The evaluation of using vegetable oils as adjuvants with Newcastle disease virus vaccine when administered by a non-injectable route

by

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INTRODUCTION

Newcastle disease (ND) is a very important viral disease of poultry. Although this disease was described in 1926, it is still prevalent throughout the world and is a constant threat to the rapidly growing poultry industry. Raising poultry has become a profitable business on a commercial basis. In the past 30 years, the poultry industry has transformed from small scale back-yard farming to a large scale industry. Rearing large poultry populations in confinement housing has added risk factors that may contribute to infectious diseases. Infectious diseases can account for large economic losses if not controlled. In the case of ND, the economic losses are due to mortality, decreased egg production, decreased egg quality and reduced growth rate. Therefore, prevention of this disease through proper sanitation, flock isolation, and systematic vaccination is the foremost concern of modern poultry growers.

Although the first vaccine used against ND was developed more than 40 years ago (1), efforts are still being made to develop better vaccines. The availability of a wide variety of live and inactivated vaccines has made it possible to protect poultry populations from this devastating disease. However, changes in poultry practices also demand changes in vaccines and vaccination techniques. No single vaccination program can be suitable for all types of poultry or poultry operations. Therefore, the types of vaccines and vaccination techniques employed should be based on the particular need and situation.

Many times, broiler chickens are immunized only once against ND with live lentogenic strain of ND vaccines by non-parenteral routes. Drinking water, spray, and aerosol methods of vaccination are most commonly used for mass vaccination in large scale operations. Intranasal-intraocular (IN-IO) and parenteral immunization requires individual handling. Laying and breeding stocks require more than one vaccination at

suitable intervals. Maintenance of an effective immune status of the entire flock at all times is essential to avoid the risk of heavy economic loss due to Newcastle disease.

The increasing cost of preventing infectious diseases is a growing concern to the modern poultry industry. Several measures have been employed to minimize the cost of vaccination. Drinking water, spray or aerosol methods are very effective mass vaccination techniques which save time and money. To date, only live vaccines have been used for mass vaccination purpose. The use of combined vaccines (bivalent or polyvalent) has also been used to minimize the cost and efforts normally associated with injectable vaccines. However, several reports (2, 3, 4) have indicated that a suppressive effect of one antigen on the other antigen occurs when a polyvalent preparation is used. Although combination vaccines are popular amongst the livestock and poultry industries, there is controversy regarding the effectiveness of each component of a polyvalent vaccine. Another method of inducing high levels of long-lasting immunity has been through the use of adjuvants in the preparation of vaccines. A wide variety of compounds have been studied for their immune enhancing properties. Freund's incomplete adjuvant, which is a mixture of mineral oil and an emulsifier, has been widely used to enhance the immunogenic properties of ND vaccines (5). Although mineral oils are very good adjuvants, they have been shown to cause granulomatous reactions and tumor formations. Since oil emulsion vaccines are injectable and require individual handling of chickens, they are not commonly used in broiler chickens or market turkeys.

Much effort has been made to develop biodegradable emulsions that are stable, effective, and non-carcinogenic (6, 7, 8, 9). A few reports are available on the adjuvant effects of vegetable oils. To date, the adjuvant effect of corn oil and soybean oil has not been reported. These vegetable oils could be potential candidates for adjuvants.

The disadvantage of using mineral oil as an adjuvant includes adverse tissue reaction at the injection site and the inefficiency of injectable administration, whereby, each bird must be handled individually. However, when live ND vaccines are used without adjuvant, by mass application methods, the immunity provided is short in comparison.

The main objective of this project was to study the adjuvant effect of vegetable oils, such as corn oil and soybean oil with ND vaccine when administered by non-injectable routes. Variables such as concentration of oil, age and route of administration were addressed.

LITERATURE REVIEW

Newcastle Disease

Newcastle disease (ND) is a highly infectious disease of domestic and wild birds capable of infecting birds of any age. It has become a major problem in many countries where poultry production is intensive.

Newcastle disease was first recorded on the island of Java in Indonesia in 1926. A disease with similar characteristics was reported the same year in the seaport town of Newcastle-upon-Tyne, England, by Doyle in 1926 (cited in (10)). ND was also recognized in middle Korea in 1926, India and Philippines in 1927, and Japan in 1929. In the United States, a relatively mild respiratory and nervous disease was reported in 1941 which later proved to be Newcastle disease (11). Although ND was first reported in 1926, within a few years it was recognized throughout the world (12).

Etiologic agent: Newcastle disease virus (NDV) is a member of the genus paramyxovirus (serotype PMV-1). It is an enveloped RNA virus which possess a non-segmented, single stranded genome of negative polarity. The nucleocapsid is arranged in a helical symmetry. The virus particles are usually roughly spherical or filamentous, with a diameter of 100-500 nm (13). The envelope is made of a lipid bilayer with surface projections consisting of hemagglutinin-neuraminidase (HN) and fusion (F) proteins. These antigenic components in the envelope of NDV stimulate the host to produce hemagglutination inhibiting and virus neutralizing antibodies (14). Since the lipid bilayer is derived from the host cell plasma membrane, its lipid composition reflects that of the plasma membrane (15). HN protein allows virus attachment. The F protein is responsible for several biological activities involving membrane fusion; penetration of a cell by fusion

of the viral envelope and cell membrane, cell fusion, and hemolysis (16). Since F protein is essential for viral penetration of the host cell and for direct intercellular spread by cell-to-cell fusion, it plays a key role in the pathogenesis of paramyxovirus infection. Paramyxovirus vaccines, to be maximally effective, must elicit antibodies against the F protein as well as against the HN protein (17). The other proteins are internal and function as major structural components of the nucleocapsid or play a role in the viral replication and assembly (18).

The hemagglutinin glycoprotein of the virus reacts with the receptors on the erythrocytes of many animal species to cause hemagglutination. The ND virus also has neuraminidase activity which allows the virus's eventual release from the surface of the erythrocyte allowing separation of the agglutinated cells. Red blood cells are not a target for the virus in the chicken, but many laboratory procedures with NDV employ the hemagglutination (HA) reaction. In particular, the hemagglutination-inhibition (HI) reaction is the most commonly used test for the detection of serologic antibody (18).

Virus replication: Replication of the NDV has been studied in cell culture systems. Initially, the virus adsorbs to the receptor on the surface of the susceptible cell. This is followed by the virus penetration which is mediated by the fusion of the viral envelope with the lipid bilayer of the target cell, this results in the delivery of the genomic material into the cell. The F protein is involved in this process. Immediately after penetration, the virus initiates transcription using the nucleocapsid as the template. The synthesis of viral RNA is regulated so that monocistronic mRNA is produced while a full-length positive stranded copy of the RNA genome serves as a template for the RNA viral progeny. Newly synthesized NDV proteins are transported to the host cell plasma membrane where they are integrated into either the nucleocapsid or the envelope of the virion. The nucleocapsid

protein (NP), phosphoprotein (P), and large protein (L) of NDV are synthesized in the cytosol, and are rapidly associated with the cytoskeletal framework and are assembled into the nucleocapsid. The newly synthesized NDV glycoproteins are inserted into the plasma membrane. All the viral components migrate to the plasma membrane of the host cell where final assembly of progeny virion takes place. The mature virion is released from the cell by budding (15).

Resistant to agents: NDV is thermolabile, and most strains are fully inactivated by incubation at 60° C for 30 minutes (19). At 37° C, hours and/or days are required to decrease infectivity, hemagglutination activity and immunogenicity. NDV is destroyed by exposure to ultraviolet light. It is stable at broad ranges of hydrogen ion concentration (pH). Infectivity is retained for many hours at pH as low as 2 and as high as 10. The virus is readily inactivated by formalin, alcohol, merthiolate, lipid solvents and cresol. Formalin, beta-propiolactone, and phenol have been used to destroy infectivity without damaging immunogenicity of the virus preparation. Environmental conditions, particularly warm temperature and solar radiation, facilitate destruction by chemicals (20).

Hosts: NDV has been recovered from a variety of avian species. The disease is observed most frequently in domestic poultry including chickens, and guinea fowls; these species are more susceptible than the turkey. Ducks, geese, partridges, and quail are relatively resistant. In pheasants and pigeons, the virus can cause severe disease (19). In humans, the virulent strains of NDV cause intense conjunctivitis.

Laboratory host systems: All strains of NDV grow in chickens and embryonating eggs. The virus can be grown in a variety of cell culture systems, the most commonly used are the chick embryo fibroblast (CEF) monolayer, the chick embryo kidney (CEK) monolayer, and baby hamster kidney (BHK) cells, either in monolayer or in suspension

culture (21). Chick embryos are preferred to cell cultures for vaccine production because of high titers achieved in embryos (18).

Pathotype and pathogenicity: Although strains of NDV are antigenically very stable, they vary remarkably in pathogenicity. Naturally occurring strains may cause peracute disease with 100% mortality or may be avirulent and cause no disease (18). A broad classification of NDV strains divides them into 3 pathotypes: (1) velogenic, (2) mesogenic, and (3) lentogenic.

Newcastle disease viruses that cause severe disease with high mortality are termed velogenic. These strains produce high mortalities, even in adult birds, and are further subdivided into neurotropic and viscerotropic strains according to their affinity for the central nervous system (CNS) or for organs of the thorax and abdomen (18).

Viscerotropic-velogenic Newcastle disease (VVND), also known as Doyle's form, was first recognized by Doyle in 1926 (cited in (10)). VVND is an acute lethal infection of chickens of all ages. Typically VVND initiates dullness that is rapidly followed by marked depression, increased rate of respiration, progressive weakness, and prostration. Diarrhea is common in the early stage of the disease. Feces are usually profuse, watery, greenish or yellowish in color and occasionally blood stained. There may be edema of the tissues around the eyes and throat. Other symptoms include clonic spasms, muscular tremors, torticollis, opisthotonos, paralysis of the legs and (occasionally) the wings. Egg production falls abruptly, and soft and/or imperfectly shelled eggs may be laid. Mortality is usually over 90% (10).

Neurotropic-velogenic Newcastle disease, also known as Beach's forms, appears suddenly and spreads rapidly. Birds undergo respiratory distress, coughing, marked gasping, a drop in appetite and egg production falls or stops. Paralysis of legs or wings

and torticollis are commonly observed with this form of disease. Mortality may be as high as 90% in immature chicks, whereas 10-50% mortality is observed in adult chickens (20).

Mesogenic Newcastle disease, also known as Beaudette's form, is an acute respiratory disease of adult chickens marked by coughing but rarely gasping. There is a drop in appetite and egg production. Within two or three weeks the respiratory symptoms usually subside and nervous symptoms may then appear. Involvement of the CNS is more common in young chicks than in older birds. Mortality may vary from 5-50% in mature birds and may exceed 50% in young chicks (10).

Lentogenic Newcastle disease (Hitchner's form) is a mild or inapparent respiratory infection of chickens caused by lentogenic strains of NDV. In mature chickens, mortality is negligible, but it may reach 30% in young chicks, particularly when complicated by other infections (20).

The asymptomatic enteric form of infection is caused by lentogenic strains that results in no clinical signs or pathology and is detectable only by virus isolation from the gut or feces, and by the demonstration of specific antibodies (20).

Strains of NDV: V4 is an avirulent strain of NDV present in Australian poultry and causes no disease when it spreads naturally between chickens (22). Hitchner B1, F, and LaSota are examples of lentogenic strains which have been extensively used as vaccines. Among the mesogenic strains that are used for vaccines the Mukteswar strain is the most invasive and therefore, provides the greatest and most durable immunity. Other mesogenic strains such as the Hertfordshire (H), Komarov (K) and Roakin strains are less pathogenic than the Mukteswar strain. Among the strains of highest virulence (velogenic) are Milano, Herts 33, and the GB-Texas; these have been used as challenge strains (19).

Pathotype and strain identification: The pathotype of a NDV isolate can be identified on the basis of the following tests: (1) Mean death time (MDT) of chick embryos; (2) intracerebral pathogenicity index (ICPI) in one-day-old chicks; (3) intravenous pathogenicity index (IVPI) in 6-week-old chickens; (4) pathogenicity for 8-week-old chickens; (5) ability to plaque on CEF with or without diethyl aminoethyl (DEAE) and magnesium ions (20, 23, 24, 25). Strains which are lentogenic can be differentiated on the basis of their rates of elution of chicken erythrocytes, thermostability of their hemagglutinin, and agglutination of mammalian erythrocytes (23, 25).

Transmission of NDV: Aerosol transmission appears to be the chief means of spread of this disease within a flock. The respiratory tract acts as a source of virus as well as a portal of entry. Birds infected with virulent virus excrete large quantities of virus in their feces. The disease is also spread by contaminated feed and water (18). Spread of the disease between countries is often mediated by importation of caged birds, racing pigeons, domestic poultry or poultry products and by migratory birds (18). Spread of the disease within a country may be due to mechanical factors associated with the transport of eggs, birds, carcasses, poultry offal, feed, vaccinating crews, and the movement of personnel (26). The disease may also be spread by the use of contaminated vaccines.

Diagnosis of ND: Since the signs of ND are relatively non-specific, diagnosis must be confirmed by the isolation and identification of the virus. The virus may be isolated from the spleen, brain or lungs of infected birds by inoculating 10-day-old embryonated eggs by the allantoic route (17). Identifying the recovered agent as NDV is accomplished by using specific antisera either with the hemagglutination-inhibition (HI) or virus neutralization (VN) tests (24). The HI, complement fixation (CF), enzyme-linked

immunosorbent assay (ELISA) and plaque neutralization tests have been used to monitor antibody to NDV (20).

Prevention and control: Since this disease is highly contagious in nature, attempts to control it by sanitary measures alone have often been unsatisfactory. The implementation of vaccines may also be needed for effective control (20). In countries where ND is a problematic disease, the techniques used to contain and eradicate this disease are usually: (1) identification of the infected premises with subsequent destruction of the entire chicken population and disinfection before repopulating; (2) enforcement of strict quarantine measures to limit the spread of the disease; and (3) use of vaccines to produce buffer areas with protected birds (18).

A wide variety of live and inactivated vaccines have been used successfully to protect poultry populations from this devastating disease. Lentogenic vaccines are mild and can be used safely in all classes of chickens, whereas, mesogenic strains are recommended only for secondary vaccination in older birds. Routes of vaccination depends on the type of vaccines, flock size, and technology available. A detailed review of ND vaccines and inoculation routes is reported separately in this chapter.

The Immune System of the Chicken

The immune system of birds differs from that of mammals in certain basic respects, most notably in the bird's possession of a bursa of Fabricius and the absence of organized lymph nodes (27). There are two parallel compartments of differentiating lymphocytes: (1) thymus derived (T) lymphocytes, the effector cells in cell-mediated immunity, and (2) bursa derived (B) lymphocytes, the precursor cells of the antibody-synthesizing plasma cells (28). Each compartment is divided into central or primary

lymphoid organs, comprising the thymus and bursa, and the peripheral or secondary tissues, notably the spleen and the lymphoid tissues along the gut, especially the caecal tonsils. The thymus and bursa produce T- and B-lymphocytes respectively. The cells which begin lymphoid development within the thymus originate in the yolk sac. Later in the development, stem cells are found in the liver and spleen. In adult life, these precursors reside primarily in the bone marrow (29).

