THE BIOLOGICAL CHARACTERIZATION OF CELLS OF A TRANSPLANTABLE TUMOR (JMV) DERIVED FROM A MAREK'S DISEASE LYMPHOMA

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
ELIZABETH ANN STEPHENS
1976

ABSTRACT

THE BIOLOGICAL CHARACTERIZATION OF CELLS OF A TRANSPLANTABLE TUMOR (JMV) DERIVED FROM A MAREK'S DISEASE LYMPHOMA

By

Elizabeth Ann Stephens

This work was designed to resolve an important and controversial problem associated with JMV tumor cells, namely, the nature of their association with Marek's disease herpesvirus and whether the lesions associated with JMV were virus-induced or the result of transplanted cells.

JMV tumor cells were inoculated into day-old chicks in order to study the mean days to death, the number of chick lethal doses necessary to kill 50 percent of the chicks and to observe enlarged leukotic livers and spleens. JMV tumor cells were also examined by co-cultivation in tissue culture, inoculation of susceptible chicks and inoculation of embryos for the presence of virus or viral antigens.

JMV tumor cells were found to be totally free of replicating Marek's disease virus by the methods employed. Cultivation of JMV affected kidney cells and co-cultivation of JMV spleen tumor cells on duck and chick embryo fibroblasts failed to reveal any cytopathologic changes in the cultured monolayers. Chicks inoculated with JMV cell culture material failed to show any signs of disease. Four-day-old

embryos inoculated by the yolk-sac route were negative for virus lesions on the chorioallantoic membrane. The absence of Marek's disease herpesvirus particles in JMV tumor cells was confirmed by electron microscopic examination.

Cell culture, indirect immunofluorescence and serum neutralization assays for viruses other than herpesvirus were also performed. There appeared to be no evidence for reticuloendotheliosis virus or lymphoid leukosis viruses of subgroups A through D. An attempt to identify unknown replicating agents was unsuccessful by the methods used.

JMV hyperimmune serum was assayed for Marek's disease virus-related antibodies by the indirect immunofluorescence, agar-gel precipitin and serum neutralization techniques. There was no evidence of reactivity with cells producing Marek's disease virus or viral antigens. The existence of tumor antibodies was demonstrated on viable cell suspensions of the MSB-1 Marek's disease lymphoblastoid cell line by the indirect membrane immunofluorescence technique.

JMV Marek's disease tumor cells were tested by indirect membrane immunofluorescence with virus specific antiserum for the presence of Marek's disease virus-specific intra-cellular and membrane antigens and with JMV anti-tumor serum for the presence of tumor specific antigens. There was no evidence of viral antigens. A distinct annular fluorescence was observed on the surface of the tumor cells stained with the anti-tumor serum. The tumor cell surface antigen was designated as a Marek's disease tumor-associated surface antigen (MATSA).

JMV tumor cells were studied by the membrane immunofluorescence technique to determine whether the proliferating lymphoid cells were of B or T cell origin. Since the tumor cells appeared to carry both antigenic determinants, this characteristic of JMV cells was not conclusive.

In an attempt to determine the transplantable nature of JMV tumor cells, the B blood group alloantigenic markers on the lymphoblastoid cells were examined. The tumor cells appeared to be of non-host origin and to carry an alloantigenic marker identical with or at least clearly related to B_{21}.

The presence of Marek's disease genome sequences was detected in the JMV tumor cells by nucleic acid hybridization technique. It was not possible to determine whether the cells contained a complete Marek's disease virus genome.

THE BIOLOGICAL CHARACTERIZATION OF CELLS OF A TRANSPLANTABLE TUMOR (JMV) DERIVED FROM A MAREK'S DISEASE LYMPHOMA

By

Elizabeth Ann Stephens

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Poultry Science

1976

To my husband Sam

ACKNOWLEDGMENTS

I wish to express my sincere thanks to Dr. Richard L. Witter, Associate Professor of Pathology and Director of the United States Department of Agriculture Regional Poultry Research Laboratory and to Dr. Theo H. Coleman, Professor of Poultry Science, for their encouragement and guidance throughout the research and preparation of this thesis. I also wish to thank Dr. Ben R. Burmester, former Director of the Regional Poultry Research Laboratory, who encouraged me to continue my education.

To the other staff members of the Regional Poultry Research Laboratory: Dr. William Okazaki, Dr. Keyvan Nazerian, Dr. Lucy F. Lee and Dr. Jagdev M. Sharma, I wish to express my appreciation for their vital contributions necessary for the completion of this work. In addition I wish to acknowledge Dr. B. W. Longenecker, Department of Immunology, University of Alberta, Edmonton, Alberta, Canada, for his assistance in designing the B blood group experiments, and Dr. J. Lloyd Spencer, Animal Pathology Division, Health of Animals Branch, Agriculture Canada, Animal Disease Research Institute, Hull, Quebec, Canada, for his helpful criticism of the JMV work.

I wish to thank Mr. Howard Stone and all the members of the genetics department at the Regional Poultry Research Laboratory for the extra effort extended in supplying me with the necessary experimental lines of chickens.

I would also like to thank Miss Linda Offenbecker and Miss Francie Gulvas for their excellent technical assistance. For their co-operation and willingness to assist me with the handling of my experimental birds, I wish to express my thanks to our animal caretakers, Mr. Robert Lowe and Mr. Chester Holton.

It is with the deepest gratitude that I acknowledge the love and understanding of my family. Through the efforts and encouragement of my husband Sam, our children: John, Jane, Daniel, Linda and Gary, and my parents, I was able to complete this work.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

INTRODUCTION

Marek's disease (MD) is an infectious, neoplastic disease characterized by infiltration and proliferation of lymphoid cells in nerves and visceral organs, resulting in paralysis and death (Biggs and Payne, 1967). The etiologic agent is a highly cell-associated herpesvirus (Churchill and Biggs, 1967; Solomon et al., 1968; Nazerian et al., 1968), which can be transmitted to susceptible chickens either by direct contact with diseased chickens or by inoculation of infected material (Sevoian et al., 1962; Biggs and Payne, 1963). Virus infection of cells may result in productive infection with antigen and virus production or abortive infection with the production of antigens and incomplete virus particles, both forms of which result in cell death; or non-productive infection, as exemplified by most lymphoid tumors or lymphoblastoid cells, which may result in transformation and proliferation of the cells without expression of virus or viral antigens (Nazerian et al., 1975).

MD lymphoma cells are non-productively infected and contain little or no infectious virus or viral antigens (Calnek et al., 1970; Nazerian, 1971). MD viral genome has been demonstrated in lymphoid tumor cells and lymphoblastoid cell lines (Nazerian et al., 1973; Nazerian and Lee, 1974; Lee et al., 1975). Tumor cells can transmit virus infection and reproduce the disease when inoculated into susceptible chickens (Calnek et al., 1970). Rapid serial passage of MD

lymphoma cells in chickens has resulted in a reduced latent period and increased mortality (Jakowski et al., 1974; Sevoian et al., 1964; R. Larose, personal communication; S. Schmittle, personal communication). One such virulent form, designated JMV, resulted from the serial passage of lymphoma cells of the JM isolate of MD (Sevoian, 1964).

The terms JM and JMV appear to be the source of some confusion. A "lymphomatosis" (MD) isolate was given the code name JM by Sevoian et al. (1962). The designation, JMV, is not an acronym but is a code for a highly lethal tumor cell preparation which resulted from serial passage of cell suspensions from an ovarian lymphoma induced by the JM isolate (Sevoian et al., 1964).

JMV tumor cells may be considered a class of MD tumor cells because of their origin and because of their immunologic relationship to MD. Vaccination with herpesvirus of turkey (HVT) (Mason and Jensen, 1971; Sevoian and Weston, 1972) or attenuated MD virus (Spencer et al., 1973) has been shown to protect chicks against JMV tumors. Likewise, JMV has been shown to protect against MD (Hong and Sevoian, 1974; Shieh and Sevoian, 1974; Kenyon et al., 1969).

The controversial issue with JMV cells has been whether the tumor cells are host lymphocytes transformed by exogenous infection with a virulent herpesvirus, or whether they are of donor origin, i.e., a transplant. The findings of a herpesvirus (Yoon and Kenyon, 1975), cell-free virus (Hong and Sevoian, 1974; Shieh and Sevoian, 1974) and virus specific antibodies (Hamdy and Sevoian, 1973; Hong and Sevoian, 1974; Shieh and Sevoian, 1974, 1975) associated with JMV tumor cells

are in contrast to reports that transmission of the tumor is dependent on viable intact cells (Jones et al., 1969; Sevoian, 1967; Spencer and Calnek, 1967).

JMV tumor cells have been widely used to challenge the immunity of chickens vaccinated against MD (Mason and Jensen, 1971; Sevoian and Weston, 1972) and as a rapid test for genetic resistance to MD (Gavora et al., 1974); and yet, very little has been done to identify the nature of JMV tumor cells. In order to use these tumor cells as a tool in the study of tumor immunology and Marek's disease immunity it became important to characterize JMV tumor cells on the basis of their association with MDV, as well as other characteristics of biological importance.

The objectives of this research were to resolve the nature of the association of Marek's disease herpesvirus with JMV tumor cells, to establish whether JMV tumors were virus-induced or transplants and to study certain biological characteristics of JMV tumor cells.

LITERATURE REVIEW

Marek's disease (MD) is a contagious neoplastic disease of domestic chickens caused by a herpesvirus (Churchill and Biggs, 1967; Solomon et al., 1968; Nazerian et al., 1968) and characterized by lymphoid infiltration in the peripheral nerves and development of lymphoid tumors in the visceral organs. Almost any organ or tissue can be involved, but the disease has a predilection for nervous tissue.

Marek's disease was first described in Hungary by Joseph Marek (1907) as a polyneuritis, but has now been recognized throughout the world. During the first two decades of the century, both in Europe and the United States, the disease appeared to be confined to the nervous system (Kaupp, 1921; van der Walle and Winkler-Junius, 1924). Pappenheimer et al. (1929) observed that about 10 percent of chickens with MD had lymphoid tumors principally of the ovary. The disease became a serious problem to the commercial poultry industry in Europe and the United States during the late 1920's and throughout the 1930's. The increase in the incidence of the disease appeared to be associated with the increase in commercial poultry farms. A highly pathogenic form of the disease appeared around 1950 in the United States (Benton and Cover, 1957) and was characterized by an early onset, high mortality and high incidence of visceral tumors. This acute form was epizootic in nature and appeared to be concentrated in

geographic areas where poultry production was prevalent. Until the advent of vaccination (Churchill et al., 1969b; Okazaki et al., 1970) the acute form was a serious cause of mortality to poultry in many countries.

Clinically the earlier or mild form of the disease is characterized by progressive paralysis of one or more of the extremities. Morbidity and mortality are usually low. Lesions are predominantly in the nerves which become enlarged due to infiltration with lymphocytes of different sizes and plasma cells. In the acute form of the disease, which appeared around 1950, tumors are more prevalent and morbidity and mortality may be as high as 30 percent or more (Biggs, 1973). The lymphoma appears to be multifocal and affects the gonad, kidney, liver, spleen, lung, muscle, skin, bursa of Fabricius or thymus. Tumors are the result of infiltration of lymphocytes, plasma cells and large lymphoblastic cells referred to as "Marek's disease cells" (Biggs, and Payne, 1967).

ETIOLOGY

The selection of several lines of chickens with genetic resistance to MD (Hutt and Cole, 1948) and adequate isolation housing (Sevoian et al., 1962; Biggs and Payne, 1963) were of great importance in the study of Marek's disease. Sevoian et al. (1962) and Biggs and Payne (1963) successfully transmitted the disease serially from bird to bird with intact blood or tumor cells. The spread of the disease to uninfected chickens in contact with infected chickens conclusively

established the contagious nature of MD (Biggs and Payne, 1963, 1967; Sevoian et al., 1963; Colwell and Schmittle, 1968).

