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Studies on the immune response to Newcastle disease virus in poultry

by

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A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Microbiology, Immunology and Preventive Medicine
Major: Veterinary Microbiology

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1996
This dissertation is dedicated to my wife,
Kholoud Maraqa
Thank you for your love, prayers and encouragement.
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GENERAL INTRODUCTION

Newcastle disease (ND) is an important viral disease of poultry which is a major economic concern. It has world-wide distribution and affects a variety of avian species. The disease is characterized by marked variation in morbidity, mortality, clinical signs and lesions (Alexander, 1991). Newcastle disease is prevented and controlled by vaccination and/or by quarantine and slaughter of diseased flocks in confirmed outbreaks (Alexander, 1991). Various types of Newcastle disease virus (NDV) vaccines, including live attenuated NDV strains, inactivated NDV strains and recombinant vaccines have been used to immunize birds (Glisson and Stantley, 1993). The relationship between the immune response that is stimulated by vaccination and protection is not clearly established. Some studies have indicated that both antibody mediated immunity and cell mediated immunity (CMI) play roles in protection against ND (Ghumman and Bankowski, 1976; Meulemans et al., 1986). Serological antibodies to NDV have been commonly used for the evaluation of ND vaccination and have been related to protection against virus challenges (Beard and Brugh, 1975). Antibodies directed towards the surface glycoproteins HN and F have been shown to play a role in neutralizing NDV *in vitro* and *in vivo* (Meulemans et al., 1986). However, a lack of correlation between the serological NDV antibody titer and protection from challenge has been reported (Gough and Alexander, 1973). In addition, with regards to protection, the role of antibodies directed against other NDV proteins has not been determined.
Cell mediated immunity has been demonstrated to be the first immunological response following ND vaccination and has been suggested to play a key role in protection (Cannon et al., 1988; Ghumman and Bankowski, 1976). It has been shown that early protection following vaccination can be demonstrated in the presence of low levels of antibody or in the absence of detectable antibodies (Allan and Gough, 1976; Ghumman and Bankowski, 1976; Gough and Alexander, 1973). Although the CMI response to NDV has been demonstrated in vaccinated birds with ND vaccine (Ghumman and Bankowski, 1976; Marino and Hanson, 1987), little is known about its role as a key component in protection. The focus of this research was to delineate the role of CMI and humoral immunity in protection against NDV.

**Dissertation Research Objectives**

The primary purpose of this research was to evaluate the role of cellular and humoral immune responses to NDV in relation to protection. The specific objectives of this study were to: (1) develop and implement methodology to evaluate specific NDV cell-mediated and humoral immune responses in birds; (2) assess the role of CMI to NDV in protection; and (3) assess the role of antibodies to NDV proteins in protection.

**Dissertation Organization**

This dissertation consists of a general introduction, a review of the literature, four manuscripts, a general summary, literature cited and acknowledgments. The format of each manuscript is presented in the style of the journal *Avian Diseases*, and is comprised of a summary, introduction, materials and methods, results, discussion, references, and
acknowledgments. Data and results of the statistical analysis for each study are appended at
the end of each manuscript. The references cited in the general introduction, literature review,
and the general summary are listed following the general summary. The Ph.D. candidate,
Anwar Maraqa, was the principal investigator for each study.
LITERATURE REVIEW

Newcastle Disease

Newcastle disease (ND) is an infectious disease affecting domestic and wild birds of any age. It is one of the most serious diseases of poultry throughout the world (Alexander, 1991).

History

Newcastle disease was first recognized in Java, Indonesia, in 1926. At about the same time, a disease with the same symptoms was observed in England, where it was recognized in Newcastle by Doyle, hence the name. Within ten years, the disease had spread to middle Korea, Japan, India, the Philippines, East Africa, and Australia. In 1930 a relatively mild-respiratory disease in chickens was reported in California, USA. This disease was first called pneumoencephalitis but later was shown to be caused by a virus which was indistinguishable immunologically from Newcastle disease virus (NDV) (Alexander, 1991). Within a few years numerous NDV isolations that produced either a mild or no disease were made around the world. Such isolates were used later as live vaccines. Newcastle disease is now recognized in most of the major poultry producing countries. Its virulent form is one of the most devastating diseases of poultry, causing 100% mortality in chickens (Kouwenhoven, 1993). No accurate estimate is available on the cost of disease to the commercial industry. However, the cost would include deaths and loss of production due to clinical disease, the total cost of
purchasing and applying vaccines, the cost of eradication and quarantine programs and the
loss of trade (Kouwenhoven, 1993).

**Etiologic agent**

**Classification.** Newcastle disease virus is classified in the paramyxoviridae family, which includes a number of important human and animal pathogens. The viruses in this family are grouped into three genera. The genus Morbillivirus consists of measles, rinderpest and canine distemper viruses. The Pneumovirus genus consists of the mammalian respiratory syncytial viruses, the mouse pneumonia virus, and an avian pneumovirus associated with turkey rhinotracheitis. The Paramyxovirus genus consists of parainfluenza virus type 1 (Sendai virus), parainfluenza virus type 2 (Simian virus 5), parainfluenza virus type 3 (shipping fever virus), parainfluenza virus type 4, mumps and nine distinct groups of avian paramyxoviruses termed PMV-1 to PMV-9 (Nagai et al., 1989). Newcastle disease viruses are grouped in the avian paramyxovirus type 1 (PMV-1) serotype on the basis of their antigenic relationship in serological tests. Some serological relationships have been demonstrated between NDV and other paramyxovirus serotypes, the most significant being the viruses of the PMV-3 serotype (Alexander, 1991).

**Properties of the virion.** Newcastle disease virus is an enveloped virion of pleomorphic shape ranging in size from 100-300 nm in diameter. The envelope is a lipid bilayer derived from the host plasma membrane and has spike glycoproteins embedded in and
protruding from it (Nagai et al., 1977). There are two sizes of surface projections, the longest (about 8 nm) consisting of a single glycoprotein with both hemagglutination and neuraminidase activities associated with it. The smaller glycoprotein is associated with the ability of the virus envelope to fuse with cell membranes and to cause the fusion of infected cells. All avian paramyxoviruses have a herring-bone nucleocapsid of about 18 nm in diameter and a pitch of 5 nm, which may be seen either free or emerging from disrupted virus particles (Alexander and Collins, 1981; Nagai et al., 1989; Schied et al., 1972).

**Properties of the genome.** The genome of NDV is a linear, single-stranded RNA of about $5 \times 10^6$ dalton in molecular weight with a negative polarity. Nucleotide sequencing of the NDV genome has shown it to consist of 15,156 nucleotides. Genes are arranged in the genomic RNA in the following order: 3'NP-P/V-M-F-HN-L. There is a leader sequence at the 3' end comprised of 53 nucleotides, which is not translated into protein. (Matsumor, 1982; Miller and Emmerson, 1988; Nagai et al., 1989). There are two consensus sequences, S and E, at the start and end of each gene, which are thought to signal the transcription initiation and polyadenylation, respectively (Nagai et al., 1989).

**Properties of the virus proteins.** The Newcastle disease virus genome encodes the following seven proteins: the nucleoprotein (NP), phosphoprotein (P), V protein (V), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large (L) proteins (Alexander and Collins, 1981; Nagai et al., 1989). The hemagglutinin-neuraminidase (HN) and fusion (F1, 2) glycoprotein are glycosylated and form spiked projections on the lipoprotein envelope of the
virion (Schied et al., 1972). The hemagglutinin-neuraminidase (HN) is the larger NDV glycoprotein and contains both hemagglutinin and neuraminidase activities. It is responsible for the initial attachment of the virus particles to the host cell receptor that contains neuraminic acid. The virus, however, elutes rapidly by the action of neuraminidase, even at low temperatures (4°C). Thus, the pattern of hemagglutination, which is caused by the virus binding to the receptor on red blood cells (RBC), is unstable (Nagai et al., 1989). This property of agglutinating RBCs, and the specific inhibition of agglutination by antiserum has proven to be a useful tool in the diagnosis of disease (Alexander, 1991). The role of neuraminidase (NA) in inducing virus fusion was addressed by Huang et al. (1980). The researchers suggested that the role of NA is to remove neuraminic acid from the primary cellular receptor following adsorption which allows the cellular and viral membranes to come closer together and thus permits membrane fusion. Neuraminidase is also considered responsible for removing neuraminic acid from glycoproteins and glycolipids synthesized in NDV infected cells (Nagai et al., 1976).

Fusion (F) protein is a small glycoprotein involved in virus penetration or fusion through the host cell membrane or between the plasma membrane of two cells, infected and uninfected (Scheid et al., 1974). The fusion protein is considered to be the major determinant of the wide-ranging virulence of NDV strains. It is synthesized as a precursor, F0, which is subsequently cleaved by cellular proteases into F1 and F2, and both are held together by disulfide linkages (Scheid and Choppin, 1981). The cleavage generates a new N-terminus of F1 which is hydrophobic and conserved among paramyxoviruses. The fusion characteristics of
the paramyxoviruses are driven by the interaction of this hydrophobic region with the lipid bilayer of the target membrane (Richardson et al., 1980). The importance of the F-cleavage event with respect to host range and tissue tropism was indicated by Nagai et al. (1977) who examined the F glycoprotein synthesized in a variety of host cell systems infected with virulent and avirulent strains. With virulent NDV strains, the cleavage of F0 occurred in all host systems analyzed. Whereas, with avirulent strains, cleavage occurred only in embryonated hens' eggs, or in cultures of chorioallantoic membrane cells, or cell cultures with trypsin. Thus, F0 molecules of virulent viruses can be cleaved by a wide range of proteases. However, F0 molecules in viruses of low virulence were restricted in their sensitivity and these viruses grew only in certain host cells (Scheid and Choppin, 1974).

The M protein is located on the inner surface of the envelope and provides structural integrity to the virion. It has a crucial role in virus assembly by locating nucleocapsid structures beneath those regions of the plasma membrane in which the F and HN glycoproteins are anchored (Mastsumor, 1982).

Nucleoprotein (NP), phosphoprotein (P), Large (L) protein and (V) protein are associated with the genomic RNA to form a ribonucleoprotein complex, termed the nucleocapsid. The NP is the structural subunit of the nucleocapsid and confers helical symmetry on the capsid. It is the most abundant viral protein and is responsible for control of genomic replication (Hamaquchi, 1983). The phosphoprotein (P) is required for viral RNA synthesis (Hamaquchi, 1983). The P gene is transcribed to give two different mRNAs. One is a copy of the P gene and leads to normal P protein production. The other has an extra
guanosine residue inserted during transcription which leads to production of V protein (Nagai et al, 1989). The Large (L) protein has an extremely high molecular weight of > 200 Kd. It is the least abundant NDV viral protein and is assumed to play a role, together with the P protein, in viral transcription and replication. In addition, the L protein may also be involved in methylation, capping, phosphorylation and polyadenylation (Hamaquchi, 1983).

Replcation. Newcastle disease virus generates three different RNA products during its infectious cycle, namely mRNA, full length antigenomic RNA and genomic RNA. Immediately after penetration, the viral transcriptase complex (L and P proteins) binds to the template at the 3' end of the genome and sequentially synthesizes the NP, P/V, M, F, HN, and L mRNAs. The transcription is discontinuous by terminating and restarting transcription at each of the gene junctions (Hamaquchi et al., 1983). Thus, NP mRNA is the most abundant while L mRNA is the least, and this is reflected in the quantities of protein synthesized (Nagai et al., 1989). The components of the virus protein are assembled at the host cell membrane and the mature virus is released by budding (Nagai et al., 1976).

Cultivation. All NDV strains replicate in embryonated chicken eggs, but vary in their capacity and time taken to kill chick embryos. Virulent strains spread quickly throughout the embryo and kill it, while less virulent strains kill embryos more slowly or not at all (French et al., 1967). The time required by an isolate to kill embryos (the mean death time, or MDT) is related to its pathogenicity. Thus, an indicator of lentogenic, mesogenic and velogenic viruses can be made by MDT. Virus titers are also influenced by strain, with the highest titers
obtainable by those avirulent strains (Hanson et al., 1974). Newcastle disease virus strains can also replicate in a great variety of cells, of both avian and mammalian origin. Infection is usually lytic, causing typical cell-rounding (Lancaster, 1966; Reeve et al., 1971; Rott, 1979), however, different cell types exhibit marked differences in cytopathic formation. BHK-21 cells, a baby hamster kidney cell line, display a cytopathic effect (CPE) as a giant syncytium, while MDBK cells, a bovine kidney cell line, exhibit CPE as rounding with no syncytium at all. Furthermore, the infection of L cells, a mouse fibroblast cell line, results in limited or abortive replication with reduced yield and cytopathogenicity (Wilcox, 1986). As a consequence, the cell may survive the infection but it will become persistently infected.

The disease

**Hosts.** Newcastle disease has been shown to occur in a variety of avian species. Over 200 species of birds have been reported to be susceptible to natural or experimental infections of NDV. Chickens, turkeys, pigeons and pheasants are more susceptible to NDV than ducks and geese, which tend to show few signs of disease even when infected with the most virulent chicken pathotypes of NDV (Alexander, 1991). A wide range of captive and free-ranging semidomestic and free-living birds, including migratory waterfowl, are susceptible and can be primary sources of infection (Kouwenhoven, 1993). Occasionally humans become infected with NDV. Newcastle disease virus infection in humans usually causes conjunctivitis but sometimes laryngitis, pharyngitis, and tracheitis occur (Kouwenhoven, 1993).
Transmission. Chickens infected with NDV shed the virus into the environment from 1 to 3 weeks following exposure (Hanson, 1974). Newcastle disease virus has been isolated from samples taken from swabs of oropharyngeal, tracheal, or cloacal excretion (Hanson, 1974). Virus containing excretions including aerosols can contaminate feed, water, clothing, equipment and other objects. Exposure of susceptible birds to any of these sources of virus can result in transmission (Tumova et al., 1979). True vertical transmission has not been documented, but disease in young chickens may originate from eggs laid by infected parents, where the virus contaminates the shell (Lancaster and Alexander, 1975). Newcastle disease virus can also cause persistent infections and has been recovered from the tracheas of infected birds at 14, 40 and 120 days following infection (Heuschele and Esterday, 1970; King, 1983).

Pathogenesis. Newcastle disease virus usually enters the body via the respiratory and/or the intestinal tract. Following multiplication of the virus at the site of introduction, it spreads rapidly to the bloodstream where the virus is carried by the blood to different organs (Alexander, 1988). The spread of the virus largely depends on the strain virulence. While lentogenic strains are present only at low titers in the circulation, mesogenic strains spread to the kidneys, lungs, bursa, and spleen. Virulent NDV can be found within 22-44 hours in nearly all tissues, with the highest titers in the thymus and lowest titers in the muscles and brain (Sing and El-Zein, 1973). During the second replication, the virus titer increases again and the virus is released into the bloodstream. This is associated with the appearance of general disease signs and virus excretion into the environment via feces and respiratory secretions. Virulent
neurotropic viruses infect the central nervous system at the same time as the respiratory and intestinal tract. Viruses pass the blood brain barrier at an accelerated rate causing severe damage to this barrier (Kouwenhoven, 1993).

**Pathotype and pathogenicity.** The disease caused by PMV-1 is characterized by marked variation in morbidity, mortality, clinical signs and lesions which depends on the age of the host, infection route, virulence of the strains, virus dose, co-infection with other organisms, environmental stress, viral tropism and host immune status (Alexander, 1988). In general, the younger the chicken the more acute the disease. Natural routes of infection (nasal, ocular, and oral) appear to emphasize the respiratory nature of the disease while intramuscular, intravenous, and intracerebral routes appear to enhance the neurological signs (Beard and Easterday, 1967). The clinical signs that have been associated with ND are: respiratory distress; diarrhea; nervous signs; depression; edema of the head, face, and wattle; and a sudden decrease in egg production together with depigmentation and loss of egg shell and albumen quality in layers and sudden death.

Beard and Hanson (1984) grouped NDV strains and isolates into five pathotypes that relate to clinical signs of the disease: (1) viscerotropic velogenic Newcastle disease form (VVND) - produces an acute lethal infection in which hemorrhagic lesions are prominent in the digestive tract; (2) neurotropic velogenic Newcastle disease form (NVND) - causes an acute often lethal infection of chickens of all ages and has been characterized by acute respiratory and nervous disorders; (3) mesogenic form - is less virulent and may cause
mortality occurring in young chickens; (4) lentogenic form - produces mild or nonapparent respiratory infections, and commonly used as live vaccines; and (5) asymptomatic enteric NDV - an avirulent virus that appears to replicate primarily in the intestinal tract.

**Diagnosis.** Since clinical signs of NDV are relatively nonspecific, a diagnosis must be confirmed by virus isolation and serology (Alexander, 1991). Virus isolation can be made by inoculation of infectious material into the allantoic cavity of embryonated eggs. The virus may be isolated from the spleen, brain, or lungs. The presence of the virus can be detected by hemagglutination and can be differentiated from other viruses by hemadsorption and a hemagglutination inhibition (HI) test using specific antisera. Determination of virulence is essential for evaluating field isolates since lentogenic strains in feral birds are widespread and lentogenic strains are commonly used as live vaccines. The mean death time in embryonated eggs, the intracerebral pathogenicity index (ICPI) in 1-day-old chicks, and the intravenous pathogenicity index (IVPI) in 6-week-old chicks are currently used for virulence assessment. The ICPI does not distinguish viruses of high virulence. The IVPI is useful for classifying moderately and highly virulent NDV but does not differentiate between low virulence viruses. Other techniques have also been used to distinguish between various strains of NDV such as plaque type and size, hemagglutinin elution rate, structural polypeptides, oligonucleotide fingerprints, and monoclonal antibody typing (Alexander, 1991).

**Serology.** Most serological techniques used for the detection of antibodies to viruses have been applied to NDV. The hemagglutination inhibition test (HI) has been the
conventional method of choice, but in more recent years several enzyme-linked immunosorbent assay (ELISA) tests have been developed and used widely for flock profiling. Virus neutralization, single radial immunodiffusion and agar gel precipitation tests have also been used for measuring antibodies to NDV (Alexander, 1991).

Prevention and control

Most countries have regulations directed at preventing the introduction of ND by quarantining infected birds or contaminated products. Restrictions may also be imposed to prohibit the movement of poultry within specified areas (Glisson and Stantley, 1993).

Vaccination. Basically, there are three types of commercially available vaccines for ND: live vaccines, inactivated vaccines and recombinant vaccines (Glisson and Stantley, 1993). Types of NDV vaccines and route of administration (eye drop, spray, drinking water and/or injection) vary depending on several factors. These factors include the level of maternal immunity, the virulence of the endemic NDV of a geographic location, the time between two successive vaccinations, and the lifespan of the birds (Alexander, 1988).

Live NDV vaccines. Live vaccines can be divided into two categories based on virulence. More virulent vaccines are classified as mesogenic strains while less virulent strains are classified as lentogenic strains. Four mesogenic strains (Herts, Mukteswar, Komarov, and Roakin) have been used in many parts of the world but presently their use is restricted to areas in Africa, Southeast Asia and the Middle East where endemic velogenic
NDV is prevalent. These vaccines are pathogenic and are not recommended for use in chickens less than 8 weeks of age, or older chickens that have not been previously vaccinated against NDV (Meulemans, 1988).

Live lentogenic NDV vaccines are the most widely used. They have been shown to have a low pathogenicity in poultry while producing an adequate immune response. Four lentogenic NDVs (LaSota, Hitchner B1, F and V4) have been developed as vaccines. Of these, B1 and LaSota are the most widely used. Both of these viruses replicate in the respiratory tract and induce local and systemic immunity. LaSota vaccines are more virulent and induce a stronger immune response than B1 vaccines. Because of the greater potential for LaSota vaccines to cause respiratory disease, they are normally used for boosting NDV vaccines in chickens previously immunized with B1 (Eidson and Kleven, 1980).

More recently, a non-pathogenic NDV has been developed for vaccine use in the USA. The VG/GA strain was isolated from commercial turkeys and found to provide protection in chickens against the lethal challenge of velogenic viscerotropic NDV (VVNDV), similar to that provided by the B1 strain (Beard et al., 1993).

Inactivated vaccines. Inactivated oil emulsions of ND vaccines are found to provide uniform and long lasting protection without post-vaccinal respiratory reactions. Since inactivated vaccines must be injected, they are used primarily to vaccinate breeders and layers that have been vaccinated previously with one or more live NDV vaccines. The humoral
immune response to inactivated NDV in breeders and layers is very high and of long duration, and provides a high level of maternal antibodies for their progeny (Eidson et al., 1982).

**Recombinant NDV vaccines.** The recombinant fowlpox virus, which expresses the HN and/or F NDV proteins, has been found to provide protection against virulent NDV challenge when it is inoculated intravenously, intramuscularly or by wingweb in birds without pre-existing antibodies. However, only partial protection is achieved when the vaccines are administered orally or ocularly (Edbauer et al., 1990; Taylor et al., 1990). Furthermore, recombinant vaccines expressing the HN and/or F glycoprotein have also been reported with a vaccinia virus (VV) (Meulemans et al., 1988), a herpes virus of turkeys (HVT) (Morgan et al., 1992), and a baculovirus (Nagy et al., 1991). Recently, a recombinant fowlpox has been licensed and recommended for use with birds having no pre-existing antibodies to NDV.

