Monoclonal antibodies were developed against USDA challenge strain of infectious laryngotracheitis virus (ILTV). Indirect immunofluorescence test was used to detect antibodies in supernatants of hybridomas. Hybridoma cells were developed by fusing Sp 2/0 myeloma cells with spleen cells obtained from mice immunized four times with partially purified USDA challenge strain of infectious laryngotracheitis virus. The supernatant of three hybridomas, designated as 2D1D8, 2E11G2, 2C6C7 were found positive for antibody activity against USDA challenge strain of ILTV. Hybridomas producing antibodies were cloned by the limiting dilution method.

All three monoclonal antibodies reacted with USDA challenge strain of ILTV, S 88 00224 strain of ILTV, and 86 1169 strain of ILTV in an indirect immunofluorescence test. None of the monoclonal antibodies reacted with avian adenovirus 301 or parrot herpes virus in an indirect
immunofluorescence test. The monoclonal antibodies were isotyped, and all three monoclonal antibodies were found to be IgM.
PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST INFECTIOUS LARYNGOTRACHEITIS VIRUS OF CHICKENS AND THEIR USE IN AN INDIRECT IMMUNOFLOUORESCENT DIAGNOSTIC TEST.

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CHAPTER 1

LITERATURE REVIEW

**Infectious Laryngotracheitis**

Infectious laryngotracheitis (ILT) is an acute disease of chickens characterized by signs of respiratory depression, gasping, and expectoration of bloody exudate. Although the disease affects all ages, the most characteristic signs are observed in adult birds.

**History**

Infectious laryngotracheitis was first described in 1925, and the outbreak on which that report was based occurred in October and November of 1923 (May & Tittsler, 1925). Some reports speculate that the disease may have existed earlier, as an unusual high mortality among live poultry was observed. Investigations were made and finally it was concluded that the losses were due to a disease which, if not new had not been previously extensively studied (Beach, 1926).

In some poultry periodicals, it has been stated that ILT was introduced into the United States by show birds from Canada in 1921 (Beach, 1926). The first authentic report of
its occurrence in the United States, involved a Rhode Island flock in November, 1923 (May & Tittsler, 1925). The etiology of the disease was undetermined, and it was thought that mortality could be due to exposure to cold (May & Tittsler, 1925). The disease was transmitted to healthy birds by means of a throat swab from dead birds (Graham, et al., 1930; May & Tittsler, 1925).

Old names for ILT include infectious bronchitis, infectious tracheitis, tracheo-laryngitis, chicken "flu" and Canadian "flu" (Beach, 1926). The term laryngotracheitis was used as early as 1930 (Beach, 1930; Graham, et al., 1930) and the name infectious laryngotracheitis was adopted in 1931 by the Special Committee on Poultry Diseases of the American Veterinary Medical Association. A mild form of ILT was reported and designated as the subacute or chronic form of the disease (Graham, et al., 1930). The cause of ILT was first shown to be a filterable virus in 1930 (Beaudette, 1930).

Classification

Infectious laryngotracheitis has been identified in most countries. It is a serious disease of chickens in the United States, Europe, and Australia. Infectious laryngotracheitis virus has all the characteristics of the family Herpesviridae. The virus is cuboidal, enveloped, ether sensitive, and contains a core composed of DNA. ILTV is a member of the subfamily Alphaherpesvirinae (Roizman, 1982). In the last 50 years, more than 80 distinct herpesviruses have
been isolated from a wide variety of animal species. These herpesviruses share structural features (Roizman, 1982). "Herpes" is a Greek word which means "creep". The term herpes was used in medicine to describe a variety of skin conditions and diseases for at least 25 centuries (Roizman, 1982). In recent years the known membership of the herpes group has increased considerably. Twenty or more members share the following properties (a) a genome of double stranded DNA (b) a regular icosahedral capsid made of 162 capsomeres and often surrounded by a lipid-containing envelope, and (c) intranuclear assembly of the capsid around the genome.

**Morphology**

Electron microscopic examination reveals icosahedral viral particles in infected chicken embryo cells. These particles are similar in structure to herpes simplex virus. The hexagonal virion is 80-100 nm in diameter. There are three main structural components: the core, the capsid, and the envelope. The envelope is 195-250 nm in diameter and has fine projections at the periphery of its membrane (Watrach, et al., 1963). The capsid has icosahedral symmetry and is composed of 162 elongated hollow capsomeres. The diameter of a complete virus particle is 195-250 nm (Cruickshank, et al., 1963; Watrach, et al., 1963).

**Virus Replication**

Replication of ILTV is similar to that of herpes simplex and pseudorabies virus. Virus enters into the cell by
pinocytosis after being adsorbed on the cell surface. Rate of adsorption depends on the volume of inoculum and cell system. Enveloped particles adsorb more readily than naked particles (Holmes & Watson, 1963).

Once inside the cells, host enzymes disrupt the envelope and capsid of ILTV and its DNA is released. Viral DNA migrates to the cell nucleus. After 10-12 hours ILTV components can be observed in the nucleus. A sharp rise in intracellular virus content occurs after 12 hours of infection and a maximum virus titer is found between 30 and 36 hours of infection. Virus titer gradually diminishes thereafter (Reynolds, et al., 1968).

The viral envelope is formed as the nucleocapsid migrates through the cell’s nuclear membrane. The enveloped virus particles accumulate in a vacuolar membrane in the cytoplasm. Then the vacuoles containing virus particles migrate to the plasma membrane and are released after disruption of the cell membrane (Nii, et al., 1968). A maximum titer of ILTV reaching $10^6$ plaque forming units/ml of tracheal exudate is found on the third day after inoculation of 8-week-old white leghorn chickens. Virus may also be isolated from the lungs and livers of infected chickens but virus titers are low in those organs (Chang, et al., 1973).