The discovery in cell culture of a highly cell-associated herpesvirus associated with MD was a major breakthrough (Churchill and Biggs, 1967; Solomon et al., 1968; Nazerian et al., 1968). Additional evidence that a herpesvirus was the causative agent accumulated with investigations of natural and experimental cases of MD (Witter et al., 1969a; Biggs et al., 1968; Ahmed and Schidlovsky, 1968; Bankowski et al., 1969; Calnek and Madin, 1969; Eidson et al., 1969). The feather follicle epithelium was found to be the maturation site of cell-free infectious virus. The transmission of the disease with cell-free feather follicle extracts provided further proof that MD herpesvirus was the etiological agent of MD. Enveloped virus was rarely seen by electron microscopy in tumors, nerves and other tissues of infected chickens (Schidlovsky et al., 1969; Calnek et al., 1970; Ubertini and Calnek, 1970) but was common in the nuclei and cytoplasmic inclusions of the feather follicle epithelium (Calnek et al., 1970; Nazerian and Witter, 1970). The highly cell-associated nature of the virus both in cell culture and most tissues characterized the virus as a type B herpesvirus (Wilner, 1969; Churchill and Biggs, 1967; Nazerian et al., 1968; Churchill, 1968; Lee et al., 1969).

The several isolates of MD, which have been obtained, varied considerably. Virulent or pathogenic isolates included JM (Sevoian et al., 1962), HPRS-16 (Biggs et al., 1965), GA (Eidson and Schmittle, 1968a) and Cal-1 (Bankowski et al., 1969). The apathogenic or mildly

pathogenic isolates included HPRS-B14 (Biggs and Payne, 1963), WSU-GF (Kenzy et al., 1964), Conn-A (Chomiak et al., 1967) and HPRS-27 (Biggs and Milne, 1972).

EPIDEMIOLOGY

Chickens appear to be the principle animate reservoir of MD infection (Witter, 1972). Environmental factors such as exposure to contaminated air for less than 30 minutes have been sufficient to induce infection in newly hatched chicks (Chen and Witter, unpublished data). Infection has been accomplished by contaminated litter and droppings (Witter et al., 1968a), dust and dander (Beasley et al., 1970) and feathers (Calnek et al., 1970). Vertical or egg transmission was proposed as a means of transmission (Sevoian, 1968); however, on the basis of extensive further evidence it may be concluded that egg transmission does not occur or occurs only very rarely (Rispens et al., 1969; Solomon et al., 1970).

The portal through which the infectious material enters the host and initiates infection is not known, but the respiratory tract is a likely route (Calnek and Hitchner, 1969; Eidson and Schmittle, 1968b; Calnek et al., 1970). The portal of exit is the feather follicle epithelium (Calnek and Hitchner, 1969; Calnek et al., 1970; Nazerian and Witter, 1970). Kenzy and Biggs (1967) reported shedding of virus by infected chickens into the environment one or two weeks after infection.

The disease spreads rapidly and the incidence of infection in a flock usually reaches 100 percent (Witter et al., 1970a); although,

clinical signs do not necessarily follow infection (Chubb and Churchill, 1968; Witter et al., 1969a). Factors influencing the progression to clinical disease are the virulence of the infecting virus, dosage and route of exposure. Virulent isolates of MD cause a higher incidence of disease than the more apathogenic isolates (Biggs et al., 1968; Purchase and Biggs, 1967). Experimentally, the incidence of the clinical disease is dose-dependent (Calnek and Witter, 1972). The route of inoculation, such as intra-abdominal, results in higher incidence and shorter latent period as opposed to the nasal or oral routes or contact exposure (Witter and Burmester, 1967).

Factors influencing the development and control of the disease include breeding and husbandry procedures. Genetic selection for resistance to MD can be accomplished in a few generations (Biggs et al., 1968b; Cole, 1968). Resistance appears to be dominant and has no correlation with other production traits or resistance to lymphoid leukosis (Cole, 1970). Control by breeding for genetic resistance can be beneficial but costly and time consuming for the commercial industry.

Conventional methods of sanitation and isolation used in prevention of infectious disease are of little use in the control of MD. Isolation type housing with filtered air positive pressure (FAPP) systems and sanitation procedures have shown success in reducing the incidence of MD under experimental conditions (Drury et al., 1969).

Three types of vaccine have been developed for use in controlling MD. These are an attenuated Marek's disease virus (MDV) (Churchill et al., 1969; Churchill et al., 1969; Biggs et al., 1970), an apathogenic MDV (Rispens et al., 1972) and an apathogenic herpesvirus

of turkeys (HVT) (Okazaki et al., 1970; Purchase et al., 1971; Purchase et al., 1972). MD appears to be the only naturally occurring lymphoma induced by a herpesvirus which is successfully controlled by vaccination. Since 1972 HVT has been in use as a commercial vaccine. Prior to its use the level of condemnations of broilers was almost 1.6 percent in the United States in 1970, the last year of statistics before the extensive use of the vaccine. By 1974 the level of condemnation was 0.29 percent, a reduction of 1.12 percent based on the assumption that without vaccination losses would be at least 90 percent of 1970 level or 1.41 percent. Annual monetary losses due to MD have been reduced from $223 million to about $43 million (Agricultural Research Service, United States Department of Agriculture, 1975).

METHODS FOR VIRUS ASSAY

MDV can be propagated and assayed by several methods. The most sensitive method for detecting virus in chicken tissues is the inoculation of one-day-old susceptible chicks; this has been reported to be 10 to more than 1000-fold more sensitive than cell culture systems (Witter et al., 1969b). Chicks are observed for any of several parameters of infection (viremia, antibodies, antigens, lesions) between 2 and 10 weeks; the usual period being from 4 to 6 weeks (Witter et al., 1969b). Virus can be detected first in the lung and lymphoid tissues as early as 1 day after inoculation and, in most tissues, can reach a maximum titer of about 100 plaque forming units (PFU)/10^6 cells after about the 10th day and may persist at a lower level of 1-10 PFU/10^6 cells for the life of the chicken (Purchase, 1974). Fully

infectious enveloped virus can be found in the feather follicle epithelium around the 2nd to 4th week in titers of about 10^3PFU/10^6 cells (Calnek et al., 1970).

Assay of MDV has been done in duck embryo fibroblasts (DEF) (Solomon et al., 1968; Witter et al., 1969a,b) or chick kidney cell cultures (Churchill and Biggs, 1967; Witter et al., 1969 a,b). Primary isolation is usually made from white blood cells, tumors or kidney cells (Solomon et al., 1971). The number of cells required to initiate a micro-plaque may vary considerable. Churchill and Biggs (1967) reported that 10^2 to 10^5 tumor cells were needed to initiate a micro-plaque in tissue culture. Witter et al. (1969b) reported that at least 10^4 to 10^5 white blood cells were required to produce one plaque in tissue culture. The limited sensitivity of cell culture assay was presumably only detecting birds with high-titer viremias (Witter et al., 1971). Therefore, the number of cells necessary to initiate a micro-plaque would undoubtedly depend on the level of viremia in the donor chicken.

Inoculation via the yolk-sac of 4-day-old chick embryos with MDV chick kidney cell culture material or blood cells from infected chickens results in pock formation on the chorioallantoic membrane (CAM) (von Bülow, 1968 and 1971). The sensitivity of the assay has been shown to be similar to assay in cell culture, particularly when embryos from antibody-free dams are used (Biggs and Milne, 1971).

The oncogenic herpesvirus of chickens is important as a model because MD tumors resemble certain tumors occurring in humans. Understanding of the animal model may serve to help in further understanding of the etiology, epidemiology, control and recovery from human cancer.

MAREK'S DISEASE TUMOR CELLS

There are several classes of tumor cells associated with MD that are available for study. Tumor cells may be obtained directly from lymphomas induced by inoculation of chickens with MD virus. Infection may induce the development of progressive lymphoma with death or, in some instances, regression; however, it must be noted that only a low percentage of infected chickens develop lymphomas (Nazerian, 1973a). Lymphoma cells induced by inoculation of chickens with MD virus are of host origin and have been shown to carry thymus (T) cell antigenic determinants on their surface (Payne and Roszkowski, 1972; Hudson and Payne, 1973; Rouse et al., 1973). The cells associated with the lymphoma are a mixture of different lymphocytes, blast cells and plasma cells (Biggs and Payne, 1967). The proportion of cells in MD lymphomas demonstrating a tumor-specific surface antigen (MATSA) ranged from 2.3 to 27.3 percent indicating that not all cells in the tumor are transformed (Witter et al., 1975).

Another class of tumor cells is represented by the lymphoblastoid cell lines established in continuous culture from lymphomas of chickens inoculated with MDV. Two cell lines established by Akiyama et al. (1973a) and Akiyama and Kato (1974) from chickens inoculated with BC-1 isolate of MD were derived from an ovarian and splenic lymphoma and were designated as MOB-1 and MSB-1, respectively. Two additional cell lines were established by Powell et al. (1974) from tumors of chickens inoculated with HPRS-16 isolate and designated as HPRS-1 and 2. The MSB-1 cell line was studied extensively (Akiyama and Kato, 1974; Nazerian and Witter, 1975) and found to be a homogeneous

population of transformed lymphoblastoid cells. The cell line appeared to express a high frequency (nearly 100 percent) of MD tumor-specific surface antigen (MATSA) (Witter et al., 1975) and T cell surface antigenic markers (Nazerian and Sharma, 1975).

A third class of tumor cells was produced as a result of a transplantable lymphoma from chickens inoculated with MDV (Jakowski et al., 1974). The virus-induced tumor cells were passaged by inoculation into the pectoralis muscle resulting in tumors at the site of inoculation as early as 5 days from donor cell proliferation and virus-induced lymphomas of the visceral organs, which appeared after 21 days from host cell proliferation. The transplanted tumor line was shown to be a mixture of medium to large undifferentiated lymphoblastic cells with areas of necrosis. It has not been shown to date whether these tumors express MD tumor-specific surface antigen (MATSA) or T cell surface antigen.

JMV tumor cells, developed by a similar rapid passage procedure, have been classed as a transplant of donor origin (Spencer et al., 1976). Cell suspensions produced from leukotic livers and spleens contained a predominance of lymphoblastoid cells (Spencer and Calnek, 1967); however, their association with MD was not clearly established.

Differences between virus-induced cells and tumor cells. MD lymphoma cells contain little or no infectious virus or viral antigens (Calnek and Hitchner, 1969; Spencer and Calnek, 1970; Schidlovsky et al., 1969; Nazerian and Witter, 1970; Purchase, 1970). Neither naked or enveloped virions could be demonstrated by electron microscopy; however,

infectivity of these cells could be demonstrated by *in vitro* cultivation or by inducing the disease when inoculated into susceptible chickens (Calnek *et al.*, 1970; Nazerian and Witter, 1970). The only cells known to produce complete enveloped virions are the cells of the feather follicle epithelium (Calnek *et al.*, 1970; Nazerian and Witter, 1970; Purchase, 1970). Virus particles are found in the nucleus and cytoplasm of these cells (Nazerian and Witter, 1970) and cell-free infectious virus is released from this site (Calnek *et al.*, 1970).

Purchase (1974), Nazerian *et al.* (1975) and Nazerian (1976) described two different forms of infection caused by MDV. In the first form, i.e., productive infection, the cell produces viral deoxyribonucleic acid (DNA), virus-induced enzymes and antigens and complete virus particles which acquire an envelope while maturing in the degenerating, stratified, squamous cells of the feather follicle epithelium. Complete enveloped virions are formed both in the nucleus and cytoplasmic inclusions. Another phase of the productive infection, i.e., abortive infection, has been found in the bursa of Fabricius, thymus, kidney and other organs without tumors in which nuclear and cytoplasmic antigens may be detected by immunofluorescence in cells undergoing degenerative changes. Naked viral particles are commonly found in these cells but enveloped virions are rare. The infectivity of these cells is cell-associated and is destroyed by killing the cells (Calnek *et al.*, 1970; Nazerian and Witter, 1970; Purchase, 1970). Cells which demonstrate abortive infection contain viral genome and viral antigens but do not synthesize infectious virus (Purchase, 1974). Productive infection therefore, ultimately results in death of the cell.

