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MEETING REPORT

Threat of infection: Microbes of high pathogenic potential – strategies for detection, control and eradication

Abstract

Infectious diseases due to microbes of high pathogenic potential remain a constant and variable threat for human and animal health. The emergence of new diseases or the re-emergence of diseases that were previously under control complicates the situation to date. Infectious disease research, which has undergone a dramatic progress in understanding disease mechanisms such as host–pathogen interactions, is now focusing increasingly on new strategies for prevention and therapy. Significant progress has been achieved in the development of delivery systems for protective heterologous protein antigens and in veterinary vaccinology. A landmark of infectious diseases research is the chemical synthesis of genomes, a major new field of research referred to as “synthetic biology”, that to date has resulted in the chemical synthesis of the poliovirus and of phage ϕ X174 genomes and their expression as infectious viruses. On the molecular level the evolution of pathogens and mechanisms of genome flexibility, which account for several pathogenic properties of infectious agents, have received increased attention. Bacterial toxins are an additional threat to human health and their interference with host cells and cellular functions is receiving more attention.

Keywords: Infectious disease; Vaccination strategies; Eradication; Bacterial evolution; Synthetic biology; Toxins

Introduction

This report highlights some of the lectures that were presented during the international symposium ‘Threat of infection: Microbes of high potential – strategies for detection, control and eradication’ in July 2004 in Würzburg (Germany). This meeting was organized by the German Academy of Natural Scientists Leopoldina (Deutsche Akademie der Naturforscher Leopoldina, Halle, Germany), the Académie des Sciences (Paris, France) and the Research Center for Infectious Diseases of the University of Würzburg together with other scientific societies (Germany). The 35 speakers and two poster sessions covered many aspects of infectious diseases, including political and social aspects. At the molecular and cellular levels, the action of virulence factors and toxins, the potential applications of RNA interference and mechanisms of genome flexibility were discussed. The reservoirs and transmission of pathogens, novel detection methods and the exploitation of mechanisms of host–pathogen interaction were further discussed for the development of novel preventive and therapeutic approaches. A comprehensive summary with papers of each of the speakers who had been invited for an oral presentation during this symposium

will be published soon in the series ‘Nova Acta Leopoldina’ (<http://www.leopoldina.uni-halle.de>).

Strategies for prevention and control of infectious diseases

Infectious diseases are the biggest killer of children and young adults, accounting for more than 13 million deaths a year and as many as one in two deaths in developing countries. Almost 90% of deaths from communicable diseases are caused by six diseases: acute respiratory and diarrhoeal infections, AIDS, tuberculosis, malaria, and measles. David L. Heymann (WHO, Geneva) reminded that many deaths occur as a result of preventable diseases such as measles, hepatitis B, diphtheria, whooping cough and, most often, because of failure to implement vaccination programs. The burden of infectious diseases is not restricted to developing countries, since previously controlled infectious diseases such as tuberculosis and diphtheria have re-emerged in Europe and other developed areas of the world. High mortality due to infectious diseases is alarming but so too is the fact that large populations in

remote areas of the developing world are at risk of disabling diseases, such as polio, leprosy, lymphatic filariasis, and onchocerciasis. As further reported by David L. Heymann these diseases cause a double economic burden by reducing the work force and undermining the financial security of already impoverished families. Most infectious diseases can be prevented with drugs or vaccines that are already available, but inadequate funding of health care in developing countries or the inability to deliver the antimicrobial drugs and antibiotics to the entire population contributes to the devastating situation (Table 1).

The emergence of severe acute respiratory syndrome (SARS), which is one of 30 diseases newly identified in recent decades, clearly demonstrated that every country is vulnerable and that the economic consequences, exaggerated by public fear of the unknown, can be felt around the world. Today, diseases can be transported from one continent to another in a matter of hours. For these reasons public health care systems have received more attention. David L. Heymann stated that global partnerships and strategies are required that increase the power of epidemic control such as real-time electronic communications.

Prevention and therapy: novel vaccination strategies

In considering novel vaccination strategies against microbes of high pathogenic potential, one has to distinguish between the case of bio-warfare and newly emerging agents with high pathogenic potential. We have to consider that, in the latter case, mass vaccination or even vaccination of high-risk individuals would be unrealistic especially since the overall risk of side effects will exceed the risk of disease. Rapid protection of individuals at risk has to be given high priority. This causes various problems, including rapid diagnosis as well as prompt induction of a highly efficacious host response, both in naive and in infected individuals, in addition to prompt chemotherapy. Therefore, classical vaccination strategies need to be complemented by generic vaccination strategies and immunomodulatory interventions. Such strategies must include both innate and acquired immunity. In his presentation, Stefan H. E. Kaufmann (Max-Planck-Institute for Infection Biology (MPI), Berlin) suggested that the following vaccination strategies against intracellular bacteria deserve consideration: (i) attenuated viable strains, (ii) naked DNA encoding protective antigens and (iii) protective antigens expressed by recombinant viable vectors (bacteria or viruses). Extracellular bacteria are controlled by antibodies – typically opsonizing antibodies – and, hence, vaccination strategies are based on killed

