RECENT ADVANCES IN AVIAN VIROLOGY

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SUMMARY

Selected, recent research on the following avian diseases, and their causative viruses, has been reviewed: chicken anaemia, infectious bursal disease, turkey rhinotracheitis, avian nephritis, fowlpox, influenza, infectious bronchitis and turkey enteritis.

INTRODUCTION

It may always have been a quiet week in Lake Wobegon but the last 12 years have certainly been eventful for the world's poultry. During this time there has been a growing appreciation of the role of chicken anaemia virus in poultry disease. Other diseases appeared for the first time, e.g. turkey rhinotracheitis, or, in the case of infectious bursal disease, returned with a vengeance. In view of the rapid advances made in our knowledge of these and other relatively new diseases and their causative viruses I have concentrated on them in this review. Even by narrowing the field in this way I cannot possibly review all the relevant literature, even for only the last 3 years or so. Therefore I have been selective. If I have whet the appetite of the reader with regard to one or more of the diseases then I would direct attention, for a thorough bibliography, to the reference sections of the most recent papers and reviews cited and to the latest edition of *Diseases of Poultry*, the bible of avian pathologists (Calnek *et al.*, 1991a).

CHICKEN ANAEMIA

Understanding of chicken anaemia (CA) has increased greatly in recent years as a result of the isolation of the causative agent, chicken anaemia agent, now known to be a virus (CAV), in 1979 by Yuasa and its propagation in certain lymphoblastoid chicken cell lines; CAV does not replicate in conventional monolayer cell cultures. It has recently been reviewed by McNulty (1991) and von Bülow (1991).

The disease has been reproduced experimentally by many workers since the early studies in Japan, permitting detailed analysis of the pathogenesis and pathology of chicken anaemia (Engstrom *et al.*, 1988; Goryo *et al.*, 1989; Jeurissen *et al.*. 1989; McNulty *et al.*, 1990a; Hoop & Reece, 1991). The effects of CAV are most

marked in very young chicks. Bone marrow atrophy is the most characteristic lesion but thymic atrophy is the most consistent. After intramuscular inoculation of day-old chicks haematocrit levels and counts of thrombocytes and red and white blood cells decline after about 8 days. The main target cells during these early days are the haemopoietic stem cells in the bone marrow and thymic lymphoblasts in the outer cortex of the thymus. In addition there is some depletion of lymphocytes in the spleen, bursa of Fabricius and caecal tonsils. Chicken anaemia is very age dependent and by 2 weeks of age birds are essentially resistant to development of the disease but not to infection (Yuasa & Imai, 1986; McNulty et al., 1989, 1990a; Rosenberger & Cloud, 1989). Age resistance may be related to increased humoral antibody production as the chicken matures (Yuasa et al., 1988). Certainly maternal antibody is protective against experimental infection (Yuasa et al., 1988) and protection of progeny against the disease has been obtained by vaccinating the parents, which develop only subclinical infections. CAV spreads both horizontally and vertically. In the field disease is first observed as the birds approach 2 weeks of age. Mortality is often 5–10% but can exceed 50%, peaking within 5–6 days of the onset of disease signs and returning to normal within the next week. Focal skin lesions occur on the wings, and elsewhere, being due to skin haemorrhages. The subsequent blue colour of the skin has led to 'blue wing disease' being one synonym for the disease. The lesions break and become infected with bacteria, leading to gangrenous dermatitis, and haematocrit values fall (Chettle et al., 1988). Subclinical (McNulty et al., 1991) in addition to clinical (McIlroy et al., submitted for publication) infections with CAV have resulted in economic losses in broilers.

A most important aspect of CAV is that it is believed to be immunosuppressive, young chicks frequently suffering from increased incidence of infection by other pathogens. CAV can also impair responses to vaccines (Box et al., 1988; Otaki et al., 1988).

There has been much activity on the composition of CAV. Electron microscopy has revealed that this non-enveloped virus is 20–25 nm in diameter, the size estimates depending on the staining procedure, the virions being composed of 32 subunits (Goryo et al., 1987; Gelderblom et al., 1989; McNulty et al., 1990c; Todd et al., 1991) (Fig. 1). Only one polypeptide, estimated at 50 000 molecular weight by electrophoresis, has been detected in virions of CAV (Todd et al., 1990) and monoclonal antibodies have been prepared (McNulty et al., 1990d). Earlier observations on the DNA genome of CAV have now been confirmed and extended by the cloning and sequencing of CAV DNA (Claessens et al., 1991; Noteborn et al., 1991). This has revealed that CAV virions contain a small, single-stranded (minus sense), circular DNA molecule of approximately 2300 nucleotides. The DNA sequence has three partially overlapping major reading frames that would encode polypeptides of approximately 52 000, 24 000 and 14 000 molecular weight. The genome probably contains only one promoter region and only one poly-adenylation signal. Transfection of cells in vitro with the cloned, circularized DNA resulted in the production of CAV which was infectious for chickens. To date CAV remains unclassified but properties of its genome and polypeptides show that it is not a parvovirus. Whether it is related to two other small icosahedral viruses with circular, single-stranded DNA genomes, porcine circovirus and psittacine beak

and feather disease virus, cannot be decided until there is further analysis of the genomes of these viruses (McNulty, 1991).