The functions of T-lymphocytes in the chicken are similar to those in mammals, and include helper and suppressor effects on antibody production, delayed hypersensitivity, graft-versus-host activity, macrophage activation, and cytotoxicity (30). The role of T-lymphocytes in the immune response of the chicken has been comprehensively reviewed by Chi et al. (31).

The main functions of the bursa are the immunological education of prebursal stem cells to form immunoglobulin-producing cells. Subsequently, the seeding of the resultant bursal stem cells to the peripheral lymphoid tissues gives rise to cells which produce immunoglobins and specific antibodies, as well as perform other immunological functions (32). Sorvari et al. (33) have reported that the bursa also functions as a peripheral lymphoid organ, involving the phenomenon of "cloacal drinking" which may be a way in which the fowl gains part of its immunity to environmental microorganisms.

The immune function of the spleen is to trap and process particles and substances capable of eliciting immune reactions and to provide a "home" for lymphocytes and macrophages. The spleen is also an important source of antibodies. Aitken (34) has reported that removing the spleen delays the peak of antibody response in young birds and depresses antibody production in older birds.

The bone marrow of fowl functions as a source of bursal and thymic stem cells, and also functions as secondary lymphoid tissue.

In mammals, the lymphocytes supplied by the thymus (T-cells) and the fetal liver and later the bone marrow (B-cells), participate in the formation of the lymph nodes. Many birds, including domestic fowl, do not possess lymph nodes. Others, such as ducks and other aquatic species, do have true lymph nodes (29).

Distribution of lymphoid tissues in the alimentary tract of the fowl has been described in detail by Payne (32). These lymphoid tissues are irregularly distributed throughout the alimentary tract from pharynx to cloaca, in the *lamina propria* and submucosa in the form of solitary and aggregated masses. Caecal tonsils and Peyer's patches are the most organized forms of lymphoid tissues in the alimentary tract of the fowl (35). The intestinal lymphoid tissue provides for the mechanisms of the local immune responses to gut antigens. In mammals, antigens pass through the specialized dome epithelium cells covering the submucosal lymphoid tissues of the Peyer's patches. IgA antibodies are produced in response and pass through the gut epithelial cells into the lumen. This mechanism of antigen sampling is similar to the mechanism of antigen uptake by the bursal epithelium. This same mechanism has also been suggested to occur in the caecal tonsils. In contrast with the bursa, the development in the caecal tonsil is dependent on the antigenic content of the intestine, since germ free chickens do not show germinal centers or plasma cells in the caecal tonsils (36). Carbon uptake and antibody production by Peyer's patches of the chicken have been reported by Burns (35).

The Harderian gland is an accumulation of lymphoid cells in the paraocular tissues. The Harderian gland is well developed in birds and the application of antigens into the eye results in antibody production, therefore, indicating its role as a peripheral lymphoid tissue

(37). The Harderian gland contains large number of plasma cells, many of which secrete IgA (29). Montgomery and Maslin (38) have shown that removal of the Harderian gland resulted in a constant decrease in the antibody level in the tears, regardless of the route of exposure. Ewert et al. (39) have studied the local antibody response against ND in chickens. They have suggested that the Harderian plasma cells are the most likely source of the antibody found in saliva.

The lacrimal gland is another orbital gland and in the bird it is less well developed than the Harderian gland. Removal of the Harderian gland increases the number of plasma cells in the lacrimal gland, possibly as a compensatory mechanism (30). These paraocular and paranasal lymphoid tissues are considered to be responsible for the local immune functions of the orbital, nasal, and upper respiratory tract areas (40).

Non-lymphoid cells contributing to immunity: In birds, non-lymphoid cells comprise macrophages, monocytes, heterophils, eosinophils, basophils, mast cells and thrombocytes.

Macrophages are involved in a great number of functions, both immune and non-immune. Non-immune functions include synthesis of complement components, transferrin, pyrogen, certain interferons, colony stimulating factors, enzymes, clotting factors, etc. The macrophage also participates in degradation of certain proteins and polysaccharides, and the elimination of necrotic cells and foreign bodies during the healing process.

Immune functions of the macrophage include phagocytosis, involvement in antibody synthesis, delayed-type hypersensitivity and tumor immunity. Lymphocyte-macrophage interactions are important in primary and secondary antibody responses, antigen recognition and proliferation of T-lymphocytes. Antigen specific activation of T-lymphocytes leads to the proliferation of the antigen specific T-helper cell and subsequent

production of antibody by B-lymphocytes. The processing of antigen by macrophages is often considered to be the initial step in many immune responses. Macrophages also mediate the antibody-dependent cell mediated cytotoxicity (41, 42).

The heterophil leukocyte of the chicken is considered to be the equivalent of the neutrophil in man. The heterophil, like the neutrophil of mammals, inactivates the invading micro-organisms by phagocytosis and renders them harmless.

Eosinophilia, in mammals, is associated with parasitic infections, allergic reactions, and some neoplastic, inflammatory, and immunodeficiency diseases; however, eosinophilia is difficult to induce in birds. Avian eosinophils do not respond to inflammatory stimuli in the same way as mammalian eosinophils (43).

Basophils and mast cells are weakly phagocytic and lack significant amounts of bacteriocidal and lysosomal enzymes. Mast cells are involved in the initiation of inflammation by releasing pharmacologically active agents which facilitate the migration of heterophils and monocytes to the site of the injury. Basophils may have some part in the early acute inflammatory response and the induction of immediate hypersensitivity reaction in chickens (41).

Thrombocytes are mononuclear cells which are thought to function in the same way as the platelets of mammals. Their primary role being blood coagulation, clotting and subsequently the disintegration of the clot. However, in addition, they are phagocytic and because of their number (three times as many as other circulating phagocytes) they may be the chief circulating phagocyte in chickens (42).

Immunoglobin isotypes: Chicken B-cells produce at least three major classes of immunoglobulin (Ig): IgG, IgM, and IgA (44). IgG is the major serum immunoglobulin in chickens. Chickens produce IgM predominantly during the primary immune response and

then switch to IgG production for the secondary immune response. A monomeric IgM can be detected in the amniotic fluid of eggs and in day-old chicks (45). Bienenstock et al. (46), and Orlans and Rose (47) have reported the existence of an Ig in chicken bile and intestinal secretion which is serologically and electrophoretically distinct from both the major serum IgG and IgM immunoglobulins. IgA is found in relatively high concentration in secretions of all mucosal surfaces and in small amounts in the serum of chickens and several other avian species. There is indirect evidence for the presence of avian homologs of mammalian IgD and IgE (48).

Immunity to Newcastle Disease

The immune response which is induced by vaccination has at least four components: (1) humoral antibody, (2) secretory antibody, (3) cellular immunity, and (4) non-specific resistance factors (49). Humoral immunity against NDV is due primarily to antibody directed against the two NDV glycoproteins: HN and F proteins (50).

Humoral antibody: All NDV strains are capable of inducing a specific antibody response in the chicken. The virus neutralizing (VN) antibody, which is also the hemagglutination inhibiting (HI) antibody, effectively blocks the ability of the virus to infect chickens, chicken embryos and cells in *in vitro* cultures. Resistance to reinfection is usually associated with the presence of moderate to high titers of neutralizing or HI antibody (20). A serum neutralization test may be performed in embryonating eggs, on tissue culture monolayers or on tissue culture plaque overlays. The speed with which synthesis of antibody is induced in birds vaccinated with live vaccine strains of NDV varies with the strain of virus and the method of vaccine administration (51).

Using the HI test, serologic antibody can usually be detected about 7-8 days after vaccination when lentogenic vaccines are given in the drinking water, when lentogenic vaccines are given by the aerosol route serologic antibody is detected in 3-4 days. Depending on the route of vaccine administration, the peak response is seen between 12 and 21 days after vaccination.

Evaluation of humoral immune response: Challenging the vaccinated birds with the virulent ND virus is the best method of evaluating the overall immune status of the bird. However, there is a good correlation between HI titers and protection from challenge (19, 52, 53). The HI test is generally considered to be a reliable, economical and a rapid means of measuring the humoral response of chickens to NDV. Allan and Gough (54) have described an automated HI test for NDV which can be used to evaluate serologic immune response of chickens against NDV, whereas, Beard and Wilkes (55) have described a simple manual micromethod to conduct the HI test.

The HI test is generally performed using a constant amount of antigen and varying the amount of serum. This method is known as the beta procedure. In the alpha procedure, a constant amount of serum is used and the amount of antigen is varied (24). Two standard methods of conducting the HI test for serologic antibody to NDV have been described by Allan and Gough (56).

Secretory antibody: Secretory IgA is present in lacrimal fluid, saliva, tracheal exudate, and bile (18). Since the primary site of infection of NDV is the respiratory tract, the secretory immunoglobulin plays a major role in the host's defense against NDV. Lymphoid tissues in the upper respiratory tract, intestine and paraocular regions are the major source of secretory antibody. Routes of vaccination influence the level of local immunity. Katz and Kohn (57), and Powell et al. (58) have reported that HI antibodies in

secretions, as measured by the HI test, reached higher levels after aerosol vaccination than after intramuscular administration, whereas in serum the situation was reversed. The bulk of an avian mucosal secretion initially consists of IgA class of antibody, although both IgA and IgG have been found in the saliva of infected chickens (59). Local replication of virus is required to stimulate local immunity which reduces or eliminates the growth of virus at the mucosal surface (39). Like serum antibodies, these local antibodies can be detected by the HI and virus neutralization tests (60).

Malkinson and Small (61) have explained the difference in the immune defense mechanisms operative at the two anatomical extremities of the avian respiratory tract i.e. the eye and the air sac. They immunized one group of 4-day-old chicks by intraocular route and another group by air sac inoculation. They found that the chicks were resistant to challenge only if they are challenged by the same route and susceptible to infection if challenged by another route. They suggested that local immunity is a major factor in the defense of chickens against ND infection and systemic antibody play a secondary role in the prevention of the natural infection.

Cell mediated immunity: Immunity to ND is not merely a function of serologic antibody, because immunity is sometimes demonstrated within a short period after vaccination before serologic antibodies are detectable (18). Cell Mediated Immunity (CMI) induced by NDV is an integral part of the host's defense in addition to local and other humoral factors which play a role in early protection.

Gough and Alexander (51), and Allan and Gough (62) have shown that early protection following vaccination can be demonstrated in the presence of low level of antibody or even in the absence of detectable antibody. They have suggested that local immunity in the respiratory tract is involved in this protective mechanism. This protection

can be explained by the rapid onset of a cell-mediated immune response. CMI is the initial immunological response and can be demonstrated as early as two days following vaccination. There is no direct correlation between CMI and serologic antibody levels (63).

Sensitized lymphocytes, when stimulated by specific antigen, release factors that are capable of inhibiting the migration of macrophages (macrophage-inhibition factor) and blood leukocytes (leukocyte-inhibition factor) (64). Zwilling et al. (65) reported that inhibition of leukocyte migration is antigen specific, reproducible, independent of antibody response and transferable to normal cells with a soluble cell-free product.

Evaluation of CMI: The Leukocyte migration inhibition test and the lymphocyte transformation test can be used to measure the CMI in chickens. There are two techniques commonly used to measure migration inhibition factors: the capillary tube method, and the agarose method (64).

Timms and Alexander (63) have used the capillary tube leukocyte migration inhibition technique to demonstrate CMI against NDV in chickens. Vlaovic et al. (66), and Timms (67) have shown that the leukocyte migration inhibition test is practical and a reproducible method of studying the role of cellular immunity in chickens. Timms et al. (68) have used the lymphocyte transformation technique to determine the CMI against infectious bronchitis virus in chickens whereas, Ghumman and Bankowski (69) have used this technique to demonstrate CMI to NDV in turkeys.

In the capillary tube method, lymphocytes and other migratory cells are placed together in capillary tubes and gently centrifuged. The cell-filled capillaries are then placed into solutions either containing antigen or containing no antigen. Antigen activated T-cells secrete leukocyte inhibitory factor (lymphokine) which inhibits the outward migration of leukocytes (64).

In the agarose method, purified leukocytes and antigens are placed in wells cut out of a layer of semi-solid agarose contained in a petridish. Leukocytes are allowed to migrate under the agarose. After staining to enhance the visualization of the cells the area of migration is measured.

The above two methods are direct methods for measuring the leukocyte migration inhibition factor (LIF). In the direct methods, lymphocytes and migratory cells from the sensitized birds are placed together with antigen, whereas, the indirect technique is a two-step procedure which involves the incubation of sensitized lymphocytes with antigen in a separate culture system. After incubation, the supernatant fluid from the culture which contains the leukocyte migration inhibitory factor is collected. This supernatant is then tested on heterogenous non-immune indicator cells for lymphokine activity (70).

The Lymphocyte blastogenesis assay involves culturing a population of lymphocytes *in vitro* either in the presence or absence of an antigen for varying periods of time. The evaluation of lymphocyte proliferation can be achieved by the addition of a radiolabelled precursor of DNA (usually tritiated thymidine) to the culture medium and subsequently detecting the amount of radioactivity which has been incorporated into the cells (71). Several techniques have been used to isolate leukocyte populations from the whole blood of chickens (67, 72, 73, 74). Although erythrocyte contamination in the leukocyte preparation does not interfere in the capillary tube method, a relatively pure leukocyte suspension is required for the lymphocyte blastogenesis assay and the agarose technique.

Non-specific resistance factors: The non-specific resistance factors include inhibition of viral replication by interferon, natural secretions of the body (such as mucus, saliva, gastric enzymes and tears), mechanical factor such as mucociliary escalator and other non-specific inhibitors of the virus. NDV is one of the well known inducers of interferon (75).

Evaluation of the total immune response of the chicken to NDV: The total immune status of the chicken against NDV can be determined by challenging the vaccinated and non-vaccinated control birds with a standard dose of virulent virus. A potent vaccine should provide full protection following a single vaccination (76).

Maternally derived antibody: The maternally derived antibodies are found in the yolk and consist of IgG. The IgM and IgA classes of antibodies have not been reported to occur in the yolk material. The yolk sac is fully absorbed within a few days after hatching. The level of passively acquired maternal antibody in the serum of a day-old chick is approximately the same as in the serum of the hen. The level of passively acquired maternal antibody in the young chick generally declines at a constant rate and has a half-life of approximately four and a half days (19). Usually, maternal antibody interferes with the development of active immunity in response to ND vaccine given by the intramuscular route (77). This interference is reflected by lower serologic response, shorter duration of refractiveness and an irregular flock response. Hitchner (1) has suggested that chicks possessing maternal antibodies can be successfully immunized by the respiratory route. The vaccination of maternal antibody positive chicks by non-parenteral route has shown to induce local immunity which provides protection against field exposure to NDV and eliminates the risk of serious losses.

