Scientific Report of the Scientific Panel on Biological Hazards on:

Food as a possible source of infection with highly pathogenic avian influenza viruses for humans and other mammals[1]

March 2006 – 21st Plenary meeting

(Question N° EFSA-Q-2005-295)

This comprehensive background document makes a scientific analysis of the possibility that food, acting as a vehicle for highly pathogenic avian influenza (AI) virus, initiates infection in mammals via the gastrointestinal tract. The paper examines in detail existing pathogenic data on AI virus infection following natural exposure and experimental inoculation in mammals. The focus is placed on virus-host interactions of H5N1 infection.

The content of this document provided by the Scientific Panel on Biological Hazards supports EFSA's present position and advice on avian influenza in relation to food safety:

"On present evidence, humans who have acquired the infection have been in direct contact with infected live or dead birds. There is no epidemiological evidence to date that avian influenza can be transmitted to humans through consumption of food, notably poultry and eggs. EFSA and other organisations such as the WHO generally support longstanding food safety advice that chicken and eggs be properly cooked in order to protect consumers from possible risks of food poisoning. Thoroughly cooking poultry meat and eggs also eliminates viruses, thereby providing further safety assurance in the unlikely event that H5N1 virus may be present in raw poultry products entering the food chain."

[1] For citation purposes: Scientific report of the Scientific Panel on Biological Hazards on "Food as a possible source of infection with highly pathogenic avian influenza viruses for humans and other mammals", *The EFSA Journal 2006, 74, 1-29*

SUMMARY

In view of the presence of H5N1 avian influenza in the EU and the heightened concern of the general public with respect to the safety of poultry products and eggs for human consumption, the European Food Safety Authority requested the preparation of a comprehensive background document on the state-of-science of the fate of highly pathogenic avian influenza (AI) viruses (mainly H5N1) in avian species and the possible transfer of the virus to other species including humans via the food chain.

Highly pathogenic H5N1 virus causes a generalised infection in several avian species with virus dissemination to all organs and virus presence in all secretions and excretions. Edible tissues from infected animals, if collected at the height of infection which is 2 to 5 days after contact with virus has taken place, thus may contain high virus quantities.

Direct transfer of H5N1 to humans occurs rarely and particularly after very close contact with infected animals. The exact entry route(s) of the virus in humans is(are) not known but it is generally accepted that respiratory and/or oropharyngeal tissues are the entry sites. However, when one considers the low number of recorded human infections in relation to the high number of people that have been exposed to H5N1 virus infected animals, it is clear that a readily accessible portal of entry does not exist. The possibility of virus entry via the gastrointestinal (GI) tract after ingestion of virus with food has been raised. So far, there is no proof that virus replicates in the human intestine. The presence of diarrhoea in several patients, the detection of viral RNA in the intestines of two patients and the demonstration of infectious virus in rectal swabs of one patient do not allow one to conclude that the GI tract is a portal of entry or a target organ. Foodborne virus might be a source of infection after ingestion but with virus uptake taking place via oropharyngeal tissues, if this site can serve as portal of entry. The existence of an undisclosed virus entry site in the intestinal tract can, however, not be ruled out at this time.

In felines, infection with H5N1 virus can occur naturally after eating infected carcasses from avian species and can be reproduced by oral feeding of infected chicks. However it is not proven that the GI tract is a portal of entry or a target organ in these species.

The pathogenetic basis for the observation that H5N1 virus causes infection in some humans and not in others remains unknown. The role of several viral and host factors such as receptors, receptor binding sites, genetic make up of viral strain, virus quantity at exposure, etc. is discussed. Further more research on this aspect is required, as is also the case with regard to the GI tract.

In several mammalian species (including felines, mice and ferrets), H5N1 shows a neurotropic character and this aspect possibly merits more attention in the pathogenesis of human infections.

The route(s) of entry and the cell type(s) that enable the virus to enter, and the mechanism of species barrier crossing, must be studied. Some mammals such as cats, ferrets or pigs may serve as a useful model for human infection. Experimental inoculation studies are needed in which different inoculation routes are used and in which sequential examinations are performed on different tissues for virus replication throughout the course of infection. Only by using this approach, can speculation on the portals of entry and routes of infection be avoided. The results obtained may provide useful information to be applied to humans.

BACKGROUND

The European Food Safety Authority (EFSA) is aware that there is no epidemiological information available to date suggesting that avian influenza (AI) - an infectious disease primarily affecting birds - can be transmitted to humans via food. Nevertheless, in view of the developing situation in relation to AI, EFSA's Scientific Panel on Biological Hazards (BIOHAZ) is keeping this issue under constant review. EFSA concurs with the advice of health authorities such as the World Health Organisation (WHO) and the European Centre for Disease Prevention and Control (ECDC) indicating that the most likely route of infection of the H5N1 bird flu virus in humans is through close contact with infected live poultry. The consumption of poultry products or eggs has not been implicated in the transmission of the H5N1 AI virus to humans. In the event of an H5N1 outbreak in poultry in Europe, stringent biosafety measures would immediately be put in place to limit the spread of infection by any means.

EFSA provided an initial statement on AI in January 2004 where it stated that there is no direct evidence to support the food chain as a possible route for transmission of the AI virus (http://www.efsa.eu.int/press_room/press_statements/40/pressrel_biohaz_ahaw_01_en_amended_27jan1.pdf). EFSA published a further statement on 12th September 2005 (http://www.efsa.eu.int/press_room/press_statements/1130_en.html) outlining its work in progress on the animal health and welfare aspects of AI and reiterating with respect to food safety the WHO recommendations on the safe handling and cooking of food in relation to AI.

On 20th September 2005 EFSA published an Opinion and Report on the animal health and welfare aspects of AI and provided information on the risks of AI entering the European Union and spreading amongst poultry. EFSA also made recommendations to prevent its introduction and spread amongst flocks in Europe. This report has provided the scientific basis for AI risk management measures already put into practice in Europe with respect to animal health (http://www.efsa.eu.int/press_room/press_release/1146_en.html).

Other organisations that have provided advice and information on the safe handling, preparation and cooking of foods are mainly food safety authorities, the World Health Organization (WHO) (http://www.who.int) and the European Centre for Disease Prevention and Control (ECDC) which has provided advice regarding the public health aspects of avian influenza (http://www.ecdc.eu.int/).

EFSA has based its previous statements on the following scientific data:

- Scientific information on AI indicates that the AI virus can be present in the meat and eggs of poultry which are infected with the H5N1 form of AI (Swayne and Beck, 2004; Swayne and Beck, 2005).
- Documented cases of certain species of animals becoming infected to varying degrees through the consumption of raw poultry meat and eggs.
    - For example, one experimental study carried out by Kuiken and co-workers (2004) showed that cats became infected when fed on infected chickens. Further documentation also indicates that tigers may have contracted the infection in a similar way as a result of being fed fresh chicken carcasses from a local slaughterhouse (Keawcharoen and co-workers, (2004). Based on these reports, cats (felines) appear to

be relatively susceptible to the H5N1 strain and may have become infected after consumption of carcasses of infected chicken.[2]

- The introduction of avian viruses to pigs is also not an uncommon occurrence (summarized in the Appendix to the EFSA Scientific report on animal health and welfare aspects of avian influenza[3]). For example, pigs kept on farms where infected poultry had been detected during the Dutch avian influenza epidemic developed antibodies which were linked in one case to the feeding of broken eggs from infected poultry (Loeffen et al, 2003, 2004).
- Likewise, the same EFSA scientific report[4] also considers that there may be sufficient virus present in infected poultry meat to infect other birds if fed in the raw state. In effect, there is much circumstantial evidence of infection of certain animal species via food and consequently and as a precautionary principle this mode of transmission to animals cannot be ruled out.