INFECTIOUS BURSAL DISEASE

First recognized as a specific disease in 1962, infectious bursal disease (IBD) is an acute, highly contagious disease of young chickens, the causative virus (IBDV) having a tropism for lymphoid tissue, in particular the bursa of Fabricius (Lukert & Saif, 1991). Consequently, in chicks, IBDV is immunosuppressive, recent experimental studies indicating that IBDV infection has exacerbated infections by a reovirus (Moradian *et al.*, 1990), *Escherichia coli* (Nakamura *et al.*, 1990), avian nephritis virus (Narita *et al.*, 1991) and *Aspergillus flavus* (Okoye *et al.*, 1991). Using bursectomized chickens Okoye & Uzoukwu (1990) showed that the bursa was not essential for the establishment of an IBDV infection but was required for the clinical infection. IBDV is a birnavirus, that is to say it has a non-enveloped, icosahedral virion, 55–65 nm in diameter, containing two pieces of double-stranded RNA and four proteins (Kibenge *et al.*, 1988). IBD returned to prominence in the second half of the last decade, both in Europe and the USA, as a consequence of the spread of antigenic and pathotypic variants. However, the nature of the variants on each side of the Atlantic would appear to be different (Snyder *et al.*, 1988; Snyder, 1989, 1990; Box, 1990).

There are two serotypes of IBDV, serotype 1 strains being pathogenic for chickens while serotype 2 strains are not. The emergence in the USA of variants of economic importance has been reviewed by Snyder (Snyder, 1989, 1990). The area concerned was the Delmarva Pennisula (i.e. portions of the eastern states of Delaware, Maryland and Virginia) where in 1984–85 there was an increase in mortality in broilers. A concerted effort revealed that young broiler chicks had poor
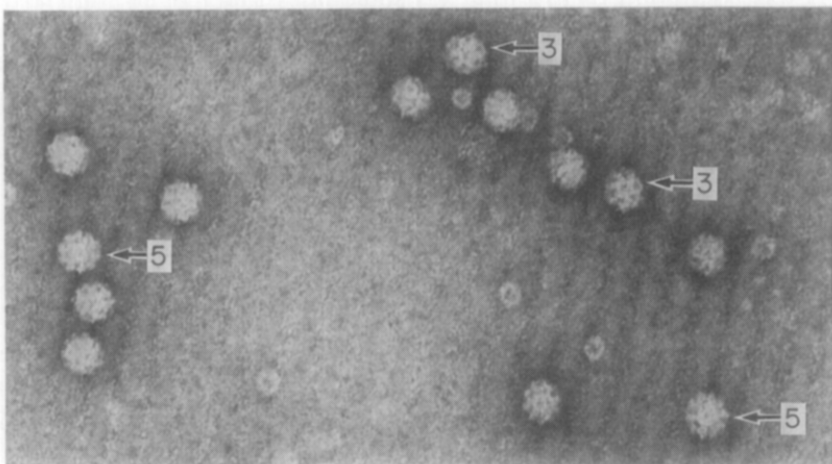

Fig. 1. Electron micrograph of chicken anaemia virus. Arrows show particles viewed down threefold and fivefold axes of symmetry. (×225 000). Courtesy of M.S. McNulty, Veterinary Research Laboratories, Belfast.

bursal integrity while not exhibiting classical IBD. Strains of IBDV were isolated that were of the same type 1 serotype as the standard IBDV strains from which vaccines had been prepared but which differed from the standard strains in causing very rapid bursal atrophy whereas classical strains initially cause an increase in bursal size followed by a decline to about one-third of original weight by day 8. Virulent standard strains are associated with mortality, haemorrhagic bursas and transudate whereas the variants produce few clinical signs.

Monoclonal antibody analysis has revealed differences between the standard and Delmarva variants and identified another variant, termed GLS (Snyder, 1990). Although not proven it is believed that the increased use of vaccines played a role in the emergence of the variants. Epidemiological evidence supports the view that these pathotypic variants were responsible for the higher incidence of IBD and losses related to immunosuppression in the eastern states compared with elsewhere. For example, in the Delmarva Peninsula classical IBDV isolates accounted for only 4% of the total, the remainder being the Delaware and GLS strains, whereas in the west and northwest states the situation was the reverse, 94% being classical isolates (Snyder, 1990).

Commercial vaccines did not give full protection against these Delaware variants and new inactivated virus vaccines were developed. Ismail & Saif (1991), using inactivated virus, have confirmed that the standard and variant strains have some protection-inducing epitopes in common. However, there were significant antigenic differences as the inactivated variant viruses induced greater protection against challenge with variant than did inactivated standard strains.

The recent resurgence of IBD in Europe appears to be distinct from that in the USA. Prior to 1987 the European strains of IBDV were of low pathogenicity, causing less than 1% mortality, although there were commercial losses due to decreased growth and immunodeficiency. Then, in 1987, the picture changed. Van den Berg et al. (1991), for example, describe how a highly pathogenic or very virulent (VV) IBDV strain spread rapidly throughout Belgium. Mortality in broilers was commonly 5–15% but was sometimes lower or as high as 25%. In laying pullets mortality reached as high as 60% and infection occurred later than in broilers. This is related to the growth rates of the birds; layers grow more slowly than broilers and their maternally derived antibody declines more slowly, protecting them for longer (Box, 1990). Similar observations to those in Belgium have been made in the Netherlands and the UK (Chettle et al., 1989; Lister, 1989; Stuart, 1989).

The VVIBDV is able to infect some chicks at only 15 days old, in the face of maternal antibody titres too high to permit the highly attenuated vaccine strains to replicate. Vaccines of intermediate virulence have been introduced in some countries in an attempt to control VVIBDV, with some degree of success (Rosales et al., 1989). These vaccines are able to be used earlier than the highly attenuated vaccine, thus indicating active immunity earlier and thus reducing the window of opportunity for VVIBDV strains (data of Wyeth & Chettle quoted in Box, 1990). There is experimental evidence that IBDV vaccines of intermediate virulence (van den Berg et al., 1991) and inactivated vaccines (Wyeth & Chettle, 1990) can reduce losses caused by VVIBDV.

