EMBRYO VACCINATION WITH INFECTIOUS BRONCHITIS VIRUS

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ABSTRACT

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Embryos were vaccinated on the eighteenth day of embryonation (ED18) against avian infectious bronchitis virus (IBV). A commercial vaccine of Massachusetts 41 strain IBV (V-IBV) was found to be highly pathogenic for embryos necessitating passage in chick kidney (CK) tissue culture in order to reduce pathogenicity. The virus became apathogenic for embryos at the 40th tissue culture passage level (P_{40}-IBV). Maternal antibody-positive or -negative chicks hatching from eggs injected with P_{40}-IBV developed antibody against IBV as determined by a constant-virus diluting-serum plaque reduction assay. When challenged at 4 weeks of age with virulent IBV (C-IBV), these chicks were protected both from clinical symptoms of IBV infection and isolation of C-IBV from the trachea. While P_{40}-IBV protected chicks when administered on ED18, this virus did not protect well if given at hatch. Primary and anamnestic antibody responses, natural killer (NK) cell activity, mitogenic response to phytohemagglutinin (PHA) and cytotoxic reactivity against IBV-infected target cells did not differ between chicks embryo vaccinated (EV) with P_{40}-IBV or those

vaccinated at hatch with V-IBV. Light microscopic, transmission electron microscopic, and scanning electron microscopic examinations demonstrated mild to moderate, transitory, inflammatory lesions affecting both the lung and trachea in P_{40}-IBV EV chicks and the trachea in chicks vaccinated with V-IBV at hatch. Early (2-4 days post-inoculation [PI]) tracheal lesions consisted of deciliation, occasional foci of low cuboidal epithelium and rarely, a mild mixed inflammatory cell infiltration of the mucosa accompanied by an acellular exudate. At 17 days PI, hyperplasia of the epithelium, vacuolation of epithelial cells and regeneration of the cilia were observed. Lung lesions consisted of small foci of increased interstitial cellularity accompanied by occasional areas of necrosis, mixed inflammatory cell infiltration and exudation into the lumens of tertiary bronchioles, atria and air capillaries. At 17 days PI, these lesions were focal, chronic and less severe. When the P_{40}-IBV embryo vaccine was combined with turkey herpesvirus (HVT), there was no interference with the protection against challenge with virulent Marek's disease virus (MDV), nor did the presence of HVT interfere with the protection against C-IBV challenge afforded by P_{40}-IBV. Thus, under laboratory conditions, P_{40}-IBV was an effective embryo vaccine against IBV that could be combined with HVT as a bivalent vaccine.

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KEY TO ABBREVIATIONS

ED 18	Eighteenth day of embryonation
IBV	Infectious bronchitis virus
V-IBV	Commercial vaccine of Massachusetts 41 strain IBV
CK	Chick kidney
P_{40}-IBV	Fortieth tissue culture passage level of V-IBV
C-IBV	Virulent IBV used for challenge
NK	Natural killer
PHA	Phytohemagglutinin
EV	Embryo vaccinated
PI	Post inoculation
HVT	Turkey herpesvirus
MDV	Marek's disease virus
CMI	Cell-mediated immunity
P-IBV	Tissue culture passaged V-IBV
MD	Marek's disease
IB	Infectious bronchitis
TEM	Transmission electron microscopy
SEM	Scanning electron microscopy
SI	Stimulation indicies
HI	Hemagglutinin inhibition
IBDV	Infectious bursal disease virus
ELISA	Enzyme-linked immunosorbent assay
15 x 7	Line $15I_5$ x 7_1
EID_{50}	50% embryo infective dose
CPE	Cytopathic effects
PFU	Plaque forming units
P_{20}-IBV	Twentieth tissue culture passage level of V-IBV
P_{30}-IBV	Thirtieth tissue culture passage level of V-IBV
cpm	Count per minute
PC	Post challenge

INTRODUCTION

Recently embryo vaccination has been explored as a technique designed to induce earlier immunity against disease and to reduce the costs of vaccination. Studies conducted using the turkey herpesvirus (HVT) as an embryo vaccination against Marek's disease (MD) have been highly successful. Vaccination with HVT primarily stimulates a cell mediated immune response and results in a persistent viremia. Therefore, it was of interest to determine if the embryo vaccination technique could also be applied to vaccination against infectious bronchitis virus (IBV), which causes a transient viremia and stimulates a humoral immune response. The purpose of this research was to 1) develop an IBV embryo vaccine, 2) compare qualitatively and quantitatively cellular and humoral immune responses in chickens vaccinated with IBV as embryos with those vaccinated at hatch, 3) compare the pathogenesis of IBV in the chicken inoculated with IBV on the 18th day of embryonation with those inoculated at hatch and 4) evaluate IBV and HVT as a bivalent embryo vaccine.

CHAPTER 1

INFECTIOUS BRONCHITIS VIRUS:

A REVIEW OF THE LITERATURE

INFECTIOUS BRONCHITIS VIRUS: A REVIEW OF THE LITERATURE

Historical Perspective

Avian infectious bronchitis (IB) was recognized as a separate disease entity in 1931 after an outbreak of acute viral respiratory disease in North Dakota.[1] The distribution is now known to be worldwide. Although observed initially as a disease of young chicks, it was subsequently recognized in growing birds and laying flocks.[2,3] In addition to causing respiratory disease, IB virus (IBV) severely affected egg production. Marked losses were incurred from reduced egg production and poor egg quality.[3-5] Chickens that contracted IBV at 3 weeks of age or earlier, but subsequently recovered, often failed to reach a satisfactory rate of egg production.[6,7] The advent of vaccines in the 1950s,[8-9] however, substantially reduced losses due to IBV. Sporadic outbreaks still ocassionally occur, and it is probable that the large number of variant IBV serotypes are responsible.[10-16]

During 1962, IBV was identified as the causative agent of episodes of renal disease which resulted in severe economic losses in endemic areas.[17,18] Although IBV strains such as the Australian T strain[19] and the American Holte and

Gray strains[17] were identified as being primarily nephrotropic, even the more common Massachusetts type was capable of inducing kidney disease.[20,21] Vaccination against the IBV nephritis syndrome was complicated by both strain variations and the discovery that even some of the vaccine strains were capable of causing renal disease.[21,22]

Currently, efforts are underway to characterize the structural composition of IBV[23-33] that may facilitate the development of genetically-engineered vaccines.

Characteristics of IBV

Infectious bronchitis virus is an enveloped coronavirus[34] with a particle size ranging from 80-120 nm and a corona of club-like projections of up to 20 nm in length.[35,36] The genome consists of a continuous single strand of 8.5×10^6 molecular weight RNA.[25,37] There are three protein structural elements: surface projections, a nucleocapsid protein and a matrix/membrane protein.[29] The virus is generally spherical and the envelope contains an essential lipid.[38]

Reports differ on the sensitivities of IBV strains to chemical and physical treatments. Most strains are resistant to trypsin and a pH of 3.0.[39] Variations between strains are commonly seen in sensitivities to ether or heating at 45° C for 90 minutes. Most strains were inactivated in 15 minutes at 56° C.[39] Ultraviolet irradiation, chloroform and sodium deoxycholate also inactivated most strains.[39]

The buoyant density of various strains of IBV also differs. Ranges from 1.16 to 1.27 have been reported.[38,40-42] Virion shape variations, types of culture medium used or differences in surface projection size may be responsible for these differences.[38]

Beaudette and Hudson[43] reported the first successful propagation of IBV outside the natural host in the embryonated chicken egg. Since that time, IBV has been successfully adapted to a variety of other animal species and tissue culture preparations. The animal species include Cynomolgus monkeys,[44] cave bats,[45] quail,[46] magpies,[47] beetles,[48] and turkey embryos.[49] Cell cultures such as avian adult and embryonic tissues,[50-63] monkey kidney (VERO) cells,[64,65] fat head minnow cells,[66] baby hamster kidney cells (BHK-21),[67] and Chinese hamster lung cells (Don)[68] have been infected with IBV. In addition, immunofluorescent methods have detected IBV intracellular antigens unaccompanied by cytopathic effect in a variety of heterologous cell systems.[69]

After serial passages in chicken embryos, IBV is capable of producing cytopathic effect in chicken embryo and adult kidney cell cultures.[38,70] The lower the passage level in chick embryos, the more difficult it is to adapt IBV to cell culture.[63] Akers and Cunningham[71] described the cytopathic effect as syncytia which necrosed and subsequently detached from the culture plate with release of infective virus. Since observation of ciliostasis in

tracheal organ cultures does not require previous passage in chicken embryos, these cultures may be preferable for assaying IBV.[58,60]

Passage of IBV in chick embryos has also been found to decrease pathogenicity, antigenicity and immunogenicity for hatched chicks while increasing the lethality for embryos.[38] These modifications are probably due to either mutation of the virus or selection of variant clones. The "adapted" viruses do not revert to their original virulence.[72]

Growth kinetics of IBV vary with passage history and strain differences.[73] Typical single cycle growth curves, however, show a 2 to 4 hour eclipse phase with a maximum yield in 16-24 hours.[52,71,74,75] Culture pH will also influence virus release and subsequent reduction in titer.[76] Alkaline pH values cause faster release and a quicker reduction in titer than acid pH values.

Electron microscopic studies have demonstrated that IBV particles enter cells by viropexis rather than by fusion of viral and cell membranes.[77,78] Two different modes of viropexis were observed: 1) engulfment of the virus and formation of phagocytic vacuoles, or 2) uptake of individual particles by micropinocytosis.[77] Previously, lysosomal enzymes were not observed to be involved in either procedure.[78] A recent report, however, stated that the virus containing electron dense particles found in cells infected with IBV are actually virus-packed secondary lysosomes.[79]

Attachment of the virus to the cell membrane occurred independently of temperature variation although viropexis usually did not occur unless preparations were warmed to 37° C.[78] Attachment sites contained at least two active chemical groups: 1) the neuraminidase sensitive group, and 2) the sulfhydryl-containing group.[80] Heat inactivation of IBV prevented attachment.[78]

Viruses replicated in the cytoplasm by budding into the cisternae of endoplasmic reticulum or into cytoplasmic vacuoles.[77] Surface projections appeared to be attached during the budding processes. Although some virus particles were released by reverse micropinocytosis after transportation to the cell surface within coated vesicles, most particles were discharged after cell lysis.

Pathogenesis

Infectious bronchitis virus is responsible for three major clinical syndromes: 1) respiratory disease, 2) oviduct lesions, and 3) renal disease. The virus is highly contagious and is rapidly transmitted. The disease has an incubation period of 18 to 36 hours.[2,5] Early experiments show a wide variation in the duration of infection of chickens with IBV.[81-87] It is possible that some of the discrepancies are due to recovery of virus from secondary infection. In experiments conducted under isolation conditions, virus could not be recovered from the trachea,

liver, spleen, bursa or kidneys longer than 29 days post-infection but was recovered from the feces of chickens for up to 20 weeks post-infection.[88]

Clinical symptoms of IBV initially begin with rales, conjunctivitis, wheezing, gasping and nasal discharge. These generally peak between the second and fourth day and resolve sometime between the seventh and fourteenth day post-infection.[2,5] Histopathologically, the respiratory lesions depend on the route of inoculation of the virus. Hofstad[89] found that the lesions were confined to the trachea in intratracheally-infected experimental birds but air sac lesions were seen in field cases. Sole infection of the trachea after intratracheal inoculation has been confirmed by other studies,[90] whereas aerosal exposure can also involve the air sacs and lungs.[91] Initially, IBV causes extensive deciliation and destruction of the epithelial lining of the trachea followed by proliferation of the remaining basal cells.[91] Infiltration of the mucosa with lymphocytes, plasma cells and histiocytes often occurs giving the mucosa a grossly thickened appearance.[92] Although Garside[92] stated that epithelial hyperplasia was not seen, other investigators have found this to be a common occurrence.[91,93] Pulmonary lesions in the primary and secondary bronchi are similar to those found in the trachea, although the lymphoid infiltration is more prolonged.[91] Air sac lesions initially begin with edema and mild inflammatory cell infiltration and progress to epithelial desquamation,

fibrinous exudation and occasionally a pseudomembrane formation.[91] Lesions in the trachea have been examined by both transmission electron microscopy (TEM)[94] and scanning electron microscopy (SEM).[95] Both studies demonstrated an extensive loss of cilia with exposure of the underlying epithelial cells. Swollen microvilli and mitochondria, enlargement of endoplasmic reticulum and Golgi apparatus, and appearance in the cytoplasm of electron-dense areas were observed with TEM. Virus particles were located both in the electron-dense areas and in the cisternae and vesicles formed from the membranes of the endoplasmic reticulum. Budding was not observed.

Although mortality from IBV respiratory disease can be high in young chicks,[4] death losses are relatively uncommon in mature birds. In these flocks the most serious economic losses are due to decreased egg production.[2,5] IBV can affect production through both early exposure of baby chicks,[4,6,7,96-99] and infection of hens during production.[3,5]

Young chicks that are exposed to IBV and recover from the clinical respiratory disease frequently become either nonlayers or poor layers with a high percentage of deformed eggs.[4,6,7,96-99] Production rarely exceeds 50% of that of uninfected flocks. The birds generally have the appearance of good layers, but some nonlaying birds may have pendulous abdomens. Microscopically, the initial changes include a lymphoid infiltration of the oviduct wall, submucosal edema

and development of lymphoid nodules.[96,98,99] These lesions progress to localized hypoplasia of the oviduct with loss of epithelial cells and eventually a complete obliteration of the oviduct lumen. Macroscopically, cysts were often found caudal to the hypoplastic areas.[4,6,96] Oviducts from chickens that became poor layers had focal areas of glandular hypoplasia within patent oviducts and a loss of demarcation between the magnum and isthmus. Reproductive tract lesions were generally more severe in young chicks than those found in birds infected during production.[97] Ultrastructural changes found in the epithelial cells of the oviduct included moderate dilations of rough endoplasmic reticulum and distended perinuclear cisterna.[97]

When laying hens are infected with IBV, they may remain non productive for an average of 35 days.[5] Lesions of the reproductive tract consist of deciliation and decreased height of the epithelial cells lining the oviduct and lymphocytic infiltration of the oviduct lamina propria and intertubular stroma. Fibroplasia and edema in these areas were also observed.