A second form of infection, i.e., non-productive infection, is found in lymphoid cells of tumors and cell lines derived from tumors. Infectivity of these tumor cells is cell-associated. The virus DNA is present in multiple copies (Nazerian et al., 1973) but is not expressed in the form of antigens or physical virious. This type of infection does not result in the death of the cell and thus provides a condition conducive to cell transformation and proliferation (Nazerian, 1976). For some reason however, in a low percentage (1-2 percent) of the lymphoblastoid cells the virus genome is spontaneously expressed and results in productive infection (Nazerian, 1976), which probably explains the occasional fluorescing cells found in tumors (Calnek et al., 1970).

Intra-cellular viral antigens. Several intra-cellular virus-related antigens are detected in cells productively infected with MDV. These antigens have been detected by immunofluorescence and immunodiffusion techniques. Combined fluorescent antibody and electron microscopy studies have demonstrated that cells positive for antigens are positive for virus particles and, conversely, cells negative for antigens are negative for virus particles (Nazerian et al., 1969; Nazerian and Purchase, 1970).

The immunofluorescence technique was also used to detect antigens induced by MDV in cell cultures and in tissues of infected birds (Spencer and Calnek, 1970; Calnek et al., 1970). Antigens were found in the nucleus and cytoplasm of infected cells.

Chubb and Churchill (1968) and Churchill et al. (1969a) using chick kidney cell cultures infected with MDV as the antigen, described

several immunodiffusion antigens associated with MD infection. The A antigens were found in culture fluids and cell extracts. Two other antigens designated B and C were found in the extracts of infected cells.

In contrast, tumor cells did not contain immunofluorescent viral antigens, precipitin antigens or infectious cell-free virus (Churchill and Biggs, 1968) and except only rarely there have been no virus particles observed (Schidlovsky et al., 1969; Calnek and Hitchner, 1969; Ubertini and Calnek, 1970; Nazerian, 1971; Akiyama et al., 1973b).

Viral-membrane antigens. Cell surface viral membrane antigen has been reported on MD infected chicken kidney cell cultures (Chen and Purchase, 1970) and on Japanese quail embryo fibroblasts infected with either MDV or the cell-free HVT (Ishikawa et al., 1972). Chen and Purchase (1970) demonstrated a bright ring of fluorescence on the surface of rounded MDV infected cells of unfixed chick kidney cell monolayers and chick kidney cells in suspension. The specificity of the reaction was determined by the fact that only membranes of altered cells within virus plaques and not membranes of surrounding uninfected cells reacted with MDV specific antiserum.

Early antigen. Nazerian (1975) reported the induction of an MDV antigen under conditions where DNA synthesis was inhibited. Lymphoblastoid cells of the MSB-1 cell line were treated with thymidine DNA analogues, 5-iodo-2-deoxyuridine (IUDR) and 5-bromo-2-deoxyuridine (BUDR). After removal of the drugs, another antigen dependent on virus DNA synthesis was induced followed by virus production. The early antigen production could not be distinguished from the late antigen production by

serological methods because most sera from infected chickens has antibodies to both antigens. The results did suggest that MDV genome was associated with a large number of cultured lymphoma cells that did not ordinarily produce virus particles.

A similarity can be drawn with the Raji cell, a human lymphoblastoid cell line derived from Burkitt lymphoma, which is negative for Epstein-Barr virus (EBV) and viral antigens but contains the EB viral genome (zur Hausen and Schulte-Holthausen, 1972). Treatment of the Raji cells with thymidine analogues has induced early antigen and virus particles (Hampar et al., 1972).

Tumor antigens. An antigen was detected on the surface of MD tumor cells and lymphoblastoid cells of lines developed from MD tumors (Powell et al., 1974; Witter et al., 1975). Tumor cells in suspension were examined for membrane antigens with anti-tumor sera by a modification of the membrane immunofluorescence technique (Möller, 1961). The tumor-specific antigen did not appear related to virus structural proteins because cells positive for the tumor antigen did not react with anti-viral sera containing antibodies for membrane and intracellular antigens, furthermore, anti-tumor sera did not react with membrane and intra-cellular antigens of MDV infected cells (Powell et al., 1974; Witter et al., 1975). The surface antigen was not detected on normal chicken lymphocytes or on cells transformed by avian ribonucleic acid (RNA) tumor viruses. The antigen did not appear to be related to embryonic or histocompatibility antigens. The antigen was designated as a Marek's disease tumor-associated surface antigen (MATSA) (Witter et al., 1975).

Virus genome in tumor cells. As mentioned previously virus particles or virus specific antigens are found only rarely in tumors. The appearance of tumor-specific antigens on the surface of MD tumor cells accompanied by the absence of virus particles supports the work of Nazerian et al. (1973) that MDV-DNA is present in tumor cells. Marek's disease virus DNA was demonstrated in tumor cells from infected chickens (Nazerian et al., 1973) and in MD lymphoblastoid cell lines by nucleic acid hybridization (Nazerian and Lee, 1974; Lee et al., 1975). Lee et al. (1975) demonstrated that all MD tumor cells, regardless of the MD isolate used, contained MDV-DNA.

Transplantability of tumor cells. The transplantibility of MD tumor cells was demonstrated by Jakowski et al. (1974) and Theis et al. (1974). A transplantable lymphoma was developed in an inbred line of chickens by intramuscular injection of leukocytes from a Marek's disease virus infected chicken and was maintained by serial passage of virus-induced tumor cells in chickens of the same B blood group histocompatibility genotype. Tumors developed at the site of inoculation and in the visceral organs. Karyotype studies, using sex chromosome markers, revealed the tumor at the site of inoculation to be of donor origin and the cells of the visceral organs to be of recipient origin (Jakowski et al., 1974). Detection of the B alloantigens of the original tumor after repeated passage in chickens of a different B genotype confirmed the perpetuation of the original B phenotype in the cells of the transplantable lymphoma (Theis et al., 1974). The pathogenicity of the transplantable lymphoma increased with each passage as indicated by an increase in the number of recipients developing tumors at the

site of inoculation and an increase in mortality with a shortened latent period. The progressive growth of the transplantable tumor cells suggested that the tumor represented a population of proliferating cells (Theis et al., 1974). Therefore, Theis et al. (1974) concluded that the recipient chickens did not contribute to the transplantable lymphoma. The occurrence of the transplant appeared to be an event of low incidence in that only 1 out of 15 attempts resulted in a continuously transplantable line. Even in histocompatible lines the successful transplantation of tumor cells may depend on optimal conditions for selection of a small population of transformed cells (Theis et al., 1974).

Because MDV was initially associated with the primary virus-induced lymphoma Theis et al. (1974) investigated the association of MDV with the transplantable lymphoma cell line. Both MD virus and antibodies were found in isolation reared recipients of the MD lymphoma cell line. Whether MDV was the only virus involved in the transformed transplantable lymphoma was not determined; however, attempts to control contamination by avian leukosis viruses were made by using COFAL negative dams (dams free of the group-specific antigens of the avian leukosis viruses).

B and T cell surface antigenic markers. Two populations of lymphocytes are recognized in the chicken; bursa-derived cells (B cells), which are responsible for humoral immunity, and thymus-derived cells (T cells) which are primarily concerned in cell-mediated immunity (Payne et al., 1974; Rouse et al., 1973). Antisera directed specifically against chicken thymus or bursa cells have been used to study the origin of the lymphoid cells in Marek's disease lymphomas.

By means of the membrane immunofluorescence technique the majority of cells in MD tumors were found to be T lymphocytes (77 percent) with only a few (3-11 percent) B lymphocytes present (Hudson and Payne, 1973; Rouse et al., 1973). Powell et al.(1974) studied the MD lymphoblastoid cell lines HPRS-1 and 2 for B and T cell antigenic markers and found that 100 percent of the cells stained for T cell determinants and 0 percent for B cell determinants. Additional work by Nazerian and Sharma (1975) with the MSB-1 cell line also indicated about 99 percent T cells and 1-2 percent chicken B cells present in the cell suspension.

The question of whether the T cells in the tumor were transformed by virus or were there as a result of a host immune response was not resolved. Hudson and Payne (1973) and Rouse et al. (1973) noted both T and B cells but did not prove whether the lymphoma cells were transformed or were normal invading cells. Hudson and Payne (1973) proposed that although T and B lymphocytes contribute to the tumor, the majority of cells were of thymus origin. Thus the lymphoid system may provide an initial target cell which, after infection, could lodge in the gonads and other sites where it could transform and express surface antigens. These altered T lymphocytes (now foreign) may provoke a host immune response and stimulate entry of antigen reactive lymphocytes into the tumor. This secondary antigen reactive response may be the result of both B and T lymphocytes in the tumor.

Payne (1972) proposed two theories by which MD lymphoproliferation could occur: (1) an "intrinsic mechanism" whereby virus within lymphoid cells causes altered cells to proliferate and form

tumors or (2) an "extrinsic mechanism" by which virus infected antigen bearing non-lymphoid cells illicit an immunological response by the host. It has not been clear whether MD lymphomas are caused by direct infection and transformation of lymphoid cells (intrinsic) or whether lymphomas are formed as a result of host reaction to virus antigens (extrinsic). Recent evidence has been presented that MD lymphoma cells (Nazerian et al., 1973) and the MSB-1 lymphoblastoid cells (Nazerian and Lee, 1974) favors the intrinsic hypothesis by showing that the cells contain a significant number of copies of MDV-DNA in the absence of virus and viral antigens. This has provided strong circumstantial evidence that true transformation may indeed occur in MD. The presence of T cell surface antigens and MDV genome associated with the tumor cells provides additional evidence that MD lymphomas are virus-induced transformed T cells. A direct in vitro transformation of chicken T cells by MDV has not been done but would help to confirm the present data.

B blood group antigenic markers. The B blood group locus of the chicken has been shown to be the major histocompatibility locus (Schierman and Nordskog, 1961). The production of transplantable MD lymphomas has depended on the use of this knowledge (Theis et al., 1974). Various reference alloantisera have been developed that are specific for the B alloantigens on the surface of erythrocytes and lymphocytes and have been used to identify chicken populations selected for such parameters as histocompatibility and resistance to MD (Pazderka et al., 1975). In addition to the technique of karyotype analysis, this technique based on the B blood group alloantigenic markers has been used to

identify cell transplants. Specific agglutination reactions of lymphocytes in the presence of specific alloantisera can identify the alloantigens expressed on cells of MD lymphomas and thereby establish whether the MD lymphoma cells are the result of virus-induced recepient cells or whether they are transplanted donor lymphoid cells (Theis et al., 1974).

Theis et al. (1974) used specific alloantisera to agglutinate specific alloantigens (B_1 and B_2) on the surface of the transplanted tumor cells formed at the site of inoculation. It was concluded that the MD lymphoma was a replicating cell line and that passage of the lymphoma cells in birds of different B genotypes did not alter the alloantigenic specificity of the cells. The visceral tumors which developed in some chickens after injection of the MD transplantable lymphoma cells were examined and the preliminary results suggested that these tumors did not develop as a result of metastasizing MD lymphoma cells.

