These vaccines are highly reactogenic due to contamination with brain proteins. This type of vaccine is still unfortunately manufactured and used in South-East Asia, but the number of countries doing so has been decreasing during the past 10 years in accordance with the WHO recommendations to replace them by cell-cultured vaccines.

The new generation of rabies vaccines is made of inactivated virus grown in cell cultures, human diploid fibroblasts (HDCV, Chiron Behring, Sanofi-Pasteur), fetal rhesus cells (Bioport), primary Syrian hamster kidney cells (PHKCV, local manufacturers), chick embryo cells (PCECV, Chiron Behring) and Vero cells (PVRV, Sanofi-Pasteur). Manufactured mainly in industrialized countries but distributed worldwide, these vaccines – which are inactivated with b-propionolactone or formalin – are safe and immunogenic.

A number of cell-culture based rabies vaccines are being developed in China and India on Vero cells, human diploid cells (HDC), or duck embryo cells. These vaccines however have not yet been prequalified by WHO and may require further assessment in terms of safety and efficacy before they can be traded internationally. Of importance for the supply of rabies vaccine is the use of the intradermal route schedule which reduces the number of vaccine vials and thereby the cost of PEP by up to 80% (US$ 5–10 for vaccine alone).

Oral vaccination of wildlife to prevent the spread of rabies, which relies on the use of baits containing either live-attenuated rabies virus strains or a live vaccinia virus recombinant expressing the gene for the G protein (VV-G), was implemented with great success in Europe. Field vaccination with VV-G has begun in the Eastern USA.

7.5.4 Passive immunization

It is well known that rabies PEP with vaccine alone is not always sufficient, especially in cases of severe exposure (category 3) where concommittant passive immunization with rabies immunoglobulins (RIG) is strongly recommended. The worldwide shortage of RIG thus represents a real public health threat and a new challenge. According to a WHO survey, the vast majority of the 7.6 million PEP undertaken worldwide in 2000 were without RIG because of their unavailability and/or unaffordability. Two types of RIGs are currently manufactured: a) human IgGs (HRIG) which are used in industrialized countries and Thailand, and b) equine IgGs (ERIG) which are either pepsin-digested (Thai Red Cross, India, and formerly Berna, Chiron, Sanofi-Pasteur) or highly purified (heat treatment and chromatography purification). Highly purified ERIGs were developed by Sanofi-Pasteur in order to meet the highly stringent regulatory environment in Europe and North America.

It is to be noted that, in view of the current cost of HRIG (over US$ 100) and ERIG (over US$ 40), the price of a cocktail of 2–3 mouse monoclonal antibodies (MRIG) might be highly competitive (expected price less than US$ 10). Interestingly, whereas ERIG can in some experimental conditions interfere with vaccine activity, MRIG were not found to be suppressive. Extensive clinical trials are needed to validate the threshold of MRIG potency for their use as adjuvant of vaccines for curative treatment. China, India and the Philippines have already expressed strong interest in developing such a technology.

8. Viral cancers

Viruses linked to cancers in humans are the Epstein-Barr virus (EBV), associated with lymphomas and nasopharyngeal cancer, hepatitis B virus (HBV) and hepatitis C virus (HCV), both associated with cancer of the liver, human papillomaviruses (HPV), associated with cancer of the cervix, human T lymphotropic virus type 1 (HTLV-1) and type 2 (HTLV-2), associated with adult T-cell leukemia and with hairy-cell leukemia, respectively, and human herpesvirus 8 (HHV-8), associated with Kaposi sarcoma. No vaccine exists against these viruses except HBV. This chapter will describe vaccines under development against EBV, HCV and HPV.