Table 1. Leading infectious killers and examples of microorganisms with high pathogenic potential

Infection or disease	Microorganism
<i>Acute respiratory infections</i>	
	<i>Streptococcus pneumoniae</i>
	<i>Haemophilus influenzae</i>
	Influenza virus
Pertussis	<i>Bordetella pertussis</i>
SARS	SARS coronavirus
	Respiratory syncytial virus (RSV)
Hantavirus pulmonary syndrome	Hantavirus
Tuberculosis	<i>Mycobacterium tuberculosis</i>
<i>Diarrhoeal diseases</i>	
	<i>Shigella dysenteriae</i>
	<i>Salmonella typhi</i>
	<i>Vibrio cholerae</i>
Malaria	<i>Plasmodium falciparum</i>
Measles	Paramyxovirus genus
	Morbillivirus
<i>Sexually transmitted diseases</i>	
Syphilis	<i>Treponema pallidum</i>
Gonorrhoea	<i>Neisseria gonorrhoeae</i>
Hepatitis B	Hepatitis B virus (HBV)
AIDS	HIV
<i>Tropical diseases</i>	
Trypanosomiasis/Chagas disease	<i>Trypanosomas cruzi</i>
Sleeping sickness	<i>Trypanosoma brucei gambiense</i>
Schistosomiasis/bilharziosis	<i>Schistosoma mansoni</i> , <i>S. japonicum</i>
Leishmaniasis	<i>Leishmania tropica/brasiliensis</i>
Lymphatic filariasis/elephantiasis	<i>Wuchereria bancrofti</i> and <i>Brugia malayi</i>
<i>Hemorrhagic fever</i>	
	Ebola virus
	Marburg virus
Lassa fever	Lassavirus
<i>Other</i>	
Pest, bubonic plague	<i>Yersinia pestis</i>
	<i>Clostridium botulinum</i>
Leprosy	<i>Mycobacterium leprae</i>
Poliomyelitis	Poliovirus
Hepatitis C	Hepatitis C virus (HCV)
	West Nile virus
Guinea-worm disease	<i>Dracunculus medinensis</i>
Onchocerciasis	<i>Onchocerca volvulus</i>

vaccines or subunit vaccines. Protection against intracellular bacteria depends on T-lymphocytes rather than antibodies (Winau et al., 2004). Although recent advances in genomics, basic immunology and molecular

biology provide the guidelines for constructing novel vaccine candidates in research programs involving stretching from basic science to applied field studies.

Werner Goebel (Microbiology, Würzburg, Germany) highlighted the development of live virulence-attenuated bacteria as delivery systems for protective heterologous protein antigens. Two different strategies were used: one that allows the release of the antigen from Gram-negative bacterial carriers via type 1 secretion system (T1SS) (Gentschev et al., 2002) and one that introduces antigen-determining plasmid DNA or RNA into mammalian cells including antigen-presenting cells via intracellular bacteria (Dietrich et al., 1998; Pilgrim et al., 2003; Schoen et al., 2005). A self-destructing variant of the Gram-positive bacterium *Listeria monocytogenes*, which activates a phage-derived lysine and lyses upon internalization, was used for efficient release of DNA or mRNA into the cytosol of host cells (Pilgrim et al., 2003; Schoen et al., submitted). Transfer of DNA and RNA was highly efficient not only into epithelial and fibroblast cell lines, but also into phagocytes including dendritic cells. The α -hemolysin (HlyA) secretion system of *E. coli* was exploited as a T1SS to deliver heterologous antigens. HlyA can be genetically fused to almost any protein antigen without drastically affecting secretion, e.g. by virulence-attenuated *Salmonella* strains including *S. typhi* ty21 (Gentschev et al., 2004). Exploitation of type III and auto-transporter secretion systems of Gram-negative bacteria for recombinant vaccine delivery into the cytosol of mammalian cells has recently been demonstrated (Rüssmann, 2003a, b; Rizos et al., 2003). Werner Goebel emphasized that these approaches might not only be useful in combating infectious diseases but might also open up new avenues in anti-tumor therapy by either designing anti-tumor vaccines or by delivering therapeutic agents.