- Amongst the reported human cases of H5N1, mainly in Asia, there are two H5N1 cases reportedly related to the consumption of infected raw duck blood products (Source: Update on Epidemiological aspects of H5N1 by P. Horby presented at the "Groupe d'étude d'information sur la grippe". 18th European Meeting on Influenza and its prevention Sep 19-20-05 in La Baule, France). However, as direct contact with infected live or undressed dead animals cannot be ruled out in these cases, epidemiological data are insufficient to confirm the consumption of infected product as the only transmission route.
- Amongst the 118 reported cases of human infection, most of the cases in Asia have been associated with direct exposure to live or dead infected poultry; however, in many instances, there is not sufficient epidemiological evidence to identify the source of infection (WHO) http://www.who.int/csr/disease/avian_influenza/avian_faqs/en/index.html
- While the level of acidity in the human stomach (pH value 1-3) does have the potential to eliminate the virus, as is widely believed to be the case for normal flu, this effect depends on several factors, such as the virus strain, quantity of virus present in the gastrointestinal tract and other local gastrointestinal factors (e.g. gastric transit time, acquired failure of the gastric acid barrier due to Helicobacter pylori infection, which is common among the healthy elderly, etc.) and the nature and composition of the gastric content. There are however few scientific data on this aspect today. What is certain is that, in the hypothetical circumstance of infectious virus being present in the food, proper cooking of poultry meat and eggs will eliminate any virus present before consumption, thereby preventing even the theoretical possibility of the virus infection being acquired through food consumption.

[2] Page 17, Appendix to the EFSA Scientific report on animal health and welfare aspects of avian influenza, 2005). http://www.efsa.eu.int/science/ahaw/ahaw_opinions/1145_en.html

3 Page 15, Appendix to the EFSA Scientific report on animal health and welfare aspects of avian influenza, 2005). http://www.efsa.eu.int/science/ahaw/ahaw_opinions/1145_en.html

4 Page 46, Appendix to the EFSA Scientific report on animal health and welfare aspects of avian influenza, 2005). http://www.efsa.eu.int/science/ahaw/ahaw_opinions/1145_en.html

TERMS OF REFERENCE

In view of the spread of H5N1 avian influenza in the EU and the heightened concern of the general public with respect to the safety of poultry products and eggs for human consumption, the European Food Safety Authority requests the preparation of a comprehensive background document on the state-of-science of the fate of highly pathogenic Avian Influenza viruses (mainly H5N1) in avian species and the possible transfer of the virus to other species including humans via the food chain. The comprehensive document should include scientific aspects of the hurdles which the virus must overcome in order to infect cross species, in particular to humans and the gastro-intestinal barriers that may inactivate the virus after consumption of contaminated products. The identification of gaps in current scientific knowledge would also be very valuable.

FOOD SAFETY ASPECTS OF HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS INFECTIONS IN HUMANS

The purpose of this monograph is to present data and reflections on the probability of human infections with avian influenza (AI) viruses after consumption of food which originates from infected avian species or which was contaminated by the virus.

Firstly, the presence of avian influenza (AI) viruses in organs or edible products during an infection will be discussed.

Secondly, available data on the pathogenesis of human infections with AI viruses subtypes H5 and H7 will be given.

Reference will be made to possible sites of virus entry with particular attention to the gastro-intestinal (GI) tract. Information from pathogenesis studies with highly pathogenic AI viruses in mammals other than humans will be reviewed because it may give insights into the possible routes of infection and target organs in humans.

Finally, the likelihood that highly pathogenic AI viruses, mainly H5N1, can start a human infection via food consumption will be discussed. Missing scientific information will be pointed out in this discussion.

1. FATE OF AVIAN INFLUENZA VIRUSES (MAINLY H5 AND H7 SUBTYPES) IN TISSUES OF AVIAN SPECIES

Avian influenza (AI) viruses are very widespread in nature and they have been detected in more than 90 different species of birds. Wild aquatic birds are natural reservoirs of these viruses and can become infected by viruses of all 16 haemagglutinin (HA) and 9 neuraminidase (NA) subtypes without showing disease.

AI viruses can be grouped in 2 pathotypes in domesticated poultry: low pathogenic avian influenza (LPAI) viruses and highly pathogenic avian influenza (HPAI) viruses. LPAI viruses mostly cause infections of the respiratory and the enteric tract and the infection is subclinical in most avian species. Infections may be characterised by mild respiratory signs, some depression and reduced egg production.

HPAI viruses not only replicate in the respiratory and enteric tract but also in endothelial cells throughout the body with spillover to adjacent parenchymal cells. Disease signs involve the respiratory and enteric tracts and many other organ systems. Lesions are characterised by multiple haemorrhages in visceral organs and the skin, and mortality rates approach 100%. Infection with HPAI viruses thus leads to wide dissemination in the body and virus presence in many organs and edible tissues.

Some, but not all, H5 and H7 subtype viruses may be highly pathogenic. Low pathogenic H5 and H7 subtypes may have the potential to convert to highly pathogenic ones. Wild birds may serve as healthy carriers of low pathogenic H5 or H7 viruses and transmit these viruses to domestic poultry. After a short or long time of circulation in the poultry population such viruses may acquire a HP phenotype by mutation. AI viruses have several avian species as natural hosts and horizontal transmission between or within these species occur rather easily. The routes of viral uptake in avians are mainly the respiratory tract (aerosols, droplets...) and the digestive tract (oro-faecal...) after direct contact between live birds or after indirect exposure (contaminated feed, water, fomites...). Virus transmission in domesticated poultry, therefore, occurs readily within infected areas. While HPAI viruses traditionally arise in poultry flocks after introduction of a LPAI virus from wild birds, recent evidence has identified a new evolution. It appears that some wild bird species are now directly spreading the H5N1 virus in its highly pathogenic form e.g. during migration. They may even show disease as was observed in central China where 6000 migratory birds died from a HPAI subtype H5N1 infection at the Qinghai lake nature reserve (Liu et al. 2005). HPAI viruses may thus be carried over long distances from infected regions to uninfected ones by migratory birds.

HPAI viruses have an HA glycoprotein that can be cleaved by ubiquitous protease(s), which are found in virtually all organs and of which furin is one of the leading candidates (Steineke-Grober et al. 1992). LPAI viruses, on the other hand, can only be cleaved by trypsin-like enzymes, which are mainly found in the respiratory and enteric tracts. The presence of multiple basic amino acids in the cleavage site of the HA is associated with the high cleavability of the HA and thus with the capacity of HPAI viruses to enter and replicate in multiple organs of domestic poultry (chicken, turkey ...), while LPAI virus replication is limited to the respiratory and digestive tracts.

1.1 VIRUS IN ORGANS OF CHICKENS AND TURKEYS

The next sections describe the fate of low and highly pathogenic AI viruses in different tissues, secretions and excretions of infected avian species. Data given here are not exhaustive and rather are presented as examples since numerous studies have been performed on pathogenesis and dissemination of HPAI viruses in avians, which serve as important providers of all kinds of food.

AI viruses have been isolated from several organs of chickens and turkeys that were naturally or experimentally infected with either LPAI or HPAI viruses. Tissue tropism, virus titres in different organs and degree of viraemia, however, may vary remarkably between different virus strains and among avian species. Most experimental trials have been performed in chickens, and less information is available for turkeys or other domesticated birds.

LPAI viruses subtype H7N2 have been isolated from the respiratory and gastrointestinal tract of experimentally inoculated chickens, but not from bone marrow, blood, breast or thigh meat (Swayne and Beck 2005).