JMV LYMPHOBLASTOID TUMOR CELLS

The JM isolate of MD, isolated by Sevoian et al. (1962) has produced a high rate of neural and visceral MD lesions in S-line chicks within 3 weeks after inoculation. Serial passage of affected tissues from experimentally infected chicks exhibiting a shorter latent period increased the pathogenicity not only in S-line chicks, but in other strains of chickens as well. Several other researchers have also noted that rapid serial passage of MD lymphoma cells in chickens resulted in a reduced latent period and increased morality (Jakowski et al., 1974

Theis et al., 1974; R. Larose, personal communication; S. Schmittle, personal communication). Sevoian's virulent isolate, derived from multiple serial passage of JM-MDV lymphoma cells in MD resistant Whiterock chickens (Sevoian, personal communication) was designated as JMV (Sevoian, 1967), because he interpreted this tumor to be induced by an especially virulent form of MD virus. This isolate has been used extensively as a challenge virus for evaluating HVT-induced immunity (Mason and Jensen, 1971; Sevoian and Weston, 1972) and as a rapid test for genetic resistance to MD (Gavora et al., 1974). JMV has recently been proposed as a potential vaccine against MD (Sevoian, personal communication).

JMV was highly lethal within 3 to 8 days after inoculation in all strains of chickens tested (Sevoian, 1967). The disease was characterized clinically by the sudden onset of generalized weakness and gasping a few hours prior to death. Enlarged livers and spleens, due to infiltration of lymphoblastic cells, occasional appearance of ruptured spleens, lymphoblastic leukemia in peripheral blood and tumors at the site of inoculation were noted on post-mortem examination (Spencer and Calnek, 1967).

As previously mentioned most lymphomas are non-productively infected and the appearance of virus or viral antigens is rare. MD lymphoma cells can effectively transmit virus infection and induce disease when inoculated into susceptible chicks. However, the association of MDV with JMV tumor cells has not been clearly shown and has been a subject of controversy. The principle question to be answered

in this study is whether JMV-induced tumors resulted from viral transformation of recipient host lymphocytes or from the growth of transplanted donor tumor cells.

Detection of virus or viral antigens. Spencer et al. (1973a) reported a bi-phasic mortality in chickens inoculated with JMV tumor cells. Death and MD lesions occurring at the 5th week after inoculation in birds surviving the initial inoculation of JMV tumor cells, appeared to be caused, most likely, by MDV carried in the inoculum cells. Spencer however, did not infer that the initial JMV lesions were caused be a virus. Hamdy and Sevoian (1973) reported virus-specific complement fixing and serum neutralizing antibodies in JMV immunized chickens. Cell-free JMV obtained from JMV infected blood and an embryo attenuated cell-free JMV, designated JMV-A (Hong and Sevoian, 1974; Shieh and Sevoian, 1974, 1975), have been reported to stimulate chicks of lines N and P to produce high levels of virus-specific neutralizing and fluorescent antibodies against the JM isolate of MD. Yoon and Kenyon (1975) reported a JMV herpesvirus isolated from spleens of infected chickens which was distinct from MDV in virulence, tumor formation and histopathology. Polykaryocytes were induced by the JMV virus in 3 days in chicken kidney cell cultures. Cytogenetic studies (Kim et al., 1972) indicated that donor genome, identified by sex chromosome markers, was not present in the cells of recipient birds, and therefore, the disease was a result of a virus associated with JMV tumor cells.

In contrast to the reports that JMV lesions are induced by a virus associated with JMV tumor cells is the lack of contagion to cagemates of birds inoculated with JMV (Sevoian, 1967; Spencer and

Calnek, 1967). The lack of cell-free transmission of the disease with serum from JMV moribund birds was observed by Jones et al. (1969). The dependency on viable tumor cells to reproduce the disease, the rapid onset of disease, tumors at the site of inoculation and the highly lethal nature of the inoculum cells were indicative of a cell transplant (Spencer and Calnek, 1967). Olmsted and Kenyon (1971) supported the transplant theory in their studies using whole-body irradiation to destroy the lymphoid element of recipient chickens. They concluded that the lymphoid proliferation must have been due to the proliferation of donor JMV lymphoblastoid tumor cells. Preliminary results by Witter et al. (1975) demonstrated that JMV tumor cells were totally free of replicating MDV as indicated by the failure to isolate virus by co-cultivation of tumor cells with DEF in vitro and by the absence of MDV specific antibodies in sera from JMV immunized chickens.

Probably the best indirect evidence available to date that JMV tumor cells are antigenically related to MD lymphomas is the immunologic response induced in chickens immunized with JMV, MDV or HVT. Mason and Jensen (1971), Sevoian and Weston (1972) and Shieh and Sevoian (1974) demonstrated that vaccination with HVT prior to challenge with JMV tumor cells protected chicks against the lethal effects of the tumor cells as measured by a reduction in mortality rate. Vaccination with HVT one week prior to inoculation of JMV tumor cells into the wing web of 5-week-old chickens significantly suppressed the development of wing web tumors (Spencer et al., 1973b). The mechanism by which HVT protects chicks against JMV is not understood. Mason and Jensen (1971) proposed that either protection was reflective of antigens

being shared between JMV or HVT, or that in some undefined manner HVT preempted cells critical to achieving JMV infection. Inoculation of low doses of JMV cells appeared to protect chicks against subsequent higher doses of JMV and MDV. As shown by Sevoian (1967) inoculation of mature dams with an initial sub-lethal dose of JMV tumor cells caused the chickens to be refractory to higher doses of JMV. Day-old progeny from these dams were resistant to challenge with JMV and MDV up to 5 weeks of age. Kenyon et al. (1969) reported that sera from JMV inoculated survivors could passively immunize chicks against JMV. Adsorption of the antiserum with MDV infected cells significantly decreased the neutralizing capacity and was comparable to the results obtained by adsorption with JMV cells. Hong and Sevoian (1974) reported the existence of maternal antibody in progeny from adult chickens vaccinated with cell-free JMV and HVT. They concluded that progeny with JMV maternal antibody had more protection against JM-MDV infection and tumor induction than progeny with HVT or MDV maternal antibody.

Tumor antigens on JMV tumor cells. As a result of the work begun in this research, which initiated further studies by Witter et al. (1975), the unexpected finding that JMV antiserum was reactive with MSB-1 cells, MD lymphoma cells and JMV cells suggested the possible existence of tumor-specific antigens related to MD. Other than that published by Witter et al. (1975) and the data presented in this thesis, there have been no other reports related to the existence of tumor antigens on JMV cells.

There is no published information indicating the presence of MD viral genome associated with JMV tumor cells. There appears to

be no information at present as to the association of bursa-derived and thymus-derived lymphoid cells with JMV tumor cell transformation. The transplantable nature of JMV has been reported but additional evidence indicating a difference between the B blood group surface antigenic markers of JMV tumor cells and those of the host lymphocytes has not been published.

The controversial issues related to JMV tumor cells which should be resolved are first, the claims that infectious virus is responsible for JMV lesions and second, whether JMV can be considered a class of Marek's disease tumor.

MATERIALS AND METHODS

Chickens and embryos. The chickens used were Single-Comb White Leghorns from the MD susceptible lines 7_2, 100 and crosses 15 x 7 and 7_2 x 100, developed at the Regional Poultry Research Laboratory, East Lansing, Michigan (Stone, 1975), and the MD resistant Cornell N line (Cole, 1968). Four-day-old embryos of line 6_3 were used for yolk-sac inoculation.

Line 7_2 is highly susceptible to MD (Stone, 1975) and is homozygous for the B^2 blood group allele (Pazderka et al., 1975). Line 100 is an inbred line developed from crossing line 6 males with line 7 females and then backcrossing to line 7 males for several generations (Stone, 1975). Line 15 has been maintained since its inception in 1939 and is susceptible to MD. Line 6 is relatively resistant to MD. The Cornell line N is homozygous for the B^{21} blood group allele (Pazderka et al., 1975). All chicks were from dams negative for MD antibodies except as otherwise specified. The chickens used in these experiments were maintained in Horsfall-Bauer isolators throughout their experimental period.

Cell culture. Direct chicken kidney cell cultures (DCK) from JMV donor chicks were prepared and maintained in basal medium, Eagle (BME), with bovine fetal serum, 25 units/ml mycostatin, 100 units/ml penicillin-streptomycin, as previously described (Churchill and Biggs, 1967; Witter et al., 1968b). Cells were counted in a hemocytometer and plated at

5×10^6 cells per 60mm tissue culture dish. CEF and DEF cultures were prepared in tissue culture medium F 10-199 mixture with antibiotics as described according to the methods of Solomon et al. (1971).

Viruses. The JM isolate of MDV (Sevoian et al., 1962) was cloned, by Dr. Richard L. Witter, Regional Poultry Research Laboratory, 3 times in DEF cultures from cell-free virus obtained from the feather tips of infected chickens and designated clone JM/102W. The material used had been passaged 43 times in chickens and 13 times in DEF cultures and consisted of infected DEF suspensions stored at -196°C. Uncloned preparations of the GA strain of MDV were also used. The reticuloendotheliosis virus (REV) strain T, was obtained from Dr. M. K. Cook (Witter et al., 1970c). Stocks of the A, B, C, and D subgroups of Rous Sarcoma virus (RSV) were kindly supplied by Dr. W. Okazaki, Regional Poultry Research Laboratory.

Virus and antibody assays. The phenotypic mixing test as previously described (Okazaki et al., 1974) was used to detect avian leukosis viruses of subgroups A, B, C, and D in stock suspensions of each JMV source, as well as in irradiated JMV-S cells. Line 100 C/O (cells susceptible to all subgroups of avian leukosis viruses) CEF monolayers in cell culture medium containing 2 ug/ml DEAE-Dextran were inoculated with at least 10^3 focus-forming units of RSV-O. The JMV test samples were added 24 hours later and maintained at 37°C in a humidified chamber under CO_2 for 7 days. Tissue culture fluids were harvested, frozen and thawed 3 times, centrifuged and inoculated at 0.5 ml supernatant fluid onto 24 hour cultures of C/E CEF monolayers. The monolayers were overlaid with agar 24 hours later and examined approximately

7 days later for RSV foci. A neutralization technique as described by Ishizaki and Vogt (1966) was used to detect the presence of antibody to RSV subgroups A, B, C, and D in JMV antiserum.

CEF monolayers infected with JMV spleen cells, maintained 7 to 21 days, were examined with REV positive antiserum by indirect immunofluorescence for the presence of REV antigen (Witter et al., 1970c). Sera were assayed for REV antibodies by indirect immunofluorescence using REV infected CEF cell culture antigen (Witter et al., 1970c).

A blind fluorescent antibody technique designed to detect unspecified replicating agents was based on indirect immunofluorescence staining with homologous hyperimmune JMV antiserum of cultures inoculated with JMV. The lack of positive staining reactions was considered evidence for the absence in test material of agents capable of replicating in the culture system and of inducing antibodies in chickens after inoculation.

MDV was assayed by induction of herpesvirus cytopathologic changes in chick kidney, CEF and DEF cultures (Witter et al., 1969b). Coverslips included at the first and third passage were fixed in acetone, and examined by indirect immunofluorescence for the presence of MD viral antigens. Four-day-old embryonated eggs of line 6_3 (negative for MD antibody) were inoculated via the yolk-sac route and examined for pocks on the CAM at 14 days after inoculation (von Bülow, 1968 and 1971). Day-old susceptible chicks were inoculated, held in Horsfall-Bauer isolators for 4 to 8 weeks and observed for MDV-specific pathologic or immunologic responses. Presumably these assay systems would also have detected the immunologically related herpesvirus of turkey (HVT) (Witter et al., 1970b).

Assay for MDV antibodies was done by several methods. Detection of precipitin antibody was done by the agar-gel precipitin test (Chubb and Churchill, 1968; Churchill et al., 1969a). Antibodies to virus-induced cellular antigen were detected by indirect immunofluorescence with acetone-fixed MDV infected chicken kidney cell coverslip antigen (Purchase and Burgoyne, 1970; Spencer and Calnek, 1970). Neutralizing antibody was assayed by using one part serum diluted 1:5 or 1:10 and then mixed with an equal part of virus suspension such that the final dilution of serum was 1:10 or 1:20. The serum virus mixture was inoculated at 0.1 ml directly onto chick kidney monolayers, incubated at 37°C for 30 minutes at which time the media was replaced. JMV hyperimmune sera were examined for MATSA antibody according to procedures previously published (Witter et al., 1975).