Chickens that develop IBV-induced nephritis may or may not also suffer from respiratory disease. Renal disease can occur in any age group although there is some evidence that the susceptibility may differ with age.[100] Certain strains of IBV may be more nephrotropic than others,[17,19] and the ensuing renal disease can be either acute[101-103] or chronic[22,104] in duration. Mortality of greater than 50% is

often seen with renal disease and is usually higher than the mortality observed in those birds affected with respiratory symptoms alone.[22,104] Replication and cytolytic action of the virus in the tubules is the direct cause of the kidney disease.[104] Acute renal disease is characterized by microscopic lesions consisting of necrosis of tubular epithelium, urate deposition and interstitial infiltration with lymphocytes and heterophils. Siller and Cumming[101] noted PAS-positive material both intracellular and extracellular in the tubular epithelium. Basophilic cytoplasmic inclusion bodies were also observed in tubular epithelial cells.[104] Ultrastructural examination revealed cytoplasmic vesicles containing virions were found in attached and desquamated cells.[104] Free virions were also seen lining the microvilli of proximal tubules.

Chronic nephritis has been experimentally induced only in Rhode Island Red chickens.[22,104] The lesions are similar to those seen in acute nephritis cases with a primarily lymphoid infiltration and tubular dilation. Neither acute nor chronic IBV renal disease affected the glomeruli.

Cold stress is often a factor in determining the severity of IBV infection. Low temperatures greatly increased mortality from both nephritis and respiratory disease.[105] This effect appears to be independent of either strain variation or route of inoculation.

Immune Responses to IBV Infection

Humoral immunity. Chickens infected with IBV develop antibodies to the virus and are resistant to reinfection with the homologous strain of virus.[106] The degree of resistance to challenge with heterologous strains vary according to the type of virus that caused the initial infection.[14]

Serum neutralizing antibodies are first detectable 2-3 weeks post inoculation (PI) and have been demonstrated in the serum for up to 1 year PI.[107] Hemagglutinating antibodies can be detected as early as 1 week PI, however.[108] Gillette[109] found that IgM antibodies attain maximum levels between 1 and 3 weeks PI and decline thereafter. IgG antibodies reach maximum levels at 3 weeks and decline after 5 weeks PI.

Passive antibodies decrease steadily from the time of hatching and disappear completely around 4 weeks of age.[110] These antibodies do not protect the chicks from either IBV-induced respiratory or kidney disease.

Many researchers have noted inconsistencies between serological response and immunity to challenge with IBV.[12,22,106,111-121] Neutralizing antibody in the serum may not prevent either respiratory disease or nephritis caused by IBV. Conversely, those birds lacking anti-IBV neutralizing antibody may be adequately protected from challenge with virulent IBV. Some authors have suggested that either local immunity[116,118,121] or cell-mediated

immunity (CMI)[122,123] may be playing an important role in resistance and current research efforts have been directed to these areas.

Local immunity. Initial experiments with tracheal explants from immune and susceptible chickens revealed no difference in susceptibility to in vitro IBV infection.[124] Subsequent investigations, however, demonstrated good correlation between ciliary activity and anti-IBV immunity in tracheal explants.[125-130] Since the IBV-immune explants remained susceptible to laryngotracheitis virus, secretion of nonspecific antiviral substances was discounted.[125] Interferon was not found in the supernatant fluid from IBV-inoculated immune or susceptible tracheal cultures.[125] Due to the specificity of the local resistance to IBV and the demonstration of IgA and IgG in tracheal fluids,[131,132] these antibodies were considered to be responsible for the local immune response. Residual serum antibodies were probably not involved because explants from passively immunized chicks with high serum antibody concentrations did not demonstrate resistance to in vitro IBV infection.[126]

Peak levels of virus neutralizing antibody in tracheobronchial secretions were reached 10-14 days after the initial IBV vaccination delivered by a combination of intraocular and intratracheal routes.[132] IgA was the predominant immunoglobulin in the secretions. Revaccination did not produce an anamnestic local antibody response. Challenge exposure resulted in predominantly IgG local

antibody levels of undetermined origin. Intranasal IBV vaccination resulted in low levels of virus neutralizing antibody in nasal secretions for up to 4-5 weeks.[133] These antibodies were either locally produced or transuded serum antibodies.

Previous investigations showed that the application of non-infectious agents to the eyeball without systemic injection stimulated the formation of antibodies in the tears.[134] The Harderian gland and the lacrimal glands were considered responsible for the local immune response. Conjunctival and intranasal infection with IBV caused a progressive infiltration of plasma cells into the Harderian gland with formation of lymphoid follicles.[135] These changes were observed despite high levels of circulating maternal antibodies. Removal of the Harderian gland in 1-day-old chicks resulted in decreased protection 3 weeks post-vaccination when challenged with virulent IBV.[136] This decrease occurred despite high serum levels of neutralizing antibody. Anti-IBV IgA antibodies were at a higher concentration in the lacrimal fluid than in the serum at 2 weeks of age after chicks were vaccinated at hatch.[137] Higher concentrations of anti-IBV IgG antibodies in the tears than in the serum were not reached until 5 weeks post-vaccination in similarly vaccinated birds. After challenge, anti-IBV IgA decreased in vaccinated chicks and increased in unvaccinated chicks indicating that the increase in IgA levels was a primary response.

Cell-mediated immunity. The role of CMI in the immunological response of chickens to IBV infection has only recently been explored.[122,123,138] A specific cell-mediated response to IBV antigen has been assayed by the lymphocyte transformation test. The nonspecific effects of IBV on mitogen responsiveness has been evaluated by the phytohemagglutinin (PHA) assay. Reactivity of cytotoxic T-cells against specific targets[139-152] and investigations into the effects of viral diseases on natural killer cell activity[153-155] have been explored with other avian diseases but have not been researched with IBV.

Blastogenic transformation of lymphocytes accompanied by an increase in DNA synthesis has been employed as an assay of CMI in a variety of avian diseases.[156-161] The transformation test detects the specific in vitro response of lymphocytes to an antigen which the cells have been exposed to in vivo. Lymphocytes from birds inoculated with the Massachusetts strain of IBV were stimulated to blastogenesis when exposed to IBV antigen in vitro.[122] The same antigen did not stimulate lymphocytes from uninoculated birds indicating that IBV did not act as a nonspecific mitogen. Positive stimulation indices (SI) were observed beginning 5 days post-vaccination, peaked at 12 days, and were virtually gone 40 days after inoculation. There was no evidence of direct correlation between SI and hemagglutination inhibition (HI) titers, but a negative correlation was observed between SI and clinical symptoms of

IBV. Revaccination and challenge produced weak secondary CMI responses.[123,138]

Phytohemagglutinin has been shown to be a good mitogen for nonspecifically stimulating avian T lymphocytes.[162-170] Lymphocytes from birds vaccinated or naturally infected with viruses have demonstrated both enhanced stimulation[157,161,171] or depressed responses[171-176] to PHA. Vaccination with either live or inactivated IBV did not have any significant effect on PHA mitogenic response,[123,138] although the ability to respond appeared to decline with increasing age.[138]

Mammalian and avian T lymphocytes may be cytotoxic for a variety of different allogeneic target cells including virus infected[152,177-181] and tumor derived cells.[139-147,149,150] Macrophages may provide assistance to cytotoxic T cells[148] and avian cells[152] appear to share the major histocompatibility complex restriction of cytotoxicity seen with mammalian cells.[179,182] Cytotoxic T cells are adherent to *V. villosa* lectin[151] but are not adherent to nylon wool.[148] The specific effects of these cytotoxic cells can be evaluated *in vitro* by a ^{51}Cr-release assay using spleen-derived effector cells directed against ^{51}Cr-labelled target cells. To date, cytotoxic cells have not been evaluated in IBV infected birds.

Natural killer (NK) cells are large granular lymphoid-type cells[183] which react to antigens regardless of previous antigenic exposure.[184] They are found in most normal

individuals and have the ability to spontaneously lyse targeted cells. Although NK cells are non-phagocytic, nonadherent and lack the typical surface characteristics of T or B lymphocytes or macrophages,[185] recent evidence supports a T-cell lineage.[186-188]

The role of NK cells appears to be multifunctional and includes the following: 1) maintenance of the initial line of defense against primary tumors, 2) participation in immune surveillance, 3) participation in defense against selected microbial agents, 4) augmentation of resistance to bone marrow transplants and participation in graft versus host disease, and 5) functions as secretory and regulatory cells.[184,189] The NK cells are often considered the immune system's first line of defense against disease.

Recently, the counterpart of the NK cell system in mammals has been discovered in birds.[190-196] Chicken NK cells are also of undetermined lineage and are thermolabile, non-phagocytic, nonadherent, radioresistant and bear receptors to the Fc portion of immunoglobulins.[190,191,194,196] Expression of NK activity is genetically controlled and levels increase with age.[190] Antigens present on tumor cells but absent on normal cells were the targets of NK activity[190] and cells from retrovirus tumor line LSCC-RP9 were the most susceptible to lysis.[196] Transfer of spleen cells from normal chickens to susceptible chickens protected the recipients from acquisition of transplantable tumors.[191,194] This resistance was not

affected by treatment with either anti-T- or anti-B-cell serum, thus indicating that NK cells were probably responsible for the resistance.

Much of the current knowledge acquired concerning the role of NK cells in avian diseases has been obtained with Marek's disease virus (MDV).[153,155,191,194] The assay system used for evaluating NK activity was a 4-hour ^{51}Cr-release assay similar to that described for cytotoxic T-cells. Infection with MDV was found to significantly reduce levels of NK activity.[153] This effect could be reversed by vaccination prior to challenge with virulent MDV. Similar studies with infectious bursal disease virus (IBDV) showed no consistent effect on NK activity.[154] Natural killer cell activity has not been evaluated in IBV infected chickens at this time.

Diagnosis of IBV

Clinical disease. Diagnosis of IBV based on clinical symptoms is not definitive due to the resemblance of IBV infection to other respiratory diseases such as Newcastle disease, infectious laryngotracheitis and infectious coryza. Involvement of the nervous system with Newcastle disease, histopathologic demonstration of intranuclear inclusion bodies with infectious laryngotracheitis and the facial swelling observed with infectious coryza may be helpful in distinguishing between these diseases. Infection with IBV may be confirmed by isolating the virus from infected

chickens or by detecting anti-viral immune responses in chickens that may no longer be viremic.

Virus isolation. Isolation of virus is considered the most stringent method of diagnosing IBV infection or evaluating vaccinal protection post-challenge. Tracheal swabs 3-5 days post-infection are the preferred source of the virus,[106,197] although kidney tissue is suitable for nephrotropic strains.[85] Broth suspensions prepared from these swabs are standardly injected into 10- to 12-day-old embryonating eggs and the embryos are observed for death, stunting and urate deposition in the mesonephrons.[198] Although kidney cell cultures have not been used for isolating field viruses because of the necessity of adapting the virus to the cell cultures, isolation of IBV in tracheal organ cultures appears to be a more rapid and reproducible method of isolation than using embryonating eggs.[58,60,199-201] Identification of IBV in infected tracheal smears by agar gel precipitin tests[202] and fluorescent antibody tests[203-205] have also been used, although these methods have not always been as reliable as isolation from tracheal swabs.[106,197] Recently, a ^{32}P-radiolabelled cloned cDNA probe complementary to IBV genomic RNA has been developed to identify virus isolates through the hybridization test.[206] This test has proven to be highly specific in identifying variant strains of IBV.

