

REVIEW ARTICLE

Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus

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Vaccines against infectious bronchitis of chickens (*Gallus gallus domesticus*) have arguably been the most successful, and certainly the most widely used, of vaccines for diseases caused by coronaviruses, the others being against bovine, canine, feline and porcine coronaviruses. Infectious bronchitis virus (IBV), together with the genetically related coronaviruses of turkey (*Meleagris gallopavo*) and ring-necked pheasant (*Phasianus colchicus*), is a group 3 coronavirus, *Severe acute respiratory syndrome (SARS) coronavirus* being tentatively in group 4, the other known mammalian coronaviruses being in groups 1 and 2. IBV replicates not only in respiratory tissues (including the nose, trachea, lungs and airsacs, causing respiratory disease), but also in the kidney (associated with minor or major nephritis), oviduct, and in many parts of the alimentary tract—the oesophagus, proventriculus, duodenum, jejunum, bursa of Fabricius, caecal tonsils, rectum and cloaca, usually without clinical effects. The virus can persist, being re-excreted at the onset of egg laying (4 to 5 months of age), believed to be a consequence of the stress of coming into lay. Genetic lines of chickens differ in the extent to which IBV causes mortality in chicks, and in respect of clearance of the virus after the acute phase. Live attenuated (by passage in chicken embryonated eggs) IBV strains were introduced as vaccines in the 1950s, followed a couple of decades later by inactivated vaccines for boosting protection in egg-laying birds. Live vaccines are usually applied to meat-type chickens at 1 day of age. In experimental situations this can result in sterile immunity when challenged by virulent homologous virus. Although 100% of chickens may be protected (against clinical signs and loss of ciliary activity in trachea), sometimes 10% of vaccinated chicks may not respond with a protective immune response. Protection is short lived, the start of the decline being apparent 9 weeks after vaccination with vaccines based on highly attenuated strains. IBV exists as scores of serotypes (defined by the neutralization test), cross-protection often being poor. Consequently, chickens may be re-vaccinated, with the same or another serotype, two or three weeks later. Single applications of inactivated virus has generally led to protection of < 50% of chickens. Two applications have led to 90 to 100% protection in some reports, but remaining below 50% in others. In practice in the field, inactivated vaccines are used in laying birds that have previously been primed with two or three live attenuated virus vaccinations. This increases protection of the laying birds against egg production losses and induces a sustained level of serum antibody, which is passed to progeny. The large spike glycoprotein (S) comprises a carboxy-terminal S2 subunit (approximately 625 amino acid residues), which anchors S in the virus envelope, and an amino-terminal S1 subunit (approximately 520 residues), believed to largely form the distal bulbous part of S. The S1 subunit (purified from IBV virus, expressed using baculovirus or expressed in birds from a fowlpoxvirus vector) induced virus neutralizing antibody. Although protective immune responses were induced, multiple inoculations were required and the percentage of protected chickens was too low (< 50%) for commercial application. Remarkably, expression of S1 in birds using a non-pathogenic fowl adenovirus vector induced protection in 90% and 100% of chickens in two experiments. Differences of as little as 5% between the S1 sequences can result in poor cross-protection. Differences in S1 of 2 to 3% (10 to 15 amino acids) can change serotype, suggesting that a small number of epitopes are immunodominant with respect to neutralizing antibody. Initial studies of the role of the IBV nucleocapsid protein (N) in immunity suggested that immunization with

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bacterially expressed N, while not inducing protection directly, improved the induction of protection by a subsequent inoculation with inactivated IBV. In another study, two intramuscular immunizations of a plasmid expressing N induced protective immunity. The basis of immunity to IBV is not well understood. Serum antibody levels do not correlate with protection, although local antibody is believed to play a role. Adoptive transfer of IBV-infection-induced $\alpha\beta$ T cells bearing CD8 antigen protected chicks from challenge infection. In conclusion, live attenuated IBV vaccines induce good, although short-lived, protection against homologous challenge, although a minority of individuals may respond poorly. Inactivated IBV vaccines are insufficiently efficacious when applied only once and in the absence of priming by live vaccine. Two applications of inactivated IBV are much more efficacious, although this is not a commercially viable proposition in the poultry industry. However, the cost and logistics of multiple application of a SARS inactivated vaccine would be more acceptable for the protection of human populations, especially if limited to targeted groups (e.g. health care workers and high-risk contacts). Application of a SARS vaccine is perhaps best limited to a minimal number of targeted individuals who can be monitored, as some vaccinated persons might, if infected by *SARS coronavirus*, become asymptomatic excretors of virus, thereby posing a risk to non-vaccinated people. Looking further into the future, the high efficacy of the fowl adenovirus vector expressing the IBV S1 subunit provides optimism for a live SARS vaccine, if that were deemed to be necessary, with the possibility of including the N protein gene.

Introduction

Severe acute respiratory syndrome (SARS) emerged in humans in Guangdong Province, China, in late 2002 and subsequently spread to other continents early the following year (Chan-Yeung & Yu, 2003; Lee *et al.*, 2003; Peiris *et al.*, 2003; Poutanen *et al.*, 2003; Tsang *et al.*, 2003). Shortly after the spread of the virus beyond China the causative agent was quickly identified as being a species of coronavirus (*SARS coronavirus*) that was previously unknown (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Marra *et al.*, 2003; Peiris *et al.*, 2003; Rota *et al.*, 2003). Indeed, the proteins of *SARS coronavirus* had such low amino acid identity with those of known coronaviruses that it has been tentatively assigned to a new coronavirus group 4. The three existing coronavirus groups (Table 1) were initially devised on the basis of a lack of antigenic relationships between the species of different groups (Pedersen *et al.*, 1978; Sánchez *et al.*, 1990; Enjuanes *et al.*, 2000; González *et al.*, 2003). It is probable that the vast majority of the human population is susceptible to *SARS coronavirus*. The SARS epidemic was contained at 'only' 8460 cases by the enormous effort, and sacrifice, of a large number of individuals associated with many public authorities. So severe were the infections, in a fully susceptible population, that 808 died; doubtless a greater proportion would have died had it not been for the medical attention focussed upon them. Sadly, a large proportion of the deaths in some countries were sustained by health care workers. In addition to the human misery, there were dire economic consequences for those countries most affected.

The last SARS cases of the early 2003 epidemic were in June 2003. The disease might arise again by further transmission from the suspected animal reservoir, or transmission from human, possibly asymptomatic, carriers. There is evidence from

studies of avian infectious bronchitis virus (IBV) and feline coronavirus that coronaviruses establish persistent infections. Chicks that had been experimentally infected with IBV at 1 day of age re-excreted virus at around 19 weeks of age (Jones & Ambali, 1987). It is suspected that the stressor of the start of egg production caused the release of the virus. Approximately 10% of cats that had been naturally infected with feline coronavirus became asymptomatic carriers, excreting virus for over 1 year (Addie & Jarrett, 2001; Addie *et al.*, 2003). Others excreted virus for periods of several months.

The World Health Organisation has called for the development of vaccines against SARS. This short review looks at the experiences with IBV vaccines over half a century, which might be instructive with regard to the development of SARS vaccines.

Table 1. *Coronavirus species*

Group 1	Porcine transmissible gastroenteritis coronavirus Canine enteric coronavirus Feline coronavirus Porcine epidemic diarrhoea coronavirus Human coronavirus 229E
Group 2	Murine hepatitis coronavirus Human coronavirus OC43 Bovine coronavirus Canine respiratory coronavirus Porcine haemagglutinating encephalomyelitis coronavirus
Group 3	Infectious bronchitis coronavirus Turkey coronavirus Pheasant coronavirus
Group 4	SARS coronavirus ^a

^a*SARS coronavirus* has provisionally been placed in group 4, based on the criteria previously used to place the other coronaviruses into groups (i.e. extremely low amino acid identity between its proteins and those of the other three groups, and nature and organization of its non-structural protein genes).

Infectious bronchitis

As suggested by its name, IBV causes respiratory disease, although its replication is not limited to the respiratory tract. It is the major respiratory virus of the chicken (the domestic fowl), as it is endemic in probably all countries that raise chickens. Its host range is considered to be limited to the chicken (Cavanagh & Naqi, 2003), although genetically very similar coronaviruses cause disease in turkeys (enteric disease; Guy, 2000; Cavanagh *et al.*, 2001) and pheasants (respiratory and kidney disease; Lister *et al.*, 1985; Gough *et al.*, 1996; Cavanagh *et al.*, 2002). IBV occurs globally, both in chickens kept on a large and a small scale (Wunderwald & Hoop, 2002). It exists as scores of serotypes/genotypes (Cavanagh, 2001; Meulemans *et al.*, 2001; Farsang *et al.*, 2002), which are problematic with regard to prophylaxis.