**Resistance to Chemical and Physical Agents**

Infectious laryngotracheitis virus particles are sensitive to lipolytic agents, heat, and various disinfectants.
A solution of 3% cresol or 1% lye can inactivate LTV in less than 1 min. ILTV can survive for a long period when lyophilized or kept at -20 to -60 °C (Goldhaft, 1961).

**Strain Classification**

Most of the ILTV strains appear antigenically similar based on virus neutralization (VN) or immunofluorescence tests (IFT) using reference-specific antiserum. Some strains, however, have been neutralized poorly by such antisera (Burnet, 1936; Pulsford & Stokes, 1953). Some other workers have also suggested there is minor antigenic variation among the strains of ILTV (Russell & Turner, 1983; Shibley, et al., 1962).

In attempts to find useful techniques for differentiating the antigenically-similar strains of ILTV, restriction endonucleases have been used to study the viral DNA. On the basis of these findings some ILTV strains have been classified. Differences in DNA fragment patterns have been found between an Australian vaccine strain and a field strain by this method (Kotiw, et al., 1982), and other workers have found differences between American and European ILTV strains and between an American field strain and a vaccine strain (Leib, et al., 1986). Differences in DNA patterns between live ILTV vaccines, reference strains of ILTV, and ILTV field isolates from North Carolina have also been studied by using restriction endonucleases (Guy, et al., 1989). Differences between DNA patterns of vaccine strains and Georgia field
isolates of ILTV were also found (Andreasen, et al., 1990). Differences among restriction endonuclease DNA fingerprints of Pennsylvania field isolates, vaccine strains, and challenge strains of ILTV have also been reported (Keller, et al., 1992). The use of cloned DNA fragments in reciprocal DNA:DNA hybridization also appears promising for differentiating ILTV strains (Kotiw, et al., 1986).

Monoclonal antibodies can be used to study the antigenic relationship among virus isolates and other isolates of the same family. Work has been done to observe antigenic relationship in paramyxoviruses, and different strains have been classified by using monoclonal antibodies in reactions such as the indirect immunoperoxidase test, haemagglutination inhibition test, and the binding pattern of monoclonal antibodies with the infected cells (Alexander, et al., 1989; Collins, et al., 1989). Monoclonal antibodies potentially could be used to distinguish strains of ILTV.

**Laboratory Host System**

Infectious laryngotracheitis virus can be propagated in embryonating chicken eggs. The virus causes formation of opaque plaques on the chorio-allantoic membrane (CAM) resulting from proliferative and necrotic lesions. Embryo deaths occur in 2-12 days postinoculation (Brandly, 1935; Brandly, 1937).

Infectious laryngotracheitis virus can also be grown in chicken embryonic liver (CEL), chicken embryo kidney (CEK),
and chicken kidney (CK) cell cultures (Atherton & Anderson, 1957). Chicken embryonic liver and CK are the preferred culture systems (Hughes & Jones, 1988). The virus can also grow in cultures of cells from the avian immune system, but only to extremely low titers. Chicken leukocytes (buffy coat) can also be used in vitro for viral growth (Chang, et al., 1977). Viral growth at low titer has been observed in macrophages cultured from bone marrow and spleen (Bulow & Klasen, 1983). Susceptibility of macrophages in vitro does not correlate with susceptibility of infection with ILTV (Louduvaris, et al., 1991).

**Genome of ILTV**

A comparative study of DNA density and behavior in tissue culture of 14 different herpesviruses found that the nucleic acid core of ILTV is DNA, with a density of 1.704 g/ml, which is consistent with DNA values of some of the other herpesviruses (Plummer, et al., 1969). A molecular weight estimation of ILTV DNA was made by summation of restriction endonuclease fragments cleaved with Bam HI (102.1 x 10^6) and Hind III (97.35 x 10^6). Difference between the estimates may indicate the presence of submolar fragments. These findings indicate that the molecular weight of ILTV DNA is approximately 100 x 10^6, with the genome having two isomeric forms (Kotiw, et al., 1982). Other workers also investigated genome isomerism of ILTV using restriction endonuclease analysis, and ILTV DNA was found to have two isomers with a
molecular weight of $109 \times 10^6$ (Leib, et al., 1986). Clones of ILTV have been used in hybridization experiments by workers and it was found that the genome is 155 kilobase pairs (Kbp) and is comprised of a long unique sequence of 120 Kbp and a short unique sequence of 17 Kbp bounded by repeat sequences each of 9 Kbp. They also found an unrelated second pair of repeat sequences located at 0.67 and 0.88 map units. A terminal repeat of the unique long region was also detected, but no isomerization of the unique long sequence was found (Johnson, et al., 1991). Infectious laryngotracheitis virus DNA has been reported to have a guanine plus cytosine percentage of 45% (Plummer, et al., 1969), which is lower than that for many other animal herpesviruses. Some workers have studied the nucleotide sequence of the ILTV gene coding the 205K complex glycoprotein (gb 205) and have found that the gene is contained within a 3 kb EcoRI restriction fragment mapping at map coordinates 0.23 to 0.25 in the unique long region of the ILTV genome and is transcribed from right to left (Kongsuwan, et al., 1991).

**ILTV Proteins and Glycoproteins**

Four major glycoproteins of molecular weights 205, 115, 90, and 60 Kd, respectively, have been located both on the virus and the surface of virus-infected cells (York, et al., 1987), and are considered to be the major immunogens of ILTV. Monoclonal antibodies (MCAs) to immunogenic glycoproteins were produced and characterized by immunoprecipitation and western
blotting. One group of MCA reacted only with the 60 Kd glycoprotein, by both techniques, while a second group reacted with the 205, 115, and 90 KD glycoproteins in immunoprecipitation and with additional bands of 85 and 160 KD in Western blotting (York, et al., 1987). Glycoproteins with molecular weights less than 60 Kd have been detected in extracts of virus-infected cells.

In a recent study, the glycoprotein B (gB) gene was identified using the polymerase chain reaction. Northern blot analysis using a portion of open reading frame as a probe identified a 2.7 kb RNA transcript in ILTV-infected chicken embryo liver cells, and analysis of the amino acid sequence of the ILTV protein indicated that it shares structural features with the gB glycoproteins of other herpesviruses (Poulsen, et al., 1991).

