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Coronavirus immunogens

Linda J. Saif

Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH USA

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ABSTRACT

Coronaviruses (CV) infect a variety of livestock, poultry and companion animals. They belong to at least five antigenic groups. CV cause localized infections of the respiratory and/or intestinal tracts, with the exception of feline infectious peritonitis virus (FIPV) and hemagglutinating encephalomyelitis (HEV) which cause systemic infections. The enteropathogenic CV infect the villous enterocytes resulting in villous atrophy leading to malabsorptive diarrhea. Several CV (bovine CV-BCV, porcine respiratory CV-PRCV, infectious bronchitis virus-IBV) cause respiratory disease.

Current evidence indicates that protection against enteric and respiratory CV infections is mediated by passive or active immunity at the primary site of CV replication. Maternal vaccination approaches to induce passive immunity include the use of inactivated and modified live viral vaccines. Modified live viruses and a Ts mutant CV (FIPV) are also used as oral or intranasal vaccines to induce active mucosal immunity. The success of these vaccines in the field is often compromised by a number of potential problems.

Coronaviruses are spherical, enveloped viruses, ranging from 80–160 nm in diameter and containing a positive-stranded RNA genome. They possess prominent surface spikes and some species display a fringe of smaller surface projections believed to be the hemagglutinin (HE). Coronaviruses possess 3 to 4 structural proteins: the spike (S) glycoprotein (150–200 kDa), the integral membrane glycoprotein (M; 20–30 kDa) and the nucleocapsid phosphoprotein (N; 43–50 kDa). A subset of CV (BCV, HEV, turkey CV) possess a third glycoprotein on the virion surface, the HE (60–65 kDa). These proteins can be quantitated using pooled monoclonal antibodies (mAb) to distinct epitopes of each protein in ELISA.

Most research has focused on the S protein as a candidate antigen for CV vaccines since it induces virus neutralizing (VN) antibodies. However the HE protein stimulates the production of VN and HE inhibiting antibodies and the M protein induces antibodies that neutralize virus in the presence of complement. Attempts to correlate in vitro VN antibody activity with in vivo protection have shown that the passive transfer of VN mAb to the S or HE protein conferred passive protection against CV challenge in some studies, but not others. Additional research has implicated a possible role for other CV proteins in immunity. Studies of mAb to the M protein of transmissible gastroenteritis (TGEV) have provided evidence for a direct role of the M protein in the induction of αIFN by porcine blood leukocytes. The potential significance of this phenomenon to immunity to TGEV is unclear. Similarly, studies of IBV have suggested that determinants recognized by T cells reside on the N protein and these determinants may be shared among heterologous strains of IBV, resulting in the induction of cross-protection. Thus epitopes on the N protein may be important for induction of

cell mediated immunity (CMI). CMI may play an important role in protection of cats against FIPV, since induction of circulating antibodies to the S protein of FIPV contributes to disease pathogenesis by the induction of immune complexes and antibody dependent enhancement of the infectivity of FIPV for macrophages.

An increased understanding of antibody and CMI responses following natural CV infections in animals is needed to identify the antigens and epitopes that induce protective immune responses. The expression of CV structural protein genes in various vectors will provide the recombinant proteins needed for future immunogenicity studies in the host species. Furthermore, live rDNA vectors that replicate in the gut and express coronavirus genes may provide a new generation of coronavirus vaccines.

ANIMAL HOSTS, ANTIGENIC RELATIONSHIPS AND DISEASES

Coronaviruses infect a variety of livestock, poultry and companion animals, causing economically important diseases in these animals. The prototype coronaviruses and their host species include swine: transmissible gastroenteritis virus (TGEV) and the respiratory variant, porcine respiratory coronavirus (PRCV), porcine epidemic diarrhea virus (PEDV) and hemagglutinating encephalomyelitis virus (HEV); cattle: bovine coronavirus (BCV); horses: equine coronavirus (ECV); chickens: infectious bronchitis virus (IBV); turkeys: turkey coronavirus (TCV) or bluecomb; dogs: canine coronavirus (CCV); and cats: feline infectious peritonitis virus (FIPV) and feline enteric coronavirus (FECV) (Saif and Heckert, 1990).