JMV tumor cells. Three sources of JMV tumor cell preparations passaged 800, 250, and 60 times in chicks were designated as JMV-S, JMV-x462 and JMV-x55, respectively. The JMV-S was obtained from Dr. M. Sevoian, Amherst, Massachusetts. The JMV-x55 and JMV-x462 were obtained from Dr. B. W. Calnek, New York State Veterinary College, Ithaca, New York, who received them from Sevoian in 1965 and 1972, respectively. All stocks were received as frozen liver suspensions and represented different passage levels of the original JM-MDV lymphoma from which JMV was derived. Stock tumor materials were prepared by inoculating day-old chicks of crosses 7_2 x 100 or 15 x 7_2 (MD antibody positive) and harvesting liver tumors from moribund chicks 7 to 12 days post-inoculation. The stock liver tumor materials were prepared as 10 percent tissue homogenates and stored with 10 percent dimethyl-sulfoxide at -196°C (Spencer and Calnek, 1967). Single cell

suspensions of JMV spleen tumors for immunofluorescence and chick inoculation were prepared by removing the splenic capsule and connective tissue and gently teasing the cells with a scalpel. The crude spleen material was allowed to stand from 3 to 5 minutes before being filtered through gauze. The cells were then washed and centrifuged at 1500 rpm for 3 minutes (2 cycles) and resuspended in tissue culture medium or phosphate buffered saline (PBS). Spleen homogenates were prepared with a Ten Broeck tissue grinder in medium F10-199 with 4 percent calf serum as 1:10 suspension on a weight per volume basis. Sonication was accomplished with a Bronwell Biosonik II cell disrupter (Bronwell Scientific, Rochester, N. Y.) for 3 one minute intervals at a setting of 70. The material was filtered through a 0.45 u filter (Nalgene Labware Division, Sybron Corporation, Rochester, N. Y.). Gamma irradiation of JMV-S spleen cells at 10^8 cells/ml was accomplished at 5000 r with a cobalt 60 source (Michigan State University, East Lansing, Michigan, Department of Food Science and Human Nutrition).

Preparation of hyperimmune sera. JMV-S and JMV-x462 hyperimmune sera were prepared by inoculation of twelve-week-old 15 x 7_2 chickens with 69 and 178 CLD_{50}, respectively, of liver homogenate, followed by two additional injections of 690 and 1780 CLD_{50} at the 15th and 18th weeks in some of the chickens. All chickens were bled at the 21st week.

Membrane immunofluorescence technique. Staining of unfixed lymphoid cells of the MSB-1 cell line and JMV spleen tumor cells in suspension was done by the indirect membrane immunofluorescence technique as previously described (Witter et al., 1975). Approximately 2 x 10^6 lymphoid cells/12 x 75 mm tube were suspended in PBS, washed and pelleted.

The cells were resuspended in 0.1 ml of 1:8 dilution of JMV hyperimmune serum and allowed to react for 30 minutes at 4°C. Cells were counter-stained with 0.1 ml of a 1:20 dilution of fluorescein-isothiocyanate conjugated horse anti-chicken globulin (Roboz Surgical Instrument Co., Washington, D. C.) for 20 minutes at 4°C. After a final wash in PBS cells were suspended in 0.1 ml PBS/tube, a drop of cell suspension placed under a coverslip and examined immediately with a Leitz fluorescence microscope with vertical illumination system for the presence of MATSA (Witter et al., 1975).

MSB-1 lymphoblastoid cell line. The lymphoblastoid cell line was obtained from Dr. S. Kato of Osaka University, Japan, and was derived from the spleen tumor of an MD infected chicken. The properties of the cell line have been previously described (Akiyama and Kato, 1974; Nazerian and Witter, 1975).

JMV assay. One-day-old MD susceptible chicks were inoculated intra-abdominally with 0.1 ml of JMV tumor cells, placed in Horsfall-Bauer isolators, and observed through an experimental period of at least 21 days. Death from JMV tumor cells occurred between the 7th and 12th days after inoculation from enlarged leukotic livers and spleens. Chickens dead of non-specific causes, as confirmed by histopathology, were excluded from the data. Positive diagnosis was based on typical gross lesions or lymphoblastic infiltration of liver and spleen as confirmed by microscopic examination. Several lots of chicks were maintained in the same isolator since no horizontal transmission to control chicks within the isolators had previously been observed by

others (Spencer and Calnek, 1967) nor in any of the experiments conducted in this research. Quantitative assays were performed for each JMV source by inoculation of serial dilutions into separate lots of chicks. Titers were expressed as chick lethal doses (CLD_{50}) according to Reed and Muench (1938).

JMV protection test. A test was designed to measure the protective ability of JMV sera against *in vivo* tumor formation and death. Protection was based on the ability of one ml of heat inactivated JMV serum to protect at least 75 percent of treated chicks against mortality resulting from inoculation of 50 CLD_{50} of JMV-S. Day-old chicks of line 100, five birds per lot, were each inoculated intra-abdominally with 1 ml of the respective serum. The chicks in each lot were then challenged with 50 CLD_{50} of JMV-S one day later. Responses were measured as described for JMV assay.

Assay for MDV virus-specific membrane antigen and MATSA. Unfixed coverslips of MDV infected DEF cell monolayer cultures and lymphoid cells in suspension were assayed for membrane antigen by indirect immunofluorescence (Nazerian, 1973b) using anti-MDV and HVT sera. Unfixed, single cell suspensions of JMV and normal spleen cells were assayed for MATSA by indirect membrane immunofluorescence as has already been described. Cells in suspension were stained with 1:8 dilution of chicken JMV hyperimmune serum containing MATSA antibody and counter stained with conjugated anti-chicken gamma globulin as previously described.

Detection of B alloantigenic markers. B_2 and B_{21} antisera were kindly supplied by Dr. B. H. Longenecker, Edmonton, Alberta, Canada. This

experiment, designed by Dr. Longenecker, was done in an attempt to identify the alloantigens on the surface of JMV tumor cells. The indirect membrane immunofluorescence technique was used to examine the cells.

B and T cell surface antigenic markers. Unfixed, single cell suspensions of normal and JMV affected spleen cells were examined for B cell and T cell surface antigens by the indirect membrane immunofluorescence method. Anti-T and anti-B sera were kindly supplied by Dr. J. M. Sharma, Regional Poultry Research Laboratory, East Lansing, Michigan. Anti-T serum was produced in a turkey by three bi-weekly intravenous injections of 10^9 thymus cells from line 6_3 chickens and adsorbed 5 times with chicken erythrocytes (1:20 v/v) and repeatedly with bursa cells until all reactivity against B cells was removed. Anti-B serum was prepared by three intravenous injections of 10^9 bursa cells from line 6_3 chickens into ducks at 3 week intervals. The anti-B serum was adsorbed 6 times with chicken erythrocytes and repeatedly with thymus cells until reactivity against T cells was reduced or removed.

DNA-cRNA hybridization. Normal spleen pools and JMV tumorous spleen pools were processed to form cell suspensions of at least 10^9 cells per pool. These cells were given to Dr. Lucy F. Lee, Regional Poultry Research Laboratory, East Lansing, Michigan, for hybridization studies. DNA from normal CEF, GA-MDV infected CEF, and spleen from normal and JMV affected chickens were extracted according to published procedure (Lee et al., 1971). Transcription of purified MDV-DNA into ^{3}H-labelled complementary RNA and the technique of DNA-cRNA hyribidization have

been published elsewhere (Nazerian and Lee, 1974). Liquid scintillation spectrometry was used to assay radioactivity on the membrane filters.

Electron microscopy. Spleens were removed from each of 5 JMV-x55 and 5 JMV-S donors that were obviously moribund. Thin sections from each spleen were given to Dr. Keyvan Nazerian, Regional Poultry Research Laboratory, for examination by electron microscopy for virus particles (Nazerian and Burmester, 1968; Nazerian, 1970).

EXPERIMENTAL DESIGN

The primary objective of this research was to resolve the nature of the association of MD herpesvirus with JMV tumor cells and to establish whether these tumor cells were virus-induced or transplants. The implication of a virus associated with JMV lesions had been reported but there was no substantial evidence that would resolve the controversy.

In order to determine whether replicating MDV was associated with JMV tumor cells the various methods presented were used in an attempt to resolve the question.

1. Cell culture techniques of the following type were used to assay for MDV.
    a. Direct kidney cultures were prepared from JMV moribund chicks and maintained and observed through a 7 day experimental period.
    b. JMV affect spleen tumor cells were co-cultivated with DEF and CEF cultures and maintained and observed through 3 passages or 21 days.
    c. The detection of MDV antigens in JMV inoculated cultures was done by the indirect immunofluorescence test.
    d. JMV inoculated cell culture material was assayed in day-old chicks which were then maintained for 21 days in isolators.

2. Four-day-old embryonated eggs were inoculated with JMV tumor material via the yolk-sac route and observed for typical MDV lesions on the CAM 14 days after inoculation.
3. Day-old chicks were inoculated with sub-lethal doses of JMV tumor cells and observed through an 8 week experimental period for any signs of a low level of MDV in the inoculum cells that would ultimately result in MD infection.
4. In order to determine whether cell-free herpesvirus was associated with JMV tumor cells, intact JMV tumor cells and various preparations of disrupted JMV tumor cells were inoculated into day-old chicks which were held in isolators for 21 days.
5. JMV tumor cells were subjected to gamma-irradiation in order to determine whether a latent herpesvirus was associated with the tumor cells. The cells were then co-cultivated on CEF and DEF cultures and observed for 21 days.

After extensive efforts failed to reveal a herpesvirus associated with JMV tumor cells the possibility of other avian tumor viruses was considered. The following methods were used to assay JMV tumor cells for other RNA tumor viruses.

1. The indirect immunofluorescence technique was used to detect the presence of REV antigens in JMV inoculated CEF cultures.
2. A blind fluorescent antibody technique was designed to detect unspecified replicating agents in cell culture and in the serum of chicks inoculated with JMV tumor cells.

3. A virus neutralization technique was used to detect the presence of RSV antibody in JMV sera.
4. The very sensitive phenotypic mixing test was used to detect the presence of leukosis viruses in all three sources of JMV liver homogenate and JMV spleen tumor cells.

Since all efforts to rescue a virus associated with JMV tumor cells had not produced positive results, the possibility of other antigens and viral genome was considered. The unexpected results of finding tumor specific antigen on the surface of MSB-1 cells stained with JMV anti-tumor sera led to the following experiments.

1. The membrane immunofluorescence technique was used to detect the presence of antibody in JMV hyperimmune sera against viral and membrane antigen in MDV infected cells and tumor antigens on JMV cells.
2. Day-old chicks were inoculated with JMV antisera to determine whether the sera could protect chicks against a lethal dose of JMV tumor cells inoculated one day later. This test was done previous to the discovery of tumor-specific antigens; however the ability of the sera to protect chicks and the presence of tumor-specific antibody appeared to be related.
3. In order to determine whether the tumor antigens observed on MSB-1 cells and JMV tumor cells was specific for MD non-productively infected cells, the technique of DNA-cRNA hybridization was used to determine whether JMV tumor cells possessed MDV genome. The presence of MDV genome had been shown in MSB-1 cells but no such evidence existed for JMV tumor cells.

4. JMV tumor cells were examined for the presence of any form of virus particle or viral antigens by the electron microscope.