Detection of antibody. Many different types of serological tests have been applied to evaluating the

humoral immune system response to IBV. These include serum neutralization tests,[53,207-218] HI tests,[108,209-227] enzyme-linked immunosorbent assays (ELISA),[131,228-234] precipitin tests,[235-239] indirect and direct complement fixation tests,[240-243] fluorescent antibody assays,[216] and immune electron microscopy.[244]

Current techniques for detecting anti-IBV serum neutralizing antibodies are plaque reduction assays, virus neutralization in embryonating eggs and microneutralization tests. Although the former two procedures have been used extensively and are more sensitive than microneutralization,[216] they are both cumbersome and expensive. For plaque reduction tests, large volumes of tissue culture are needed for processing multiple serum samples at one time. Serum neutralization in embryonating eggs is time consuming and often requires the use of pooled samples.[208] Microneutralization techniques have eliminated most of these disadvantages. The results obtained with microneutralization tests correlate well with neutralization in embryonating eggs, plaque reduction assays and ELISAs.[215,216,218,245]

Recently, certain strains of IBV were found to hemagglutinate chicken erythrocytes after sucrose gradient purification of the virus and incubation with phospholipase C.[246] The HI test was then developed as a rapid and inexpensive method for field diagnosis of IBV.[219,220] Hemagglutination-inhibition titers are roughly parallel to

serum neutralization titers, although hemagglutinating antibodies generally first become detectable 1 week PI, whereas neutralizing antibodies cannot be detected until 2-3 weeks PI.[107,218] Because there are more cross reactions between heterologous and homologous strains with the HI test than with plaque reduction neutralization tests, selection of an appropriate antigen for the HI test is important.[219,221-223,226] Antibodies from the egg yolk also can be used in the HI test.[248]

The ELISA conditions for IBV were established relatively recently.[228] In comparison to the HI and neutralization tests, the ELISA was the most sensitive of the three assays and detected antibody earlier and at higher titers.[230] At the present time, IBV serotyping results with the ELISA test have not been satisfactory due to ready detection of serotype common antigens.[229] Although the use of ELISA for IBV testing under field conditions has not been established yet, the economy, speed, reliability and the small amounts of serum required make the assay system appealing for diagnostic use. The ELISA can also be automated for screening of large numbers of samples,[249] and can be adapted to detect different classes of antibodies.[233,350] In addition to serum, whole blood dried on filter paper and egg yolk can be employed in the ELISA.[234,248]

Precipitin tests for IBV have provided a simple method of serological diagnosis for many years. With the advent of

other serologic techniques such as microneutralization, HI and ELISA, the less sensitive agar gel precipitin tests have lost their popularity.[107,245]

Complement fixation, fluorescent antibody and immune electron microscopy assays have been used infrequently for diagnosis of IBV and currently have not been applied to field situations.

Assays of local immune response. Most assays of local immune response have concentrated on tracheal resistance to IBV infection. Currently popular assays involve removal of tracheal explants from vaccinated and unvaccinated chicks, either post-IBV-challenge or post-IBV-vaccination, and examination of these explants for ciliostasis.[124-130] Ciliary activity correlated well with virus isolation and assays of humoral immunity.[129,130] In addition, cross protection studies involving heterologous and homologous IBV challenge were faster and more reliable using tracheal organ explants as the assay system.[127] Local antibody response has also been assayed using tracheobronchial secretions[131,132] and nasal secretions.[133]

Assays of CMI. Currently only the lymphocyte transformation test has been used to evaluate the CMI response to IBV infection.[122,123,138] Specific CMI responses were associated with either recovery from or resistance to IBV and the blastogenic assay was considered appropriate as an additional method of evaluating the immune response to IBV.

Prevention and Control

Vaccination against IBV is the only acceptable method of preventing the spread of IBV. Currently, two categories of vaccines are available: the modified live vaccine and the inactivated vaccine.[106] Both types are available commercially in a number of different strains depending on the area of use. The IBV vaccines are frequently combined with Newcastle disease vaccines despite evidence of interference between the two viruses.[251-253]

Oil emulsion inactivated vaccines were developed primarily as long lasting vaccines for laying hens.[254] Despite the good protection and induction of antibody observed under laboratory conditions,[254-259] results of field trials have been disappointing.[260] Currently, inactivated vaccines have limited commercial application.

Live virus vaccines are modified by serial embryo passage of 25 or more times, although many still retain pathogenicity under certain circumstances.[8,9,12,21,119,261,262] The Massachusetts type virus vaccines have been most widely used due to their ability to generate the best immune response to challenge with heterologous IBV strains.[12,112,263-266] Combinations of vaccine strains often incite a greater and more prolonged vaccine reaction than when the same strains are used singly.[267,268]

The severity of vaccine reactions depends to a great degree on the method of vaccine administration.[106,116,264,269-274] Aerosol exposure to IBV vaccines usually incites a greater respiratory reaction than administration in the drinking water and often is reserved for revaccination rather than for primary vaccination.[106,116,264,270] Drinking water application of the vaccine, however, is subject to more environmental variables.[275]

Chicks can be vaccinated successfully at 4-5 days of age despite the presence of maternal antibodies, although the virus neutralizing responses may be reduced.[106,115,269,272,276-278] Revaccination of broilers is conducted at 4 weeks of age and layers are often vaccinated again at 2-4 months.[279]

Embryo vaccination is a new technique recently developed using the turkey herpesvirus (HVT) vaccine against MDV.[280] Chicks vaccinated as embryos with HVT successfully resisted early challenge with MDV without adverse effects on either hatchability or survival. The embryo vaccinated (EV) chicks acquired the virus via the respiratory tract and had higher virus recovery from infected tissues than that obtained with posthatch vaccinated chicks.[281] Embryo vaccination did not cause progressive lesions or deleteriously affect the immune status of chicks after hatch.[281] In addition, MDV serotypes 1 and 2 were also successfully administered as embryo vaccines.[282] Although

the presence of anti-MDV maternal antibodies did reduce the efficacy of embryo vaccination, protection against challenge was still better than that acquired by posthatch vaccinated chicks.[283]

Recently, the embryo vaccination technique has been applied to vaccination with IBDV.[284] Chicks vaccinated as embryos against IBDV were successfully protected against challenge with virulent IBDV at 3 weeks of age. Embryo vaccination did not affect hatchability or survival or induce progressive histologic lesions in the bursa of Fabricius. Maternal antibodies interfered with protection in embryos vaccinated with certain vaccine strains. Bivalent HVT-IBDV vaccination of embryos resulted in protection against challenge with both MDV and IBDV. At this time, the embryo vaccination technique has not been applied to vaccination against IBV.

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CHAPTER 2

EMBRYO VACCINATION OF CHICKENS WITH INFECTIOUS BRONCHITIS VIRUS

EMBRYO VACCINATION OF CHICKENS WITH INFECTIOUS BRONCHITIS VIRUS

Summary

A commercial infectious bronchitis virus vaccine (V-IBV) of the Massachusetts 41 strain was injected in embryonated chicken eggs on embryonation day (ED) 18. The V-IBV was pathogenic for embryos and was therefore passaged in chick kidney (CK) tissue culture in order to reduce the pathogenicity. At the 40th tissue culture passage (P_{40}-IBV), the virus became apathogenic for the embryos. Maternal antibody-positive or -negative chicks hatching from eggs injected with P_{40}-IBV developed antibody to IBV and were protected against challenge at 4 weeks of age with virulent Massachusetts 41 IBV (C-IBV). While P_{40}-IBV protected chicks when administered on ED18, this virus did not protect well if given at hatch. When combined with the turkey herpesvirus (HVT), P_{40}-IBV given on ED18 did not interfere with the protection against challenge with virulent Marek's disease virus (MDV), nor did the presence of HVT interfere with protection by P_{40}-IBV. Thus, under laboratory conditions, IBV vaccine could be combined with HVT as a bivalent embryo vaccine.

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Introduction

Recent studies with Marek's disease (MD) have shown that inoculating MD vaccine into embryonated chicken eggs on embryonation day 18 (ED18) resulted in resistance of hatched chicks to challenge with virulent MD virus (MDV).[1] When used in chickens as a vaccine against MD, the turkey herpesvirus (HVT) causes a persistent viremia[2-5] and provides protection primarily through cell-mediated immunity.[6-9] Efficacy of embryo vaccination with other avian viruses is currently being tested.[10]

Infectious bronchitis virus (IBV) causes a highly contagious respiratory disease and occasionally a nephrosis/nephritis syndrome in chickens.[11-14] Economic losses can be incurred both from mortality and weight loss in young chicks and from decreased production and poor egg quality in laying flocks.[15-17] The virus is highly transmissible, with vaccination being the only effective means of prevention.[18,19] Some vaccines that are commercially available are capable of producing clinically significant disease and thus may be undesirable for use in young chicks or high producing laying flocks.[20,21]

The objective of the present study was to examine the possibility of immunizing chickens against infectious bronchitis (IB) by embryo vaccination. Infectious bronchitis virus was chosen for two reasons: 1) It causes a transient viremia and primarily elicits a humoral immune response.[22-24] Therefore, it was of interest to determine

if a protective response could be induced by injecting IBV vaccine in the embryos as is possible with HVT, which principally protects through the cell-mediated immune system.[6-9] 2) Infectious bronchitis is a disease of practical importance that must be protected by vaccination.[25-28] In commercial flocks, vaccination against IB is usually done after hatch either by administering the vaccine in the drinking water or by spray.[29,30] Successful embryo vaccination with IBV could induce early protection and reduce the costs involved in posthatch vaccination. The feasibility of combining IBV with HVT as an efficacious bivalent embryo vaccine was also explored.

Materials and Methods

Chickens and embryonated eggs. Line P and line $15I_5$ x 7_1 (15 x 7) White Leghorn chickens and embryonated eggs were obtained from a flock maintained at this laboratory. This flock was free of exposure to IBV, MD, HVT, avian leukosis viruses, reticuloendotheliosis virus and to other common bacterial and viral poultry pathogens. Embryonated eggs bearing maternal antibodies to IBV were obtained from a commercial broiler flock vaccinated at hatch against IBV, MD and Newcastle disease. The titers of anti-IBV neutralizing antibody of five progeny chicks examined at hatch ranged from 80-320, as determined by the constant-virus diluting-serum plaque reduction assay. Maternal antibody titers against other pathogens were not determined. The chickens were hatched and raised in positive pressure plastic canopy

isolators supplied with biologically filtered air. After virus challenge, the birds were maintained for 5 days in open pens in a clean environment.

Viruses. A commercial IBV vaccine (V-IBV) of Holland strain, Massachusetts type, with a titer of 10^6 50% embryo infective dose (EID_{50})/ml was used. This vaccine was used either without treatment or after serial passages in chicken kidney (CK) cell cultures,[31] as previously described.[32] Serial passages were conducted every 24-32 hours. The cytopathic effects (CPE) of the virus, first detected at the third serial passage, were usually quite extensive within 24 hours of inoculation. Various cell culture passage levels of IBV (P-IBV) were used for vaccination. Passage levels were designated as follows: passage 20 - P_{20}; passage 30 - P_{30}; and passage 40 - P_{40}. The IBV challenge virus (C-IBV) was a virulent Massachusetts 41 strain, with a titer of $10^{7.5}$ EID_{50}/ml.[a] This virus was used at a 1:100 dilution in sterile tryptose phosphate broth. Titration of V-IBV was conducted in 9- to 12-day-old chicken embryos and the titer was expressed as EID_{50}, calculated by the method of Reed and Muench.[33] After cell culture passage, the virus was titrated in CK and the titer was expressed as plaque-forming units (PFU). For assaying P-IBV, CK monolayers were inoculated with serial dilutions of the virus and overlayed

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[a]Dr. Wenger, National Veterinary Services Laboratories, Ames, IA.

with agar medium, as previously described.[34] Virus plaques were counted 36-48 hours postinoculation (PI). The FC126 strain of HVT, propagated in chicken embryo fibroblasts, was used as a cell-associated vaccine against MD.[35] Each embryo was vaccinated with 1,000 PFU of HVT. A cloned preparation of the JM strain of MDV was propagated in duck embryo fibroblasts and used as the MD challenge virus.[36]

Embryo and posthatch vaccination. Embryo vaccination with IBV was done as described previously for HVT.[1] Briefly, eggs on ED18 were inoculated with a 1 1/4-inch-long 22 gauge needle. The large end of the egg was cleaned with 80% ethyl alcohol, a small hole was punched in the egg shell and the entire length of the needle was inserted through the hole in the egg. The inoculum consisted of 0.1 ml of the desired virus dilution. Control embryos were inoculated with 0.1 ml of the diluent. Vaccinated and control chicks were hatched in separate isolators.

Posthatch vaccination with IBV was conducted at one day of age. One drop of inoculum containing the appropriate virus concentration was deposited intraocularly. The birds were then carefully observed for swallowing and subsequent absorption of the vaccine droplet before they were released.

Infectious bronchitis virus challenge. Chickens were removed from the isolators at 4 or 6 weeks of age, bled for antibody and inoculated intraocularly with 0.03 ml of C-IBV. After inoculation, the chickens were moved to an open room and individually observed daily for clinical respiratory

symptoms. On the 5th day, the chickens were killed and an approximately 1/2-inch length of the lower half of the trachea from each bird was aseptically removed, suspended in 2 ml of tryptose phosphate broth and examined for virus isolation in 9- to 12-day-old chicken eggs.[37] Briefly, the tracheal tissue was crushed with a Ten Broeck grinder, centrifuged at 250 x g for 5 minutes, and 0.1 ml of the supernatant was injected into each embryonated egg. Embryos were examined for positive evidence of virus, as previously described.[37] If the test was not clearly positive or negative, the allantoic fluid of 1 or more eggs injected with the test inoculum was inoculated into 5 additional 9- to 12-day-old eggs. The percent protection against IBV challenge for both clinical symptoms and virus isolation was calculated as the number of birds protected against IBV challenge divided by the number of birds tested and multiplied by 100.

Mareks disease virus challenge. At 11 days of age, each chicken was injected intraabdominally with 1,000 PFU of MDV and observed for 8 weeks. Chickens that died and those surviving at the end of 8 weeks were necropsied and inspected for gross lesions of MDV.[38] The percent protection against MDV challenge was calculated as described for IBV challenge.

Antibody analysis. Serum samples were assayed for antibodies to IBV by a plaque reduction assay in CK cultures.[39,40] The indicator virus was P_{20}-IBV or P_{30}-IBV.

Serum samples were screened at a dilution of 1:20. An 80% or greater reduction in PFU was considered positive for the presence of IBV antibody.