Veterinary vaccines

Infected animals are not only a major economic factor in animal husbandry but also have a high impact as reservoirs of human pathogens. Veterinary vaccines have to be cost-efficient, easy to administer and efficacious within a short period of time after, preferably, one immunization. This implies a rather exclusive reliance on live vaccines. Fox rabies, a major threat for human health, will serve as an example. Initiated by Swiss researchers, oral immunization schedules have been devised which were initially based on the use of baits composed of chicken heads filled with blisters containing live-attenuated rabies vaccine (Steck et al., 1978). Further research led to the development of industrially produced bait casings that are attractive enough to be eaten by foxes (Schneider, 1989). Bait

distribution over large areas eradicated rabies or drastically reduced its incidence in several European countries (Pöttsch et al., 2002). The successful oral immunization against rabies in foxes was followed by a similar approach against classical swine fever (CSF) in wild boar. CSF is a highly contagious disease of high pathogenic potential that is contained within the European countries by elimination of all pigs on infected holdings. Most of the sporadic occurrences of CSF in domestic pig can be epidemiologically linked to wild boar. Therefore, elimination of CSF virus from the wild boar population is a major prerequisite for protecting domestic pig farms. A corn-based bait that contains a blister filled with the live-attenuated CSFV C-strain vaccine has all but eliminated CSFV, which once was quite common in the German wild boar population (Kaden et al., 2002).

Veterinary vaccinology has recently achieved spectacular success by introducing the first genetically engineered live viral vaccines and marker technology able to differentiate between infected and vaccinated animals (DIVA or marker concept). Thomas C. Mettenleiter from the Friedrich-Loeffler-Institute (Insel Riems, Germany) illustrated the success of disease control by applying the DIVA concept. The DIVA concept (van Oirschot, 1999) has been successfully employed for the elimination of Aujeszky's disease (AD) in pigs caused by the alphaherpesvirus pseudorabies virus (PrV; Mettenleiter, 2000). Based on the initial finding that several live-attenuated PrV vaccine strains lack a major surface antigen (glycoprotein E, gE) which is invariably present in all field strains (Mettenleiter et al., 1985), a simple ELISA system has been developed that is able to specifically detect the presence or absence of anti-gE antibodies in the animal (van Oirschot et al., 1986). Based on this concept, eradication programs allow the use of gE-deleted ('Marker') vaccines combined with sero-surveillance using detection of anti-gE antibodies. Using this approach, Germany has been declared free of AD by February 26, 2003 (Müller et al., 2003). It is important to mention that during the AD eradication campaign, genetically modified live vaccines were used for the first time in Europe on a wide scale. A similar eradication program has recently been started to eliminate bovine herpesvirus 1 infections in cattle (Beer et al., 2003; Kaashoek et al., 1994, 1995).

The less complex RNA viruses generally do not specify a protein product like the glycoprotein E of PrV and, therefore, require different DIVA approaches (Scawah (Scientific Committee on Animal Health and Animal Welfare), 2003). The DIVA concept is also applicable to the control of foot-and-mouth-diseases virus (FMDV), a picornavirus of the genus aphthovirus. Only four virally encoded structural proteins are present within the mature virion, whereas in infected cells (and animals), non-structural proteins also act as antigens.

Thus, infected animals produce antibodies against both structural and non-structural proteins, whereas animals vaccinated with the inactivated virions will only produce antibodies against virion components. A recombinant swine fever virus (CSFV) vaccine (de Smit, 2000) with an antigenetically different E₂ glycoprotein (from bovine viral diarrhoea virus) induces excellent protection against CSFV challenge (Reimann et al., 2004). Likewise, a DIVA strategy is based on differential detection of antibodies against a heterologous neuraminidase in avian influenza. These examples, presented by Thomas C. Mettenleiter, illustrate the success of the DIVA concept in cost-efficient control and eventual elimination of specific infectious agents in livestock.

Successful eradication of infectious diseases: smallpox

A virgin population is particularly threatened by unintentional or intentional re-introduction of eradicated pathogens, such as variola virus. Release of smallpox virus, the only virus to be successfully eradicated, would be catastrophic, since 75% of the population is susceptible to the disease and nobody under the age of 25 years has ever been vaccinated (Fenner et al., 1988). The last natural case of smallpox occurred in Somalia in 1977. Routine vaccination programs were stopped and vaccine producers ceased making vaccines. Approximately 100 million died from the disease prior to its eradication. The eradication strategy comprised widespread vaccination of at least 80% of the population, and surveillance for the early detection of cases and control of outbreaks. Smallpox virus officially still exists in two laboratories of the WHO, one in Atlanta, Georgia, USA and one in Novosibirsk, Russia (and possibly unofficially in others). In these laboratories, research is continuing. The former deputy director of the Soviet Union biological weapons program declared in 1992 that smallpox would be developed as a weapon and that an annual stockpile of 20 ton variola virus would be maintained (Alibek and Handelman, 1999). Bioterrorism has considerably dampened the hopes of positive medical and economic benefits resulting from the global eradication of a disease. In his presentation, Reinhard Kurth (Robert-Koch Institute, Berlin, Germany) asked whether we can really eradicate infectious diseases. The development of a new vaccine or improvement of the old vaccine face challenges: the clinical efficacy of a new vaccine cannot be tested in humans (Rosenthal et al., 2001) and the side-effects of the old one will be not accepted by the community. Most countries, however, have no vaccine and will be dependent on others in the

case of emergency as reported by Donald L. Henderson (Johns Hopkins Center for Civilian Biodefense Studies, Baltimore, USA).