In an attempt to determine whether JMV cells were the result of a transplant the following assumption was made. Assuming that a transplanted cell would maintain the same B blood group histocompatibility antigen of the host in which it was produced, regardless of the number of times it was passaged in a host of another B genotype, an attempt was made to determine whether JMV tumor cells were a replicating transplantable cell line. JMV tumor cells were examined for the presence of the particular B antigen of the host into which the cells had been inoculated. The absence of membrane staining with a specific alloantiserum homologous to the host genotype would imply that JMV was indeed a transplant and would probably express a different B alloantigen, namely that of the original host.

As an additional biological characteristic JMV tumor cells were examined by membrane immunofluorescence for the presence of B and T cell surface antigenic markers. Since MD lymphoma cells had been shown to express T cell surface markers it was of interest to determine whether JMV cells expressed a similar marker.

RESULTS

Biological characteristics. Day-old chicks inoculated intra-abdominally with JMV tumor cells died between the 7th and 12th day depending on the dilution of the inoculum cells. The latency of the disease was increased approximately 1 day for each log dilution of JMV tumor cells (Figure 1). Prior to death the chicks were generally weak, emaciated and showed signs of respiratory difficulty. Affected livers and spleens were generally twice their normal size (Figure 2). In some cases spleens were ruptured and tumors were noted at the site of inoculation, especially with JMV-x55. The predominant cell in JMV lesions was a lymphoblast (Figures 3 and 4). The appearance of small lymphocytes in the lymphoblastic lesions (Figure 5) was not completely understood, but may be indicative of a host response to the transformed cells. There was no evidence of virus or viral antigens in any of the tumor cells examined by methods to be discussed. These characteristics were quite different from those associated with MD, such as longer latent period, the lack of tumors at the site of inoculation and a mixed population of lymphocytes, blast cells and plasma cells associated with the MD lesions (Calnek and Witter, 1972).

The quantitative biological characterization of JMV tumor cells is presented in Tables 1 through 3 with the mean days to death for each dilution and the respective CLD_{50} for each JMV source presented.

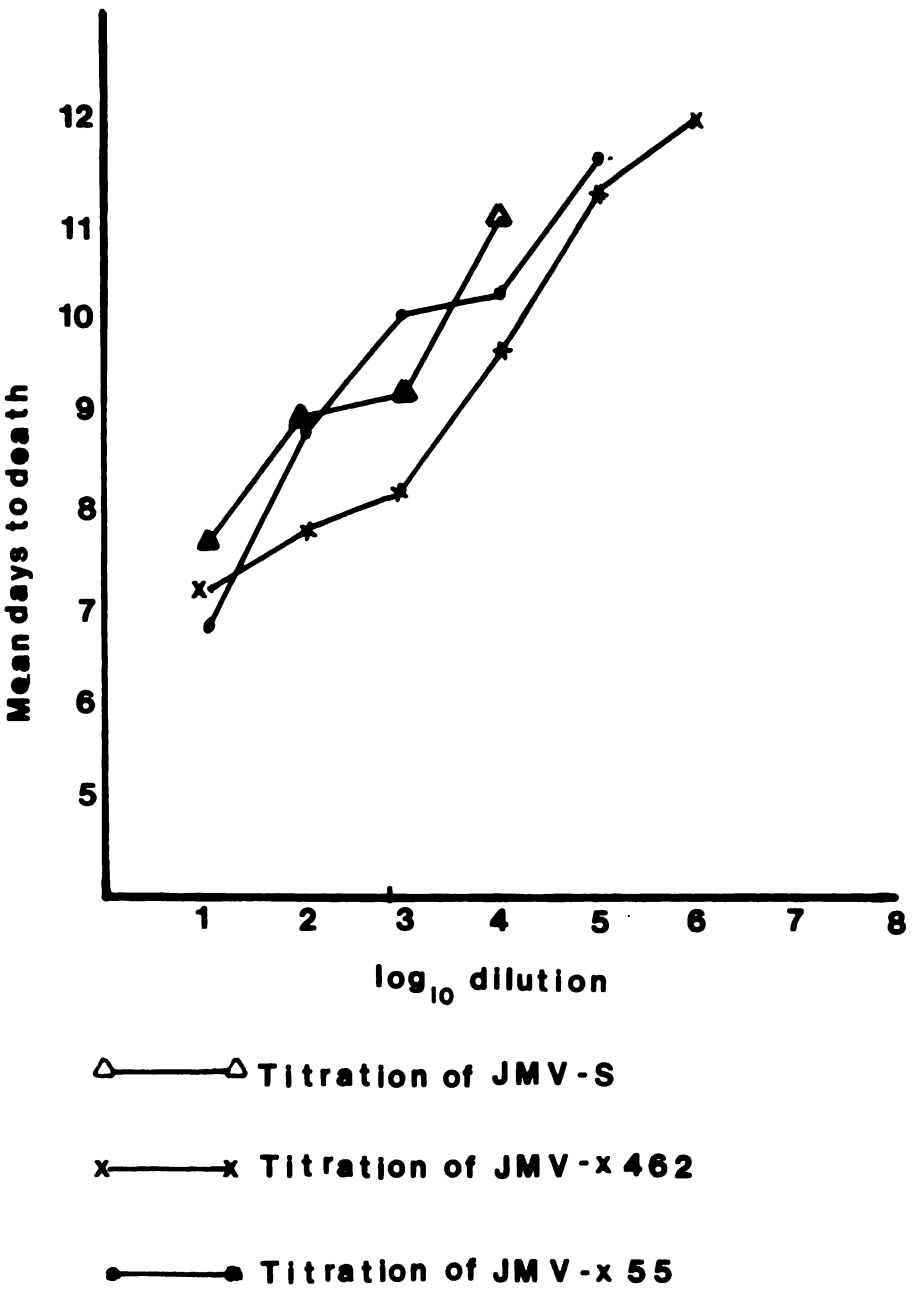


Figure 1. Comparative titrations of three JMV sources demonstrating dose dependent latency effect. The data were obtained from the results in Tables 1, 2 and 3.

Figure 2. Enlarged tumorous spleen of a line 7_2 chicken 8 days after inoculation with JMV-S.

Figure 3. Smear preparation of JMV-S spleen tumor cell suspension showing typical lymphoblastic cells. Wright X1580

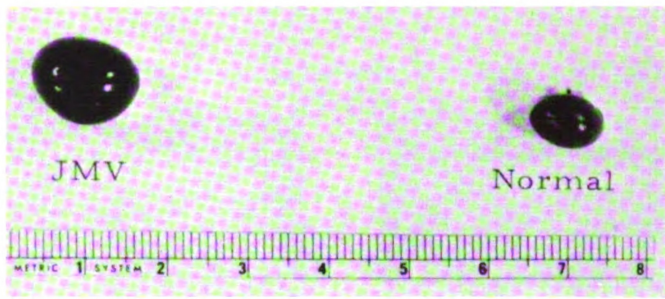


Figure 2

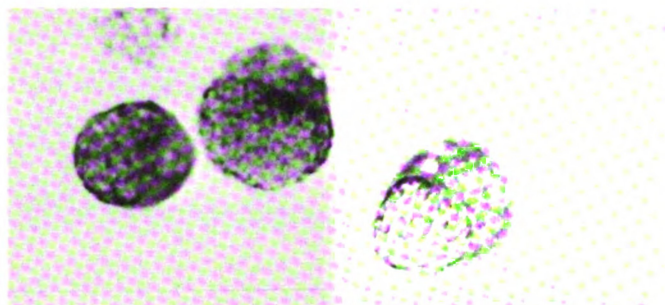


Figure 3

Figure 4. Liver of chicken inoculated with JMV-S; a focus of lymphoblastic tumor cells is shown. H & E X 390

Figure 5. Liver of chicken inoculated with JMV-S; a focus of lymphoblastic tumor cells (curved arror) infiltrated with small mononuclear cells (straight arrow) is shown. H & E X 630

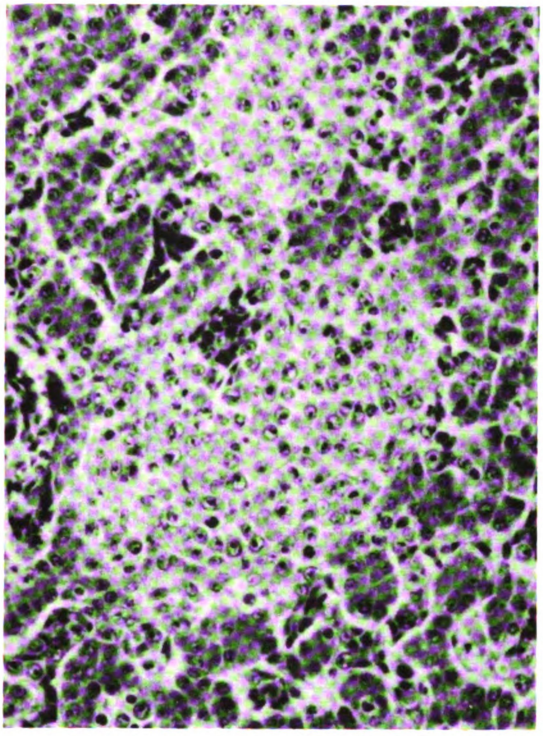


Figure 4

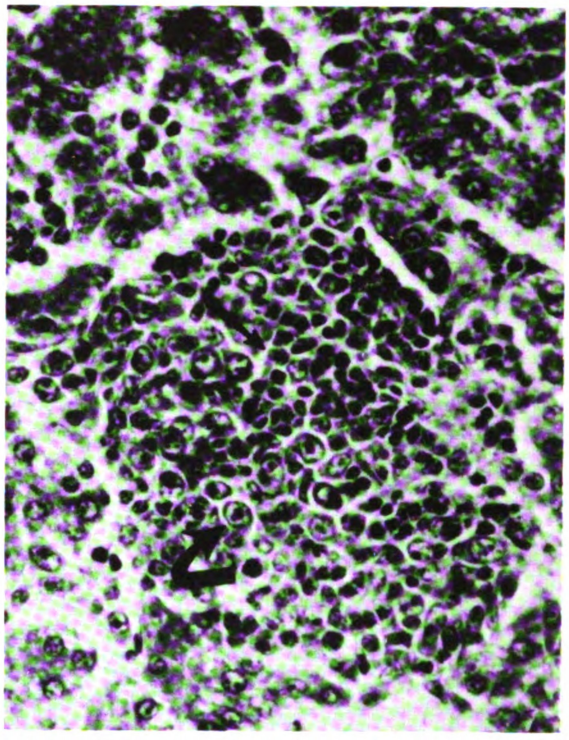


Figure 5

Table 1. Titration of the JMV-S stock liver homogenate stored at -196°C.

Dilution	Host line	# Pos./ total	Mortality Mean days to death	Range (days)
10^{-1}	SPF 7_2	7/7	7.7	7 - 9
10^{-2}		7/7	9.0	8 - 10
10^{-3}		5/7	9.2	9 - 10
10^{-4}		2/7	11.0	
10^{-5}		0/7	21.0[a]	
10^{-6}		0/7	21.0	
None		0/7	21.0	

SPF = Negative for antibody to MDV.

a. Experimental period was for 21 days.

Titer: CLD_{50} = 3.46/0.1 ml

Antilog = 2884 cld_{50}/0.1 ml

Table 2. Titration of the JMV-x462 stock liver homogenate stored at -196°C.

Dilution	Host line	# Pos./ total	Mortality Mean days to death	Range (days)
10^{-1}	SPF 7_2	4/4	7.2	7 - 8
10^{-2}		5/5	7.8	7 - 8
10^{-3}		4/4	8.2	8 - 9
10^{-4}		5/5	9.6	9 - 10
10^{-5}		3/5	11.3	11 - 12
10^{-6}		1/5	12.0	
None		0/5	21.0[a]	

SPF = Negative for MD antibody.

a. Experimental period was for 21 days.

Titer: CLD_{50} = 5.25/0.1 ml

Antilog: = 178,000 cld_{50}/0.1 ml

Table 3. Titration of the JMV-x55 stock liver homogenate stored at -196°C.