IBV causes deciliation of the ciliated epithelia of the nose and trachea, clinical signs being similar to those of the common cold caused in humans by the group 1 and group 2 human coronaviruses (HCoV-229E and HCoV-OC43, respectively; Cavanagh, 2000, 2004). Chickens, especially those of only a few days or weeks of age, exhibit nasal discharge, snicking (similar to sneezing), watery eyes and lethargy (Dhinakar Raj & Jones, 1997; Cavanagh & Naqi, 2003). Some chickens exhibit r les, a vibration emanating from lower in the respiratory tract, and small areas of pneumonia may be observed in the lungs (Dhinakar Raj & Jones, 1997). Notwithstanding this, and the fact that titres of virus in the lungs can be similar to those in the nose and trachea, bronchitis is not considered to cause pneumonia.

From a welfare point of view the hardest hit are chicks of only a few days or weeks of age. Some may die directly from the viral infection but a greater number die following secondary bacterial infection. An overall slowing down of growth causes further economic losses. Juvenile and mature birds suffer less from IBV infection although the economic consequences of infection in egg-laying stock can be disastrous, as egg production drops precipitously and usually does not rise back to normal in the flock as a whole.

As discussed further later, IBV grows at many epithelial surfaces beyond the respiratory tract (reviewed by Dhinakar Raj & Jones, 1997). Although many alimentary tract tissues are susceptible to IBV, infection of enteric tissues usually does not manifest itself clinically. Recently a strain of IBV has been associated with disease of the proventriculitis (the cranial glandular compartment of the stomach, adjacent to the gizzard, which is the caudal muscular compartment) (Yu *et al.*, 2001a). Nephritis is not uncommon in a proportion of naturally IBV-infected meat-type birds, environmental factors playing a role. Some IBV strains are intrinsically nephropathogenic; that is, they repro-

ducibly cause nephritis when inoculated experimentally into specific pathogen free chickens (Lambrechts *et al.*, 1993; Pensaert & Lambrechts, 1994; Cook *et al.*, 2001; Li & Yang, 2001). The oviduct is also susceptible to IBV, which may contribute to diminished egg production.

Infectious bronchitis virus

IBV contains the same number of structural proteins (i.e. those present in virions) as the group 1 coronaviruses; that is, a nucleocapsid protein associated with the 27.6 kb single-stranded, positive-sense RNA genome, a large spike glycoprotein (S), a smaller integral membrane glycoprotein (M) and a few molecules of the envelope protein (Lai & Cavanagh, 1997; Enjuanes *et al.*, 2000; Gonz lez *et al.*, 2003). It does not contain a haemagglutinin esterase glycoprotein such as is found in group 2 coronaviruses. The coronavirus S protein is a dimer or trimer (Cavanagh, 1983; Gallagher & Buchmeier, 2001; Lewicki & Gallagher, 2002). The S glycopolyptide of IBV, like that of group 2 coronaviruses, is cleaved into amino-terminal S1 (approximately 520 amino acid residues) and carboxy-terminal S2 (approximately 625 residues) glycopolyptides (Lai & Cavanagh, 1997). The IBV S protein has been demonstrated to be a determinant of the host cell range *in vitro*. When the S protein gene of an infectious cDNA clone of the Beaudette strain (Casais *et al.*, 2001), which replicates in mammalian Vero and BHK cells, was replaced by that of the M41 strain, which does not replicate in those cell lines, the recombinant virus was unable to replicate in them (Casais *et al.*, 2003). The recombinant did replicate in primary chick kidney cells, as did both the Beaudette and M41 strains. Whether the S protein of IBV is a determinant of tissue tropism *in vivo* remains to be determined.

Tissue tropism of IBV

IBV initially infects the upper respiratory tract. Titres of live virus are maximal in the nose and trachea by 3 days post inoculation (by eye-drop and intranasally) and remain so for 2 to 5 days further, depending on the strain (Table 2) (Ambali & Jones, 1990; Hofstad & Yoder, 1966). Thereafter the titre falls to below detectable levels, although virus was still detectable at 14 days in some studies (Ambali & Jones, 1990). The virus is restricted to the ciliated and mucus-secreting cells (reviewed by Dhinakar Raj & Jones, 1997). Similar virus titres occur in the lungs and airsacs, sometimes peaking a little after those in the nose and trachea. Small areas of pneumonia may be observed in the lungs (Dhinakar Raj & Jones, 1997).

Lucio & Fabricant (1990) investigated the growth of several isolates of IBV in chickens.

Table 2. *Titres of IBV (strain G) in respiratory and non-respiratory tissues following intranasal and eye-drop inoculation of 1-day-old chicks (from Ambali & Jones, 1990)*

Tissue	Virus titre (log ₁₀ CD ₅₀ /g) ^a on the following days after inoculation					
	1 day	3 days	5 days	7 days	10 days	14 days
Non-enteric						
Trachea	5	5	5	5	3	2
Kidney	3	4	5	5	4	3
Upper gut						
Proventriculus	5	3	3	3	3	3
Duodenum	4	4	3	3	3	3
Jejunum	0	2	2	0	0	0
Lower gut						
Rectum	3	5	6	4	4	4
Bursa	2	3	5	4	3	3
Caecal tonsil	2	3	4	4	4	4
Ileum	3	5	3	3	3	3

^aCD, ciliostatic dose; the virus was titrated in tracheal organ cultures, the presence of virus being indicated by cessation of ciliary activity. For simplicity, titres have been rounded to the nearest log value.

Although they did not quantify the virus that they recovered, they showed that infectious virus was recovered from the trachea, bronchus, lung, oesophagus, proventriculus, duodenum, jejunum, caeca (caecal tonsils), kidney and cloaca. The longest periods of isolation were in respect of the kidney and caecal tonsils, the latter being at the distal end of the alimentary tract in birds.

Ambali & Jones (1990) used another strain (strain G) of IBV that produced high titres in the kidney, similar to those in the trachea (Table 1). Various alimentary tract tissues (proventriculus, duodenum, ileum, caecal tonsils, bursa of Fabricius and rectum) had peak titres the same as in the trachea and kidney, or within 10-fold of that amount (Table 1). Fluorescence revealed the location of the virus, in epithelial cells. Several other isolates were compared with the G strain; they were isolated much less frequently or not at all from kidney and rectal contents (El-Houadfi *et al.*, 1986). Organ culture studies showed that while all strains studied by Bhattacharjee & Jones (1997) grew in the proventriculus, bursa and kidney explants, some did not grow in the caecal tonsil or rectal explants.

In the experiments of Hofstad & Yoder (1966), titres in the kidney and bursa of Fabricius were approximately two orders of magnitude lower than in the respiratory tissues for a number of IBV strains of low passage number in embryos. High egg-passaged virus (85 passages) was almost undetectable in the kidney and bursa, and gave reduced titres in the trachea and lung.

The most intensively studied tropism, apart from the upper respiratory tract, has been that for the kidney. Although only a small proportion of IBV isolates exhibit high nephropathogenicity, these can cause up to 44% mortality within 3 weeks of intratracheal inoculation (Pensaert & Lambrechts, 1994) in the absence of vaccination. Histopathological and immunochemical studies of the process

showed that the MA-87 strain of virus attacked mainly the lower nephron down to the collecting duct epithelial cells (Chen & Itakura, 1996; Chen *et al.*, 1996). An ultrastructural investigation revealed that the virus replicated in all segments of tubules and ducts, but more frequently in the epithelial cells of the collecting ducts, collecting tubules, distal convoluted tubules and Henle's loops (Chen, 1996). The extent of mortality is age related (greatest in chicks; Lambrechts *et al.*, 1993) and varies according to the line of bird; for example, 18% and 44% of an egg layer-type bird and a meat-type bird, respectively (Pensaert & Lambrechts, 1994).

Birds have a small lymphoid organ, the Harderian gland, in the eye-socket that is a major contributor to locally produced antibody for protecting oculonasal mucosae (reviewed by Dhinaraj & Jones, 1997). Inoculation of a mild vaccinal strain of IBV by eye-drop resulted in replication of virus in the gland and partial damage to it (Toro *et al.*, 1996). There was an increase in the number of plasma cells and enlargement of lymphoid foci. IBV has also been isolated from another lymphoid organ, the bursa of Fabricius (Table 1) (El-Houadfi *et al.*, 1986; Ambali & Jones, 1990), with gross and histopathological lesions occurring, even after infection with vaccinal strains.

Most recently, Yu *et al.* (2001a) have studied the pathogenesis of three isolates of IBV from the proventriculus of chicken flocks that had affected proventriculi. Experimental infections with these strains resulted in age-dependent mortality (75 to 100% in 2-week-old birds, 0 to 25% in 16-week-old chickens). Necropsy of sick chickens revealed that the mucosa of the proventriculus was thickened and exuded a milky fluid when squeezed, and the entire proventriculus was enlarged.

Thus, IBV strains as a group productively infect a large range of epithelial surfaces, literally from the top to the bottom of the chicken. Isolates differ in their extent of replication in the non-respiratory

tissues, and some produce clinical disease in non-respiratory tissues, most notably the kidney and proventriculus. The pantropism of IBV might be the case for *SARS coronavirus*, as the latter has not only been associated with pneumonia, but also with diarrhoea (although it remains to be demonstrated whether the SARS virus is replicating in enteric tissues; Peiris *et al.*, 2003). A point of difference is that whereas SARS virus is associated with severe clinical signs in both the respiratory and enteric tracts, IBV is usually limited to disease in the respiratory tract.