In contrast to the USA, the European IBDV variants appear not to differ sub-

stantially in antigenic terms from the classical strains (van den Berg *et al.*, 1991; Öppling *et al.*, 1991a). What is the molecular basis behind the changes observed in IBDV in the USA and Europe? It has been established that IBDV-neutralizing antibodies are induced by virus protein 2 (VP2) (Becht *et al.*, 1988; Fahey *et al.*, 1989). This protein and VP3 and VP4 are derived by cleavage of a large precursor protein which is encoded by the larger, A, of the two RNA segments of the virus (Kibenge *et al.*, 1988). Neutralizing monoclonal antibodies have been raised against VP2 and have been able to passively protect chickens (Fahey *et al.*, 1991). A complementary DNA (cDNA) sequence encoding VP2 has been inserted into the DNA genome of fowlpox virus which was then used to vaccinate chickens (Bayliss *et al.*, 1991). Expression of VP2 in the chickens protected them from mortality, but not bursal damage, when substantially challenged with virulent IBDV. VP2 has also been expressed in yeast cells (Jagadish *et al.*, 1991), the product being able to induce anti-VP2 neutralizing antibodies when injected into laying chickens (MacReadie *et al.*, 1990). The chicks derived from these hens had passively transferred antibody which protected them from challenge with IBDV. These results confirm the important role of VP2 for the induction of protective immune responses and are promising for the development of genetically engineered vaccines; currently IBDV vaccines are developed from the bursae of IBDV-infected chicks.

Monoclonal antibodies have been used to analyse the epitopes of some of the IBDV proteins. Öppling *et al.* (1991b) have detected two non-overlapping epitopes on VP3, one of which is common to both serotypes, the other being different in serotype 1 and 2. One epitope on VP2 is common to both serotypes (Becht *et al.*, 1988). The antigenic domain of VP2 which induces neutralizing antibody is conformation-dependent and is composed of at least three independent epitopes (Fahey *et al.*, 1991; Lee *et al.*, 1991; Öppling *et al.*, 1991a; Reddy & Silim, 1991). The sequence of VP2 has been determined for several serotype 1 strains (Bayliss *et al.*, 1990; Kibenge *et al.*, 1990; Heine *et al.*, 1991) and one serotype 2 strain (Kibenge *et al.*, 1991). This has shown that VP2 of serotype 1 strains has approximately 490 amino acids which includes a hypervariable region, known to contain a conformation dependent, neutralizing antibody-inducing epitope (Azad *et al.*, 1987), approximately compromising amino acids 240–330. Serotype A strains have 97% or greater amino acid identity in VP2 as a whole. Half of the amino acid differences fall within the hypervariable region. The VP2 of a serotype 2 strain had 90% amino acid identity with that of the most distantly related serotype 1 strain (Kibenge *et al.*, 1991).

So, what of the difference between standard strains and the variants? Heine *et al.* (1991) have sequenced VP2 of the Delaware E variant. It differed from a consensus sequence of six standard strains at eight amino acids, all of which were located in the hypervariable region. Changes in the two hydrophilic regions at either end of this region were unique to the Delaware variant and were crucial for its ability to escape neutralization by monoclonal antibodies produced against a classical strain. The authors suggested that it was changes in the second hydrophilic region in particular which accounted for the resistance of the E variants to neutralizing antibodies which had been induced by classical vaccine strains. It was also observed that the sequence Ser-Trp-Ser-Ala-Ser-Gly-Ser, which occurs within the

VP2 variable region next to the second hydrophilic sequence was conserved only in virulent strains and it was suggested that this seven residue sequence might be in some way related to the virulent nature of the Delaware E strain.

In contrast to the American variants, monoclonal antibody studies have indicated that major antigenic changes were not present in the European VVIBDV strains (Öppling *et al.*, 1991a; van den Berg *et al.*, 1991). That is, the ability of the VVIBDV strains to replicate in the face of maternally derived antibody appears not to be due to antigenic changes which would enable the virus to circumvent neutralization by these antibodies. Further investigations are required to explain the field observations.

TURKEY RHINOTRACHEITIS (TRT)

The demise of the turkey industry in South Africa was due in no small part to the disease turkey rhinotracheitis. The disease, in which young poults had respiratory signs, was first observed by Buys & Du Preez in 1978 and reported in a trade journal in 1980. Morbidity was usually in excess of 90% and mortality, generally caused by secondary bacterial infection, was up to 30%. Buys and Du Preez isolated a virus (TRTV), which by electron microscopy (Fig. 2) resembled ortho- and para-
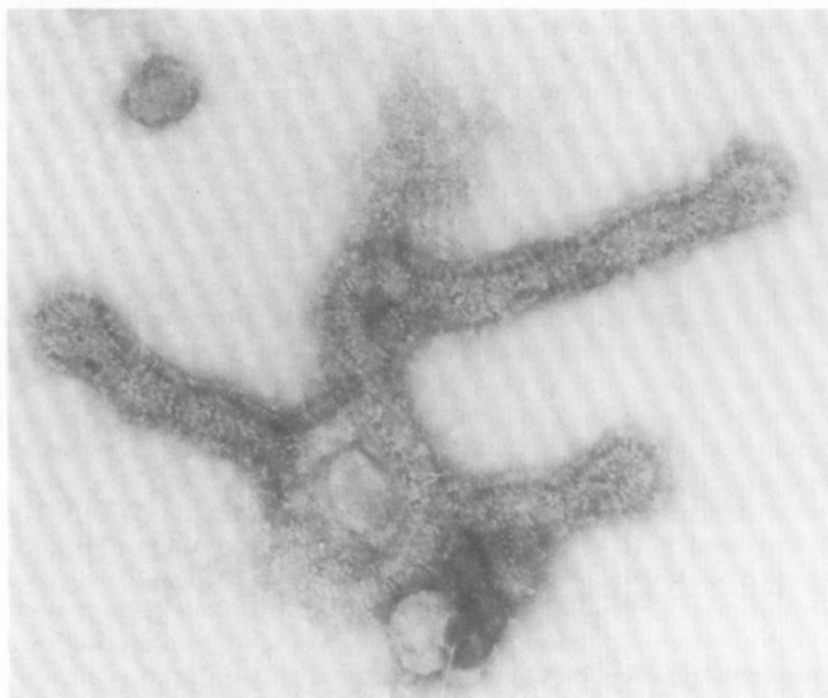