HPAI viruses, on the other hand, have been demonstrated in the respiratory and gastrointestinal tracts, bone marrow, muscle tissue, blood and several internal organs (Mase et al. 2005a, Swayne and Beck 2005, Muramoto et al. 2006). In one study, the H5N1 strain examined replicated to higher virus titres than the H5N2 strain (Swayne and Beck 2005). Virus titres in thigh and breast muscles were as high as $10^{7.3}$ EID_{50} per gram tissue with the H5N1 strain, while the H5N2 strain yielded peak virus titres of $10^{2.7}$ per gram breast meat and $10^{3.2}$ EID_{50} per gram thigh meat. Virus titres in lungs and blood reached up to $10^{6.0}$ EID_{50} per gram tissue and $10^{1.4}$ EID_{50} per ml respectively with the H5N2 strain, but they were not examined for the H5N1 virus.

Virus titres in internal organs of chickens inoculated with HP H5N1 isolates from Japan reached up to $10^{7.5}$ EID_{50} per gram tissue between 2 and 4 days post virus inoculation (Mase et al. 2005a). Virus titres in blood of chickens inoculated with a reassortant virus carrying the HA of the Hong Kong/97 H5N1 virus were as high as $10^{8.0}$ EID_{50} per ml at 24 hours after inoculation (Muramoto et al. 2006).

After experimental inoculation of turkeys with 10 LPAI viruses belonging to various HA subtypes, most isolates were isolated from the kidney, liver, bursa and ileocecal junction, but only some of them were found in the brain, thymus, lung, spleen, pancreas and jejunum (Laudert et al. 1993). These results may not reflect the natural infection because the animals were inoculated intravenously, thus causing artificial viraemia and allowing the viruses to reach all tissues.

Intranasal inoculations of turkeys with a HP H5N1 virus from the 1997 Hong Kong outbreak revealed the presence of infectious virus in the upper respiratory tract, lung, heart, brain, pancreas, bone marrow, bursa, thymus, spleen, testicles, adrenal glands and feather follicles, but virus titres were not determined (Perkins and Swayne 2001).

1.2 VIRUS IN ORGANS OF DUCKS AND GEESE

HPAI viruses may also cause disease in domesticated and wild waterfowl, but usually with a markedly less fulminating character. Even then, they can be isolated from several tissues, including edible products. Duck meat has been found positive for HPAI viruses during routine surveillance of imported poultry. H5N1 virus, for example, was detected in frozen meat of subclinically infected ducks imported from China into South Korea (Tumpey et al. 2002) and Japan (Mase et al. 2005a). These viruses were later used for inoculation trials in ducks, chickens and mice (Tumpey et al. 2003, Mase et al. 2005a). The virus strain recovered from duck meat in South Korea caused 100% mortality in chickens and 22% in mice, while ducks remained healthy even though they contained high virus titres in the brain, lung, kidney (as high as $10^{6.8}$ EID_{50} per gram tissue) and muscle (reaching $10^{5.5}$ EID_{50} per gram) (Tumpey et al. 2002, 2003). The duck meat isolate in Japan was highly pathogenic to chickens, replicated well in the lungs of mice and spread to the brain, but was not as pathogenic in mice as H5N1 human isolates (Mase et al. 2005b).

The H5N1 isolate from duck meat imported into Japan replicated in multiple organs in ducks after experimental inoculation and induced neurological signs in some of the inoculated

animals (Kishida et al. 2005). Virus titres in blood amounted to $10^{0.7}$ to $10^{2.3}$ EID_{50} per ml and those in liver and kidney up to $10^{4.5}$ to $10^{7.5}$ EID_{50} per gram tissue. The above information clearly shows that HPAI viruses may end up in all edible products of ducks, even though the animals are not ill at slaughter.

Experimental inoculations of geese with a HP H5N1 virus from 1997 showed virus replication in brain, lungs and kidneys (Perkins and Swayne 2002). At day 4 post inoculation, virus titres up to $10^{6.7}$ EID_{50} per gram brain, $10^{2.8}$ EID_{50} per gram lungs and $10^{3.6}$ EID_{50} per gram kidney were found.

HPAI viruses are thus widely disseminated in the body of domesticated poultry including geese and ducks. When infected birds are slaughtered during the height of infection, virus can be present at high quantities in all raw edible tissues and in blood.

During an outbreak of highly pathogenic H5N1 influenza in waterfowl (geese, ducks and swans) and other wild birds (flamingos, teals and herons) in Hong Kong in 2002, samples were collected from over 80 dead birds (Ellis et al. 2004). The virus was isolated from lung, brain, cloacal or oropharyngeal swabs in 68 out of 88 birds examined, indicating that edible products from wild-hunted avian species may also contain high titres of HPAI viruses.

1.3 VIRUS IN EGGS

Virus has been isolated from albumen, yolk samples and from the shell surface of eggs during the 1983 HPAI (H5N2) outbreak in Pennsylvania (Cappucci et al. 1985), but not during another HPAI (H7N2) outbreak in 2001 – 2002 (Lu et al. 2004). HPAI viruses have also been detected in eggs of experimentally infected chickens and turkeys (Moses et al. 1948, Narayan et al. 1969, Beard et al. 1984, Starick and Werner 2003), both on the egg surface and in egg contents. Virus detected on the egg surface probably represents a fecal contamination resulting from passage of the egg through the cloaca. Virus detected in egg contents presumably results from viraemia or virus replication in the oviduct because such eggs were positive particularly when laid 3 to 4 days post inoculation. However, the acute course of the infection, the sudden egg drop and the rapid mortality in laying chickens minimize the risk that infected eggs are laid.

In contrast to HPAI viruses, LPAI viruses have not been isolated from egg shell swabs, albumen or yolk samples of naturally infected chickens (Lu et al. 2004). However, LPAI virus has been isolated from the oviduct of chickens during an H7N2 outbreak in Pennsylvania (1996-1998) and acute salpingitis was commonly reported, which suggests that LPAI virus may have the potential to be deposited in eggs (Ziegler et al. 1999).
No information is available on the presence of virus in eggs from AI virus infected ducks.

1.4 VIRUS IN EXCRETIONS AND SECRETIONS

Considering the sites of AI virus replication and the generalised character of the infection with HPAI viruses, different secretions and excretions can be expected to carry virus sometimes in high quantities.

Chickens inoculated with a highly pathogenic H5N2 virus excreted virus from the nares, mouth, conjunctiva and cloaca. Virus titres on day 3 post inoculation amounted to $10^{4.2}$-$10^{6.5}$ EID_{50} per ml of oropharyngeal secrete and to $10^{4.5}$ EID_{50} per ml faeces (Swayne and Beck 2005).

Chickens inoculated with a HP H5N1 virus excreted virus quantities up to $10^{4.6}$ EID_{50} per ml in the oropharynx and $10^{4.5}$ EID_{50} per ml faeces within 2 to 3 day post inoculation (Tian et al. 2005). In another experiment titres up to $10^{7,5}$ EID_{50} per ml faeces were found in H5N1 inoculated chicks killed at 24 hours post inoculation (Rimmelzwaan et al. 2006).

Experimental inoculations of mallard ducks with 4 LP AI viruses revealed virus titres as high as $10^{5.8}$ EID_{50} per ml in both tracheal and cloacal swabs at 2 to 4 days post inoculation (Webster et al. 1978). Ducks and geese experimentally or naturally infected with H5N1 HPAI viruses have also displayed high virus quantities both in oropharyngeal and cloacal swabs (Perkins and Swayne 2002, Tian et al. 2005, Ellis et al. 2004).

These data show that faecal material can contain very high quantities (titers) of both LP and HP AI viruses. Faecal material can, therefore, be an important source of avian influenza viruses in different avian species. Faeces excreted from acutely infected birds may readily contaminate all types of food products and water. However, virus quantities on contaminated objects will be lower than in organs from infected animals.

1.5 CONCLUSIONS

HPAI virus strains are widely disseminated in the body of diseased avians (chickens, turkeys) as well as in subclinically infected species such as ducks. All edible products derived from such animals can be considered as carriers of virus in variable quantities.