8.1. Epstein-Barr virus

In the 1950s, Denis Burkitt described the existence of B-cell lymphomas in 2–14 year-old African children from malaria endemic areas. In 1964, continuous B-lymphocyte cell lines derived from these tumors were found by Epstein and Barr to spontaneously release a herpesvirus. It was Gertrud and Werner Henle who demonstrated that the Epstein-Barr virus (EBV) is ubiquitous in the human population where it is usually the cause of infections that are not apparent though it may cause infectious mononucleosis. The more severe, albeit rare, result of EBV infection is malignant transformation and cancer development in various forms, including Burkitt's lymphoma and nasopharyngeal carcinoma, one of the most common cancers in China.

8.1.1. Disease burden

The primary site of Epstein-Barr virus (EBV) infection is the oropharyngeal cavity. Children and teenagers are commonly afflicted usually after oral contact, hence the name "kissing disease". Based on serology, about 95% of the world adult population has been infected with EBV and, following primary infection, remains lifelong carriers of the virus. In developed countries, exposure to EBV occurs relatively late: only 50–70% of adolescents and young adults are EBV seropositive. About 30% of the seronegative group will develop infectious mononucleosis as a result of primary EBV infection. The disease is characterized by fever, sore throat, generalized lymphadenopathy, splenomegaly, intense asthenia, hyper-lymphocytosis (>50%) with atypical lymphocytes and elevated transaminase levels. In developing countries, EBV antibodies are acquired early in life and the disease is mostly asymptomatic.

EBV is associated with Burkitt's B-cell lymphoma and nasopharyngeal carcinoma. Burkitt's lymphoma (BL) is a malignant form of tumor associated with EBV that is endemic to central parts of Africa and New Guinea with an annual incidence of 6–7 cases per 100 000 and a peak incidence at 6 or 7 years of age. The epidemiological involvement of EBV in Burkitt's lymphoma is based on the recognition of the EBV viral genome in tumor cells, associated with an elevated antibody titre against EBV

viral capsid antigen (VCA). The highest prevalence of BL is found in the "lymphoma belt," a region that extends from West to East Africa between the 10th degree north and 10th degree south of the equator and continues south down the Eastern coast of Africa. This area is characterized by high temperature and humidity, which is probably the reason why an association of malaria with BL was suspected at one time. In African countries such as Uganda, in the lymphoma belt, the association of BL with EBV is very strong (97%), whereas it is weaker elsewhere (85% in Algeria; only 10–15% in France and the USA).

Nasopharyngeal cancer (NPC) incidence rates are less than 1 per 100 000 in most populations, except in populations in southern China, where an annual incidence of more than 20 cases per 100 000 is reported. Isolated northern populations such as Eskimos and Greenlanders also show high incidence. There is a moderate incidence in North Africa, Israel, Kuwait, the Sudan and parts of Kenya and Uganda. Men are twice as likely to develop NPC as women. The rate of incidence generally increases from ages 20 to around 50. In the USA, Chinese-Americans comprise the majority of NPC patients, together with workers exposed to fumes, smoke and chemicals, implying a role for chemical carcinogenesis. Studies related to nutrition and diet have shown an association between eating highly salted foods and NPC. Vitamin C deficiency at a young age also may be a contributing factor. Finally, a study of HLA haplotypes revealed a genetically distinct subpopulation in southern China, with an increased frequency of haplotype A-2/B-Sin-2 which may account for the higher disease incidence in the area.

Recent studies have shown that EBV also is associated with B-cell malignancies such as Hodgkin's lymphoma (HL) and lymphoproliferative disease in immunosuppressed patients, as well as with some T-cell lymphomas and other epithelial tumors such as gastric cancers. These tumors are characterized by the presence of multiple extrachromosomal copies of the viral genome in tumor cells and the expression of part of the EBV genome.