Fig. 2. Electron micrograph of turkey rhinotracheitis virus, a pneumovirus, grown in tracheal organ cultures. Extremely elongated forms like the one shown are frequently observed. Roughly spherical particles, 80–100 nm in diameter but also larger, are also commonly seen. (× 110 400). Courtesy of J. K. A. Cook, AFRC Institute for Animal Health, Houghton Laboratory.

myxoviruses, reproduced the disease experimentally and produced a live vaccine. However, this work went largely unnoticed; it was not reported fully until some 10 years later (Buys et al., 1989a), encouraged by developments in the UK in 1985.

Disease observations similar to the South African ones were made in Israel and in several European countries in the late 1970s and early 1980s (Alexander, 1991). A number of viruses and bacteria were suspected of being the cause but the work was inconclusive. The situation was resolved in 1985 when the disease made its landfall in the UK. Turkey flocks in the eastern counties were the first to be hit, after which the disease spread rapidly throughout England and Wales; Scotland was spared. In addition to mortality, up to 60% as a result of secondary bacterial infection, there were marked drops in egg production. Several groups of researchers in the UK and one in France isolated a virus (TRTV), in which process tracheal organ cultures made from turkey or chicken embryos played an important role, and reproduced the disease (Giraud et al., 1986; Jones et al., 1986; McDougall & Cook, 1986; Wilding et al., 1986; Wyeth et al., 1986). Analysis of the proteins and mRNAs of TRTV (Cavanagh & Barrett, 1988; Collins & Gough, 1988; Ling & Pringle, 1988) and subsequent cloning of the fusion (F) and matrix (M) protein genes (Yu et al., 1991, 1992) has shown that TRTV is a member of the genus *Pneumovirus* of the Paramyxoviridae family, Paramyxovirinae subfamily. The TRTV F and M proteins have approximately 40% amino acid identity with those of human respiratory syncytial virus, the type species of the pneumoviruses, although there are some differences in gene order (Yu et al., 1992).

TRTV can replicate in the ciliated epithelial cells of the nares and trachea and in lung and air sac tissues and in the epithelium of the uterus and other regions of the oviduct (Jones et al., 1986, 1988; Cook et al., 1991b). TRTV was not isolated from heart, liver, spleen, kidney or caecal tonsils unless the turkeys had been simultaneously infected with *Bordetella avium* and a *Pasteurella*-like organism (Cook et al., 1991b).

A vaccine to combat TRT was clearly needed and the effort to produce one was given added impetus with the accumulation of evidence that TRTV could replicate and produce disease in chickens (O'Brien, 1985; Jones et al., 1987; Picault et al., 1987; Wyeth et al., 1987; Cook et al., 1988; Gough et al., 1988; Buys et al., 1989b; O'Loan et al., 1990). Live attenuated vaccines have been developed in the UK and France and some are available commercially (Cook et al., 1989a, b; Cook & Ellis, 1990; Williams et al., 1991).

TRTV can also infect guinea fowl and there is some experimental evidence that the virus can replicate in pheasants (Picault et al., 1987; Gough et al., 1988). In South Africa TRTV was isolated from broiler flocks in which more than 50% of the birds had respiratory symptoms and about 1% exhibited swollen heads (Buys et al., 1989b). Unlike the 1978 South African TRTV isolate the 1988 one was able to produce disease in broiler birds under experimental conditions. It is important to confirm this observation which has clear potential implications in respect of the hazard posed by TRTV to the poultry industry. Serum antibody studies by O'Loan et al. (1990) suggest that viruses related to TRTV may have been in the UK, without causing disease, for several years before the outbreak of TRT in the UK in 1985. More light needs to be thrown on this and on whether TRTV, or closely related pneumoviruses, are present in feral birds. Monoclonal antibodies and mol-

ecular techniques are currently being used to assess to what extent TRTV exhibits variation.

AVIAN NEPHRITIS VIRUS AND OTHER PICORNAVIRUSES

For some time the only virus of the domestic fowl that was considered to be a member of the enterovirus genus of the picornaviruses was avian encephalomyelitis virus (AEV) (Calnek et al., 1991b). There has been a resurgence of interest in avian viruses considered to be picornaviruses and probably of the enterovirus genus. The virus studied in greatest detail is avian nephritis virus (ANV), which was first isolated in Japan in 1976 from the rectal contents of apparently normal broiler chicks, to give isolate G4260. The virus spreads readily and typically causes a subclinical disease of chicks, causing lesions in the kidneys initially involving degeneration of proximal tubules and later interstitial nephritis, gouty nodules being present and plasma urate levels raised (Narita et al., 1990a, b; Takase et al., 1990; Imada & Kawamura, 1991). Some chicks had visceral urate deposits or urate deposits in the digital joints (Shirai et al., 1990).

Antibodies to ANV have been found to be widespread in chicken and turkey flocks, including specified-pathogen-free chickens (Connor et al., 1987; Nicholas et al., 1988). ANV may simply occupy just one sector of a spectrum of avian picornaviruses, perhaps very different from AEV, with some differences in tropism and pathogenicity. A number of enterovirus-like isolates have been divided into various groups by serological methods (Decaesstecker & Meulemans, 1989; McNulty et al., 1990b; Shirai et al., 1990).