**Immunity to Newcastle Disease**

**Humoral immunity**

The role of humoral immunity against lethal infections of chickens with NDV has been reported (Umino et al., 1987; Vasington et al., 1960; Wills and Luginbuhl, 1963). It has been found that chickens inoculated with egg yolk or plasma from hyperimmunized birds raised against NDV were protected when challenged after three weeks of administration (Vasington et al., 1960; Wills and Luginbuhl, 1963). The same protection was found when 9-day-old
chickens were passively immunized with hyperimmune serum against NDV (Umino et al., 1987). Hyperimmune serum was also found to reduce mortality when injected 72 hours after infection (Umino et al., 1987).

The role of antibodies in early protection following vaccination against NDV has also been reported. Khare et al. (1976) reported that protective immunity against NDV is due primarily to antibodies. Circulating antibodies protect the host from reinfection, starting as early as four to six days after infection. In the early phase of vaccination IgM is involved and followed by IgG. Titers and protection peak three weeks after vaccination, and then gradually decline if there is no boosting. A number of reports have indicated that concurrent outbreaks of infectious bursal disease virus (IBDV), which has an immunosuppressive effect in chickens, reduces the serological response to ND vaccination as measured by the hemagglutination-inhibition (HI) test. This has also been shown to increase the incidence of ND and prolonged virus excretion (Allan et al., 1972; Faragher et al., 1974; Pattson and Allan, 1974).

**Maternally derived antibodies.** Immunoglobulins in birds are deposited into the egg yolk and chicks absorb these antibodies into their developing systems. The yolk contains mainly IgG which becomes the circulating antibody in the chick, while the albumen contains predominantly IgA which is swallowed by the developing chick, thus coating its mucous membranes with the IgA (Brierly, 1956; Heller et al., 1977). The levels of passively acquired maternal antibodies in the serum of a day-old chick are approximately the same as those found in the serum of the hen (Allan et al., 1978). The level of passively acquired maternal
antibodies found in young chicks generally declines at a constant rate, and has a half-life of approximately 4 1/2 days (Allan et al., 1978). Maternal immunoglobulins (IgG) to NDV have also been detected in tears, the trachea and lungs (Russell and Koch, 1993).

Maternal antibodies play an important role in protecting the chick early in its life. Umino (1987) demonstrated that chickens hatched from eggs laid by hens vaccinated with live attenuated NDV possessed antibodies to the various components of the virus and were resistant to challenges with lethal doses of virulent strains of NDV administered intramuscularly. The maternal antibodies were found to interfere with active immunization by sequestering vaccine antigens or restricting the replication of vaccinal viruses (Beard and Brugh, 1975).

Local immunity. The development of a local antibody immune response to NDV has been demonstrated in both the respiratory and intestinal tracts. After vaccination, both IgA and IgG specific to NDV have been found in the trachea, lungs, saliva, lacrimal fluid, the Harderian glands and the intestine (Powell et al., 1979; Russell and Koch, 1993). The administration of NDV vaccines by the intra-ocular route results in the development of high levels of IgM, IgG and IgA antibodies in the Harderian gland and in lacrimal fluids due to NDV replication in the Harderian gland (Russell, 1993).

The importance of a local antibody immune response in protection against infection by NDV has been indicated by a lack of correlation between serum antibody concentrations and resistance to challenge (Beard and Easterday, 1967). It has been reported that birds with large
quantities of passively administered antibodies failed to protect the tracheal epithelium upon aerosol challenge (Beard and Easterday, 1967). On the other hand, it has also been demonstrated that newly hatched chicks possessing maternal antibodies when immunized by eye-drop, were protected against intranasal challenge as effectively as antibody-free chickens similarly immunized and challenged (Powell and Aitken, 1979). Furthermore, the vaccination of chickens via the airsacs protected them against airsac challenge but not against intraocular challenge (Malkinson and Small, 1977). These results suggest that local immunity may be operating in the respiratory tract independently of systemic immunity (Holems, 1979).

Neutralizing antibodies, mainly IgA and IgG, were found in the bile of vaccinated birds (Lee and Hanson, 1975). These antibodies, together with an inhibitory effect of the low pH in the gizzard, were found to protect the birds from repeated low levels of ingested virulent virus (Kohn, 1958).

The role of NDV polypeptides in protection. Antibodies directed to the surface glycoproteins (HN and F) have been shown to play a major role in neutralizing NDV \textit{in vitro} and \textit{in vivo} (Merz et al., 1981; Meulemans et al., 1986). Antibodies to the HN glycoprotein were found to inhibit attachment to the cell host receptor, and inhibit adsorption to erythrocytes and neuraminidase activities (Merz et al., 1981). Seven antigenic sites on the HN have been identified. Three of the antigenic sites have been found to overlap the other sites. Antibodies to each site exhibit specific neutralizing capacities of that site (Iorio et al., 1984, 1989). Meulemans (1986) reported that MAbs with a high virus neutralizing activity directed
to one antigenic site delayed virus growth and significantly prolonged survival time, even though all the chickens succumbed to infection; however, a combination of these monoclonal antibodies neutralized the lethal NDV infection.

Antibodies directed to the F glycoprotein were found to inhibit the hemolysis of chicken erythrocytes and neutralize the effectiveness of the virus (Abenes et al., 1986; Avery and Niven, 1979; Merz et al., 1981). Merz et al., (1981) demonstrated the effectiveness of anti-F antibodies in vitro in neutralizing paramyxovirus infectivity when the virus and antibodies were mixed before infection. The same result was found when anti-F sera were added after infection because these antibodies were capable of preventing the spread of infection from cell to cell (Merz et al., 1981). Abenes et al. (1986) identified four antigenic sites (I-IV) on the F protein molecule. Monoclonal antibodies directed to sites I, II and III inhibited both virus-induced hemolysis of chicken erythrocytes and syncytium formation of BHK-21 cells. Umino et al. (1990) reported that MAbs directed to two antigenic sites of the F protein completely suppressed virus growth and prevented the death of chickens, although the neutralizing activities of these anti-F MAbs were lower than those of anti-HN MAbs. Similar findings have also been reported by Meulemans et al. (1986) who demonstrated that antibodies directed against the F protein of NDV were more capable of protecting chickens against viral challenge than HN antibodies.

The importance of two glycoproteins (HN and F) as a major target of humoral immunity has also been examined by using recombinant Newcastle disease vaccines. Protection studies have indicated that 1-day-old-birds with no prior immunity to NDV can be
protected against velogenic NDV challenge by vaccination with a recombinant fowlpox virus (FPV) (Boursnell et al., 1980; Edbauer et al., 1990; Taylor et al., 1990), a vaccinia virus (VV) (Meulemans et al., 1988), a herpes virus of turkeys (HVT), or a baculovirus (Nagy et al., 1991) expressing the NDV HN, and/or F polypeptides (Morgan et al., 1992). The antibody titers induced by these recombinant vaccines were low as measured by the HI assay and the ELISA (Edbauer et al., 1990; Taylor et al., 1990).

**Cell mediated immunity**

Cell mediated immunity (CMI) and protection from NDV have also been reported (Agrawal and Reynolds, 1991; Cannon and Russell, 1988; Ghumman and Bankowski, 1976; Lam and Hao, 1987; Marino and Hanson 1987; Perey et al., 1975; Timms and Alexander, 1977). Ghumman and Bankowski (1976) used a lymphocyte blastogenic transformation assay to measure the CMI of chickens. Lymphocyte proliferation occurred as early as the 2nd day after a primary or secondary vaccination. It was also found that the degree of lymphocyte stimulation varied with the antigen used for vaccinating the birds. The highest and earliest responses were found in groups vaccinated with live NDV via the intramuscular route, whereas the lowest response was detected in groups previously vaccinated by the aerosol route. Antibody response in this study was detected five days after vaccination. Ghumman and Bankowski concluded that there was no correlation between the CMI and HI antibody response.
Cutaneous delayed hypersensitivity reactions were observed in chickens after ND vaccination and challenge. These reactions were diminished during clinical ND which was attributed to the extensive damage of macrophages and lymphoid cells, a characteristic of virulent NDV infections (Cheville and Beard, 1972; Turner et al., 1979).

Timms and Alexander (1977) used a leukocyte migration inhibition test (LMI) to assess CMI and its relationship to humoral response following vaccination with both live and inactivated ND vaccines. The highest LMI values appeared in birds that had received live vaccines while the lowest LMI values were found in birds not vaccinated. While the response was found to be higher in birds vaccinated with live vaccines than those vaccinated with inactivated vaccines, it was diminished when birds were challenged with live viruses. There was no correlation between LMI values and HI titers. Agrawal and Reynolds (1991) also reported similar findings.

Marino and Hanson (1987) used bursectomized chickens to study the role of humoral and cellular immunity in vaccinated and challenged chickens. Bursectomized birds that had been vaccinated had a lower detectable antibody titer than nonbursectomized vaccinated birds. In contrast, the birds had a significantly higher CMI response as estimated by a blastogenic assay using specific NDV antigen for stimulation. Both vaccinated bursectomized and non bursectomized birds survived the virus challenge, however, the challenge virus was isolated from the majority of the vaccinated bursectomized birds until 16 days after the challenge.
Cell mediated immunity in other viruses

There have been a number of reports regarding the importance of the cellular immune response in controlling other paramyxoviruses (Bennink et al., 1984; Koszinowski and Simon, 1979; Pemberton et al., 1987; Randall et al., 1988). Randall et al. (1988) demonstrated that adoptive transfer of immune splenocytes isolated from mice previously infected with Simian virus 5 (SV5) to immunocompromised mice increased the rate of virus clearance. In contrast, passive transfer of neutralizing monoclonal antibodies against HN and F glycoproteins of SV5 did not have a significant effect on the rate of virus clearance.

The importance of viral glycoproteins as a major antigen for inducing CMI has also been demonstrated with the Sendai virus (McGee et al., 1980), respiratory syncytical virus (Pemberton et al., 1987), influenza virus (Bennink et al., 1984) and measles virus (Wild et al., 1990). In most cases, cytotoxic T lymphocytes (CTLs) were elicited when the viral proteins were incorporated into the plasma membrane. The F and HN proteins of Sendai virus were shown to be capable of inducing CTLs when presented to the host animal or isolated lymphocytes in the phospholipid environment or in a liposome (McGee et al., 1980).

Internal proteins have also been shown to act as target antigens for inducing cellular immune response. Studies on the influenza virus (Townsmed et al., 1984), Sendai virus (Melief and Kast, 1987) vesicular stomatitis virus (Yewedell et al., 1986), rabies virus (Dietzschold et al., 1987) and the measles virus (Stewn et al., 1987) have shown that ribonucleoproteins can induce protective immunity. Evidence has been presented by Melief and Kast (1987) that the primary line of defense against Sendai virus infection is mediated by
CTL by recognizing the internal NP protein. The immunodominant polypeptide of the NP of the Sendai virus has been identified and used successfully to vaccinate mice against virulent Sendai virus infection. In addition, the immunization of immunocompetent mice with solid matrix antibody antigen (SMAA) complexes, containing either surface proteins or internal virus structural proteins, has been shown to induce cell-mediated immune responses that enhance the speed of viral clearance. Immunization with SMAA complex (exogenous antigen) was found to induce both humoral and cytotoxic T-cell (CD8\(^+\)) responses (Randall et al., 1988). Adoptive transfer experiments of splenocytes isolated from mice immunized with internal structural proteins to immunocompromised mice have demonstrated that the virus could be cleared in the absence of detectable levels of serum neutralizing antibodies (Randall et al., 1988).

Cyclophosphamide

A tumoricidal agent, cyclophosphamide (CY), has a nonspecific immunosuppressive effect. Cyclophosphamide acts as an alkylating agent, causing damage to nucleic acids, including breaks in DNA strands, cross-links between two DNA strands and cross-linking between DNA and RNA or protein (Bach, 1975). Linking results in a major disruption of the DNA function and subsequent interference with normal cell mitosis and kills cells undergoing multiplication. Cyclophosphamide appears to be selectively cytotoxic for lymphocytes at intermediate doses (Petrov et al., 1971), but it does have some effect on all rapidly dividing tissues, such as the cells of the bone marrow and gastrointestinal tract (Bach, 1975).
Cyclophosphamide has been used experimentally as an immunosuppressant in numerous studies to determine some aspects of the immune response such as the role of cell mediated immunity in protection (Corrier et al., 1991; Kibenge et al., 1987; Nathanson et al., 1980; Okoye et al., 1992). The susceptibility of B and T lymphocytes to CY has been studied (Eskola and Toivanen, 1974; Lerman and Weidanz, 1970). Early observations in chickens revealed that B cells were affected to a much greater extent than T cells, especially when small doses were used. It has been reported that CY treatment causes suppression of the primary antibody response and a profound deficiency in the humoral immunological function (Lerman and Weidanz, 1970; Truk and Poulter, 1972). Eskola and Toivanen (1974) found that CY given in ovo caused a long-lasting humoral immunodeficiency when administrated on days 14-16 or on days 16-18 of incubation. The suppression was reversible, and the functional and morphological recovery of the antibody producing organs occurred after 10 weeks. However, Abdul-Aziz (1981) observed that the bursa of Fabricious regenerated 35 days after CY treatment. The mechanism of CY action is based on destroying lymphoid cells in both central and peripheral lymphoid organs that have been seeded with bursa-derived cells during embryo development. Cyclophosphamide treatment leaves the bursal reticulum intact and affects only the lymphoid cells. In addition, very few germinal centers and plasma cells are found within the spleen, cecal tonsils or in other lymphoid tissues even after an antigenic stimulation (Eskola and Toivanen, 1974).

When given in large doses, CY could suppress the T-lymphocyte response (Winklestein et al., 1972). Cyclophosphamide destroys the lymphoid cells in the thymic cortex
and, to some extent, causes depletion of the thymus-derived T lymphocytes in the spleen. Stevenson et al. (1980) found that T-helper cells and cytotoxic T cells are more resistant to the action of CY than B cells and suppressor T cells. A larger dose of CY is required to suppress generation of cytotoxic T cells in mice (Ferguson and Simmon, 1978). Delayed hypersensitivity reaction is enhanced due to the elimination of suppresser T cells (Grill and Liew, 1978). Most evidence indicates that CY suppresses cell mediated immunity in chickens temporarily and regeneration occurs within 2 weeks following treatment (Lerman and Weidanz, 1970).
A TECHNIQUE FOR INDUCING B-CELL ABLATION IN CHICKENS
BY IN OVO INJECTION OF CYCLOPHOSPHAMIDE

Manuscript for submission to Avian Diseases
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SUMMARY

The effect of cyclophosphamide (CY) treatment in ovo on avian B and T cells was studied. Cyclophosphamide was injected in ovo on the 16th, 17th and 18th day of incubation. Similarly, control eggs were injected with PBS. Blood samples were collected periodically from CY-treated and non-treated birds and were used to measure blood lymphocyte responses to the T-cell and B-cell mitogens, Con A and LPS, respectively. Additionally, flow cytometric analysis was used to determine the presence of B and T cells in peripheral blood. The results demonstrated that CY treatment reduced hatchability by 35-40%, increased mortality by 3-5% and induced a significant retardation in the gain of body weight. The majority of deaths occurred within two weeks following hatchings. At 2 weeks of age, approximately 50% of CY-treated birds were devoid of B-cell mitogenic responsiveness while demonstrating significant T-cell mitogenic responsiveness. B-cell responses were observed at 4 and 6 weeks from only a small percentage of birds that were originally T-cell responsive and B-cell nonresponsive at 2 weeks of age. Flow cytometric analysis of peripheral blood lymphocytes revealed that CY-treated birds had significantly less B cells (or were devoid of B cells) than
the corresponding nontreated control birds. However, no significant differences in the T-cell percentage were observed between CY-treated and CY-nontreated birds.

Humoral immune response was also evaluated by vaccinating T-cell positive/B-cell negative (CY-treated) birds with Newcastle disease vaccine, and comparing them with non CY-treated control birds. The results revealed that CY-treated birds did not produce detectable antibodies specific for NDV during the first and second week post-vaccination, as demonstrated by HI assay. However, antibodies were detected in a small number of CY-treated birds one week post-booster. Those responding birds were found to be the same birds that had subsequently responded to the LPS mitogen on the blastogenesis microassay. This study indicates the importance of monitoring the B- and T-cell responses in CY-treated birds to identify those birds in which B-cell regeneration may have occurred.

**Keywords:** cyclophosphamide, Newcastle disease virus, humoral immune response, immunosuppressive.

**Abbreviations:**

B  bursa-dependent lymphocyte
CMF-PBS  calcium and magnesium free-phosphate buffered saline
CMI  cell-mediated immunity
CY  cyclophosphamide
Con A  concanavalin A
HI  hemagglutination inhibition
INTRODUCTION

Cyclophosphamide (CY), a tumoricidal agent, is a nonspecific immunosuppressant agent affecting primarily antibody-mediated immunity (7, 8). It has been used experimentally as an immunosuppressant to elucidate some aspects of the immune response such as the role of cell mediated immunity (CMI) in protection (6, 9, 12, 13, 15, 16). In the chicken, CY given in newly hatched birds leads to severe and permanent deficiency in the humoral immune response. Eskola et al. (1974) found that CY given in ovo caused severe humoral immunodeficiency when administered on days 14-16 or on days 16-18 of incubation. The suppressive effect, however, was found to be reversible and the functional and morphologic recovery of antibody producing tissue occurred after 10 weeks following treatment (7, 14). Cell mediated immunity was also found to be initially affected, but regeneration of the T-cell
response occurred more rapidly. T-cell responsiveness was reported to be suppressed for less than two weeks (10, 14).

The CY method for ablating B cells has been shown to be limited by the early recovery of antibody producing organs, thus providing only temporary immunosuppression (1, 12, 15). Schlink (1986) reported that CY is not effective in depleting all the B cells and a high dose of CY with bursectomy is required for ablating B cells from the immune system.

The objective of this study was to better characterize and describe the procedure by which CY induces B-cell ablation when administered in ovo. Such a model may be useful for deriving birds devoid of B cells to determine the protective components of the immune response in vivo against avian pathogens. The procedure was accomplished by treating chick embryos with CY during incubation. After hatching, blood samples were collected and evaluated by flow cytometry, blastogenesis response to B- and T-cell mitogens and specific antibody response to the Newcastle disease vaccine.

**MATERIALS AND METHODS**

**Eggs.** Specific pathogen-free (SPF) white leghorn eggs were purchased (Hy-Vac Co., Gowrie, IA). Birds that hatched were reared on wire-floored cages for 3 weeks. Feed and water were provided ad libitum. The birds were also provided with antibiotics (aureomycin, chlortetracycline hydrochloride), 3 gram/liter in water, until they were 2 weeks of age.
Medium and reagents. A preparation of cyclophosphamide (CY) (Cytoxan, Mead Johnson & Company, Evansville, IN) was obtained in a dry form containing active ingredients. An aqueous solution was prepared by reconstituting 2 grams of CY in 100 ml (20 mg/ml) of calcium and magnesium-free phosphate-buffered saline (CMF-PBS) and filtered through a 0.22 μm syringe filter. RPMI 1640 supplemented with 25 mM HEPES and L Glutamine (Sigma Chemical Co., St. Louis, MO), penicillin (200 μg/ml), streptomycin (200 μg/ml) was used for washing and resuspending cells, diluting the mitogens and antigens, and culturing the cells.

Concanavalin A (Con A; Sigma Chemical Co., St. Louis, MO) was used as the T-cell mitogen. An aqueous solution was prepared by dissolving 100 mg in 10 ml of CMF-PBS and filtered through a 0.22 μm syringe filter. Con A was used at a concentration of 50 μg/ml for whole blood. Lipopolysaccharide (LPS) from Salmonella typhimurium (Sigma Chemical Co., St. Louis, MO) was used as the B-cell mitogen. It was prepared in CMF-PBS as 1 μg/μl. All stock solutions were dispensed into small aliquots and stored at -20 C until used. LPS was used at a concentration of 1 μg/ml for whole blood response. An MTT (3-[4,5-Dimethylthiazole-2-yl],2-5-diphenyltetrazolium bromide; Sigma Chemical Co., St. Louis, MO) solution was prepared by dissolving 10 mg of MTT in 1 ml of CMF-PBS and solubilized by sonication. The solution was then filtered through a 0.45 μm syringe filter and stored at 4 C in a dark bottle. The HCl-isopropanol (0.04 N HCl-isopropanol) solution was prepared by adding 40 ml of 1 N HCl to one liter of isopropanol. The HCl-isopropanol was stored at room temperature in a light-proof bottle.
Cyclophosphamide treatment. In the first trial, 164 eggs were injected with CY at 16, 17 and 18 days of embryonic development. In addition, 30 eggs were injected with phosphate buffered saline (PBS). This was done by candling eggs to identify the air sac of the embryo. The area of the shell above the air sac was disinfected with 3% tincture of iodine. A drill was used to make a small hole in the eggshell. An aqueous solution of CY (0.1 ml, 20 mg/ml) was injected into the air cell membrane by inserting a 25-gauge, 5/8-inch (16 mm) needle attached to a syringe. The hole was then sealed with transparent tape and the eggs were returned to the incubator. The same procedure was used to inject the control eggs with CMF-PBS at day 16 of incubation and on the two succeeding days. The same procedure was repeated in the second trial in which 144 eggs were injected with CY and 50 eggs were injected with CMF-PBS.