Statistical analysis. Data were compared with the use of a two-tailed Chi square test, using Yates correction factor.[41]

Experimental Design

Serial passage of V-IBV in CK culture and the response of maternal antibody-negative embryos to the passaged vaccine (Experiments 1-3). In experiment 1, unpassaged V-IBV was inoculated into line P eggs on ED18 and the embryos were observed for hatchability. In experiment 2, P_{20}-IBV and P_{40}-IBV were inoculated into line P eggs on ED18 and the chicks were hatched and raised in separate isolators. At 4 weeks of age, the chickens were bled and sera were analyzed for anti-IBV antibody. In experiment 3, P_{20}-IBV was injected into eggs of lines P and 15 x 7 on ED18 and the response of the two lines was compared.

Protection against C-IBV following embryo vaccination with P_{20}-IBV, P_{30}-IBV or P_{40}-IBV (Experiments 4-8). Experiments 4 and 5 were similar in design. Line P chickens lacking maternal antibodies to IBV were vaccinated with P_{40}-IBV or V-IBV either as embryos or at hatch. Vaccinated and unvaccinated control chickens were raised separately under isolation. At 4 weeks of age, the chickens were bled and then challenged with C-IBV. Chickens were observed daily for 5 days for clinical symptoms of respiratory disease. At

the end of the observation period, all surviving chicks were examined for virus isolation from the trachea. Because in experiments 4 and 5 posthatch vaccination with 100 PFU of P_{40}-IBV protected chicks poorly, the dosage was increased in experiments 6 and 7. In experiment 7, seven maternal antibody-negative line P chicks (five from the group post-hatch vaccinated with 10,000 PFU P_{40}-IBV and two from the 100 PFU P_{40}-IBV embryo vaccinated [EV] group) were removed from the isolators 5 days PI for virus isolation studies. The remaining chicks were subsequently challenged at 4 weeks of age, as above. Protective ability of increasing doses of P_{40}-IBV were compared. Chickens and embryonated eggs used in experiment 8 were derived from a commercial breeding flock that had been immunized against IBV. P_{20}-IBV, P_{30}-IBV and P_{40}-IBV were injected on ED18 and V-IBV was injected at hatch. Chickens of each vaccinated group and of an unvaccinated control group were examined for resistance to IB by challenge with C-IBV at 4 or 6 weeks of age.

Protection against C-IBV and MDV following embryo vaccination with a bivalent vaccine containing HVT and P_{40}-IBV (Experiment 9). Groups of commercial chickens bearing maternal antibodies to IBV, HVT and MDV were vaccinated on ED18 as follows: group 1 received P_{40}-IBV, group 2 received HVT and a third group received a bivalent vaccine containing IBV and HVT. An additional group of chickens was left unvaccinated and served as controls. One half of the chickens in each group were challenged at 11 days of age

with MDV and observed for 8 weeks. These birds were then terminated and examined for gross lesions of MDV. The remaining birds in each group were challenged at 4 weeks of age with C-IBV and were observed for 5 days at which time they were terminated and tracheal sections were removed for virus isolation.

Results

Effect of serial tissue culture passages of V-IBV on pathogenicity for embryos. In experiment 1, V-IBV was found to be highly pathogenic for embryos as measured by both hatchability and survival (Table 2-1). Only 17% of the embryos hatched and none of the chicks survived when given the standard chick dose of V-IBV (10^5 EID_{50}). Chicks that hatched succumbed to severe IBV respiratory disease or were euthanized when they became moribund. Dilution of V-IBV from 10^5 EID_{50} to 10^2 EID_{50} did not significantly ($p<0.05$) improve either hatchability (48%) or survival (0%). In experiment 2, inoculation of various cell culture passages of IBV on ED18 indicated that as the tissue culture passage level increased, the pathogenicity of the virus for embryos decreased (Table 2-1). Hatchability improved to 25/40, or 63%, by passage level 9 (data not shown) and became comparable with that of unvaccinated control chicks by passage level 20 (75-90%). Survival of chickens following embryo vaccination also improved with increasing passage levels, although not as rapidly as hatchability. Survival

Table 2-1. Effect of V-IBV and P-IBV on hatchability, survival and antibody production in line P chickens inoculated on ED18 (Experiments 1 and 2).

Expt. #	IBV Type	IBV Dose per embryo	Hatchability: No. eggs hatch/ No. inoculated	Hatchability: % hatched	Survival of hatched chicks*: No. survived/ No. raised	Survival of hatched chicks*: % survival	No. chicks with anti-IBV antibody†/ No. tested
1	V-IBV	10^5 EID_{50}	1/6[a]	17	0/1[a]	0	NT
		10^4 EID_{50}	3/15[a]	20	0/3[a]	0	NT
		10^2 EID_{50}	10/21[a]	48	0/10[a]	0	NT
2	P_{20}-IBV	100 PFU	15/20[b]	75	5/15[a]	33	NT
		10 PFU	36/40[b]	90	12/30[a]	40	12/12[b]
	P_{40}-IBV	100 PFU	31/40[b]	78	25/31[b]	81	24/24[b]
		50 PFU	32/40[b]	80	28/32[b]	88	26/28[b]
		5 PFU	16/20[b]	80	20/20[b]	100	NT
1 & 2	None		52/65[b]	80	32/33[b]	97	0/35[a]

*Survival to 4 weeks of age.
†Titer $\geq$ 20 at 4 weeks of age.
NT = Not tested.
Values with the same superscript (within experiments) do not differ ($p<0.05$).

of chickens injected on ED18 with P_{20}-IBV was significantly ($p<0.05$) lower (33-40%) than the survival of chickens inoculated with P_{40}-IBV (81-100%). However, preliminary data (not shown) indicated that increasing the dosage of P_{40}-IBV from 100 PFU to 500 PFU significantly ($p<0.05$) affected survival, with all 11 chicks succumbing to IBV respiratory disease. All of the chicks tested that were vaccinated with P_{20}-IBV or P_{40}-IBV had anti-IBV antibody at 4 weeks of age.

Response of 2 lines of chickens to embryo vaccination with P_{20}-IBV. Embryos from lines P and 15 x 7 were compared for hatchability and survival following embryo vaccination with P_{20}-IBV to ascertain whether there was a difference in response between the two strains of chickens. Survival was not improved with line 15 x 7 (3/9 survived) as compared to line P (3/15 survived), and hatchability decreased with line 15 x 7 (9/20 hatched vs. 18/20 that hatched with line P) (data not shown). Line 15 x 7 was not used in succeeding experiments.

Comparative protective efficacy of embryo vaccination and posthatch vaccination. Results of similar experiments 4 and 5 shown pooled in Table 2-2 indicated that line P chickens EV with P_{40}-IBV were well protected against challenge with C-IBV. The level of protection was similar to that obtained in chickens vaccinated at hatch with V-IBV. Antibody response of chickens given P_{40}-IBV on ED18 and of those given 10^5 EID_{50} of V-IBV at hatch were comparable.

Table 2-2. Effect of embryo and posthatch vaccination with P_{40}-IBV and V-IBV on resistance of line P chickens to challenge with C-IBV (pooled data from Experiments 4 and 5).

Vaccine (dose)	Age at vaccination	Antibody response*		C-IBV challenge response†			
				No. positive/tested		% Protection against	
		No. positive/ tested	% positive	Respiratory symptoms‡	Isolation of C-IBV	Respiratory symptoms‡	Isolation of C-IBV
P_{40}-IBV (10^2 PFU)	18-day embryo	23/23[b]	100	2/23[a]	1/23[a]	91	96
	at hatch	4/31[a]	13	13/32[b]	14/32[b]	59	56
V-IBV (10^5 EID_{50})	at hatch	31/32[b]	97	4/32[a]	5/32[a,b]	87	84
Unvaccinated	---	0/28[a]	0	28/28[c]	28/28[c]	0	0

*Titer $\geq$ 20 at 4 weeks of age.
†Challenged with C-IBV at 4 weeks of age.
‡Respiratory symptoms included wheezing, gasping or coughing.
Values with the same superscript do not differ ($p<0.05$).

Unvaccinated control chickens had no antibody response and were highly susceptible (100%) to challenge with the C-IBV. Chickens that were vaccinated at hatch with P_{40}-IBV had poor antibody response (13%) and were significantly ($p<0.05$) more susceptible to challenge with C-IBV than chickens EV with P_{40}-IBV either as determined by respiratory symptoms (41% had respiratory signs) or by isolation of C-IBV (44% had positive virus isolation). Increasing the dosage levels of P_{40}-IBV given at hatch did not consistently increase protection against challenge in hatched chicks (Table 2-3). Virus could not be isolated 5 days post-vaccination from 5 chicks posthatch vaccinated with 10,000 PFU P_{40}-IBV. In contrast, virus was readily isolated from 2 chicks EV with 100 PFU P_{40}-IBV that were also terminated 5 days postvaccination (data not shown). Therefore, P_{40}-IBV did not protect chickens vaccinated at hatch as well as it did when given on ED18.

Efficacy of embryo vaccination in chickens bearing maternal antibodies to IBV. Vaccination of maternal antibody-positive embryos with P_{20}-IBV, P_{30}-IBV and P_{40}-IBV induced antibody and resistance to challenge with C-IBV (Table 2-4). Chicks vaccinated on ED18 with all passage levels of P-IBV were protected against challenge comparable to those vaccinated at hatch with V-IBV. In addition, P_{20}-IBV did not decrease survival in maternal antibody-positive chicks as it did in antibody-negative chicks. Unvaccinated hatchmates in all trials lacked anti-IBV antibody at the

Table 2-3. Protective ability of P_{40}-IBV vaccine given at hatch or on ED18 (pooled data from Experiments 6 and 7).

Dose of P_{40}-IBV (PFU/chick or embryo)	Age at vaccination	Antibody response*		C-IBV challenge response†			
				No. positive/tested		% Protection against	
		No. pos./ tested	% pos.	Respiratory symptoms‡	Isolation of C-IBV	Respiratory symptoms‡	Isolation C-IBV
100	18 day embryo	16/16[c]	100	0/16[a]	1/16[a]	100	94
100	at hatch	15/31[b]	48	22/31[b]	13/21[b]	29	38
1,000	at hatch	13/25[b]	52	11/25[b]	15/21[b,c]	56	29
10,000	at hatch	21/29[b,c]	72	12/29[b]	16/21[b,c]	59	24
Unvaccinated	-	0/34[a]	0	33/34[c]	21/21[c]	NA	NA

*Titer $\geq$ 20 at 4 weeks of age.
†Challenged with C-IBV at 4 weeks of age.
‡Respiratory symptoms included wheezing, gasping or coughing.
NA = Not applicable.
Values with the same superscript do not differ ($p<0.05$).

Table 2-4. Embryo vaccination in chickens bearing maternal antibody to IBV (Experiment 8).

Vaccine (dose)	Age at vaccination	% Eggs hatched	% Survival of hatched chicks*	Antibody response†		C-IBV challenge response‡			
						No. pos./No. tested		% Protection against	
				No. pos./ tested	% pos.	Respiratory symptoms**	Isolation of C-IBV	Respiratory symptoms**	Isolation of C-IBV
P_{20}-IBV (1,000 PFU)	18-day embryo	81	100	15/18[b]	83	0/6[a]	0/5[a]	100	100
P_{30}-IBV (1,050 PFU)	18-day embryo	77	71	10/12[b]	83	1/5[a]	0/5[a]	80	100
P_{40}-IBV (100 PFU)	18-day embryo	92	100	12/14[b]	86	2/14[a]	7/14[a]	86	50
V-IBV (10^5 EID_{50})	at hatch	100	100	10/14[b]	71	5/14[a]	10/14[a,b]	64	29
Unvaccinated	--	80	84	0/25[a]	0	17/17[b]	17/17[b]	0	0

*Survival to at least 4 weeks of age.
†Titer $\geq$ 20 at 6 weeks of age (P_{20}-IBV and P_{30}-IBV) or 4 weeks of age (P_{40}-IBV and V-IBV).
‡Challenged with C-IBV at 4 weeks of age (P_{40}-IBV and V-IBV) or at 6 weeks of age (P_{20}-IBV and P_{30}-IBV).
**Respiratory symptoms included wheezing, gasping and coughing.
Values followed with the same superscript do not differ ($p<0.05$).

time of challenge and were highly susceptible to respiratory symptoms and isolation of challenge virus.

Embryo vaccination with a bivalent vaccine containing HVT and P_{40}-IBV. Commercial chickens bearing maternal antibody to IBV and HVT were used. The bivalent vaccine containing 1,000 PFU of HVT and 100 PFU of P_{40}-IBV, injected on ED18, protected chickens against challenge with both virulent MDV and C-IBV (Table 2-5). The protective levels of the bivalent vaccine were similar to those levels achieved by either 100 PFU P_{40}-IBV or 1,000 PFU HVT injected singly. Hatchability and survival of chickens given bivalent or monovalent vaccine on ED18 were greater than 90% (data not shown). Unvaccinated hatchmates and chickens EV with HVT alone were free of anti-IBV antibody and were highly susceptible to challenge with virulent IBV.

Upon challenge with virulent MDV, neither the chickens EV with HVT alone, nor the chickens dually-vaccinated with the HVT plus P_{40}-IBV bivalent vaccine had gross lesions of MDV. Comparable gross lesions of MD were found in unvaccinated hatchmates and chickens EV with P_{40}-IBV alone. However, the incidence of MD lesions in unvaccinated-MDV challenged chickens was low (38%). This result indicated the relative resistance of commercial chickens to MD.