Eradication as a future challenge

Poliomyelitis is the only disease worldwide that is currently being seriously targeted for eradication with a possibility of success. The number of cases decreased from over 350,000 cases in 125 countries to 748 cases in six countries between 1988 and 2003. Although in the majority of regions poliovirus, a human enterovirus of the Picornaviridae, has already been eliminated (Rasch et al., 2001), the global eradication campaign will be in danger in those African countries which have for economic reasons reduced the vaccination campaigns after achieving polio elimination. The termination has led to an increase of susceptible persons. Ninety percent of polio infections are asymptomatic. Transmission can, therefore, easily occur without detection and even new outbreaks are at high risk. This problem is present in Africa where sudden new cases are seen after many years of being polio-free. New problems will arise in the post-eradication period, including those posed by the new threat of bioterrorism. The live attenuated virus used as a vaccine can be transmitted and recombine with other enteroviruses, most likely C-cluster coxsackie viruses. The result can be a vaccine virus – vaccine-derived poliovirus (VDPV) – with increased virulence and transmissibility. Long-term circulation of such VDPV has, in the past, occurred in countries with suboptimal oral polio vaccine (OPV) vaccination rates (Kew et al., 2004). This occurred independently in Egypt (1983–1993), Hispaniola (2000–2001), Philippines (2001), and Madagascar (2001–2002). The live attenuated (Sabin) OPV was developed in the 1950s. To minimize the circulation of vaccine viruses in, e.g. immunodeficient or unvaccinated individuals, it is essential that the WHO initiates an early and synchronized stop of live attenuated (Sabin) OPV vaccinations (MacLennan et al., 2004). After the confirmed global eradication it is up to the individual countries to stop vaccinating or to switch to using inactivated (Salk) polio vaccine (IPV). At least for those countries in which IPV is produced, the manufacturers have to maintain large quantities of the wild-type virus and the corresponding seed virus stocks. Even after the cessation of vaccination it will be essential to conserve the wild-type virus in case of emergency to fall back on these vaccines (Henderson, 2002). The problems with the containment of wild-type poliovirus would be reduced if the production of IPV could be successfully based on strains derived from the Sabin vaccine virus (S-IPV, Doi et al., 2001).

Pathogenesis of poliomyelitis

Most of the basic mechanisms of poliovirus pathogenesis are still obscure. This is true for the replication of poliovirus in the gastrointestinal tract after oral infection and the mechanism by which the virus spreads to the central nervous system (CNS). Another mystery is the cunning preference of the virus to destroy motor neurons in the CNS, especially in the spinal cord, a specificity leading to paralysis and possibly death – poliomyelitis. A major determinant of poliovirus pathogenesis is the CD155 receptor (Koike et al., 1990; Mendelsohn et al., 1989). Analysis of human and primate specimens of the gastrointestinal tract revealed strong expression of CD155 in enterocytes, in follicular-associated epithelial cells and M-cells of Peyer's patches (PPs), which represent an important site of replication during first stage of infection (Minor, 1997; Iwasaki et al., 2002). Endothelial cells in the interfollicular region of PPs were also found to express CD155, an observation that leaves open the possibility that poliovirus may enter the bloodstream via vessel damage in these tissues (Iwasaki et al., 2002). Entry into the CNS can occur by passage through the blood-brain barrier or at neuromuscular junctions, followed by retrograde axonal transport to the motor neuron cell body. The latter is the route of CNS invasion in "provocation poliomyelitis", e.g. injury enhanced disease progression (Gromeier and Wimmer, 1998). A cargo protein of the dynein motor, Tctex-1, was discovered to have affinity to the C-terminal intracellular domain of CD155 (Mueller et al., 2002). Therefore, a new model of retrograde transport was proposed: once the virus has been internalized at the neuromuscular junction, it is transported to the cell body of motor neurons in cargo vesicles along axonal microtubules, driven by the dynein motor complex (Mueller et al., 2002).

"Lego biology": chemical synthesis of poliovirus and other infectious agents

The chemical synthesis of poliovirus in the absence of natural template (Cello et al., 2002) indicated that a virus can no longer be considered extinct when its genome sequence is known. A poliovirus-specific double-stranded DNA ("cDNA") that was assembled from synthetic desoxy-oligonucleotides was transcribed with an enzyme of bacteriophage T7 (T7 RNA polymerase) yielding poliovirus RNA. This RNA was identical to the genomic RNA of 7500 nucleotides except for the 5' end (Cello et al., 2002; Van der Werf et al., 1986). The sequence of the synthetic poliovirus (sPV) cDNA was designed such that it contained 27 "silent" mutations as identity markers (Cello et al., 2002). The sPV RNA was

incubated in a cell-free extract of uninfected HeLa cells that directs virus specific translation, genome replication and virion assembly (Molla et al., 1991). This via synthetic cDNA *outside* living cells generated polio virus has a plaque phenotype similar to that occurring in nature. The sPV is growing in tumor cells but is highly attenuated (*att*) in CD155 transgenic mice (Cello et al., 2002).