Virus quantities will be highest when the sample materials (blood, meat, secretions, excretions, viscera...) have been collected during the height of the infection, which is 2 to 5 days after virus contact. Considering the acute course of the infection in chickens, virus quantities can be high at the end of the incubation period and shortly before the animals become sick. HPAI viruses are excreted in high quantities during the acute stages of disease. Excretions, particularly faeces, can thus contaminate all types of food and water.

LPAI viruses have a more restricted infection pattern, with the respiratory and enteric tracts as the main target organs. The probability of virus presence in edible products is, therefore, low but nevertheless real.

1.6 MISSING SCIENTIFIC INFORMATION

In fact, there is no missing scientific information on this point. Virus quantities present in food of avian origin will vary considerably among virus strains, avian species, type of organs, degree of viraemia and several other factors such as the stage of infection at which the products were collected. Such biological variation will always be present and cannot be specifically determined for each variable. Also, quantities of virus will be influenced by possible physical or chemical treatments that are carried out on edible products (heating or cooking) or physiological processes that may occur (e.g. pH changes in meat after slaughter).

2. INFECTION OF MAMMALS WITH HPAI VIRUSES

Almost all cases of AI virus infection in humans have been caused by highly pathogenic H5 or H7 viruses that were directly transmitted from infected birds to humans. While such human infections generally result from direct and intensive contact with infected or diseased poultry, other routes of infection such as consumption of edible tissues from infected avians or contact

with contaminated water have been suggested as possible sources of infection. Consideration of pathogenetic aspects of the infection in different mammalian species may give some insights on possible routes of infection and target organs in humans.

2.1 INFECTION AND PATHOGENESIS IN HUMANS

The 1957 and 1968 pandemics of human influenza arose through reassortment between the circulating human influenza virus and an avian virus. Until recently, the ability of HPAI viruses to directly infect mammals was not considered. However, during the past decade, 3 different subtypes of HPAI viruses, H5N1, H7N3 and H7N7 have been isolated from humans. These viruses were identified as entirely avian influenza viruses and epidemiological evidence has shown that they were transmitted directly from birds to humans. H5N1 has caused many cases of severe illness and death, whereas the H7 subtypes were mainly associated with conjunctivitis. These viruses, therefore, can now be considered as posing a real zoonotic threat. Confirmed disease cases and seropositivity have been directly linked to outbreaks of illness in poultry caused by certain HPAI virus strains such as H7N7, H7N3, H5N1 but not by others such as H7N1 and H5N2, which indicates that the species barrier is crossed more readily by some virus subtypes than by others (Hayden and Croisier 2005). Most severe cases of disease have been encountered with H5N1. The occurrence of the zoonoses has to be viewed against the background of exposure of large population of people with millions of infected birds and resulted in a limited number of infected cases.

The outbreak of H7N7 in poultry in the Netherlands resulted in infections in 86 humans who handled infected poultry and 3 of their family members, including one death. Clinically apparent infections due to this H7 subtype typically involved conjunctivitis with higher loads of virus in the eye than in the pharynx (Koopmans et al. 2004), pointing towards the eye or nose as a site(s) of initial virus entry.

With H5N1, more than 156 cases of infection including 86 deaths have occurred in humans in Southeast Asia (The World Health Organization). Very recently, 12 cases including 4 deaths have been reported in Turkey and one person died of H5N1 in Iraq. These cases of infection and the relatively high mortality rate have created much concern. Human infections and disease have mainly occurred following close contact with infected live, dying or dead chickens (Hayden and Croisier 2005). High risks were linked to slaughtering, defeathering, butchering and preparation of infected birds for consumption. Contact usually had taken place within a week before the onset of clinical signs (de Jong and Hien 2006). Inhalation of infectious droplets or self-inoculation of the conjunctival or upper respiratory tract mucosae are considered the most common routes of infection. Exposure to chicken faeces in areas with free ranging poultry has also been proposed as a source of infection. No significant risk has been related to travel in countries with infected poultry or the consumption of poultry products. However, in 33% of the H5N1 infected patients in Vietnam there was no history of direct exposure to poultry, or the source of infection was unknown (Horby, personal communication). This leaves the possibility for speculation about the existence of modes of virus entry in humans other than the respiratory and conjunctival routes. Consumption of edible tissues from infected avians, and oral contact with contaminated water, have been suggested.

Two confirmed human cases of H5N1 infection in Vietnam occurred shortly after consumption of a popular dish (tiet canh) that consists of raw duck blood pudding (Horby 2005). One patient in Thailand showed diarrhoea as the initial symptom (Apisarnthanarak

2004). Diarrhea was also a feature in 7 out of 10 well studied H5N1 infected patients in Vietnam (Tran et al. 2004). Another fatal case of H5N1 infection in Vietnam showed severe diarrhoea and possible intestinal involvement (de Jong and Hien 2006). In this latter case, epidemiologic investigation did not reveal exposure to ill poultry but the patient had regularly swum in a canal inhabited by ducks and the water of this canal was proposed as the possible source of infection. While the possibility exists that contaminated water used for bathing or swimming was orally ingested and that oral uptake of the virus resulted in infection and disease, it is not excluded that virus could have been deposited intranasally or on the conjunctival tissues.

Thus, questions arise as to whether or not some infections may have been initiated by virus-carrying water or food products, when eaten raw, and thus after oral uptake. The major questions are whether the oral uptake can serve as route of virus entry and whether the gastrointestinal tissues can serve as sites of replication, once the virus has reached the enteric tract.

In this part of the monograph, the most relevant information on human and mammalian infections with H5N1 will be given, with special interest to pathogenesis and to oral uptake of the virus. It should be mentioned that tissue tropisms and pathogenesis of influenza H5N1 infection in humans are not well defined.

In all cases of human infection with highly pathogenic H5N1 avian influenza viruses in South East Asia, the lungs were clearly the major site of virus replication and viral pathology (Uiprasertkul et al. 2005). Most patients died as a result of acute respiratory distress syndrome followed by multi-organ failure but, unlike the case in poultry, there is no firm evidence for a systemic infection in humans. In one case of fatal H5N1 infection in a boy in Thailand, positive-stranded viral mRNA was not only detected in the lung but also in the small and large intestine, suggesting productive viral infection of the gastrointestinal tract (Uiprasertkul et al. 2005). No viral RNA was found in plasma, adrenal glands, brain, bone marrow, kidneys, liver and pancreas. The spleen only contained negative-stranded genomic RNA and no positive-stranded messenger RNA, showing that there had been no influenza virus replication. On the other hand, examination of the intestines did not reveal viral antigen-positive cells or histopathological changes and the patient did not show diarrhoea. In the lungs, viral antigen-positive type II pneumocytes were present and characteristic lesions such as diffuse alveolar damage, interstitial pneumonia, focal hemorrhage and bronchiolitis were observed.

In Vietnam 2 fatal cases of H5N1 infection presented with severe diarrhoea and encephalitis without respiratory disease (de Jong et al. 2005). Only one of the patients was virologically examined and the virus was isolated from throat and rectal swabs, serum and cerebrospinal fluid.

Some of these data suggest that the gastrointestinal tract may be another site of H5N1 virus replication in humans. It must be stressed, however, that viral antigen-positive intestinal cells have not been demonstrated in the intestines of humans. Definitive evidence of H5N1 virus replication in the intestines of humans is, therefore, still lacking despite the detection of viral nucleic acid by RT-PCR. The detection of viral RNA in intestinal samples could be an indication of local virus replication or may also be the result of virus swallowed as a result of throat infection.

While infection of the gastrointestinal tract is a constant finding in several avian species, it has never been virologically confirmed in classical influenza infections in humans. Also, in natural and experimental infections of swine and horses with their species-specific influenza viruses, intestinal involvement has not been shown. Non-natural hosts, however, may show intestinal involvement following experimental infection with influenza viruses and this will be discussed later.