The above viruses belong to five distinct antigenic groups that share within a group common antigens recognized in immunofluorescence, ELISA and immunoblotting assays (Saif and Heckert, 1990: Wege et al., 1982: Horzinek et al., 1982). These groups include: group 1: TGEV, PRCV, CCV, FIPV, and FECV; group 2: BCV and HEV; group 3: IBV; group 4: TCV; and group 5: PEDV. It has been suggested that the members of group 1 may represent host range mutants of a single virus strain (Horzinek et al., 1982). Within this group, the virus pairs TGEV and PRCV and FIPV and FECV may represent variants of one another with altered tissue tropisms. For most of the above coronavirus species, only a single serotype is recognized. An exception is IBV for which at least five serotypes are described (Kusters et al., 1987). Although FIPV and FECV strains at present comprise a single serotype, strains vary greatly in infectivity and virulence and constitute multiple biotypes (Pedersen, 1987a,b; Scott, 1987; Mostl, 1990).

The coronaviruses that infect the above species cause localized infections of the respiratory and/or intestinal tracts, with the exception of IBV strains which also infect the kidneys and oviduct (King and Cavenagh, 1991) and FIPV and HEV which cause systemic infections (Saif and Heckert, 1990; Wege et al., 1982; Mostl, 1990). The enteropathogenic coronaviruses (TGEV, PEDV, BCV, ECV, TCV, CCV, FECV) infect the villous enterocytes resulting in villous atrophy and consequently a malabsorptive diarrhea (Saif and

Heckert, 1990; Mostl, 1990). These infections are most severe in young animals, but bloody diarrhea associated with coronavirus shedding in feces has been reported in adult cattle with winter dysentery (Saif, 1990). The pneumoenteric coronavirus (BCV) and the respiratory coronaviruses, PRCV and IBV induce respiratory infections in cattle, swine and chickens, respectively. The latter two coronaviruses replicate in the nasal mucosa, trachea and lungs whereas BCV replicates in these respiratory tissues and the distal small intestine and colon (Saif and Heckert, 1990; Saif et al., 1986; Pensaert and Cox, 1989). Although respiratory disease is commonly associated with infection of chickens by IBV strains (King and Cavenagh, 1991; Kusters et al., 1987), most respiratory infections associated with BCV and PRCV are commonly mild or subclinical (Heckert et al., 1990; Heckert et al., 1991; Pensaert and Cox, 1989).

Although both FECV and FIPV strains initially replicate in the epithelium of the oropharynx and small intestine, their subsequent disease pathogenesis differs (Stoddart et al., 1988; Pedersen, 1987a,b). Whereas FECV strains, like other enteric coronaviruses, are restricted in their replication to mature villous epithelial cells of the small intestine and cause a transient enteritis, FIPV strains can cross the mucosal barrier via a cell-associated viremia in macrophages and cause disseminated disease (Petersen, 1987a,b). Such generalized infections are characterized by an inflammatory exudate in the body cavities (effusive FIP) or disseminated pyogranulomas (non-effusive FIP) (Petersen, 1987a,b). A broad spectrum exists in the virulence of FIPV strains, with the ability of strains to infect and replicate in macrophages and spread systemically correlated with virulence (Stoddart et al., 1989). Serum antibodies accentuate the pathogenesis of FIP via the generation of immune complexes which are deposited in affected organs and via mediation of antibody-dependent enhancement (ADE) of FIPV infection of macrophages (Scott, 1987; Olsen et al., 1992).

CONCEPTS OF IMMUNITY TO ANIMAL CORONAVIRUSES

Current evidence indicates that protection against enteric and respiratory coronavirus infections is most effectively mediated by passive or active mucosal immunity at the primary site of viral replication (Saif and Jackwood, 1990; Saif and Wesley, 1992). The presence of serum antibodies usually fails to correlate with protection against enteric coronavirus infections (Hooper and Haelterman, 1966; Heckert et al., 1990). However in one study conventional newborn calves with low titers of IgA BCV antibodies in serum had significantly greater days with diarrhea than those calves with high titers of serum IgA antibodies (Heckert et al., 1990). As noted previously for FIPV infections, high titers of serum antibodies actually exacerbate FIP and sensitize cats to subsequent viral exposure (Pedersen, 1987a,b; Scott, 1987). A

new approach for vaccination against FIPV is based on the intranasal administration of a live temperature sensitive (TS) strain of FIPV which replicates primarily in the upper respiratory tract (Gerber et al., 1990). The rationale for this approach is to stimulate local mucosal immunity (SIgA and CMI) but only low levels of humoral antibodies.