IBV exists as dozens of serotypes

It is not appropriate in this short review to go into detail about the extensive antigenic variation exhibited by IBV (Darbyshire *et al.*, 1979; Ignjatovic & Mcwaters; 1991; Cook *et al.*, 1999; Cavanagh, 2001; Meulemans *et al.*, 2001; Farsang *et al.*, 2002); it is an issue that may not arise with *SARS coronavirus*. That said, only a few amino acid differences in the S1 protein of two strains of IBV are sufficient to change the serotype.

The large spike glycoprotein (S) comprises a carboxy-terminal S2 subunit (approximately 625 amino acid residues), which anchors S in the virus envelope, and an amino-terminal S1 subunit (approximately 520 residues), believed to largely form the distal bulbous part of S (Cavanagh, 1995; Lai & Cavanagh, 1997). It is the S1 subunit that induces virus-neutralizing (VN) antibody. This has been demonstrated in various ways: failure to induce VN by virus from which S1 had been removed (Cavanagh *et al.*, 1986); induction of VN antibody by S1 released from virions by treatment with urea (Cavanagh *et al.*, 1986), by non-ionic detergent, followed by affinity chromatography (Ignjatovic & Galli, 1994) and by expression from a vaccinia virus recombinant (Tomley *et al.*, 1987); by production of monoclonal antibody-resistant mutants, the mutations being in the S1 part of the spike protein gene (Cavanagh *et al.*, 1988; Koch *et al.*, 1990; Kant *et al.*, 1992).

Failure of serotypes to induce cross-protective immunity

Experimental vaccination studies usually involve application of virus to the nostrils and by eye-drop, sometimes by direct application to the trachea. Protection is determined by challenge, 3 weeks after vaccination, the challenge virus being applied by the same route. Three main approaches to the assessment of protection have been: (1) observation of clinical signs (not commonly used); and removal of the trachea at 4 or 5 days after challenge followed by either (2) quantitative assessment of ciliary activity or (3) detection of live challenge virus, usually by inoculation of embryonated eggs.

The second and third methods result in similar deductions being made as regards protection (Marquardt *et al.*, 1982), although in some studies challenge virus was isolated from some individuals whose ciliary activity was unaffected or little affected. Arguably, declaring a chicken as non-protected because of the recovery of small amounts of challenge virus, when ciliary activity is normal, is being too stringent.

Most IBV serotypes differ from each other by 20 to 25% of S1 amino acids (Adzhar *et al.*, 1997; Kingham *et al.*, 2000), although some differ by up to 50% (Cavanagh *et al.*, 1997; Gelb *et al.*, 1997). (Differences between the other IBV proteins are in the region of 10%, rarely exceeding 15%; Cavanagh *et al.*, 2001, 2002.) Generally speaking, the immunity induced by inoculation with one serotype protects poorly against infection by heterologous serotypes.

With regard to protection of the respiratory tract, in some experiments some heterologous challenge viruses broke through in 100% of the chickens (i.e. 0% cross-protection) (Hofstad, 1981; Marquardt *et al.*, 1982; Picault *et al.*, 1986). Other challenge viruses, known now to differ by 20% or so in S1 from the virus used to initially infect the chickens, broke through immunity in 30 to 70% of cases (Rosenberger *et al.*, 1976; Hofstad, 1981). Collectively these and other reports show that cross-protection can range from very poor to moderate (poor for commercial purposes), although in some cases the differences among these reports may be also be due to differences in stringency by which protection was assessed (discussed later). When Winterfield *et al.* (1976) used clinical criteria to assess cross-protection, a vaccinal strain of the Massachusetts serotype was concluded to protect all chickens (in groups of 10) against challenge with serotypes now known to differ by 20% or so in their S1 proteins. Protection was assessed as being less when re-isolation of challenge virus was used as the criterion for protection. There is no doubt that in the field the application of a vaccine that is homologous to the prevailing field strain has given better protection than an heterologous vaccine.

Cross-protection against nephritis is also usually poor, although some strains are more cross-protective than others (Pensaert & Lambrechts, 1994; Cook *et al.*, 2001).

Monoclonal antibody-resistant mutant investigations have revealed that many of the amino acids involved in the formation of VN epitopes are located within the first and third quarters of the linear S1 polypeptide (Cavanagh *et al.*, 1988; Koch *et al.*, 1990; Kant *et al.*, 1992). Sequence analysis of variants that are genetically very similar (>95% amino acid identity in S1) has shown that most of the differences are within these two regions (Cavanagh *et al.*, 1992; Adzhar *et al.*, 1997). Moreover,

some variants that differed by only 2 to 3% of S1 amino acids (10 to 15 residues) behaved as different serotypes (Cook, 1984; Cook & Huggins, 1986; Cavanagh *et al.*, 1992).

Would small S1 differences between SARS coronavirus isolates be of practical significance?

It is conceivable that there are variants (e.g. with small differences in S1) of *SARS coronavirus* in the field (i.e. within its natural host(s)). If this were to be the case, and if variants infected humans, would the variants be able to break through the immunity induced by a vaccine based on a strain isolated from humans in early 2003? Unfortunately there have been few protection studies based on closely related variants of IBV.

Cavanagh *et al.* (1997) inoculated groups of 10 chickens with the virulent UK/6/82 isolate and challenged with isolates that differed by up to 4% of S1 amino acids (some of which were different serotypes; Cook, 1984; Cook & Huggins, 1986), protection being assessed on retention of ciliary activity in trachea. Challenge with two variants (98% S1 identity with UK/6/82) resulted in challenge scores (a relative measure of retention of ciliary activity) virtually the same as with the homologous challenge. Challenge with two others isolates (96% and 98% S1 identity, respectively), resulted in a higher challenge score, indicative of less cross-protection, although the numbers were not statistically significantly different. Also, in three experiments one of each group of 10 birds initially inoculated with the UK/6/82 virus was clearly not protected against homologous challenge. The chickens had been individually inoculated with a high dose (5 to 6 log₁₀ infectious virus) of the primary inoculum. Therefore, this lack of protection in 10% of birds was unlikely to have been due to a failure to initiate infection with the primary virus, but rather to heterogeneity of the immune response.

In other studies approximately 10% of chickens in a group, which had been inoculated and challenged with the same strain or with one of the same serotype, were not fully protected (Winterfield *et al.*, 1976; Hofstad, 1981; Picault *et al.*, 1986; Parsons *et al.*, 1992; Cavanagh *et al.*, 1997; Cook *et al.*, 1999; Nix *et al.*, 2000) (using virus isolation or tracheal ciliary activity as the criterion). These results show that chickens (out-bred, although with restricted sets of parental breeding stocks) are not uniform in their response to IBV vaccination. One would imagine that the human population might be even more heterogeneous in response to vaccination against SARS.

Nix *et al.* (2000) inoculated chickens with several virulent isolates that had 94% or greater amino acid identity in S1. All were assessed as being of the same serotype, Arkansas, but some cross-reacted in a VN test much less than others. Some isolates

induced 100% protection, assessed by re-isolation of challenge virus, a stringent test, against challenge, including against challenge with a strain that was of lower serological identity. However, two other isolates induced cross-protection of only 55 to 58% of the chickens when the challenge virus was of lower serological identity.

Clearly, the induction of protective immunity to IBV is complex. It not only depends on the antigenic relatedness of the primary infecting strain and a subsequent challenge strain, but also on the response of individual chickens; approximately 10% of chickens in some experiments failed to develop a protective immune response. Furthermore, while breeds of chicken are similar in that they are all efficiently infected by IBV, resulting in similar titres of virus, they differ in their capacity to clear the infection (Otsuki *et al.*, 1990). Small (~5%) differences in S1 amino acids would appear to be sometimes responsible for poor cross-protection. It should be borne in mind that while the focus has been on the relatedness of the S1 protein, other viral proteins may have a role in protective immunity; for example, the nucleocapsid protein, of which more later. Recently, we (T. Hodgson, R. Casais, B. Dove, P. Britton & D. Cavanagh, manuscript in preparation) have substituted (swapped) the spike protein gene of our Beaudette infectious clone (receiver strain) with that of the M41 strain (donor strain). Both strains are of the Massachusetts serotype and have 95.0% S1 amino acid identity. The Beaudette and M41 strains are attenuated and pathogenic for chickens, respectively. When chickens were inoculated with the donor, receiver or spike-swapped recombinant IBV and challenged with M41, the receiver strain induced almost no tracheal protection (assessed by ciliary activity) while the recombinant IBV induced protection almost as good as the donor strain. This was not due to an increased virulence of the receiver strain, as the spike-swapped recombinant was as non-pathogenic as the Beaudette receiver strain, and both replicated poorly. As the receiver strain and recombinant virus had identical proteins except for the spike protein, it would appear that the poor immunity induced by the receiver strain against the donor strain was due to some of the 5.0% of amino acid differences (27 different residues) in S1. This supports the findings of earlier work that small differences in S1 can contribute to poor cross-protection (Cavanagh *et al.*, 1997; Nix *et al.*, 2000).