Newcastle Disease Vaccines

Vaccine efficacy depends on many variables such as the age of the bird at the time of vaccination, immune and health status of the bird, the type of vaccine used, the virus titer of the vaccine and the route of vaccination. A wide variety of live and inactivated vaccines have been used successfully against ND.

Live vaccines: The efficacy of a live vaccine depends on its invasiveness and its power to multiply sufficiently within the chicken to set up an adequate immune response (78). Live ND vaccines are generally prepared in embryonated eggs. The most widely used lentogenic vaccine strains are: F strain (79), Hitchner B1 strain (80), and LaSota strain (81), whereas, the mesogenic vaccine strains include: Roakin (82), Komarov (83), Hertfordshire (84), and Mukteswar (85).

Live lentogenic vaccines: The Hitchner B1, F, and LaSota strains are strains of choice and have proven to be highly efficacious on a world wide base (78). When first introduced, the B1 strain was used primarily for vaccination of baby chicks, but because of its safety and its ability to stimulate a good immune response in older birds, it has been widely used in birds of all ages.

In general, the LaSota strain gives better protection than the B1 strain, however, this strain induces a slightly greater respiratory reaction and also has a greater tendency than the B1 strain to spread from bird to bird within a house (78). A common practice is to use the B1 strain for primary vaccination and the LaSota strain for subsequent booster vaccinations because the LaSota strain produces a more severe vaccine reaction after the primary vaccination than does the B1 strain (86).

Strain F closely resembles the B1 strain in many of its properties and has been found suitable for vaccination of chickens of all ages (87). The F and B1 strains usually cause little or no vaccine reaction.

Sagild and Haresnape (88) have successfully used an Australian isolate referred to as V4 as a vaccine strain in Malawi where the LaSota and the Komarov vaccines were often not effective in controlling ND. The success of the V4 vaccine was attributed to its thermostability, ease of administration and transmissibility. Ideris et al. (89) have studied

the efficacy of a pelleted form of ND vaccine prepared from V4 strain which can be used in the feed. They have shown that this food pellet vaccine can protect chickens against NDV challenge.

A number of reports on the duration of immunity to NDV after initial vaccination of young chicks with live lentogenic vaccines have been summarized by Lancaster (87). The duration of immunity to NDV may vary from 8 weeks to 16 weeks depending on a number of factors such as age and immune status of the bird, virus titer of the vaccine and route of vaccination.

Live mesogenic vaccines: Live mesogenic vaccines such as the Roakin, Komarov, and Mukteswar strains are still widely used throughout Africa, the middle East and SE Asia. These strains are pathogenic for chicks under 8 weeks of age. These mesogenic vaccines are not recommended for adult birds which have not been previously immunized using lentogenic vaccines (78). In young chicks, the Mukteswar strain of ND vaccine has been reported to produce a severe vaccine reaction with as high as 30% mortality. Paralysis has also been observed in about 2% of the young birds vaccinated with the Mukteswar strain vaccine. As with other mesogenic vaccines, the Mukteswar strain causes a reduction in egg production usually lasting for a period of 1-3 weeks (85).

Live tissue culture vaccines: Several studies have indicated that the hemagglutinin develops poorly and that some loss of antigenicity occurs when the lentogenic strains are propagated in tissue culture. However, no loss of antigenic properties were found when the mesogenic strains were propagated in tissue culture. Pig kidney monolayers and bovine kidney monolayers have been used successfully to propagate the Mukteswar and the Komarov strains of NDV respectively. Live mesogenic vaccines produced in tissue culture have shown to provide long lasting immunity (87).

Inactivated ND vaccines: The inactivated ND vaccines are generally administered by intramuscular or subcutaneous route. Oil-based inactivated ND vaccines have been shown to be more immunogenic than aluminum hydroxide inactivated vaccines (90). The efficacy of oil-emulsion ND vaccines depends on various factors such as emulsifier content, aqueous-to-oil ratio, and antigen concentration (90-94). Chickens vaccinated with a live ND vaccine and then subsequently revaccinated with an inactivated oil-emulsion ND vaccine have higher and more persistent HI serologic antibody titers and lay more eggs than birds vaccinated using only the live ND vaccines (95, 96).

Inactivation of NDV is most commonly achieved by the use of formalin or beta propiolectone (BPL). Crystal violet, phenol, heat, ultra-violet light, and ultra-sonic treatment have also been used to inactivate the NDV. Dardiri et al. (97), Hofstad (98), Legenhausen et al. (99), and Waller and Gardiner (100) have demonstrated that a high degree of protection against NDV can be produced with inactivated vaccines. However, the level of protection and the duration of immunity depend on a number of variables such as: (1) the strain of the virus; (2) the selection and concentration of the inactivating agent; (3) the type and concentration of the adjuvant used; (4) the age of the bird; (5) the immune and health status of the bird; and (6) the route and dosage schedule.

Beard and Mitchell (101) have reported that inactivated ND vaccines induce higher serologic titers at hot (26.6-40.7° C) and moderate (18.3-32.3° C) environmental temperatures, whereas, the live ND vaccines induce low serologic titers at these temperatures. Inactivated vaccines have been found to be safe in the young bird as well as in older birds. The vaccination of a laying flock with an inactivated vaccine caused no significant effect on egg production or any respiratory symptoms (102).

One criticism against using adjuvants with vaccines for meat birds has been the persistence of the adjuvant in the muscle tissue. In addition, certain oils when used as adjuvants have caused severe granuloma formation at the site of injection leading to carcass down grading and processing losses (87, 103).

Since there is no virus replication with inactivated vaccines, a higher concentration of antigen is required with inactivated vaccines than with live vaccines. The inactivated vaccines are administered by intramuscular or subcutaneous route which require individual handling of the birds and because of the cost of individual vaccination, oil-based inactivated ND vaccines are used mainly for revaccinating laying chickens and breeding stocks.

ND vaccines have been combined with other vaccines such as infectious bronchitis (IB), fowl pox (FP), infectious bursal disease (IBD) and infectious laryngotracheitis (ILT) in order to save vaccination time and expense. However, the efficacy of all these agents incorporated into a single product has been questioned on the grounds that antigenic competition may prevent satisfactory immunity (1, 104).

Liposomes are small spherical sacs which consist of a lipid bilayer enclosed aqueous compartment. The liposome can be unilamellar (single lipid bilayer) or a multilamellar (many layered). Efforts are being made to use liposomes as adjuvants with ND vaccines. Liposome-adjuvanted experimental ND vaccine has been found to be potent and safe in chickens and turkeys.

There are several advantages of genetically engineered vaccines over the conventional vaccines including: the lack of possible reversion, purity, lack of vaccine reactions following administration and the possibility of differentiating between vaccinal response and seroconversion due to field strains (78).

Since the sequence of the HN and F proteins of different NDV strains are now known, the genes coding for these immunizing proteins can be cloned using recombinant DNA technology. Expression of these genes in various vectors could lead to the production of genetically engineered vaccines. Meulemans et al. (50) have emphasized the role of F protein in the immunity against NDV. Recently, Meulemans et al. (105) have demonstrated that chickens can be protected against ND using F protein expressed from a recombinant vaccinia virus (Vaccinia-Italien-F). A close correlation has been observed between the presence of F antibodies and the resistance to challenge. Vaccination against ND using a recombinant virus expressing only the F protein would be of great interest as it would allow the differentiation between the immunological response induced by a vaccine or a field virus (78). They have suggested that this differentiation would allow the joint application of a vaccination and eradication program for NDV.

Route of ND Vaccine Administration

The route of ND vaccine administration plays a major role in the type and degree of immunity developed in chickens (106, 107). ND vaccine can be administered by a variety of techniques including: (1) intramuscular or subcutaneous injection; (2) intraocular or intranasal instillation; (3) beak dipping; (4) drinking water; (5) food pellet; and (6) aerosol or spray application.

The first three methods listed above are performed on the individual bird. These immunization methods produce a more uniform immune response in the flock than the mass vaccination methods. However, individual inoculation methods are not economically feasible in broilers and are therefore limited to vaccination of replacement layers and breeders (78).

In order to determine how different routes of vaccine administration influence the immune response, comparative studies have been made by using live ND vaccines administered by various routes such as intraocular (108), intranasal (80, 106), intramuscular (87), intravenous (cited in (87)), drinking water and spray (109), and dust (97, 109).

The drinking water method is the most common and easiest method of ND vaccine administration. Factors such as impurities in the water, management and environmental conditions can influence the effectiveness of the drinking water method. The drinking water vaccination induces a minimum vaccine reaction as compared to the more severe vaccine reactions observed after aerosol vaccination (86). Parental immunity against ND can interfere with the drinking water vaccination (110).

Most lentogenic vaccines have an affinity for the respiratory epithelium and are more effective when applied individually via the respiratory tract. Therefore, the eye drop method of vaccination results in a higher antibody response than that attained by vaccination via the drinking water. In addition, the eye drop method results in a longer duration of immunity and a higher degree of flock protection (19).

The major advantages of aerosol and spray vaccination are that mass application makes it possible to vaccinate large number of chickens in a minimum period of time. Some disadvantages of this technique are the difficulties of standardization and risk of severe vaccine reactions, especially in mycoplasma positive flocks (111). Vaccine reactions also depend on the strain of the ND virus used. Allan and Borland (112) have compared 14 different lentogenic strains to measure the stress caused by aerosol exposures. Gough and Allan (113) have studied the effect of aerosol exposure using the Ultster strain vaccine against ND. They found that the Ulster strain of ND vaccine, when administered by

aerosol technique, induced a higher degree of immunity in chickens with less reaction in the respiratory tract than in those birds which were vaccinated with other strains of ND vaccine by the same route.

The site of deposition of inhaled particles in the respiratory tract of chickens depends on the size of particles (114). Particles of 3.7 to 7μm are deposited in the upper respiratory tract, whereas, smaller particles are deposited in the lower respiratory tract.

Several factors such as the vaccine diluent (115, 116), particle size (117, 118), and virus concentration (116) can influence the success of the aerosol method. Villegas and Kleven (115), and Yadin (116) have demonstrated that distilled water produces fine droplets which are more immunogenic and stable than the droplets derived from vaccines containing tap water or gelatin as diluents.

Beard and Easterday (106), and Partadiredja et al. (107) have shown that chickens that were vaccinated with live ND vaccine by the aerosol method developed greater levels of HI or VN antibodies than chickens which were vaccinated by the intramuscular, intraocular, intranasal or drinking water methods. Giambrone (119) has compared the three most commonly used commercial vaccination techniques (Spra-Vac, Beak-O-Vac, and drinking water) in the field and observed that resistance to challenge with the virulent NDV was greater in birds which were vaccinated by a coarse spray method using a Spra-Vac machine. Chickens vaccinated by the aerosol method have been shown to be more refractory to challenge than other birds which were vaccinated by other techniques. The birds vaccinated by the intramuscular route were resistant to intramuscular challenge but were susceptible to aerosol challenge two weeks after vaccination, whereas, those birds vaccinated by aerosol exposure were resistant to both challenge techniques (106). Aerosol exposure also produces a better local antibody response in the respiratory tract. Vaccine

virus can be recovered from the tracheas of chickens after aerosol exposure but not after intramuscular injection.

Adjuvants

Substances that enhance the immune response when administered with antigens are called adjuvants. A wide variety of compounds have been found to enhance the immune response of chickens. These compounds are diverse in both substance and function. The diversity of adjuvants have presented difficulties in their classification (120). Aluminium compounds, calcium phosphate, levamisol, dextran sulphate, oil-emulsion, liposomes, synthetic polymers, polynucleotides, vitamins A and E, lanolin, certain bacteria, bacterial toxins, and viruses are examples of chemical and biologic substances that have adjuvant activity. Lipopolysaccharides (LPS), and muramyldipeptides (MDP) are chemically defined microbial products. Thymic hormones, lymphokines, and cytokines also have significant effect on the immune system (121).

Mechanism of adjuvant activity: A wide diversity of substances possess adjuvant properties and the mechanism of action of one adjuvant may differ from another. Adjuvants may act on specific antigens or on the host's cells involved in the immune response.

Adjuvants may modify the antigen by conformational changes or possibly altering the net electrical charge of the antigen molecules (122). Neter et al. (123), Nossal et al. (124), and Draper and Hirata (125) have suggested that one role of the adjuvant could be to change the antigen conformation. Thus, soluble antigen could have a non-antigenic conformation, however, aggregation of several non-immunogenic molecules recovers the antigenic site and the immunogenicity. The adjuvant could give the right shape to the

antigenic site and this lead to immunogenicity. Jolles and Paraf (122) have suggested that poorly immunogenic molecule could become strongly immunogenic following a change in its net electrical charge or a change in the distribution of the charge. They have suggested that oily adjuvants with a large hydrophobic moiety, could induce change in net electrical charge in a protein. Depending on the hydrophillic or hydrophobic environment, a protein with a hydrophobic cavity may unfold and this change in conformation may expose a new electrical charge.

Adjuvants may transform a non-immunogenic hapten into an immunogen. Certain antigens can be denatured by emulsification or made particulate by adsorption onto alum, bentonite or other particles. Denatured or particulate antigens readily become associated with the membranes of macrophages and dendritic cells of the lymph node and spleen. This may facilitate the presentation of antigen to lymphocytes (121).

Adjuvants such as aluminum salts and water-in-oil emulsions trap the antigen and cause the formation of depots from which the antigen is released slowly over a prolonged period. This may induce a secondary immune response. Adjuvants may also modify the catabolism of the antigen by the host.

Certain adjuvants cause the sequestration of lymphocytes in lymphoid organs. This lymphocyte trapping encourages optimal contact between the antigen and immunocompetent cells.

Accumulation of large numbers of macrophages and lymphocytes around a focus of infection is called a granuloma. A classic granuloma contains the agent in the center surrounded by macrophages that is surrounded first by lymphocytes and then by connective tissue. Some adjuvants recruit macrophages, lymphocytes, and other cells to the site of inoculation to form a local granuloma. This type of granuloma provides an

effective means of localizing the agent at the site and allowing other inflammatory and immunologic mechanisms to act for longer periods of time.

Many adjuvants such as endotoxins, mycobacteria (wax D), vitamin A (retinol), silica, beryllium, cationic detergents and saponin have surface activity which may facilitate contact between collaborating cells such as macrophages and lymphocytes. These substances have been reported to facilitate the release of lymphokines and monokines (126).