The duration of immunity to coronavirus infections has not been well-studied. Repeated or recrudescence of respiratory infections with BCV, PRCV or IBV have been reported in calves, sows and chickens, respectively (Heckert et al., 1990; Heckert et al., 1991; Callebaut et al., 1990; Jones and Ambali, 1987). Whether respiratory immunity to coronavirus infections is short-lived or antigenic variants of coronaviruses frequently arise is unknown and warrants further study.

Because young animals are usually at the greatest risk of developing severe disease from coronavirus infections, many studies have focused on passive immunity derived from the mother to prevent enteric viral infections. Maternal vaccination approaches that induce protective levels of SIgA antibodies in the milk of monogastrics or IgG_1 antibodies in mammary secretions of cows provided effective passive immunity against enteric coronaviruses when evaluated under experimental challenge conditions (Saif and Jackwood, 1990; Saif and Wesley, 1992). These antibodies provide lactogenic immunity in the intestine whereas passive serum antibodies are usually not protective (Hooper and Haelterman, 1966).

The ability of passive serum antibodies transferred to the neonate via colostrum to prevent respiratory coronavirus infections is unclear. The presence of low maternal antibody titers to PRCV in pigs or BCV in calves did not prevent viral replication in the respiratory tract (Cox et al., 1990; Heckert et al., 1991). However in the calf study, there was a significant correlation between IgG_1, IgG_2 and IgA antibody titers to BCV in serum and number of respiratory sick days (Heckert et al., 1991). These results are similar to those observed in studies of bovine respiratory syncytial virus in calves, which indicated that although maternal antibodies do not prevent this respiratory disease, they may moderate its severity (Kimman et al., 1988).

A respiratory variant of TGEV (PRCV), has recently emerged which is serologically indistinguishable from TGEV, but possesses an altered pathogenicity and a tropism for respiratory tissues (Pensaert and Cox, 1989). Comparative studies of mucosal immunity to attenuated and virulent TGEV and PRCV in pigs provide an ideal model to explore the concept of a common mucosal immune system in pigs. At present, evidence is contradictory as to whether sows previously infected with PRCV passively protect their pigs against TGEV (Bernard et al., 1989; Paton and Brown, 1990; Callebaut et al., 1990). Results from several researchers indicate that pigs previously infected with PRCV or nursing PRCV-exposed dams are only partially protected upon challenge with virulent TGEV (Bernard et al., 1989; Van Nieustadt et al.,

1989; Paton and Brown, 1990). Preliminary data from our laboratory (Van Cott et al., 1993) based on enumeration of antibody secreting cells (ASC) from mucosal tissues by ELISPOT, indicate that only virulent TGEV and to a lesser extent high titer attenuated TGEV stimulate ASC in the mesenteric lymph nodes (MLN) of infected pigs. Few if any ASC were detected in the MLN after PRCV infection, but high numbers of ASC were present in the bronchial lymph nodes of these pigs. Such findings may explain the lack of immunity to TGEV observed in PRCV-infected pigs (Van Nieustadt et al., 1989). In a recent report, IgA TGEV antibodies were detected in the milk of only about 30% of PRCV-infected sows, but in 100% of TGEV-infected sows (Callebaut et al., 1990). The authors noted that IgA antibodies in the milk of the PRCV-infected sows may be contingent upon their reinfection with PRCV.