Vaccination with live-attenuated vaccines

Meat-type chicks (broilers) are usually vaccinated on the day of hatch, in the hatchery, by spray. The birds are vaccinated at this very early age in part for logistical/economic reasons. Also, because IBV is endemic and ever-present in many poultry-rearing parts of the world, protection is needed as early

as possible. The consequences of IBV infection are greater for chicks than older birds. Also, the modern broiler goes for processing at only 6 weeks of age, so vaccination has to be early.

Many studies have been undertaken during the past 30 years or so to examine the degree of protection induced by single inoculations of various serotypes of IBV. In many of the studies referred to in the previous section, the initial inocula were virulent strains, not attenuated derivatives. This was an advantage in that the degree of protective immunity induced by a virus is in part dependent on its virulence. Notwithstanding, the overall conclusions reached, already described, were similar, irrespective of whether the initial inoculum virus was virulent or attenuated.

Duration of respiratory tract immunity following live attenuated vaccination

Protection of the respiratory tract following a single live attenuated virus vaccination has been found to be short-lived; Gough & Alexander (1979) and Darbyshire & Peters (1984) reported a decline in the number of protected chickens at 6 and 9 weeks after vaccination, respectively. In regions where a given serotype of IBV vaccine is consistently not giving acceptable protection against field challenge, the birds might be revaccinated 2 to 3 weeks after the first application. Sometimes this is with the same vaccine preparation, sometimes with another product of the same serotype, and sometimes with a vaccine of a heterologous serotype. The latter approach sometimes gives protection against a broader range of serotypes (Cook *et al.*, 1999).

Protection against nephritis by live vaccines

Some strains of IBV are markedly nephropathogenic. For example, experimental tracheal infection of commercial meat-type birds with the B1648 nephropathogenic strain resulted in the death of approximately one-third of a group of 68 chickens within 9 days. Vaccination, by coarse spray, with the homologous attenuated strain completely protected against mortality upon challenge 4 weeks later (Pensaert & Lambrechts, 1994). Vaccination with non-homologous, commercially available vaccines markedly, although not completely, reduced mortality. The homologous vaccine had prevented detectable growth of the challenge virus in the trachea, as ascertained by virus isolation, but the heterologous vaccines did not do so. Challenge virus in the kidney was assessed by immunofluorescence. By this criterion the number of chicks with detectable IBV in the kidney was reduced by 84% by vaccination with the homologous vaccine, and not at all by the heterologous vaccines.

Vaccination with inactivated vaccines

Inactivated oil-emulsion IBV vaccines were developed during the 1960s and 1970s. The objective was to make a vaccine that would give long-lasting immunity to the hen bird, to protect against drops in egg production. Single applications of inactivated virus induced little or no protection against egg loss (McDougall, 1969; Box *et al.*, 1980; Muneer *et al.*, 1987) and no protection against loss of ciliary activity in the trachea (Martins *et al.*, 1991).

However, immunization of 11 chickens with a single dose of inactivated IBV strain M41 resulted in 36 to 45% (depending on the criterion used) developing respiratory tract protection (Cavanagh *et al.*, 1986). A dose of 100 µg was not more efficacious than 5 µg. In a second experiment, 29% (based on retention of ciliary activity in the trachea) to 76% (based on not isolating challenge virus) of 17 chickens were protected after a dose of 10 µg. In a third experiment, 40% of 10 chickens were judged protected (retention of ciliary activity) following a single 10 µg dose. It is possible that larger amounts of virus was used in these three experiments than by previous workers, and that this was the reason for the partial success.

As part of a study of the role of the IBV nucleocapsid protein (N) protein in protective immunity (discussed later), Boots *et al.* (1992) immunized chickens with inactivated IBV at 4 weeks of age and again 6 weeks later. After challenge, 1/8 and 8/8 of the two groups of chickens, respectively, were protected, by assessment of ciliary activity in trachea. The degree of protection was assessed as being slightly less (88%) when immunofluorescence was used to detect infected tracheal cells.

Ignjatovic & Galli (1994) gave four doses, each of 200 µg, of inactivated IBV; 40% and 20% of 10 birds had developed protection (assessed by non-re-isolation of challenge virus) in the trachea and kidney, respectively, after three doses. These percentages did not increase after four doses. Song *et al.* (1998) immunized chickens up to three times with an inactivated nephropathogenic strain. Protection (assessed by non-re-isolation of challenge virus) of the kidney and trachea was 50% and 13%, respectively, of eight chickens, after two inoculations, rising to 88% and 50%, respectively, after three inoculations.

To see whether two doses of inactivated vaccine would be protective against loss of egg production, McDougall (1969) vaccinated birds at an interval of 8 weeks and challenged them 18 weeks later. Only a few of the vaccinated birds had respiratory signs and these lasted for less than 3 days, compared with 8 days for the unvaccinated controls. The birds that had received the inactivated vaccine were largely protected against a drop in egg production. Gough *et al.* (1977) demonstrated a

poor serum antibody response to a single application of inactivated infectious bronchitis vaccine but titres increased markedly upon revaccination 10 weeks later.

The approach commonly used in the poultry industry today is to vaccinate young females two or more times with live vaccine, followed by one dose of inactivated vaccine as the birds come into lay. The live vaccines serve to give protection to the young bird and to prime the immune response to the later inactivated vaccine (Gough *et al.*, 1977; Box *et al.*, 1980; Finney *et al.*, 1990). Gough *et al.* (1977) demonstrated that the chickens that had received one dose of live vaccine followed by inactivated vaccine 10 weeks later, were protected against challenge 10 weeks later, as assessed by re-isolation of challenge virus from trachea, oviduct and kidney. Box & Ellis (1985) reported that the response to the inactivated vaccine was poor if given within 8 weeks of a live vaccination.

Passive immunization

Macdonald *et al.* (1981) inoculated 12-week-old chickens with 2 ml convalescent IBV serum. Two days later they were challenged by intravenous application of the H52 vaccinal strain, a procedure known to result in nephritis. The birds were protected against kidney disease, as assessed by lack of watery droppings, macroscopic and microscopic pathology in the kidney, in contrast to control birds to which non-immune serum had been given. The passive application of immune serum did not protect against respiratory infection, although onset was delayed and of shorter duration.

Variation among genetic lines of chicken in their susceptibility to IBV

Smith *et al.* (1985) and Cook *et al.* (1986) demonstrated varying mortality among different breeds of chicken, both when inoculated with a mixture of IBV isolates alone and when in conjunction with a pool of *Escherichia coli* strains. The IBV predisposed the birds to infection with the bacterium, resulting in increased mortality. Bumstead *et al.* (1989) continued with this IBV/*E. coli* infection, showing marked variation in mortality among several inbred lines of White Leghorn chickens. More detailed analysis of two of these lines, line 15I (highly susceptible) and line C (relatively resistant), showed that the rate of virus production and titres in the respiratory tract were similar during the first 4 days after infection (Otsuki *et al.*, 1990). Thereafter, however, titres declined much more slowly in the susceptible line 15I, taking two-fold longer to decline to undetectable levels. In tracheal explants of the two lines there was no difference in the replication profile of the virus, showing that the difference in the birds

themselves was not because of an intrinsic difference in the capacity of the trachea to support the replication of IBV. The underlying cause of the difference *in vivo* might be immunological.

The extent to which infection by a nephropathogenic strain caused mortality was also dependent on the type of chicken (Pensaert & Lambrechts, 1994). Thus, 0% and 2% of a specified pathogen free flock died within the periods 5 to 7 days and 7 to 9 days, respectively, after tracheal inoculation of the B1648 strain. In contrast, 15% and 21% of commercial meat-type birds died in these periods.

Presence and duration of antibody in the serum, trachea and nasal secretions

The humoral immune response to IBV vaccination has mostly been investigated by measuring antibody levels in serum, using enzyme-linked immunosorbent assay (ELISA), VN or haemagglutination-inhibition (HAI) tests. (IBV haemagglutinates very poorly until treated with enzyme preparations containing neuraminidases; Alexander *et al.*, 1983; Schultze *et al.*, 1992, and references therein.) However, there have also been a few studies of IBV antibodies in the nose and trachea. It should be stated at the outset that many studies have shown that the presence or absence or titre of serum antibody to IBV does not correlate with protection; that is, vaccinated chickens may be protected against respiratory disease IBV irrespective of the titre of serum antibody (for example, Raggi & Lee, 1965; Ignjatovic & Galli, 1994).