Adjuvants can also act on the cells involved in the immune response. The cellular response may depend on the type of adjuvant and route of adjuvant administration. Adjuvants can affect humoral immunity, cell-mediated immunity and antibody dependent cell-mediated immunity. Dresser and Phillips (127) have studied the adjuvants which have selected effect on T-cell or B-cell activity. Warren et al. (120) have shown that adjuvants can have an effect on the class or subclass of antibody which is produced. Benedict and Yamaga (128) have reported that immunizing chickens with antigens incorporated in Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) resulted in a biphasic antibody response. The second phase was most pronounced in birds injected with FCA; moderate in those given FIA. This second phase was not detected without antigen.

Severe local reactions, hypersensitivity, and tumor inducing effects are major adverse reactions of adjuvants. Other unfavorable effects include: (1) persistence of material in food animals that can not safely be ingested by humans; (2) increased vascular permeability and inflammatory reaction; (3) pyrogenicity; (4) induction of autoimmune responses; (5) CNS and untoward behavioral effects; (6) impairment of growth; and (7) arthritis (129). Development of tumors have been observed in mice which were given mineral oils (130).

Efforts have been made to develop biodegradable emulsions that are stable, effective, and non-carcinogenic. Woodhour et al. (6) have developed adjuvant 65, a preparation of peanut oil, aluminium monostearate stabilizer, and Arlacel A emulsifier. Unlike mineral oil, adjuvant 65 contains only components that are metabolizable or excreted by the body. When an influenza vaccine containing adjuvant 65 was used to vaccinate humans no local or systemic reactions were observed (7). The adjuvant 65 has been shown to be extremely effective in eliciting a rapid and long lasting antibody response to influenza virus vaccine (131).

Efforts have also been made to develop a safe and effective adjuvant using almond oil (8) and sesame oil (9). Highly refined peanut oil was found to be devoid of adverse effects. Although the vegetable oils have proven to be safe and do enhance the immune response, the potency of the vegetable oil adjuvant has been found to be less than that of mineral oil (122). Other adjuvants commonly used in veterinary medicine are aluminum hydroxide, aluminum salts and oil emulsion.

Live and inactivated oil adjuvant ND vaccines when inoculated simultaneously in one-day-old chicks induced a good level of protection against the challenge virus (95, 132, 133). Brugh et al. (134) have compared 9 inactivated ND vaccines containing different adjuvant emulsions and found that the adjuvant activity of vegetable oil adjuvants were lower than that of mineral oils. Stone et al. (91) have compared three oil-emulsion inactivated ND vaccines in white leghorn pullets and observed that despite their equal antigen content and their desirable physical characteristics, the three oil-emulsion vaccines induced different levels of serum HI antibody. They have concluded that the immunopotentiating effect of oil-emulsion adjuvant varies depending on the emulsion composition. In an effort to maximize the adjuvant effect of oil-emulsion ND vaccine and

avian influenza vaccine, Stone (93) evaluated the efficacy of oil-emulsion vaccines in white rock chickens with a surfactant hydrophile-lipophile balance (HLB) value between 4.3 and 9.5. The HLB is an expression of the relative simultaneous attraction of an emulsifier for water and for oil. The HLB value of 7 was found to induce highest HI antibody titer. He suggested that increased adjuvant effect at certain HLB value may be related to an increased rate of release of the aqueous phase content.

MATERIALS AND METHODS

Chickens

One-day, 2-week or 6-week-old specific pathogen free (SPF) white leghorn chickens were used for this study. Fertile SPF eggs, obtained from HY-VAC Laboratory Eggs Company, Gowrie, IA 50543, were hatched at the SPF facility at the Veterinary Medical Research Institute (VMRI). All the chicks were maintained in the SPF facility at the VMRI until the beginning of the experiment.

One day before the beginning of each trial, a group of SPF chicks was transferred to a separate presterilized containment isolator and reared in the same isolator throughout the trial period. The chickens were provided feed and water *ad libitum*.

Embryonated Chicken Eggs

11-day-old SPF chicken embryos were used to propagate and titrate the virulent NDV which was used to challenge the vaccinated birds, and to propagate the B1 strain of NDV which was used as antigen in the leukocyte migration inhibition assay.

Vaccines

The B1 strain of live virus ND vaccine (Ceva Laboratories, Inc. Overland Park, KS 66212) was used for primary vaccination and the LaSota strain of live virus vaccine (Ceva Laboratories) was used for secondary vaccination.

Vegetable Oils

Corn oil (Mazola brand 100% pure corn oil; Best Foods, CPC International, Inc. Englewood Cliffs, NJ 07632) or soybean oil (Crisco brand 100% pure soybean oil; Procter and Gamble, Cincinnati, Ohio 45202) was mixed with the ND vaccine to prepare an oil adjuvant ND vaccine.

Challenge Virus

The GB-Texas strain of ND virus was used to challenge the vaccinated and control birds. The virus was propagated in 11-day-old SPF embryonated eggs after first being passaged in 6-week-old chickens. The allantoic fluid was clarified by low speed centrifugation at 4080 x g for 30 minutes. The titration of the ND virus in the clarified allantoic fluid was carried out in embryonated eggs. The embryo lethal dose 50 percent end point (ELD_{50}) was calculated by the Spearman-Karber method (135). Purified allantoic fluid was dispensed in small plastic vials and stored at -70° C until further use. Birds were challenged with 10^6 or 10^7 ELD_{50} per bird by intramuscular or intranasal-intraocular (IN-IO) routes as described below in experiment 9.

Erythrocyte Suspension

Whole blood was collected in equal volume of Alsever's solution from a turkey pre-screened for non-specific hemagglutination. The red blood cells were washed three times by centrifugation in phosphate buffered saline (PBS). A red blood cell concentration of 0.5% was used in the HI assay.

Vaccine Preparation

Preparation of 70% oil adjuvant vaccine and aqueous vaccine: One vial (1000 doses) of vaccine was first diluted with PBS so that 1 drop contained 3.3 doses. Half of this PBS diluted vaccine was further diluted in PBS so that 1 drop equaled 1 dose and was used as the aqueous ND vaccine. The remaining half of the PBS diluted vaccine was diluted with vegetable oil in a 7:3 ratio of oil to water resulting in one drop being equal to one dose of a 70% oil adjuvant ND vaccine.

Preparation of 90% oil adjuvant vaccine and aqueous vaccine: One vial (1000 doses) of ND vaccine was diluted with PBS so that 1 drop contained 8.3 doses. Half of this PBS diluted vaccine was further diluted in PBS so that 1 drop contained one dose and was used as the aqueous ND vaccine. The remaining half of the PBS diluted vaccine was mixed with vegetable oil in a 9:1 ratio of oil to water so that one drop provided one dose of 90% oil adjuvant ND vaccine.

The oil adjuvant vaccine was prepared by mixing the vegetable oil and the PBS diluted ND vaccine in a double-hubbed emulsifying syringe. No emulsifier or stabilizer was added. The emulsified vaccine was used immediately after preparation.

Preparation of oil adjuvant vaccine with emulsifiers: Oil adjuvant vaccine used in experiments 7 and 8 was prepared by adding emulsifiers as described by Stone et al. (136). The aqueous-phase emulsifier Tween 80 (Sigma Chemical Company, St. Louis, MO 63178) was mixed with the PBS diluted ND vaccine, and the oil-phase emulsifier Arlacel™ A (Sigma Chemical Company) was mixed with the corn oil separately. The final oil-emulsion vaccine was prepared by mixing the aqueous-phase and the oil-phase components in a VirTis® 45 homogenizer (The VirTis Company, Gardiner, NY 12525).

The oil-phase component contained 10% Arlacel™ A. The total amount of Tween 80 incorporated into the vaccine preparation was 10% of the total amount of Arlacel™ A. These concentrations of emulsifiers were chosen in order to achieve the hydrophile-lipophile balance (HLB) value of 7 which has been shown to influence the HI response in chicken (93). The oil-to-aqueous ratio 7:3 was used for both trials.

For experiment 8, the 70% corn oil adjuvant ND vaccine was prepared in such a way that 0.5 ml of the vaccine preparation contained 1 dose of vaccine when administered by subcutaneous route. The LaSota strain vaccine was used for parenteral administration.

Vaccination

Group 1 in each experiment was vaccinated with one chick dose of oil adjuvant ND vaccine by the intranasal-intraocular (IN-IO) route. Group 2 in each experiment was vaccinated with one dose of aqueous ND vaccine by the IN-IO route. Group 3 was not vaccinated and was considered as unvaccinated control.

In experiment 8, group 1 was vaccinated with a 70% corn oil adjuvant ND vaccine by the subcutaneous (SC) route, whereas, group 2 was vaccinated with an aqueous ND vaccine by the SC route.

Secondary vaccination was performed 2 weeks after the primary vaccination. The LaSota strain of ND vaccine was used for the secondary vaccination. The route of administration for the secondary vaccination was always the same as for the primary vaccination.

Hemagglutination-inhibition Test

Two weeks after the primary and the secondary vaccination, blood was collected from each bird to measure the hemagglutination-inhibition (HI) antibody titer in the serum. Blood samples were collected from the wing vein using microhematocrit capillary tubes (cat.no. 02-668-66; Fisher Scientific, Pittsburgh, PA 15219). Three capillary tubes filled with whole blood was found to be sufficient for performing the HI test (50μl/sample). The capillary tubes were centrifuged in a microhematocrit centrifuge and serum samples were collected on wax paper by cutting the capillaries at the cell/serum interface.

The HI test was performed using the beta procedure as described by Beard and Wilkes (55). Two-fold serial dilution of serum was made in 96-well, U-bottom microtiter plates containing 50μl of PBS in the first row and 50μl of antigen (8 HA units) in the remaining 11 rows. The antigen-serum mixture was allowed to react for 30 minutes at 37° C. A positive reference serum, a negative reference serum, antigen and erythrocyte controls were included in each HI procedure. After the addition of 50μl of a 0.5% erythrocyte suspension, the plates were reincubated for 30 minutes at 37° C. The highest dilution of serum causing complete inhibition was considered the end point. The results were expressed as the $\log_2$ mean of the HI titers.

Preparation of Agar Plates for the LMI Test

Agar plates for leukocyte migration inhibition test were prepared at least 24 hours prior to the procedure. Agar was prepared as follows:

Solution A: 1.6 grams of agarose (Indubiose 45, cat.no. 60113, Gallard-Schlesinger Industries, Inc. 584 Mineola Av., Carle Place, NY 11514) was dissolved in 160 ml of sterile distilled water by heating in a boiling water bath for 10-15 minutes. The dissolved agar mixture was autoclaved for 1 minute and then maintained at 52° C.

Solution B: Solution B was prepared by mixing 20 ml of fetal bovine serum (cat.no. 110-1120, JR Scientific, Inc. PO Box 1937, Woodland, CA 95695), 18 ml of 10X M-199 medium with Hanks salts (cat.no. 56-329, Hazletone Research Products, Box 72, Denver, PA 17517), 2 ml of penicillin-streptomycin solution (cat.no. P 0781, Sigma Chemical Company), and 1 ml of 7.5% sodium bicarbonate solution. The final pH was adjusted to 7.2 and this solution was warmed to 52° C.

Solutions A and B were mixed together and maintained at 52° C in a water bath. The final agar medium consisted of 0.8% agarose, 10% fetal bovine serum, and 1% penicillin-streptomycin solution. Five ml of agar medium was dispensed in each 60x15 mm tissue culture petri dish (Falcon no. 1007, Becton Dickinson Labware, 2 Bridgewater Lane, Lincoln Park, NJ 07035). After the agar was allowed to solidify the plates were stored at 4° C in a humidified condition.

Preparation of Leukocytes

Two weeks after the primary and the secondary vaccination, blood samples were collected from each bird for the leukocyte migration inhibition (LMI) assay. Blood samples (10-15 ml) were collected by cardiac puncture using a syringe containing sodium heparin (20 U/ml of blood). Leukocytes were isolated as described by Andreasen and Latimer (74) with slight modification. In this procedure, blood samples from 2-3 chickens were pooled in a disposable tube and centrifuged at 150 x g for 15 minutes. The buffy coat was collected and suspended in 4 volumes of PBS.

A two-step discontinuous Ficoll-Hypaque gradient was prepared by using commercially available reagents. Three mls of Histopaque®-1119 (specific gravity 1.119, cat.no. 1119-1, Sigma Chemical Company) was placed in a 15 ml disposable centrifuge

tube. Three mls of Histopaque®-1077 (specific gravity 1.077, cat.no. 1077-1, Sigma Chemical Company) was layered over the Histopaque®-1119. Six mls of the PBS diluted buffy coat suspension was carefully layered over the Histopaque®-1077 and centrifuged at 300 x g for 40 minutes at room temperature.

When mammalian blood was centrifuged using the two-step discontinuous gradient technique, there was a formation of two distinct layers of cells. The first layer which formed at the plasma/Histopaque®-1077 interface contained mainly mononuclear cells and platelets, and the second layer which formed at the Histopaque®-1077/Histopaque®-1119 interface contained granulocytes. Instead of forming a distinct layer at the Histopaque®-1077/Histopaque®-1119 interface the chicken heterophils diffused throughout the Histopaque®-1119. After centrifugation, the cellular layer at the plasma/Histopaque®-1077 interface and the heterophil rich Histopaque®-1119 layer was transferred to a sterile centrifuge tube. The leukocytes were washed three times in M-199 medium containing Hank's salts by centrifugation at 225 x g for 10 minutes. The final pellet was resuspended in 1 ml of M-199 medium and the total leukocyte count was estimated using the improved Neubauer hemocytometer. Leukocyte viability was determined by the trypan blue dye exclusion test. The viability was always greater than 90%. The final leukocyte concentration was adjusted to 5×10^7 cells per milliliter.

Preparation of ND Antigen for the LMI Test

The B1 vaccine strain of NDV was propagated in 11-day-old SPF embryonated chicken eggs. Three days post inoculation, the virus was harvested by chilling the embryos overnight and collecting the allantoic fluid. The virus was purified as described by Reeve and Alexander (137). Briefly, allantoic fluid was centrifuged at low speed (4080

x g) for 30 minutes to remove cellular debris. The supernatant was pelleted by ultracentrifugation using a SW 28 rotor at 72000 x g for 2 hours. The pellet was resuspended in about 1 ml of cold PBS. A discontinuous sucrose gradient was made using 20% and 50% sucrose solutions (w/v) in Tris saline. The resuspended pellet was centrifuged onto the sucrose gradients at 72000 x g for 2 hours. The opaque band at the interface between the 20% and 50% sucrose solutions was collected and a HA titer was determined.

The protein concentration of the antigen was determined by the method described by Lowry et al. (138) using a Gilford spectrophotometer (model 250, Gilford Instrument Laboratories, inc. Oberlin, Ohio 44074). The protein concentration was adjusted to 4 mg/ml and the virus was inactivated by incubating at 56° C for 30 minutes.