Virus. The lentogenic strain B1, B1 of Newcastle disease virus (NDV) was propagated in 9-day-old embryonated SPF eggs. Embryonated eggs were inoculated by the chorioallantoic route and incubated for 5 days at 37 C. The allantoic fluid was harvested and clarified by centrifugation at 3000 x g for 30 minutes. The virus was purified and concentrated from the allantoic fluids (see below). The purified virus was then inactivated by exposure to ultraviolet light for 40 minutes and evaluated to assure loss of infectivity in embryonic eggs. The inactivated NDV was used as an inoculum at 40 µg per bird.

Virus propagation and purification. Newcastle disease virus purification was based on the method of Alexander and Collins (3). The B1 strain of NDV was grown in 9-day-old embryonated chicken SPF eggs. The embryonated eggs were inoculated by the chorioallantoic
route and incubated for 5 days at 37 C. The allantoic fluid was harvested and clarified by centrifugation at 3000 x g for 30 minutes. The virus was pelleted by centrifugation at 50,000 x g for two hours. The pellet was resuspended in 0.01 M Tris-NaCl pH 7.2 and applied to a discontinuous sucrose gradient made from 14 ml 50% w/v and 21 ml 20% w/v in 10 mM Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4. Following centrifugation for 2 hours at 50,000 x g, a virus band was observed at the sucrose gradient interface. The virus band was collected and pelleted at 50,000 x g for 2 hours. The pellet was resuspended in PBS. The purified virus was assayed for total protein concentration by Bio-Rad assay (Bio-Rad Laboratories, 2000 Alfred Nobel Dr., Hercules, CA 94547), and the purity was assessed by Coomassie blue-stained polyacrylamide gel electrophoresis.

Whole blood. Whole blood was collected from the wing vein of the chickens using a syringe containing heparin (20 unit/ml). The whole blood was used for the colorimetric blastogenesis microassay.

Colorimetric blastogenesis assay. The procedure for the blastogenesis microassay was performed similarly to that previously described (11). Briefly, the assay was carried out in 96-well flat-bottomed tissue culture plates (Corning Laboratory Sciences Co., Corning, NY). Two hundred microliters of RPMI 1640 containing Con A 50 µg/ml or LPS 1 µg/ml or media without mitogens (control wells) were dispensed in each well. Ten microliters of whole blood were added in each well. The plate was incubated at 37 C for 93 hours in a humid atmosphere of 5% CO₂. At 93 hours of the incubation period, 20 µl of MTT (10 mg/ml) were added in each well and the plate was reincubated for three hours. At 96 hours of incubation the plate
was centrifuged at 1000 x g for 10 minutes at room temperature. The supernatant was
removed carefully and 150 µl of a 10% saponin solution were dispensed into each well of the
plate to lyse the cells. The plate was shaken for 20 minutes on a plate shaker (mini-orbital
shaker, Bellco Biotechnology, Vineland, NJ) and the cells were thoroughly resuspended by a
multiple pipetting with a micropipette. The plate was centrifuged at 1000 x g for 10 minutes
and the supernatant was carefully removed. One hundred seventy-five ml of 1N HCl-
isopropanol were added to each well to dissolve the formazan crystals and the plate was
shaken, resuspended and centrifuged as above. One hundred microliters of supernatant were
transferred to the corresponding wells of a new 96-well plate. The absorbance of each well
was measured using a microtiter ELISA reader (Model EL310, BIO-TEK Instruments, Inc.,
Winooski, VT 05404) at a wavelength of 550 nm.

Flow cytometric analysis. An anti-CD3 mouse monoclonal antibody (MAb)
(Southern Biotechnology Associates, Inc., Birmingham, AL 35226) was used at 1:200
dilution. Anti-mouse IgG (Fc specific) conjugated to fluorescein isothiocyanate (FITC)
(Sigma Chemical Company, St. Louis, MO), was used at 1:500 dilution. Anti-mouse IgM
polyclonal antibody (Fc specific) conjugated to FITC (Nordic Immunological Laboratories,
Drawer, Capistrano Beach, CA 92624-0517) was used at a concentration 1:200. The single
color staining method was used to stain T and B lymphocytes as described by Chan et al. (5).
Briefly, three milliliters of blood were collected from each chicken by venipuncture in a
syringe containing 20 units/ml of heparin. The blood was diluted 1:1 with CMF-PBS. Then it
was layered on Lymphoprep™ (Accurate Chemical & Scientific Corporation, Westbury, NY)
and centrifuged at 800 x g for 15 minutes at room temperature. The cellular band at the medium/Lymphoprep™ interface was collected and washed twice with CMF-PBS. The viable lymphocytes were counted by trypan blue dye exclusion after the addition of a 10 μl solution of trypan blue in 90 μl of physiological saline (0.15 M NaCl). After the cell concentration was adjusted to 5 x 10⁶ lymphocytes/ml in CMF-PBS, 200 μl of purified lymphocytes were added into each of three 1.5 ml microfuge tubes. The first tube contained 200 μl of diluted anti CD3 MAb, the second tube contained 200 μl anti-mouse IgM conjugate FITC and the third tube contained CMF-PBS (negative control). The three tubes were incubated for 30 minutes on ice. Following incubation, 200 μl of CMF-PBS were added to each tube and then the tubes were centrifuged at 500 x g for 5 minutes. The supernatant was discarded and 200 μl of anti-mouse IgG conjugated FITC were added only to the tubes containing anti-CD₃ and reincubated for 30 minutes on ice. After the incubation period these tubes were rinsed as before. The remaining cells were rediluted to the final volume of 200 μl in CMF-PBS. The staining percentages for each sample preparation were determined by flow cytometric analysis on a profile (Coulter Corp., Hialeah, FL).

Hemagglutination inhibition (HI) test. The hemagglutination inhibition test was performed as described (4). Briefly, twofold serial dilutions of serum were made in a 96 well, round-bottom microtiter plate containing 50 μl of PBS in the first row and 50 μl of NDV antigen (10 HA units) in the remaining 11 rows. Serum dilutions ranged from 1:2 to 1:2048. The antigen serum mixture was incubated for 30 minutes at 37 C. Then, 50 μl of a 0.05%
turkey erythrocyte suspension were added to each well and reincubated for 30 minutes. A positive serum, a negative serum, erythrocytes and antigens were also included as a control. The highest dilution of serum causing complete inhibition was considered the endpoint. The geometric mean titer was expressed as reciprocal $\log_2$ values of the highest dilution that displayed HI.

Experimental design. At two weeks of age all in ovo CY-treated and CY non-treated birds were bled and the MTT blastogenesis microassay were performed using B- and T-cell mitogens. Statistical analysis using a t-test was performed to select those birds that had significant T-cell response and no significant B-cell response. At 3 weeks of age CY-treated birds that had significant Con A (T-cell) response and no significant LPS (B-cell) response were divided into two groups. The birds in group 1 were each vaccinated subcutaneously (SC) with approximately 40 $\mu$g/birds with UV inactivated NDV (CY-NDV). The birds in group 2 served as unvaccinated control birds and were injected with PBS (CY-PBS). Similarly, the CY-untreated birds that were con A (T-cell) and LPS (B-cell) responsive were divided into two groups (group 3 and 4). Group 3 was vaccinated subcutaneously with approximately 40 $\mu$g/bird of UV inactivated NDV (C-NDV). The birds in group 4 were injected with PBS (C-PBS) and served as untreated, unvaccinated controls. The chickens were boostered at 5 weeks of age and blood samples were collected weekly. The MTT blastogenesis microassay and flow cytometric analysis were performed 1 week after vaccination and one week following the booster. The samples used for flow cytometric
analysis were pooled samples from 4 chickens within each group. The antibody response to NDV for each bird was evaluated weekly by using HI.

Two trials were conducted. In the first trial, 10 birds (CY-NDV) were used from first group and 5 chickens from groups 2, 3, and 4. In the second trial, 12 chickens were used per group.

Statistical evaluation. To study the mitogenic response of each bird to B- and T-cell mitogens, analysis of variance (ANOVA) and t-tests were performed. A comparison was made between the absorbencies from 4 wells of cultured lymphocytes response without mitogen and the absorbencies from 4 wells of cultured lymphocytes responding to each mitogen (LPS, Con A) for each bird. Differences were determined as highly significant (P<0.01), significant (P<0.05), or not significant (P>0.05). The average body weight in the CY-treated group was compared with that of the CY-nontreated group using ANOVA. The percentage of B-cell and T-cell staining was compared between CY-treated birds and CY-nontreated birds using ANOVA.

RESULTS

Effect of in ovo cyclophosphamide treatment on hatchability, mortality, and body weight. Cyclophosphamide treatment in ovo resulted in decreased hatchability and increased mortality, as determined at 2 weeks post-hatching (see Table 1). In ovo
cyclophosphamide treatment also significantly depressed body weight at 3 weeks of age compared to CY-nontreated control birds (see Table 1).

**Whole blood blastogenesis microassay.** Figure 1 depicts the responses of PBL of one-day-old birds hatched from CY-treated eggs and CY-nontreated eggs to Con A and LPS. The results show that reduced responses to both T- and B-cell mitogens were observed in all of the CY-treated birds. Significant PBL mitogenic responses in both T- and B-cell mitogens were observed in all CY-nontreated birds. Table 2 shows the responses of two-week-old birds to Con A and LPS. Approximately 50% of the birds were T-cell positive and B-cell negative at two weeks of age. A number of birds that had T-cell responses but not B-cell responses were retained in the study for continued monitoring of the B-cell response, whereas those birds responding to Con A and LPS were eliminated from the study.

The blastogenesis results at 4 weeks of age indicated that all birds responded to Con A and 1 and 2 CY-treated birds responded to LPS in the 1st and 2nd trial, respectively. At 6 weeks of age, all the birds continued to respond to Con A and three of the CY-treated birds responded to LPS, with a total of 4 of 15 birds responding in the 1st trial. Similarly, in the 2nd trial, 2 birds responded to LPS at 4 weeks in addition to the 3 birds responding at 6 weeks, giving a total of 5 of 24 birds responding.
Table 1. The effect of \textit{in ovo} cyclophosphamide treatment on hatchability, mortality and body weight.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatment</th>
<th>Hatchability</th>
<th>Mortality$^C$</th>
<th>Body weight$^D$ (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CY$^A$</td>
<td>106/164 (64%)</td>
<td>13/106 (12.3)</td>
<td>ND$^E$</td>
</tr>
<tr>
<td></td>
<td>C$^B$</td>
<td>30/30 (100%)</td>
<td>0/30 (0%)</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>CY</td>
<td>90/144 (62.5%)</td>
<td>5/90 (3.3%)</td>
<td>142.07*</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>48/50 (96%)</td>
<td>0/48 (0%)</td>
<td>197.400</td>
</tr>
</tbody>
</table>

$^A$ Birds hatched from eggs injected \textit{in ovo} with cyclophosphamide.
$^B$ Birds hatched from eggs injected \textit{in ovo} with PBS.
$^C$ Cumulative mortality at two weeks of age.
$^D$ Average body weight of group at three weeks of age.
$^E$ ND = Not done.
* Statistically significant difference from control group ($P < 0.005$).

\textbf{Antibody response to Newcastle disease vaccine.} The results of the NDV vaccination are shown in Table 3. Chickens hatched from eggs treated with CY and vaccinated with UV-NDV did not produce detectable antibodies during the 1st and 2nd weeks post-vaccination. However, 3 birds from the CY-treated group vaccinated with UV-NDV developed detectable antibodies during the 1st week post-booster. Antibody titers were detected in some of the CY-nontreated birds that were vaccinated with UV-NDV by 2 weeks post-vaccination and all produced antibody titers following boosters.
Figure 1. Mitogenic response of peripheral blood lymphocytes (PBL) of one-day-old birds hatched from CY-treated eggs (A) and CY-nontreated eggs (B) to Con A and LPS. The bar represents the average absorbencies from four replicates and the error bars represents the standard deviation. A single asterisk (*) denotes a significant difference (P < 0.05) and two asterisks (**) denotes a highly significant difference (P < 0.01) in the mitogenic response when compared to the control treatment (no Con A or LPS).
Table 2. Mitogenic responses of PBL of 2-, 4- and 6-week-old birds hatched from CY-treated eggs and CY-nontreated eggs to ConA and LPS.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Week</th>
<th>No.</th>
<th>Treatment</th>
<th>ConA</th>
<th>LPS</th>
<th>ConA(+) &amp; LPS(-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>92</td>
<td>CY</td>
<td>75/89 (84%)</td>
<td>36/89 (40%)</td>
<td>46/89 (51%)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>CY</td>
<td>10/10 (100%)</td>
<td>10/10 (100%)</td>
<td>0/10 (0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>15</td>
<td>CY</td>
<td>15/15 (100%)</td>
<td>1/15 (6%)</td>
<td>14/15 (93%)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>C</td>
<td>10/10 (100%)</td>
<td>9/10 (90%)</td>
<td>1/10 (10%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>15</td>
<td>CY</td>
<td>15/15 (100%)</td>
<td>4/15 (26%)</td>
<td>11/15 (73%)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>C</td>
<td>10/10 (100%)</td>
<td>8/10 (80%)</td>
<td>2/10 (20%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>85</td>
<td>CY</td>
<td>75/85 (88%)</td>
<td>27/85 (31%)</td>
<td>48/85 (56%)</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>C</td>
<td>36/36 (100%)</td>
<td>36/36 (100%)</td>
<td>0/36 (0%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>24</td>
<td>CY</td>
<td>24/24 (100%)</td>
<td>2/24 (8%)</td>
<td>22/24 (91%)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>C</td>
<td>24/24 (100%)</td>
<td>24/24 (100%)</td>
<td>0/24 (0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>24</td>
<td>CY</td>
<td>24/24 (100%)</td>
<td>5/24 (20%)</td>
<td>19/24 (79%)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>C</td>
<td>24/24 (100%)</td>
<td>24/24 (100%)</td>
<td>0/24 (0%)</td>
<td></td>
</tr>
</tbody>
</table>

A CY = cyclophosphamide-treated birds in ovo; C = cyclophosphamide-nontreated birds injected with PBS in ovo

B Number of the birds that had a statistically significant response to Con A (P < 0.05).

C Number of the birds that had a statistically significant response to LPS (P < 0.05).

D Number of birds that had a statistically significant response to Con A but not to LPS (P < 0.05).

Effect of CY on T and B-cell populations in PBL. Table 4 shows the results from trial 1 and 2. Data from the ANOVA for flow cytometric analysis are given in the Appendix (Tables A1 - A6). In the first trial, the flow cytometric analysis indicated that the percentage of lymphocytes expressing CD3 in the CY-treated group was significantly less than CY-
Table 3. Antibody response to UV-NDV vaccination from CY-treated and CY-nontreated birds.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatment</th>
<th>Post-vaccination</th>
<th>Post-booster</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8 days</td>
<td>14 days</td>
<td>10 days</td>
</tr>
<tr>
<td>1</td>
<td>CY-NDV</td>
<td>0/10</td>
<td>0/10</td>
<td>3/10 (2.37)</td>
</tr>
<tr>
<td></td>
<td>CY-PBS</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>C-NDV</td>
<td>0/5</td>
<td>3/5 (3)</td>
<td>5/5 (5.4)</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>2</td>
<td>CY-NDV</td>
<td>0/12</td>
<td>0/12</td>
<td>3/12 (2.6)</td>
</tr>
<tr>
<td></td>
<td>CY-PBS</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>C-NDV</td>
<td>0/12</td>
<td>4/12 (3)</td>
<td>12/12 (6.2)</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
</tbody>
</table>

A CY-NDV = CY-treated birds vaccinated with NDV; CY-PBS = CY-treated birds injected with PBS; C-NDV = CY-nontreated birds vaccinated with NDV; C-PBS = CY-nontreated birds injected with PBS.

B Number of birds having HI titer > 1:2.

C Numbers in parentheses indicate, the geometric mean titer of responding birds to NDV expressed as reciprocal $\log_2$.

nontreated groups. No significant differences were found in the percentage of CD3 positive cells in either the CY-treated birds or the CY-untreated birds in the second trial.

There were significant differences in the percentages of IgM positive cells between CY-treated birds and CY-nontreated birds. The staining percentages derived from CY-nontreated birds were found to be higher than CY-treated birds.
Table 4. Effect of in ovo CY treatment on the percentage of B and T lymphocytes from the peripheral blood as determined by flow cytometric analysis.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Week</th>
<th>Treatment</th>
<th>Anti-IgM</th>
<th>Anti-CD3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>CY</td>
<td>1.0*</td>
<td>66.7*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>8.2</td>
<td>85.9</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>CY</td>
<td>1.3*</td>
<td>70 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>17.9</td>
<td>83.2</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>CY</td>
<td>1.35*</td>
<td>73.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>13.5</td>
<td>78.15</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>CY</td>
<td>0.8*</td>
<td>85.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>9.5</td>
<td>83.05</td>
</tr>
</tbody>
</table>

A Asterisk indicates the value is significantly different from the control group (P < 0.05).
B CY = cyclophosphamide treated birds; C = cyclophosphamide nontreated birds.
C Percentage of staining B-Lymphocytes with anti-IgM.
D Percentage of staining T-Lymphocytes with anti-CD3.

DISCUSSION

Cyclophosphamide treatment has been used as a means of abrogating the humoral immunity in order to determine the role of T and B cells in protective responses to infectious pathogens (9, 12, 13, 15). Cyclophosphamide injected during embryonic development has been reported to destroy both B and T lymphocytes. Subsequently, T cells were found to regenerate and repopulate lymphoid tissue in the majority of the treated chickens by two weeks of age. However, B-cell regeneration remained suppressed for longer periods (7, 14).
The results of this study corroborate the effect of CY treatment reported in previous studies (7, 14). Similar patterns of immunosuppressive effects induced by CY-treatment were observed, whereby following hatch a severe lymphoid depletion of B and T cells was evident in the PBL of CY-treated birds and functional recovery of T lymphocytes was observed in the majority of CY-treated birds by 2 weeks of age. However, the immunosuppressive effect of the CY on B cells was not detected in all CY-treated birds and the recovery of antibody producing cells may probably have occurred earlier than 2 weeks of age. One of the main reasons for the disparity between the studies may have been due to the route of CY injection into the eggs. Eskola and Toivanen (7) used intravenous injections of CY at 16 to 18 days of embryonic development to ensure a uniform distribution of the drug through the embryo. However, injecting CY by this method reduced hatchability, dramatically increased post-hatch mortality, and for those few birds that survived, there was a failure to regenerate T cells. Lymphocyte responses to PHA and Con A were significantly reduced at 44 days of age which may have been due to the severe damage of the epithelial component of the thymus induced by the intravenous injection of CY. However, injecting into the allantoic fluid as described in this study, was found to be more convenient, time efficient, applicable for larger quantities of eggs, and yielded a substantially lower mortality rate when compared with the intravenous method.

The toxic effect of the CY-treatment on hatchability, mortality, and body weight has been reported (1, 6, 7, 14, 15). Schlink et al. (1986) found that high doses of CY administration destroyed both the B and T lymphocytes for long periods of time and increased
the mortality rate. Low doses of cyclophosphamide, however, reduced mortality but were not sufficient to permanently affect the morphology or immunocompetency of the lymphoid organs of the bird (15). Other factors may also influence the immunosuppressive effect of CY such as strains of chickens, environmental factors and the origin of CY. These observations may emphasize the importance of determining the optimal dose of CY to be used since there was a great variability in the results among investigations.

The flow cytometric analysis indicated that there was a significant reduction of B cells in CY-treated birds as compared with the CY-nontreated birds. The percentage of anti-IgM stained cells in the CY-treated birds were within the background level of the assay. Therefore, although there was a low percentage of anti-IgM stained cells, this percentage was within the experimental error of the assay and could be indicative of total B-cell ablation. The effect of CY treatment on the T cells was also demonstrated in CY-treated groups in the 1st trial. The percentage of T cells in CY-treated birds that were identified by CD3 markers were found to be approximately 13% less than that of CY-nontreated birds. However, these differences were not detected in the 2nd trial in which the percentage of CD3 was not significantly different from control group. The difference between the results in the 1st and 2nd trials might be due to the variation among the samples that were analyzed.

The immunosuppressive effect of the CY treatment on the antibody response to NDV was in agreement with the birds’ responses to LPS mitogen. Only those birds that had significant responses to LPS at six weeks of age developed detectable specific NDV antibodies.
The fact that a few of the 2-week-old T-cell positive/B-cell negative birds responded at 4 and 6 weeks reveals that the immunosuppressive effects of CY on the B-cell system is transient and functional recovery of antibody-producing cells could occur at any time, thus demonstrating the need for continuous monitoring. This might be achieved by employing a technique that would enable monitoring the humoral immune response in the CY-treated birds. In this regard, the suppressive effect of CY might be evaluated through flow cytometric analysis, blastogenesis response to B-cell mitogens and specific antibody response to pathogens. Those birds that demonstrate a functional and/or morphological recovery of B cells should be identified and noted.