Based on the above information, it must be stated that the role of the gastrointestinal tract as a possible portal of entry or site of replication for H5N1 virus is still uncertain. If, however, oral uptake of H5N1 virus were able to initiate the infection, it does not necessarily imply that the lower gastrointestinal tract is involved. Virus ingested with food may use the oropharyngeal and upper respiratory tract tissues as portals of entry rather than the lower gastrointestinal tract itself. In H5N1 infected humans, pharyngeal swabs may yield higher viral loads than nasal swabs and this contrasts with the findings during normal human influenza virus infections. This indicates that the oropharynx can be a very important target tissue for H5N1 (Beigel et al. 2005, de Jong et al. 2005). It is, therefore, possible that initiation of viral infection after oral virus uptake is a consequence of throat infection rather than of a primary gastro-intestinal infection. Also, if intestinal tissues were to allow active virus replication, intestinal invasion may have been the result of viraemia, considering that infectious H5N1 virus or viral RNA has been demonstrated in the serum of some patients (Beigel et al. 2005, de Jong et al. 2005).

Taken all together, there is insufficient evidence so far in humans to state that H5N1 virus, if it were to reach the intestinal lumen upon oral uptake or consumption of infected or contaminated food, could initiate infection via intestines or could actually replicate in intestinal tissues.

2.2 INFECTION AND PATHOGENESIS IN MAMMALS OTHER THAN HUMANS

Natural and experimental infections with AI viruses, particularly H5N1, have been performed in several mammalian species other than humans and these studies have created some insights into the pathogenesis in these unnatural hosts. Different routes of inoculation were used. While there is definitely much variation in the response of different mammalian species to such inoculations, some findings may be relevant to the pathogenesis of H5N1 in humans. Since the present document mainly refers to oral uptake or gastro-intestinal involvement, most attention will be given to these sites in different mammalian species.

2.2.1 Natural infection

Feeding of raw infected chicken carcasses from a local slaughterhouse to tigers and leopards in Thai zoos led to outbreaks of respiratory disease and deaths (Keawcharoen et al. 2004, Thanawongnuwech et al. 2005). This occurred at a time when many chickens in the area were dying with respiratory disease and neurological signs due to H5N1 virus. These feline species showed a generalised course of disease and lesions were present in the lungs, heart, thymus, stomach, intestines, liver, lymph nodes and brains. Virus replication was demonstrated in bronchiolar epithelium, hepatocytes and cerebral neurons. Clinical symptoms included fever, respiratory distress and neurological signs. These observations show that H5N1 virus infection can occur after oral uptake of contaminated avian carcasses. The site of viral entry upon the oral ingestion of H5N1 virus in tigers and leopards was not determined and no virological examinations of the intestines were performed. The authors suggested that the positive staining for virus nucleoprotein in hepatocytes may have resulted from a heavy virus

load that had passed through the digestive tract after carcasses were eaten (Thanawongnuwech et al. 2005). Involvement of the liver as part of the digestive tract, however, may well have been the result of oropharyngeal or respiratory infection followed by generalisation of the infection rather than initiation in the intestinal tract itself.

Recently confirmed natural cases of infection with HP H5N1 virus, presumably as a consequence of feeding on infected birds, have been reported in 3 cats and in a stone marten in Germany (WHO) http://www.who.int/csr/don/2006_03_09a/en/index.html.

Pigs are also susceptible to avian H5N1 influenza virus in nature. Serological evidence of H5N1 infection was found in a very small population of pigs (8 of 3175 pigs tested, or 0,25%) in Vietnam in 2004, where H5N1 has hit hardest, and no virological examinations were performed (Choi et al. 2005).

During the epidemic of HPAI in poultry in The Netherlands in 2003, the causative H7N7 virus was also introduced into the swine population (Loeffen et al. 2004). Antibodies to H7N7 were found in 13 out of 46 mixed herds with swine and infected poultry, but not in mixed herds where poultry was preventively culled, or in swine herds without poultry. In one of the infected herds, the farmer had been feeding broken eggs to the pigs, suggesting that pigs may have become infected via contaminated food. However, the virus was unable to establish itself in the swine population after the removal of the source of infection (i.e. infected poultry).

2.2.2 Experimental inoculation

Oral inoculations

Infection of 3 domestic cats was experimentally established by oral feeding of chick carcasses, which chicks had been inoculated with H5N1 virus 24 hours earlier (Rimmelzwaan et al. 2006). In all 3 cats, virus replicated not only in the respiratory tract but also in multiple extra respiratory tissues including the central nervous system. Viral antigen expression and virus-associated ganglioneuritis were observed in the submucosal and myenteric plexi in duodenum and ileum. Virus was detected consistently and at high titres in pharyngeal and nasal swabs, but virus titres were highly variable in rectal swabs. This study showed that H5N1 infection in felids could be established via oral uptake but, again, whether the infection was initiated in the oropharyngeal or gastro-intestinal tissues was not determined. The authors suggested that lesions found in the neural plexi of Meisner and Auerbach may reflect uptake of virus via the intestinal lumen. The suggestion was made because myenteric plexi were not involved in cats which, in a similar experiment, had been inoculated intratracheally with the same virus strain. Viral uptake via intestinal nerve endings was proposed, a mechanism that was shown to occur with herpes simplex virus type 1 (Gesser and Koo 1996), but other routes of entry were also considered. The neurotropism observed with H5N1 virus in cats was striking and neural pathways of virus spread and dissemination must be considered, as further discussed for H5N1 inoculated mice and ferrets.

Intranasal and intratracheal inoculations

Intratracheal H5N1 virus inoculations by the use of a catheter were performed in 3 cats as part of the experiments with orally inoculated cats described above (Kuiken et al. 2004, Rimmelzwaan et al. 2006). These cats showed clinical signs similar to the cats that had been orally fed carcasses of infected chicks. Lesions were found in the respiratory tract and extra

respiratory tissues including the brain but not in the intestinal plexi. Virus was isolated from several internal organs tested, which showed that the infection had a generalised character. In these cats, infectious virus was readily detected in pharyngeal and nasal swabs and also in rectal swabs, but the latter were less frequently positive and virus was present at much lower titres. This experiment also demonstrated that highly pathogenic H5N1 virus was more pathogenic for cats than other influenza viruses, since earlier experiments in which inoculation of cats had been performed with LPAI viruses resulted in a transient virus excretion without clinical signs (Hinshaw et al. 1981), while a human H3N2 virus strain caused no infection at all (Kuiken et al. 2004).

In a limited experimental study, intranasal inoculation of pigs with 2004 Asian H5N1 viruses resulted in virus isolation from the respiratory tract (tonsils, trachea and lungs) but not from the intestines (Choi et al. 2004). Virus was also isolated from the liver of 2 out of 4 pigs, despite the absence of detectable viraemia or virus in spleen and kidney. Although this experiment confirmed the susceptibility of pigs for H5N1, there was no virus transmission between experimentally inoculated and in-contact pigs.