Determination of the Working Antigen Titer for the LMI Test

Blood samples from 12 6-week-old non-vaccinated chickens were collected and leukocytes were isolated as described before. A serial dilution of the ND antigen was made in M-199 medium containing 5 x 10^7 leukocytes per ml and incubated at 37° C for 30 minutes. Three wells of an agar plate were filled with the leukocyte suspension containing each dilution of antigen. A cell suspension devoid of antigen was also included in the test as a control. The plates were incubated at 37° C in a humidified atmosphere containing 5% CO_2. After 18 hours of incubation, the plates were removed and fixed by flooding them with 8% glutaraldehyde solution for 1 hour. The agar was removed and the plates were stained with Wright's modified stain (cat.no. WS16, Sigma Chemical Company). The area of leukocyte migration was measured using an inverted microscope.

The lowest dilution of antigen which induced less than 20% inhibition of non-sensitized cells was considered as the working titer of antigen for the LMI test. The leukocyte suspensions containing 75μg, 100μg, and 150μg per ml caused less than 20% inhibition of migration. The antigen concentration of 100μg/ml was considered as the working antigen titer for this study.

Leukocyte Migration Inhibition Test

Each sample used in the LMI test was a pooled sample of leukocytes from 2-3 chickens. The samples were diluted to contain 5×10^7 cells/ml and then were divided into two sterile tubes. The ND antigen was added to the first tube (100μg/ml) and an equal amount of M-199 medium to the control tube. All the tubes containing leukocyte suspensions with or without antigen were incubated at 37° C for 30 minutes.

Six wells were made in each of the agar plates using a punch and punch guide. The punch and punch guide were made locally by the Engineering Research Institute, ISU, Ames,Iowa, as described by Nelson et al. (139). Three wells of the agar plate were filled with cell suspensions containing antigen and the remaining three wells were filled with cell suspensions containing no antigen. A suspension of leukocytes collected from non-vaccinated control birds were also included in each experiment. All plates were incubated at 37° C for 18 hours in a humidified atmosphere containing 5% CO_2. After incubation, the plates were fixed with 8% glutaraldehyde solution for 60 minutes. The hardened agar was removed and the adhering cells were stained with Wright's modified stain. After washing with distilled water, the plates were allowed to dry. The diameter of the cellular migration was measured using an inverted microscope and the area of migration was calculated using the following formula:

$$\text{Area of a circle} = \pi r^2$$

where $\pi = 3.14$

r = radius of the circle or diameter/2

The average migration area in the presence or absence of antigen was compared and the percentage of migration was calculated as follows:

% migration =

$$\frac{\text{mean of migration in the presence of antigen}}{\text{mean of migration in the absence of antigen}} \times 100$$

The percentage of inhibition of migration was determined as follows:

$$\% \text{ inhibition} = 100 - \% \text{ migration}$$

Generally, greater than 20% inhibition in the presence of antigen represents significant leukocyte inhibition factor (LIF) activity (63, 64, 70).

Experimental Design

A total of 9 experiments were carried out to study the adjuvant effect of vegetable oils with ND vaccine. The experiments differed from one another by variation of the following parameters: age of the chickens, vegetable oil used, concentration of the oil used, whether or not any emulsifier was incorporated into the vaccine, and route of inoculation.

All chicks used in any given experiment were from the same hatch. Each experiment consisted of 3 groups: group 1 was vaccinated with an oil adjuvant ND vaccine; group 2 was vaccinated with an aqueous ND vaccine; and group 3 was not vaccinated. In all experiments, the B1 strain of ND vaccine was used for primary vaccination and the LaSota strain of ND vaccine was used for secondary vaccination. Vaccines were prepared immediately before use as described above.

HI and LMI tests were performed two weeks following the primary and the secondary vaccination to evaluate the humoral and cell mediated immunity respectively. In experiment 9, each group was challenged with a virulent ND virus to evaluate the protective immunity.

Experiments 1, 2, and 3: The total number of one-day-old SPF chicks in each group was 20 in experiment 1, and 30 in experiments 2 and 3. The 70% corn oil adjuvant ND vaccine was prepared without adding any emulsifier and one-day-old chicks were vaccinated by the IN-IO route. LMI test was performed in experiments 2 and 3 as described before.

Experiment 4: This experiment was identical to experiment 1, 2, and 3 except that 6-week-old SPF chickens were used instead of day-old chicks. The HI and LMI tests were performed as before.

Experiments 5 and 6: In experiments 5 and 6, the concentration of oil combined with the vaccine was increased to 90%. In experiment 6, two-week-old SPF chicks were vaccinated with 90% soybean oil adjuvant ND vaccine instead of corn oil adjuvant ND vaccine. Birds were vaccinated by the IN-IO route. The 90% corn oil or soybean oil ND vaccine was prepared without the incorporation of any emulsifier as described above.

Experiments 7 and 8: In both experiments, the 70% corn oil ND vaccine preparation was prepared by adding Arlacel™ A as the oil-phase emulsifier and Tween 80 as the aqueous-phase emulsifier. Two-week-old SPF chicks were inoculated with one-chick-dose of emulsified vaccine by the IN-IO route in experiment 7 and by the SC route in experiment 8.

Experiment 9: In experiment 9, each of the 3 groups (groups A, B and C) consisted of 25 2-week-old chickens. All chickens in group A were vaccinated with the 70% corn oil adjuvant ND vaccine by the IN-IO route at 2 weeks of age. The 70% corn oil adjuvant ND vaccine was prepared without adding any emulsifier. Similarly, all chickens in group B were vaccinated with the aqueous ND vaccine by the IN-IO route at 2 weeks of age. The chickens in group C were not vaccinated and were considered as control birds. Each group was again subdivided into 3 subgroups (Table 1). Two weeks following the primary vaccination, only subgroup 3 of groups A and B were revaccinated as before. Subgroups 1 and 2 did not receive a second dose of the ND vaccine.

Ten days following the primary vaccination, blood samples from each chicken of subgroup 1 of each group were collected for the HI test and each chicken was challenged with 10^6 ELD_{50} of the GB-Texas strain of ND virus by the intramuscular route. Similarly, three weeks following the primary vaccination, blood samples were collected from each chicken of subgroup 2 of each group and were used for the HI test. Each chicken was then challenged with 10^7 ELD_{50} of the GB-Texas strain of ND virus by the IN-IO route. Two weeks following the secondary vaccination, blood samples from revaccinated chickens (subgroup 3) were collected for the HI test and each chicken was challenged with 10^7 ELD_{50} GB-Texas strain of ND virus by IN-IO route. Necropsy findings of the mortalities as well as the euthanized birds were recorded.

A comparative analysis of data obtained from the HI, LMI, and the challenge tests of all the experiments was made. The data were examined for statistical significance using the Student's t-test (140).

The complete experimental plan is summarized in Table 2.

TABLE 1. Summary of Experiment 9

Group	Sub group	No. of chicken per grp.	Primary vaccination	Secondary vaccination	Time of HI test and challenge	Route of challenge
A	1	5	+[a]	—[b]	10 days after primary vaccination	IM[c]
	2	10	+	—	3 weeks after primary vaccination	IN-IO[d]
	3	10	+	+	2 weeks after secondary vaccination	IN-IO
B	1	5	+	—	10 days after primary vaccination	IM
	2	10	+	—	3 weeks after primary vaccination	IN-IO
	3	10	+	+	2 weeks after secondary vaccination	IN-IO
C	1	5	—	—	10 days after primary vaccination	IM
	2	10	—	—	3 weeks after primary vaccination	IN-IO
	3	10	—	—	2 weeks after secondary vaccination	IN-IO

[a]Vaccinated.

[b]Not vaccinated.

[c]Intramuscular.

[d]Intranasal-intraocular.

TABLE 2. Summary of Experimental Plan

Expt. no.	Total no. of chicken per grp.	Age of chickens	Vegetable oil used	Conc. of oil	Emulsifiers used	Route of inoculation	HI[a] test	LMI[b] test	Challenge test
1	20	one-day	corn oil	70%	—	IN-IO[c]	+	—	—
2	30	one-day	corn oil	70%	—	IN-IO	+	+	—
3	30	one-day	corn oil	70%	—	IN-IO	+	+	—
4	10	6-week	corn oil	70%	—	IN-IO	+	+	—
5	24	2-week	corn oil	90%	—	IN-IO	+	+	—
6	24	2-week	soybean oil	90%	—	IN-IO	+	+	—
7	25	2-week	corn oil	70%	+[d]	IN-IO	+	+	—
8	25	2-week	corn oil	70%	+[d]	SC[e]	+	+	—
9	25	2-week	corn oil	70%	—	IN-IO	+	—	+

[a]Hemagglutination inhibition.

[b]Leukocyte migration inhibition.

[c]Intranasal-intraocular.

[d]Aqueous-phase emulsifier Tween 80, and oil-phase emulsifier Arlacel A used.

[e]Subcutaneous.

RESULTS

The immune response of chickens vaccinated with vegetable oil adjuvant ND vaccine, aqueous ND vaccine or unvaccinated control chickens was evaluated by the hemagglutination inhibition test, the leukocyte migration inhibition test and the challenge test.

Humoral Immune Response

The mean HI antibody titers of each group is presented in Table 3. The HI antibody titer is expressed as the $\log_2$ of the end point of the serum dilution. Since the unvaccinated control chickens were negative for HI antibody, the HI titer of the control group is not shown in Table 3. The minimum and maximum HI titer in each group is shown in the parentheses.

Effect of vegetable oils: The humoral immune response of chickens following primary and secondary vaccination by the IN-IO route was always higher when the corn oil adjuvant ND vaccine was used than when the aqueous ND vaccine was used (see Figures 1, 2 and 3). However, as the results from experiment 8 (Figure 3) indicate, when the corn oil adjuvant ND vaccine was prepared by using an emulsifier and administered by the subcutaneous route it induced a lower HI antibody response than did the aqueous ND vaccine administered by the same route. In this experiment, the lower HI antibody response to the corn oil adjuvant ND vaccine was evident following both the primary and secondary vaccinations.

In experiment 6, soybean oil was used instead of corn oil. The vaccine contained 90% soybean oil and was administered by the IN-IO route. As indicated in Figure 2, the antibody titers of chickens vaccinated with the soybean oil adjuvant ND vaccine was slightly lower than those titers of birds vaccinated with the aqueous ND vaccine.

Although the corn oil adjuvant ND vaccine always induced a higher HI antibody titer, the difference between the antibody titers of the two groups was not statistically significant in any of the experiments.

Effect of concentration of vegetable oil: Corn oil adjuvant ND vaccines contained 70% oil in all the experiments except in experiment 5 where 90% corn oil was used. As indicated in Figures 1, 2 and 3, the HI antibody titers following both the primary and secondary vaccination with the 70% and the 90% corn oil ND vaccine was higher than the antibody titers produced with the aqueous ND vaccine. However, the difference in the HI titers of chickens vaccinated with the oil adjuvant ND vaccine when compared to the chickens vaccinated with the aqueous ND vaccine was not statistically significant. No significant difference in HI titer was observed between the chickens vaccinated with 70% or 90% corn oil adjuvant ND vaccine. The 90% soybean oil ND vaccine (experiment 6) induced slightly lower HI antibody response than did the aqueous ND vaccine.

Effect of age of chicken: The difference in humoral immune response between those chickens which were one-day-old (experiments 1-3) and those chickens which were two-week-old (experiments 5-9) was not significant. However, the humoral immune response of chickens vaccinated at 6 weeks of age (experiment 4) with either the oil adjuvant or the aqueous ND vaccine was significantly higher ($P<0.05$) when compared to the day-old and two-week-old chickens.

Effect of route of vaccination: The ND vaccine was administered by the IN-IO route in all the experiments except in experiment 8 where either the oil adjuvant or the aqueous ND vaccine was administered by the subcutaneous route. In this experiment, the oil adjuvant ND vaccine was prepared by using an emulsifier to make a stable water-in-oil emulsion. Contrary to what resulted when the IN-IO route was used, the HI antibody

titer of chickens vaccinated with the oil adjuvant ND vaccine by the subcutaneous route (experiment 8) was lower than the titers of those chickens vaccinated with aqueous ND vaccine administered by the same route. The anamnestic immune response was significantly greater ($P<0.05$) in the chickens vaccinated with the oil adjuvant ND vaccine by the subcutaneous route. This was not observed in chickens vaccinated with the aqueous ND vaccine.

Cell Mediated Immune Response

The cell mediated immune response of vaccinated as well as unvaccinated chickens was evaluated by the leukocyte migration inhibition (LMI) test. It has been determined that if inhibition of leukocyte migration is greater than 20% then the inhibition of migration is due to an inhibitory factor produced by the cells involved in the cell mediated immune response. Therefore, the degree to which leukocytes fail to migrate is an indication of the cell mediated immune response. The mean migration inhibition of each group is presented in Table 4. The minimum and maximum range of inhibition in each group is shown in parentheses.

As shown in Figures 4, 5 and 6, both the oil adjuvant and the aqueous ND vaccines induced significant cellular immune response in all the experiments. The mean migration inhibition in unvaccinated control birds was always less than 15%.

Leukocyte migration inhibition after primary vaccination with the oil adjuvant ND vaccine was higher in all the experiments except in experiment 8 where the inhibition in both groups was nearly the same. In experiments 2, 3, 4, and 6, the LMI level after secondary vaccination was lower than the level of inhibition after primary vaccination, whereas, in experiments 5, 7, and 8, the LMI level remained nearly the same. In chickens

vaccinated with the oil adjuvant ND vaccine at one-day of age (experiment 3), the LMI after secondary vaccination was significantly lower ($P<0.05$) than the primary vaccination.

In experiment 6, the LMI after primary vaccination was significantly higher ($P<0.05$) in those chickens vaccinated with the 90% soybean oil adjuvant ND vaccine than in those chickens vaccinated with the aqueous ND vaccine. This significant difference between the two groups was not observed in any other experiments. In chickens vaccinated with the oil adjuvant or the aqueous ND vaccine by the subcutaneous route (experiment 8), the level of leukocyte migration inhibition in both groups was nearly the same and remained unchanged after secondary vaccination.

Results of the Challenge Study

The results of the challenge test is summarized in Table 5.

Ten days following the primary vaccination (subgroups 1), the HI antibody titers ranged from 4-7 in the chickens vaccinated with the 70% corn oil adjuvant ND vaccine and 4-6 in those birds vaccinated with the aqueous ND vaccine. All the unvaccinated chickens were negative for HI antibody and died within 6 days following the challenge. The vaccinated chickens did not show any signs of ND infection and remained healthy until the termination of the experiment.

Three weeks following the primary vaccination (subgroups 2), the HI antibody titers ranged from 5-8 in those chickens vaccinated with the 70% corn oil adjuvant ND vaccine and 4-7 in those birds vaccinated with the aqueous ND vaccine. HI antibody was not detected in unvaccinated control chickens. Clinical signs of ND were observed in all the chickens of the unvaccinated group. Nine from a group of 10 unvaccinated chickens

died due to ND infection within 12 days of challenge. One unvaccinated chicken displayed clinical signs of ND but recovered and survived until the termination of the experiment. The vaccinated chickens in both subgroups did not show any signs of ND infection and remained healthy until the termination of the experiment.