8.1.2. Virology

EBV, together with HHV-8 (Kaposi sarcoma-associated virus), belongs to the genus Lymphocryptovirus, in the subfamily *Gammaherpesvirinae,* family *Herpesviridae*. These are complex enveloped DNA viruses, which multiply in the nucleus of the host cell *(see 5.3).* EBV infects resting human B-lymphocytes and epithelial cells, multiplies in the latter and establishes latent infection in memory B-lymphocytes. Thus, infected individuals may produce virions, carry virus-specific CTLs, produce EBV-specific antibody, and yet harbor latently infected memory B-cells. These maintain the latent EBV genome as an episome that expresses only part of its genetic information, including EBV nuclear antigens EBNA-1 (a latent DNA replication factor), EBNA-2 (a transcriptional activator) and EBNA-3A and -3C (involved in the establishment of latency), together with integral membrane proteins LMP-1 and LMP-2 which play major roles in maintenance of latency and escape from the immune response of the host. Latently infected cells do not produce the B7 coactivator receptor and, therefore, are not killed by CTLs. When peripheral blood from an infected individual is cultured, latently infected B-cells begin to replicate and yield immortalized progeny lymphoblasts that can be indefinitely propagated in the laboratory.

The major EBV external surface glycoprotein is a 350 kD antigen, gp350/220, which binds the CD21 receptor on B-cells. Another envelope glycoprotein, gp42, is responsible for the fusion between the virus envelope and the host cell membrane. The EBV genome, a 172 kbp linear double-stranded DNA molecule, becomes circular for replication and latency. Viral capsid antigens (VCA) are late gene products.

8.1.3. Vaccines

The development of an EBV vaccine could protect individuals against primary infection and hence presumably reduce the burden of EBV-associated cancers.

The principal target of EBV neutralizing antibodies is the major virus surface glycoprotein gp350/220. Several vaccine candidates based on gp350/220 have been developed. Live recombinant vaccinia virus vectors have been used to express the gp350/220 antigen and were found to confer protection in primates and elicit antibodies in EBV-negative Chinese infants.

Soluble recombinant gp350/220 produced in CHO cells was found to be safe in humans but needed strong adjuvants to elicit acceptable immunogenicity (co-development by MedImmune, GSK and Henogen). Phase II clinical trials of this candidate vaccine are under way.

Clinical trials of an EBNA-3A peptide are being conducted in Australia.

8.2. Hepatitis C

The majority of the worldwide hepatitis burden, with subsequent chronic hepatitis, cirrhosis and liver cancer is due to hepatitis virus B (HBV), which kills 4000 to 5000 Americans each year, and about 1.2 million people worldwide. Approximately 350 million people have chronic hepatitis B infection, with endemic areas primarily in Africa and Asia. Fortunately, the global burden of hepatitis B should eventually decrease as affordable recombinant subunit vaccines based on the surface antigen of the virus (HBsAg) and effective control strategies are deployed to control the disease on a global basis. Infants in developing countries begin their HBV immunization at birth; this has resulted in dramatic reductions in virus transmission in high-risk populations, and in decrease in incidence of liver cancer, as seen in China (Province of Taiwan).

Other viral hepatitis, initially regrouped under the designation “Non A-non B” hepatitis and against which there is still no vaccine, include hepatitis C and hepatitis E. The search for a possible “non A-E” virus which would be responsible for the 4% acute cases of hepatitis of undiagnosed origin led to the successive identification of the HGV/GBV-C, TTV and SEN-V viruses, none of which appear to be the right candidate.

8.2.1. Disease burden

Hepatitis C has been compared to a "viral time bomb". WHO estimates that about 180 million people, some 3% of the world's population, are infected with hepatitis C virus (HCV), 130 million of whom are chronic HCV carriers at risk of developing liver cirrhosis and/or liver cancer. It is estimated that three to four million persons are newly infected each year, 70% of whom will develop chronic hepatitis. HCV is responsible for 50–76% of all liver cancer cases, and two thirds of all liver transplants in the developed world. Disease prevalence is low (< 1%) in Australia, Canada and northern Europe, about 1% in countries of medium endemicity such as the USA and most of Europe, and high (>2%) in many countries in Africa, Latin America and Central and South-Eastern Asia. In these countries, prevalence figures between 5% and 10% are frequently reported. The extremely high seroprevalence of HCV in the Nile delta of Egypt was found to increase with age from 19% in those 10–19 years of age to about 60% in 30 year-old persons, and is thought to be the major cause of the high prevalence of liver cirrhosis in the country.