Pathogenesis

The chicken is the primary natural host affected by ILTV, but several workers have described a form of ILT in pheasants and pheasant-chicken crosses (Kernohan, 1931; Crawshaw & Boycott, 1982). Lesions were experimentally induced in the upper respiratory tract of young turkeys (Winterfield & So, 1968). Starlings, crows, doves, ducks, pigeons, and guinea fowl are not susceptible to ILTV (Beach, 1931; Brandly & Bushnell, 1934), although experimental infection of ducks has been reported (Yamada, et al., 1980).

Natural infections by ILTV occur through upper
respiratory and ocular routes (Beaudette, 1930; Beaudette, 1937). Ingestion can also produce infection, although exposure of nasal epithelium following ingestion is required with this route (Robertson & Egerton, 1981). Transmission occurs more readily from acutely infected birds than through contact with clinically recovered carrier birds.

Viral replication is limited to upper respiratory tract tissues, with little or no evidence of viremia normally occurring (Bagust, et al., 1986; Hitchner, et al., 1958). Affected tracheal mucosa becomes swollen and edematous, resulting in erosion and hemorrhage. Infectious laryngotracheitis virus is usually present in tracheal tissues and secretions for only 6-8 days postinoculation (Bagust, et al., 1986; Hitchner, et al., 1977; Robertson & Egerton, 1981). Mechanical transmission can occur by use of contaminated equipment and litter (Kingsbury & Jungherr, 1958; Dobson, 1935; Beaudette, 1937). Egg transmission of virus has not been demonstrated, and ILTV infected embryos die before hatching (Jordan, 1966).

A recent study suggests a possible association of the chicken major histocompatibility complex (MHC) with susceptibility and resistance of chickens to ILTV (Loudovaris, et al., 1991).

Characteristic signs of an acute infection are nasal discharge, and moist rales followed by coughing and gasping (Beach, 1926; Kernohan, 1931). In severe forms of the disease
there is severe dyspnea and expectoration of blood-stained mucus (Beach, 1926; Hinshaw, et al., 1931; Seddon & Hart, 1935). Mild enzootic forms of the disease have been described in Britain, Australia, the United States, and New Zealand (Cover & Benton, 1958; Pulsford & Stokes, 1953; Seddon & Hart, 1935). In mild forms, signs are unthriftiness, reduction in egg production, watery eyes, conjunctivitis, swelling of infraorbital sinuses, persistent nasal discharge, and hemorrhagic conjunctivitis (Seddon & Hart, 1935; Pulsford & Stokes, 1953; Cover & Benton, 1958). Most chickens recover in 10-14 days, but extremes of 1-4 weeks have also been reported (Hinshaw, et al., 1931; Beach, 1926). Reactivation of latent ILTV has not been achieved by using immunosuppressive drugs (Hughes, et al., 1987, Bagust, 1984).

The incubation period is 6-12 days following natural exposure (Kernohan, 1931; Seddon & Hart, 1935). Intratracheal exposure results in a shorter incubation period of 2-4 days (Seddon & Hart, 1935; Benton, et al., 1958). Mortality varies from 5-70% (average 10-20%) in epizootic form of the disease, which spreads rapidly in susceptible chickens (Beach, 1931; Hinshaw, et al., 1931; Seddon & Hart, 1935). In the benign forms of the disease seen in recent years in the USA, Australia, and the UK, morbidity is low and mortality varies from 0.1-2% (Curtis & Wallis, 1983; Davidson & Miller, 1988).

**Immunity**

Chickens gain resistance after being infected; the
duration of resistance induced from a natural infection appears to be a year or more. Immunity induced by vaccination is of variable duration. Cell-associated (chicken embryo fibroblast cells) vaccine prepared from an attenuated ILTV provides adequate protection when given at 1 day of age intramuscularly or subcutaneously, and the chickens acquire immunity within 6 days after vaccination. The protection rate remains above 60% until 10 weeks post-vaccination (Taneno, et al., 1991). Infectious laryngotracheitis virus infection causes the production of antibodies that can neutralize virus (Bagust, et al., 1986). The humoral response associated with infection is not a primary mechanism of protection against ILTV infection. There is a poor correlation between serum antibody titers and the immune status of a flock (Jordan, 1981). Resistance to ILTV due to cell-mediated responses is of greater importance. Bursectomized chickens, which can not produce a humoral response, can develop immunity following ILT vaccination (Robertson, 1977). Some workers have reported transmission of maternal antibodies to ILTV through the egg to the offspring (Benton, et al., 1960). The antibody does not interfere with vaccination, and does not provide a long-lasting protection. Older birds respond well to vaccination as compared to chickens less than two weeks of age (Alls, et al., 1969; Cover, et al., 1960; Gelenczei & Marty, 1965), although chickens can be successfully vaccinated as early as 1 day old (Sincovic & Hunt, 1968).
Diagnosis

Although some acute signs of the disease are characteristic, signs are often similar to other respiratory diseases, so ILT can not be reliably diagnosed by observation of signs and lesions. Laboratory confirmation is required for certain diagnosis.

Virus can be isolated from tracheal and lung tissue by chicken embryo inoculation, by inoculation of trachea, and infraorbital sinuses of susceptible chickens (Hitchner & White, 1958), or by cell culture inoculation (Chomiac, et al., 1960). Inoculation of ILTV is achieved by inoculation of suspension from tracheal exudate, tracheal tissue, or lung tissue onto the CAM of 9-12 day old embryonated egg or into susceptible cell culture. Cytopathic effect (CPE) can be observed 24-48 hours later (Hitchner & White, 1958). Chicken embryo liver cells and chicken kidney cells are the laboratory systems of choice for the isolation of ILTV (Hughes & Jones, 1988).