Discussion

Attempts at embryo vaccination as a mode of vaccine delivery resulted from the observation that chickens develop certain immunologic functions before hatch.[42] Chickens

Table 2-5. Embryo vaccination with P_{40}-IBV and HVT vaccines given singly or in combination (Experiment 9).

Vaccine (dose)	IBV antibody response*		C-IBV challenge response† No. pos./No. tested		% Protection against IBV		MDV challenge response**	
	No. pos./ tested	% pos.	Respiratory symptoms‡	Isolation of C-IBV	Respiratory symptoms‡	Isolation of C-IBV	No. dead or with gross lesions/ No. tested	% Protection against MDV
P_{40}-IBV (100 PFU)	12/16	75	6/16	5/16	63	68	4/17	NA
P_{40}-IBV (100 PFU) & HVT (1,000 PFU)	9/16	56	5/16	8/16	69	50	0/17	100
HVT (1,000 PFU)	0/13	0	13/13	13/13	0	0	0/12	100
Unvaccinated	0/13	0	12/12	12/12	0	0	5/13	NA

*Titer $\geq$ 20 at 4 weeks of age.
†Challenged with C-IBV at 4 weeks of age.
‡Respiratory symptoms included wheezing, gasping or coughing.
**Challenged with pathogenic MDV at 11 days of age.
NA = Not applicable.

vaccinated on ED18 with HVT developed lasting resistance to posthatch challenge with virulent MDV.[1] In this study, we have shown that chickens may also be successfully immunized by embryo vaccination against IB, another naturally occurring, highly contagious viral disease. As in the chicken, prenatal vaccination against pathogens has also been successfully attempted in mammalian features.[43-45]

Preliminary trials indicated that V-IBV was pathogenic for embryos after inoculation on ED18. Because V-IBV was an egg-propagated commercial vaccine, its pathogenicity for embryos was not unexpected. Previously, it had been demonstrated that passaging IBV in tissue culture or in chick embryos reduced its pathogenicity for chickens.[25] Consequently, we used this approach for preparing the embryo vaccine. We passed V-IBV 40 times and the passaged virus (P_{40}-IBV) was found to be non-pathogenic for embryos at a dosage of 100 PFU per embryo. Inoculation of P_{40}-IBV on ED18 did not affect hatchability or survival of hatched chicks. The P_{40}-IBV vaccine also induced antibody and resistance to challenge in EV chickens. Therefore, P_{40} was chosen for additional investigation. It is possible that tissue culture passage may have altered the vaccine virus either through selection of apathogenic clones or viral mutation. Similar modification of other vaccine viruses may be necessary to make them suitable for embryo vaccination.

The P_{40}-IBV vaccine retained infectivity and immunogenicity for embryos but not for the hatched chicks.

The reduced ability of hatched chicks to respond immunologically to P_{40}-IBV was not overcome by increasing the dosage from 100 to 10,000 PFU per chick. In comparison with EV chicks in which P_{40}-IBV could be readily isolated from the trachea 5 days postvaccination, virus could not be isolated from the trachea following posthatch vaccination with the highest dosage of P_{40}-IBV tested. This result indicated that P_{40}-IBV vaccine did not establish infection in chicks if the vaccine was administered after hatch. This observation is of importance because it showed that one must carefully monitor the modification by cell culture passages of virus vaccines for embryo use. Excessive tissue culture passages may lead to lack of infectivity of the virus.

We also examined the efficacy of P_{40}-IBV vaccine in embryos possessing maternal antibodies to IBV. Although maternal antibodies are known to interfere with the production of vaccinal immunity to IBV,[46] successful vaccination of chicks bearing anti-IBV maternal antibodies has been accomplished.[47,48] The successful induction of protective immunity is possibly due either to the virus overcoming the presence of circulating antibody or to the predominant stimulation of local immunity in the upper respiratory tract. Maternal antibodies interfered with protective ability of P_{40}-IBV as they did with protective ability of V-IBV. Thus, embryo vaccination did not alter the relationship between maternal antibodies and vaccinal immunity. Similar relationships were observed with embryo

vaccination and posthatch vaccination against MD and infectious bursal disease.[10,49] Of interest was an apparent higher level of protection of maternal antibody-positive chicks with P_{20}-IBV and P_{30}-IBV than with P_{40}-IBV. However, a direct comparison of P_{20}-IBV and P_{30}-IBV with P_{40}-IBV was not possible because of different dosage levels and challenge times used. Possibly a higher dose or a lower passage level of the vaccine virus is necessary to induce optimum vaccinal immunity in maternal antibody-bearing embryos than in maternal antibody-lacking embryos.

Under commercial broiler industry conditions, IBV vaccine is often given to chicks at hatch along with HVT. We determined if both vaccines could be given simultaneously in the embryo. Results revealed that embryo vaccination with a bivalent vaccine containing HVT and IBV induced resistance in hatched chicks against both virulent MDV and IBV. Neither virus interfered with the protective ability of the other. Thus, HVT may be safely combined with IBV and given as a dual vaccine on ED18. Similarly, HVT may also be combined with infectious bursal disease vaccine.[10]

Although we examined the response of commercial broilers to embryo vaccination with IBV, all studies presented here were conducted under laboratory conditions. Whether embryo vaccination shown to be highly successful under a laboratory setting will also be equally successful under commercial conditions needs to be determined. The commercial use of the embryo vaccination procedure is

attractive not only because of early posthatch resistance, which may be critical in diseases like MD, but also because several vaccines may be combined and given in one injection in the embryo. This may save the cost of labor necessary to give each vaccine individually. Further savings may be realized if automated mechanized procedures can be developed that may facilitate multiple injections in embryonated eggs. Such possibilities are currently being considered.

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CHAPTER 3

EMBRYO VACCINATION OF CHICKENS WITH INFECTIOUS BRONCHITIS VIRUS: HISTOLOGIC AND ULTRASTRUCTURAL LESION RESPONSE AND IMMUNOLOGIC RESPONSE TO VACCINATION

EMBRYO VACCINATION OF CHICKENS WITH INFECTIOUS BRONCHITIS VIRUS: HISTOLOGIC AND ULTRASTRUCTURAL LESION RESPONSE AND IMMUNOLOGIC RESPONSE TO VACCINATION

Summary

Chickens were vaccinated on embryonation day 18 (ED18) or at hatch with a commercial infectious bronchitis virus vaccine (V-IBV) or the 40th tissue culture passage of this vaccine (P_{40}-IBV). Immunologic responses and pathologic changes of the vaccinated groups were compared. Histologic, transmission electron microscopic (TEM) and scanning electron microscopic (SEM) evaluation of lung and tracheal sections from chickens given P_{40}-IBV on ED18 demonstrated transient mild to moderate tracheal and pulmonary lesions. The tracheal lesions were characterized by deciliation, occasional foci of flattened, simplified squamous epithelium and rarely, a mild, mixed inflammatory cell infiltration accompanied by an acellular exudate. Small foci of increased interstitial cellularity and occasional areas of necrosis, mixed inflammatory cell infiltration and exudation into the lumens of tertiary bronchioles, atria and air capillaries were observed in the lung. Birds vaccinated with V-IBV at hatch had similar tracheal lesions. Embryo vaccination with P_{40}-IBV or post-hatch vaccination with V-IBV did not consistently influence the response of whole

blood cells to phytohemagglutinin (PHA) or the natural killer (NK) cell reactivity of spleen effector cells. Embryo vaccination and post-hatch vaccination induced similar primary and secondary antibody responses in chickens. Effector cells cytotoxic to IBV-infected target cells were not detected in chickens vaccinated on ED18 with P_{40}-IBV or at hatch with V-IBV and challenged at 4 weeks with virulent IBV (C-IBV).

Introduction

Avian infectious bronchitis virus (IBV) is a coronavirus[1] which causes a highly transmissible respiratory disease in chickens.[2] In addition, some strains are capable of inciting a severe nephrosis/nephritis syndrome.[3,4] Chickens are infected by horizontal transmission with respiratory symptoms usually appearing 18 to 36 hours post-exposure.[5,6] Infectious bronchitis (IB) is an economically important disease due to both high mortality in chicks under 1 week of age and disruption of production in laying flocks.[2,5-8] The disease is contagious and untreatable, with vaccination being the only means of protection.[9,10]

Vaccination against IBV is generally initiated during the first week of life and is often repeated in laying flocks.[9,11,12] The vaccine is routinely administered either in the drinking water or by aerosol spray.[9,13-15] Recently, we have noted that chickens may be protected against several diseases including IB by injecting the vaccines on ED18 rather than at hatch.[16-21] The chickens embryo vaccinated

(EV) with IBV developed antibody and resisted challenge with virulent IBV at 4 weeks of age. The purpose in this study was to examine the immunologic and pathological responses of chickens injected as embryos with a tissue culture propagated IBV vaccine (P_{40}-IBV).

Materials and Methods

Chickens and embryonating eggs. Line P White Leghorn chickens and embryonated eggs were obtained from a flock maintained at the Regional Poultry Research Laboratory. This flock was held in isolation and was free of exposure to IBV, Marek's disease virus (MDV), turkey herpesvirus (HVT), avian leukosis viruses, reticuloendotheliosis virus and other common bacterial and viral poultry pathogens. The chickens were hatched and reared in positive pressure plastic canopy isolators supplied with biologically filtered air. After virus challenge, the birds were maintained for 5 days in open pens in a clean environment. Specific pathogen-free chickens of line 15 x 7 were used as the source of allogeneic chicken kidney (CK) cell cultures.

Viruses and inoculation procedures. A commercial IBV vaccine (V-IBV) of Holland strain, Massachusetts type, with a titer of 10^6 50% embryo infective dose (EID_{50}) per ml was used. The vaccine virus was either untreated or was serially passaged 40 times (P_{40}-IBV) in CK cell cultures,[22] as previously described.[21] The IBV challenge virus (C-IBV) was a virulent Massachusetts 41 strain, with a titer of

$10^{7.5}$ EID_{50}/ml.[a] This virus was used at a 1:100 dilution in sterile tryptose phosphate broth. The viruses were titrated as described previously.[21]

Embryo vaccination with IBV has been described previously.[21] Briefly, eggs were inoculated on ED18 with 0.1 ml of the desired virus dilution or virus-free diluent (control embryos). The large end of the eggs was cleaned with 80% ethyl alcohol, a small hole was punched in the egg shell and the entire length of a 1 1/4-inch-long 22 gauge needle was inserted through the hole in the egg.

Posthatch vaccination with IBV was conducted at 1 day of age. One drop of inoculum containing either diluent for control birds or the appropriate virus concentration was deposted intraocularly. After the inoculum was deposited in the eye, the birds were carefully observed for swallowing and for subsequent absorption of the droplet before they were released.

Chickens were challenged at 4 weeks of age by intraocular inoculation with 0.03 ml of C-IBV. After inoculation, the chickens were moved to an open room.

Antibody analysis. Serum samples were assayed for antibodies to IBV by a constant virus-diluting serum plaque reduction assay in CK cell cultures as previously described.[23,24] The indicator virus was the 20th or the 30th CK cell culture passage of V-IBV. An 80% or greater

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[a]Dr. Wenger, National Veterinary Services Laboratories, Ames, IA.

reduction in plaque forming units by a 1:20 dilution of the serum was considered as evidence for the presence of IBV antibody.

Tissue collection and processing. Embryos and hatched chicks were euthanized (by decapitation) and lungs, kidneys and distal tracheas were immediately recovered for fixation and processing. Tissues for light microscopy were fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned at 6 μm thickness and stained with hematoxylin and eosin.[25] Tissues for electron microscopy were fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded ethanols and stored. Prior to examination by transmission electron microscopy (TEM), the tissues were embedded in epon-araldite, sectioned with glass knives at 1 μm thickness, and stained with toluidine blue.[26] Thin sections of 90 nm thickness were then prepared for TEM viewing.[b] After dehydration, sections for scanning electron microscopy (SEM) were critical point dried, mounted on aluminum stubs, sputter-coated with gold and viewed with a scanning electron microscope.[c,26]

Cell-mediated immune assays. The 4-hour ^{51}Cr-release assay for natural killer (NK) cell activity was conducted by reacting spleen effector cells against LSCC-RP9 target cells at target to effector cell ratios of 1:100 and 1:200 in all

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[b]Elektronenscopen EM 952, Carl Zeiss, Hamburg, Germany.

[c]Model JSM 35, JEOL, Tokyo, Japan.

trials and additionally at 1:400 in some trials.[27,28] With the exception of two trials with spontaneous release levels of 32 and 33 percent, respectively, the spontaneous release was always less than 26 percent of the total releasable label. A similar 4-hour ^{51}Cr-release assay was used to determine presence in vaccinated chickens of cytotoxic effector cells reactive against IBV-infected target cells. The virus infected target cells were prepared as follows: CK cell monolayers grown in 60 mm petri dishes were inoculated at 2×10^6 plaque forming units per dish of the 31st serial cell culture passage of V-IBV. At 72 hours when cytopathic effect was extensive, the cells were trypsinized, suspended in Basal Medium Eagle[d] containing 2% bovine fetal serum and 1×10^7 cells were labelled for 1 hour at 37° C with 0.25 mCi of ^{51}Cr. At the conclusion of the labelling period, the cells were washed three times and used at 5×10^3 cells per well. Cells in each well had an average count per minute (cpm) value of $7\text{-}8 \times 10^3$ and upon 4-hour incubation alone or in combination with thymocytes from normal chickens at up to 1:400 target cells to thymocyte ratio, released 15-28 percent of the input label. The cytotoxic activity of effector cells from the spleens of vaccinated chickens was examined against virus-infected allogeneic and syngeneic target cells. In each test, effector to target ratios of 100:1 and 200:1 were examined

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[d]Gibco Laboratories, Detroit, Michigan.

using triplicate wells for each ratio. Background release of the label was determined by reacting target cells with thymocytes from normal chicks, as previously described.[28] Percent cytotoxicity was calculated as follows: % cytotoxicity = cpm in target cells mixed with effector cells - cpm in target cells mixed with normal thymus cells ÷ cpm incorporated in target cells - cpm in target cells mixed with normal thymus cells x 100. Blastogenic response of whole blood cells to phytohemagglutinin (PHA)[e] was examined as previously described.[29]

Statistical analysis. Data were compared with the use of a Student's t-test.