Ferrets are known to be naturally susceptible to infection with human influenza viruses, as well as to some swine, equine and avian influenza viruses, and they are a widely used influenza model. Ferrets have been intranasally inoculated with H5N1 viruses isolated in Hong Kong in 1997 (Zitzow et al. 2002) and in other Southeast Asian countries in 2003 and 2004 (Govorkova et al. 2005, Maines et al. 2005). H5N1 isolates from humans can induce more severe respiratory disease, fever, lethargy and weight loss than common human H3N2 influenza viruses. Diarrhoea and neurological signs were also seen following exposure to the H5N1 isolates and many infectious were fatal. The H5N1 isolates of human origin caused a systemic infection in ferrets, with high virus titres in the blood, spleen and liver. Only some H5N1 isolates were isolated from the intestine, at virus titres that were at least 100 to 1000 times lower than those in the respiratory tract. Histopathological examinations showed mononuclear cell infiltration in the interalveolar septa in the lungs and in the meninges, choroid plexus and brain parenchyma. Viral antigen-positive cells were only found in the lung alveoli and bronchioli and in neurons in the same areas of the brain where inflammation was present, but not in the intestines, despite the presence of diarrhoea in some ferrets. While the human H5N1 viruses had an obvious neurotropism, virus was also isolated from the brains of ferrets inoculated with normal human H3N2 influenza viruses, which do not spread beyond the respiratory tract in humans, and failed to induce neurological signs in ferrets. Because influenza virus titres in the olfactory bulb of ferrets peaked early in infection, the authors suggest that the virus reaches the central nervous system via the olfactory nerves and ethmoid plate after intranasal inoculation. The H5N1 virus was also isolated from the brains of ferrets without detectable viraemia or replication in internal organs, so that it is unlikely that the virus reaches the nervous system via the circulation. Some pathogenetic events in these ferrets inoculated with H5N1 resemble those in cats, particularly the involvement of extra-respiratory organs and the strong neurotropic character. It should be mentioned that involvement of the central nervous system has also been reported in 2 human cases of H5N1 (de Jong et al. 2005). In contrast to H5N1 viruses obtained from humans, most avian H5N1 isolates induced only mild disease in ferrets and they replicated only in the respiratory tract.

Most experimental infection studies with H5N1 have been performed in BALB/c mice. H5N1 viruses isolated from humans in Hong Kong in 1997 showed two distinct phenotypes in mice (Gao et al., Lu et al. 1999). Some viruses were low pathogenic: they replicated exclusively in

the respiratory tract, were generally non lethal and animals had cleared the virus by 6 to 9 days post inoculation. Other viruses were highly pathogenic: they replicated in multiple organs in addition to the respiratory tract and caused mortality in the mouse model. Most human isolates from 2003 and 2004, in contrast to avian isolates, were also highly pathogenic for mice (Maines et al. 2005). One of such highly pathogenic viruses was isolated from the colon of infected mice, at a low virus titre ($10^{2.5}$ PFU per gram) (Gao et al. 1999). In another mouse infection study with similar H5N1 isolates, the stomach, duodenum and large intestine tested negative for influenza viral antigen positive cells (Dybing et al. 2000). It is very clear, in any case, that the respiratory tract and nervous system are the major sites of H5N1 replication in mice. According to some studies, viraemia is unlikely to contribute to the dissemination of virus to other organs in mice, and this is clearly different in chickens (Tanaka et al. 2003, Nishimura et al. 2000). One of the Hong Kong/97 (H5N1) isolates was found to replicate first in epithelial cells of the nasal mucosa, bronchi and alveoli, thereafter it appeared to spread via extensions of the nervus vagus and/or nervus trigeminus to the brain stem and later to the cerebral cortex (Tanaka et al. 2003). Virus spread to the central nervous system may also be virus dose-dependent, as a less virulent Hong Kong/97 (H5N1) isolate was isolated from the brain after intranasal inoculation with a high virus dose but not with a low virus dose.

Multiple inoculation sites

Macaques are considered among the most reliable animal models used to study the pathogenesis of influenza in humans. In experimental infection studies, cynomolgous macaques were inoculated with a human Hong Kong/97 H5N1 isolate via intratracheal and oropharyngeal and intraconjunctival inoculation routes (Rimmelzwaan et al. 2001, Kuiken et al. 2003). The clinical signs – fever and acute respiratory distress - of H5N1 infection in macaques resembled those in humans and were more severe than those seen with a common human H3N2 influenza virus. Unlike in cats, mice or ferrets, neurological or gastrointestinal symptoms did not occur in H5N1-inoculated macaques. Another difference with the situation in cats, mice or ferrets, was the lack of evidence for replication of H5N1 outside the respiratory tract. The lungs were clearly the major site of H5N1 virus replication. Multiple organ dysfunction was observed without evidence of virus replication in the brain, spleen, liver or kidney, and was probably due to diffuse alveolar damage and acute respiratory distress syndrome. To date no investigations have been reported as to whether or not H5N1 can replicate in the intestines of macaques.

2.3 CONCLUSIONS

Infection of humans with HPAI viruses subtypes H5N1 and H7N7 has occurred. The majority of human cases have a record of close contact with infected poultry and infection presumably occurs via conjunctival or upper respiratory tract tissues. However, the possibility that the gastrointestinal tract is another site of virus entry, replication and shedding, particularly with H5N1 virus, cannot be excluded. Oral uptake of H5N1 has led to infection in cats and tigers but the portal of entry of the virus - oropharynx, upper respiratory tract tissues or intestinal tract - is under discussion. In fatal human cases of AI due to H5N1, viral replicating RNA was found in the intestines by RT-PCR and infectious virus was found in rectal swabs. However, this replicating RNA may have been produced in oropharyngeal or respiratory tissues and swallowed, and may not have originated from replication in the gastrointestinal tract itself.

Virus may have reached the intestinal plexi of orally inoculated cats via the intestinal lumen, but it is also possible that spread via neural pathways has occurred. Another virus, the porcine

haemagglutinating encephalitis virus belonging to the Coronavirus family, is known to affect the same plexi. Pathogenesis studies showed that the intestinal plexi of pigs were reached after replication in intestinal epithelial cells and local sensory ganglia; thereafter the virus spread to the spinal cord (Andries and Pensaert 1980). Neural tropism of H5N1 virus has been shown in several experimentally inoculated mammals such as cats, ferrets and mice and was also confirmed in one human case in Vietnam. The possibility exists, therefore, that H5N1 viruses also have neurotropic characteristics in several mammals and that entry and spread in the body may occur via nerves. Neural spread of H5N1 was shown in mice (Tanaka et al. 2003) while entry via nerve endings in the intestine was suggested in cats (Rimmelzwaan et al, 2006). Viral entry via nerve endings or neuroepithelial cells and invasion in the body via nerves has often been described for *Alphaherpesvirinae* (Mettenleiter 2003).

2.4 MISSING SCIENTIFIC INFORMATION

The pathogenesis of HPAI viruses in humans particularly with regard to the gastrointestinal tract as a possible portal of entry and site of replication needs more study. Features may differ from one mammalian species to another and from one strain to another. From public health and food safety points of view, it is important to know if consumption of raw food containing HPAI virus can lead to human infection and if so, how and under which conditions. This information will be useful and will make clear which precautions need or need not to be taken when consuming poultry products in or from infected areas. It requires to be determined if the initiation of infection after oral virus uptake occurs via upper respiratory, oropharyngeal and/or gastrointestinal tract tissues, and what type of cells serve as entry routes for HPAI viruses. Animal models in which the pathogenetic events resemble those in humans will be needed. Detailed infection studies in cats, in which several inoculation approaches (e.g. directly in the stomach or intestinal lumen) are used, may provide useful information. It must be clarified if intestinal involvement occurs at the start of the infection, or merely results from a generalised dissemination of the virus to many types of organ tissues.

More attention is required to be given to the neurotropism of H5N1 in different mammals, including felids and mice, and to the possibility that nerve endings or neuroepithelial cells serve as portal of entry in humans. This might offer one explanation for the exceptionally low frequency with which direct transmission of H5N1 to humans occurs.

3. VIRAL AND HOST FACTORS POSSIBLY INVOLVED IN AI INFECTIONS IN HUMANS (WITH REFERENCE TO THE GASTRO-INTESTINAL TRACT)

It has long been thought that the lack of suitable receptors for AI viruses on mammalian cells restricts their ability to infect humans. Apart from the host receptors and the receptor-binding site of the virus, other viral genetic characteristics will also determine the chance for avian viruses to replicate in mammals. These aspects will be discussed in the present section.