The first biologically active cDNA synthesized was that of RNA phage Q β (Taniguchi et al., 1978). This landmark accomplishment heralded the birth of reverse genetics in virology. In contrast to Cello et al. (2002) who synthesized virus from chemical information only, naturally occurring RNA were required as templates for the enzymatic reactions preparing Q β cDNA. This is fundamentally different and, as *proof of principle*, has far reaching consequences as stated by Eckard Wimmer (Stony Brook, NY, USA). The chemical synthesis of genomes of viruses or bacteria is no longer a dream. This research referred to as "synthetic biology" has already become a new hot field. Consequently, a paper by Smith et al. (2003) described the synthesis of a chemical mixture of phage ϕ X174, 5386 bp long, in only 2 weeks. The re-programming of microorganisms by existing functional modules ("lego biology") may yield novel microbes capable of functioning as highly specific and efficient mini-factories (Ferber, 2004).

Bacterial evolution and mechanisms of genome flexibility

Genetic flexibility is a phenomenon that accounts for the pathogenic properties of many infectious agents. Therefore, one focus of genomic research has been the characterization of mechanisms that are involved in genomic variability and evolution of bacterial pathogens. A new type of genomic entity – known as pathogenicity islands – has been shown to contribute to the rapid evolution of bacterial pathogens by horizontal gene transfer (for review, see Dobrindt et al., 2004). Another flexible genetic element is represented by integrons, which were first identified as the primary mechanism for antibiotic resistance gene capture and dissemination among Gram-negative bacteria.

Studies on *Yersinia* and *Bartonella* have further shown that numerous gene rearrangements, massive gene inactivations and loss of gene functions are at least as important determinants of pathogenicity as gene acquisitions. Siv G.E. Andersson (Uppsala, Sweden) presented that the deterioration of the α -proteobacterial human pathogen genomes, which could mean eliminations of a few thousand genes, shifted the bacterial lifestyle to intracellular animal environments and

vector-mediated transmission pathways. A computational approach was used to infer ancestral gene sets and to quantify the flux of genes along the branches of the α -proteobacterial species tree (Boussau et al., 2004). The analysis suggests that *Rickettsia* and *Wolbachia* represent the earliest diverging lineage in the tree, followed by *Caulobacter* (Boussau et al., 2004). The analysis indicated a loss of more than 2000 genes prior to the divergence of *Rickettsia* and *Wolbachia*, followed by another loss of about 500 genes prior to or associated with the emergence of the typhus pathogen. *R. conorii* contains more than 500 genes not present in *R. prowazekii*, the causative agent of epidemic typhus. Eighty percent of the 500 genes are orphans and not identified in any other species. Genomic analysis suggests further a massive loss of more than 2000 genes immediately prior to the divergence of *Bartonella* and *Brucella*. This is followed by yet another major loss of some 1500 genes prior to the divergence of *B. henselae* and *B. quintana*. The genome sequence of the louse-borne *B. quintana* comprises 1,581,384 bp, and that of the zoonotic agent *B. henselae* comprises 1,931,047 bp (Alsmark et al., 2004). A genome comparison elucidated a high degree of overall similarity between the two genomes. *B. quintana* and *B. henselae*, which cause trench fever and cat-scratch disease, respectively, are unique among bacteria in their ability to induce tumor-like lesions of the skin (bacillary angiomatosis) as well as the liver and spleen (bacillary peliosis) of immunocompromised individuals. Both are transmitted by insect vectors, using mammalian reservoirs, and infecting similar cell types. However, *B. quintana* is a specialist, using only humans as a reservoir, whereas *B. henselae* is more promiscuous. The difference in gene content is mainly due to the presence of several genomic islands in *B. henselae* that are not present in *B. quintana*. Around 50% of the unique genes in *B. henselae* were attributed to a prophage region of 55 kb and three genomic islands of 72, 34 and 9 kb, respectively. The prophage is an evolutionary mosaic with genes of different origins that are interspersed with genes showing sequence similarities to a putative phage in *Wolbachia*. Potential virulence features located on the *B. henselae* genomic islands are multiple copies of *hecB* and *fhaB* putatively coding for a hemolysin transporter and a filamentous hemagglutinin, respectively. These two proteins form a two-partner secretion system, where the *hecB* gene product may mediate the transport of filamentous hemagglutinin that is required for attachment to the host cell (Alsmark et al., 2004).