Experimental design. Because P_{40}-IBV does not replicate well when given at hatch,[21] the responses of birds given P_{40}-IBV on ED18 were compared with the responses of chickens given V-IBV at hatch. In experiment 1, 60 18-day-old embryonated eggs and 60 newly hatched chicks were each divided into three equal groups and were inoculated as follows: Group 1 (embryos) and group 4 (chicks) received 0.1 ml of diluent, group 2 (embryos) and group 5 (chicks) received 100 pfu P_{40}-IBV and group 3 (embryos) and group 6 (chicks) received 10^5 EID_{50} V-IBV. On PI days 2, 4, 11 and 17, five embryos or chicks from each group were sampled and sections of the lung, trachea and kidney were placed in formalin for histopathologic examination.

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[e]Difco Laboratories, Detroit, Michigan.

In experiment 2, 18 18-day-old embryos were divided into three equal groups and were inoculated as follows: Group 1, 0.1 ml of diluent (negative control); group 2, 100 pfu P_{40}-IBV; and group 3, 2 EID_{50} V-IBV (positive control). On PI days 2 and 4, three chicks or embryos from each group were killed and sections of the lung and trachea were placed in both formalin and glutaraldehyde for either histopathologic or TEM/SEM examination.

Experiments 3 and 4 were similar in design with the only differences being in the PI or post-challenge (PC) sample collection dates. In both experiments, 18-day-old embryos were divided into two groups and inoculated as follows: Group 1, 0.1 ml of diluent; and group 2, 100 pfu P_{40}-IBV. Day-old chicks were simultaneously inoculated intraocularly with 10^5 EID_{50} V-IBV. On PI days 5, 7 and 35 (experiment 3) and PI days 5, 7 and 31 (experiment 4), five chicks were sampled from each group, and were examined for cytotoxic activity of spleen cells against IBV infected target cells and serum anti-IBV antibody. At 4 weeks of age, all of the vaccinated and half of the unvaccinated control chickens were challenged with C-IBV. Cytotoxic reactivity and antibody analysis were examined on PC days 4, 7 and 14 in experiments 3 and cytotoxic reactivity was examined on PC days 3, 8 and 16 in experiment 4.

In experiments 5 and 6, 18-day-old embryos and newly hatched chicks were divided and inoculated with P_{40}-IBV or V-IBV as in experiments 3 and 4. On PI days 6, 8 and 31

(experiment 5) and 5, 7 and 28 (experiment 6), 5 chicks were sampled from each group. Whole spleens and whole blood were removed from each chick. Spleen cells were examined for NK cell activity and the peripheral blood cells were assayed for mitogenic response to PHA. Challenge with C-IBV was administered at 4 weeks of age and 5 chicks sampled on PC days 7 and 14 in experiment 5 and PC days 5, 9 and 14 in experiment 6 were examined for NK cell activity and mitogenic response.

Results

Lesion response to vaccination (Experiments 1 and 2).

Histologic changes: Histopathologic changes in all tissues were similar in experiments 1 and 2. No lesions were found in the kidneys of infected or control groups in either experiment.

P_{40}-IBV vaccinated chickens: Lesions were only observed in chicks injected with the vaccine as embryos (experiments 1 and 2, group 2) and not in those injected at hatch (experiment 1, group 5). In embryo inoculated chicks, microscopic changes in the trachea first detectable at 2 days PI persisted until 17 days PI, the longest time tested (Table 3-1), although the changes on the 17th day PI were regenerative. The lesions were confined to the tracheal mucosa and consisted of deciliation, occasional foci of flattened, simplified squamous epithelium and rarely, a mild, mixed inflammatory cell infiltration accompanied by an acellular exudate (Figure 3-1a) in comparison to the tall,

ciliated columnar pseudostratified epithelium of the control birds (Figure 3-1b). At 11 and 17 days PI, hyperplasia of the epithelium, vacuolation of the epithelial cells and regeneration of the cilia were also observed. Lung lesions appeared early, 2 days PI, and were still observed in 1 bird at 17 days PI (Table 3-1). Although the microscopic changes mainly consisted of small foci of increased interstitial cellularity (Figure 3-2a) when compared to the open, lacelike appearance of the lungs of control birds (Figures 3-2b, 3-3c), occasional areas of necrosis, mixed inflammatory cell infiltration and exudation into the lumens of tertiary bronchioles, atria and air capillaries were observed (Figures 3-3a,b). By 11 and 17 days PI, the lesions had become focal and less severe.

V-IBV vaccinated chickens: In experiment 1, either 18-day-old embryos (group 3) or day-old chicks (group 6) were vaccinated with the standard chick dose of V-IBV (10^5 EID_{50}) used for field application. With the exception of 1 small focus of inflammatory cells surrounding a major bronchus in the lung of 1 bird at 17 days PI, lesions were confined to the trachea for posthatch vaccinated chicks and were similar to those observed in P_{40}-IBV EV chicks (Figure 3-1c, Table 3-1). Microscopic changes first appeared at 4 days PI and were observed throughout the test period. At 17 days PI, the changes seen were mainly regenerative. In experiment 1, pathologic changes in both the trachea and lungs of V-IBV EV birds were severe and culminated in death of all birds by

Table 3-1. Histopathologic evaluation of lung and tracheal lesion development in chickens vaccinated with IBV as embryos or at hatch (Experiment 1).

Vaccine (dose)	Age of inoculation	Tracheal lesions: Days post-vaccination 2	4	11	17	Lung lesions: Days post-vaccination 2	4	11	17
None	-3d	*0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	1d	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
P_{40}-IBV (100 pfu)	-3d	3/5	4/5	2/4	1/3	2/5	3/5	3/4	1/3
	1d	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
V-IBV (10^5 EID_{50})	-3d	5/5	5/5	D	D	5/5	5/5	D	D
	1d	0/5	2/5	2/5	2/5	0/5	0/5	0/5	1/5

D = All birds deceased.
*# Chicks with lesions/# tested.

Figure 3-1a. Tracheal epithelium from a P_{40}-IBV EV bird 4 days PI (experiment 1, group 2, bird 7). There is deciliation and increased cellularity of the epithelium. H & E stain; X40.

Figure 3-1b. Normal tracheal epithelium from a control bird 4 days PI (experiment 1, group 1, bird 6). Note the prominent cilia on the luminal surface. H & E stain; X40.

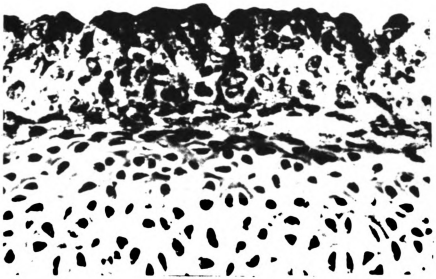

Figure 3-1a

Figure 3-1b

Figure 3-1c. Tracheal epithelium from a V-IBV posthatch vaccinated bird 4 days PI (experiment 1, group 6, bird 10). Although the normal columnar epithelium has been retained, there is patchy deciliation and mild infiltration with heterophils (arrows). H & E stain; X40.

Figure 3-1d. Tracheal epithelium from a V-IBV EV bird 4 days PI (experiment 1, group 3, bird 9). The normal columnar epithelium has been replaced by a nonciliated squamous epithelium. H & E stain; X40.

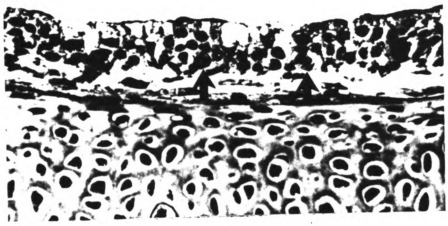

Figure 3-1c

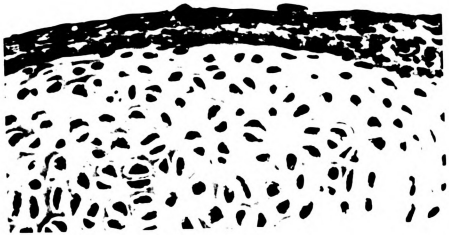

Figure 3-1d

Figure 3-2a. Lung tissue from a P_{40}-IBV EV bird 2 days PI (experiment 1, group 2, bird 3). Occasional foci of increased cellularity are present. H & E stain, X4.

Figure 3-2b. Normal lung tissue from a control bird 2 days PI (experiment 2, group 1, bird 2). The lung has an open, lace-like appearance. H & E stain; X4.

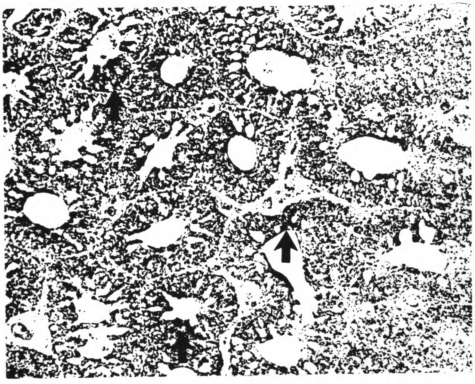

Figure 3-2a

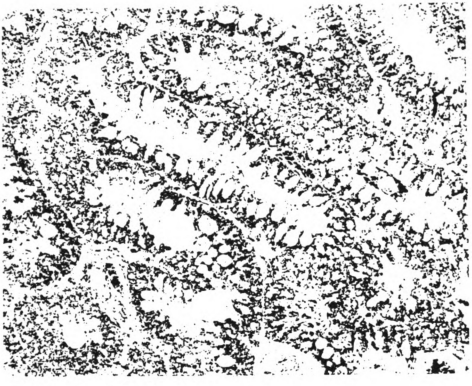

Figure 3-2b

Figure 3-2c. Lung tissue from a V-IBV EV bird 2 days PI (experiment 1, group 3, bird 5). The lung parenchyma contains areas of consolidation (arrows) with loss of lace-like architecture. H & E stain; X4.

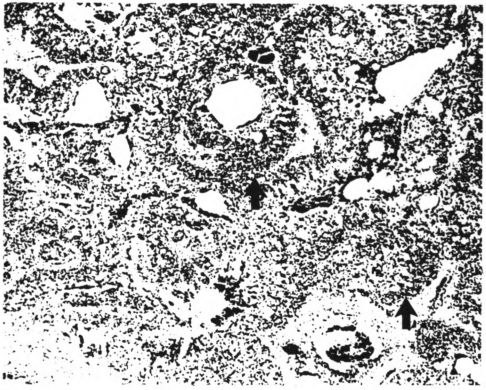

Figure 3-2c

Figure 3-3a. Lung tissue from a P_{40}-IBV EV bird 2 days PI (experiment 1, group 2, bird 3). The tertiary bronchi (T) contain a heterophilic inflammatory exudate with hemorrhage and there is swelling of the atrial and air capillary walls and increased cellularity of lung parenchyma (arrows). H & E stain; X10.

Figure 3-3b. A higher magnification view of Figure 3-3a shows a tertiary bronchus with an accumulation of erythrocytes (single arrow) and inflammatory cells (double arrow) in the lumen (L) and mild inflammation of the bronchial wall. H & E stain; X40.

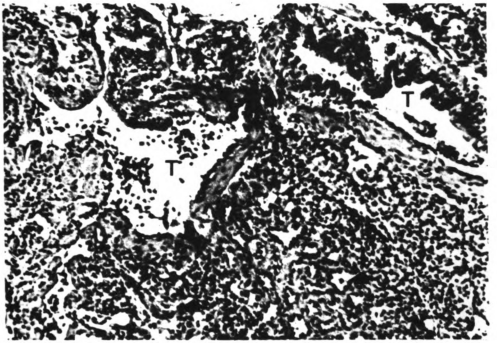

Figure 3-3a

Figure 3-3b

Figure 3-3c. Lung tissue from a control bird 2 days PI (experiment 2, group 1, bird 2). The tertiary bronchus (T) is lined by a thin layer of cuboidal epithelium with underlying bands of smooth muscle. From large atria (arrows) arise numerous air capillaries. H & E stain; X10.

Figure 3-3d. Lung tissue from a V-IBV EV bird 2 days PI (experiment 1, group 3, bird 5). The entire lumen of this tertiary bronchus is occluded with necrotic debris and inflammatory cells (arrow). Atria and air capillaries are also similarly involved leading to parenchymal destruction. H & E stain; X10.