Two weeks following the secondary vaccination (subgroups 3), the range of HI antibody titer was 5-9 in those birds vaccinated with the 70% corn oil adjuvant ND vaccine and 4-8 in those birds vaccinated with the aqueous ND vaccine. All the vaccinated chickens resisted the challenge, whereas, all unvaccinated chickens displayed the signs of ND infection. Within 12 days of challenge, 8 unvaccinated birds died and 2 birds recovered from the infection and survived until the termination of the experiment.

Muscular tremors, leg paralysis, torticollis and gasping were observed in infected chickens. At necropsy, no gross pathological changes were observed in the vaccinated chickens, whereas, profuse hemorrhage or accumulation of bile in the gizzard was found in the birds that died.

TABLE 3. Geometric Mean HI Titer of Chickens Vaccinated with Vegetable Oil Adjuvant ND Vaccine and Aqueous ND Vaccine

Expt.no.	Geometric mean HI titer[a]			
	O 1[b]	A 1[c]	O 2[d]	A 2[e]
1	5.9* (4-7)[f]	5.5** (4-7)	7.0* (5-8)	6.6** (5-8)
2	5.9 (4-9)	5.5 (4-8)	6.5 (4-9)	6.4 (4-8)
3	6.4 (4-9)	6.0 (4-9)	6.7 (5-9)	6.3 (5-8)
4	7.4 (5-9)	7.1 (5-9)	7.6 (6-9)	7.2 (6-8)
5	6.4 (6-8)	5.9 (5-7)	6.6 (5-8)	6.2 (5-7)
6	5.5 (4-7)	5.9 (5-7)	6.1 (5-7)	6.2 (5-7)
7	6.6 (5-8)	6.1 (5-7)	7.1 (6-8)	6.7 (5-8)
8	5.3*** (4-9)	6.5 (5-11)	7.2*** (5-9)	7.5 (5-9)
9	6.0 (5-8)	5.6 (4-7)	6.6 (5-9)	6.0 (4-8)

[a]Expressed as the $\log_2$ of the end point of the serum dilution.

[b]Primary vaccination with vegetable oil adjuvant ND vaccine.

[c]Primary vaccination with aqueous ND vaccine.

[d]Secondary vaccination with vegetable oil adjuvant ND vaccine.

[e]Secondary vaccination with aqueous ND vaccine.

[f]Minimum and maximum HI titer in the group.

*Values having same number of asterisks are significantly different ($P<0.05$).

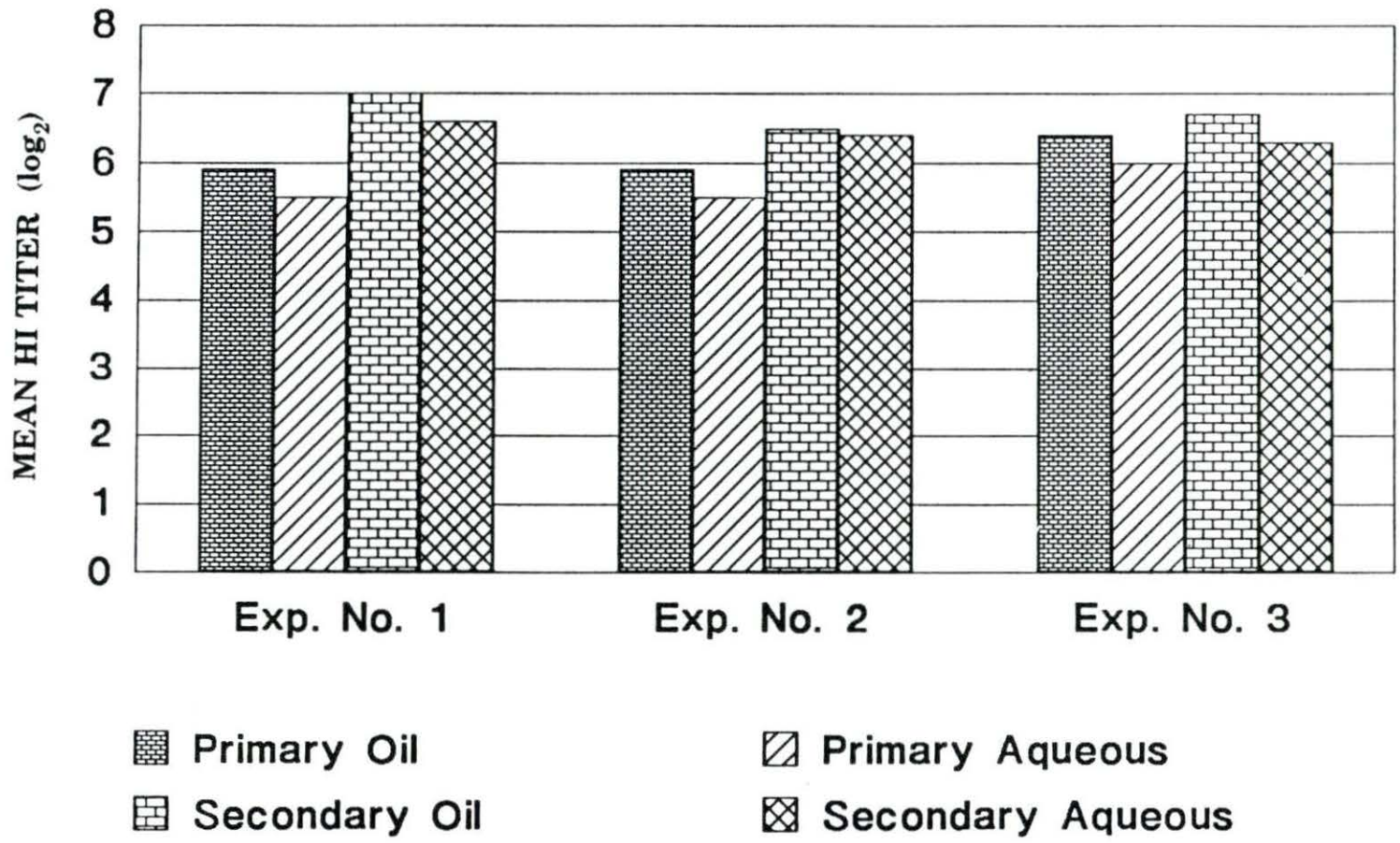

FIGURE 1. Geometric Mean HI Titer of Chickens Vaccinated with Vegetable Oil Adjuvant ND Vaccine and Aqueous ND Vaccine (Experiments 1-3)

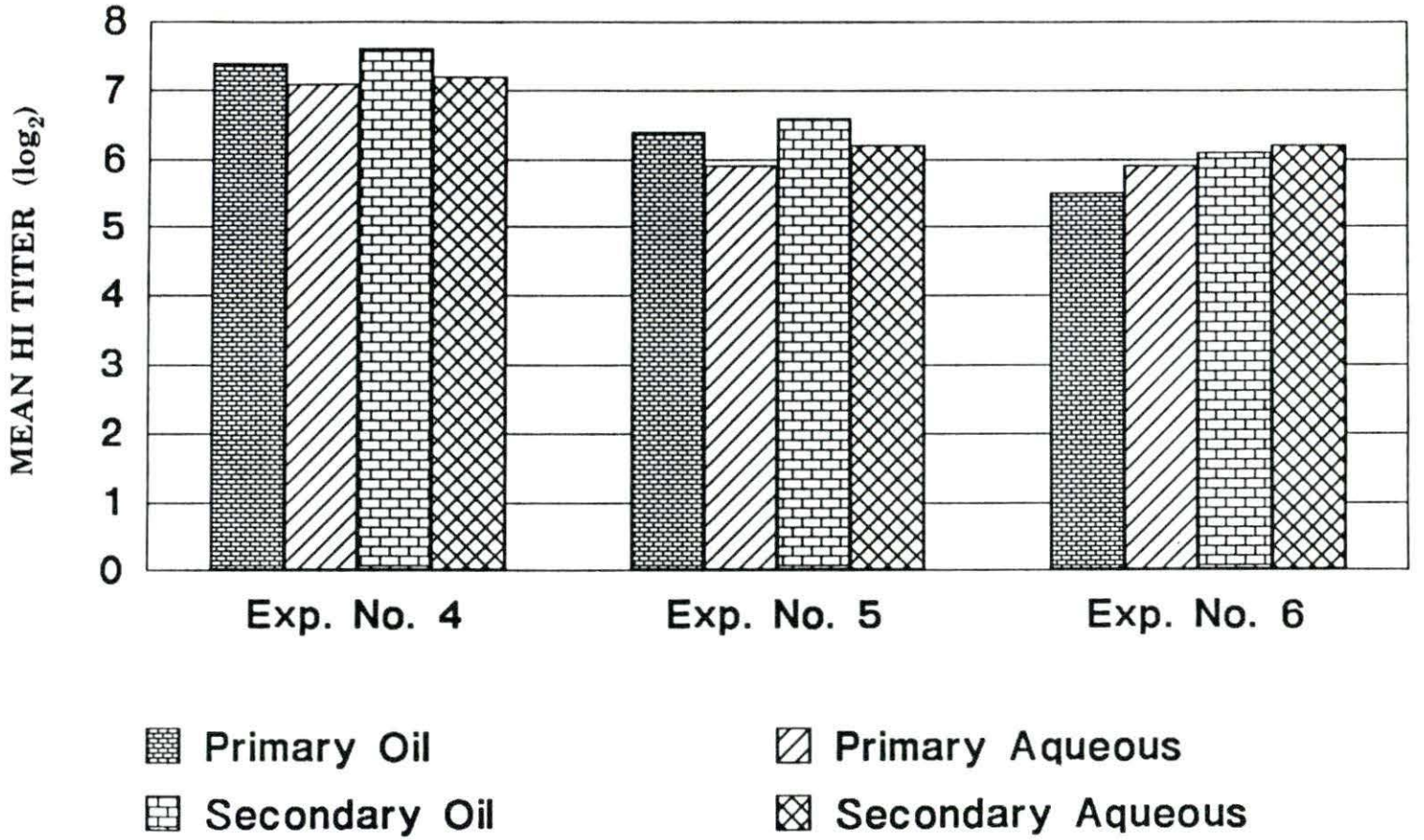

FIGURE 2. Geometric Mean HI Titer of Chickens Vaccinated with Vegetable Oil Adjuvant ND Vaccine and Aqueous ND Vaccine (Experiments 4-6)

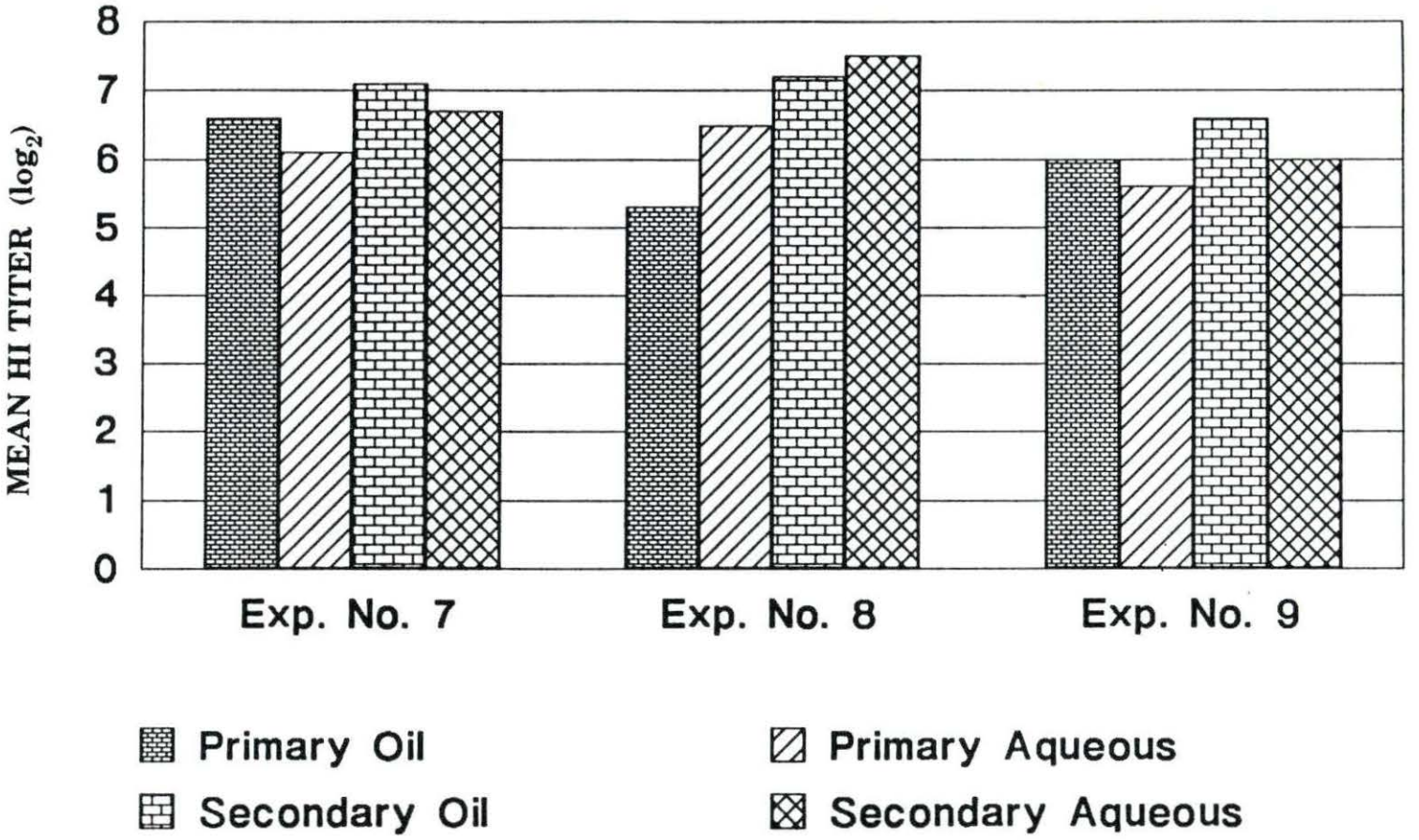

FIGURE 3. Geometric Mean HI Titer of Chickens Vaccinated with Vegetable Oil Adjuvant ND Vaccine and Aqueous ND Vaccine (Experiments 7-9)

TABLE 4. Mean Migration Inhibition of Peripheral Leukocytes from Vaccinated and Unvaccinated Chickens

Expt. no.	Mean migration inhibition in percent					
	O 1[a]	A 1[b]	C 1[c]	O 2[d]	A 2[e]	C 2[c]
2	25.7 (23.6-27.7)[f]	20.5 (19.6-21.3)	9.3	24.4 (19.8-29.0)	21.3 (20.8-21.8)	8.0
3	26.8 (25.2-27.8)	25.9 (23.8-28.7)	9.8 (9.3-10.2)	21.6 (20.8-22.4)	27.5 (23.5-31.4)	10.7 (7.8-14.3)
4	27.5 (27.0-27.9)	24.1 (21.9-25.9)	14.6 (13.3-15.9)	25.8 (23.3-28.5)	21.3 (20.2-21.8)	9.3 (7.8-10.7)
5	29.9 (27.2-32.5)	23.8 (23.7-23.8)	11.0 (8.8-13.1)	29.9 (28.2-32.9)	27.9 (26.5-29.3)	11.3 (9.0-13.8)
6	32.4* (32.0-32.7)	23.8* (23.7-23.8)	11.0 (8.8-13.1)	27.8 (25.6-30.0)	27.9 (26.5-29.3)	11.3 (9.0-13.8)
7	27.8 (25.3-30.7)	23.9 (20.8-27.4)	13.4 (12.1-14.6)	27.6 (25.0-30.2)	27.7 (25.6-29.0)	10.2 (10.0-10.3)
8	23.6 (21.1-27.4)	23.0 (20.4-26.1)	13.4 (12.1-14.6)	23.1 (21.0-25.5)	22.6 (21.0-24.2)	10.2 (10.0-10.3)

[a]Primary vaccination with oil adjuvant ND vaccine.