IMMUNOGENS OF CORONAVIRUSES AND THEIR ROLE IN IMMUNITY

Coronaviruses are spherical, enveloped viruses, ranging from 80–160 nm in diameter and containing a positive-stranded RNA genome. They possess prominent surface spikes and some species display a fringe of smaller surface projections believed to be the hemagglutinin (HE). Coronaviruses possess 3 to 4 structural proteins: the spike (S) glycoprotein (150–200 kDa), the integral membrane glycoprotein (M; 20–30 kDa) and the nucleocapsid phosphoprotein (N; 43–50 kDa). A subset of coronaviruses (BCV, HEV, TCV) possess a third glycoprotein on the virion surface, the HE (60–65 kDa) (Wege et al., 1982; Spaan et al., 1988; Saif and Heckert, 1990).

S glycoprotein
Most research has focused on the S protein as a candidate antigen for coronavirus vaccines since it induces virus neutralizing (VN) antibodies. This was established initially by inoculating animals with purified S proteins obtained following disruption of the virion (Garwes et al., 1978; Cavanagh et al., 1984). More recently, the IBV and TGEV S proteins expressed in recombinant viral vectors have also been shown to induce neutralizing antibodies in rodents (Pulford et al., 1990; Tomley et al., 1987; Godet et al., 1991). Recent evidence has shown that the S protein of TGEV, IBV, FIPV and BCV induces neutralizing monoclonal antibodies (mAb). Using mAb, four major antigenic sites (A–D) have been delineated that cluster in the amino terminal portion (presumably the globular part) of the S protein of TGEV (Delmas et al., 1990; Enjuanes et al., 1990). Most of the neutralization-mediating determinants were localized in the A–B domain, were conformation dependent and were highly conserved among TGEV strains and the antigenically-related (group 1) coronaviruses (PRCV, FIPV, and CCV) (Delmas et al., 1990; Simkins et al.; 1989; Zhu et al., 1990). mAbs to Site D (designated site B by Correa et al., 1988) did not cross-react with other group 1 coronaviruses in-

cluding PRCV (Delmas et al., 1990; Simkins et al., 1992). Non-neutralizing mAb to site D/B have been used in blocking ELISA to differentiate sera from pigs infected with TGEV or PRCV (Callebaut et al., 1988). Site C was composed of linear epitopes and exhibited antigenic variability among TGEV strains (Delmas et al., 1990).

Unlike TGEV, the S protein of BCV and IBV is cleaved into an amino terminal subunit (S1) and a carboxy terminal subunit (S2) (Laude, 1990; Spaan et al., 1988; Vautherot, et al., 1990). For both viruses, the S1 subunit is the major inducer of neutralizing mAb and for IBV it is also the major site of antigenic variation (Mockett et al., 1984; Laude, 1990; Vautherot, et al., 1990). On the other hand, the S2 subunit of BCV may be involved in membrane fusion during infection, as reflected by its mediation of membrane fusion in insect cells infected with a recombinant baculovirus expressing the S2 BCV gene (Yoo et al., 1991). The S protein of IBV also exhibits HE activity (Mockett et al., 1984).

The evidence for a direct role of the S protein in the induction of protective immunity to coronaviruses is contradictory. Purified S protein from IBV or vaccinia virus recombinants expressing the S gene were unable to induce complete respiratory tract protection in chickens (Cavanagh et al., 1984; Spaan, 1990; Tomley et al., 1987). Kittens immunized with vaccinia virus recombinants expressing the FIPV S gene became sensitized to FIPV and died after challenge (Vennena et al., 1990).

Attempts to correlate in vitro neutralizing antibody activity induced by the S protein with in vivo protection have also led to conflicting results. In one study, the administration into ligated intestinal loops of a mixture of BCV with a neutralizing mAb to the A domain of BCV S (S1 subunit) or HE proteins, conferred passive protection in calves against BCV-induced villous atrophy (Deregt et al., 1989). However neutralizing mAb to the S (site A or undefined sites) or M proteins of TGEV failed to confer passive protection in pigs against TGEV challenge, although polyclonal porcine antibodies were protective (Wesley et al., 1988). Reasons for these discrepancies are uncertain, but may relate to the overall experimental design, the binding affinity, epitope specificity and in vivo stability of the mAbs used and the virus challenge dose. More research is needed to clarify the degree of in vivo protection provided by neutralizing mAbs to the S protein of coronaviruses. In a recent report, an adverse effect of neutralizing mAbs to the S protein of FIPV was shown. These mAbs were found to mediate ADE of the infectivity of FIPV for macrophages in vitro (Olsen et al., 1992). Such results correlate with the ADE of FIPV infection observed in cats vaccinated with a recombinant vaccinia virus expressing the S protein of FIPV (Vennema, et al., 1990).