The largest unique island in *B. quintana* is only 9 kb and contains a gene with sequence similarity to *yopP* in *Yersinia enterocolitica* and *yopJ* in *Yersinia pseudotuberculosis*. Sequences in *B. quintana* located at positions corresponding to islands in *B. henselae* contain remnants of phage and island genes, suggesting that these were

present in the common ancestor of the two species. In total, the Siv G.E. Andersson's group has identified 128 pseudogenes and extensively degraded gene sequences in *B. henselae* (including 23 tandem duplication remnants) as compared to 175 fragmented genes in *B. quintana*. The rearrangements flank islands uniquely present in *B. henselae*, indicative of a link between rearrangement and insertion/deletion events. Remnants of genes present in the *B. henselae* islands have been identified at the breakpoint positions in *B. quintana*. As reported by Siv G. E. Andersson both of the two *Bartonella* genomes contain numerous scars of putative phage and plasmid integrations, indicative of exposure to genetic parasites in the evolutionary past. However, the human pathogen specialist *B. quintana* has lost most of these sequences and seems no longer to accumulate genetic parasites. Thus, both of the two human pathogen specialists, *R. prowazekii* and *B. quintana*, have experienced an enhanced frequency of genome degradation as compared to their closest relatives with other host-vector reproduction strategies.

Interestingly, the high pathogenic potential of *Yersinia pestis* is not solely attributable to the presence of two additional plasmids as shown by Elisabeth Carniel (Institut Pasteur, Paris). A comparative analysis with the ancestor *Y. pseudotuberculosis* revealed 32 chromosomal genes in addition to the plasmids that represent the new genetic material in *Y. pestis*. In contrast, 149 other pseudogenes and 317 genes absent from *Y. pestis* were detected, indicating that as many as 13% of *Y. pseudotuberculosis* genes no longer function in *Y. pestis* (Chain et al., 2004). It appears that loss of function is at least as important as gene acquisition in the evolution of *Y. pestis*.

Human infections emerging from animal reservoirs

High genetic variability is particularly dangerous if pathogens originate from animal reservoirs. An example is pandemic influenza which is a zoonotic disease caused by the transfer of influenza A viruses or virus gene segments from aquatic bird reservoirs to humans and domestic animals. In their natural reservoir these viruses are in evolutionary stasis – they are in equilibrium with their natural host and cause no disease. But after transfer to new hosts they evolve rapidly posing a constant threat to human and animal health as told by Robert G. Webster (St. Jude Children's Research Hospital, Memphis, USA). Widespread infections of poultry with H5N1 viruses in Asia have caused increasing concern that this subtype forms the basis for human-to-human spread and interspecies transmission. Multiple opportunities for the successful

mammalian transmission of H5N1 influenza viruses are provided by their continuing evolution in Asia, their propensity for re-assortment, the generation of multiple genotypes of H5N1 viruses (Matrosovich et al., 1999), antigenic drift in the HA gene of H5N1 viruses, and the acquisition of high pathogenicity for aquatic birds. If an opportunity for re-assortment with human influenza strains occurs, then the likelihood of successful transmission between humans is high.

Marburg and Ebola viruses, family Filoviridae, re-emerge periodically from an unknown animal reservoir. They cause severe hemorrhagic fever in humans and non-human primates. Disturbances of the blood tissue barrier, primarily controlled by endothelial cells, and immune suppression seem to be the key pathogenic factors of the disease as reported by Heinz Feldmann (National Microbiology Laboratory, Winnipeg, Canada). The endothelium is affected in two ways: directly by virus infection leading to activation and lytic replication and indirectly by a mediator-induced inflammatory response. These mediators originate from virus-activated cells of the mononuclear phagocytic system which are the primary target cells. Immune suppression may result from lytic infection of circulating and sessile cells of the mononuclear phagocytic system, inactivation of neutrophils by secreted viral proteins, necrosis of antigen-presenting cells, and lymphoid depletion. In addition, the virion structural protein 35 (VP35) has been identified as an interferon antagonist (Basler et al., 2000). Despite being clearly immunosuppressive, there is good evidence for protective immunity during filovirus hemorrhagic fever. In contrast to survivors and asymptomatic cases that show IgM and IgG antibody responses to viral antigens, fatal infections usually end with high viremia and little evidence of a humoral immune response. Neutralizing antibodies are directed against the transmembrane glycoproteins, which are responsible for virus entry. However, infectivity-enhancing antibodies directed against the transmembrane glycoprotein have also been described. The transmembrane glycoprotein can be used to provoke a protective immune response in animal models including non-human primates. Several distinct approaches including DNA vaccination or replication-deficient and replication-competent recombinant viruses can achieve this. Thus, the transmembrane glycoprotein serves as the most promising vaccine candidate for filoviral hemorrhagic fever (Feldmann et al., 2003).