Dilution	Host line	# Pos./ total	Mortality / Mean days to death	Range (days)
10^{-1}	SPF 100	5/5	6.8	6 - 7
10^{-2}		5/5	8.8	8 - 10
10^{-3}		4/4	10.0	
10^{-4}		4/4	10.2	10 - 11
10^{-5}		4/5	11.5	10 - 13
10^{-6}		0/5	21[a]	
None		0/5	21	

SPF - Negative for MD antibody.

a. Experimental period was for 21 days.

Titer: CLD_{50} = 5.375/0.1 ml

Antilog: = 237,000 cld_{50}/0.1 ml

Herpesvirus isolation. In order to determine whether JMV tumor cells were actively infected with a herpesvirus, extensive efforts were made to isolate an MD-type herpesvirus from the three sources of JMV tumor cells. Direct kidney cell cultures of 5×10^6 cells per plate and 2×10^6 to 10^7 spleen tumor cells were co-cultivated on CEF and DEF, respectively, and were found negative for typical herpesvirus morphological changes throughout their experimental period (Table 4). Since 10^2 to 10^5 MD tumor cells were shown to be sufficient to induce microplaques in cell culture (Churchill and Biggs, 1967), the number of JMV tumor cells presumably would have been enough to initiate cytopathological changes. To demonstrate that the cultures were capable of expressing MDV plaques, control cultures of each type were superinfected with 1×10^3 PFU of JM/102W and were shown to develop characteristic foci. Coverslips included at the first and third passages were negative for MDV antigen when stained by indirect immunofluoresence with MDV or HVT antisera. Tissue cultures inoculated with JMV tumor cells failed to reproduce JMV or MDV lesions in day-old chicks, indicating that the infectious property associated with JMV tumor cells was not being propagated or maintained in cell culture after 7 or 21 days. There were no typical lesions of herpesvirus MD infection throughout the 21 day experimental period as determined by gross and histological examination. Gamma-irradiated tumor cells also failed to yield a latent herpesvirus based on the methods used in these experiments.

The development of virus lesions on the CAM of chick embryos inoculated with MDV material (Figure 6) via the yolk-sac has been

Table 4. Footnotes.

[a]Donor chicks were inoculated at 1-day of age and tissues harvested at 7 days after inoculation from moribund chicks.

[b]Kidneys were removed from moribund chicks 7 days after inoculation, trypsinized and plated in 5 dishes at 5 x 10^6 cells per dish and maintained for 7 days. Spleens were removed from the same donors, single cell suspensions prepared by gently teasing the tissue with a scalpel. The cells were inoculated on to CEF and DEF 24 hour monolayers at 2 x 10^6 and 1 x 10^7 spleen cells, respectively, 5 replicate plates per donor spleen and maintained through 3 passages or 21 days.

[c]Coverslips were included in each culture and examined at the first and last passage for evidence of MD antigen.

[d]Spleen cell suspensions were prepared from 9 moribund donors at 7 days after inoculation, pooled and adjusted to 1 x 10^8 cells/ml and irradiated at 5000r for 13.1 minutes at 1 meter from a cobalt 60 source. The irradiated cells were then inoculated on to 5 replicate dishes each of CEF at 2 x 10^6 and DEF at 1 x 10^7 cells per plate.

[e]Clone JM/102W was stored as DEF suspension at -196°C. DEF 24 hour monolayer cultures containing coverslips were inoculated with 10^3 PFU per dish, 5 dishes per vial tested.

Abbreviations:

CKC = Chick kidney cell culture

CEF = Chick embryo fibroblasts

DEF = Duck embryo fibroblasts

PFU = Plaque forming units

FA = Fluorescent antibody test

MDV = Marek's disease virus

HVT = Herpesvirus of turkey

CPE = Cytopathologic effect

Symbol:

γ = Gamma-irradiation

Table 4. Attempts to rescue an MD-like herpesvirus from JMV tumor cells.

Agent	Number of tumor donors[a]	Type of Tissue harvested	Techniques for detecting virus from harvested tissue				
			Cell type[b]	CPE	FA[c] MDV	FA[c] HVT	In vivo assay of cell culture material in 1-day old line 100 chicks
JMV-S	3	Kidney	CKC	-	-	-	0/5
		Spleen	CEF	-	-	-	0/5
			DEF	-	-	-	0/5
JMV-x462	6	Kidney	CKC	-	-	-	0/5
		Spleen	CEF	-	-	-	0/5
			DEF	-	-	-	0/5
JMV-x55	5	Kidney	CKC	-	-	-	0/5
		Spleen	CEF	-	-	-	0/5
			DEF	-	-	-	0/5
γ JMV-S[d]	9	Spleen	CEF	-	-	-	ND
			DEF	-	-	-	ND
JM/102W[e]	4	DEF	DEF	+	+	+	ND

Figure 6. Virus induced lesions on the CAM of line 6_3 embryo inoculated at 4 days of age via the yolk-sac with Marek's disease virus.

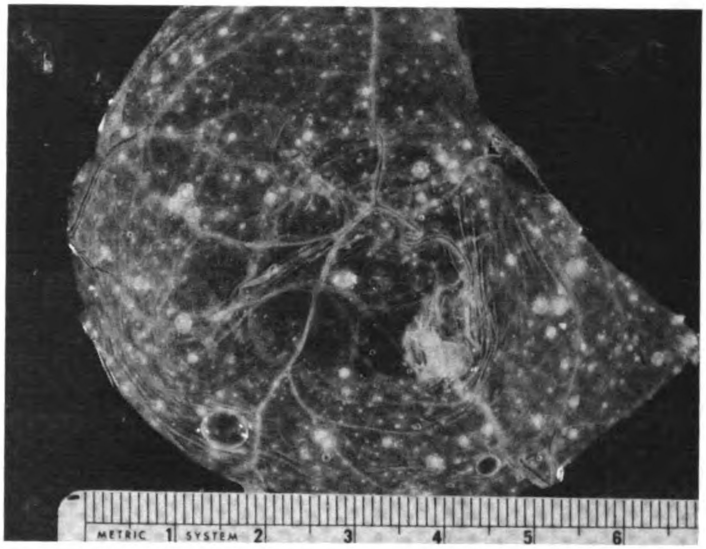


Figure 6

previously reported (von Bülow, 1968, 1971; Biggs and Milne, 1971; Churchill, 1968), and has been shown to be nearly as sensitive as cell culture assay (Biggs and Milne, 1971). Stock liver homogenates of JMV-S and JMV-x55 failed to produce lesions on the CAM (Table 5).

An 8 week in vivo experiment was designed to detect the presence of MDV in the JMV stocks (Table 6). Chicks surviving an initial low dose at 1-day of age were given a second challenge with 10^4 viable JMV tumor cells at 4 weeks of age. There were no chickens diagnosed for MD throughout the 8 week experimental period. Sera obtained at 8 weeks were negative for MD specific precipitin and fluorescent antibodies. All lots were maintained in separate isolators. Only lot 5, which had been inoculated with 10^3 PFU of JM/102W, was positive for MD antibody. Lot 4 inoculated with 10 PFU JM/102W and 5.7×10^4 JMV tumor cells simultaneously was included to determine whether a low dose of MDV would have sufficient time to infect contact chicks in the same isolator before the high dose of JMV tumor cells killed the chicks. The results indicate that a low level of MDV would not be detected in birds dying from the JMV tumor cells during the first 7 days, but would possibly be detected in those birds surviving a sub-lethal dose of JMV tumor cells.

Attempts to transmit the disease with cell-free filtrates of JMV-S, JMV-x462 and JMV-x55 spleen tumor cells and feather tip filtrates from JMV donors were unsuccessful (Table 7). The infectivity of JMV tumor cells appeared to be highly dependent upon intact cells. Similar material from JM-MDV infected birds did demonstrate the cell-free infectious virus associated with the feather follicle epithelium.

Table 5. Demonstration of the inability of JMV spleen cells to induce MD herpes-like lesions on the chorioallantoic membrane of line 6_3 (MD antibody negative) embryos.

Route of Inoculation	Duration of test	Tumor material			Number of embryos examined	Lesion response
		Agent	Inoculum	Dose[b]		
Yolk-sac[a]	14 days	JMV-S	Liver	$10^{2.8}CLD_{50}$	10	0/10
		JMV-x55	Liver	$10^{4.4}CLD_{50}$	10	0/10
		JMV-x55	Liver	$10^{3.4}CLD_{50}$	10	0/10
		JM/102W	Blood	0.01 ml	7	7/7
		None	Blood	0.01 ml	7	0/7

[a]Yolk-sac inoculation of 4-day-old embryonated eggs has been used as a standard assay for virus (von Bülow, 1971).

[b]The dose of liver tumor cells was based on previous titration in chicks (Tables 1 and 3) and a preliminary titration in embryos. The dose for JM-MDV and normal blood was based on previous data (Stone, unpublished).

Table 6. Attempts to demonstrate MDV herpesvirus and antibody in chicks inoculated with JMV tumor cells.

Agent	Lot	Duration of test	Inoculum cells	Dose[a]	MDV antibody in survivor serum AGP	FA
JMV-S	1	8 weeks	Liver	10^4	-	-
JMV-x462	2		Liver	10^4	-	-
JMV-x55	3		Liver	10^4	-	-
JMV-S and JM/102W[b]	4		Liver DEF	5.7×10^4 10 PFU	-	-
JM/102W	5		DEF	10^3 PFU	+	+

[a]JMV tumor cells were given at 4-weeks of age after an initial sublethal dose of 0-600 cells or about 0-50CLD_{50} for each JMV source.

[b]JMV and JM/102W were inoculated simultaneously.

Table 7. Cell-associated nature of JMV spleen tumor cells and feather follicle extract.

Agent	Donor bird		Spleen cells			Feather tip extract
	Age(days)	Gross	Intact	Sonicate	Sonicate-filtrate	
JMV-S	7	+	4/5[a]	0/4	0/5	0/4
JMV-x462	7	+	ND[b]	0/5	0/3	0/4
JMV-x55	7	+	4/5	0/5	0/5	0/5
JM/102W	22	+	1/1	1/5	0/5	4/5

[a]The fraction indicates the number of line 100 (MD antibody negative) chicks dead or killed with typical lesions of JMV tumor or MD per total chicks at risk. Chicks dead from nonspecific causes were excluded from the data. The experimental period was for 28 days.

[b]ND means no data available because all inoculated chicks died of nonspecific causes.

Assays for other viruses. Tissue cultures inoculated with JMV tumor cells (described in Table 4) were examined for other avian tumor viruses. Attempts to detect REV antigen by indirect immunofluorescence with positive REV serum were unsuccessful. All three stocks of JMV tumor cells, as well as gamma-irradiated JMV-S cells were examined for avian leukosis viruses and found to be negative by the phenotypic mixing (PM) test. Sera obtained from hyperimmunization with JMV tumor cells were also negative for antibodies to RSV subgroups A through D as indicated by the inability of the serum to neutralize virus. The sera were also negative for REV antibodies by the indirect immunofluorescence test on positive acetone-fixed coverslip antigen. Hyperimmune anti-JMV serum did not react with acetone-fixed coverslip antigen from JMV inoculated tissue cultures as might have been expected if the JMV stocks contained an agent capable of replicating in both cultures and the host chicken (Table 8).

Analysis of anti-JMV sera for herpesvirus antibodies. Sera from birds hyperimmunized with JMV tumor cells were analyzed by various methods (Table 9). The sera were negative for MDV specific antibody as indicated by the indirect immunofluorescence, agar-gel precipitin and serum neutralization tests. Viable cells of the MSB-1 cell line stained with certain of the JMV sera by membrane immunofluorescence of unfixed cells in suspension exhibiting a distinct annular fluorescence. A small number of the cells did not stain. Those sera expressing MATSA antibody with MSB-1 cells also appeared to protect chicks inoculated at 1-day of age with 50 CLD_{50} of the homologous JMV liver suspension (Table 9).