The profile of the serum antibody response depends on the method used to detect it. Following infection of chickens with a virulent strain of IBV, specific antibody was first detected by ELISA (plates coated with IBV), a majority of the birds having detectable antibody by 6 days after infection, with titres maximal within 21 days and tending to decline shortly afterwards (Mockett & Darbyshire, 1981). VN antibody was delayed in comparison with ELISA-detected antibody, being first detected in the period 9 to 21 days after infection, peaking within 35 days and remaining level for the remainder of the sampling period (63 days after inoculation), although in 25% (2/8) of the birds the VN antibody was still increasing at 63 days, when sampling ended. The HAI antibodies were first detected at 9 days, peaking at 14 to 17 days and then declining, although in one bird the HAI titre did not peak until much later (as with the VN titre in the same bird).

Mockett (1985) also compared ELISAs using purified S and M proteins with the conventional virus-coated ELISA; the primary and secondary antibody response profiles were very similar, VN antibody again later than ELISA antibody.

Following infection with a live IBV vaccinal strain there was a good primary immunoglobulin (Ig)M response (Mockett & Cook, 1986; Martins *et*

al., 1991). As expected, the primary IgM response peaked, and declined, before that of the IgG response (Figure 1) (Martins *et al.*, 1991). The secondary IgM response (i.e. in response to a second (challenge) infection) peaked at the same time as that of IgG, but declined faster (Figure 1). Mockett & Cook (1986) suggested that measurement specifically of serum IgM would be useful in defining recent infection. In contrast to the response after IBV infection, vaccination with inactivated virus produced almost no IgM response, as well as a poor IgG response (Figure 1) (Martins *et al.*, 1991).

A single and double vaccination with inactivated IBV resulted in HAI titres of 1/28 and 1/219, respectively, 4 and 2 weeks after the respective vaccinations (Gough *et al.*, 1977). A single inoculation of 3 µg purified IBV induced maximum titres of HAI and ELISA antibody of 6 log₂ and 12 log₂, respectively, by 3 to 4 weeks after vaccination (Cavanagh *et al.*, 1986). VN antibodies peaked 1 week later, at 5 log₂. Titres decreased eight-fold within 4 weeks of peaking.

Holmes (1973) measured VN antibody in nasal washings after challenge of chickens that had received either a single live virus infection or two intra-muscular inoculations (4 weeks apart) with inactivated IBV. Serum antibody levels rose rapidly in both groups. Nasal antibody rose more slowly in the group that had received inactivated virus than in the group that had received a primary inoculum of live virus.

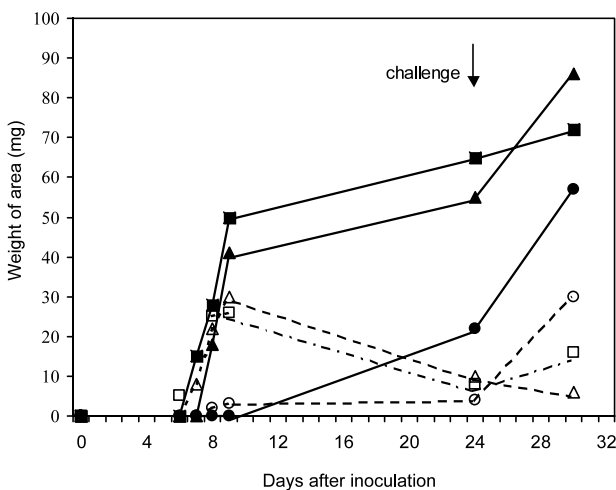


Figure 1. Antibody induction by inactivated and live IBV vaccination, from Martins *et al.* (1991). Profile of IBV-specific IgM and IgG responses of unprimed 14-week-old chickens to live attenuated vaccine (H120) given by eye-drop or intramuscularly (i.m.), and to inactivated vaccine (oil emulsion) given i.m. The IgM and IgG were measured using an ELISA. The ELISA results (as absorbance values) are plotted graphically, and the areas corresponding to each immunoglobulin class were cut out and weighed. The chickens were challenged with virulent IBV 24 days after vaccination. IgG: live vaccine, eye-drop (■); live vaccine, i.m. (▲); inactivated vaccine, i.m. (●). IgM: live vaccine, eye-drop (□); live vaccine, i.m. (△); inactivated vaccine, i.m. (○).

Induction of protection by subviral and vectored vaccines

There is no doubt that the IBV S protein, when inoculated on its own, can induce protective immunity. The proportion of chickens being protected may be dependent on the manner by which the S protein is presented to the host. There is also evidence that the N protein can prime protective immune responses, and one report that the N protein on its own induced protective immunity.

Immunization with the S protein

Whereas a single inoculation of 10 µg IBV induced tracheal protection in 40% of 10 chickens, all birds were susceptible after immunization with IBV from which the S1 subunit had been removed by urea, indicating that the S1 subunit was required to induce protective immunity (Cavanagh *et al.*, 1986). The S1 that had been released by treatment with urea was dialysed against decreasing concentrations of urea, and eventually in the absence of urea; this might have enabled some renaturation of the protein. Sedimentation analysis indicated, as expected, that the S1 that had been released by urea was in monomeric form; that is, it was no longer a multimeric (dimer or trimer) molecule with quaternary structure. Notwithstanding, 22% and 44% of 10 chickens that had been inoculated on four occasions with 3 µg S1 developed VN and HAI antibody, respectively. There were no positives following three inoculations. Protective immunity was not assessed in that experiment.

Ignjatovic & Galli (1994) dispersed the proteins of a nephropathogenic IBV using non-ionic detergent and purified S1, N and M by affinity chromatography using monoclonal antibodies. Chickens were inoculated on four occasions, 50 µg for each dose. Chickens that had been immunized with N or M proteins did not develop protective immunity (assessed by non-recovery of challenge virus) to either the trachea or kidney following challenge. However, 71% and 86% of 10 chickens inoculated with S1 developed tracheal and kidney immunity after four inoculations. The respective percentages were 70% and 10% after three inoculations.

The S1 protein has also been expressed in *Spodoptera frugiperda* cells from a recombinant *Autographa californica* baculovirus (Song *et al.*, 1998). When chickens were inoculated with these cells containing expressed S1, 43% and 0% of seven birds were protected (assessed by non-re-isolation of challenge nephropathogenic virus) in the kidney and trachea, respectively. Protection rose to 50% and 25%, respectively, of eight chickens after three inoculations.

The S1 protein has also been expressed *in situ* from fowlpoxvirus and adenovirus vectors. Three inoculations, in the wing web, of a recombinant

fowlpoxvirus expressing S1 induced IBV-specific antibodies (Wang *et al.*, 2002). After challenge with IBV, there was somewhat less recovery of challenge virus and less tracheal damage than in controls, and only mild clinical signs. Greater protection was achieved following a *single* oral application of a fowl adenovirus expressing S1 (Johnson *et al.*, 2003). Protection (assessed by non-re-isolation of challenge virus) was obtained in 90% and 100% of 10 to 13 chickens in two experiments.

Immunization with the N protein

The first study of the role of the IBV N protein in immunity was by Boots *et al.* (1992). The N protein was in the form of a bacterially expressed fusion protein with β -galactosidase. Initial experiments were with mice, inoculated in the footpad. A single inoculation resulted in popliteal lymph node cell proliferative responses to IBV. Delayed-type hypersensitivity responses were obtained when mice had been inoculated with: (a) inactivated IBV on two occasions; (b) inactivated IBV followed by N protein; and (c) N protein followed by inactivated IBV.

Experiments were subsequently undertaken with chickens. Following a single intramuscular inoculation of inactivated IBV, 1/8 birds were protected against respiratory challenge, as assessed by ciliary activity in the trachea, and none were protected in a group inoculated with N protein. Other chickens were inoculated with either inactivated virus or N protein, and then inoculated a second time, 6 weeks later, with inactivated IBV. After challenge, 8/8 and 8/10 chickens in the two groups, respectively, developed protective immunity (Table 3). The extent of protection was assessed as being slightly less when immunofluorescence was used to detect challenge virus in tracheal cells (Table 3). In a third group in which birds had been inoculated with β -galactosidase without any N protein, and then inoculated with inactivated IBV, some chickens had protective immunity, induced by the secondary inoculation with inactivated IBV (Table 3). Linear regression analysis showed that this was statistically less than in the group that had been primed with the N protein. The authors concluded that im-

munization with the N protein had induced protective immunity by activation of cytotoxic or helper T-cell responses.

Two intramuscular immunizations of chickens with a plasmid expressing the N protein, or a fragment of the N protein, induced immune responses that protected the birds from infection by IBV (applied by eye-drop and intranasally), as evidenced by mark reduction in replication of the challenge virus (Seo *et al.*, 1997b). A fragment of the N protein comprising the carboxyterminal 120 amino acid residues was sufficient to induce protection. Yu *et al.* (2001b) expressed this fragment in chickens using a single inoculation of a fowlpoxvirus recombinant. Upon challenge with a homologous strain and one heterologous strain of IBV, the birds did not develop clinical signs, although some replication of challenge virus was detected by an ELISA. The birds were not protected against challenge with a different heterologous strain.