Yellow fever virus and Lassa virus, an arenavirus, are zoonotic viruses that both cause 150,000–200,000 clinically apparent infections per year in West Africa, carrying a mortality of 10–20%. While the transmission, epidemiology and immunology of rodent-borne Lassa fever is different from mosquito-borne Yellow fever, the current re-emergence of both diseases is influenced likewise by the ecology of the viral reservoirs as well

as the immunity of the susceptible human populations. Herd immunity to Yellow fever virus which was achieved in the 1950s through mass-vaccinations is generally fading, since immunization programs have been discontinued. Humoral cross-immunity due to heterologous flavivirus infections can mitigate severe disease, but cannot prevent Yellow fever epidemics, which are again threatening the urban centers of West Africa. No vaccine has ever been developed against the mainly T-cell-controlled Lassa virus, but outbreaks of the disease have been rare because of focally restricted contact with infected rodents in West Africa. Migration of susceptible human populations and severe changes in the habitat of the rodent reservoir have now led to increased transmission of the virus and export of Lassa fever cases to European countries (J. ter Meulen, Institute of Virology, University of Marburg, Germany).

The flavivirus family comprises about 70 different viruses, most of which are transmitted to their vertebrate hosts by mosquitoes or ticks. Several flaviviruses are important human pathogens – including the Yellow fever, Dengue, Japanese encephalitis, West Nile, and tick-borne encephalitis viruses – and some of them have proven to have the characteristics of newly emerging pathogens. As pointed out by Franz X. Heinz (Institute of Virology, Medical University of Vienna, Austria) enormous progress has been made in recent years in the elucidation of the molecular structure of flaviviruses, which has greatly enhanced our understanding of specific functions during entry and assembly, viral pathogenicity, antigenic relationships, and immunogenicity. Tick-borne encephalitis virus is endemic in many European countries and large parts of Asia, including Northern China and Northern Japan. Three closely related subtypes can be discerned (European, Siberian, Far Eastern), which circulate in their natural foci between different mammalian species and ticks (mainly *Ixodes ricinus* and *Ixodes persulcatus*). Humans are infected only accidentally and do not play any role for maintaining the natural cycle of the virus. Both the virus and the geographic distribution of natural foci are remarkably stable and exhibit relatively little variation, suggesting relative evolutionary stasis of the virus and a delicate balance of environmental factors required for this maintenance in nature. A highly purified formalin-inactivated whole virus vaccine has been in widespread use in Europe and proven to be an excellent means for the prevention of tick-borne encephalitis in humans.

The recent introduction of West Nile virus to Northern America is a dramatic example of the potential of flaviviruses to conquer new territories within a short period of time. Although the same virus sporadically occurs in Europe and also causes rare infections in humans, it has never been able to establish endemicity similar to what has occurred in America, where the virus

has apparently found ideal ecological conditions for its maintenance and spread. So far human vaccines are not yet commercially available but are being tested in human trials. The four types of Dengue viruses can cause severe forms of Dengue fever (hemorrhagic fever and shock syndrome) and pose an enormous health threat to tropical and subtropical countries around the globe. These severe forms of the disease are apparently promoted during secondary infections with a certain Dengue virus type by immunological enhancement phenomena triggered through the pre-existing immunity against a different type. This phenomenon has severely hampered the development of Dengue vaccines, because of concerns that vaccination could induce a similar immunopathological state in vaccinated individuals.

Pathways used by protein toxins into cells

Bacterial toxins represent important and highly efficacious virulence determinants of pathogens and are, therefore, a threat to human health in connection with infectious diseases (Bradberry et al., 2003; O'Loughlin and Robins-Browne, 2001; Sandvig and van Deurs, 2002). Elucidating the mode of action of toxins is important to understand pathogenicity and will help to prevent and treat diseases (Karmali, 2004). Substantial progress has been made in elucidating the mechanisms by which bacterial toxins, such as tetanus, botulinum, anthrax and Shiga toxin, invade cells and interfere with vital cellular functions. Kirsten Sandvig (Institute for Cancer Research, Norwegian Radium Hospital, Oslo) introduced the pathways used by Shiga toxin. The bacterial Shiga toxin produced by *Shigella dysenteriae*, Shiga-like toxins (Stxs) produced by *Escherichia coli*, cholera toxin (*Vibrio cholerae*), and certain plant toxins such as ricin act on cells after entry of an enzymatically active part of the molecule into the cytosol. In general, the toxins have one moiety which binds to cell surface receptors and another, enzymatically active moiety, which enters the cytosol and exerts the action of the toxin. Studies of toxin entry into cells also provide us with basic knowledge about endocytosis mechanisms, intracellular transport and membrane translocation of proteins in cells (Iversen et al., 2003; Lauvrak et al., 2004; Llorente et al., 2003; Sandvig and van Deurs, 2002). Shiga toxin was the first molecule found to move all the way from the cell surface to endosomes, and then retrogradely to the Golgi apparatus and the ER (Sandvig et al., 1992). A similar trafficking has been shown also for toxins such as cholera toxin, ricin and others (Sandvig and van Deurs, 2002). On the other hand a number of protein toxins travel a much shorter distance before being translocated to the cytosol (Sandvig, 2003). Endocytosis is mediated