Oral inoculation of 1-day-old commercial broiler chicks with many but not all of the isolates produced little or no growth retardation. Decaesstecker et al. (1989) found that the isolates replicated in the cytoplasm of enterocytes but, unlike the others, ANV did not produce histological lesions in the intestines but principally induced kidney lesions. Histological lesions were produced by some isolates in pancreas, proventriculus and kidney.

Three embryo-lethal enterovirus-like viruses were isolated in England from broilers exhibiting either stunting syndrome or baby chick nephropathy (Frazier et al., 1990). Experiment showed that all three isolates caused nephritis and growth suppression when inoculated into 1-day-old chicks and one of them caused an increased incidence of baby chick nephropathy. Two of the viruses were serologically related to ANV G4260. Interestingly the ability of G4260 to produce a cytopathic effect and plaques in chick kidney cell monolayers was related to the strain of bird from which the cells had been derived. Similarly, the lesions induced on chorioallantoic membranes following embryo inoculation with these isolates was related to the breed of bird. Shirai et al. (1990) showed that one strain of ANV produced growth retardation in one chicken line (151) but not another (PDL-1).

Reece & Frazier (1990) have described the histopathology associated with field cases of infectious stunting syndrome, from which enteroviruses were isolated. There was degeneration and necrosis of enterocytes in the crypts of Lieberkuhn. Pancreatic degeneration was observed in 35% of cases. Ultrastructural studies (Frazier & Reece, 1990) revealed membrane-bound cytoplasmic inclusions con-

taining picornavirus-like particles in mesenchymal cells and macrophages in the lamina propria, and occasionally in enterocytes.

Analysis of avian enteroviruses would benefit from the production of monoclonal antibodies. There has been no application of molecular biological techniques to the analysis of avian picornaviruses and little of a biochemical nature. Indeed, for most of the viruses discussed in this section there has been no direct proof that these 'enterovirus-like' viruses are picornaviruses. It is not possible to say whether the enterovirus-like viruses isolated in recent years are genetically very similar or poles apart, whether the variations in tissue tropism and pathogenicity result from great or small differences in the viruses or, indeed, in different breeds of chicken.

FOWLPOX VIRUS

Fowlpox virus has shot to fame not because of the disease it causes (Tripathy, 1991) but because of the diseases it may help to combat. I refer, of course, to the use of attenuated fowlpox virus as a vector for the protection-inducing genes of other pathogens with a view to producing a live, genetically manipulated vaccine. This application of fowlpox virus (the name is used here to refer specifically to the pox virus which infects chickens and turkeys) and also canary pox (Taylor et al., 1991) and pigeonpox (Letellier et al., 1991) viruses is based on the pioneering work of Moss and colleagues with vaccinia virus (Brown et al., 1986; Tartalia & Paoletti, 1988). I shall concentrate here on the results obtained with prototype vaccines produced specifically to combat avian disease (Cavanagh, 1988; Tomley, 1991).

The haemagglutinin (HA) gene of influenza virus was among the first genes to be inserted into the genome of vaccinia virus and the HA protein (H5 subtype) has subsequently been expressed using fowlpox virus recombinants (Beard et al., 1991; Tripathy & Schnitzlein, 1991; Webster et al., 1991). Chickens which were vaccinated with the recombinant subsequently resisted challenge with a virulent H5 strain, that is they did not develop clinical signs and none died, in contrast to the controls. Wing web inoculation was far superior to comb scarification.

Fowlpox and pigeonpox viruses have been used to express the fusion (F) and haemagglutinin-neuraminidase (HN) proteins of Newcastle disease virus (NDV) in chickens (Boursnell et al., 1990a, b, c; Yanagida et al., 1990; Letellier et al., 1991). Both NDV proteins expressed in this way induced protective immune responses. The VP2 protein of infectious bursal disease virus has also been expressed using fowlpox virus. This protected chickens against lethal challenge but not against bursal damage (Bayliss et al., 1991).

A most exciting prospect for avian pox viruses is that they may be used to make recombinant vaccines to vaccinate mammals. It has been found that while inoculation of several mammals with a fowlpox virus recombinant carrying the G protein gene of rabies virus did not result in the production of infectious virus, some fowlpox virus proteins were produced and so was the rabies virus G protein (Taylor et al., 1988, 1991). The immune response against the G protein was such that the animals were protected against challenge. The non-production of pro-

geny fowlpox virus in mammals is a highly desirable outcome, meaning that the infection is self-limiting with no possibility of spread to other animals and, presumably, little chance that fowlpox virus will adapt to growth in mammals, an occurrence that is certainly not desirable.

AVIAN INFLUENZA VIRUSES

Recent research has shown that the dividing line between 'avian' and 'mammalian' influenza A viruses can be thin. Of the many studies on avian influenza viruses (AIVs) (serotype A) published in recent years (reviewed by Easterday & Hinshaw, 1991) I am going to concentrate on those which have addressed the question of the relationship between AIVs of feral birds and those isolated from domestic birds and from various mammals (Fig. 3). The evidence that AIVs are promiscuous (i.e. that they, or at least some of their genes, have frequently crossed species barriers) derives largely from an intensive use of monoclonal antibodies and nucleotide sequencing but also includes some direct animal experimentation. Preceding the work in the laboratory was a great deal of walking and wading to collect virus samples from a variety of species ranging across the world.