Demonstration of intranuclear inclusion bodies in tracheal and conjunctival tissues stained with Giemsa stain is also diagnostic of ILTV (Cover & Benton, 1958; Beveridge & Burnet, 1946). Intratracheal inoculation with tracheal exudate or tissue suspension from recently affected birds can provide a reliable diagnostic procedure (Pulsford & Stokes, 1953). Electron microscopic (EM) examination of tracheal scrapings and recognition of herpes virus particles is also a
way of rapid diagnosis (Van Kammen & Spradbrow, 1976). The fluorescent antibody test may also be used for identification of virus (Braune & Gentry, 1965). Infectious laryngotracheitis virus was detected in tracheal tissue from day 2 to day 14 postinoculation using fluorescein-labelled anti-LTV globulins (Bagust, et al., 1986; Hitchner, et al., 1977; Wilks & Kogan, 1979). Recently, an enzyme-linked immuno-sorbent assay (ELISA) using monoclonal antibodies was developed for rapid detection of ILTV antigen in tracheal exudate (Van Kammen & Spradbrow, 1976). Enzyme-linked immunosorbent assay systems for detection of ILT antibodies have also been developed (Ohkubo, et al., 1988; York & Fahey, 1988; York, et al., 1983). Virus neutralization (VN) can be measured by using the CAM-pock counting technique, and VN antibodies can also be detected in cell culture monolayers (Robertson & Egerton, 1977; Churchill, 1965).

Infectious laryngotracheitis virus DNA was also detected in tracheal samples in a DNA hybridization assay by using a non-radioactive probe, and this technique has been found very effective in detecting field outbreaks and also latent ILTV infections (Keam, et al., 1991).

Infectious laryngotracheitis virus antigen has also been detected in frozen tissue section by an indirect immunoperoxidase (IP) procedure using monoclonal antibodies (Guy, et al., 1992).
Treatment, Prevention and Control

No drug has yet been found effective in reducing the severity of ILT. Vaccination of chickens for ILT has proven to induce adequate protection. Live attenuated vaccines which are given in drinking water and by eye drops are very effective against ILT. Mild field isolate SA-2, which has been used for vaccination in Australia, is able to establish latent infections (Bagust, 1986). Cell-mediated immune responses are thought to be protective against infection after vaccination. Bursectomized chickens were found resistant against a challenge infection, while passive transfer of hyperimmune serum failed to protect against the infection (Fahey, et al., 1983). Infectious laryngotracheitis virus attenuated by passage in cell cultures (Izuchi, et al., 1984; Izuchi, et al., 1983; Gelenczei & Marty, 1964), or embryonated eggs, and selected mild enzootic strains (Pulsford & Stokes, 1953) are capable of inducing acceptable protection and are used in vaccines. Virulent strains of ILT vaccine should be administered with caution, because vaccination may result in some carrier birds. Inoculation of infraorbital sinuses (Shibley, et al., 1962), intranasal installation (Benton, et al., 1958), feather follicle inoculation (Molgard & Cavett, 1947), eye drop (Sinkovic & Hunt, 1968), and oral administration through drinking water (Hilbink, et al., 1981) have been used to vaccinate chickens against ILTV. Most layer or breeder chickens are now vaccinated by the eye drop method.
using attenuated ILTV vaccine. When broiler vaccination is required, spray or drinking water application is usually used. Successful vaccination through the drinking water depends upon ILTV contacting the epithelium of the nasal cavity during drinking.

Glycoproteins of ILTV, purified from detergent extracts of virus-infected cells by lectin affinity chromatography, have also been used for vaccination of chickens, and have been proven to protect up to 83% of chickens against ILTV. Both neutralizing antibody and delayed-type hypersensitivity responses were induced by vaccination with glycoprotein preparation, but neither correlated with protection (York and Fahey, 1991).

Proper management procedures help prevent the disease, including sound sanitation and hygiene measures, control of movement of staff, service workers, feed, equipment and birds, as well as rodent and dog control measures (Kingsbury, et al., 1958).

A resolution pertaining to the proposed eradication program for ILT was unanimously passed by a United States Animal Health Association committee. The proposed ILT eradication program consists of four phases (Proc. USAHA; 1990). These four phases cover all aspects of a complete eradication program, including diagnostic capabilities, vaccination, surveillance, biosecurity, and treatment of the infected flocks and infected houses.
Monoclonal Antibodies

Monoclonal antibodies are a homogenous population of identical antibodies with a defined specificity secreted by a B-cell clone after fusion with tumor cells. Monoclonal antibodies (MCAs) do not differ structurally from naturally-occurring polyclonal antibodies. The property which makes MCAs unique is that all the molecules in any single preparation are identical. Monoclonal antibodies have same specificity of reaction with any defined antigen every time when ever reacted.

Since 1975 MCAs have been considered an important discovery in the field of science. On August 7, 1975, Kohler and Milstein published in Nature a report describing "continuous cultures of fused cells secreting antibody of predefined specificity" (Kohler & Milstein, 1975). The first workshop on lymphocyte hybridomas was held on April 3-5, 1978, in Bethesda, Maryland. Kohler and Milstein (1975) observed that somatic cell hybridization could be used to generate a continuous "hybridoma" cell line producing a monoclonal antibody. Immunologists have since produced large amounts of homogenous monoclonal antibodies against a wide variety of antigens.

Monoclonal antibodies have been produced against histocompatibility, differentiation, tumor or other cell-surface antigens, as well as viral and bacterial antigens and certain single antigenic determinants on a wide variety of
proteins, nucleic acids, and sugars. Monoclonal antibodies to certain specific antigens are available commercially.

The primary advantage of MCAs as diagnostic reagents is their specificity of binding to a single epitope, producing highly specific diagnostic test reagents. Monoclonal antibodies are used for many research purposes. Examples include construction of antigenic maps of the haemagglutinin molecule of influenza virus, in vitro estimation of mutation rate or genetic changes of influenza virus by culturing virus in presence of a single neutralizing monoclonal antibody, use of monoclonal antibodies in passive protection against different antigenic determinants of influenza virus, and ability of monoclonal antibodies to separate individual antigens from a complex mixture (Antczak, 1982; Re, 1987).