Current estimates in the USA are that 3.9 million Americans are chronically infected with HCV, with prevalence rates as high as 8–10% in African Americans. Haemodialysis patients, haemophiliacs, drug addicts and people transfused with blood before 1990 are particularly affected by the disease. Injectable drug use remains the main route of transmission, accounting for nearly 90% of new HCV infections. Sexual transmission is thought to be relatively unfrequent.

Mother-to-child HCV transmission has been widely documented. The risk of perinatal infection ranges from 3–15% in different populations. Transmission is believed to occur in utero, as a consequence of a high viral load in the mother. However, correlates of transmission remain to be defined and targeted studies are needed to provide adequate counseling to HCV-infected pregnant women and to identify possible preventive measures.

HCV infection is asymptomatic or paucisymptomatic in 90% of cases. In contrast with viral hepatitis A or B, jaundice is relatively rare, and fulminant hepatitis forms are rarely observed. In 50–80% of adult cases, the immune system is nevertheless unable to eliminate the virus and the disease becomes chronic, with persistent asthenia and vascularitis, porphyria cutanea, glomerulonephritis and others. The patients usually show elevated transaminase levels and mixed cryoglobulinemia. Chronic hepatitis C disease is the first cause of liver transplantation in developed countries. Furthermore, about 20–50% of chronically infected persons will eventually develop cirrhosis or cancer of the liver. Incidence rates of hepatocellular carcinoma among patients with HCV-related cirrhosis is highest in Japan. It has been estimated that only about 50% of HCV-infected persons are diagnosed in most developed countries and that two-thirds of them need to undergo antiviral treatment.

8.2.2. Virology

HCV belongs to the genus Hepacivirus in the family *Flaviviridae*. There are 6 HCV genotypes and more than 100 subtypes. In addition, HCV, very much like HIV, is characterized by the continuous emergence of virus variants, thus making a moving target for vaccine design. Like other flaviviruses, HCV is an enveloped virus with an icosahedral capsid that contains a 9.6 kb-long, single-stranded, positive sense genomic RNA. The virus does not grow in cell culture; this made its initial

identification in 1989 a molecular biology *tour de force* and does not facilitate the selection of attenuation mutants or the titration of neutralizing antibodies. Its envelope contains two glycoproteins, E1 and E2, which form heterodimers at the surface of the virion. The genomic RNA is translated into a viral polyprotein which is cleaved by cellular proteases to generate the capsid protein (C), the two glycoproteins E1 and E2, a small protein the role of which is unclear (p7), viral proteases NS2 and NS3 and nonstructural proteins NS4A and 4B and NS5A and 5B, which are required for viral RNA replication. The putative HCV receptor has recently being identified as protein CD81.

8.2.3. Vaccine development

The development of an HCV vaccine is an obvious necessity as an overall 50% of treated patients do not experience significant long-term benefits from the current pegylated interferon and ribavirin-based combination therapy. Such a development, however, meets with many obstacles. Chimpanzees remain the only animal model for HCV infection, but they are an endangered species and difficult to work with because of high costs and other restrictions. Even though HCV infection generates antibodies, none of these seem capable of resolving the infection. One reason might be that the virus does not appear to circulate as free virions but is always found in association with lipoprotein particles or immune complexes. Recovery from acute hepatitis is typically associated with broad and early class II-CD4+ responses and class I-CD8+ responses to HCV. A vaccine, to be successful, will presumably need to elicit strong CTL and T helper cell responses. It also will have to face high variability of the virus favouring immune evasion.

No vaccine is yet available. Several vaccine approaches, essentially therapeutic, are currently in development.