REFERENCES


ACKNOWLEDGMENTS

The authors thank Dr. Jagdev Sharma for his advice and consultation, Dr. Ali Akbar, Sevinc Aknic, and Joan Oespar for their technical assistance, and Ms. L. L. Wu for her valuable assistance in the statistical analysis of the data.
APPENDIX: DATA AND ANALYSIS

Table A1. Data from the first trial represent the effect of cyclophosphamide treatment on the percentage of T and B lymphocytes in peripheral blood lymphocytes. The values given are pooled samples from 4 birds collected at 4 and 6 weeks of age.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Groups</th>
<th>Weeks</th>
<th>B Cell</th>
<th>T Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CY</td>
<td>4</td>
<td>1</td>
<td>63.9</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>4</td>
<td>1.2</td>
<td>69.9</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1</td>
<td>CY</td>
<td>6</td>
<td>2.6</td>
<td>68.4</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>6</td>
<td>0</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>23</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12.8</td>
<td>90</td>
</tr>
</tbody>
</table>

Table A2. Analysis of variance for data on the effect of cyclophosphamide treatment on percentage of B cells from the first trial.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY</td>
<td>1</td>
<td>310.889</td>
<td>310.887</td>
<td>158.62</td>
<td>0.0504</td>
</tr>
<tr>
<td>Birds</td>
<td>2</td>
<td>6.67</td>
<td>3.3</td>
<td>1.7</td>
<td>0.4767</td>
</tr>
<tr>
<td>Week</td>
<td>1</td>
<td>38.5067</td>
<td>38.50</td>
<td>19.65</td>
<td>0.1413</td>
</tr>
<tr>
<td>CY*Week</td>
<td>1</td>
<td>71.053</td>
<td>71.05</td>
<td>36.25</td>
<td>0.104</td>
</tr>
<tr>
<td>Error</td>
<td>1</td>
<td>1.9600</td>
<td>1.9600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected total</td>
<td>6</td>
<td>429.0743</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table A3. Analysis of variance for data on the effect of cyclophosphamide treatment on the percentage of T cells from the first trial.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY</td>
<td>1</td>
<td>431.573</td>
<td>431.573</td>
<td>1726.29</td>
<td>0.0153</td>
</tr>
<tr>
<td>Birds</td>
<td>2</td>
<td>70.2916</td>
<td>35.1458</td>
<td>140.58</td>
<td>0.0595</td>
</tr>
<tr>
<td>Week</td>
<td>1</td>
<td>0.015</td>
<td>0.015</td>
<td>0.06</td>
<td>0.8471</td>
</tr>
<tr>
<td>CY*Week</td>
<td>1</td>
<td>45.63</td>
<td>45.63</td>
<td>182.52</td>
<td>0.047</td>
</tr>
<tr>
<td>Error</td>
<td>1</td>
<td>0.250</td>
<td>0.250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected total</td>
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<td>547.76</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table A4. Data from the second trial represent the effect of cyclophosphamide treatment on the percentage of T and B lymphocytes in the peripheral blood lymphocytes. Values given are the pooled samples from 4 birds collected at 4 and 6 weeks of age.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Groups</th>
<th>Weeks</th>
<th>B Cell</th>
<th>T Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>CY</td>
<td>4</td>
<td>0.8</td>
<td>73.1</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13.9</td>
<td>73.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>82.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>1.8</td>
<td>89.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>10.3</td>
<td>76.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.8</td>
<td>82.8</td>
<td></td>
</tr>
</tbody>
</table>
Table A5. Analysis of variance for data on the effect of cyclophosphamide treatment on percentage of B cells from the second trial.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY</td>
<td>1</td>
<td>213.2115</td>
<td>213.2115</td>
<td>222.97</td>
<td>0.0045</td>
</tr>
<tr>
<td>Birds</td>
<td>2</td>
<td>1.5025</td>
<td>0.75125</td>
<td>0.79</td>
<td>0.5600</td>
</tr>
<tr>
<td>Week</td>
<td>1</td>
<td>9.03125</td>
<td>9.03125</td>
<td>9.44</td>
<td>0.0916</td>
</tr>
<tr>
<td>CY*Week</td>
<td>1</td>
<td>6.30125</td>
<td>6.30125</td>
<td>6.59</td>
<td>0.124</td>
</tr>
<tr>
<td>Error</td>
<td>2</td>
<td>1.9125</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Corrected total 7 231.958

Table A6. Analysis of variance for data on the effect of cyclophosphamide treatment on the percentage of T cells from the second trials

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY</td>
<td>1</td>
<td>1.361125</td>
<td>1.36125</td>
<td>0.17</td>
<td>0.7225</td>
</tr>
<tr>
<td>Birds</td>
<td>2</td>
<td>68.0825</td>
<td>34.04125</td>
<td>4.17</td>
<td>0.1933</td>
</tr>
<tr>
<td>Week</td>
<td>1</td>
<td>113.25125</td>
<td>113.251</td>
<td>13.89</td>
<td>0.0651</td>
</tr>
<tr>
<td>CY*Week</td>
<td>1</td>
<td>61.05125</td>
<td>61.05125</td>
<td>7.49</td>
<td>0.1117</td>
</tr>
<tr>
<td>Error</td>
<td>2</td>
<td>16.3125</td>
<td>8.15625</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Corrected total 7 260.058
STUDIES ON NEWCASTLE DISEASE IN CHICKENS:
THE ROLE OF CELL MEDIATED IMMUNITY IN PROTECTION

Manuscript for submission to Avian Diseases
A. D. Maraqa and D. L. Reynolds

SUMMARY

The role of cell mediated immunity (CMI) in protection of birds from Newcastle disease (ND) was investigated by two different strategies in which only NDV specific CMI was conveyed without a humoral response. In the first strategy, selected 3-week-old pathogen-free (SPF) birds were vaccinated with either live Newcastle disease virus (LNDV), UV inactivated NDV (UVNDV), SDS treated NDV (SDSNDV), or phosphate buffered saline (PBS) (negative control) by the subcutaneous route. Birds were booster vaccinated 2 weeks later and challenged with the Texas GB strain of NDV 1 week after the booster. All vaccinated birds had specific CMI responses to NDV as measured by a MTT blastogenesis microassay. Specific antibody response was detected in birds vaccinated with LNDV and UVNDV. However, birds vaccinated with SDSNDV developed antibodies which were detected by the Western blot analysis but not by the HI test or VN test. Protection from challenge was only observed in those birds which had NDV specific antibody response. That is, birds with demonstrable CMI and a specific antibody response were protected, whereas birds with demonstrable CMI but no specific antibody response were not protected. In the
second strategy, birds from SPF embryos were treated in ovo with cyclophosphamide (CY) to deplete B cells. At 2 weeks of age, the birds were monitored and selected for the presence of T-cell activity and the absence of B-cell activity. Birds that had a significant T-cell response but not a B-cell response were vaccinated with either LNDV, UVNDV or PBS at 3 weeks of age along with the CY-untreated control birds. The birds were booster vaccinated at 5 weeks of age and were challenged with the Texas GB strain of NDV at 6 weeks of age. All birds vaccinated with LNDV or UVNDV had a specific CMI response to NDV. Specific NDV antibodies were detected in all CY-nontreated vaccinated birds and some of the CY-treated vaccinated birds that were found to have regenerated their B-cell function at one week post-booster. The challenge results clearly revealed that CY-treated birds that had specific CMI and antibody responses to LNDV or UVNDV were protected as were the CY-nontreated vaccinated birds. However, birds that had an NDV-specific CMI response but did not have NDV-specific antibodies were not protected from challenge. The results from both strategies indicate that specific CMI to NDV by itself is not protective against virulent NDV challenge. The presence of specific antibodies is important in providing protection from Newcastle disease.

**Keywords:** cyclophosphamide, Newcastle disease virus, cell mediated immune response.
Abbreviations:

B  bursa-dependent lymphocytes
CMF-PBS  calcium and magnesium free-phosphate buffered saline
CMI  cell-mediated immunity
Con A  concanavalin A
EID$_{50}$  embryo infectious dose of 50 percent
HI  hemagglutination inhibition
IBD  infectious bursal disease virus
LNDV  live Newcastle disease virus.
LPS  lipopolysaccharide
MTT  3-[4,5-Dimethylthiazole-2-yl],2,5-diphenyltetrazolium bromide
NDV  Newcastle disease virus
PBL  peripheral blood lymphocytes
PBS  phosphate-buffered saline
SDS  sodium dodecyl sulfate
SV5  Simian virus 5
SI  stimulation index
SPF  specific pathogen free
VN  virus neutralization
UVNDV  ultraviolet inactivated Newcastle disease virus
T  Thymus-dependent lymphocytes
INTRODUCTION

Newcastle Disease (ND) is an economically important viral disease of poultry and is of major concern worldwide (2). It occurs in a variety of avian species and may cause respiratory distress, diarrhea, cessation of egg production, nervous signs, depression and high morbidity and mortality if not controlled (2). The severity of the disease produced by a virulent strain of NDV has been found to be greatly influenced by the immune status of the host. It was reported that the level of immunity against ND determines the severity of the disease (5).

Both cellular and humoral immune responses have been suggested to play important roles in the host's defense against NDV infection (6, 10, 11, 16, 18, 20). Antibodies directed against surface glycoproteins of NDV have been reported to inactivate or neutralize the free virus. These antibodies inhibit virus attachment to the cell host receptors and prevent the spread of the virus from cell to cell (18). The antibody response to NDV occurs rapidly with detectable neutralizing antibodies, usually detected in the serum of birds within 4 to 6 days following vaccination with live attenuated vaccines (2).

Cell mediated immunity (CMI) has been suggested to be an important factor in the development of protection in chickens vaccinated against ND (6, 10, 16, 18, 20). Cell mediated immunity has been reported as the first immunological response being detected as early as 2 to 3 days following ND vaccination (10). It has been shown that early protection following vaccination can be demonstrated in the presence of low levels of antibodies or in the absence of detectable antibodies (11). Furthermore, recent studies with other paramyxoviruses...
have demonstrated that protective immunity is mediated by CMI whereas serum neutralizing antibodies have been found to play a minor role in protection (23). While there have been suggestions of a protective role for cellular immunity, a definitive relationship between protection and the cellular immune response has not been determined in the absence of a detectable antibody response (16, 20).

The objective of this study was to ascertain whether cell mediated immunity is a key component in the protection of chickens against Newcastle disease. Two strategies were used to achieve this objective. The first strategy was to destroy neutralizing epitopes on the virus by treating NDV with a denaturing agent such as SDS. By this method, NDV-specific neutralizing antibody was not induced, but NDV specific CMI was induced in immunocompetent birds. The second strategy was to deplete the humoral immune response of the bird while retaining the T-cell response and use intact NDV as an immunogen. This was achieved by chicken embryo in ovo treatment with cyclophosphamide (CY) from specific pathogen-free (SPF) chickens eggs (9).

MATERIALS AND METHODS

Eggs. Specific pathogen-free (SPF) white leghorn eggs were purchased (Hy-Vac Co., Gowrie, IA). The chickens were hatched and housed in a facility designed for maintaining SPF status.
Medium and reagents. Cyclophosphamide (CY) (Cytoxan, Mead Johnson & Company, Evansville, Indiana) was obtained in a dry form containing active ingredients. An aqueous solution was prepared by reconstituting 2 grams in 100 ml (20 mg/ml) of calcium and magnesium-free phosphate-buffered saline (CMF-PBS) and filtered through a 0.22 μm syringe filter.

A solution of RPMI 1640 supplemented with 25 mM HEPES and L Glutamine (Sigma Chemical Co., St. Louis, MO), penicillin (200 μg/ml), and streptomycin (200 μg/ml) was used for washing and resuspending the cells, diluting the mitogens and antigen, and culturing the cells. Concanavalin A (Con A; Sigma Chemical Co., St Louis, MO) was used as the T-cell mitogen. An aqueous solution was prepared by dissolving 100 mg in 10 ml of CMF-PBS and filtering it through a 0.22 μm syringe filter. Con A was used at a working concentration of 50 μg/ml for whole blood and at a concentration of 6 μg/ml for purified lymphocytes. Lipopolysaccharide (LPS) from *Salmonella typhimurium* (Sigma Chemical Co., St. Louis, MO) was used as the B-cell mitogen. It was prepared in CMF-PBS as 1 μg/μl. All stock solutions were dispensed into small aliquots and stored at -20 C until used. LPS was used at a working concentration of 1 μg/ml for the whole blood blastogenesis microassay. A solution of MTT (3-[4,5-Dimethylthiazole-2-yl]2,5-diphenyltetrazolium bromide; Sigma Chemical Co, St. Louis, MO) was prepared by dissolving 10 mg of MTT in 1 ml of CMF-PBS and solubilized by sonication. The solution was then filtered through a 0.45 μm syringe filter and stored at 4 C in a dark bottle. The HCl-isopropanol (0.04 N HCl-isopropanol) solution was
prepared by adding 40 ml of 1 N HCl to one liter of isopropanol. The HCl-isopropanol was stored at room temperature in a light-proof bottle.

**Virus propagation and purification.** Newcastle disease virus purification was based on the method of Alexander and Collins (4). The lentogenic strain B1, B1 of NDV, was propagated in 9-day-old embryonated SPF chicken eggs. The embryonated eggs were inoculated by the chorioallantoic route and incubated for 5 days at 37 C. The allantoic fluid was clarified by centrifugation at 3000 × g for 30 minutes. The virus was pelleted by centrifugation at 50,000 × g for 2 hours. The pellet was resuspended in 0.01M Tris-NaCl pH 7.2 and applied to a discontinuous sucrose gradient made from 14 ml 50% w/v and 21 ml 20% w/v in 10 mM Tris, 0.1M NaCl, 1mM EDTA, pH 7.4. Following centrifugation for 2 hours at 50,000 × g, a visible band was observed at the sucrose gradient interface. The virus band was collected and pelleted at 50,000 × g for 2 hours. Then the pellet was resuspended in PBS. The purified virus was assayed for total protein concentration by Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA) and the purity was assessed by Coomassie blue-stained polyacrylamide gel electrophoresis. The purified virus was inactivated by exposure to ultraviolet light for 40 minutes and tested for virus replication in the embryonic eggs. A concentration of 0.3 μg/ml of ultraviolet light inactivated NDV (UVNDV) was used for measuring cell mediated immunity in the blastogenesis micro-assay and 40 μg/bird for vaccination. The purified NDV was treated with an equal volume of 4% SDS and heated at 100 C for two minutes. The SDS-treated NDV was mixed with incomplete Freund's adjuvant and used for vaccination.
The velogenic Texas GB strain of NDV was propagated in embryonic eggs and used for challenge at $10^2$ ELD$_{50}$/birds.

**Preparation of purified lymphocytes.** Three milliliters of blood were collected from each chicken by venipuncture in a syringe containing 20 units/ml of herpin. The blood was diluted 1:1 with an RPMI 1640 medium. The blood was layered on Lymphoprep™ (Accurate Chemical & Scientific Corporation, Westbury, NY) and centrifuged at 800 × g for 15 minutes at room temperature. The cellular band at the medium/Lymphoprep™ interface was collected and washed twice with RPMI 1640 medium. The viable lymphocytes were counted by trypan blue dye exclusion after the addition of a 10 μl solution of trypan blue in 90 μl of physiological saline (0.15 M NaCl). After the cell concentration was adjusted to $5 \times 10^6$ lymphocytes/ml in RPMI 1640, the purified lymphocytes were used for measuring CMI to NDV.

**Cyclophosphamide treatment.** The procedure for CY injection was performed similarly to that previously described (15). Briefly, eggs were injected with CY during day 16, 17 and 18 of embryonic development. This was done by candling the eggs to identify the air sac of the embryo. After the area of the shell above the air sac was disinfected with a 3% tincture of iodine, a drill was used to make a small hole in the eggshell, and an aqueous solution of CY (0.1 ml, 20 mg/ml) was injected into the air cell membrane by insertion into the hole with a 25-gauge 5/8-inch (16 mm) needle attached to a syringe. Then the hole was sealed with transparent tape and the eggs were returned to the incubator. The same procedure was
used to inject the control eggs with CMF-PBS at day 16 of embryonic development and on the two succeeding days.

**Colorimetric blastogenesis assay.** The procedure for the blastogenesis microassay was performed similarly to the procedures previously described (17). Briefly, the assay was carried out in a 96-well flat-bottomed tissue culture plate (Corning Laboratory Sciences Co., Corning, NY). Two hundred microliters of RPMI containing Con A 6 µg/ml or 0.3 µg/ml UV-NDV or media without antigen (control well) were dispensed in each well and 10 µl of purified lymphocytes were added to each well. The plate was incubated at 37 C for 93 hours, in a humid atmosphere of 5%CO₂. At 93 hours of incubation period, 20 µl of MTT (10 mg/ml) were added in each well and the plate was reincubated for 3 hours. At 96 hours of incubation the plate was centrifuged at 1000 × g for 10 minutes at room temperature. The supernatant was removed carefully and 175 µl of 1N HCl-isopropanol were added into each well to dissolve the formazan crystals. Then the plate was shaken for 20 minutes on a plate shaker (mini-orbital shaker, Bellco Biotechnology, Vineland, NJ) and the blood lymphocytes were thoroughly resuspended by multiple pipettings with a micropipetter. The plate was centrifuged at 1000 × g for 10 minutes. One hundred microliters of supernatant were transferred to the corresponding wells of a new 96-well plate. The absorbance of each well was measured by a microtiter ELISA reader (Model EL310, BIO-TEK Instruments, Inc., Winooski, VT 05404) at a wavelength of 550 nm. The response was reported as a stimulation index (SI) as calculated by the following formula:
Western blot. Purified NDV was solubilized in a gel sample buffer containing 0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.001% bromophenol blue and heated at 100°C for 2 minutes. The proteins were separated on a 10% polyacrylamide gel according to the Laemmli procedure (14) and the gel was electrophoresed at a constant voltage of 150 V for 4 hours. Part of the gel was stained with Coomassie brilliant blue G 250 (Pharmacia Biotech, Piscataway, NJ) destained with methanol and fixed. The remaining portion of the gel was prepared for transfer onto a 0.45 μm nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad Laboratories, Hercules, CA) for a Western blot analysis. The transfer was carried out for 4 hours in a transfer buffer consisting of 25 mM Tris, 192 mM Glycine (pH 8.3) and 20% v/v methanol at a constant voltage of 60 V. After transfer the membrane was stained with 0.05% Ponceau S dye (Fisher Biotech, Fisher Scientific, Fair Lawn, NJ) in a 1% acetic acid solution for band visualization. The nitrocellulose membrane containing the separated proteins was cut into 7 mm strips and each strip was placed into individual wells in an immunoblotting plate. An avidin-biotin immunobinding assay was used for developing the blot as previously described (7). Briefly, a nitrocellulose membrane containing the separated proteins was blocked with 3% skim milk in TBS (500 mM NaCl, 20 mM Tris, pH 7.5) for 1 hour. Antiserum was diluted 1:400 in TBS containing 0.3% skim milk and added to each strip. After one hour of incubation, secondary biotinylated antibodies (biotinylated anti-chicken IgG, vector Laboratories, Burlingame, CA) at a dilution of 1:5000, strept-avidin at a dilution of

$$SI = \frac{(\text{mean absorbance of stimulated culture}) - (\text{mean absorbance of unstimulated culture})}{(\text{mean absorbance of unstimulated culture})}$$
1:2000 and the chromogen (4-chloro-1-Naphthol; Sigma chemical Co., St. Louis, MO) were subsequently added. Washing and blocking the nitrocellulose membrane were performed between each step. Then the immunoblotting plate was incubated in the dark until a color reaction developed against the white (nitrocellulose) background, and the reaction was stopped by washing with TBS.

Sample collection for virus isolation. Cotton-tipped applicators were used for swabbing the trachea. Following tracheal swabbing, each cotton applicator was placed into 2 ml of tryptose phosphate broth containing penicillin and streptomycin and then frozen (-70 C). On the day of egg inoculation, the tubes were thawed and the swabs were removed. The remaining fluid was centrifuged at 1000 × g for 10 minutes and the supernatant was collected for embryo inoculation. Five 9-day-old embryonated eggs for each tracheal swab sample were inoculated via the chorioallantoic sac with 0.1 ml per embryo. Then the eggs were incubated at 37 C for 7 days. The presence of NDV was detected by hemagglutination (HA) using 1% turkey erythrocytes.

Hemagglutination inhibition (HI) test. The HI test was performed as described (3). Briefly, twofold serial dilutions of serum were made in a 96 well, round-bottom microtiter plate containing 50 µl of PBS in the first row and 50 µl of NDV antigen (10 HA units) in the remaining 11 rows. Serum dilutions ranged from 1:2 to 1:2048. The antigen serum mixture was incubated for 30 minutes at 37 C. Then, 50 µl of a 0.05% turkey erythrocyte suspension were added to each well and reincubated for 30 minutes. A positive serum, a negative serum, erythrocytes and antigens were also included as controls. The highest dilution of serum
causing complete inhibition was considered the endpoint. The geometric mean titer was expressed as reciprocal Log_2 values of the highest dilution that displayed HI.

**Virus neutralization (VN) test.** The virus neutralization test was performed as described (3), except the ST cell line was used instead of chicken embryo fibroblasts. Briefly, 50 microliters of media were added into an empty 96-well flat-bottomed tissue culture plate (Corning Laboratory Sciences Co., Corning, NY). Another 50 μl of the serum sample were added into the first column. Twofold serial dilutions were made across the plate to achieve dilutions from 1:2 to 1:1024. One hundred tissue-culture infectious doses (TCID_{50}) of the Texas GB strain of NDV in 50 μl were mixed with equal volumes of the serum dilutions. Then the plate was incubated for one hour at 37 C in a humidified 5% CO_2 incubator. Fifty μl of media without antibodies were mixed with the virus suspension for the virus control, and 100 μl of medium without antibodies or virus were used for a cell control. Positive and negative serum controls were also included in the experiment. All samples were tested in quadruplicate. Following a 1-hour incubation period, 100 μl of the virus-serum mixture were transferred into 96-well culture plates containing monolayers of a swine testicular (ST) cell line and incubated at 37 C for 72 hours. The plate was examined for cytopathic effect (CPE) to confirm the presence of the virus. The 50% neutralizing endpoint (ED_{50}) was calculated by the method of Reed and Muench (21).