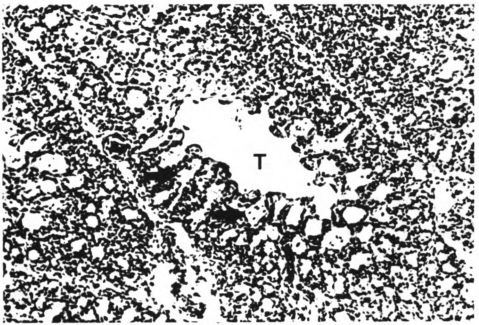

Figure 3-3c

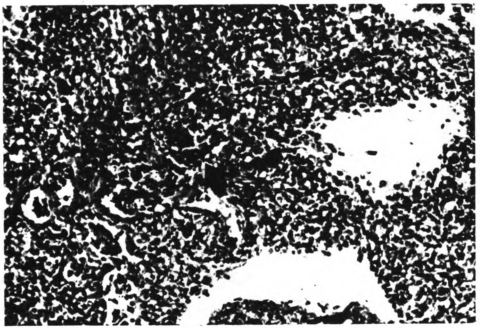

Figure 3-3d

the 11th day PI (Table 3-1). Similar lesions were seen in positive control birds from experiment 2 (group 3) that were EV with 2 EID_{50} V-IBV. The normal ciliated columnar tracheal epithelium (Figure 3-1b) was completely replaced by a nonciliated squamous epithelium (Figure 3-1d) accompanied by occasional submucosal foci of mononuclear inflammatory cells. In the lung there were large areas of complete loss of the normal lace-like architecture due to consolidation and inflammatory cell infiltration (Figures 3-2c). The walls of tertiary bronchi, atria and air capillaries were often necrotic and proteinaceous exudate and an admix of inflammatory cells occluded the lumens (Figure 3-3d).

Ultrastructural changes. Examination by TEM and SEM in experiment 2 supported the light microscope observations.

P_{40}-IBV EV chickens (Group 2): Scanning electron microscopic examination revealed tracheal sections denuded of cilia making the underlying epithelial cells readily apparent (Figures 3-4a,b). Tracheal sections from control chickens had a normal ciliated epithelial surface covered by a thin layer of mucus (Figures 3-5a,b). Transmission electron microscopic examination revealed tracheal sections from control chicks with ciliated epithelial cells, tight intracellular junctions and occasional artifactual vacuolation (Figure 3-6). In comparison, TEM examination of tracheas from P_{40}-IBV EV birds revealed deciliation with a few remaining swollen microvilli adhered to the luminal surface and a heterophilic infiltrate in the epithelium

(Figure 3-7). Degeneration and vacuolation of epithelial cells characterized by dilation of the cisternae and early cytoplasmic disruption were also observed. The subbasal area was edematous and contained distended capillaries. Sections of lung examined by TEM from P_{40}-IBV EV birds appeared similar to the lung sections from control birds consisting of tertiary bronchioles that gave rise to atria lined with intact cuboidal to squamous epithelium and leading to numerous branching air capillaries (Figure 3-8).

V-IBV EV chickens (Group 3): Complete deciliation and exposure of the epithelial cells was observed with SEM examination of the tracheas from V-IBV EV chickens (Figures 3-9a,b). Inflammatory cells were seen adhering to the naked epithelial surface. The epithelial cells were simplified cuboidal cells with only remnants of microvilli remaining on the surface (Figures 3-10a,b) when compared by TEM with sections from control birds (Figure 3-6). The cells were widely separated by intercellular edema and infiltrating heterophils. Coronavirus particles consistent with IBV were seen in the cisternae, vesicles and free in the lumen. Ultrastructural changes in lung sections examined by TEM were extensive. The epithelial surfaces of tertiary bronchioles were ulcerated and infiltrated with inflammatory cells (Figure 3-11). Cellular debris and hemorrhage filled the lumen and the remaining cells were widely separated by edema. Rare swollen microvilli remained attached to the luminal surface. Infectious bronchitis virus particles were

Figure 3-4a. Tracheal mucosa from a P_{40}-IBV EV bird 2 days PI (experiment 2, group 2, bird 2). There is extensive cilial loss exposing the underlying epithelium. Bar = 10 µm SEM

Figure 3-4b. Higher magnification of Figure 3-4a. Inflammatory cells (1 arrow) and erythrocytes (2 arrows) are also adherent to the epithelial surface. Bar = 10 µm SEM

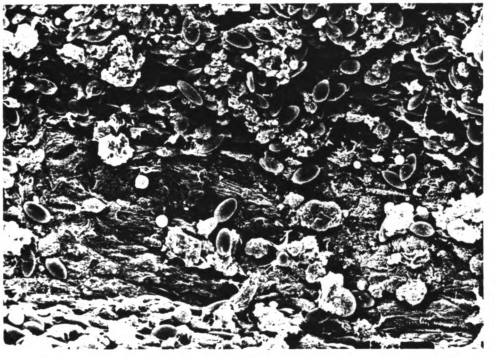

Figure 3-4a

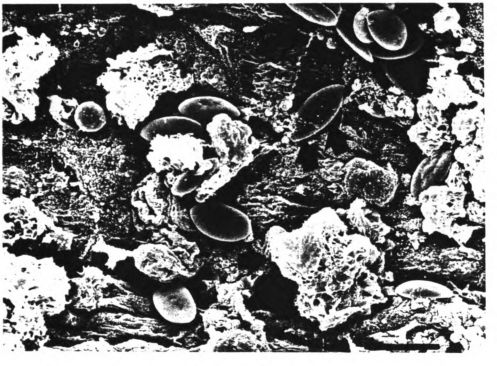

Figure 3-4b

Figure 3-5a. Tracheal mucosa from a control bird 2 days PI (experiment 2, group 1, bird 2). Abundant cilia are present covered by a thin layer of mucus. Bar = 10 μm SEM.

Figure 3-5b. Higher magnification of Figure 3-5a. A few erythrocytes lie on the luminal surface (arrow). Bar = 10 μm SEM.

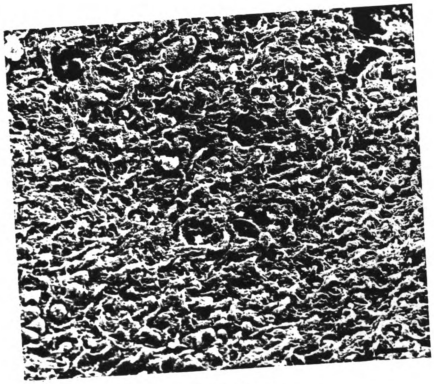

Figure 3-5a

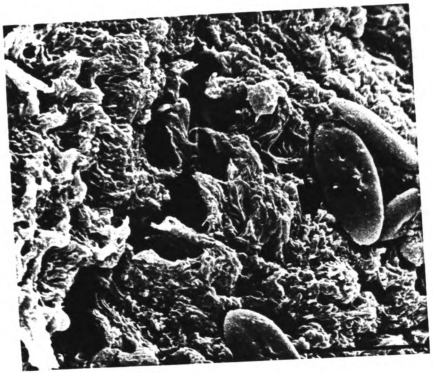

Figure 3-5b

Figure 3-6. Normal tracheal mucosa from a control bird 2 days PI (experiment 2, group 1, bird 2) comprised of columnar epithelium. The luminal surfaces (L) of the cells are covered by cilia interspersed by microvilli. The vacuolation of epithelial cells is an artifact. TEM X3000.

Figure 3-7. Tracheal mucosa from a P_{40}-IBV EV bird 2 days PI (experiment 2, group 2, bird 2). The luminal surfaces are naked except for a few swollen microvilli (arrows). The mucosal surface contains degenerate vacuolated epithelial cells and is infiltrated with heterophils (H). The subbasal capillary (C) is congested and there is submucosal edema. TEM X1500.

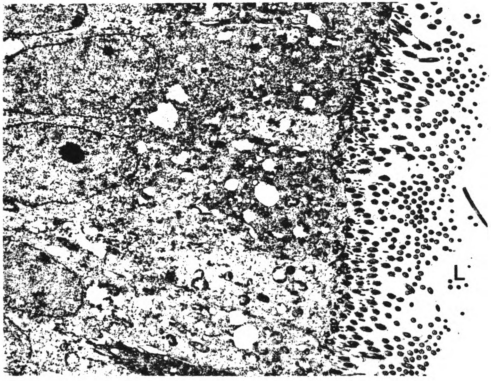

Figure 3-6

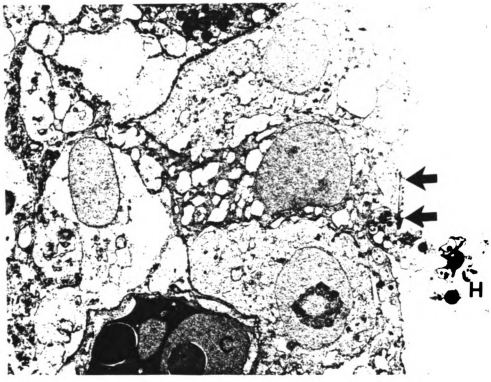

Figure 3-7

Figure 3-8. Lung tissue from a control bird 2 days PI (experiment 2, group 1, bird 2). Small airway epithelium with numerous microvilli on the luminal surface. Normal tight junctions are evident between cells (arrow) and no intercellular edema is evident. Vacuolation is an artifact. TEM X3000.

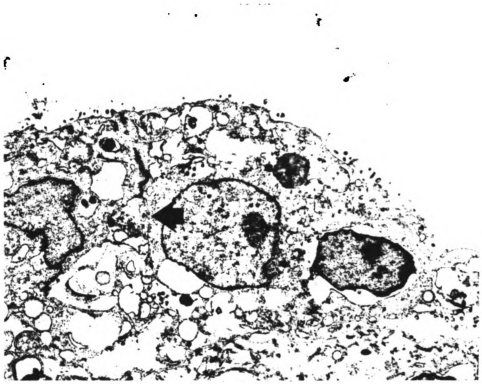

Figure 3-8

Figure 3-9a. Tracheal mucosa from a V-IBV EV bird 2 days PI (experiment 2, group 3, bird 3). There is complete deciliation and exposure of underlying epithelial cells. Bar = 10 μm SEM.

Figure 3-9b. Higher magnification of Figure 3-9a. Inflammatory cells (arrows) are adhered to the deciliated epithelial surface. Bar = 10 μm SEM.

Figure 3-9a

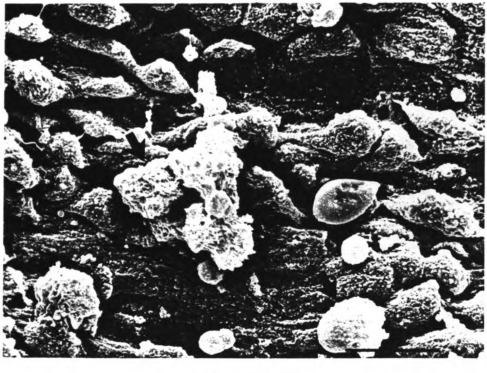

Figure 3-9b

Figure 3-10a. Tracheal mucosa from a V-IBV EV bird 2 days PI (experiment 2, group 3, bird 1). The normal epithelium has been replaced by a less complex cuboidal epithelial cell population. Individual cells are separated by severe intercellular edema (arrows). Remnants of microvilli remain on the luminal surface (L). TEM X2000.

Figure 3-10b. Higher magnification of Figure 3-10a. Heterophils (arrows) have infiltrated to the epithelial surface. TEM X3000.

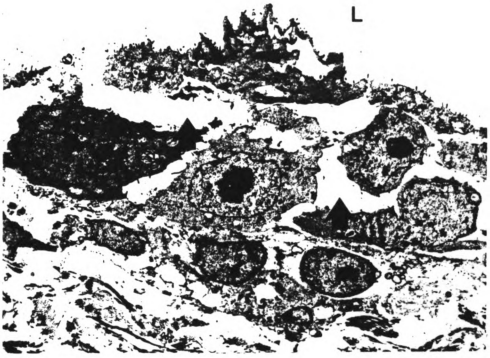

Figure 3-10a

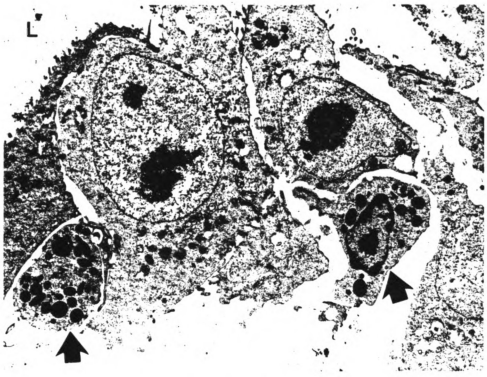

Figure 3-10b

Figure 3-11. Lung tissue from a V-IBV EV bird 2 days PI (experiment 2, group 3, bird 3). Epithelial cells (arrows) are necrotic and some have sloughed off the basement membrane of a tertiary bronchus. The lumen (L) contains cellular debris and erythrocytes (E). The remaining epithelium is infiltrated with inflammatory cells (H) and is edematous. A few swollen microvilli are present on remaining epithelial cells. TEM X1500.

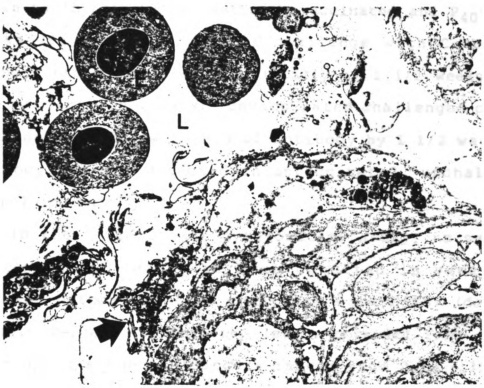

Figure 3-11

also seen in the cisternae and vesicles of the epithelial cells and free in the lumen.