- Native heterodimer complexes comprising both envelope glycoproteins E1 and E2 have been produced in CHO cells and used as a subunit vaccine added with the MF59 adjuvant (Chiron). The vaccine elicited high titre antibodies and CD4+ T-cell responses and provided nonsterile protection against challenge in 50% of the vaccinated chimpanzees. Phase I trials of this vaccine are in progress.
- A vaccine candidate based on recombinant E1 in alum, developed by Innogenetics in Europe, has reached Phase II trials in non-responders to interferon treatment. Results showed that it is well tolerated and seems to slow down the progression of liver fibrosis, but no changes in plasma HCV loads were detected, despite decreased antigen levels in the liver and strong antibody and cellular responses to E1. An additional Phase II randomized study should be completed in 2005.
- VLPs (HCV-LPs) were produced in insect cells using a recombinant baculovirus expressing the cDNA of the HCV structural proteins C, E1 and E2. This approach is attractive because particulate structures are more immunogenic than soluble proteins. HCV-LPs resemble the putative HCV virions and induce strong HCV-specific immune responses in mice and baboons, including antibodies to HCV structural proteins and IFN-γ CD4+ and CD8+ T-cell responses. The immunogenicity of the HCV-LPs was only marginally enhanced by the addition of CpG oligonucleotides or the ASO1 formulation as adjuvants.

- An immunostimulatory complex formulation (ISCOMATRIX) with the HCV core antigen has been studied by CSL in Phase II trials in Australia, in collaboration with Chiron.
- HLA-A2-restricted core epitope peptides formulated with influenza virosomes as carriers are being developed by Berna/Pevion in Switzerland for both therapeutic and prophylactic vaccine strategies. This formulation is in preclinical studies. To improve the induction of T-cell immunity, the aminoacid sequence of the peptides was modified so as to increase their affinity for the HLA molecule (epitope enhancement).
- Several other vaccine projects are at an early preclinical stage, including HCVACC and Innogenetics in Europe and GenPhar, Epimmune, and Merix in the USA.
- Two Chinese teams also are making significant progress in HCV vaccine research: Fudan University and the National Taiwan University, Taipei, China (Province of Taiwan).
- Finally, an MVA-based live recombinant vaccine expressing three NS protein genes should reach the clinics by the end of 2005 (BioMérieux, France).

8.3. Human papillomavirus

8.3.1. Disease burden

Human papillomavirus (HPV) causes cervical cancer, the second biggest cause of female cancer mortality worldwide with an estimated 240 000 deaths yearly. The prevalence of genital HPV infection in the world is around 440 million, and that of clinical infections about 160 million. Genital HPV infection is extremely common and most often remains subclinical, but a proportion of the infected individuals with low-risk HPV types such as HPV-6 or HPV-11 will develop genital warts, whereas a subset of women with high-risk HPVs such as HPV-16 or HPV-18 will develop preneoplastic lesions of cervical intraepithelial neoplasia (CIN). Low-grade cervical dysplasias are common and most regress spontaneously. In contrast, the minority of lesions that progress to high-grade dysplasias tend to persist and/or progress to carcinomas in situ before becoming invasive cancers. The majority of adenocarcinomas of the cervix and of squamous cell cancers (SCC) of the vulva, vagina, penis and anus are caused by HPV-16 (57% of cases) and HPV-18 (14% of cases), the remaining 30% being due to less prevalent high-risk HPV types (HPV-31, -33, -35, -39, -45,-51, -66, etc.).

About 490 000 cases of cervical cancer are reported each year, nearly 80% of which from developing countries, where neither population-based routine screening (Papanicolau smear test) nor optimal treatment is available; estimated figures are 265 000 cases in South-East Asia, 79 000 in Africa, and 72 000 in Latin America. The highest yearly incidence of cancer of the cervix is found in some countries of Central and South America (e.g. Haiti), southern Africa (e.g. Tanzania), and Asia (India). Epidemiological studies in the USA have reported that 75% of the 15–50 year-old population is infected with genital HPV over their lifetime, 60% with transient infection (antibodies), 10% with persistent infection (detection of DNA), 4% with mild cytological signs, and 1% with clinical lesions.