**Experimental design.** In the first experiment, 4 groups of 3-week-old SPF chickens were injected subcutaneously with approximately 40 μg/bird of either LNDV, UVNDV, SDS-treated NDV or PBS. The birds were booster vaccinated at 5 weeks of age. Blood samples
were collected weekly for an evaluation of cellular and humoral immune response. The birds were challenged 1 week following booster vaccinations with $10^2 \text{EID}_{50}$ of Texas GB strain NDV which was administered intramuscularly. The birds were observed for 2 weeks following challenge. Tracheal swabs were taken at 4 days post-inoculation and used for virus isolation. Two trials were conducted, with 12 birds per group used in the 1st trial and 10 birds per group in the 2nd trial.

Birds hatched from SPF in ovo CY-treated and CY-nontreated eggs were used in the second experiment. At 2 weeks of age, blood samples were taken from all in ovo CY-treated and CY-nontreated birds and the MTT blastogenesis microassay was performed using B- and T-cell mitogens. Statistical analysis were performed using t-tests to select those birds that had a significant T-cell response and no significant B cell response. At 3 weeks of age CY-treated birds that had a significant Con A (T-cell) response and no significant LPS (B cell) response were vaccinated subcutaneously (SC) with approximately 40 μg/bird, with either LNDV, UVNDV or injected with PBS. Similarly, the CY-untreated birds that were Con A (T cell) and LPS (B cell) responsive were vaccinated subcutaneously with 40 μg/birds with either LNDV, UVNDV or PBS. The chicks were booster'd at 5 weeks of age and blood samples were collected weekly for evaluation of cellular and humoral immune response. Challenge testing was administered intramuscularly one week post-booster with $10^2 \text{EID}_{50}$ of the Texas GB strain of NDV per bird, and the chicks were observed for 2 weeks afterward. Tracheal swabs were taken 4 days post-inoculation and used for virus isolation.
Two trials were conducted. In the 1st trial 10 CY-treated chickens were vaccinated with UVNDV and 5 were used for other groups. Birds vaccinated with LNDV were not included in the 1st trial. In the 2nd trial, 12 birds were used per group.

Statistical evaluation. T-tests were conducted to test the mitogenic response of each CY-treated bird and CY-nontreated bird to ConA and LPS mitogens. Stimulation index (SI) values were used for statistical evaluation of cell-mediated immune response. The SAS statistical software (SAS Institute, Inc., Cary, NC) was used to compare the average SI of 4 birds from each group vaccinated with NDV vaccine and those from unvaccinated groups. Duncan's multiple range test was used to determine the differences in CMI among vaccinated and control groups, and statistical significance was expressed as highly significant (P < 0.01), significant (P < 0.05), or not significant (P > 0.05). Correlations between HI, CMI and protection for the two experiments were determined by linear regression analysis and expressed as a correlation coefficient (r) for which r > 0 indicated a positive linear relationship and r < 0 indicated a negative relationship.

RESULTS

Humoral immune response. The results from trials 1 and 2 of experiment 1 are shown in Table 1. The results revealed that chickens vaccinated with LNDV and UVNDV produced detectable antibody titers as measured by the HI test, with antibody titers increasing subsequent to booster. However, chickens vaccinated with SDS treated NDV did not produce
Table 1. Antibody titers to NDV as measured by the hemagglutination inhibition (HI) and virus neutralization (VN) assays of birds vaccinated with live, UV inactivated or SDS-treated NDV preparations.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatment</th>
<th>HI titers&lt;sup&gt;B&lt;/sup&gt;</th>
<th>VN titers&lt;sup&gt;B&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Post-vaccination</td>
<td>Post-booster</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10days</td>
<td>14 days</td>
</tr>
<tr>
<td>1</td>
<td>LNDV</td>
<td>4.4 (3-6)&lt;sup&gt;C&lt;/sup&gt;</td>
<td>6.1 (6-7)</td>
</tr>
<tr>
<td></td>
<td>UVNDV</td>
<td>0</td>
<td>3 (0-4)</td>
</tr>
<tr>
<td></td>
<td>SDSNDV</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>LNDV</td>
<td>5 (5-6)</td>
<td>7.3 (6-9)</td>
</tr>
<tr>
<td></td>
<td>UVNDV</td>
<td>0</td>
<td>2.8 (2-5)</td>
</tr>
<tr>
<td></td>
<td>SDSNDV</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>A</sup> LNDV = Live NDV; UVNDV = UV inactivated NDV; SDSNDV = SDS treated NDV; C-PBS = PBS control.

<sup>B</sup> Geometric mean titers of responding birds expressed as reciprocal Log₂.

<sup>C</sup> Numbers within parentheses indicate the range of HI titers within the group.

detectable levels of specific antibody to NDV as measured by the HI assay. Similarly, the virus neutralization results shown in Table 1 revealed that virus neutralizing antibodies were detected in all the chickens inoculated with LNDV and UVNDV but not in chickens vaccinated with SDSNDV or uninoculated control.
Western blot tests revealed that birds vaccinated with LNDV, UVNDV and SDSNDV had positive sera that reacted with NDV polypeptides. However, not all birds vaccinated with NDV treated with SDS had the same reaction to all the NDV polypeptides (Figure 1).

Table 2 shows the HI titers of sera from trials 1 and 2 of experiment 2. The results revealed that in ovo CY-treated birds that were vaccinated with UVNDV did not produce detectable antibody titers during the first and second weeks post-vaccination. However, antibody titers were detected in 3 birds at one week post-booster, and in all CY- nontreated birds that were vaccinated with LNDV and some of the birds vaccinated with UVNDV by 2 weeks post-vaccination. In addition, antibody titers increased in all NDV vaccinated, CY-nontreated birds subsequent to booster vaccinations.

The virus neutralization results (Table 2) also showed that all CY-treated vaccinated birds, with the exception of those birds that responded to the HI test, had no serum antibody response. However, a substantial response was detected in the CY-nontreated vaccinated birds.

**Blastogenesis response.** The results from the blastogenesis microassays that were performed on the samples of four birds from each group are displayed in Tables 3 and 4. The statistical analysis of the CMI responses is shown in the Appendix (Table A7-A10). The results of the first experiment revealed that those birds vaccinated with LNDV, UVNDV and SDSNDV had a significant CMI response to NDV as compared with the control groups (Table 3). There were no significant differences between blood lymphocyte responses isolated from the three inoculation groups. Control birds did not produce a specific response to NDV.
Figure 1. Western blot test results from 10 individual birds vaccinated with SDS-treated Newcastle disease virus.
Table 2. Antibody response of CY-treated and CY-nontreated birds vaccinated with live or UV inactivated NDV.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatment</th>
<th>HI titters</th>
<th>VN titters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Post-vaccination</td>
<td>Post-booster</td>
</tr>
<tr>
<td>1</td>
<td>CY-UVNDV</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>CY-PBS</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>C-UVNDV</td>
<td>0/5</td>
<td>3/5 (3)</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>2</td>
<td>CY-LNDV</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>CY-UVNDV</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>CY-PBS</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>C-LNDV</td>
<td>12/12</td>
<td>12/12 (6.1)</td>
</tr>
<tr>
<td></td>
<td>C-UVNDV</td>
<td>0/12</td>
<td>4/12 (3)</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>0/12</td>
<td>0/12</td>
</tr>
</tbody>
</table>

$^A$ CY-LNDV = CY-treated birds vaccinated with LNDV; CY-UVNDV = CY-treated birds vaccinated with UVNDV; CY-PBS = CY-treated birds injected with PBS; C-NDV = control birds vaccinated with LNDV; C-UVNDV = control birds vaccinated with UVNDV; C-PBS: CY-control birds injected with PBS.

$^B$ Number of birds having HI titters > 1:2.

$^C$ Geometric mean titer of responding birds expressed as reciprocal Log$_2$.

$^D$ Numbers in parentheses indicate the geometric mean titer of responding birds expressed as reciprocal Log$_2$.

The results of the second experiment revealed that the birds vaccinated with UVNDV or LNDV had specific CMI responses to NDV that were significantly higher than that of the PBS injected birds (Table 4).

**Challenge results.** Tables 5 and 6 show the challenge results from the first and second experiments. The results revealed that the birds which did not produce a specific antibody
Table 3. Lymphocyte blastogenesis results from birds vaccinated with live, UV inactivated or SDS-treated NDV preparations.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatment^</th>
<th>Mean SI^B ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LNDV</td>
<td>0.640^ ± 0.233^*</td>
</tr>
<tr>
<td></td>
<td>UVNDV</td>
<td>0.5746^ ± 0.153</td>
</tr>
<tr>
<td></td>
<td>SDSNDV</td>
<td>0.436^ ± 0.052</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>0.07^b ± 0.195</td>
</tr>
<tr>
<td>2</td>
<td>LNDV</td>
<td>0.7151^b ± 0.443</td>
</tr>
<tr>
<td></td>
<td>UVNDV</td>
<td>0.250^ ± 0.156</td>
</tr>
<tr>
<td></td>
<td>SDSNDV</td>
<td>0.4575^ ± 0.059</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>0.043^b ± 0.052</td>
</tr>
</tbody>
</table>

^ LNDV = birds vaccinated with live NDV; UVNDV = birds vaccinated with UVNDV; SDSNDV = birds vaccinated with SDS treated NDV; C-PBS = control birds injected with PBS

^ Average stimulation index of four birds.

* Values within the same column and having different superscripts are significantly different (P<0.05) as measured by Duncan’s multiple range test.

response to ND developed clinical signs of NDV and died by three or four days post-challenge. Due to mortality, tracheal swab samples could not be taken from birds that perished. The LNDV and UVNDV groups did not develop clinical signs of ND nor were viruses demonstrated from their tracheal swabs.

Statistical analyses using correlation coefficients from the two experiments are given in the Appendix (Table A1-A6). The results indicated that there was a high correlation between the presence of a specific antibody response as measured by HI and protection (0.91), whereas
Table 4. Lymphocyte blastogenesis test results from CY-treated and CY-nontreated birds vaccinated with live or UV inactivated NDV preparations.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatment^</th>
<th>Mean SI^B ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CY-UVNDV</td>
<td>0.5217±0.0519*</td>
</tr>
<tr>
<td></td>
<td>CY-PBS</td>
<td>0.0325±0.0178</td>
</tr>
<tr>
<td></td>
<td>C-UVNDV</td>
<td>0.7772±0.442</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>0.045±0.032</td>
</tr>
<tr>
<td>2</td>
<td>CY-LNDV</td>
<td>0.610±0.108</td>
</tr>
<tr>
<td></td>
<td>CY-UVNDV</td>
<td>0.77805±0.173</td>
</tr>
<tr>
<td></td>
<td>CY-PBS</td>
<td>0.0645±0.142</td>
</tr>
<tr>
<td></td>
<td>C-LNDV</td>
<td>0.640±0.233</td>
</tr>
<tr>
<td></td>
<td>C-UVNDV</td>
<td>0.575±0.1539</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>0.070±0.195</td>
</tr>
</tbody>
</table>

^ CY-LNDV = CY-treated birds vaccinated with live NDV; CY-UVNDV = CY-treated birds vaccinated with UVNDV; CY-PBS = CY-treated birds injected with PBS; C-LNDV = control birds vaccinated with live NDV; C-UVNDV = control birds vaccinated with UVNDV; C-PBS = control birds injected with PBS

^B Average stimulation index of four birds.

* Values in the same column having different superscripts are significantly different (P<0.05) as measured by Duncan's multiple range test.
Table 5. Challenge test results of chickens vaccinated with live, UV inactivated or SDS treated NDV after challenge with the Texas GB isolate of NDV.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Group $^A$</th>
<th>No.$^B$</th>
<th>Mortality$^C$</th>
<th>Virus shedding$^D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LNDV</td>
<td>12</td>
<td>0/12</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>UVNDV</td>
<td>12</td>
<td>0/12</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>SDS-NDV</td>
<td>12</td>
<td>12/12</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>12</td>
<td>12/12</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>LNDV</td>
<td>10</td>
<td>0/10</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>UVNDV</td>
<td>10</td>
<td>0/10</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>SDS-NDV</td>
<td>10</td>
<td>10/10</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>10</td>
<td>10/10</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^A$ LNDV = birds vaccinated with live NDV; UVNDV = birds vaccinated with UV-NDV; SDS-NDV = birds vaccinated SDS treated NDV; C-PBS = control birds injected with PBS

$^B$ Number of the birds in each group.

$^C$ Number of dead birds from total challenged birds.

$^D$ Neg. = No virus shedding; ND = Not done.
Table 6. Challenge test results of CY-treated and CY-nontreated birds vaccinated with LNDV or UVNDV. Birds were challenged with the Texas GB strains of NDV.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Group A</th>
<th>No. B</th>
<th>Mortality ^</th>
<th>Virus shedding °</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CY-UVNDV</td>
<td>10</td>
<td>7/10</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>CY-PBS</td>
<td>5</td>
<td>5/5</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>C-UVNDV</td>
<td>5</td>
<td>0/5</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>5</td>
<td>5/5</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>CY-LNDV</td>
<td>12</td>
<td>9/12</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>CY-UVNDV</td>
<td>12</td>
<td>9/12</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>CY-PBS</td>
<td>12</td>
<td>12/12</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>C-LNDV</td>
<td>12</td>
<td>0/12</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>C-UVNDV</td>
<td>12</td>
<td>0/12</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>C-PBS</td>
<td>12</td>
<td>12/12</td>
<td>ND</td>
</tr>
</tbody>
</table>

CY-LNDV = CY-treated birds vaccinated with live NDV; CY-UVNDV = CY-treated birds vaccinated with UVNDV; CY-PBS = CY-treated birds injected with PBS; C-LNDV = control birds vaccinated with LNDV; C-UVNDV = control birds vaccinated with UVNDV; C-PBS = control birds injected with PBS

A Number of the birds in each group.
B Number of dead birds from total challenged birds.
C Neg. = No virus shedding; ND = Not done
an intermediate correlation was found between the presence of specific CMI to NDV and protection (0.47).

**DISCUSSION**

Two approaches have been commonly used for identifying the specific components of the immune system involved in protection (20, 23). One approach has been to destroy one component of the immune system and then demonstrate the protective immunity of the other component (16, 20, 23). Another approach is to passively transfer immunity from an immune donor to a nonimmune animal recipient (23). Cyclophosphamide was used in this study as an immunosuppressive agent to deplete B lymphocytes and suppress humoral immunity (9). In addition, immune-competent birds were vaccinated with denatured NDV proteins to elicit a specific CMI response without inducing a specific HI or neutralizing activity. The results of this study indicated that there was a high positive correlation between the presence of specific NDV antibodies and protection from virulent NDV challenge. The presence of an NDV-specific CMI response in the absence of NDV specific antibodies did not protect birds against virulent NDV challenge. Cyclophosphamide-treated birds that were vaccinated with LNDV or UVNDV at three weeks of age and developed a specific CMI response did not produce specific antibodies and were susceptible to virus challenge. It should be noted that a small number of CY-treated birds that survived the virus challenge had developed an antibody response to NDV one week post-booster. The antibody titers to NDV in the CY-treated birds
that were protected was found to be low as measured by the HI test. It has been reported that protection can be demonstrated in the presence of very low levels of antibodies (5). Gough and Alexander (11) found 100% protection in chickens with low antibody titers (from less than 1:2 to 1:32) as measured by HI.

The results of this study also demonstrated the importance of the protective epitopes that induce specific antibody response to NDV in protection. All sera from chickens immunized with SDS-treated NDV had antibodies that were detected by the Western blot analysis but not with the HI test or VN test. Those birds not having NDV-specific HI or VN antibodies were not protected from challenge. It was hypothesized that SDS treatment of the virus may have destroyed the conformational epitopes needed for inducing virus-neutralizing activity. This result has also been reported to occur with other viruses. For example, immunizing chickens with denatured proteins of IBDV rendered the birds incapable of inducing virus-neutralizing antibodies (13).

A lymphocyte blastogenesis microassay was used in this study to measure cell mediated immunity. It was evident in the experiments that CMI was induced when the chickens were vaccinated with ND vaccine. All birds, including the CY-treated and CY-nontreated birds that were vaccinated with UVNDV, LNDV or SDSNDV, elicited specific CMI as measured by the MTT blastogenesis microassay. No statistical differences were observed among any of the vaccinated groups. The results of the blastogenesis assays in the present study support the conclusions of previous studies (12, 10). Ghumman and Bankowski (10) found that birds vaccinated with LNDV or inactivated ND vaccine elicited CMI as early
as the second day following vaccination. Denatured protein was found to be capable of inducing mitogenic responses of PBLs from cattle immunized with bovine herpes virus 1 (BHV-1) as detected in the lymphocyte proliferation assay (12).

The quantity of NDV antigens that was used in the *in vitro* blastogenesis microassay was less than that reportedly used in other studies (10, 16). Preliminary studies performed in our laboratory indicated that using a low concentration (0.3-0.6 µg/ml) of purified UVNDV produced a specific response without producing a nonspecific response, thus averting the need to eliminate any nonspecific reactivity when using NDV with the blastogenesis microassay as an indicator for NDV specific CMI (22).

One interpretation from the results of this study (see above) is that antibodies are key components for protective immunity to ND. This interpretation might be considered contradictory to other reports that have emphasized the importance of cell mediated immunity as a key component in protection (10, 11, 16, 20). For example, Marino and Hanson (16) reported that *in ovo* bursectomized birds vaccinated with NDV were protected against virus challenge. While their findings suggested a protective role for cellular immunity, they may have overlooked the importance of antibody-mediated protection. In their study, vaccinated bursectomized birds also developed antibodies, but at much lower titers than control non-bursectomized vaccinated birds. The low titer in bursectomized birds was found to be significantly lower and assumed to be non-protective. Therefore, protection was attributed to CMI.
The importance of humoral immunity as a key component of protection against ND is supported by studies involving infectious bursal disease virus (IBDV). It was shown that infection with IBD at one day of age causes a reduction in serological response to ND vaccination, thereby prolonging virus excretion and increasing the susceptibility and severity of ND (1, 19).

Several research findings have supported the importance of CMI in controlling other paramyxovirus infections. Young et al. (23) found that the immune response to Simian virus 5 (SV5) was interposed entirely by cell mediated immunity and serum-neutralizing antibodies which played only a minor role in protection. This may be explained in part by the fact that SV5 establishes persistent non-lethal infections in infected cells, and in vivo spread of the virus occurs by cell-to-cell fusion. In such circumstances, the cytotoxic T cell (Tc cell) is required to lyse the infected cells and clear the infection. Thus, the net effect of Tc cell activity is preventing further spread of the virus and terminating infection. This mechanism may not be applicable to NDV because NDV replicates rapidly, enabling large amounts of the infectious virus to be released quickly from infected cells before an effective immune response can be made. With such an infection, the cellular-immune response is unlikely to be sufficiently rapid to significantly alter the peak titer of the virus achieved in the body, whereas neutralizing antibodies which are more successful in restricting the replication of viral infection and preventing the spread of virus within infected tissues is more likely to be protective (2). Furthermore, recent work with the influenza virus has demonstrated that animals rendered deficient in cytotoxic (Tc) or helper (Th) cell responses were able to clear the influenza virus
infection in a manner similar to their fully immunocompetent counterparts, suggesting that antibodies might participate in the clearance of viral infection (8).

In conclusion, the data from this study support the concept that humoral immunity to NDV is a key component in the protection against Newcastle disease. Therefore, vaccination programs should be directed towards eliciting and maintaining high antibody levels to NDV in flocks of birds.

REFERENCES


ACKNOWLEDGMENTS

The authors thank Dr. Ali Akbar, Sevinc Akinc and Joan Oesper for their technical assistance, and Ms. L. L. Wu for her assistance in the statistical evaluation of the data.
**APPENDIX: DATA AND ANALYSIS**

Table A1. Data of blood lymphocyte blastogenesis assay (CMI), hemagglutination inhibition (HI) test and challenge results in birds vaccinated with either live, ultraviolet inactivated, SDS-treated NDV preparation or PBS. Data are from the first trial in the first experiment.

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* 0 = No protection, 1= protection
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Table A3. Data of blood lymphocyte blastogenesis assay (CMI), hemagglutination inhibition (HI) test, and challenge results in CY-treated and CY-nontreated birds vaccinated with either ultraviolet inactivated NDV or PBS. Data are from the first trial in the second experiment.

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* 0 = No protection; 1 = protection
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Table A5. Analysis of variance of protection results from the four trials.

Dependent variable: protect

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</table>

Corrected total 71

Pearson Correlation Coefficients / Prob > R under Ho: Rho=0

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<th>HI</th>
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<td>Protection</td>
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<td>0.90650</td>
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Table A7. Blood lymphocyte response to UVNDV in CY-treated and CY-nontreated birds vaccinated either with live, ultraviolet inactivated NDV or PBS. The values represent the average stimulation index (SI) for each bird calculated using the formula defined in the experimental design section. Mean SI values for each group with different superscripts indicate a significant difference at the P < 0.05 level.