If an AI virus were to reach the GI tract of humans, it would be exposed to numerous adverse conditions in the stomach and intestines, which may affect the infectivity of the virus. If the GI tract were to serve as a possible portal of entry, then high virus quantities may be required in the food in order to reach a minimal infectious dose. Some of these factors influencing the fate of the AI virus in the food after consumption will be discussed.

3.1 INFLUENZA VIRUS RECEPTORS AND RECEPTOR-BINDING SITES

It is generally accepted that host specificity of influenza viruses is controlled by a variety of viral genes and their interactions and role in different cellular environments (Zambon 2001).

The HA mediates the binding of an influenza virus to receptors on the host cell, while the NA breaks the binding between the HA and its receptor which is required for the release of new progeny virus particles. The combination of viral HA receptor specificity and NA cleavage specificity is, therefore, important for the host specificity, tissue tropism and virulence of an influenza virus.

The influenza virus receptor consists of sialic acid attached to galactose. Influenza viruses recognise two types of sialic acid (N-acetylneuraminic acid and N-glycolylneuraminic acid), which are attached to galactose by an α-2,3 or an α-2,6 linkage (reviewed by Suzuki 2005). The ability of an influenza virus to replicate in a certain host species is influenced by both the sialic acid and linkage types in that host and by the receptor-binding site of the viral HA. The epithelial cells of duck intestine, for example, predominantly express α 2,3-linked sialic acid and influenza viruses isolated from aquatic birds possess HAs with high affinity for this type of sugar (Ito et al. 1998). In contrast, α 2,6-linked sialic acids predominate in the human respiratory tract (Baum and Paulson 1990) and influenza viruses circulating in humans favour binding to this type of sialic acid (Connor et al. 1994). Small mutations in the receptor binding site of the HA, particularly in amino acids 226 and 228, have been shown to cause a change in receptor specificity from 2-6 to 2-3 siallyl linkages and *vice versa* (reviewed by Suzuki 2005). Both types of receptors have been reported in the pig trachea and their presence supports the possibility that pigs are susceptible to both avian and human influenza viruses (Ito et al. 1998).

Recent *in vitro* studies on primary human airway epithelial cells have somehow changed our understanding of the interaction between sialic acids and influenza viruses (Matrosovich et al. 2004). A first surprising finding was that these cells contained both types of receptors, with 2-6 linkages predominantly found on non-ciliated and 2-3 linkages predominantly on ciliated cells. This suggests that the type of sialic acid linkage may be cell type- rather than species-dependent. Secondly, both human (H3N2) and avian H5N1 influenza viruses were able to replicate in these differentiated human airway epithelial cells. During the first cycle of virus replication human viruses preferentially infected non-ciliated cells and avian viruses mainly infected ciliated cells, which correlates with the expression of the corresponding types of sialic acid receptors. In later cycles of virus replication, the human but not the avian virus infected both ciliated and non-ciliated cells. Most avian H5N1 viruses that were isolated from humans in South East Asia had an avian virus-like receptor specificity (Matrosovich et al. 1999), which shows that avian-to-human transmission can occur despite the predominance of human receptors in humans. These data indicate that the particular role of receptor-binding sites as a barrier for the replication of AI viruses in humans is often oversimplified.

H5N1 viruses with no differences in their receptor-binding site differed strongly both in their replication ability and efficiency in the ferret model (Govorkova et al. 2005). This finding indicates that factors other than HA receptor specificity may restrict infection of mammals with AI viruses.

Because the respiratory tract is considered the major target of influenza virus replication in mammals, receptor studies have been performed exclusively in this organ system. Therefore,

there is no information on the types of receptors and the ability of AI viruses to replicate in the mammalian intestinal tract.

3.2 VIRUS CHARACTERISTICS AND GENETIC MAKE-UP

The genetic constellation of influenza viruses may also play an important role in the ability to cross the species barrier. The first H5N1 influenza virus that infected humans in Hong Kong in 1997 appeared to be a reassortant with the HA gene from Goose/Guangdong/96 and the other genes from different (non-H5) avian influenza viruses (Webster et al. 2002). The Hong Kong /97 (H5N1) viruses were rapidly eliminated by the culling of millions of poultry, but its precursors continued to persist in geese in southern China. Highly pathogenic H5N1 influenza viruses have been circulating in wild geese in China since 1996 and they have gone through several reassortment events by which they acquired new internal genes in various combinations from influenza viruses circulating in aquatic birds (Sims et al. 2005). From 2001 onwards, H5N1 viruses were not only isolated from aquatic birds but also from terrestrial poultry. Seven distinct H5N1 genotypes were identified in terrestrial poultry in 2001 and another 5 genotypes in 2002. Compared to the original Goose/Guangdong/96 virus, most genotypes had deletions in the stalk of the neuraminidase (NA) and in the non-structural NS1 protein. The so-called "Z" genotype, which contains both deletions, has become dominant in aquatic and terrestrial poultry since its emergence in 2002. The H5N1 isolates from humans since 2003 and 2004 had the same gene constellation as genotype Z but lacked the NA stalk deletion and were designated genotype "Z+".

It must be recognized that, since 2004, H5N1 isolates have infected the greatest number of humans ever, as well as felidae and pigs. Some believe, therefore, that the evolution of H5N1 viruses in recent years has been associated with increasing transmissibility to humans. On the other hand, it must be considered that the increased number of human infections may merely reflect the more frequent and more extensive exposure due to the large epidemics that have occurred in poultry since 2003 and that there is in fact no proof of an increase in the ability of the virus to replicate in mammals between 1997 and now. Taken together, the molecular determinants of H5N1 transmission to humans remain unclear and this is also true for the replication efficiency in cells of humans, including cells of the intestine.

3.3 FACTORS INFLUENCING HOST-VIRUS INTERACTIONS

3.3.1 Some considerations

In general terms, the direct transmission of H5N1 to humans must still be considered as an exceptional event since the number of confirmed human infections is less than 200 and thus still low in relation to the large population of people that have been exposed to the millions of infected birds. The factors that allow the virus to start the infection in some humans and not in others are still unclear. The relatively high number of cases in healthy children and young adults raises questions on possible host factors or circumstances that allow the exceptional virus uptake and crossing of the species barrier. The question arises as to whether or not the genetic make-up of the virus (or of some isolates in the viral population) has changed to facilitate virus invasion in the human body.

Practically all human cases studied to-date point towards the oropharynx and/or respiratory tract as the portal of entry and major site of replication. If, however, cells in these readily accessible mucosal surfaces were fully susceptible to the virus, then more human infections would be expected to take place. It is possible that entry routes and/or target cells exist other

than those traditionally considered, or that the virus can enter via an undisclosed target site or target cell type. The GI tract has been put forward as a possible portal of entry upon intake of contaminated food even though there is no sound evidence for virus replication in this organ system. It must be taken into account that, when ingestion of virus occurs, contact with oropharyngeal or upper respiratory tract tissues cannot be avoided and that these tissues may serve as entry sites.

The finding of intestinal involvement in some mammalian species is interesting. In cats, nerve plexi in the intestinal wall were infected after oral administration of H5N1 and virus uptake via nerve endings in the intestinal lumen was proposed, an entry mechanism that has been described for the human herpes simplex virus type 1 (Gesser and Koo 1996). It has become clear that H5N1 virus shows neurotropic characteristics in different mammalian species, while there has been only one reported case of H5N1 associated encephalitis in humans (de Jong et al. 2005). A study in mice demonstrated that H5N1 replicated in the respiratory tissues, ascended to the brain stem via the vagus and trigeminal nerves, but not via the blood, and then spread to other areas of the brain (Tanaka et al. 2003). One should be careful, of course, when extrapolating pathogenetic features of H5N1 viruses in mice to humans. However, neurotropic variants of H5N1 were rapidly selected after virus passage in mice and the possibility of a similar rapid selection of variants in humans or other mammals should be considered (Lipatov et al. 2003). The question arises whether H5N1 in mammals may use neural pathways to enter the body with or without prior replication in epithelial-like target cells in mucosal sites. Neuro-epithelial cells located in the upper respiratory tract could also serve this purpose. This potential mode of virus uptake needs further study in mammals and certainly deserves more attention in human cases.