HE protein
The HE protein stimulates the production of virus neutralizing and hemagglutination-inhibiting mAb to BCV (Deregt et al., 1989; Vautherot et al.,

1990). In at least one study, neutralizing mAb to the HE protein also conferred passive protection against BCV infection in calves (Deregt et al., 1989). Recently the HE protein of BCV was cloned, expressed and shown to exhibit extensive amino acid sequence homology and binding and enzymatic properties similar to the HE of influenza C virus (Parker et al., 1989).

M protein

The M protein of TGEV induces mAb that neutralize virus in the presence of complement (Woods et al., 1987). In one study, in which a single neutralizing mAb to the M protein of TGEV was tested, this mAb failed to provide passive protection in pigs against challenge with TGEV (Wesley et al., 1988). Other studies of mAb to the M protein of TGEV and mAb selected M protein mutants have provided evidence for a direct role of the M protein in the induction of α interferon by porcine blood leukocytes (Charley and Laude, 1988; Laude et al., 1992). However the potential significance of this phenomenon in regard to immunity to TGEV is unclear.

N protein

The N protein binds to virion RNA and provides the structural basis for the helical nucleocapsid. The deduced amino acid sequences have been reported for the N proteins of BCV, TGEV and IBV (Spaan et al., 1988). Studies of IBV have suggested that determinants recognized by T cells reside on the N protein (Boots et al., 1990). Furthermore these determinants were shared among certain heterologous strains of IBV, and may explain the cross-protection induced between certain heterologous IBV strains (protectotypes). Immunization of mice with a pEX nucleocapsid expression product induced CMI responses measured in a lymphocytes proliferation test: no CMI responses were found after immunization with pEX S protein expression products (Boots et al., 1990). Thus epitopes on the N proteins of coronaviruses may be important for induction of CMI. The CMI responses are postulated to be especially important in protection of cats against FIPV (Gerber et al., 1990), and circulating neutralizing antibodies to the S protein of FIPV actually accentuate the pathogenesis of the disease (Vennema et al., 1990).

CURRENT CORONAVIRUS VACCINES AND THEIR POTENCY TESTING

A number of modified live and killed coronavirus vaccines are currently licensed by the USDA. The modified live viruses (BCV, TGEV, IBV) and a Ts mutant (FIPV) are usually administered orally or intranasally to induce active mucosal immunity. Killed vaccines administered parenterally with adjuvant are available to induce active or passive immunity to BCV, TGEV, CCV and IBV. The success of these vaccines in the field may be compromised by a number of potential problems (Saif and Jackwood, 1990; Saif and Wes-

ley, 1992). For live virus vaccines to effectively induce active immunity they must replicate to an optimal degree at a site relevant for the induction of mucosal immunity. A factor which interferes with their efficient replication is the presence of maternal antibodies which may neutralize the vaccine virus. Secondly, attenuated strains may fail to replicate to the extent required to induce protective immunity at the site of infection of the virulent virus. A further possible complication with live coronavirus vaccines, especially when given to highly susceptible neonatal animals, includes their possible reversion to virulence or the presence of adventitious agents in the vaccine. Much experimental evidence exists to support the concept of a common mucosal immune system. Enteric infections induce immune responses which are protective at respiratory mucosal surfaces. However, the converse may not be true in the case of agents which cause localized respiratory infections. Thus PRCV strains which replicate almost exclusively in the respiratory tract fail to induce complete protection against TGEV challenge (Bernard et al., 1989; Van Nieustadt et al., 1989; Callebaut et al., 1990; Paton and Brown, 1990).