For production of MABs, a suitable host is immunized with an antigen. Antibody-secreting B cells are obtained from the spleen of the immunized host and fused with myeloma cells in medium containing hypoxanthine, aminopterin, thymidine (HAT medium). Normal cells have two pathways for nucleotide synthesis, de novo pathway and salvage pathway. Myeloma cells do not have hypoxanthine-guanine phosphoribosyl transferase (HGPRT) which is responsible for the salvage pathway, so because of the absence of this enzyme they are sensitive to aminopterin, which inhibits dihydrofolate reductase and hence blocks de novo purine and thymidilate synthesis. Fused cells are thrown in the salvage pathway, being provided with
hypoxanthine and thymidine in the medium. Fused cells have HGPRT which utilizes the hypoxanthine for purine synthesis and also have thymidine kinase which utilizes the thymidine for thymidylate synthesis. Both of these enzymes are provided by the spleen cells after fusion. Unfused B cells die naturally in the media because they are not immortal like myeloma cells. Because of the effect of aminopterin, unfused myeloma cells will be killed too. Only fused cells can survive in this HAT medium because of HGPRT and thymidine kinase provided by the B cells, which is responsible for the salvage pathway, and immortalization provided by the myeloma cells. Positive hybridomas producing the desired antibody are identified, and cloning is done to get specific monoclonal antibodies (Goding, 1986; Harlow & Lane, 1988). Enzyme-linked immuno-sorbent assay and indirect immunofluorescence tests are most commonly used for identification of positive hybridomas. There are different ways of cloning, but a common way is cloning by limiting dilution at the rate of <1 cell/well.

Four animal species can be used for MCA production: mouse, rat, hamster, and rabbit. Rabbit MCAs have recently been described (Raybould & Takahashi, 1988). Mice are the best choice for immunization and production of MCAs because mice are easier to handle and there are many anti immunoglobulin (Ig) reagents available that are specific for each mouse Ig isotype. Also, mouse MCAs are easier to purify than rat.
Female BALB/c mice are preferred if the SP2/0-Ag14 myeloma cell line is used as a fusion partner because the hybridomas resulting from this fusion will be entirely of BALB/c origin. Such hybridomas can grow well in BALB/c hosts for the production of ascites fluid. The sex of the mice does not matter except that females are easier to handle and are less aggressive.
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CHAPTER II

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST THE USDA
CHALLENGE STRAIN OF INFECTIOUS LARYNGOTRACHEITIS VIRUS
Introduction

Infectious laryngotracheitis virus (ILTV) is assigned to the subfamily Alphaherpesvirinae, of the Herpesviridae family on the basis of its rapid replication and latency in neurons. It is an enveloped virus, icosahedral, with double stranded DNA which has a density of 1.704 g/ml, similar to the DNA of other herpes viruses (Plummer, et al., 1969). Four major glycoproteins with molecular weights 205, 115, 90, and 60 kD have been described in the SA-2 strain of ILTV (York, et al., 1987). These glycoproteins are considered to be the major immunogens of ILTV and are present on the virus envelope and on the surface of virus-infected cells. Infectious laryngotracheitis virus DNA has a molecular weight of about $100 \times 10^6$, with the genome having two isomeric forms (Kotiw, et al., 1982; Leib, et al., 1987). Some workers have reported that ILTV has a guanine plus cytosine percentage of 45% which is lower than other animal herpesviruses (Plummer, et al., 1969).

The envelope glycoproteins are important immunogens capable of inducing humoral and probably cellular immune responses. The involvement of other virion components in inducing immune responses has not been investigated (Watari, et al., 1987).

Chicken antiserum and rabbit antiserum have been used to immunoprecipitate four major glycoproteins of 205, 115, 90, and 60kD molecular weight. Some additional glycoproteins also
have been recognized by immune chicken and rabbit antisera in western blotting using a glycoprotein fraction which was purified from virus-infected cells (York, et al., 1987). The same workers have also produced monoclonal antibodies (MCAs) and have characterized them by immunoprecipitation and western blotting. They have defined 5 groups of MCAs. Group 1 reacted with the 60kD by both techniques, and group 2 MCAs reacted with the 205, 115, and 90kD glycoproteins in immunoprecipitation. The reactivity of group 1 and group 2 MCAs in immunofluorescence suggests that the antigens are present on the surface of virus infected cells. Group 2 antibodies recognized at least three epitopes, which was confirmed by ELISA additivity assays. Group 3, 4, and 5 antibodies recognized several proteins with lower molecular weights from 45 to 24kD. Furthermore, on the basis of immunofluorescence studies, it was found that the antigens recognized by groups 3, 4, and 5 were nuclear and cytoplasmic antigens that were not present on the surface of virus infected cells (York, et al., 1990).

All strains of ILT virus isolated throughout the world so far are antigenically homogenous on the basis of their reactions in virus neutralization tests. However, restriction endonucleases have been used to study the DNA patterns and some workers have found differences among some strains of ILTV (Kotiw, et al., 1982; Leib, et al., 1986).

Once monoclonal antibodies are produced, they can be used
in various diagnostic tests such as the indirect immunofluorescent antibody test (IFAT), virus neutralization (VN), enzyme linked immunosorbent assay (ELISA), etc. Virus neutralization activity has been assayed by some workers using the plaque reduction method (York, et al., 1989). There are various techniques to characterize these antibodies such as western blotting, immunoprecipitation test, IFAT, ELISA.

The objective of the present research was to produce monoclonal antibodies against the USDA challenge strain of infectious laryngotracheitis virus and to perform an initial investigation of their use in an indirect immunofluorescence diagnostic test.