Nature of protective immune responses to IBV

Collisson and colleagues (Seo & Collisson, 1997b; Pei *et al.*, 2003) have shown that cytotoxic T-cell responses in chickens to IBV infection correlated with initial decreases in infection and clinical signs. Cytotoxic T-cell activity was major histocompatibility complex restricted, and lysis was mediated by CD8⁺CD4⁻ cells. Adoptive transfer of IBV-infection-induced $\alpha\beta$ T cells bearing CD8 antigen protected chicks from challenge infection (Seo *et al.*, 2000; reviewed in Collisson *et al.*, 2000). Earlier work has been reviewed by Dhinakar Raj & Jones (1997). Tissues from chickens infected with two field strains of IBV were assayed for interferon, which was readily detected in the trachea and lung but at only low levels in the plasma, kidney, liver and spleen (Otsuki *et al.*, 1987).

Final comments

If there were to be a resurgence of SARS in humans, one would anticipate that the authorities

Table 3. Priming of tracheal protection by primary immunization of chickens with IBV N protein (modified from Boots *et al.*, 1992)

Primary immunization	Number of protected chickens			
	Ciliary activity assay		Immunofluorescence assay	
	Challenge after primary immunization ^a	Challenge after secondary immunization ^b	Challenge after primary immunization ^a	Challenge after secondary immunization ^b
Inactivated IBV	1/8	8/8	ND ^c	7/8
β -galactosidase-IBV N protein fusion protein	0/8	8/10	ND	7/10
β -galactosidase	0/8	8/12	ND	4/12

^aChickens were challenged by eye-drop 4 weeks after primary immunization.

^bThe secondary immunization was inactivated IBV. Chickens were challenged by eye-drop 4 weeks after secondary immunization.

^cND, not done.

would rapidly put into operation the various surveillance and control measures that proved successful in early 2003. If vaccines had been developed, vaccination might be included, especially for health care workers and contacts in the wider community. Coronaviruses can establish persistent infections, in at least a proportion of their hosts, resulting in chronic asymptomatic shedders, with subsequent problems for containment of the disease. For this and other reasons, a decision to apply a SARS vaccine would not be taken lightly. Notwithstanding, what type of vaccine might be used?

The poultry industry prefers to use live vaccines rather than inactivated ones. The former are cheaper to make and buy, and easier/cheaper to apply. These financial considerations would presumably not be paramount in the case of a resurgence of SARS in humans. Efficacy would be an important criterion for use of a particular vaccine, although that also applies in the poultry industry, of course. Safety of a SARS vaccine might be deemed more important than efficacy, assuming that a vaccine had an acceptable, if not complete, efficacy. In general, inactivated vaccines are considered to be safer than live attenuated vaccines, although the safety of inactivated vaccines cannot be taken for granted.

Unless a future SARS outbreak were to get rapidly out of control, it would seem unlikely that a conventional live attenuated vaccine would be used; the potential risk of vaccine-related problems (reactions) might be deemed too high. Genetic manipulation, in addition to traditional approaches, will be used as a means of attenuating *SARS coronavirus* (Kuo *et al.*, 2000; Thiel *et al.*, 2001; de Haan *et al.*, 2002a,b; Casais *et al.*, 2003; Ortego *et al.*, 2003). However, a desirable outcome cannot be guaranteed and fears of vaccine-related problems will still be harboured. However, given the almost total susceptibility of the global human population, the lethal outcome of 10% of cases, and the huge strain put on the health care services, and economies, a killed vaccine, subunit vaccine or vectored vaccine might be used in the first instance, among selected groups of people.

The experience of single applications of inactivated IBV is not greatly promising in the context of SARS; a single application of inactivated IBV protected <50% of chickens, sometimes much less than 50%. However, even a poorly efficacious single application of an inactivated SARS vaccine might help to reduce the spread of the virus, by reducing the number of susceptible people. At the first sign of a resurgence of SARS, the authorities might decide to vaccinate key personnel, with a view to revaccinating them. The outcome of some experiments with IBV in this regard is promising. In a number of studies, two vaccinations with inactivated IBV produced protective immunity in

>85% of chickens. That said, in some studies even >2 inoculations of high doses of inactivated IBV gave protection in only 50% or so of chickens. It might be that the criterion of protection used in these studies—non-re-isolation of challenge virus—might be too stringent. That is, some amelioration of the clinical effects of infection might be obtained even if there is some detectable replication of the challenge virus. In the case of infection by *SARS coronavirus*, if a vaccine only protected against the worst outcomes—pneumonia, death—then it might be deemed to have been successful.

Experimental IBV subunit vaccines, in the form of the S1 protein, were efficacious, but, as expected, not more so than inactivated virus. In two studies, protection of the trachea was achieved after three vaccinations in 70% and 59% of chickens, respectively. Protection of the kidney was less efficient (10% and 25%, respectively).

Vectored SARS vaccines might be considered likely to be less potentially problematic, with regard to safety, than attenuated *SARS coronavirus*. Perhaps such a vaccine might be used if an outbreak developed into an epidemic or pandemic. In this regard the results of the investigation by Johnson *et al.* (2003) are very promising; a single oral application of a non-pathogenic fowl adenovirus expressing the IBV S1 protein gave 90+% protection, as good as is obtained by conventional attenuated IBV.

In the event that any type of live vaccine was used against SARS, an inactivated SARS vaccine might subsequently be applied, to build upon the initial vaccination and perhaps provide at least medium-term protection. The experience with IBV is very good in this regard.

If *SARS coronavirus* were to re-emerge in humans, its S1 protein might not be the same as that of the 2002/2003 outbreak. Research with IBV has indicated that differences of only 5% of S1 protein amino acid can reduce cross-protection. Consequently, S1 differences among *SARS coronavirus* isolates must not be viewed complacently. The finding that the IBV N protein plays a beneficial role in immunity suggests that the SARS virus N protein should not be overlooked in a SARS vaccine development programme.

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RÉSUMÉ

Développement de vaccin contre le SARS (Syndrome Respiratoire Aigu Sévère): Expériences acquises en vaccination contre la bronchite infectieuse à coronavirus.

On peut dire que les vaccins contre la bronchite infectieuse (IB) des poulets (*Gallus gallus domesticus*) ont été ceux qui, parmi les vaccins contre les maladies à coronavirus, ont eu le plus de succès; et certainement ceux qui ont été les plus utilisés au niveau mondial, les autres étant ceux à coronavirus bovins, canins, félins et porcins.

Le virus de l'IB (IBV), le coronavirus de la dinde (*Meleagris gallopavo*) et celui du faisan de Colchide (*Phasianus Colchicus*) avec lesquels il y a des relations génétiques font partie du groupe 3 des coronavirus; le coronavirus SARS a été provisoirement classé dans le groupe 4 et les autres coronavirus des mammifères ont été classés dans les groupes 1 et 2.

L'IBV se multiplie au niveau des tissus respiratoires (incluant le nez, la trachée, les poumons, les sacs aériens) causant des symptômes respiratoires, mais également au niveau des reins (associé à une néphrite mineure ou majeure), de l'oviducte et en différents endroits du tractus alimentaire: œsophage, proventricule, duodénum, jéjunum, bourse de Fabricius, amygdales caecales rectum et cloaque, sans causer de troubles cliniques. Le virus peut persister puis être re-excrété au début de la

ponte (à l'âge de 4 à 5 mois) passant pour être une conséquence du stress au moment de l'entrée en ponte.

Des lignées génétiques de poulet réagissent différemment à l'IBV notamment en ce qui concerne la mortalité chez les poulets et la clearance du virus après la phase aiguë.

Les souches d'IBV atténuées (par passage sur œufs embryonnés) ont été utilisées comme vaccins dans les années 1950 puis une vingtaine d'années plus tard des vaccins inactivés ont été développés pour booster la protection des animaux en ponte. Les vaccins à virus vivants sont généralement administrés à l'âge d'un jour chez les poulets de chair. Dans les conditions expérimentales, ceci peut entraîner une immunité stérile lors d'une épreuve à virus virulent homologues. Bien que 100% des poulets peuvent être protégés (contre les signes cliniques et la perte de l'activité ciliaire au niveau de la trachée), il arrive que 10% des poulets vaccinés ne présentent pas de réponse immunitaire protectrice. La protection conférée par les souches vivantes est courte, elle commence à décliner neuf semaines après la vaccination pour les vaccins préparés avec des souches très atténuées. L'IBV présente de nombreux sérotypes (définis par séroneutralisation), la protection croisée est souvent faible. En conséquence, les poulets doivent être revaccinés avec le même ou un autre sérotype deux ou trois semaines plus tard.

Une simple administration de vaccin inactivé entraîne généralement une protection des sujets inférieure à 50%. D'après certaines publications, deux administrations entraînent 90 à 100% de protection, mais pour d'autres la protection est inférieure à 50%. Sur le terrain, les vaccins inactivés sont administrés aux futures adultes qui ont reçu au préalable deux ou trois vaccins à virus vivants atténués. Ceci augmente la protection des animaux adultes contre les pertes de protection en œufs et induit des anticorps sériques à un niveau soutenu qui sont transmis à la descendance.