HPV infection also can lead to papillomas in the oral cavity and in the upper respiratory tract. In HIV-infected individuals, HPV infection appears to cause extensive warts and severe and rapidly progressing disease.

8.3.2. Virology

HPV belongs to the family *Papovaviridae.* These are small nonenveloped icosahedral viruses with an 8 kbp-long double-stranded circular DNA genome. More than 100 different HPV types have been identified on the basis of genomic nucleotide sequence homology, some 40 of which can infect the ano-genital mucosa. The papillomavirus genome comprises early and late genes that encode early proteins E1–E7 and late proteins L1–L2. The early proteins are nonstructural proteins involved in replication and transcription of the genome (E1–E5) or in host cell tumoral transformation (E6 and E7), whereas L1 and L2 are the structural capsid proteins of the virion. The low-grade cervical dysplasias correspond to productively infected cells that actively shed virus, whereas high-grade dysplasias and cancers do not produce virions: viral gene expression in these cells is limited to the E6 and E7 oncogenes that are transcribed from randomly integrated viral DNA. The E7 protein is thought to induce cell proliferation and disrupt the cell cycle regulation by inactivation of the Rb family proteins, whereas E6 blocks cell apoptosis by directing the p53 tumor suppressor protein to the proteasome.

8.3.3. Vaccines

Prophylactic HPV vaccine candidates are based on recombinant capsid protein L1 and aim to elicit neutralizing antiviral antibodies to protect against infection, while therapeutic vaccine candidates are based on viral oncogenic proteins E6 and E7, and aim to induce cell-mediated immune responses to eliminate the transformed tumor cells.

8.3.3.1. Preventive vaccines

The most advanced and promising approach for a prophylactic vaccine involves the use of noninfectious virus-like particles (VLPs) which self-assemble spontaneously from pentamers of the L1 capsid protein. These VLPs can be produced in baculovirus-infected insect cells or in yeast. They induce high titres of virus-neutralizing antibodies even in the absence of an adjuvant. In preclinical studies, vaccination of animals resulted in excellent protection from homologous virus challenge, and passive transfer of antibodies from the vaccinated animals also conferred protection.

Two prophylactic vaccine candidates are at the level of Phase III clinical evaluation. GSK is focusing on a bivalent HPV-16,-18 VLP vaccine candidate, based on baculovirus technology, and Merck is developing a tetravalent vaccine based on VLPs from HPV-6, -11, -16, and -18, using yeast-recombinant technology. A pilot efficacy trial of the monovalent HPV-16 vaccine showed that after 17 months follow-up, none of the 768 vaccinated young women acquired persistent HPV infection, whereas 41 of the 765 placebo recipients became persistently infected with HPV-16, 5 of whom were at CIN-1 grade and 4 at CIN-2/3 grade. A comparable study by GSK has reported similar results with 100% efficacy against HPV-16 and -18 persistent infections. A Phase III multicentre clinical trial of the tetravalent vaccine has been launched by Merck to define efficacy against HPV-6/11-related genital warts, HPV-6/11/16/18-related CIN-1 lesions, and HPV-16/18-related CIN 2/3 lesions. The VLP vaccines are expected to be on the market by 2007.

A recombinant attenuated *Salmonella typhimurium* that expresses a HPV-16 L1 capsid gene whose codon usage was optimized to fit with the most frequently used codons in *Salmonella* was engineered at the University of Lausanne, Switzerland, and found to induce high titres of HPV neutralizing antibodies in mice after a single nasal or oral immunization with live bacteria. Testing of the vaccine in human volunteers is at the planning stage.

8.3.3.2. Therapeutic vaccines

Therapeutic vaccine candidates also have been developed, several of which have undergone Phase I/II clinical evaluation.