Whether entry of AI viruses via nerve endings can occur in the intestine is rather doubtful, taken into account the many barriers the virus must overcome to reach the intestinal lumen while maintaining its infectivity in sufficient quantity. Nerve endings and neuro-epithelial cells are more numerous and more accessible in the oropharynx and respiratory tract than in the intestinal system (Pick et al. 2005, Steven and Lowe 2005).

At any rate, the fact that human infections with H5N1 are rare despite frequent exposure in heavily infected areas could be due to a lack of readily accessible primary replication sites or target cells at mucosal surfaces. Another option is that the virus replicates in epithelial cells of the respiratory tract, as human influenza viruses do, but that replication in these cells is hampered by some as yet unknown conditions. One possibility is that most of the AI virus particles fail to undergo a complete replication cycle in epithelial cells, so that there is no or minimal production of infectious progeny virus particles. This is in agreement with the finding that the H5N1 isolates from humans have a deletion in the stalk of the NA and that a short-stalked NA is inefficient in disaggregating progeny virus from infected cells. Another possibility is that innate immune proteins in respiratory secretions prevent initial infection with AI viruses in humans. The pulmonary surfactant proteins A and D and the scavenger receptor-rich glycoprotein 340, for example, have influenza A virus neutralizing and aggregating activity (White et al. 2005). Studies on the effect of these proteins on AI viruses are still lacking, and it remains to be seen whether they exert more potent effects on avian than on human influenza viruses. Finally, it cannot be excluded that existing immunity to human H1N1 and H3N2 influenza viruses confers a partial protection against infection of humans with H5N1. Despite the lack of serological cross-reaction between the HAs of these viruses, antibodies to the N1 of H1N1 and/or cell-mediated immunity could reduce the chance

for infection with H5N1. This might also explain why children and young adults, who have a less solid immune status to human influenza viruses than older subjects, are most prone to infection with H5N1.

3.3.2 Virus quantity at exposure

Most human infections with H5N1 occurred after close and intensive contact with avian species. There is thus circumstantial evidence that virus quantity at exposure must be high for the infection to become established. Again, this implies that the target cells or entry sites for the virus may not be easily reached. Also, an AI virus must overcome several unidentified barriers to infect humans. Whether high quantities of infectious virus reach in the intestinal lumen after consumption of infected food is doubtful, considering the many adverse conditions to which the virus is exposed in the GI tract. After being swallowed, the virus will adsorb on to tissues in the upper digestive tract such as those in the oesophagus and the stomach, and it will be diluted during the digestive processes. Its infectivity titre may be reduced by the extremely low pH of gastric secretions and by the effects of bile, when passing the stomach and the duodenum respectively. Inactivating effects of bile were demonstrated with several enveloped viruses including Newcastle disease virus (Lee and Hanson 1975). The chance of high amounts of virus reaching the intestinal lumen is thus rather low, unless abnormally high virus titres are taken up (such as when eating raw blood pudding from viraemic ducks) or when the virus is protected from inactivation (such as in bone marrow of bones eaten by carnivores).

Based on these data infection of the GI tract is considered to have a low probability of occurring under natural circumstances. Still, it cannot be fully overlooked and more studies to examine the possibility of gastrointestinal replication are required. Cats, ferrets, mice and possibly pigs could serve as useful animal models but it should be realised that the human infection may differ in several aspects.

3.3.3 Effect of low pH on AI viruses.

Acid lability of influenza viruses and virus inactivation by the low pH in the stomach has long been presumed to be a reason for the lack of intestinal replication of influenza viruses in mammals. However, 6 of 14 influenza viruses examined, including 2 LPAI viruses, were isolated from one or another part of the intestinal tract of ferrets that had been intranasally inoculated (Kawaoka et al. 1987). In the same study, one of 4 influenza viruses tested was also isolated from the faeces of intranasally inoculated pigs, whereas the jejunum, ileum and colon were negative.

Studies on the acid resistance of influenza viruses have yielded somewhat contrasting results. In one study (Webster et al. 1978), influenza viruses of ducks were relatively more acid stable than human viruses and it was claimed that this permits them to pass the acid pH of the gizzard and to replicate in the cells lining the intestinal tract, which is the major site of replication in several avian species.

In more recent studies (Lu et al. 2003), 4 H7N2 strains were examined for acid stability at pH 2 and infectivity was lost in less than 5 minutes. Still another study (Scholtissek 1985) showed great differences in pH sensitivity among different subtypes. All H3 strains were relatively stable against low pH (5.1-5.4) independent of the species of origin. All H7 and H5 strains were relatively labile at pH 5.5-6.0. H1 strains showed an intermediate sensitivity. Low pH values appear to affect mainly the HA and NA. Interestingly, the NA activity of AI

viruses of the H1N1 subtype was found to be more resistant to acid pH than the NA of human or swine H1N1 viruses (Fiszon et al. 1989), which may explain why AI viruses retain their functional activities in the low pH values in the upper digestive tract.

Acid stability of AI viruses appears to be a multifactorial issue in which not only the virus strain but also the virus quantity, the embedding medium, the pH value and the duration of exposure will play a role. Whether influenza viruses will be inactivated by exposure to the low pH of the gastric secretions after passing through the stomach is thus unpredictable and cannot be guaranteed

3.4 CONCLUSIONS

It is unlikely that the lower GI tract (stomach and intestines) could serve as a portal of entry of H5N1 virus in humans after consumption of food products from infected animals. There is no evidence that the virus replicates in the human intestine or that GI symptoms are due to direct effects of the virus in that organ. Even if virus uptake in the GI tract were possible, e.g. via nerve endings as was suggested in the experimentally inoculated cats, the chances that these endings would be hit are very slim. A minimal infectious dose of the virus must be able to reach the intestinal lumen considering the many barriers which have to be overcome. Diarrhoea, which was seen in some of the human H5N1 cases, may be a non-specific symptom. Similarly, the presence of viral RNA or even of infectious virus in rectal swabs does not allow one to conclude that the GI tract is a portal of entry or target organ for H5N1 virus. Viral RNA or infectious virus may have been produced in throat or respiratory tissues and swallowed, or could have reached the intestine after generalisation from other infected targets. Food that contains virus could be a source of infection if viral uptake were to occur via oropharingeal tissues. Also, the existence of an undisclosed target cell in the intestinal tract cannot be ruled out. The exact portal(s) of entry of the virus in humans have not been localised or identified. Epidemiological evidence suggests that infection in humans occurs rarely and occurs particularly after very close contact with infected animals. It is, therefore, likely that a high dose of virus may be needed to initiate an infection and that a readily accessible entry route for the virus does not exist. Experimental studies on the exact route(s) of H5N1 virus entry in mammals are needed and may provide useful information for the human infection.

3.5 MISSING SCIENTIFIC INFORMATION

It is clear that more research is needed on the significance of total gene constellations and of specific genes or gene products in order to gain insights into the molecular basis of the host restriction of H5N1 influenza viruses.

The pathogenetic basis of the observation that the H5N1 virus causes infection in some individuals and not in others remains unknown. The route(s) of entry and/or the cells that allow the virus to enter and the mechanism of species barrier crossing must be studied based upon experimental inoculation of mammalian species which can be useful as models for the human infection. It remains to be determined if the lower GI tract can serve as portal of entry. Different inoculation routes require to be studied and sequential examinations of different tissues for virus replication throughout the course of infection must be performed.

Only this way, speculations on the routes of infection in humans will turn into knowledge.

4. SCIENTIFIC PANEL MEMBERS

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