via both a clathrin-dependent and -independent mechanism. The experiments with ricin indicated that the endocytosis can be independent of lipid rafts and can even under some circumstances operate without GTP hydrolysis (Garred et al., 2001). Shiga toxin can also enter cells by clathrin-independent mechanisms (Lauvrak et al., 2004; Nichols et al., 2001; Saint-Pol et al., 2004). However, the fraction of toxin exploiting the different pathways is likely to be cell-type dependent. Similarly, cholera toxin can be endocytosed by different mechanisms (Shogomori and Futerman, 2001; Singh et al., 2003; Torgersen et al., 2001.) and most probably cell-type-dependent differences exist. Shiga toxin and ricin seem to be transported from the early endosomes by a Rab9-independent process, either directly to the Golgi or via the perinuclear recycling compartment (Iversen et al., 2001; Sandvig et al., 2002; Johannes and Goud, 2000; Sannerud et al., 2003). For Shiga toxin Rab11 is implied in the transport and clathrin is required for efficient endosome-to-Golgi transport (Johannes and Goud, 2000; Lauvrak et al., 2004; Saint-Pol et al., 2004). Both molecules are not required for ricin transport (Iversen et al., 2001). After arrival in the trans-Golgi network the toxins are transported to the ER. Neither Shiga toxin nor ricin contain a KDEL sequence that could facilitate retrograde transport by the KDEL receptor and the COPI-coated vesicles. The KDEL receptor-dependent transport has been shown for *Pseudomonas* exotoxin A (Jackson et al., 1999). In the case of Shiga toxin there is evidence for a Rab6, COPI-independent pathway, but not all details are known (Johannes and Goud, 2000; Sandvig and van Deurs, 2002; Sannerud et al., 2003). In summary, Kirsten Sandvig indicated that more work is required to understand the retrograde transport and that the answers are important and might help to exploit the toxins in medical therapy.

Concluding remarks

Despite significant progress was made in controlling infectious diseases and understanding disease mechanisms with special emphasis on the molecular level, we face to date several new threats due to infectious agents. Vaccination programs aimed to eradicate high pathogenic microorganisms with high mortality rates. Nevertheless, the emergence of newly identified diseases such as SARS and the re-emergence of diseases has become a great public health concern worldwide. A further threat is the dramatic increase of antimicrobial resistance of pathogens. Antimicrobial resistance against antibiotics and other drugs has a deadly impact on the control of diseases such as tuberculosis, malaria, cholera, dysentery, and pneumonia. The basis to control infectious diseases and epidemics is the installation of worldwide

accessible communication platforms by public health care systems. The interchange and storage of our current know-how and experience of successfully employed eradication programs and disease treatments is required to combat infectious diseases.

A landmark in modern molecular microbiology and combinatorial chemistry is the chemical synthesis of microorganisms. This provides not only the possibility to construct novel delivery systems or vaccines but may also lead to an improved understanding of key molecules and events in the development of diseases. On the other hand, this so-called “Lego biology” may represent a tool for bioterrorism in terms of constructing infectious agents with high pathogenic potential.

The microbial evolution and genomic variability is accompanied with horizontal gene transfer. However, novel studies have indicated that not only gene acquisition but also loss of gene functions represents important determinants of pathogenicity. Comparative genome analysis of microorganisms with special emphasis on intracellular pathogens will provide insights whether the evolution of these microorganisms is also associated with genome reduction by gene loss and resulted in a highly specified pathogenic potential.

The developments in vaccinology in veterinary medicine highlight the potential and application of vaccine research. The successful disease control in animals by veterinary vaccination campaigns offers new perspectives to implement vaccination programs in low- or middle-income countries or even after bioterrorism attacks. Veterinary vaccines have to be cost-efficient, easy to administer and efficacious within a short period of time. A single immunization is preferred. To reach and maintain high vaccination rates, novel vaccines have to fulfil these requirements. These vaccines should be ideally not based on live vaccines as used for veterinary vaccinology. Instead, preferable oral immunizations with subunit vaccines and novel antigen-delivery systems have to be implemented in order to reduce the burden of infectious diseases worldwide.

Acknowledgements

The authors wish to express their appreciation for the helpful contributions and permissions of the speakers on (parts of) this report which gives an insight of presentations held during the Symposium “Threat of infection” in July 2004 in Würzburg, Germany. The authors are grateful to Dr. Anthony P. Pugsley (Paris) for helpful discussions and suggestions.

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