- A live recombinant vaccinia virus expressing modified versions of the E6 and E7 genes from HPV-16 and -18 (TA-HPV) has been tested by Xenova in two open-label Phase IIa trials in women with high grade vulvar intraepithelial neoplasias (VIN). A single immunization with TA-HPV induced at least 50% reduction in lesion size in 44% of the vaccinated patients. An additional study evaluated the combination of TA-HPV with TA-CIN, a recombinant fusion protein made up of the L2, E6 and E7 proteins of HPV-16, produced in *E. coli*. Three immunizations with TA-CIN followed by a single immunization with TA-HPV resulted in 23% of the patients experiencing a >50% reduction in VIN lesion size.
- Another recombinant bacterial fusion protein of HPV-16 E6 and E7 formulated with the ISCOMATRIX adjuvant has been made by CSL and shown to elicit good immune responses in a Phase I study.
- Transgene is developing a MVA-based vaccine that expresses modified HPV-16 E6 and E7 proteins, as well as the IL-2 cytokine. The vaccine is aimed at treating cervical as well as ano-genital dysplasias. In an initial Phase II clinical trial in women with CIN2/3, 43% of the patients receiving the highest dose of the vaccine showed clinical improvement within 6 weeks. A second trial is now under way, using this high dose in 18 women with CIN2/3 who will be followed for a 6-month period.
- Stressgen has conducted a number of Phase II clinical trials with a fusion protein made of E7 and heat shock protein (HspE7). In a Phase II study on 133 patients with anal dysplasia, there was no difference in adjudicated pathological response between vaccine and placebo recipients, although a significant effect was noted by the treating physician in "global assessment" scoring. The HspE7 vaccine was also shown to induce a 40% response rate within 8 weeks in a trial in 21 women with high grade dysplasia. Elucidation of the full extent and duration of the clinical benefit will require additional long-term follow-up.
- Finally, Zycos Inc. (now MGI Pharma) is developing a DNA plasmid-based therapeutic vaccine which, in a Phase II study, provided resolution of 43% pre-cancerous lesions caused by HPV in vaccinated women as compared to 23% in placebo recipients.

Medigene, in partnership with Schering AG, has developed a "chimeric" VLP vaccine (CVLP) using L1 or L2 recombinant proteins fused to modified E7 or E2 oncogenic antigens. This technology allows the combination of both prophylactic and therapeutic components in the same immunogen. The safety of these vaccine candidates has been successfully tested by Medigene but their reported immunogenicity and efficacy were unsatisfactory.

The World Health Organization has managed cooperation with its Member States and provided technical support in the field of vaccine-preventable diseases since 1975. In 2003, the office carrying out this function was renamed the WHO Department of Immunization, Vaccines and Biologicals.

The Department's goal is the achievement of a world in which all people at risk are protected against vaccine-preventable diseases. Work towards this goal can be visualized as occurring along a continuum. The range of activities spans from research, development and evaluation of vaccines to implementation and evaluation of immunization programmes in countries.

WHO facilitates and coordinates research and development on new vaccines and immunization-related technologies for viral, bacterial and parasitic diseases. Existing life-saving vaccines are further improved and new vaccines targeted at public health crises, such as HIV/AIDS and SARS, are discovered and tested *(Initiative for Vaccine Research).*

The quality and safety of vaccines and other biological medicines is ensured through the development and establishment of global norms and standards *(Quality Assurance and Safety of Biologicals).*

The evaluation of the impact of vaccine-preventable diseases informs decisions to introduce new vaccines. Optimal strategies and activities for reducing morbidity and mortality through the use of vaccines are implemented *(Vaccine Assessment and Monitoring).*

Efforts are directed towards reducing financial and technical barriers to the introduction of new and established vaccines and immunization-related technologies *(Access to Technologies).*

Under the guidance of its Member States, WHO, in conjunction with outside world experts, develops and promotes policies and strategies to maximize the use and delivery of vaccines of public health importance. Countries are supported so that they acquire the technical and managerial skills, competence and infrastructure needed to achieve disease control and/or elimination and eradication objectives *(Expanded Programme on Immunization).*

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