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<thead>
<tr>
<th>Groups</th>
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<tr>
<td>CY-UVNDV</td>
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<td>0.609</td>
<td>0.486</td>
<td>0.511</td>
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<td>CY-PBS</td>
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<td>0.777a</td>
</tr>
<tr>
<td>C-PBS</td>
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<td>0.02</td>
<td>0.01</td>
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<td>Second trial</td>
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<td></td>
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<tr>
<td>CY-LNDV</td>
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<td>0.567</td>
<td>0.778</td>
<td>0.511</td>
<td>0.610a</td>
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<tr>
<td>CY-UVNDV</td>
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<td>0.672</td>
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<tr>
<td>CY-PBS</td>
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<td>C-LNDV</td>
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<td>0.640a</td>
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<tr>
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<td>-0.02</td>
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Table A8. Analysis of variance of data on cellular immune response of CY-treated bird experiments

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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
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<td>0.06530983</td>
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<tr>
<td>Vaccine* CY</td>
<td>2</td>
<td>0.04235725</td>
<td>0.02177862</td>
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<tr>
<td>Error</td>
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<td>corrected total</td>
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<td>8.73761553</td>
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Table A9. Blood lymphocyte response to UVNDV in birds vaccinated with either live, ultraviolet inactivated, SDS-treated NDV preparation or unvaccinated control. The values represent the stimulation index (SI) for each bird calculated using the formula defined in the experimental design section. Mean SI values for each group with different superscripts indicate a significance at the $P < 0.05$ level.

<table>
<thead>
<tr>
<th>Groups</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Average</th>
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<td></td>
</tr>
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<td>0.605</td>
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<td>0.07 b</td>
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<tr>
<td><strong>Second Trial</strong></td>
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<td>0.01</td>
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Table A10. Analysis of variance of lymphocyte blastogenesis response to UVNDV in birds vaccinated with either live NDV, UVNDV, SDSNDV or PBS.

<table>
<thead>
<tr>
<th>Source</th>
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<th>Mean Square</th>
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<td>Error</td>
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THE ROLE OF ANTIBODIES TO NEWCASTLE DISEASE VIRUS
POLYPEPTIDES IN PROTECTION

Manuscript for submission to Avian Diseases
A. D. Maraqa and D.L. Reynolds

SUMMARY

Studies were undertaken to determine whether passive immunization utilizing
hyperimmune sera that were specific for Newcastle disease virus (NDV) proteins conferred
protection against virus challenge. Six groups of 3-week-old chickens were passively
immunized with antiserum against either HN/F, NP/P, M, a mixture of all proteins (ALL),
intact UV, inactivated NDV, or negative sera. Blood samples were collected two days post-
immunization and the birds were challenged with Texas GB strain of NDV. Antibody titers to
HN/F, ALL and UV-NDV were detected in the recipient birds by a hemagglutination
inhibition (HI) test, an ELISA, and a virus neutralization (VN) test. Whereas antibodies to
NP/P and M were detected only by ELISA, antibody titers in the recipient birds dropped by
two dilutions (Log₂) after two days post-injection. Protective immunity revealed that birds
passively immunized with antisera against HN/F, ALL and UV-NDV were resistant to the
challenge virus, whereas chickens passively immunized with antisera against NP/P and M
proteins and SPF sera developed clinical signs of Newcastle disease. The challenge virus was
recovered from all passively immunized groups. It was concluded that the presence of neutralizing antibodies to NDV provided protection from clinical disease but was unable to prevent virus shedding.

**Keywords:** Newcastle disease virus, passive immunization, humoral immune response, protection

**Abbreviations:**
- CPE: cytopathic effect
- CMF-PBS: calcium and magnesium free-phosphate buffered saline
- F: fusion protein
- EDTA: ethylenediaminetetraacetic acid
- ELD₅₀: embryo lethal dose
- ELISA: enzyme linked immunosorbent assay
- IBV: infectious bronchitis virus
- HI: hemagglutination inhibition test
- HN: haemagglutinin-neuraminidase
- L: large protein
- M: matrix protein
- MTT: (3-[4,5-Dimethylthiazole-2-yl], 2-5-diphenyltetrazolium bromide
- NDV: Newcastle disease virus
- NP: nucleoprotein
INTRODUCTION

Newcastle disease (ND) is a highly contagious viral disease of poultry (2). It causes hemorrhagic intestinal lesions, severe respiratory distress, nervous disorders, decreased egg production and high mortality (2).

Newcastle disease virus (NDV) is the prototype member of the paramyxovirus family and is designated as PMV1. It is an enveloped virus with negative-stranded RNA (2). The NDV virion contains six proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large (L) proteins. The HN and F proteins are glycosylated and form two projections on the lipoprotein envelope of the virion. They have been found to play a role in neutralizing NDV in vivo and in vitro by preventing virus attachment and cell fusion activity, respectively (2). The M protein is located on the inner
surface of the envelope and provides structural integrity for the virion. The NP, P and L proteins are associated with genomic RNA to form the nuclecapsid (2). The role of antibodies to NP/P and M proteins in protection against NDV infection is unclear.

Following vaccination with NDV, antibodies directed towards the various components of the virus are produced to protect the birds against ND infection (3). Although high levels of antibodies have always been associated with protection against ND (2, 4), reports have suggested that serum antibodies are not directly correlated with the resistance of chickens to experimental NDV challenge (7, 8, 9, 11). One study found that a low level of antibodies was capable of preventing infection (9), while another (11) found that chicks vaccinated with an inactivated NDV vaccine given intramuscularly induced little resistance despite a high concentration of serum antibody. Studies with other paramyxoviruses have indicated that neutralizing antibodies may not be sufficient to protect against the disease (16, 25). It has also been reported that the presence of neutralizing antibodies, although they may lessen the severity of disease, are unable to prevent infection (16).

The objective of this study was to determine the role of passively administered antisera directed against individual NDV polypeptides (or whole virus) in protection.
MATERIALS AND METHODS

Embryonated eggs and chickens. Specific pathogen-free (SPF) eggs (HY-Vac Co., Gowrie, IA) were used for virus propagation and hatching. Chicks were reared on wire-floored cages for three weeks, and feed and water were provided ad libitum.

Media and reagents. A solution of minimum essential medium (MEM) (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml), and fungizone (0.5 µg/ml) was used to culture swine testicular (ST) cells. The MEM was used without serum as a maintenance media. A calcium and magnesium free-phosphate buffered saline (CMF-PBS) was used for washing monolayers.

Virus strains. The lentogenic strain B1 of NDV was propagated in nine-day-old SPF-embryonated eggs by the chorioallantoic route. The allantoic fluid was harvested and centrifuged at 3000 x g for 30 minutes. Then the virus was purified and concentrated from the allantoic fluids.

The velogenic Texas GB strain of NDV was propagated in embryonic eggs and used as an inoculum at 10^2 ELD_{50}/bird and used as the challenge virus (see below).

Virus propagation and purification. Newcastle disease virus purification was based on the method of Alexander and Collins (3). The lentogenic strain B1 of NDV was grown in 9-day-old embryonated chicken eggs for 5 days at 37 C. The allantoic fluid was clarified by centrifugation at 3000 x g for 30 minutes. The virus was pelleted by centrifugation at 50,000
x g for 2 hours in a SW28 rotor (Beckman). Then the pellet was resuspended in 0.01 M Tris- 
NaCl pH 7.2 and applied to a discontinuous sucrose gradient made from 14 ml 50% w/v and 
21 ml 20% w/v in 10 mM Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4. Following centrifugation 
for 2 hours at 50,000 x g, a visible band was observed at the sucrose gradient interface. After 
the virus band was collected and pelleted at 50,000 x g for 2 hours, the pellet was 
resuspended in PBS. The purified virus was assayed for total protein concentration using the 
Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) and the purity was assayed by 
Coomassie blue-stained polyacrylamide gel electrophoresis. The purified virus was inactivated 
by exposure to ultraviolet light for 40 minutes and evaluated to assure the loss of infectivity in 
the embryonic eggs. Then the inactivated NDV (UVNDV) was used as an inoculum at 40 μg 
per bird.

Preparation of NDV proteins. The procedure for isolating NDV glycoproteins 
(HN/F), nucleoprotein and nucleocapsid associated protein (NP/P), and the matrix (M) from 
the NDV virus was performed as described previously (23). Briefly, purified NDV 
(approximately 0.5 mg/ml) was diluted 1:5 (v/v) in 4% Triton X-100 (Sigma) in 0.01 M Tris 
buffer (pH 7.2) in the presence of 1 M KCl. The solution was mixed gently at room 
temperature for 45 minutes. The suspension was centrifuged at 10,000 x g for 35 minutes, 
and a clear supernatant fluid containing HN, F and M polypeptides, and a pellet containing 
NP/P was obtained. The pellet was washed repeatedly in PBS and pelleted by centrifugation at 
10,000 x g for 1 hour at 4 C. The supernatant from the 10,000 x g centrifugation was 
centrifuged at 200,000 x g for one hour to remove any remaining nucleocapsid or
incompletely disrupted virus. Then the pellet was discarded and the supernatant fluid was dialyzed against 0.01 M PBS for 16 hours in order to remove potassium chloride. After dialyzing, the matrix protein (M) was separated from the glycoproteins (HN and F) by centrifugation at 10,000 × g for 30 minutes. The pellet (M protein) was suspended in PBS. The proteins which remained in the supernatant fluid after removal of M protein were centrifuged repeatedly at 10,000 × g for 10 minutes at 4 C to remove any other protein contaminates. The purity of the collected proteins was assayed by Coomassie blue-stained polyacrylamide gel electrophoresis.

**Preparation of antiserum.** Hyperimmune serum to purified NDV proteins were prepared. The separated HN/F, NP/P and M polypeptides were emulsified by mixing with an equal volume of incomplete Freund’s adjuvant. The inoculum was injected subcutaneously into 3-week-old SPF chickens (two birds per group). The chickens were boosterod 2 weeks later and then blood was collected weekly for 5 weeks.

Hyperimmune serum to purified ISIDV were also prepared by subcutenous injection of 40 μg/ml of UVNDV emulsified with incomplete Freund’s adjuvant into three-week-old SPF chickens. The chickens were boostered 2 weeks later and then blood was collected weekly for 5 weeks.

**ELISA.** An avidin-biotin dot immunobinding assay was used as previously described (6). Briefly, 0.12-inch nitrocellulose membrane disks (Trans-Blot Transfer Medium, Bio-Rad Laboratories, Hercules, CA) were cut and placed at the bottom of each of the wells of a 96-well, flat-bottom tissue culture plate (Becton Dickinson and Company, Lincoln Park, NJ).
One microliter of purified NDV (100 μg/ml) was dotted in the middle of each disk and the plate was incubated over night at 37 C to dry the dot. One hundred microliters of diluent buffer were added to each well. Then, 100 μl of 1:100 diluted serum were added to each well of the first column of wells. Afterward, twofold serial dilutions were performed across the plate to achieve a dilution range of 1:100 to 1:204,800. Positive and negative sera were also included in the test. After one hour of incubation, the secondary biotinylated antibodies (biotinylated anti-chicken IgG, Vector Laboratories, Burlingame, CA) at a dilution of 1:5000, strept-avidin at a dilution of 1:2000, and the chromogen (4-Chloro-l-Naphthol; Sigma chemical Co., St. Louis, MO) were subsequently added. The nitrocellulose membrane was washed and blocked between each step. Finally, the plates were incubated in the dark until dark purple dots appeared against the white (nitrocellulose) background, which was considered a positive reaction.

**Western blot.** Purified NDV was solubilized in a gel sample buffer containing 0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.001% bromophenol blue and heated at 100 C for two minutes. The proteins were separated on a 10% polyacrylamide gel according to the procedure of Laemmli (15). The gel was electrophoresed at a constant voltage of 150 V for four hours. Part of the gel was stained with Coomassie brilliant blue G 250 (Pharmacia Biotech, Piscataway, NJ) destained with methanol, and fixed. The remaining portion of the gel was prepared for transfer onto a 0.45 μm nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad Laboratories, Hercules, CA) for Western blot analysis. The transfer was carried out in a transfer buffer consisting of 25 mM Tris, 192 mM glycine (pH 8.3) and 20%
v/v methanol at a constant voltage of 60 V for 4 hours. After transfer the membrane was stained with 0.05% Ponceau S dye (Fisher Biotech, Fisher Scientific, Fair Lawn, NJ) in 1% acetic acid for band visualization. The nitrocellulose membrane containing the separated proteins was cut into 7 mm strips and each strip was placed into an individual well on an immunoblotting plate. The ELISA procedure was used for developing the blot.

**Hemagglutination inhibition (HI) test.** The HI test was performed as described (3). Briefly, twofold serial dilutions of serum were made in a 96 well, round-bottom microtiter plate containing 50 μl of PBS in the first row and 50 μl of NDV antigen (10 HA units) in the remaining 11 rows. Serum dilutions ranged from 1:2 to 1:2048. The antigen serum mixture was incubated for 30 minutes at 37 C. Then, 50 μl of a 0.05% turkey erythrocyte suspension were added to each well and reincubated for 30 minutes. A positive serum, a negative serum, erythrocytes and antigens were also included as controls. The highest dilution of serum causing complete inhibition was considered the endpoint. The geometric mean titer was expressed as reciprocal Log₂ values of the highest dilution that displayed HI.

**Virus neutralization (VN) test.** The virus neutralization test was performed as described previously (1), except the ST cell line was used instead of chicken embryo fibroblasts. Briefly, 50 microliters of media were added into an empty 96-well flat-bottomed tissue culture plate (Corning Laboratory Sciences Co., Corning, NY). Another 50 μl of the serum sample were added into the first column. Twofold serial dilutions were made across the plate to achieve dilutions from 1:2 to 1:1024. One hundred tissue-culture infectious doses (TCID₅₀) of the Texas GB strain of NDV in 50 μl were mixed with equal volumes of the
serum dilutions. Then the plate was incubated for one hour at 37 C in a humidified 5% CO₂ incubator. Fifty μl of media without antibodies were mixed with the virus suspension for the virus control, and 100 μl of medium without antibodies or virus were used for a cell control. Positive and negative serum controls were also included in the experiment. All samples were tested in quadruplicate. Following a 1-hour incubation period, 100 μl of the virus-serum mixture were transferred into 96-well culture plates containing monolayers of a swine testicular (ST) cell line and incubated at 37 C for 72 hours. The plate was examined for cytopathic effect (CPE) to confirm the presence of the virus. The 50% neutralizing endpoint (ED₅₀) was calculated by the method of Reed and Muench (21).

**Sample collection for virus isolation.** A cotton-tipped applicators were used for swabbing the trachea. The applicators were then placed into 2 ml a tryptose phosphate broth containing penicillin and streptomycin and frozen (-70 C). On the day of inoculation, the tubes were thawed and the swabs were removed. The remaining fluid was centrifuged at 1000 x g for 10 minutes, and the supernatant was collected and used for embryo inoculation. Five 9-day-old embryonated eggs per tracheal swab sample were inoculated via the chorioallantoic route with 0.1 ml of inoculum per embryo. The eggs were incubated at 37 C for 7 days. Then the presence of NDV in the harvested chorioallantoic fluid was determined by hemagglutination (HA) testing using 1% turkey erythrocytes.

**Experimental design.** Six groups (10 chickens/group) of 3-week-old SPF chickens were passively immunized subcutaneously with 4 ml with either anti-HN/F sera, anti-NP/P sera, anti-M sera, a mixture of all NDV proteins sera (ALL), anti-UVNDV sera, or negative
SPF sera. Blood samples were collected 2 days post-immunization and the birds were challenged with $10^2 \text{ELD}_{50}$ of the Texas GB strain of NDV per bird administered intramuscularly. Tracheal swabs were taken at 4 days post-inoculation and used for virus isolation. All the chickens were observed for 14 days following challenge. Two trials were conducted and in each trial 10 birds per group were used.

RESULTS

The antibody titers of birds vaccinated with various NDV polypeptides are shown in Table 1. Those birds that were vaccinated with the HN/F glycoproteins and UVNDV had detectable levels of specific antibodies as measured by the HI, ELISA and VN tests, whereas birds vaccinated with NP/P and M proteins had antibodies that were detected by the ELISA but not by the HI or the VN tests. A combination of anti-HN/F, NP/P and M sera had antibodies that were detected by the HI, ELISA and VN tests. The Western blot results revealed that all vaccinated groups had a positive antibody response to their respective polypeptides to which they had been vaccinated (Figure 1).

Table 2 displays the results of serum titers from trials 1 and 2 of the recipient birds two days post-administration. The results revealed that recipient birds which were passively immunized with anti-HN/F sera, a combination of ALL sera, or UVNDV sera had detectable antibody titers as measured by the HI, ELISA and VN tests, whereas recipient birds passively immunized with anti-NP/P or anti-M sera had detectable antibody titers only in the ELISA
Table 1. Antibody titers of donor sera obtained from chickens vaccinated with NDV or various NDV polypeptide preparations.

<table>
<thead>
<tr>
<th>SeraA</th>
<th>Antibody titer reciprocal Log2</th>
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</thead>
<tbody>
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<td></td>
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<td>ELISA^C</td>
</tr>
<tr>
<td>HN/F</td>
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<td>8</td>
</tr>
<tr>
<td>NP</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>M</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>ALL</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>UV-NDV</td>
<td>7</td>
<td>9</td>
</tr>
</tbody>
</table>

^A Sera collected from birds vaccinated with HN/F, NP/P, M or UVNDV.
^B Hemagglutination inhibition-geometric mean titers expressed as reciprocal Log2.
^C Enzyme-linked immunosorbant assay. ELISA was performed as reciprocal Log2 dilution x 100.
^D Virus neutralization assay. The neutralization titer was the reciprocal of the dilution of antisera which neutralized 50% of virus.

dilutions lower than the original sera used for administration.

The challenge results from trials 1 and 2 are shown in Table 3. The results revealed that the majority of recipient birds passively immunized with anti-HN/F sera, ALL sera and UVNDV sera were protected from challenge. However, birds passively immunized with anti-NP/P sera, anti-M sera and negative SPF sera developed clinical signs of Newcastle disease. Clinical signs of ND and/or death appeared by the third day post-challenge in birds that were
Figure 1. Immunoblot results of chickens vaccinated with: (1) UV-inactivated NDV; (2) NDV-HN and F glycoproteins; (3) NDV-NP/P proteins, and (4) NDV-M proteins.
Table 2. Antibody titers of recipient birds passively immunized with NDV hyperimmune sera.

<table>
<thead>
<tr>
<th>Trial</th>
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<th>Antibody titer Log&lt;sub&gt;2&lt;/sub&gt;</th>
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<td></td>
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<td>No.</td>
</tr>
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</tr>
<tr>
<td></td>
<td>NP</td>
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</tr>
<tr>
<td></td>
<td>M</td>
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</tr>
<tr>
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<td>ALL</td>
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<tr>
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<td>UVNDV</td>
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<td>M</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>ALL</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>UVNDV</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>A</sup> Donor sera obtained from SPF chickens immunized with separated NDV proteins, UV-NDV or unimmunized SPF negative sera.

<sup>B</sup> Hemagglutination inhibition-geometric mean titers expressed as reciprocal Log<sub>2</sub>.

<sup>C</sup> Enzyme-linked immunosorbant assay. Geometric mean titer expressed as reciprocal Log<sub>2</sub> dilution x 10.

<sup>D</sup> Virus neutralization assay. The neutralization titer was the reciprocal Log<sub>2</sub> of the last dilution of antisera which neutralized 50% of the virus.
Table 3. Results of challenge and virus isolation from birds passively immunized with NDV antisera preparations.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Group</th>
<th>No.</th>
<th>Morbidity</th>
<th>Mortality</th>
<th>% Protection</th>
<th>Virus shedding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HN/F</td>
<td>10</td>
<td>3/10</td>
<td>1/10</td>
<td>70%</td>
<td>8/10</td>
</tr>
<tr>
<td></td>
<td>NP</td>
<td>10</td>
<td>0/10</td>
<td>8/10</td>
<td>20%</td>
<td>2/2</td>
</tr>
<tr>
<td>1</td>
<td>M</td>
<td>10</td>
<td>0/10</td>
<td>9/10</td>
<td>10%</td>
<td>1/1</td>
</tr>
<tr>
<td></td>
<td>ALL</td>
<td>10</td>
<td>4/10</td>
<td>1/10</td>
<td>60%</td>
<td>5/10</td>
</tr>
<tr>
<td>1</td>
<td>UVNDV</td>
<td>10</td>
<td>3/10</td>
<td>0/10</td>
<td>70%</td>
<td>6/10</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>10</td>
<td>0/10</td>
<td>10/10</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>HN/F</td>
<td>10</td>
<td>3/10</td>
<td>1/10</td>
<td>70%</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
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<td>10</td>
<td>0/10</td>
<td>10/10</td>
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<td>ND</td>
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<tr>
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<td>10</td>
<td>0/10</td>
<td>10/10</td>
<td>0</td>
<td>ND</td>
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<tr>
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<td>0/10</td>
<td>70%</td>
<td>8/10</td>
</tr>
<tr>
<td>2</td>
<td>UVNDV</td>
<td>10</td>
<td>2/10</td>
<td>0/10</td>
<td>80%</td>
<td>7/10</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>10</td>
<td>0/10</td>
<td>10/10</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

^Number of birds in each group
B Number of birds displaying clinical signs of NDV.
C Percentage of surviving birds that did not display clinical signs of disease.
D Number of isolations/number of samples.
E ND: Not done.
not protected. The challenge virus was recovered from all groups of passively immunized birds.