**Materials and Methods**

**Source of Virus:** Infectious laryngotracheitis virus strains include the United States Department of Agriculture (USDA) challenge strain, provided by the National Veterinary Services Laboratory in Ames, Iowa; 86-1169, a Georgia field isolate; and S 88 00224, a California field isolate. Other viruses including a parrot herpesvirus (Pacheco's disease virus) and avian adenovirus 301, were obtained from The Avian Serology Laboratory, Veterinary Diagnostic Laboratory, Oregon State University. The parrot herpesvirus was isolated in chicken embryo liver cells from the liver of a parrot that died acutely.

**Virus Growth and Purification:** Chicken embryo liver cells were made from 14-day old chicken embryos. The livers
were removed aseptically and placed in phosphate-buffered saline (PBS) (0.16% sodium phosphate dibasic, 0.05% potassium phosphate monobasic, and 0.73% sodium chloride; pH 7.2) in a beaker. Calcium and magnesium free PBS was used. After chopping with scissors, livers were washed 5-6 times with PBS to eliminate the red blood cells (RBCs) and other fibrous tissue. Versene-trypsin (Sigma, St. Louis, MO 63178 USA; Trypsin 20 mg/ml, Versene 2 mg/ml) was used to separate the cells. Trypsinization was stopped by adding sufficient chilled fetal bovine serum (FBS) to produce an 8% concentration, and cells were centrifuged at 750 X g for 5 minutes. Packed cells were diluted at the rate of 1:150 in minimal essential medium (MEM) (Sigma, MO643) containing 10% FBS, gentamicin (50 ug/ml), and Amphotericin-B (2.5ug/ml) (pH 7.0-7.1).

Five ml of diluted cells were added to 60-mm tissue culture plates and were kept in a 5% CO₂ incubator at 37C overnight until a liver cell monolayer formed. Each tissue culture plate with a grown monolayer was infected with 0.1 ml of 1:10 dilution of ILT cell culture supernatant. After infection, cells were incubated 1 hour at 37C in a 5% CO₂ atmosphere to allow virus adsorption. Infected plates were fed with growth medium containing 4% FBS and incubated at 37C and 5% CO₂ for 24 hours. Only the cells which were infected with the USDA challenge strain of ILTV were used for the purification of virus for monoclonal antibody production.
Other viruses including Parrot herpesvirus, and adenovirus 301 were also used to infect the cells for certain comparative studies.

Cells having a good growth of virus, as determined by extensive cytopathic effect (CPE) characteristic of ILTV, were washed with TEN buffer (0.01 M tris, 0.001 M EDTA, 0.1 M sodium chloride in distilled water) (pH 7.2). Cells were scraped from the tissue culture plates and collected in a 50 ml tube. Virus particles were released by sonification and the solution was centrifuged at 5930 X g for 25 minutes to separate the cellular debris. The supernatant (32 ml) was retained, and 16 ml of the supernatant was layered onto discontinuous sucrose gradients in centrifuge tubes containing 8 ml 65% (w/w) sucrose under 10 ml 30% (w/w) sucrose. Supernatant was centrifuged through this discontinuous sucrose gradient at 100,000 X g for 1 hour. Visible virus bands were collected (15 ml) from the 30%-65% sucrose interface. Two ml of this fluid was added to the top of each tube (6 tubes) containing continuous sucrose gradients. The continuous sucrose gradient was made from four different concentrations of sucrose in the same tube: 60% sucrose (w/w) was at the bottom of the tube under 50%, 40%, and 30% sucrose (w/w) respectively. Virus was centrifuged through the gradient at 100,000 X g for 19 hours. Visible virus bands were collected (15 ml). The refractory index of the virus-sucrose solution was 1.410 and the density was 1.20 g/cm³.
After diluting the virus and sucrose in 15 ml TEN buffer, the solution was placed in a concentrator (100,000 MW) (Amicon, Beverly MA 01915 USA) and centrifuged at 1250 X g for 45 minutes. This centrifugation process was repeated 3 times to eliminate sucrose. Dialysis was performed by keeping dialysis tubing containing virus-sucrose solution in TEN buffer for 48 hours to completely eliminate sucrose. The dialysis tube has a semi-permeable membrane which allows only sucrose and TEN buffer to pass through the membrane. Dialysis was followed by another three centrifugations in a concentrator. Five ml of purified ILTV was obtained.

Virus particles were seen in the solution by electron microscopy. Spectrophotometry was used to measure protein concentration as 68.117 ug/ml and nucleic acid concentration as 9.8948 ug/ml. Purified virus was stored at -70C.

**Mouse Immunization:** Purified virus was thawed in a water bath at 37 C, and 0.8 ml of virus was mixed with 0.8 ml of Freund's complete adjuvant (Sigma) by sonification. Five 10-week-old female BALB/c mice were immunized intraperitoneally (IP) with 0.2 ml of emulsion containing virus and Freund's complete adjuvant. The mice were marked by ear punch for individual identification. Three weeks later a second injection (0.2 ml, IP) of ILTV antigen, consisting of incomplete Freund's adjuvant mixed in equal amount with the purified virus was given to all 5 mice.

Three weeks after the first injection of antigen, blood
was obtained from the conjunctival venous sinus of each mouse using 100 ul heparinized capillary tubes. Serum was separated by centrifugation. Serum 1:100 dilution was checked for antibody against ILTV by the indirect immunofluorescence test. Subsequently serum was checked every week for antibody against ILTV until fusion. Mice 1, 3, and 5 continued to respond best. Four weeks after the first injection a third injection (0.2 ml, IP) of ILTV in incomplete Freund’s adjuvant was given to all 5 mice. An intravenous booster injection of the purified virus without any adjuvant was given to mice 1, 3, and 5 four days before fusion, that is 8 weeks after the first injection. Five female BALB/c mice of the same age and sex were kept as unimmunized control mice for a source of negative control sera. Serum from mice responding to the viral antigen was also collected for use as positive control serum before mice were sacrificed.