It had been suspected for many years that outbreaks of influenza in domestic birds were caused by AIVs present in feral birds, especially in water fowl. The affected domestic flocks (chickens, turkeys, ducks) were commonly under migration
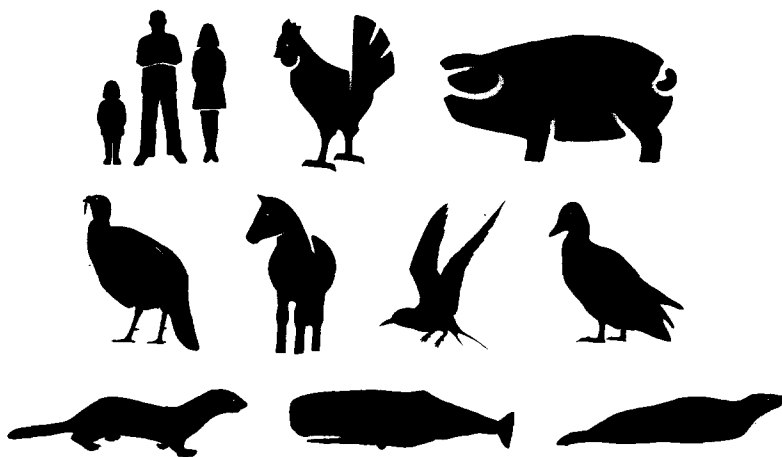

Fig. 3. Influenza A viruses have been isolated from a wide range of terrestrial and marine mammals and from many birds of different habitats. There is now much strong evidence to show that influenza A viruses can spread from one species to another, including from birds to mammals. Adaptation of the virus to another host may on some occasions simply involve nucleotide substitutions, deletions or insertions in some virus genes. In other instances influenza virus particles have been produced during mixed infections; influenza viruses have a separate RNA segment for each gene, permitting progeny (reassortants) with a combination (constellation) of genes different from that of both parents. Some gene constellations may equip a reassortant for replication in another host, although additional point mutations are often additionally involved in the adaptation process.

flyways or adjacent to water where water fowl abounded. Epidemiological, molecular and experimental (Kawaoka *et al.*, 1987b) investigations have provided strong evidence for the transfer of AIV from wild water fowl to nearby domestic ducks in Ireland and from them to neighbouring turkeys, causing serious disease. In experiments the virus was apathogenic for ducks but killed turkeys and chickens. Some AIVs from shorebirds and gulls can replicate in chickens and domestic ducks (Kawaoka *et al.*, 1988) while AIV from chickens can infect starlings and sparrows (Nestorowicz *et al.*, 1987). An AIV isolated from turkeys could circulate among quails, without causing disease, but killed in-contact chickens (Tashiro *et al.*, 1987).

The haemagglutinin (HA) glycoprotein of influenza virus is a primary determinant of pathogenicity and must be cleaved into two subunits to be active (reviewed by McCauley, 1987). In addition to a readily cleavable HA, influenza viruses require a suitable constellation of the other seven RNA segments for virulence. Webster *et al.* (1989) have shown that a larger proportion of viruses from shorebirds and from chickens in city markets, compared with wild ducks, had gene constellations which, when associated with the gene for a readily cleavable HA, produced viruses which were highly pathogenic for chickens. This work showed that since mixed infections with influenza viruses can produce reassortant viruses—having RNA segments from each parent—there is great potential for apathogenic strains to become virulent upon receipt of the gene for a readily cleavable HA.

Southern China is believed to be an influenza epicentre (Kida *et al.*, 1988; Shortridge *et al.*, 1991; Yasuda *et al.*, 1991). In a study of two influenza viruses from domestic ducks and one from a goose, all with an HA of the H3 subtype (as possessed by many isolates from humans) sequencing revealed that the HAs had sequences more than 98% identical with the H3 HAs of some isolates made from wild ducks and from pigs. This and other data support the view that in an environment, such as southern China, where there are large, close populations of wild and domestic birds and other mammals, including man, there has been a transfer of influenza viruses from wild birds to domestic birds and to pigs. It is proposed that the pig was a 'mixing vessel', producing a new human pandemic strain (Hong Kong 'flu' of 1968) by genetic reassortment. The swine influenza viruses of the H1 subtype which have been circulating in Europe since 1979 are derived from an AIV gene pool but the classical swine viruses from early this century evolved by a different route (Schultz *et al.*, 1991).

Analysis of another influenza virus gene, that for the nucleoprotein (NP), has been used to examine influenza virus isolates from whales, seals, gulls and terns. The evidence points to the 'relatively recent' transmission of the virus from the birds to these marine mammals (Mandler *et al.*, 1990). Li *et al.* (1990) have gone one stage further. They took a seal influenza isolate, apathogenic for chickens, and passaged it in chick embryo kidney cells. Variants adapted to these cells had an HA which had additional basic residues at the cleavage site; these variants were pathogenic for chickens. This was the first demonstration that an influenza virus isolate from a mammal could be modified by passage in a new cell type so as to be pathogenic for an avian species.

Finally, it has been shown that AIVs can replicate in ferrets, in both the respirat-

ory and intestinal tracts (Kawaoka *et al.*, 1987a) and in humans infected with AIVs of the H4, H6 and H10 subtypes (Beare & Webster, 1991). To date human isolates of influenza virus have been of only three H subtypes (1–3) whereas there are 14 known H subtypes of AIV. Beare and Webster suggest that it is possible that more avian HA subtypes might enter the human influenza virus gene pool through reassortment between avian and circulating human viruses.