**DISCUSSION**

Previous studies addressing the role of cell mediated immunity (CMI) in protection from ND have indicated that specific CMI to NDV by itself was not protective against a virulent NDV challenge (18). These studies have indicated that the presence of specific antibodies, as determined by the HI or the VN tests to NDV, is important in protection from ND. A high correlation was found between the presence of specific HI or neutralizing antibody response to NDV and protection. In this study, a passive immunization approach was used to determine whether antibodies to NDV conferred protection. In addition, the role of antibodies directed against specific NDV polypeptides in protection was determined. The results of this study revealed that birds passively immunized with anti-HN/F sera, a mixture of all (ALL) sera, and anti-UVNDV sera were protected from a lethal challenge of NDV, whereas birds passively immunized with anti-NP/P and anti-M sera were not protected. Similar results have been reported in other studies (12, 19, 20, 24). Umino et al. (24) found that passive administration of antiserum against intact NDV, or the surface glycoproteins HN/F, provided protection to susceptible chickens against NDV challenge. However, there were have been no reports of the protective role of the other NDV polypeptides. In studies using Simian virus 5 (SV5) Randall et al. (20) found that *in vitro* neutralization was provided
by antibodies directed against the surface HN glycoprotein but neutralization was not achieved when antisera against the internal proteins NP, P or M were used. Furthermore, similar findings have been reported for infectious bronchitis virus (IBV). It was found that immunizing birds with purified nucleocapsid (N) and membrane (M) proteins of the IBV did not protect them against virulent challenge despite high antibody titers, whereas birds immunized with the surface S1 glycoprotein were protected (14).

The results of this study corroborate previous findings in which humoral immunity was considered a key component in protective immunity (18). Similar findings supporting the role of antibodies in preventing the clinical signs of ND were observed in this study. However, passively acquired antibodies were found to have little effect on the ability to prevent tracheal viral shedding. The challenge virus was detected in the majority (but not all) of passively immunized protected birds four days post-challenge. The differences between the two studies may be due to the amount of antibodies that may have been transported to the mucosal surface of the respiratory tract. Yoshida (26) reported that the amount of neutralizing antibody titers were very low in tracheal samples from birds that had been passively immunized with anti-NDV hyperimmune sera. The transport of antibodies from the circulatory system was also different between the upper and lower respiratory tracts. The transudation of serum antibodies into the upper respiratory tract was reported to be very limited in chickens administered high-titered antiserum to avian IBV. However, transudation of antibodies to the lung correlated with the concentration of serum antibodies (11). Similar observations have been reported following influenza virus infections of neonatal ferrets. Husseini (10) found that
ferrets with high maternal antibodies demonstrated complete protection in their lungs, however, virus replication occurred in their upper respiratory tract.

Many studies have addressed those conditions required to obtain the presence of antibody in the respiratory tract (7, 8, 11). Holmes (12) reported that either direct or indirect exposure of the respiratory tract to an antigen was necessary for inducing neutralizing antibody. In birds having maternal antibodies, it was found that the lacrimal fluid antibody titers ranged from 1-9% of the serum antibody titers. Following intraocular vaccination with live NDV the lacrimal fluid antibody titers increased significantly. The increase in antibody titers were attributed to local replication of NDV in the Harderian gland (22). Ewert et al. (8) reported that birds which had been passively immunized with antisera to NDV showed a sharp rise of antibodies in the trachea four days following intratracheal viral inoculation. This was found to be a result of transudation of antibodies from the serum which coincided with the course of viral pathology observed in the trachea of the infected birds.

The importance of the presence of a local antibody response in the respiratory tract of NDV-vaccinated birds in protection has been documented (5, 10, 11, 12, 16). It has been reported that vaccination with NDV via aerosol administration protected them against challenge given by the respiratory route, but not against intramuscular challenge, whereas the vaccination of chickens via the airsac protected the birds against airsac challenge but not against intraocular route (5, 16). The replication of the virus in the respiratory tract in the presence of circulating antibodies may provide ample antigenic stimulation to confer "full protection" to the host, that is, no clinical disease or virus replication in the trachea. It was
found that vaccinated birds derived from maternally-immune chickens which had been vaccinated with NDV were more resistant to challenge than were corresponding unvaccinated birds. Surprisingly, there was no difference in the serum antibody response titers between vaccinated and unvaccinated birds (7).

In conclusion, the results of this study indicated the importance of the presence of antibodies to the HN and F polypeptides of NDV in resistance to NDV challenge. In addition, the demonstration of the challenge virus from the tracheas of protected birds is suggestive of the importance of the local immune response in protection.

REFERENCES


ACKNOWLEDGMENTS

The authors thank Dr. Ali Akbar, Sevinc Akinc and Joan Oesper for their technical assistance.
A RAPID VIRUS NEUTRALIZATION ASSAY FOR
NEWCASTLE DISEASE VIRUS USING THE SWINE TESTICULAR
(ST) CONTINUOUS CELL LINE

Manuscript for submission to *Avian Diseases*
A. D. Maraqa and D. L. Reynolds

**SUMMARY**

Five continuous cell lines, swine testicular (ST), human rectal tumor (HRT 18), fetal rhesus monkey kidney (MA104), bovine turbinate (BT) and quail tracheal (QT35) were evaluated and compared with chicken embryo fibroblasts (CEF) for their ability to propagate B1 or Texas GB strains of Newcastle disease virus (NDV). The NDV Texas GB strain was found to replicate in all the continuous cell lines used in this study. Only the ST and QT35 cells produced a cytopathic effect (CPE) similar to that produced in CEF. However, the ST cell line remained attached while displaying CPE, whereas infected QT35 cells detached as did the CEF. The B1 strain of NDV was found to replicate in ST cells, MA104 cells and CEF but with less CPE as compared to the Texas GB strain. Pretreatment with trypsin did not enhance CPE with either NDV strain. Sera evaluated for neutralizing antibody titers to NDV were found to be significantly higher in titers when the ST cell line was used and compared to CEF. A high correlation was found between microscopic examinations and MTT microassay...
methods for determining the viral neutralization endpoint, thus suggesting the ST cell line and MTT assay could be used as an alternative to CEF and microscopic examination for evaluating neutralizing antibodies titers to NDV.

**Keywords:** Newcastle disease virus, virus neutralization, continuous cell line, MTT assay.

**Abbreviations:**
- **BT** bovine turbinate cells
- **CEF** chick embryo fibroblast
- **CMF-PBS** calcium and magnesium free-phosphate buffered saline
- **CPE** cytopathic effect
- **DMEM** Dulbecco's modified eagle's medium
- **FBS** fetal bovine serum
- **EDTA** ethylenediaminetetraacetic acid
- **HEPES** N-2 hydroxy-ethylpiperazine-N’2-ethanesulfonic acid
- **HRT 18** human rectal tumor cell
- **MEM** minimum essential medium
- **MA 104** monkey kidney cell line
- **NDV** Newcastle disease virus
- **PBS** phosphate buffer saline
- **QT35** quail trachea
INTRODUCTION

The humoral immune response to Newcastle disease virus (NDV) is commonly evaluated by hemmagglutination inhibition (HI), ELISA, agar gel precipitation (AGP), and/or the virus neutralization (VN) test (1). Conventionally, the HI test has been widely used and is an acceptable serological technique for monitoring antibody levels against NDV in poultry (1). However, a lack of correlation has been reported between serological responses, as measured by the HI test, and protection against virus challenge following vaccination with ND vaccines (6). It has been reported that the HI assay could not detect low levels of circulating antibodies (6). Beard and Brugh (3) indicated that neutralizing antibodies to NDV in vaccinated birds could be demonstrated several months after vaccination by a variety of procedures even though the HI titers were negative. These results suggest that a more sensitive method for measuring the humoral antibody response is required for NDV to predict protective immunity.

Virus neutralization (VN) is a test used frequently to determine the ability of serum antibodies to neutralize the infectious agent after vaccination (5). The most commonly used method to evaluate neutralizing antibody titers to NDV is based on the presence or absence of cytopathic effect (CPE) in chicken embryo fibroblast cells (1, 2). Such an assay requires a
constant source of specific pathogen-free (SPF) embryos, and the preparation and maintenance of primary and secondary chicken embryo fibroblasts (CEF) (4). Additionally, CEFs have a limited period of use. Typically, the neutralizing endpoint is determined by a microscopic examination of individual culture wells and an estimation is made of the extent of CPE. Consequently, this methodology is time-consuming and the results have an inherent subjectivity in endpoint determination (7).

Recently, several microneutralization immunoassays have been described for quantitating neutralizing antibody titers (7, 8, 12, 13) which have been employed in various methodologies for automation. Many assays have used an ELISA reader in place of microscopic examinations to obtain quantitative results. This saves time and labor, and produces an objective determination of a neutralizing antibody titer endpoint.

The objectives of this study were to identify a continuous cell line capable of supporting NDV replication which could be used in a VN microassay and to adopt rapid methodology by utilizing ELISA reader technology.

**MATERIALS AND METHODS**

**Media and reagents.** Minimum essential medium (MEM), Dulbecco’s modified eagle’s medium (DMEM) (Life Technologies, Inc., Gaithersburg, MD), and Hams F10-medium 199 mixture (1:1) were supplemented with 10% fetal bovine serum (FBS) (Atlanta Biological, Norcross, GA). Pencillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone
(0.5μg/ml) were used to culture different cell lines. The maintenance media for propagating viruses or maintaining cells were the same as the growth medium but without FBS. A calcium and magnesium-free-phosphate buffered saline (CMF-PBS) was used for washing the monolayers. A solution of 0.05% trypsin supplemented with 0.53 mM ethylenediaminetetraacetic acid (EDTA) was used for cell passage. An MTT (3-[4,5-Dimethylthiazole-2-yl]2-5-diphenyltetrazolium bromide; Sigma Chemical Co., St. Louis, MO) solution was prepared by dissolving 10 mg of MTT in 1 ml of CMF-PBS and solubilized by sonication. The solution was then filtered through a 0.45 μm syringe filter and stored at 4 °C in a light-proof bottle. The HCl-isopropanol (0.04 N HCl-isopropanol) solution was prepared by adding 40 ml of 1 N HCl to one liter of isopropanol. Then the HCl isopropanol solution was stored at room temperature in a light-proof bottle.

**Viruses.** The lentogenic strain B1, B1 and the velogenic Texas GB strains of NDV were grown in nine-day-old embryonated SPF chicken eggs. Embryonated eggs were inoculated by the chorioallantoic route and incubated for 5 days at 37 °C. The allantoic fluid was harvested and then clarified by centrifugation at 1000 × g for 30 minutes. The infectivity of the virus was determined by establishing an EID₅₀ in embryonated eggs. The allantoic fluid was aliquoted and stored at -70 °C.

**Cell culture.** Chicken embryo fibroblasts (CEF) and the following cell lines were used in this study: MA104 fetal rhesus monkey kidney, bovine turbinate (BT), HRT18 (human rectal tumor cells), swine testicular (ST) and QT35 (quail tracheal). Chicken embryo fibroblasts were prepared from nine-day-old SPF chicken embryos (Hy-Vac Co., Gowrie, IA).
and were cultured in DMEM containing 10% FBS. The MA104, HRT18 and BT cells were grown in DMEM, whereas the ST cells were grown in MEM medium. The F10-199 medium was used to culture QT35 cells. All of these cells were grown to 80-90% confluency and maintained in 25 cm² culture flasks (Corning Laboratory Sciences Co., Corning, NY) at 37 °C in 5% CO₂. Cells were passaged every 4 days using trypsin-EDTA, and placed into new 25 cm² culture flasks (2 × 10⁴ cells) and incubated at 37 °C in 5% CO₂.

**Virus inoculation.** Flasks containing monolayers of either chicken embryo fibroblasts or one of the continuous cell lines were washed twice with their respective serum-free media and were inoculated with 10⁴ EID₅₀/ml (1 ml/per flask) of NDV. Following a one-hour adsorption period at 37 °C, the cell monolayers were washed twice with their respective serum free media and 10 ml of maintenance medium was added. Additionally, NDV was pre-treated with 10 μg/ml of trypsin for 60 minutes at 37 °C and used as an inoculum at a final concentration of 0.5 μg/ml (10⁴ EID₅₀ /ml). Cells without virus and cells with 0.5 μg/ml trypsin were used as controls. The cell culture flasks were observed daily for 5 days for cytopathic effects (CPEs).

**Serum samples.** A number of serum samples, derived from SPF chickens previously vaccinated with NDV, with different antibody titers were used in this study. The sera were heat-inactivated at 56 °C for 30 minutes and stored at -70 °C until used. Based on the HI results, the sera were placed into low, medium and high titers groups, respectively. Another eight serum samples were used to determine the effect of serum concentration on cell survival.
Virus titration in CEF and ST cells. Chicken embryo fibroblasts and ST cells were prepared in 96-well flat bottomed tissue culture plates (Corning Laboratory Sciences Co., Corning, NY). Ten-fold serial dilutions of the virus preparation were made in the respective FBS-free media. The virus titration ranged from $10^1$ to $10^{10}$. One-hundred microliters of each virus dilution were added per well using 12 replicate wells per dilution. The inoculated cultures were then incubated at 37 C for three days and examined for CPE. The TCID$_{50}$ was calculated according to the method of Reed and Muench (10).

Virus neutralization (VN) assay. Twelve sera samples with different HI antibody titers were used for assessing the VN assay. Microscopic examinations were made to determine the amount of CPE. Alternatively the tetrazlium dye, MTT, was used to measure the CPE. Fifty microliters of media were added into empty 96-well flat-bottomed tissue culture plates (Corning Laboratory Sciences Co., Corning, NY). Fifty microliters of the serum to be tested were added to the wells of the first column. Then, twofold serial dilutions were made across the plate to achieve a dilution range from 1:2 to 1:1024 (column 1-10). A solution of 100 TCID$_{50}$ of the Texas GB strain of NDV, in a volume of 50 |\mu|l, was mixed with an equal volume of the diluted serum. The plates were incubated for one hour at 37 C in a humidified 5% CO$_2$ incubator. A 50-\mu|l medium without serum was mixed with the virus suspension (column 11) for the virus control, representing a 0% neutralization. For the cell control, representing 100% neutralization, a medium without serum or virus was used (column 12). One hour following incubation, 100 |\mu|l of the virus-serum mixture were transferred into 96-well culture plates containing ST cells or CEF monolayers and incubated
at 37 C for 72 hours. At 69 hours of the incubation period, a microscopic examination of the plate was made before adding MTT. Following that, 10 μl of MTT (10 mg/ml) were added to each well and the plate was reincubated for three hours. At 72 hours of incubation, the plate was centrifuged at 1000 × g for 10 minutes at room temperature. The supernatant was removed and 100 μl of 1N HCl-isopropanol were added to each well to aid in dissolving the formazan crystals. The plate was shaken for 10 minutes on a plate shaker (mini-orbital shaker, Belco Biotechnology, Vineland, NJ) and the cells were thoroughly resuspended by repeated pipetting with a micropipetiter. The absorbance of each well was measured using a microtiter ELISA reader (Model EL310, Bio-Tek Instruments, Inc., Winooski, VT 05404) at a wavelength of 550 nm.

All samples were tested in quadruplicate. The average of the absorbance was calculated for each serum dilution, the virus control, and the cell control wells. The absorbance value which was used to calculate the 50% neutralizing endpoint (VN50) for each sera was determined as follows:

\[
\text{(average absorbance of cell control without virus) + (average absorbance of virus control)} / 2
\]

Then the average absorbance of each serial dilution was plotted for each sample tested. The serum dilution, which corresponded to a 50% neutralizing endpoint as calculated above, was determined from the neutralization curve or by using the method of Reed and Muench (10).

**Serum effect on the MTT VN microassay.** The purpose of this experiment was to determine whether the serum had an effect on the VN assay. Fifty microliters of media were
added to the first 4 rows of the empty microtiter plate (Corning Laboratory Sciences Co., Corning, NY). An additional 50 μl of 1:2 diluted serum were added into the first well of each row. Twofold serial dilutions were then performed across the plate to achieve a dilution range from 1:4 to 1:8190 (from column 1 to column 12). Subsequently, 50 μl of media were added to the first 4 rows and 100 μl of media were added to the remaining 4 rows. The sera dilutions were transferred into the CEF or ST cell line in 96-well tissue culture plates and incubated at 37°C for 72 hours. At 69 hours of incubation, MTT was added and the virus neutralization procedure was performed as described above. A statistical analysis was conducted to determine if the serum had an effect on the cells.

**Statistical evaluation.** Statistical analyses were conducted using the SAS statistical software package (SAS Institute Inc., Cary, NC). Statistical significance was expressed as highly significant (P < 0.01), significant (P < 0.05), or not significant (P > 0.05). An analysis of variance (ANOVA) using Duncan’s multiple comparison procedure was performed to determine the differences between neutralizing antibody titers as determined by the two methods (microscopic examination and MTT microassay) in both CEF and ST cell lines. The correlation between neutralizing antibody titers for the two methods was evaluated by assessing the significance using the Pearson correlation coefficient (r), where r > 0 indicates a positive linear relationship and r < 0 indicates a negative relationship.

To ascertain whether the presence of serum had any effect in determining the 50% cell survival endpoint (VN50) in CEF, multiple linear regressions were used to relate absorbance values to dilution, allowing different serum samples to have different intercepts. The
hypothesis of no serum effect was tested by comparing the fit of two regressions: one assuming no serum effect and the other allowing for a serum effect using a F test. An ANOVA was also used to analyze the ST cell line.

RESULTS

Growth of Texas GB and B1 strains of NDV in CEF and continuous cell lines.

Microscopic examinations of cell cultures infected with lentogenic B1 strain and the velogenic Texas GB strain of NDV revealed that the Texas GB strain replicated in all the continuous cell lines evaluated in this study. Replication of Texas GB strain produced distinctive CPE. However, only the ST and MA104 cell lines, in addition to the CEF, supported the B1 strain of NDV. Less CPE was observed when compared to Texas GB strain. No CPE was detected when the B1 was inoculated into the other cell lines.

The extent of CPE induced by different NDV strains varied considerably (Table 1). The CPE appeared sooner with the virulent strain (Texas GB) than the B1 strain. Typically, cell cultures which were infected with Texas GB displayed CPE between 48-72 hours after infection, with the notable exception of HRT18 cells in which CPE appeared after 96 hours. The greatest amount of cellular destruction was observed in CEF, QT35 and ST cell lines infected with the Texas GB. In contrast, those cell cultures infected with the B1 strain (CEF, ST and MA104) showed less cell destruction and, at 96 hours after infection, much of the cell culture remained intact and not infected (Figure 1).
Table 1. Cytopathic effect induced by the NDV strains: B1, B1 and Texas GB in chicken embryo fibroblasts and various continuous cell lines.

<table>
<thead>
<tr>
<th>Cell&lt;sup&gt;A&lt;/sup&gt;</th>
<th>B1&lt;sup&gt;B&lt;/sup&gt;</th>
<th>B1 + T&lt;sup&gt;C&lt;/sup&gt;</th>
<th>TxGB&lt;sup&gt;D&lt;/sup&gt;</th>
<th>TxGB + T</th>
<th>C&lt;sup&gt;E&lt;/sup&gt;</th>
<th>C + T</th>
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</tr>
<tr>
<td>ST</td>
<td>+</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MA104</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
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<td>–</td>
</tr>
<tr>
<td>QT35</td>
<td>–</td>
<td>–</td>
<td>+++</td>
<td>++++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HRT18</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BT</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>A</sup> CEF = Chicken embryo fibroblast, ST = swine testicular cell line, MA104 = Monkey kidney cell line, QT35 = Quail trachea cell line; HRT18 = Human rectal tumor cell line, BT = Bovine turbinate cell line.

<sup>B</sup> B1 = Lentogenic NDV strain B1 type B1.

<sup>C</sup> T = NDV strain pretreated with trypsin.

<sup>D</sup> TxGB = Velogenic NDV strain Texas GB.

<sup>E</sup> C = No virus, negative control cell culture.

+ less than 50% cytopathic effect
++ from 60- 70% cytopathic effect
++++ from 70- 80% cytopathic effect
+++++ from 80- 100% cytopathic effect
- no cytopathic effect
Figure 1. Cytopathic effect of NDV in the ST cell line at 96 hours post-inoculation. (A) uninfected control monolayer (50X). (B) ST cells infected with B1, B1 strain of NDV (50X). (C) ST cells infected with Texas GB strain of NDV (50X).
Cell cultures infected with B1 and Texas GB which were pre-treated with trypsin were not found to be different from the culture in which untreated viruses were used.

**Titration of Texas GB strain of NDV in CEF and ST.** The Texas GB strain of NDV was titered in the CEF and ST cell lines. The titers of Texas GB in CEF were $10^{8.23}$ TCID$_{50}$/ml, whereas the titers in the ST were $10^{7.56}$ TCID$_{50}$/ml.

**Virus neutralization assay.** Results of the comparison of NDV neutralizing antibody titers, as determined by microscopic examination and MTT assay in both CEF and ST cell lines are shown in Table 2. The ANOVAs are shown in the Appendix (Table A1 and A2). Neutralizing antibody titers in the ST cell line were found to be slightly, but significantly, higher than the CEF as determined by microscopic examination or MTT-microassay. However, the titers determined by the two methods (microscopic examinations and MTT microassay) in both CEF and ST cell lines were highly correlated ($r = 0.967$ for CEF, $r = 0.974$ for ST cell line) (Appendix, Table A3).