[b]Primary vaccination with aqueous ND vaccine.

[c]LMI of nonvaccinated control chicken leukocytes.

[d]Secondary vaccination with oil adjuvant ND vaccine.

[e]Secondary vaccination with aqueous ND vaccine.

[f]Minimum and maximum inhibition in the group.

*Values having same number of asterisks are significantly different ($P<0.05$).

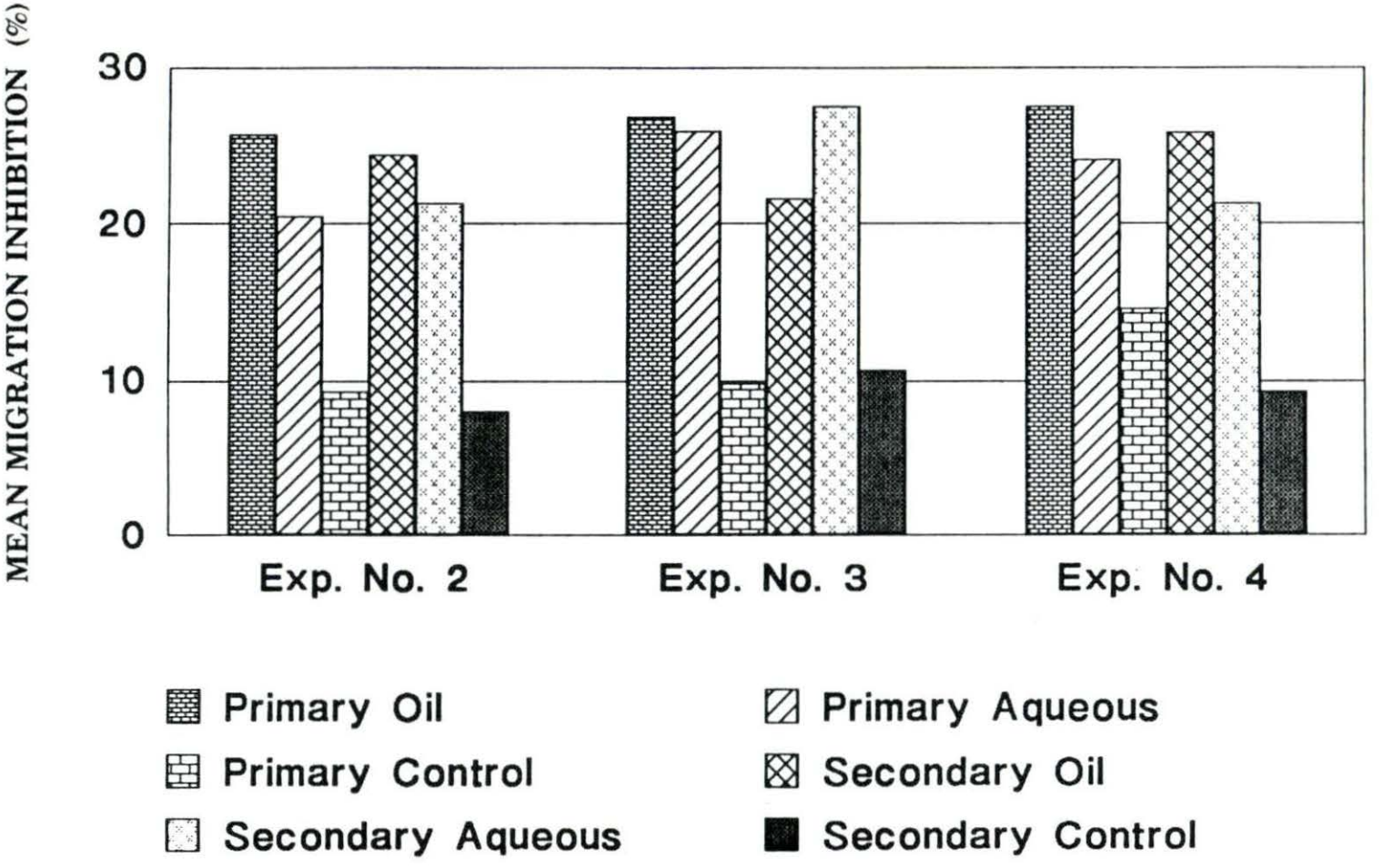

FIGURE 4. Mean Migration Inhibition of Peripheral Leukocytes of Vaccinated and Unvaccinated Chickens (Experiments 2-4)

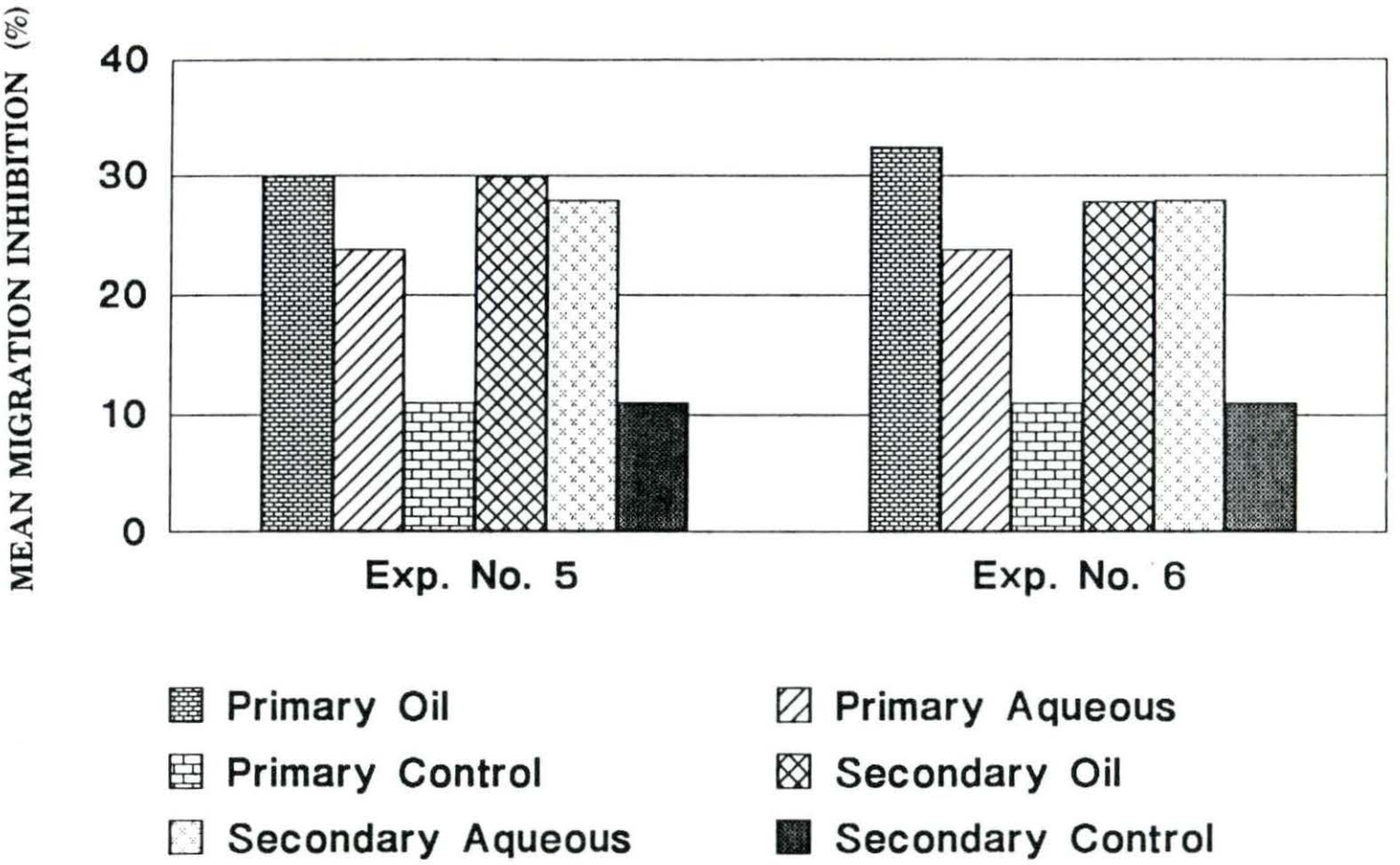

FIGURE 5. Mean Migration Inhibition of Peripheral Leukocytes of Vaccinated and Unvaccinated Chickens (Experiments 5 and 6)

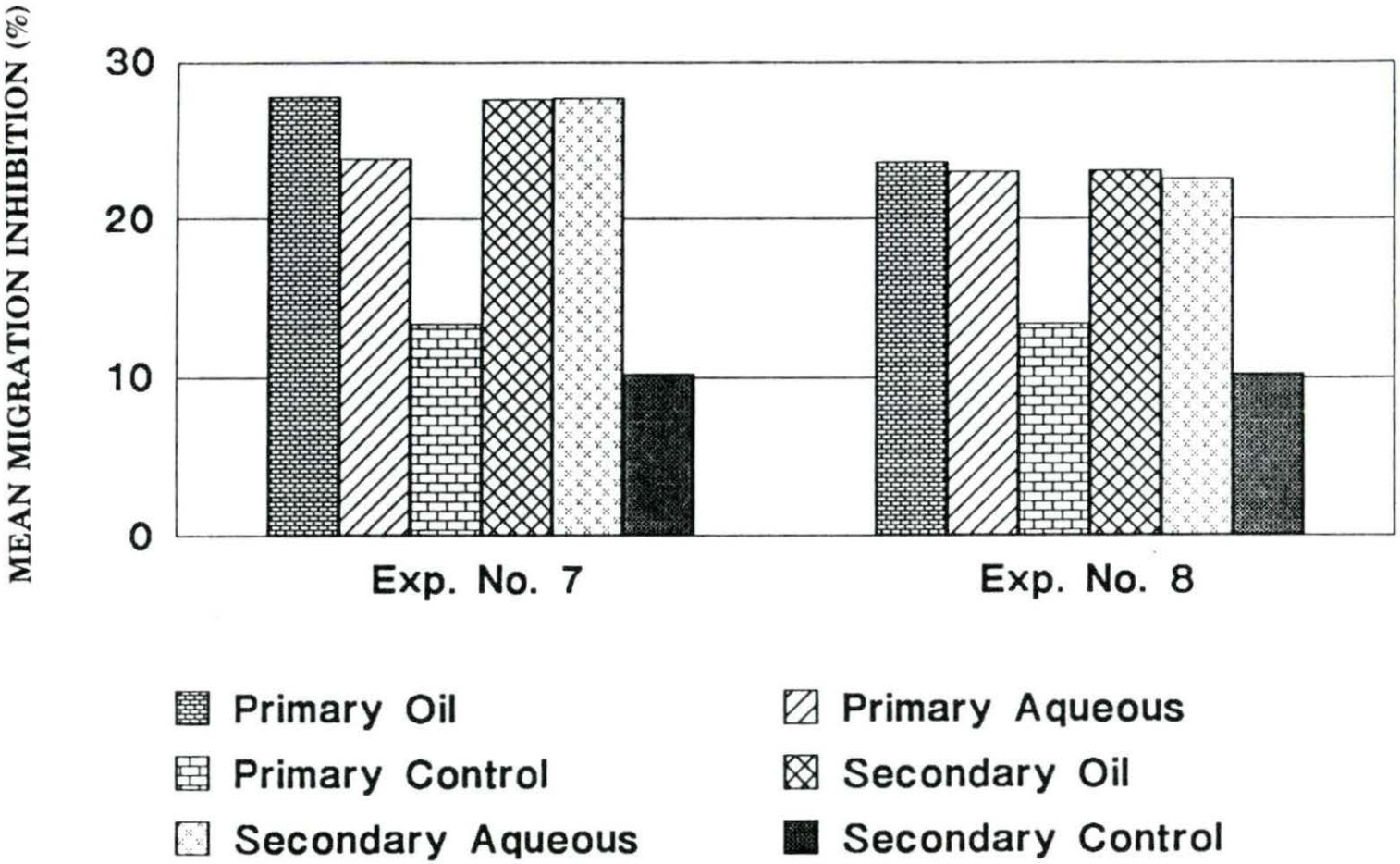

FIGURE 6. Mean Migration Inhibition of Peripheral Leukocytes of Vaccinated and Unvaccinated Chickens (Experiments 7 and 8)

FIGURE 7. Demonstration of the Leukocyte Migration Inhibition Assay, Illustrating the Migration Inhibition of Peripheral Leukocytes of Vaccinated and Unvaccinated Chickens [Antigen Treated (Ag+), Antigen Untreated (Ag-)]