**Fusion and Cloning:** SP2/0-Ag14 myeloma cells were fused with spleen cells of the mice immunized with the USDA challenge strain of ILTV for production of monoclonal antibodies (Kohler & Milstein, 1975; Current Protocol in Immunology, 1991). One day before fusion, feeder plates were made from spleen cells of a female BALB/c mouse that had not experienced any antigen exposure. Mice were killed by dislocating the cervical vertebrae and disinfected with 70% alcohol before placing them in a sterile cabinet.

Spleens from three immunized mice were removed
aseptically, minced, and washed twice in complete Dulbecco’s Modified Eagle’s medium (DMEM) (Sigma) without serum, containing 1 ml penicillin/streptomycin (100X) in 100 ml of DMEM, 1 ml of L-glutamine (100X) in 100 ml of DMEM. The medium (DMEM) was prepared according to supplier’s directions (Sigma, pH 7.2). The medium was filtered and kept refrigerated. Spleen cells were mixed by repeatedly and gently pipetting them with the media. The spleen cells were centrifuged at 750 X g for 5 minutes. Cells were counted with a hemocytometer. The spleen cells were mixed with SP2/0-AG 14 mouse myeloma cells at the ratio of 1:1 (Schulman, et al., 1978). The combined cells were centrifuged at 750 X g for 5 minutes, the supernatant was discarded, and 1 ml of 50% polyethylene glycol (PEG) (Sigma) was added to the cells as a fusion reagent (Poste & Allison, 1973). Cells were mixed very slowly in 2 ml of serum free DMEM and then in 7 ml of serum free DMEM by stirring the cells very gently with the pipet. Cells were centrifuged and resuspended in hypoxanthine aminopterin thymidine (HAT) (Sigma) media containing 20% FBS, HAT (5 x 10^{-3} M hypoxanthine, 2 x 10^{-5} M aminopterin, 8 x 10^{-4} M thymidine), pen/strep (10,000 u penicillin/ml, 10 mg streptomycin/ml), L-glutamine (200mM), hepes buffer (Sigma) (1 M), and OPI (Sigma) (0.15 g oxaloacetate, 0.5 g pyruvate, and 0.0082 g bovine insulin per 10 ml).

One hundred ul of cell suspension was added to each well
of 96 well tissue culture plates (feeder plates) and incubated at 37°C in a 5% CO₂ atmosphere. Cells were fed every 2 days. For the first feeding, the wells were fed with the media containing HAT, while for the second feeding hypoxanthine thymidine media (HT media) was used. After that, wells were fed with complete DMEM containing 10% serum. Screening normally begins 10-14 days after fusion. Supernatant fluids from wells were screened by IFAT for specific ILTV antibodies when the culture supernatant became acidic (yellowish) and the hybridomas were grown enough to produce specific antibodies. The hybridomas grow in the form of a clump of cells which is visible using a microscope. The hybridomas that were found to be producing antibodies were cloned by limiting dilution calculated to give <1 cell/well. Ten cells per ml were calculated by serial dilution and were plated onto feeder plates at the rate of 100 ul/well to give <1 cell/well. Cloning was done twice.

Fusion was done according the procedure given in Current Protocols in Immunology, Green Publishing Associates and Jhon Wiley and Sons, 1991., with slight modification.

Production of Cloned Antibodies: Monoclonal antibodies were produced by growing cloned hybridomas in 80 cm² tissue culture flasks in complete DMEM containing 10% FCS. Extinct cultures of the cells were made by incubating the cells at 37°C in a 5% CO₂ atmosphere for 20 days without providing any media after the cells were sufficiently grown. This gave a very
high titre of antibody in the cell supernatent. After 20 days the cells were centrifuged and the supernatant was collected in small aliquots and stored at -20C.

**Screening By Using Indirect Immunofluorescence Test (IFAT):** Infectious laryngotracheitis virus infected cells and uninfected cells were collected from tissue culture flasks. The cells were washed with PBS, and were centrifuged at 450 X g. The supernatant was discarded and the cell pellet was suspended in 0.01M FA buffer (containing Na₂HPO₄, NaH₂PO₄·H₂O, and NaCl) with 2% bovine serum albumin (BSA) and centrifuged as before. The supernatant was discarded and the cell pellet was resuspended in FA buffer containing 2% BSA. One microliter of the ILTV-infected cells was smeared on each top-row well of teflon-coated slides (Cel-line Associates, Inc. P.O. Box 35. Newfield, NJ 08344). Slides for other virus-infected cells such as ILTV S 88 00224, ILTV 86-1169, adenovirus 301, and parrot herpes virus were made in the same way. Uninfected cells were smeared on the wells on the bottom row of the slide to serve as negative cell controls. All slides were fixed in acetone for 10 minutes. Twenty microliters of undiluted hybridoma supernatant was added to infected and uninfected wells. Negative control sera (1:5 dilution) and positive control sera (1:100 dilution) were also used at the time of screening of hybridomas for comparison. After putting hybridoma supernatant or control sera on infected and uninfected cells, the slides were incubated for
20 minutes at 37°C in a 5% CO2 atmosphere. Slides were washed with FA buffer and were soaked in FA buffer for 20 minutes. Slides were washed with double distilled water, rinsed, and air dried completely. Twenty microliters of rabbit anti-mouse immunoglobulin (Sigma; Jackson Immunoresearch Laboratories, Inc, 872 Baltimore Pike, PO Box 9, West Grove, PA 19390) (1:200 dilution) was added to each well of infected and uninfected cells and incubated for 20 minutes. Slides were washed with FA buffer and soaked in FA buffer for 20 minutes. Slides were washed with double distilled water, rinsed, and counterstained for 5 minutes with 0.0001% Evan’s blue. Slides were air dried, mounted with mounting fluid (Difco Laboratories, Detroit, Michigan USA) having a pH of 9.0, and were examined under a fluorescent microscope.