Immune responses to vaccination (Experiments 3 and 4). In experiment 3, antibody was detected at the second week PI and reached a maximum titer between 4 and 5 weeks PI before declining in both V-IBV posthatch vaccinated and P_{40}-IBV EV chicks (Figure 3-12). After challenge with C-IBV, IBV antibody titer increased dramatically by 1 1/2 weeks PC in both vaccination groups. Unvaccinated challenged control birds developed low levels of antibody by 1 1/2 weeks PC. Antibody was not detected in unvaccinated, unchallenged control birds.

In the ^{51}Cr-release cytotoxicity assay, no specific cytotoxic cell reactivity was detected in vaccinated chickens before or after challenge with C-IBV (data not shown). Percent cytotoxicity levels obtained from control birds using syngeneic targets ranged from 0 to 4.38±3.88, and from 0 to 7.03±4.89 using allogeneic target cells. Values from infected chickens were not consistently different from those obtained from control birds at any observation point.

Effect of vaccination on immunocompetence (Experiments 5 and 6). Immunocompetence was examined by determining the NK reactivity of spleen cells and mitogenic response of whole blood cells of the vaccinated chicks. Vaccination on ED18 or at hatch or challenge of vaccinated chicks did not significantly alter ($p<0.05$) the NK cell level when compared

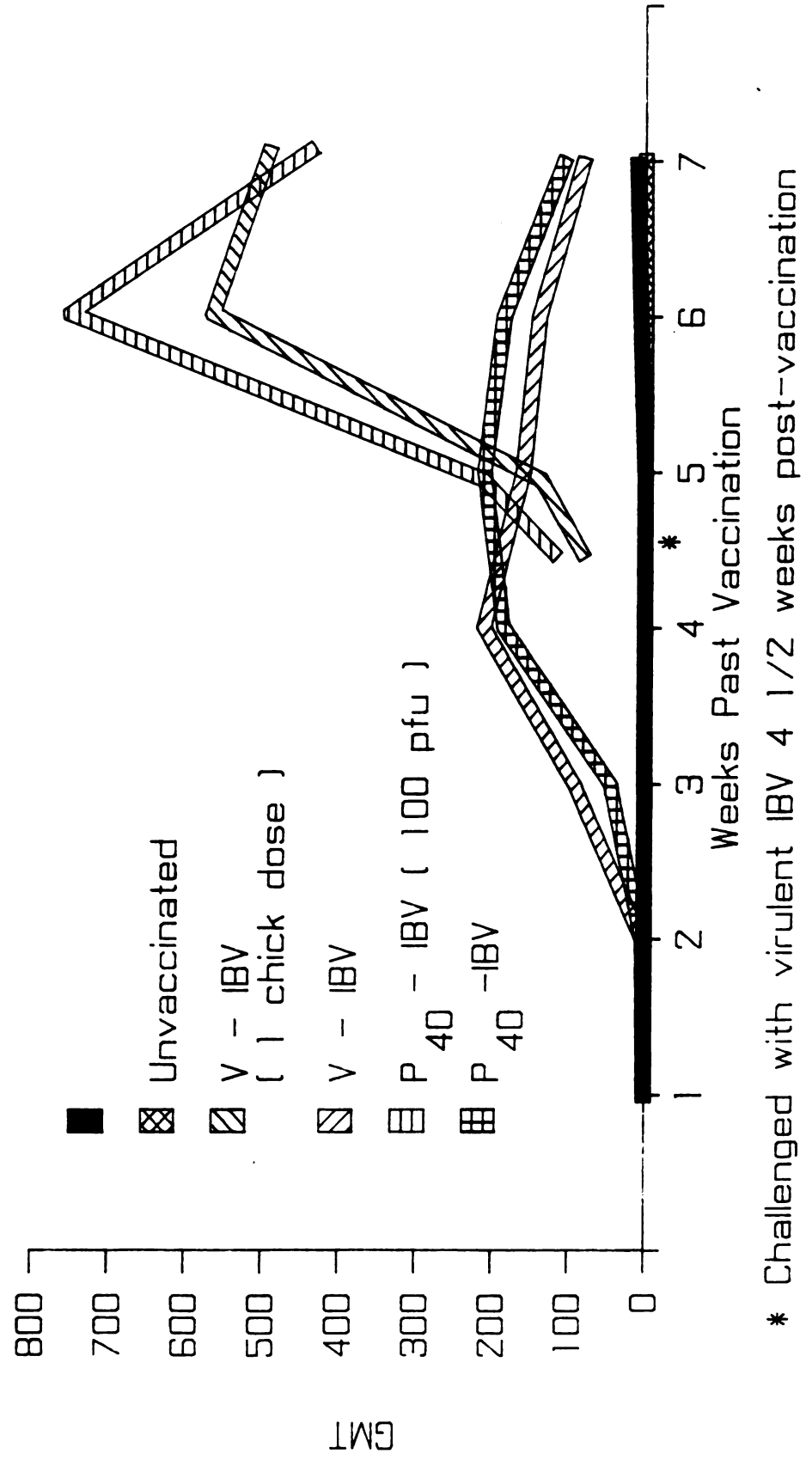

Figure 3-12. Geometric mean titers of line P chickens vaccinated with IBV as embryos or at hatch (experiment 3).

with the level in age-matched uninoculated control chickens (data not shown). The NK cell cytotoxic level in control chickens ranged from 0-9.6±0.6%.

Mitogenic response of whole blood was also not consistently affected by embryo or posthatch vaccination with IBV (data not shown). Average Log_{10} cpm values in PHA stimulated whole blood cells of uninoculated chicks in experiments 5 and 6 ranged from 2.3±0.2 to 4.0±0.1. Inoculation of unvaccinated control chickens with C-IBV at 4 weeks of age significantly ($p<0.01$) reduced the mitogenic response of whole blood cells (cpm value 3.0±0.2). The reduction in mitogenic response was transitory and was apparent at 3 days but not at 7 or 19 days after inoculation with C-IBV. Inoculation of C-IBV into EV and posthatch vaccinated chickens did not result in detectable reduction in mitogenic response of whole blood cells.

Discussion

Chickens given P_{40}-IBV on ED18 developed lesions in the lungs and the trachea indicating that injection of IBV on ED18 resulted in infection of the respiratory tract with the vaccine virus. The lesions noted in EV chicks were also found in chicks post hatch vaccinated with V-IBV. The lesions in post hatch vaccinated birds were confined to the trachea, however. Previous investigators have shown that the severity and location of both pathogenic and vaccine IBV induced respiratory lesions greatly depend on the route of

inoculation of the virus.[30-32] Sole infection of the trachea, as determined histologically, was typically seen after intratracheal, intraocular or intranasal inoculation whereas aerosol exposure often involved the air sacs and lungs.[30,31] Embryo vaccination seems to correspond with the aerosol route of exposure.

Although IBV infection of embryos at 10-12 days of embryonation produced lesions of stunting and urate deposition in the mesonephros,[33] chickens given P_{40}-IBV or V-IBV on ED18 showed no evidence of kidney lesions. Vaccination of 18-day-old embryos with IBV induced tracheal and pulmonary lesions that were similar to those previously described in birds infected with IBV at hatch.[30,31,34] The lesions in EV chickens, as in chickens given modified live commercial IBV vaccines at hatch, were transitory. In addition, the nature of lesions seen at 17 days PI indicated that the lesions were in the process of being resolved. The lack of detectable lesions in chickens given P_{40}-IBV at hatch supported our previous observation that this virus replicates poorly if given at hatch.[21]

The tracheal lesions detected by TEM and SEM in EV chicks were similar to those previously noted with pathogenic IBV.[35,36] This study represented the first attempt to examine lungs by TEM following IBV inoculation. Examination by TEM of the lungs of chickens given V-IBV on ED18 revealed extensive pathological alterations. The lesions consisted of disruption of the epithelial lining of

the pulmonary airways characterized by deciliation, necrosis, ulceration and inflammation. The presence of extensive TEM lesions in the lungs of V-IBV but not P_{40}-IBV birds inoculated on ED18 confirmed previous data[21] that V-IBV is highly pathogenic when injected on ED18.

Embryo vaccination with P_{40}-IBV did not cause immunodepression in chickens detectable by mitogenic response of whole blood to PHA or NK cell reactivity of spleen effector cells. Vaccination at hatch with live or inactivated IBV vaccines has been previously shown not to reduce mitogenic response of chickens.[37,38] Inoculation of chickens with virulent C-IBV caused significant depression of mitogenic response of whole blood cells. Embryo vaccination with P_{40}-IBV or vaccination at hatch with V-IBV prevented this immunodepressive effect.

Specific immunity generated by cytotoxic T cells against virus infected target cells has proven to be an important mediator of recovery from certain viral infections.[39-42] Virus infected target cells may be destroyed early in infection by cytotoxic T cells, thus preventing formation of infectious virions and subsequent cell to cell transfer of infection.[40] In this study, an *in vitro* ^{51}Cr-release assay was developed using IBV infected target cells and concerted efforts were made to detect cytotoxic cells in chickens following vaccination with IBV. Cytotoxic reactivity against allogeneic or syngeneic virus-infected target cells was not detected following vaccination

or challenge. These preliminary data suggested that development of cytotoxic effector cells may not be an important aspect of the response of chickens to infection with IBV.

Previous reports have shown that serum neutralization antibodies against IBV first appeared at 2 weeks PI, increased steadily until 3 weeks PI, and declined after 5 weeks PI.[43,44] In this study, chickens vaccinated on ED18 with P_{40}-IBV or at hatch with V-IBV followed a similar general pattern of antibody development. There were no appreciable differences in chronology or levels of antibody between chickens vaccinated as embryos or at hatch. The anamnestic antibody response in both vaccinated groups was also of a similar magnitude. Thus, humoral immune response of chickens to embryo vaccination was quite comparable to the response seen routinely following vaccination at hatch.

We have shown previously that embryo vaccination with P_{40}-IBV resulted in protection against challenge with virulent IBV.[21] The magnitude of protection was similar to that obtained by vaccinating chickens with commercial IBV vaccines at hatch. Results of this study indicated that embryo vaccination with P_{40}-IBV did not cause immunodepression in chickens and induced humoral immune response comparable to the response elicited by a commercial IBV vaccine given at hatch. Thus, embryo vaccination may be an effective alternative method of vaccination to protect chickens against IB.

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SUMMARY

The purpose of this research was to 1) find a suitable infectious bronchitis virus (IBV) vaccine for use in eighteen day old embryos, 2) compare the immune responses in IBV embryo vaccinated (EV) chicks with those vaccinated against IBV at hatch, 3) compare the pathogenesis of IBV in EV chicks with those vaccinated at hatch and 4) explore the feasibility of combining IBV and the turkey herpesvirus (HVT) vaccine against Marek's disease (MD) as a bivalent embryo vaccine.

Initially chicks were vaccinated on embryonation day (ED) 18 with a commercial vaccine strain of IBV (V-IBV). The V-IBV was found to be highly pathogenic for embryos and required alteration by serial passage through chicken kidney tissue culture. At passage level forty (P_{40}-IBV), the pathogenicity of V-IBV was sufficiently reduced for embryos while still retaining immunogenicity. Chicks vaccinated with P_{40}-IBV on ED18 acquired anti-IBV antibody and resisted challenge with virulent IBV (C-IBV) at 4 weeks of age. While P_{40}-IBV protected chicks when administered on ED18, this virus did not protect well if given at hatch. Embryo vaccination with P_{40}-IBV also protected chicks bearing maternal antibodies to IBV.

Embryo vaccination with P_{40}-IBV or vaccination with V-IBV did not cause permanent impairment of phytohemagglutinin (PHA) mitogenic response of whole blood cells or of natural killer (NK) cell reactivity of spleen effector cells. Both V-IBV hatch vaccinated and P_{40}-IBV EV birds mounted similar primary and anamnestic antibody responses. In vitro effector cell cytotoxicity to virus-infected target cells was not detected in chickens vaccinated on ED18 with P_{40}-IBV or at hatch with V-IBV and subsequently challenged with C-IBV.

Histologic, transmission electron microscopic (TEM) and scanning electron microscopic (SEM) evaluation of lung and tracheal sections from chickens given P_{40}-IBV on ED18 demonstrated transient mild to moderate tracheal and pulmonary lesions. The tracheal lesions were characterized by deciliation, occasional foci of flattened, simplified squamous epithelium and rarely, a mild, mixed inflammatory cell infiltration accompanied by an acellular exudate. Small foci of increased interstitial cellularity and occasional areas of necrosis, mixed inflammatory cell infiltration and exudation into the lumens of tertiary bronchioles, atria and air capillaries were observed in the lung. Birds vaccinated with V-IBV at hatch had similar tracheal lesions.

When combined with HVT, P_{40}-IBV given on ED18 did not interfere with the protection against challenge with virulent MD virus, nor did the presence of HVT interfere

with the protection afforded by P_{40}-IBV. Thus, IBV vaccine may be combined with HVT as a bivalent embryo vaccine.

In conclusion, the original purpose of this research has been satisfied. Embryo vaccination with IBV has proven to be successful under laboratory conditions and the efficacy, immunologic effects and pathologic effects of the vaccine are similar to those obtained with commercial IBV vaccination after hatch. Since the research presented here was conducted under laboratory conditions, the performance of an IBV embryo vaccination in a commercial setting remains to be determined. The commercial use of the embryo vaccination procedure is attractive not only because of early post-hatch resistance, which may be critical in diseases like MD, but also because several vaccines may be combined and given in one injection in the embryo. This saves the cost of labor necessary to give each vaccine individually. Thus, the embryo vaccination technique may be an attractive alternative to vaccination after hatch.