**Serum effect.** The multiple regression data for both CEF and ST cell lines indicated that the relationship between absorbance value and dilution was not the same for the cells with serum and cells without serum (Appendix, Table A4 and A5). The estimated relationship indicated that cells with serum were significantly lower than cells without serum at middle plate dilutions (see Figure 2).
Table 2. Neutralizing antibody titers against NDV in 12 sera as determined by microscopic examination and MTT microassay in CEF and ST cell line.

<table>
<thead>
<tr>
<th>Groups</th>
<th>No.</th>
<th>HI^</th>
<th>CEF-MICR®</th>
<th>CEF-MTT^</th>
<th>ST-MICR°</th>
<th>ST-MTT^</th>
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<td>3.66</td>
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<td>3</td>
<td>3.5</td>
<td>3.54</td>
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<tr>
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<td>3</td>
<td>3.5</td>
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<td>3.66</td>
<td>4.7</td>
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<tr>
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<td>5</td>
<td>5.78</td>
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<td>7.54</td>
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</tr>
<tr>
<td></td>
<td>8</td>
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<td>9.09</td>
<td>9.33</td>
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</tr>
<tr>
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<td>8</td>
<td>8.67</td>
<td>9.52</td>
<td>9.33</td>
<td>10.34</td>
</tr>
</tbody>
</table>

^ Hemagglutination inhibition titer
® Virus neutralization titer in CEF. The 50% neutralization endpoint was determined based on microscopic examination.
^ Virus neutralization titer in CEF. 50% neutralization endpoint was calculated based on MTT microassay.
° Virus neutralization titer on ST cell line. The 50% neutralization endpoint was determined based on microscopic examination.
^ Virus neutralization titer on ST cell line. The 50% neutralization endpoint was determined based on MTT microassay.
A. Serum effect on the chicken embryo fibroblasts

B. Serum effect on the ST cell line

Figure 2. The effect of the presence of serum in determining a 50 % neutralizing endpoint in: A. chicken embryo fibroblasts and B. the ST cell line. The lines represent the average of the absorbancies from eight serum samples. Error bars denote the standard deviation.
DISCUSSION

In the present study, different continuous cell lines were evaluated to identify a suitable continuous cell culture system that has the capacity to titer the NDV virus and determine the system's potential for use in a rapid neutralization microassay. Use of the swine testicular cell line afforded NDV replication with observable CPE of the Texas GB strain of NDV. Cytopathic changes were observed after two days of cultivation and were found to be similar to that produced by CEF. It was also found that the ST cell line was convenient and easy to maintain in a 96-well plate cell culture system.

A comparison of the capacity of the Texas GB and B1 strains of NDV to replicate and display CPE revealed that the B1 strain replicated in MA104 cells, ST cells and CEFs but with much less CPE as compared to the Texas GB strain. In addition, the lentogenic B1 strain of NDV failed to produce detectable cytopathic effects in all other cell lines evaluated (see Table 1). Reeve and Poste (11) found that the capacity of different NDV strains to induce CPE in different cells was related directly to their virulence for chicks and fertile eggs. Trypsin pretreatment of NDV did not increase CPE in the method employed in this study. This suggested that there was little, if any, effect on the NDV replication cycle of the B1 or Texas GB strains. However, Nagai et al. (9) reported that the presence of trypsin in the culture media was required for efficient replication of the lentogenic strains of NDV in continuous cell lines. In their study, 2.5-10 μg/ml of trypsin were used to support lentogenic strains of NDV replication in various cells. This represents 5-20 times more than that used in the present study.
and may explain the difference in the results. However, Nagai et al. (9) could not apply the same procedure with all continuous cell lines (e.g., BHK21-F) because the cells could not be maintained as monolayers if such high levels of trypsin were present in the medium. The present researchers' experiences were similar, thus it was elected to use a small amount of trypsin in order to maintain the cell monolayer. Unfortunately, the B1 strain did not display CPE under these conditions.

The ability of the ST cells to propagate Texas GB to high titer levels is an additional reason for utilizing the ST cell line in a virus neutralization assay. However, NDV grew to higher titers in CEFs than in the ST cell line. A possible explanation for this titer difference between CEFs and ST cells may be due to differences in the sensitivity of the cells to the virus. Newcastle disease virus titers in the ST cell line might be increased by adopting NDV to the ST cell line by increasing the number of serial passages and/or by lengthening the incubation time, although the latter approach might reduce the sensitivity of the test due to nonspecific cell death.

Neutralizing antibody titers determined in the ST cells were significantly higher than their paired counterparts evaluated in CEFs (see Table 2). The difference between the ST cells and CEFs may have been due to the difference in the way the two cell types support virus growth and the fact that the ST cell line has a longer lifespan than CEFs (4). The results of this study also revealed that there was a high correlation found between the virus neutralization assay using microscopic examination and the MTT assay. However, there were no significant differences between the microscopic examination procedure and the MTT
microassay procedure for determining the virus neutralization titer. These results support using the MTT assay as an alternative to microscopic examination. The MTT microassay would have distinct advantages for use with large numbers of sera samples. For example, the results are objective and lend themselves to automation since they are obtained using an ELISA reader instead of visual examination. In addition, the data are easily transferred and analyzed by electronic means. This assay is less laborious, less time-consuming and the results are obtained in a short time.

In the present study, the 50% virus-neutralizing endpoint (VN50) that was used for determining the viral neutralization titer in the MTT microassay was ascertained by using the mean absorbance of the cell control (without serum) as representing 100% neutralization. This method has also been used by other investigators and has been found to be an applicable method when measuring a large number of serum samples (12, 13). However, Haddad et al. (7) employed another approach for estimating the VN50 in which diluted serum without virus was added to the plate of the cells and used to calculate the percentage of virus neutralization for each serum dilution. Through this method, any serum effect which might influence the true value of VN50 was avoided. In the study by Haddad et al. (7), the first dilution of each serum was used to represent the 100% neutralization value. This method seemed to be appropriate for positive sera, however, there was no explanation given when a negative serum was used (i.e., the first dilution would not be 100% neutralizing). The results from the present study confirmed the existence of a serum effect. It is not known from this study how the effect of sera would influence the true VN50 as determined in the study. In order to determine whether
there is a significant effect, an experiment utilizing paired sera samples could be evaluated by using the estimated value (as in this study) and a method by which diluted sera without virus, are incorporated into the plate design and used to determine a 100% neutralization and a 0% neutralization value. It will be important to compare the two procedures in order to determine whether the true value of VN90 is significantly different from the estimated value.

In conclusion, the ST cell line was found to be a suitable alternative for CEF for diagnostic and serological assays. This cell line was easy to maintain, was more resistant to the toxic effects of serum, supported the growth of NDV and lent itself to adaptation with the MTT/ELISA rapid methodology.

REFERENCES


ACKNOWLEDGMENTS

The authors thank Dr. Hal Sterne and Ms. L. L. Wu. for their valuable assistance in the statistical evaluation of the data. The authors also thank Dr. Ali Akbar, Sevnic Aknic and Joan Oespar for their technical assistance.
APPENDIX: DATA AND ANALYSIS

Table A1. Analysis of variance neutralizing antibody titers to NDV determined by microscopic examination and MTT microassay in both CEF and ST cell lines.

<table>
<thead>
<tr>
<th>Sources</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Prob. &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level</td>
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<tr>
<td>Cell line</td>
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<td>5.619951</td>
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<tr>
<td>Methods</td>
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<td>0.495259</td>
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<td>0.1578</td>
</tr>
<tr>
<td>Cell * Methods</td>
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<td>0.12043</td>
<td>0.120430</td>
<td>0.50</td>
<td>0.4822</td>
</tr>
<tr>
<td>Error</td>
<td>42</td>
<td>10.058941</td>
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</tbody>
</table>

Table A2. Duncan multiple range comparison between CEF and ST cell lines, and between the microscopic examination and MTT microassay. The mean value of each variable with asterisks indicates a significant difference at the P < 0.05 level.

<table>
<thead>
<tr>
<th>Groups</th>
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<td>6.5665</td>
</tr>
<tr>
<td>ST cell line</td>
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</tr>
<tr>
<td>Microscopic</td>
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</tr>
<tr>
<td>MTT assay</td>
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</table>
Table A3. Pearson correlation coefficients between the neutralizing antibody titers for the two methods (microscopic examination and ST cell line) in both CEF and the ST cell line.

<table>
<thead>
<tr>
<th></th>
<th>CEF MICR</th>
<th>CEF MTT</th>
<th>ST MICR</th>
<th>ST MTT</th>
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<tr>
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<td>0.982</td>
<td>0.973</td>
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<tr>
<td>CEF MTT</td>
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<td>0.980</td>
<td>0.963</td>
<td></td>
</tr>
<tr>
<td>ST MICR</td>
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<td>0.909</td>
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<tr>
<td>ST MTT</td>
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Table A4. Analysis of variance representing the effect of the presence of serum in chicken embryo fibroblasts in determining the 50% cell survival endpoint (VN50).

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<tr>
<th>Source</th>
<th>Degree of Freedom</th>
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
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<td>45163.89</td>
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<tr>
<td>Corrected total</td>
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</table>

Table A5. Analysis of variance of the effect of the presence of serum in the ST cell line in determining the 50% cell survival endpoint (VN50).

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
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GENERAL SUMMARY

Newcastle disease (ND) is a viral disease of poultry that has economic importance throughout the world. The clinical disease varies in morbidity, mortality, clinical signs and lesions. A paramyxovirus, Newcastle disease virus (NDV) is an enveloped virus with negative-stranded RNA. The NDV genome encodes the proteins: nucleoprotein (NP), phosphoprotein and V protein (P/V), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and large (L) proteins. Various types of NDV vaccines, including live attenuated, inactivated and recombinant vaccines, have been used successfully for reducing losses and providing protection from the disease (Alexander, 1991). However, ND continues to be of economic significance in poultry production, requiring the development of better methods for its control (Bernard and Easterday, 1981).

There have been conflicting reports about the nature of the immune response required for protection against NDV. Although high levels of antibodies have been associated with protection against NDV (Beard and Bruge, 1975), a lack of correlation between humoral immune response and protection has also been reported (Allan and Gough, 1976; Gough and Alexander, 1973). It has been suggested that more than humoral immunity is required for protection (Ghumman and Bankowski, 1976; Marino and Hanson, 1986). Additionally, little is known about the role of cell-mediated immunity in protection. Thus, understanding the avian immune mechanism required for protection against NDV and identifying the polypeptides of NDV involved in the immune response may facilitate better protection strategies. The
objectives of this research were to evaluate the role of cellular and humoral immunity to NDV in relation to protection.

In the first study, the effect of *in ovo* cyclophosphamide (CY) treatment on B and T cells was studied. Evaluation methods were concerned with chicken survival and growth, peripheral blood lymphocyte blastogenic responses to LPS (B cell mitogen) and Con A (T cell mitogen), antibody response and flow cytometeric analysis of blood lymphocytes. The results of this study indicated that *in ovo* CY-treatment decreased hatchability and increased mortality (determined at two weeks post-hatching). Blastogenesis results revealed that approximately 40% of the birds were T-cell positive and B-cell negative at two weeks of age. Regeneration of B lymphocytes occurred at four and six weeks of age in a low percentage of those birds that were previously T-cell responsive but not B-cell responsive.

Antibody response to NDV in those birds that had T-cell responses, but not B-cell responses, was severely depressed when the birds were vaccinated at three weeks of age with the B1 strain of NDV. Most birds failed to produce detectable specific antibodies to NDV, however, a low percentage from both trials were found to have low levels of HI antibodies one week post-booster. Birds that responded were found to be the same ones that responded to the LPS mitogen. The selective nature of CY suppression in humoral immune response was also detected in flow cytometeric analysis. There was a significant decline or even the absence of lymphocytes expressing IgM in the CY-treated birds compared with untreated birds. It was concluded from this study that the immunosuppressive effect of CY-treatment in the birds was reversible and recovery of the antibody producing organs occurred as early as
two weeks. The results also revealed the necessity for continued monitoring of B and T cell responses in CY-treated birds to identify those birds in which B-cell regeneration may have occurred.

The second study was concerned with the role of cell mediated immunity as a key component in providing protection to chickens against ND. In this study, two different strategies were used. In the first strategy (virus treatment), NDV was treated with 4% SDS in which the preparation was heated to allow the virus to break into polypeptides. This preparation was then mixed with incomplete Freund's adjuvant and used as an inoculum to elicit a specific CMI response without inducing a specific NDV antibody response. Four groups of three-week-old SPF chickens were injected with live NDV (LNDV), UV-inactivated NDV (UVNDV), SDS-treated NDV (SDSNDV) and PBS. The birds were boostered at five weeks of age and challenged one week post-booster. Blood samples were collected weekly for humoral and cellular immune response evaluation.

The results of this study demonstrated that all vaccinated birds had specific CMI responses to NDV as measured by a MTT blastogenesis microassay. A specific antibody response was detected in birds vaccinated with LNDV and UVNDV, whereas an antibody response in birds vaccinated with SDSNDV was detected by Western blot analysis but not by HI test and VN assay. The results of this study also revealed that only those groups of birds with a specific antibody response to the NDV (LNDV and UVNDV groups) were protected. However, birds with CMI-specific NDV responses without a specific antibody response to the NDV, (SDSNDV group) were not protected. The results from this study suggested that the
presence of an NDV-specific CMI response by itself was not protective against ND. Additionally, the study also emphasized the importance of the protective epitopes that induce a specific antibody response to NDV.

In the second strategy, the immunosuppressive agent, cyclophosphamide (CY) was used to deplete B cells in chickens to evaluate the importance of CMI in protection against ND. Groups of three-week-old in ovo CY-treated birds were vaccinated with either LNDV, UVNDV or PBS. Similarly, CY-untreated groups from the same hatch were vaccinated with either LNDV, UVNDV or PBS. All birds were vaccinated at three weeks of age, boosted at five weeks of age and challenged at six weeks of age. Blood was collected weekly for humoral and cellular immune response evaluation. The results from this study indicated that all of the birds vaccinated with LNDV and UV-NDV had specific CMI responses to NDV. Specific NDV-antibody responses were also detected in all of the CY-untreated birds and some of the CY-treated birds that were found to have a regenerated B-cell function at one week post-booster. The challenge results revealed that birds that did not produce a specific antibody response to NDV developed clinical signs of ND, whereas the birds with specific antibodies neither developed clinical signs nor exhibited the presence of virus in tracheal swabs. In conclusion, the results from this study also suggested that the presence of specific antibodies is important in determining the ability of chickens to resist ND infection. Furthermore, the results were also in agreement with other reports in which protection against Newcastle disease virus was demonstrated in the presence of low antibody levels (Gough and Alexander, 1973).
The third study investigated the protective effect of humoral immunity against lethal ND in chickens. Specific antibodies to NDV polypeptides HN/F, NP/P, M, and whole virus (UVNDV) were prepared and used for passive administration. Six groups of three-week-old chickens were passively immunized with antiserum against either HN/F, NP/P, M, a mixture of proteins (ALL), intact UV-NDV, or negative sera. Blood samples were collected two days post-immunization and the birds were challenged intramuscularly with $10^3 \text{ELD}_{50}$ Texas GB strain of NDV. The serological results revealed that the antibodies from donors birds had a positive antibody response to their respective polypeptides as determined by the Western blot analysis. In the recipient birds, antibody titers to HN/F, ALL and UVNDV were detected by the HI, ELISA and VN tests, whereas antibodies to NP/P and M were detected by ELISA but not by the HI and VN assays even though the antibody titer was very high as determined by ELISA. The level of antibody titers in the recipients was found to be between two and three dilutions ($\log_2$) lower than the titers in the donor sera. This was hypothesized to be due to the dilution effect of the serum when distributing itself throughout the body.

Studies in protective immunity revealed that passive administration of antiserum raised against HN/F glycoproteins, ALL proteins, and intact UVNDV provided susceptible chickens protection when they were exposed to a virulent NDV challenge by the intramuscular route. On the other hand, birds that had been passively immunized with antisera against NP/P or M proteins and SPF sera developed clinical signs of ND or died. Neurological signs and/or death were detected by three days post-challenge. The virus was recovered at four days post-challenge from the trachea of the majority of passively immunized recipient birds. This study
indicated that the presence of neutralizing antibodies to NDV, even at low titers, was successful in protecting chickens from the clinical disease but not from NDV infection.

Although the movement of antibodies from the circulation to the upper respiratory tract in passively immunized birds has been well documented (Russell, 1993), the transport amount of antibodies has been very low (Holmes, 1973). Furthermore, the local immune response in the respiratory tract was found to be influenced by the type and the class of the antibody produced. The protection of the respiratory tract correlated well with an increased level of the secretory IgA. Passively immunized mice injected intravenously with polymeric IgA anti-influenza antibodies were protected against nasal challenge, whereas an intravenous injection of similar virus neutralizing doses of anti-influenza IgG did not reduce virus shedding in the majority of infected mice (Renger and Small, 1991a). This was correlated with the amount of antibodies that was transported into the respiratory tract. In addition, it has been reported that polymeric IgA was transported ten to one-hundred fold more efficiently than IgG on the mucosal surface of the respiratory tract (Renger and Small, 1991a).

In conclusion, the results from this study indicated the importance of the presence of specific antibodies to the HN and F polypeptides of NDV in protection from NDV challenge. In addition, the demonstration of the challenge virus in the trachea of challenged birds may be an indicator of the importance of the local immune response in protection.

The purpose of the fourth study was to find a suitable continuous cell line capable of supporting NDV replication which could be used for an ND viral neutralization assay when using MTT in a 96-well plate microassay. In this study, five continuous cell lines (MA104,
BT, HRT18, QT35 and ST cells) were evaluated and compared with CEFs for their ability to propagate B1 or Texas GB strains of NDV. The Texas GB strain was found to replicate in all continuous cell lines used in this study. Only the ST and QT35 cells produced CPE similarly to that produced in CEF. The most distinctive of CPE emerged between 48 and 72 hours in the ST, QT35 and CEF. In addition, high titers of NDV were detected in the ST cell line as well as the CEFs. These observations provided a rationale for utilizing the ST cell line as an alternative to CEFs for virus neutralization assay. In contrast, the B1 strain of NDV was found to replicate only in the ST and MA104 cell lines in addition to the CEFs. Less discernible amounts of CPE were observed in those cell lines when compared to the Texas GB strain. At 96 hours, many of the cell monolayers were still intact and not infected.

A comparative study was performed using paired serum samples to determine NDV neutralization titers in CEFs and ST cells using an MTT microassay. Additionally, a conventional microscopic examination was performed and the neutralizing antibody titers of each sample were compared with those measured by the MTT microassay procedure. The results from this study revealed that neutralizing antibody titers in the ST cell line were slightly, but significantly higher than in the CEFs as determined by either microscopic examination or MTT-microassay. However, the titers determined in both CEF and ST cell lines by the two methods were highly correlated.

The results from this study suggested that the ST cell line can be a suitable alternative for CEFs in NDV VN assays. This cell line was easy to maintain, more resistant to the toxic effects of serum and supported the growth of NDV with adequate CPE. In addition, the
MTT-microassay procedure could be substituted for a microscopic examination when evaluating neutralizing antibody titers to NDV. This method is less laborious, saves time, and is convenient for a large number of serum samples. In addition, the results are obtained by objective rather than subjective methods and the data are easily transferred and processed by electronic methods.

In conclusion, a technique was developed for inducing B-cell ablation in chickens by *in ovo* cyclophosphamide injection. A colorimetric blastogenic microassay for detecting specific cellular immune responses to NDV was also developed and implemented. These techniques were applied to determine the role of cell mediated immunity in protection against NDV as well as the role of antibodies to NDV polypeptides in protection. In addition, the quantification of neutralizing antibodies to NDV in ST cell line was evaluated using a colorimetric 96-well plate microassay. Finally, this study revealed the importance of antibodies as a key component in protection.
LITERATURE CITED


King, J. D. Virus isolation from tracheal explant cultures and oropharyngeal swabs in attempts to detect persistent Newcastle disease virus infections in chickens. Avian Dis. 29(2):297-311. 1983.


ACKNOWLEDGMENTS

I wish to express my sincere gratitude to my major professor, Dr. Don Reynolds, for his patience, guidance, assistance and encouragement throughout my graduate study. I am also very grateful for his helpful advice to improve my scientific writing ability and for proofreading my manuscripts.

I would like to thank my committee members: Dr. Gray Osweiler, Dr. Prem Paul, Dr Merlin Kaeberlae and Dr. Jerry Sell for their suggestions and encouragement throughout this research.

Deep appreciation is extended to my best friend, Dr. Ali Akbar, for his generous assistance in the collection and analysis of clinical specimens for my research. I would also like to thank Dr. Pawan Agrawal, Dr. Rhonda Johnson, Dr. Ratree Platt and the laboratory technicians Sevinc Akinc and Joan Oesper for their friendship and assistance during my experimentation.

I also wish to acknowledge Ms. Lie-Ling Wu for her statistical analysis, and Pat Hahn for her professional editing of the completed manuscripts of the dissertation.

To my mother, brothers, and sisters, I thank you for your prayers and regular communication which gave me support and encouragement during many long hours of research and study. You have blessed me and my young family by the close ties we have been able to maintain in spite of the great distance that has physically separated us during our five years at Iowa State University, half-way around the world from our true home.
I am indebted to my lovely wife, Kholoud Maraqa, and sweet daughter, Noor, for their patience and tireless support. You both have given me so much joy and many reasons to laugh in the midst of many long days and even longer nights. To the newest member of our family, Mohanned, welcome to our precious family. Your timely arrival on the day of my concluding seminar has truly been a blessing from God.