INFECTIOUS BRONCHITIS

During the 1980s as interest in coronaviruses grew so, apparently, did the virus. I refer to the fact that up until about 1985 it was believed that the size of the coronavirus genome was between 15 000 and 18 000 nucleotides. This was based on estimates of the size of coronavirus genomes determined by gel electrophoresis. Cloning was then commenced at the 3' end of the positive-stranded RNA genome and when 20 000 bases had been cloned and sequenced it was clear that the 5' end was, quite literally, not in sight. Eventually the whole genome had been cloned and found to comprise 27 600 nucleotides (Boursnell *et al.*, 1987). The RNA polymerase of IBV was found to be encoded by a massive 20 000 nucleotides. Translation of this enormous gene involves a process called ribosomal frameshifting in which ribosomes, having translated about 12 000 nucleotides, stop, shift backwards and then continue translation in a different reading frame (Brierley *et al.*, 1987, 1989). The control of this process has been found to involve a complex secondary RNA structure called a pseudoknot (Brierley *et al.*, 1991). Molecular aspects of IBV have been reviewed by Boursnell *et al.* (1989) and disease aspects by King & Cavanagh (1991).

The large spike (S) glycoprotein (approximately 1160 amino acids) comprises two subunits, the amino-terminal S1 and the carboxyterminal, membrane-anchoring S2. It is S1 that induces neutralizing antibody, the basis of IBV serotyping (Cavanagh *et al.*, 1988; Koch *et al.*, 1990, 1991). In the late 1970s and early 1980s Dutch and British workers isolated many strains of IBV from diseased chickens which were serologically distinct from each other and the other serotypes which had been discovered earlier in the USA. Existing vaccines did not give full protection against these other serotypes and new live and inactivated vaccines have subsequently been developed. Sequencing revealed why there were so many serotypes of IBV; the spike proteins (S) of some serotypes differed by 22–49% of the 520 or so amino acids in S1 (Binns *et al.*, 1986; Kusters *et al.*, 1989). However, some serotypes were found to be extremely similar to each other, differing by less than 3% (Cavanagh *et al.*, 1992).

Competition ELISAs with many neutralizing antibodies have revealed five neutralizing antibody inducing antigenic sites, three of which overlap, all in the S1 subunit (Koch *et al.*, 1990). Extensive sequencing of the S genes of monoclonal antibody resistant mutants has revealed that these sites are located mainly in the first and third quarters of S1 (Koch *et al.*, 1991). All the S1 neutralizing antibody sites are conformation dependent and removal of the oligosaccharides from this glycosylated protein disrupts the interaction with many neutralizing antibodies (Koch & Kant, 1991). Strains which differ by as few as about 16 amino acids

among the five antigenic sites of S1 can greatly diminish the binding of polyclonal antibodies raised against other IBV strains of similar sequence. These amino acid differences in naturally occurring isolates are located in the same regions that have been identified using monoclonal antibodies (Cavanagh et al., 1988, 1992).

Recombination is believed to be another mechanism by which IBV produces new strains. Recombination has been demonstrated experimentally for murine coronavirus and analysis of the sequence of the S and membrane (M) protein genes of many isolates of IBV has produced circumstantial evidence for IBV recombination in the field (Cavanagh & Davis, 1988; Kusters et al., 1989, 1990; Cavanagh et al., 1991).

The relative importance of humoral and cell mediated immunity in protection against IBV has received more attention. Otsuki et al. (1990) and Nakamura et al. (1991) found that one line (15I) of chicken had clinical signs and released infectious virus for a much longer period than another line (C). Experiments pointed to differences in immune responses to IBV being responsible for these findings and bursectomy resulted in more severe disease and a longer virus-release period, indicating a role for antibody in protection and recovery from IBV (Cook et al., 1991a). In some lines of chickens IBV alone caused considerable mortality in young chicks, whereas in other lines it was necessary for certain strains of Escherichia coli to be present. Even then, lines of birds varied greatly with respect to mortality in the face of dual infection (Bumstead et al., 1989).

The field situation is not static. In 1984 nephropathogenic isolates of IBV were isolated in Belgium and quickly spread throughout France. Mortality was up to 30% (Lambrechts et al., 1991). In 1990 nephropathogenic IBV, associated with 2–12% mortality in 3–4-week-old pullets, was isolated in California for the first time (Kinde et al., 1991). Mortality following infection with nephropathogenic IBV is greatly increased by cold stress and diets high in animal protein (Cumming, 1991). Some IBV isolates have been shown to grow in tissues of the alimentary tract, including proventriculus, caecal tonsil, ileum, rectum and in the bursa of Fabricius (Ambali & Jones, 1990; Lucio & Fabricant, 1990). IBV can be re-excreted at point of lay, presumably related to hormonal changes (Jones & Ambali, 1987). Might this, the re-excretion of IBV around the time when offspring appear, be a means whereby the virus can persist in a bird community, especially when chickens are kept non-intensively?

TURKEY ENTERITIS CORONAVIRUS

Turkey coronavirus (TCV) has an amazing resemblance to bovine coronavirus and very little with avian infectious bronchitis virus (IBV). TCV causes an acute and highly infectious enteritis in turkey poults (Pomeroy & Nagaraja, 1991). The disease was first recorded 40 years ago in the USA and was a major problem until an eradication programme brought the disease under control. Then in the mid-1980s there was a resurgence of the disease, this time in Canada, which led to the first detailed analysis of the causative virus. The proteins of TCV have been identified (Dea et al., 1989a, b). Even at the simple level of polyacrylamide gel electrophoresis an important difference is revealed between TCV and IBV, namely the

possession by TCV of a protein not present in IBV. This is the haemagglutinin-esterase (HE) protein, so-called because it binds to erythrocytes and has a receptor-destroying enzyme with acetylesterase activity (different from the neuraminidase possessed by NDV and influenza virus) (Fig. 4). An HE protein is possessed by BCV and has been studied in detail (Vlasak *et al.*, 1988a, b; Schultze *et al.*, 1991).