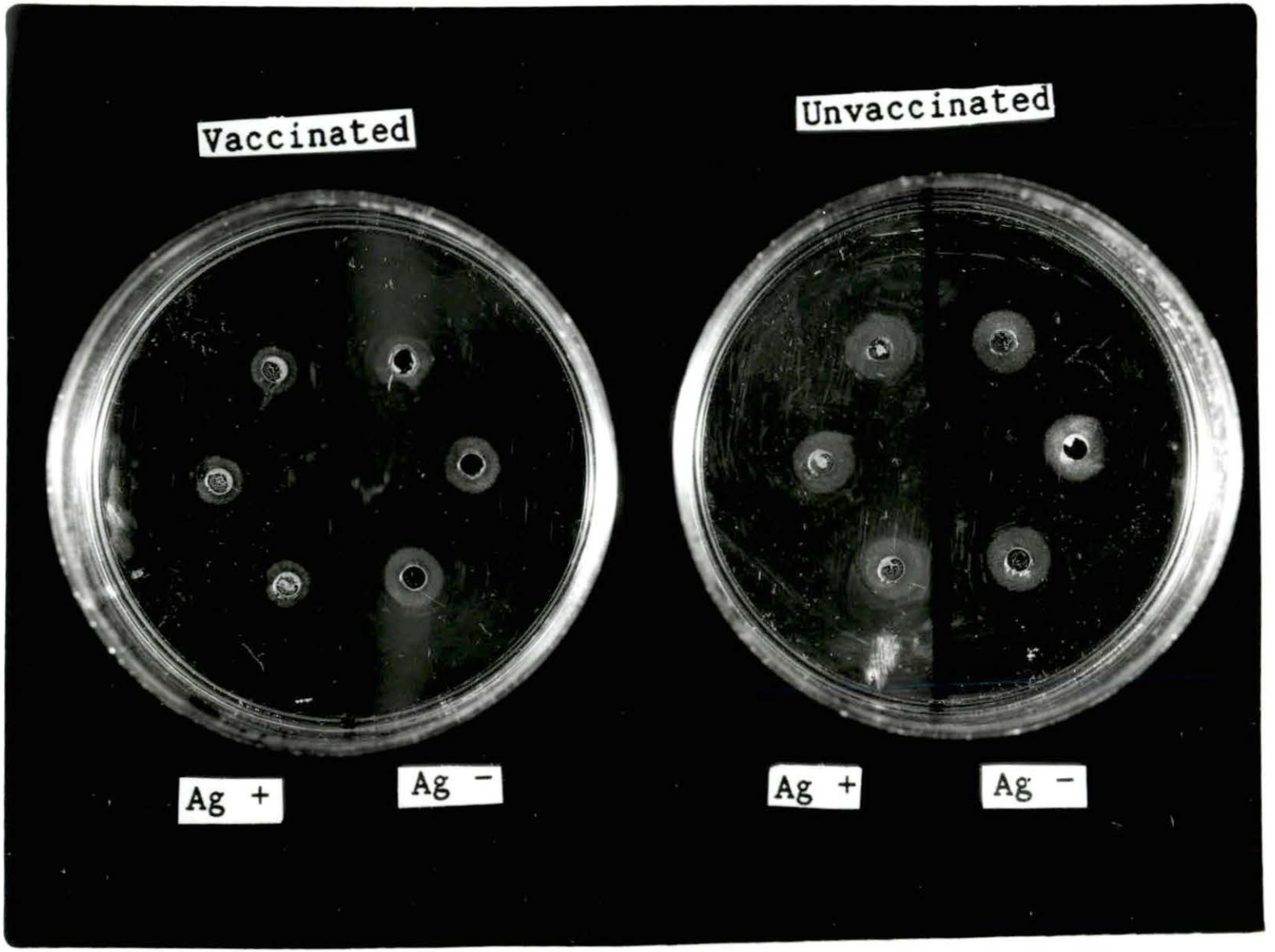
Vaccinated
Unvaccinated
Ag +
Ag −
Ag +
Ag −

TABLE 5. Results of Challenge Test in Experiment 9

Time of challenge	Type of vaccine used	No. of chicken in each group	GMT HI titer[a]	No. dead/no. challenged
10 days after[b] primary vaccination	oil adjuvant	5	5.6	0/5
	aqueous	5	4.8	0/5
	unvaccinated	5	—[c]	5/5
3 weeks after[d] primary vaccination	oil adjuvant	10	6.0	0/10
	aqueous	10	5.6	0/10
	unvaccinated	10	—[c]	9/10
2 weeks after[d] secondary vaccination	oil adjuvant	10	6.6	0/10
	aqueous	10	6.0	0/10
	unvaccinated	10	—[c]	8/10

[a]Geometric mean HI titer expressed as the $\log_2$ of the end point of the serum dilution.

[b]Each chicken was inoculated with 10^6 ELD_{50} GB Texas strain ND virus by intramuscular route.

[c]Unvaccinated SPF chickens were negative for HI antibody.

[d]Each chicken was inoculated with 10^7 ELD_{50} GB Texas strain ND virus by IN-IO route.

DISCUSSION

The results obtained from these studies indicated that the vegetable oil adjuvant ND vaccine induced higher geometric mean HI antibody titers than the aqueous ND vaccine in all the experiments except experiments 6 and 8. In experiment 6, 90% soybean oil adjuvant ND vaccine was used and in experiment 8 the oil adjuvant vaccine was prepared by using an emulsifier and was administered by the subcutaneous route. However, the difference in the HI titers of birds vaccinated using the oil adjuvant ND vaccine and those birds vaccinated with the aqueous ND vaccine was not statistically significant.

In experiment 8, the LaSota strain ND vaccine was used both for primary and secondary vaccination and was administered by subcutaneous injection. This was done because the LaSota strain vaccine was initially developed for injectable administration (1) and has been shown to induce a better immunity than the B 1 Strain. Subsequently, this strain has been shown to be equally immunogenic when administered by other non-parenteral methods.

Contrary to the results obtained in experiment 8, it was observed that 70% corn oil adjuvant ND vaccine (experiment 7) which was also prepared by adding emulsifiers, induced higher antibody titers than did the aqueous ND vaccine. Since this vaccine was prepared by using an aqueous-phase emulsifier, Tween 80, and an oil-phase emulsifier, Arlacel A, the water-in-oil emulsion may have caused a slow release of the ND antigen. Although initial levels of HI antibody obtained from birds in experiment 8 were low, the possibility exists that the duration of humoral immunity might have been longer lasting in these birds. Because these experiments were performed in containment isolators, which limit the number of birds and length of experiment due to the growing size of the chickens,

volume of accumulated droppings, etc., it was not possible to continue these experiments for a period longer than 2 weeks following secondary vaccination. Therefore, whether or not the oil adjuvant ND vaccine induced HI antibody levels for a longer period of time than did the aqueous ND vaccine was not determined in these studies.

The anamnestic immune response of chickens vaccinated with a second dose of oil adjuvant ND vaccine by the subcutaneous route (experiment 8) was significantly higher ($P<0.05$) than those birds vaccinated with the aqueous ND vaccine. A significant increase in antibody titer following secondary vaccination was also observed in experiment 1.

The cellular immune response, as measured by the leukocyte migration inhibition test, was found to be highest following primary vaccination when the oil adjuvant vaccine was used. This was observed in all experiments except in experiment 8, where migration inhibition in both groups was nearly the same. The LMI following the second dose of vaccine was lower than the LMI following primary vaccination in experiments 2, 3, 4 and 6, and was nearly unchanged in experiments 5, 7, and 8. In chickens vaccinated with the oil adjuvant ND vaccine at one day of age (experiment 3), the LMI following the secondary vaccination was significantly lower ($P<0.05$) than the LMI following the primary vaccination. This is in contrast with the HI test results, whereby, the HI antibody levels following secondary vaccination were always increased. These results suggest that there is no correlation between the geometric mean titer of HI antibody and the level of cell mediated immunity (as measured by the LMI assay). Timms and Alexander (63) have also reported similar findings.

The 90% soybean oil adjuvant ND vaccine (experiment 6) induced slightly lower antibody titers in chicks, whereas, the 90% corn oil adjuvant ND vaccine (experiment 5) induced higher HI antibody titers after both primary and secondary vaccinations. No

significant difference was found in the humoral or the cellular immune response of chickens when the 70% corn oil adjuvant ND vaccine was compared to chickens vaccinated with the 90% corn oil adjuvant ND vaccine. Contrary to HI antibody titers, the LMI level in chickens vaccinated with the 90% soybean oil adjuvant ND vaccine by the IN-IO route was significantly higher ($P<0.05$) than the LMI levels in chickens vaccinated with the aqueous ND vaccine. These observations suggest that the corn oil adjuvant ND vaccine may induce a greater humoral immunity, whereas, the soybean oil adjuvant ND vaccine may induce a better cell mediated immunity.

No detectable differences were observed between the immune response of one-day-old SPF chicks (experiments 1-3) and 2-week-old SPF chickens (experiments 5-9). Since these chickens were ND antibody free, this model may not be accurately simulating field conditions. In the field, maternal antibodies may be present in day-old chicks. Chicks are usually vaccinated by the spray or drinking water method to induce local immunity which has been shown to be very important in the prevention of ND infection in young chicks (106).

Higher antibody titers (7.1-7.6) were observed in chickens vaccinated by the IN-IO route at 6 weeks of age than those chicks vaccinated either at one day of age or 2 weeks of age (5.5-7.1). The cell mediated immune response was also found to be significantly higher after both primary and secondary vaccination in 6-week-old chickens. This suggests that both humoral and cell mediated immune responses are better in older birds than younger birds.

In all experiments, except experiments 7 and 8, the oil adjuvant ND vaccine was prepared without adding any emulsifiers so that the adjuvant effect of the vegetable oil only could be studied. These oil adjuvant ND vaccines were used immediately after

preparation. No significant difference was observed in the HI antibody titer or cell mediated immune response of chickens vaccinated with oil adjuvant ND vaccine prepared without using emulsifiers and those chickens vaccinated with oil adjuvant ND vaccine containing emulsifiers. However, in the field condition, the commercially available oil adjuvant ND vaccines are generally used several months after production. Therefore, for a better and more uniform immune response in all the chickens vaccinated with an oil adjuvant preparation, the antigen contained in the aqueous phase should remain uniformly dispersed in the suspending phase (oil) throughout the life of the preparation. The stability of the emulsified vaccine is an important factor in the efficacy of a vaccine (136).

No difference in the resistance to challenge was observed between the two groups vaccinated with either the oil adjuvant ND vaccine or the aqueous ND vaccine. Both vaccines were fully protective against challenge.

The GB-Texas strain of ND virus induced acute disease and 100% mortality in the unvaccinated chickens when inoculated by the intramuscular route. All the unvaccinated chickens which were inoculated by the intramuscular route died within 6 days post inoculation, whereas, inoculation of this virulent virus by the IN-IO route induced disease of variable intensity. Some infected chickens survived for longer periods of time than the chickens which were challenged by the intramuscular route. The IN-IO challenge caused lower mortality (80-90%) in the unvaccinated chickens than the intramuscular challenge which caused 100% mortality. This indicates that non-specific resistance factors such as mucus, saliva, gastric enzymes, tears, mucociliary escalator and other non-specific inhibitors of the virus play some role in the resistance to infectious diseases.

On necropsy, profuse hemorrhage or accumulation of bile in the gizzard was observed only in those chickens which remained infected for longer periods of time. No

inflammatory response at the site of inoculation was observed after vaccination with the vegetable oil adjuvant ND vaccine by the subcutaneous or intranasal-intraocular route. Persistence of the vegetable oil or any tissue change at the site of inoculation in chickens vaccinated with the vegetable oil adjuvant ND vaccine by subcutaneous route was not observed at the time of necropsy. This indicates that the vegetable oil which was incorporated into the vaccine was metabolized and did not cause any local tissue reactions.

This study suggests that vegetable oils when used as adjuvants with ND vaccine, induces a better immune response. However, the difference in the immune response (as measured by HI, LMI and challenge tests) of birds vaccinated with the oil adjuvant ND vaccine was not statistically different than those birds vaccinated with the aqueous ND vaccine. Although mineral oils are certainly better adjuvants than the vegetable oils for injectable vaccines, the absence of adverse effects when vegetable oils were used still qualify them as potential candidates for adjuvants. These studies were performed on small groups of chickens. However, if studies were performed employing larger numbers of birds, the results may show statistical significance. Further studies using various concentrations of vegetable oils with and without emulsifier may also prove significant if performed in larger numbers of birds. Among the non-parenteral routes of ND vaccine administration, the IN-IO method requires individual handling of birds. In this study, the vegetable oil adjuvant ND vaccine and the aqueous ND vaccine were administered by the IN-IO route. Due to technical difficulties, the vegetable oil adjuvant ND vaccine was not applied by the spray or aerosol method. Therefore, further studies using a spray or aerosol method of vaccine administration are suggested.

SUMMARY

A total of nine experiments were conducted to study the effects of vegetable oils when used as adjuvants with ND vaccine. Experiments differed from one another with respect to the age of the chickens, the type of vegetable oil used, the concentration of vegetable oil used, the emulsifier used, and the route of inoculation.

The adjuvant effect of 70% corn oil incorporated ND vaccine was studied in one-day, 2-week, and 6-week-old SPF chickens. The 70% corn oil adjuvant ND vaccine was prepared with and without emulsifiers. A comparison was also made between the effect of using a 90% corn oil adjuvant ND vaccine and a 90% soybean oil adjuvant ND vaccine in 2-week-old SPF chickens. Evaluating the route of inoculation was compared by administering a 70% corn oil adjuvant ND vaccine containing emulsifiers by the IN-IO route in one group of chickens and by the subcutaneous route in another group of chickens.

The humoral immune response of vaccinated and unvaccinated chickens was evaluated by the hemagglutination inhibition (HI) test. The cell mediated immune response of vaccinated and unvaccinated chickens was evaluated by the leukocyte migration inhibition (LMI) test. The HI and LMI tests were performed 2 weeks after the primary and the secondary vaccination. Protective immunity was evaluated by challenging the chickens with the GB Texas strain of ND virus.

The results obtained from this study indicated that the use of corn oil adjuvant ND vaccine induced higher geometric mean HI antibody titers after primary and after secondary vaccination by the IN-IO route when compared to those birds that received aqueous ND vaccine. However, the difference between the HI titers of the 2 groups was not usually significant. The HI antibody response of chickens vaccinated with a 90% soybean oil adjuvant ND vaccine by the IN-IO route and chickens vaccinated with a 70%

corn oil adjuvant ND vaccine by the subcutaneous route was lower than the HI antibody response of chickens vaccinated with an aqueous ND vaccine. The anamnestic immune response of chickens vaccinated with a second dose of oil emulsion vaccine by the subcutaneous route was significantly higher.

Although both vaccines induced significant levels of cell mediated immunity (CMI) after primary vaccination, the CMI following secondary vaccination was lower than the level achieved following primary vaccination. No correlation was found between the level of HI titers and the level of CMI. In this study, no significant difference in the HI antibody titers and CMI levels was found between the chickens vaccinated with 70% and 90% corn oil adjuvant ND vaccines. No significant difference in the HI antibody titers and CMI levels was observed between one-day and 2-week-old chickens. Higher antibody titers were observed in 6-week-old chickens than the one-day and 2-week-old chickens. Both the experimental oil vaccine and the aqueous ND vaccine provided full protection against challenge with the GB Texas strain of ND virus.

Therefore, from the results of this study, it was concluded that corn oil adjuvant ND vaccine induced a better humoral immune response than the aqueous ND vaccine, however, the difference was not usually significant. Further studies using various formulations of vegetable oils and vaccination techniques are suggested.

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