La glycoprotéine de spicule (S) comprend une sous-unité S2 carboxy-terminal (environ 625 résidus d'acides aminés) qui permet l'encrage de S dans l'enveloppe du virus et une sous-unité S1 amino-terminal (environ 520 résidus) considérée être la forme bulbueuse importante et distale de S. La sous-unité S1 (purifiée du IBV, exprimée en baculovirus ou chez des oiseaux ayant reçu le vecteur viral variole aviaire) induit des anticorps neutralisants (VN). Des réponses immunes et protectrices ont été induites, mais plusieurs inoculations sont nécessaires et pour autant le pourcentage de poulets protégés est trop faible (< 50%) pour être utilisés sur le terrain. Il est à souligner que l'expression de S1 chez des oiseaux après administration d'un vecteur adénovirus aviaire non pathogène a induit une protection de 90% et de 100% des poulets au cours de deux expérimentations. Des différences aussi faibles que 5% entre des séquences de S1 peuvent entraîner une protection croisée faible. Des différences de 2 à 3% au niveau de S1 (correspondant à 10 ou 15 acides aminés) peuvent changer le sérotype, suggérant qu'un petit nombre d'épitopes sont immunodominants en ce qui concerne les anticorps neutralisants.

Des études préliminaires sur le rôle de la protéine de Capside (N) de l'IBV au niveau immunitaire ont suggéré que l'immunisation avec N exprimé en bactérie, bien que n'induisant pas directement une protection, a amélioré l'induction de la protection lors de l'inoculation ultérieure d'un IBV inactivé. Dans une autre étude, deux immunisations intramusculaires d'un plasmide exprimant N ont induit une immunité protectrice.

La base de l'immunité de l'IBV n'est pas encore parfaitement connue. Les taux d'anticorps sériques ne sont pas corrélés avec la protection, même si les anticorps locaux jouent un rôle. Des transferts de cellules T alphabéta induisant l'infection de l'IBV, portant l'antigène CD8 ont protégé des poulets contre une épreuve virulente.

En conclusion, les vaccins vivants atténués de l'IBV induisent une bonne protection bien que de courte durée vis-à-vis d'une épreuve homologues, même si une minorité d'individus ont des réponses faibles. Les vaccins inactivés de l'IBV sont insuffisamment efficaces quand ils sont administrés une seule fois en absence de vaccins à virus vivants administrés préalablement. Deux administrations de vaccin inactivé d'IBV sont beaucoup plus efficaces bien que cela ne soit pas une proposition valable pour l'industrie avicole. Quoiqu'il en soit, le coût et la logistique de l'administration multiple de vaccin inactivé du SARS devraient être beaucoup acceptables pour la protection des populations humaines principalement s'il s'agit de groupes ciblés, tels que le personnel soignant et les contacts à risque élevé. L'administration d'un vaccin contre le SARS est certainement mieux si elle est limitée à un petit nombre d'individus ciblés qui pourront être suivis. En effet, les

personnes vaccinées peuvent, si elles sont infectées par le *coronavirus* du SARS, devenir asymptomatiques excrétrices de virus et ainsi présenter un risque vis-à-vis des personnes non vaccinées. Pour l'avenir, le vecteur adénovirus aviaire exprimant la sous-unité S1 de l'IBV qui présente une bonne efficacité, permet d'être optimiste pour un vaccin vivant contre le SARS, si ceci s'avère nécessaire, avec la possibilité d'inclure le gène de la protéine N.

ZUSAMMENFASSUNG

Entwicklung einer Vakzine gegen SARS: Erfahrungen mit der Vakzination gegen das aviäre infektiöse Bronchitis Coronavirus

Von den Impfstoffen gegen Coronavirus-induzierte Erkrankungen sind wohl die Impfstoffe gegen die infektiöse Bronchitis (IB) des Huhnes (*Gallus gallus domesticus*) die erfolgreichsten und sicherlich die am häufigsten eingesetzten, wobei die anderen Impfstoffe gegen Rinder-, Kaninchen-, Katzen- und Schweinecoronaviren gerichtet sind. Das IB-Virus (IBV) ist zusammen mit den genetisch verwandten Coronaviren der Pute (*Meleagris gallopavo*) und des Ringfasans (*Phasianus colchicus*) ein Coronavirus der Gruppe 3, während das SARS Coronavirus vorläufig in die Gruppe 4 eingeordnet wurde und die anderen bekannten Mammalier-Coronaviren zu den Gruppen 1 und 2 gehören.

Das IBV vermehrt sich nicht nur im Respirationstrakt (einschließlich Nase, Trachea, Lungen und Luftsäcken verbunden mit Verursachung einer respiratorischen Erkrankung) sondern auch in Niere (assoziiert mit gering- bis hochgradiger Nephritis), Ovidukt und in vielen Teilen des Verdauungstrakts - Ösophagus, Proventriculus, Duodenum, Jejunum, Bursa Fabricii, Zäkalkonsillen, Rektum und Kloake, dort jedoch gewöhnlich ohne klinische Auswirkungen. Das Virus kann persistieren und wird mit Beginn der Legetätigkeit (im Alter von 4-5 Monaten) wieder ausgeschieden, wobei die Virusaktivierung als Konsequenz aus dem damit verbundenen Stress angesehen wird.

Verschiedene genetische Hühnerlinien unterscheiden sich im Ausmaß der durch IBV verursachten Mortalität sowie hinsichtlich der Virus-Clearance nach der akuten Infektionsphase.

In den 1950iger Jahren wurden (durch Passagen in embryonierten Hühneriern) attenuierte IBV-Stämme als Lebendvirusvakzinen eingeführt. Einige Jahrzehnte später folgten Inaktivatvakzinen zur Boosterung der Schutzwirkung bei Legehennen. Lebendvirusimpfstoffe werden gewöhnlich bei Masthühnern am ersten Lebensstag appliziert. Unter experimentellen Bedingungen kann dies zu einer sterilen Immunität führen, wenn die Tiere mit einem virulenten homologen Virus belastungsinfiziert werden. Obwohl 100 % der Tiere geschützt sein können (gegen klinische Symptome und Zilienverlust in der Trachea), zeigen manchmal 10 % der Küken keine schützende Immunantwort. Die Schutzwirkung ist kurzfristig. Neun Wochen nach der Vakzination mit hoch attenuierten Impfstämmen wird der Beginn des Abfalls sichtbar. Das IBV besitzt eine Vielzahl von Serotypen (definiert durch Neutralisationstests), wobei die Kreuzschutzwirkung oft gering ist. Daraus folgt, dass Hühner zwei bis drei Wochen nach der Erstvakzination mit dem gleichen oder einem anderen Serotyp revakziniert werden sollten.

Einmalige Applikation von inaktiviertem Virus hat allgemein zum Schutz von 50 % der Hühner geführt. Zwei Applikationen riefen laut einiger Veröffentlichungen einen 90-100%igen Schutz hervor, in anderen Untersuchungen blieb er jedoch unter 50 %. Unter Praxisbedingungen im Feld werden Inaktivatimpfstoffe bei Legehennen eingesetzt, die vorher zwei- oder dreimal mit attenuierten Lebendvirusvakzinen geimpft worden sind. Dies erhöht die Schutzwirkung bei den Legehennen gegen Legeleistungseinbrüche und induziert einen anhaltenden Antikörperlevel, der an die Nachkommen weitergegeben wird.

Das große Spikeprotein (S) umfasst eine S2-Untereinheit am Carboxy-Ende (ungefähr 625 Aminosäuren), die das S in der Virushülle verankert, und eine S1-Untereinheit am Amino-Ende (ungefähr 520 Aminosäuren), von der angenommen wird, dass sie größtenteils den distalen Bulbusteil des S formt. Die S1-Untereinheit (gereinigt aus dem IBV, exprimiert unter Verwendung von Baculovirus oder exprimiert in Hühnern mit Hilfe eines Vektor-Hühner-Pockenvirus) induzierte Virusneutralisierende (VN) Antikörper. Obwohl eine schützende Immunantwort induziert wurde, waren mehrfache Inokulationen erforderlich und der Prozentsatz geschützter Hühner war zu niedrig (< 50 %) für die kommerzielle Anwendung. Bemerkenswerterweise induzierte die Expressierung von S1 in Hühnern unter Verwendung eines apathogenen

Vektor-Hühner-Adenovirus in zwei Experimenten eine Schutzwirkung bei 90 bzw. 100 % der Tiere. Unterschiede von weniger als 5 % en las S1-Sequenzen können in geringen Kreuzschutzwirkungen resultieren. Unterschiede im S1 von 2-3% (10-15 Aminosäuren) können den Serotyp ändern, was vermuten lässt, dass una pequeña cantidad de epítopos inmunodominantes es hincapié de neutralizantes anticuerpos.