For killed vaccines, which are usually administered parenterally, problems are frequently encountered in inducing high titers of local SIgA antibodies, CMI responses are often poor and the duration of immunity may be short-lived. On the other hand, killed vaccines may be effective in boosting antibody titers in animals recovered from natural infection with coronaviruses (Saif and Jackwood, 1990; Saif and Wesley, 1992). For killed vaccines, the inactivating agent, viral dose and adjuvant are all important variables to evaluate.

At present, the potency of live coronavirus vaccines is assessed by in vitro titration of the viral infectious dose in cell culture. This titer should be correlated with the minimum viral titer required to induce protective immunity against experimental challenge and also natural challenge under field conditions.

The potency of killed vaccines is assessed by vaccination/challenge tests using different doses of the vaccine. Certain titers of neutralizing antibodies induced by inoculation of laboratory animals with the vaccine are accepted if proven to correlate with protective immunity. Other correlates to establish, include quantitation of particular viral antigens associated with the induction of neutralizing antibodies and protection against challenge. The amounts of the viral structural proteins present in a killed or subunit vaccine can be quantitated using specific mAbs in ELISA. However, it is important to establish that the mAbs react with relevant epitopes present on intact virus. Using a pool of mAbs to distinct viral epitopes on each protein may increase the assay sensitivity (Simkins et al., 1989). In both scenarios the assumption is made that the neutralizing antibodies in serum correlate with protection.

NEW STRATEGIES FOR CORONAVIRUS VACCINES

Application of recombinant (r) DNA techniques to coronaviruses has resulted in the cDNA cloning of several genes and has opened new avenues of vaccine biotechnology. Most studies have focused on the S glycoprotein because it induces neutralizing antibodies, although as reviewed, the other structural proteins may also play a role in immunity. A variety of expression systems have been developed for the production of coronavirus antigens. Because the coronavirus S protein is glycosylated, eucaryotic expression systems were used to obtain recombinant proteins. Recently the BCV HE protein (Parker et al., 1989) and BCV and TGEV S glycoproteins have been expressed in baculovirus recombinant expression systems (Parker et al., 1990; Godet et al., 1991). The expressed TGEV S glycoprotein induced neutralizing antibodies in rats, but no studies were done in swine. In general, parenteral or oral administration of soluble proteins induces poor mucosal immune responses or results in tolerance induction (Andre et al., 1973; Rubin et al., 1981). Therefore oral administration of subunit coronavirus vaccines will require new mucosal adjuvants and delivery systems to stimulate mucosal immunity.

A second strategy using rDNA technology involves the development of live vaccines. Deletion of certain non-essential portions of the viral genome (deletion mutagenesis) has been used to attenuate viral pathogens. However to accomplish this for RNA viruses, an infectious cDNA clone is required which is not yet available for coronaviruses. The subsequent insertion of new genetic material into the altered virus genome could provide a marker to identify this type of vaccine virus strain.

Another approach involves the use of live viruses and bacteria vectors to express viral genes coding for protective antigens. These vectors have one or more nonessential regions capable of accommodating the insertion of foreign genes. At present, live vectored vaccines for coronaviruses have been limited to recombinant vaccinia viruses. Chickens, mice and gnotobiotic pigs immunized with vaccinia virus recombinants expressing the S gene of IBV or TGEV, respectively developed neutralizing antibodies to the homologous virus (Cavenagh et al., 1984; Hu et al., 1985; Tomley et al., 1987; Pulford et al., 1990). However the immunized chickens were only partially protected against challenge (Spaan, 1990). Two potential vectors which may hold promise for development of live recombinant DNA vaccines for coronaviruses include adenoviruses and *Salmonella*. An attenuated adenovirus has been developed as an oral vaccine in humans (Chanock et al., 1988). Attenuated adenoviruses exist in other species which might also be developed for live rDNA vectors permitting expression of the coronavirus gene inserts in the intestine. Similarly, attenuated strains of *Salmonella* are attractive as potential live vectors for enteric viruses such as coronaviruses because they have the potential

to target the expressed proteins directly to lymphoid tissues of the gut (Clements et al., 1986; Curtiss and Kelly, 1987). Although only in the early stages of development, such live rDNA vectors may play a key role in the generation of future coronavirus vaccines.

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