**Immunoglobulin Subclass:** The isotypes of the monoclonal antibodies were determined by a mouse monoclonal antibody isotyping kit (Sigma) containing rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA following manufacturer’s procedures. The immunotype kit consists of nitrocellulose strips on which the entire assay is completed. The precoated strip captures the relevant mouse immunoglobulin and reveals the isotype by self-description.
RESULTS

**Monoclonal Antibodies.** Of 234 growing hybridomas, 15 showed positive reactions with cells infected with ILTV USDA challenge strain and no reaction with uninfected control cells. Five of the 15 died during the process of expansion and cloning. Four of the remaining 10 proved to give negative reactions, and 3 reacted both with infected and non-infected cells on the second screening after cloning by limiting dilution. Three hybridomas were found positive against the USDA challenge strain of ILTV and were designated as 2D1D8, 2E11G2, 2C6C7.

**Isotypic Characterization.** Monoclonal antibodies secreted by the three hybridomas were isotyped using the isotyping kit, and all three hybridomas 2D1D8, 2E11G2, and 2C6C7 were found to secrete IgM (Table 1).

**Indirect Immunofluorescence Assay.** All three monoclonal antibodies 2D1D8, 2E11G2, 2C6C7 reacted in an identical manner, fluorescence being found mainly along the peripheries of the infected cells. Monoclonal antibodies were also examined against other viruses including S 88 00224 isolate of ILTV, 86-1169 isolate of ILTV, a parrot herpesvirus, and avian adenovirus 301 (Table 2). The other two strains of ILTV reacted in the same manner as the USDA challenge strain of ILTV against all three monoclonal antibodies. Fluorescence was observed mostly at the peripheries of the infected cells (Fig.1). Cells infected with parrot herpesvirus and avian
adenovirus 301 did not produce any fluorescence with any of the monoclonal antibodies.

The reaction of all three monoclonal antibodies with the different isolates of ILTV and production of fluorescence was almost identical. This suggests that the other isolates of ILTV share the same antigen and the same epitope as the USDA challenge strain of ILTV.
Table 1. Isotypes of monoclonal antibodies secreted by the three cloned hybridomas producing ILTV-specific antibodies.

<table>
<thead>
<tr>
<th>Isotypes</th>
<th>Monoclonal antibody supernatants</th>
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<tbody>
<tr>
<td></td>
<td>2D1D8</td>
</tr>
<tr>
<td>IgM</td>
<td>+</td>
</tr>
<tr>
<td>IgG1</td>
<td>-</td>
</tr>
<tr>
<td>IgG2a</td>
<td>-</td>
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<td>IgG2b</td>
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<td>IgG3</td>
<td>-</td>
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<tr>
<td>IgA</td>
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Table 2. Reaction of monoclonal antibodies with different strains of ILTV and some other viruses as determined by indirect immunofluorescence.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain</th>
<th>Monoclonal antibody supernatant</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td><strong>2D1D8</strong></td>
</tr>
<tr>
<td>ILTV USDA</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>ILTV S 88 00224</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>ILTV 86-1169</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Avian Adenovirus 301</td>
<td></td>
<td>-</td>
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<tr>
<td>Parrot herpes virus</td>
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</tbody>
</table>
Figure 1. Indirect immunofluorescence staining of cells infected with USDA challenge strain of infectious laryngotracheitis virus with 2D1D8 monoclonal antibody.
Figure 2. Indirect immunofluorescence staining of cells infected with avian adenovirus 301 with 2D1D8 monoclonal antibody.
Discussion

Three monoclonal antibodies designated 2D1D8, 2E11G2, and 2C6C7 were obtained. Although not yet characterized, these monoclonal antibodies probably are directed against proteins or against glycoproteins which are considered as major immunogens (York, et al., 1987). Western blotting can be used to characterize MCAs which are directed against viral proteins or against glycoproteins. All three MCAs produced nearly identical reactions with ILTV-infected cells. Fluorescence was observed mainly at the surface of the virus-infected cells and fluorescence was also observed within the nucleus. These patterns of fluorescence by the monoclonal antibodies suggest the reaction of monoclonal antibodies against glycoproteins and against viral proteins because cytoplasmic fluorescence suggests the presence of viral antigens expressed on the surface of virus-infected cells, whereas nuclear fluorescence suggests the reaction of monoclonal antibodies against viral structural proteins (York, et al., 1987). Monoclonal antibodies specific for glycoproteins have been used to investigate the role of glycoproteins in adsorption and penetration of cells, and cell fusion and neutralization (Spear, 1985).

Each of the three monoclonal antibodies cross-reacted with the other strains of ILTV, and the fluorescence was nearly identical to that observed against the USDA challenge strain. The fluorescence patterns were similar to those seen
with the USDA challenge strain. All three monoclonal antibodies were found to not react with avian adenovirus 301 or parrot herpes virus.

While screening, some of the hybridoma supernatants were found to react both with infected and non-infected cells, which indicates that some spleen cells were also producing antibodies against chicken embryo liver cells or cell proteins. This suggests that some cell antigens remained after the virus purification steps and were injected with the virus at the time of immunization. This non-specific reaction was also observed when the immunized mouse serum was checked using the indirect immunofluorescent test before the mice were sacrificed. All 5 mice showed this serum antibody reaction before being sacrificed.

Monoclonal antibodies 2D1D8, 2E11G2, and 2C6C7 were each found to be of isotype IgM. It is possible that the cells that were responsible for producing antibodies other than IgM could not fuse or could not survive during the process of fusion, expansion, and cloning.

Monoclonal antibodies are an important tool for certain other assays. Glycoproteins are considered to be the major immunogens for ILTV. Identification of ILTV glycoproteins has been done in Australia using different ILTV strains, and minor differences have been found. The largest glycoprotein that has been detected measures 205Kd (York, et, al., 1987). Identification of similar glycoproteins in ILTV strains found
in the United States may be possible if the monoclonal antibodies produced by this project are directed against those glycoproteins. The further characterization of these MCAs remains to be done.

Different isolates of ILTV can not be differentiated by conventional serological assays (Cover and Benton, 1958). It is possible that specific monoclonal antibodies could be produced which could differentiate among virus isolates.
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