Anfangsuntersuchungen zur Bedeutung des IBV-Nukleokapsidproteins (N) für die Immunität legten nahe, dass die Immunisierung mit in Bakterien exprimiertem N, obwohl nicht direkt Schutz auslösend, die Induktion der Schutzwirkung bei einer nachfolgenden Inokulation mit inaktiviertem IBV verbesserte. In einer anderen Studie führten zwei intramuskuläre Immunisierungen mit einem N exprimierenden Plasmid zu einer schützenden Immunität.

Die Grundlagen der Immunität gegen IBV sind nicht gut erforscht. Serumantikörper korrelieren nicht mit der Schutzwirkung, doch lokale Antikörper werden eine wichtige Rolle zugesprochen. Die adoptive Übertragung von durch IBV-Infektion induzierten alpha/beta-T-Zellen mit CD8-Oberflächenantigenen schützten Hühnerküken gegen eine Belastungsinfektion.

Zusammenfassend lässt sich sagen, dass attenuierte Lebendvirusimpfstoffe einen guten, aber kurzfristigen Schutz gegen homologe Belastungsinfektionen hervorrufen, wobei eine Minderheit der Tiere eine schlechte Immunantwort zeigen können. IBV-Inaktivativakzinen sind nur ungenügend wirksam, wenn sie nur einmalig und ohne Vorvaksinierung mit einer Lebendvirusvakzine angewendet werden. Zwei Applikationen von inaktiviertem IBV sind viel effizienter, doch dies ist ein kommerziell nicht rentabler Vorschlag für die Geflügelindustrie. Für den Schutz der menschlichen Bevölkerung jedoch dürften die Kosten und die Logistik für eine mehrfache Applikation einer SARS-Inaktivativakzine akzeptabler sein, zumal wenn die Anwendung auf bestimmte Zielgruppen wie Beschäftigte im Gesundheitswesen und hoch gefährdete Kontaktpersonen beschränkt wird. Die Applikation eines SARS-Impfstoffs sollte möglichst limitiert bleiben auf eine minimale Anzahl von Zielpersonen, die überwacht werden können, da einige geimpfte Individuen, wenn sie sich mit dem SARS-Coronavirus infizieren, asymptomatische Virusausscheider werden können und somit ein Risiko für die nicht vakzinierte Bevölkerung darstellen können. Beim Blick auf die Zukunft stimmt die hohe Wirksamkeit des IBV-S1-Subunit-exprimierenden Vektor-Hühner-Adenovirus optimistisch für eine SARS-Lebendvirus-Vakzine mit der Möglichkeit der Inkorporierung des N-Proteingens, falls das für nötig erachtet werden sollte.

REVISION

Desarrollo de vacunas frente al SARS: experiencia de la vacunación frente al coronavirus de la bronquitis infecciosa aviar

Las vacunas frente a la bronquitis infecciosa (IB) de pollos (*Gallus gallus domesticus*) han sido las más exitosas y ciertamente las más comúnmente usadas de las vacunas frente a enfermedades causadas por coronavirus, siendo las otras frente a coronavirus bovino, canino, felino y porcino. El virus de IB (IBV), junto con otros coronavirus relacionados genéticamente como el del pavo (*Meleagris gallopavo*) y faisán común (*Phasianus colchicus*), forman el grupo 3 coronavirus, mientras que el coronavirus del SARS se encuentra en el grupo 4 y el resto de coronavirus de mamíferos en los grupos 1 y 2.

El IBV se replica no únicamente en tejidos del aparato respiratorio (incluida la cavidad nasal, tráquea, pulmones y sacos aéreos, causando enfermedad respiratoria), sino también en el riñón (asociado con nefritis de diferente intensidad), oviducto, y en varias partes del tracto digestivo – esófago, proventrículo, duodeno, yeyuno, bolsa de Fabricio, tonsilas cecales, recto y cloaca, habitualmente sin efectos clínicos. El virus puede persistir, siendo reexcretado al inicio de la puesta (a los 4 o 5 meses de edad), probablemente como consecuencia del estrés de la entrada en puesta.

Las diversas líneas genéticas de pollos presentan diferencias respecto a la mortalidad causada por la infección con IBV en pollos y respecto a la eliminación del virus tras la fase aguda.

Las cepas vivas atenuadas de IBV (mediante pases en huevos de pollo embrionados) fueron introducidas como vacunas en 1950s, y un par de

décadas después fueron seguidas por las vacunas inactivadas para prolongar la protección en aves de puesta. Las vacunas vivas se aplican normalmente en aves de carne al día de edad. En condiciones experimentales esto puede resultar en una inmunidad estéril cuando los animales son desafiados con un virus homólogo. Aunque el 100% de los pollos puede estar protegido (frente a la sintomatología clínica y a la pérdida de actividad ciliar de la tráquea), en ocasiones el 10% de las aves vacunadas no responden con una respuesta inmune protectora. La protección suele durar poco y empieza a declinar a las nueve semanas tras la vacunación con vacunas basadas en cepas muy atenuadas. Existen varios serotipos de IBV (definidos mediante técnicas de neutralización), pero la protección cruzada suele ser pobre. En consecuencia los pollos son revacunados con el mismo u otro serotipo, dos o tres semanas después.

Las aplicaciones únicas de virus inactivado normalmente protegen a < 50% de los pollos. Se ha descrito que dos aplicaciones pueden llegar a proteger del 90 al 100% de las aves, pero en otras ocasiones se ha descrito una protección por debajo del 50%. En la práctica de campo, las vacunas inactivadas se utilizan en aves de puesta que han sido previamente vacunadas con dos o tres vacunas de virus vivo atenuado. Esto aumenta la protección en las aves de puesta frente a las pérdidas de producción de huevos e induce una nivel de anticuerpos séricos sostenido y que pasa a la progenie.

La glicoproteína de la espícula (S) comprende una subunidad carboxi/terminal S2 (de aproximadamente 625 aminoácidos), que enclava la S en el envoltorio viral, y una subunidad aminoterminal S1 (de aproximadamente 520 aminoácidos) que forma la parte distal de la parte bulbosa de la S. La subunidad S1 (purificada de un virus de IBV, expresada en baculovirus o en aves mediante un vector de virus de viruela aviar) induce anticuerpos neutralizantes (VN). Aunque se indujeron respuestas inmunes protectoras, se requirieron múltiples inoculaciones y el porcentaje de pollos protegidos fue demasiado bajo (< 50%) para poder ser aplicada comercialmente. Cabe remarcar, que la expresión de S1 en aves mediante un vector de adenovirus aviar no patógeno indujo una protección de entre 90 y 100% de los pollos en dos experimentos. Diferencias de tan sólo el 5% en las secuencias de la S1 resultaron en una protección cruzada baja. Diferencias de entre el 2 y el 3% (de 10 a 15 aminoácidos) en la S1 pueden cambiar el serotipo, lo que sugiere que un número bajo de epítopos son inmunodominantes respecto a los anticuerpos neutralizantes.

Los estudios iniciales sobre el papel que juega la proteína de la nucleocápside (N) de IBV en la inmunidad sugieren que la inmunización con proteína N expresada en bacterias, aunque no induce protección directamente, mejoró la protección inducida por una inoculación subsiguiente con IBV inactivado. En otro estudio, dos inmunizaciones intramusculares con un plásmido que expresaba N indujeron una inmunidad protectora. La base de la inmunidad frente a IBV no está totalmente clara. Los niveles de anticuerpos en suero no se correlacionan con la protección, aunque se reconoce que los anticuerpos locales juegan un papel importante. La transferencia de linfocitos T CD8 alfa/beta inducidos mediante una infección con IBV, protegieron a los pollos frente a una infección experimental.

En conclusión, las vacunas vivas atenuadas inducen una buena protección, aunque de corta duración, frente a la infección experimental homóloga, pero una minoría de individuos puede responder pobremente. Las vacunas de IBV inactivadas no son suficientemente eficaces cuando se administran únicamente una vez y no se realiza una primovacunación con una vacuna viva. Dos aplicaciones de una vacuna inactivada de IBV son mucho más eficaces, aunque no parece una propuesta viable desde el punto de vista comercial para la industria avícola. Aún así, el coste y la logística de una aplicación múltiple de una vacuna inactivada frente al SARS serían más aceptables para la protección de una población humana, especialmente si está limitada a grupos de alto riesgo, como trabajadores relacionados con temas sanitarios o personas con alto riesgo de contacto. La aplicación de la vacuna frente al SARS es quizás mejor limitarla a un número de individuos de riesgo que puedan ser monitorizados, ya que algunas personas vacunadas podrían, al estar infectadas con el coronavirus del SARS, volverse excretoras asintomáticas del virus, siendo un riesgo para la población no vacunada. De cara a un futuro próximo, la alta eficacia de un vector de adenovirus aviar que expresa la subunidad S1 del IBV proporciona optimismo con respecto a una vacuna viva de SARS, y si se cree necesario podría haber la posibilidad de incluir el gen de la proteína N.