However, there are other features of TCV and BCV which are much more remarkable. Cloning and sequencing has shown that the amino acid sequences of the nucleocapsid (N) and membrane (M) proteins of these two viruses are 99% identical (Verbeek & Tijssen, 1991). These results were confirmed by sequencing cDNA clones derived by the polymerase chain reaction and TCV RNA from TCV-infected tissue from turkeys. The spike (S) proteins were also extremely similar, even in the S1 half (97% identity) which is the most variable protein throughout the coronaviruses (Tijssen *et al.*, 1991). This similarity between the S proteins of TCV and BCV is all the more remarkable when one considers that some IBV isolates differ by 22–49% of their S1 amino acids (Binns *et al.*, 1986; Kusters *et al.*, 1989). Analysis of several field strains of TCV revealed that some had truncated HE genes and were less cytopathic. These results, and those obtained with other coronaviruses, indicate that the different host range of TCV and BCV are probably related to differences in S although an involvement of the HE protein cannot be disregarded at the present time.

In view of the extremely close sequence similarity of TCV and BCV it is perhaps not surprising that BCV can replicate in turkey poults. However, there were no clinical signs and no lesions such as are caused by TCV (Dea *et al.*, 1991). Similarly, in view of the close similarity of the sequences of the TCV and BCV proteins, it is no longer surprising that many monoclonal antibodies raised against BCV bind to TCV (Dea et al., 1990). ·Further work is under way by Tijssen and colleagues to determine those essential differences which account for TCV being a pathogen of turkeys and not of cattle and *vice versa* with respect to BCV.
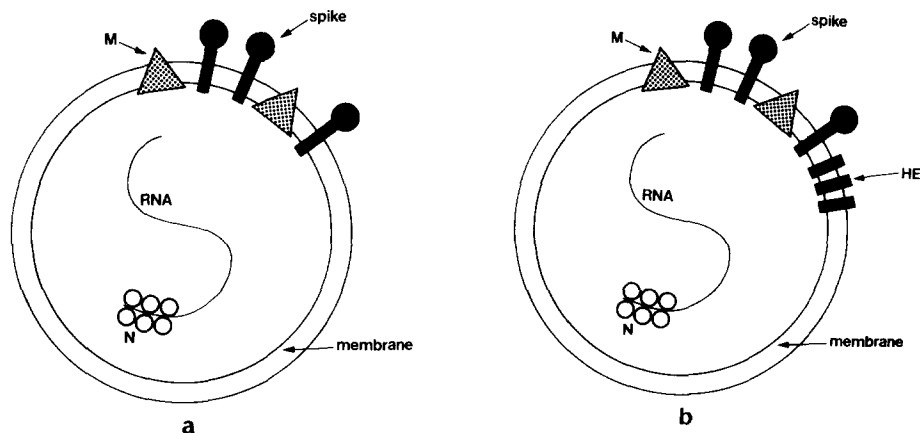

Fig. 4. Diagrammatic representation of virions of two coronaviruses: (a) infectious bronchitis virus and (b) turkey coronavirus. The viruses have three proteins in common (S, M and N) but turkey coronavirus posesses an additional membrane-associated glycoprotein HE (haemagglutinin-esterase).
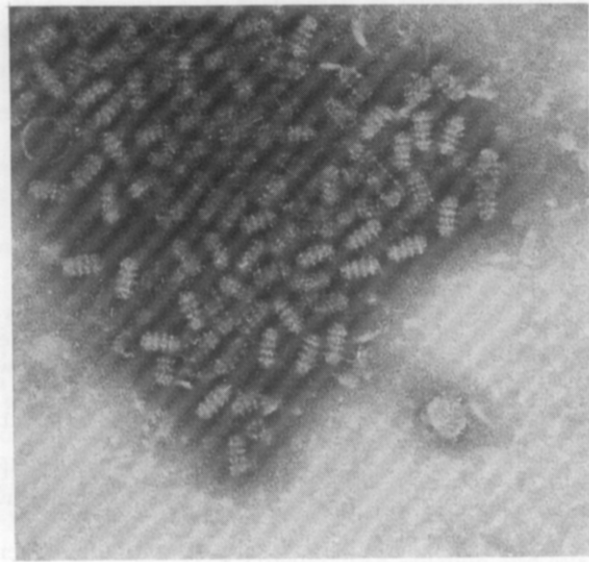
Fig. 5. Electron micrograph of 'rod-shaped-virus-like' particles isolated from the intestines of a pheasant (×139 500). Courtesy of M. Collins, Central Veterinary Laboratory, Weybridge.

OTHER VIRUSES—AND UNIDENTIFIED FLYING OBJECTS

Payne *et al.* (1991a) have isolated an avian leukosis virus with a wide host range belonging to a new subgroup for chickens. It appeared to behave like an exogenous leukosis virus and was shown to induce a relatively high incidence of myeloid leukosis and renal tumours, particularly in meat-type chickens (Payne *et al.*, 1991b). Pheasants have attracted microbiological attention in the UK during the 1980s, as the number of birds reared increased. Although infectious bronchitis virus (IBV) has been isolated from pheasants, on seven other occasions coronavirus-like viruses were isolated from pheasants and these viruses did not appear to be IBV (Gough & Alexander, 1991). The viruses were associated with egg production problems and low mortality in adult birds and higher mortality in younger birds. Severely swollen kidneys were frequently observed and visceral gout reported.

A number of other viruses have been detected by electron microscopy in the intestinal contents of game birds, mostly pheasants and partridges. Among the isolates were 'fimbriated' virus-like particles, superficially resembling paramyxoviruses, and rod-shaped, virus-like particles (Gough *et al.*, 1990). The latter particles (Fig. 5) are particularly intriguing, having been observed in game birds and chickens (Collins *et al.*, 1988, 1989; Lavazza *et al.*, 1990). The particles were frequently 40–55 nm in length, although longer ones have been seen (M. Collins, personal communication), and 15–18 nm in diameter. They do not resemble any known virus; indeed it is not certain if they are viruses.

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