Table 8. Summary of tests for detection of viral agents other than herpesvirus in JMV tumor cells.

Source material tested	Tests Conducted					
	PM[a]	SN[b]	CPE[c]		FA[d]	
			REV	Unspecified	REV	Unspecified
JMV-S	-	-	-	-	-	-
γ JMV-S	-	ND	-	-	-	-
JMV-x462	-	-	-	-	-	-
JMV-x55	-	-	-	-	-	-
Positive controls	+	+	+	NA	+	NA

[a]The phenotypic mixing test (PM) was done to detect the presence of lymphoid leukosis viruses. A positive test would be any plate containing foci as compared with the A, B, C, and D subgroups serving as positive controls. RSV-Q (negative control) on C/E cells gave no foci count.

[b]A serum neutralization test was done to detect antibody to RSV of subgroups A through D in JMV hyperimmune sera. The sera did not neutralize virus of subgroups A through D as compared with positive anti-A through D sera.

[c]Spleen inoculated CEF cell cultures were examined through 3 passages (see Table 1) for any evidence of REV or an unspecified agent replicating in cell culture. No evidence of CPE was observed.

[d]Acetone-fixed coverslips from the third passage were examined by indirect fluorescent antibody technique with positive RE serum diluted 1:20 and unknown JMV antiserum diluted 1:20. REV positive CEF antigen stained with RE positive serum at 1:640, but not with negative serum diluted 1:20. There was no fluorescence observed with anti-JMV serum at 1:20.

NA = Not applicable

ND = Not done

Table 9. Analysis of hyperimmune sera to detect antibodies induced in response to antigens associated with JMV tumor cells.

Immunization[a]			Serology				
			MDV antibody[b]			MSB-1	Protection
Agent	Donor number	Number of inoculations	FA	AGP	SN	cells[c]	test[d]
JMV-S	1	3	<20	<1	<20	128	+
	2	3	<20	<1	<20	8	-
	3	3	<20	<1	<20	64	+
	4	1	<20	<1	<20	8	-
	5	1	<20	<1	<20	8	-
	6	1	<20	<1	<20	4	-
JMV-x462	7	3	<20	<1	<20	32	+
	8	3	<20	<1	<20	128	+
	9	3	<20	<1	<20	256	+
	10	3	<20	<1	<20	16	+
	11	1	<20	<1	<20	<4	-
	12	1	<20	<1	<20	<4	-
	13	1	<20	<1	<20	<4	-

[a]Twelve-week old 15 x 7 chickens maintained in Horsfall-Bauer units were inoculated with 69 and 178 CLD_{50} of JMV-S and JMV-X462 liver homogenate, respectively. Two additional inoculations were given with 100-fold greater doses at the 15th and 18th weeks after the initial inoculation. All chicks were bled at the 21st week.

[b]Reciprocal of the highest dilution of serum staining acetone-fixed MDV infected monolayer chick kidney cultures by immunofluorescence (FA), reacting with MDV precipitin antigen (AGP) or neutralizing 50 percent or more of cell-free feather follicle derived MDV at a final dilution of 1:20 (SN).

[c]The data represent the reciprocal of the highest dilution of serum to give detectable membrane fluorescence on MSB-1 cells in suspension (MATSA).

[d]Protection indicated by the ability of one ml of undiluted hyperimmune (MATSA positive) sera given at 1 day of age to prevent death in at least 75 percent of chicks challenged at the 2nd day with 50 CLD_{50} of the homologous JMV liver suspension. Death in control groups receiving normal serum before JMV challenge was 100 percent.

The actual results of the protection test are presented in Tables 10 and 11. The specificity of the sera for MD tumor cells and the MSB-1 cells has recently been reported (Witter et al., 1975) and suggests the possible existence of tumor-specific antigens common to both types of tumor cells.

Analysis of JMV tumor cells for herpesvirus associated antigens. JMV tumor cells were examined for membrane antigens (MA) and for MATSA by indirect membrane immunofluorescence (Table 12). JMV tumor cell preparations were negative for virus specific MA but did express a distinct annular surface fluorescence when stained with MATSA positive JMV-S antiserum (Figure 7). Staining was confined to the large lymphoblastic cells of the JMV and MSB-1 cell suspensions. Sera specific for MDV and HVT antigens did not stain the tumor cells but their specificity was shown by their reactivity with virus infected DEF cell culture antigen. Normal spleen cells in the preparation of JMV tumor cells did not stain with MATSA serum, nor did normal serum react with JMV or MSB-1 cells, thus excluding the possibility that the reaction is due to immunoglobulin producing cells.

B and T cell surface markets. JMV-S tumor cell suspensions were stained with antisera produced specifically against chicken B and T cells (Table 13). In three trials the JMV cells were stained strongly with anti-B serum and were equal in intensity to the staining observed with anti-B serum against bursa cells. Anti-T serum also stained JMV cells but to a lesser degree than the homologous system with anti-T serum and thymus cells. Although the intensity of staining seemed to

Table 10. Protection test with JMV-S hyperimmune sera.

Serum No.	No. of immunizations	JMV response[a]	Mortality		No. of Survivors	Protection[b]
			Mean days	Range		
10538	3	0/4			4	+
10539	3	2/4	12		2	-
10540	3	0/2			2	+
10543	1	1/4	10		3	+
10544	1	3/5	12		2	-
10545	1	4/4	11.7	11-13	0	-
Negative		3/3	10		0	-
None[c]		5/5	10		0	-

[a]The JMV response was measured in MD antibody negative line 100 chicks.

[b]Protection was based on 75 percent survivors as compared with the negative serum. Lack of sufficient serum prevented larger numbers of chicks per group.

[c]JMV challenge at 2-days of age was 50 CLD_{50} for all groups. Chicks were given one ml of the respective antiserum at 1-day of age.

Table 11. Protection test with JMV-x462 hyperimmune sera.

Serum No.	No. of immunizations	JMV response[a]	Mortality		No. of Survivors	Protection[b]
			Mean days	Range		
10524	3	1/5	13		4	+
10525	3	1/4	13		3	+
10526	3	4/4	10.7	9-12	0	-
10528	3	0/2			2	+
10529	3	0/3			3	+
10533	1	3/4	11.3	11-12	1	-
10534	1	5/5	10.2	9-12	0	-
10535	1	5/5	10.6	11-13	0	-
Negative		6/6	9.7	9-11	0	-
None[c]		5/5	10.0		0	-

[a]The JMV response was measured in MD antibody negative line 100 chicks.

[b]Protection was based on 75 percent survivors as compared with the negative serum control. Lack of sufficient serum prevented larger numbers per group.

[c]JMV challenge at 2-days of age was 50 CLD_{50} for all groups. Chicks were given one ml of the respective antiserum at 1-day of age.

Table 12. Analysis of JMV cells for membrane antigen and tumor associated membrane antigen.

Cells	Percent lympho-blasts[a]	Percent of cells fluorescing after treatment with: anti-MDV serum (MA)	anti-HVT serum (MA)	anti-JMV serum (MATSA)	normal serum
JMV-S spleen	74	0	0	85.3 ± 5.0[b]	0
JMV-x462 spleen	57	0	0	59.3 ± 1.7	0
JMV-x55 spleen	47	0	0	57.0 ± 7.7	0
MSB-1	100	0	0	91.2 ± 3.4	0
Normal spleen	ND	0	0	0	0
JM/102W DEF[c]	ND	++[d]	+	0	0

Abbreviations:

MA = MDV membrane antigen
MATSA = Marek's disease tumor-associated surface antigen
ND = Not done

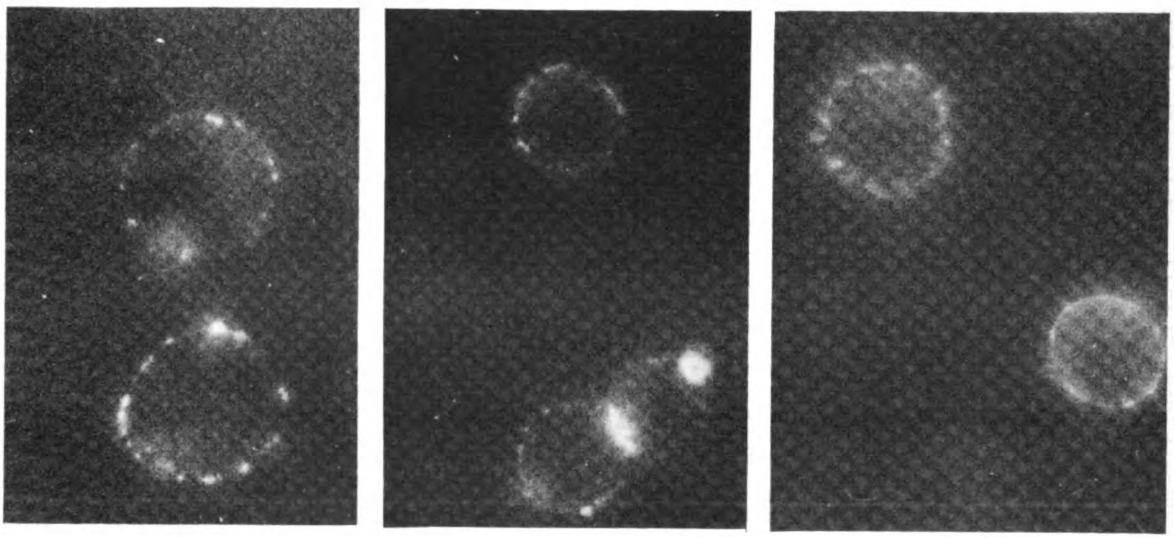
[a]Number of cells estimated by counting large lymphoblastic cells in a hemocytometer.

[b]Mean percent positive cells in 5 replicate samples of at least 50 cells ± the standard deviation.

[c]Monolayer cells not counted.

[d]Counts on individual cells not possible since immunofluorescence was done on unfixed monolayer cultures. Staining scored as ++ = very strong; + = positive; and 0 = negative.

Figure 7. A, B, and C: Three fields illustrating Marek's disease tumor-associated surface antigen (MATSA) on lymphoblasts from spleen of a chicken inoculated with JMV-S and stained with anti-JMV serum by the indirect membrane immunofluorescent technique. X1580



a b c

Figure 7

Table 13. Analysis of JMV lymphoblasts for B and T cell surface antigenic markers.

Serum	Trial	Percent cells fluorescing (relative intensity)[a]			
		JMV tumor suspension[b]	Bursa cells[c]	Thymus cells[c]	Normal spleen cells[c]
Anti-B	1	48.6 (3+)	79.2 (3+)	0.0	16.0 (3+)
	2	92.5 (3+)	93.0 (3+)	0.0	20.4 (3+)
	3	95.3 (3+)	79.0 (3+)	0.0	ND
		$\bar{X}$ 82.7	$\bar{X}$ 74.3	$\bar{X}$ 0.0	$\bar{X}$ 18.2
Anti-T	1	67.2 (±)	4.5 (3+)	78.6 (3+)	15.2 (3+)[d]
	2	64.6 (±)	12.9 (3+)	67.4 (3+)	76.5 (3+)
	3	89.2 (±)	0.0	88.7 (3+)	ND
		$\bar{X}$ 59.9	$\bar{X}$ 5.0	$\bar{X}$ 78.2	$\bar{X}$ 45.8
Anti-JMV	1	84.2 (3+)	0.0	0.0	0.0
	2	65.3 (3+)	1.7 (3+)	0.0	0.0
	3	76.9 (3+)	1.3 (3+)	0.0	ND
		$\bar{X}$ 68.2	$\bar{X}$ 1.0		
Normal	1	0.0	0.0	0.0	0.0
	2	0.0	0.0	0.0	0.0
	3	0.0	0.0	0.0	0.0

[a]At least 100 cells counted per sample per trial. Relative intensity based on a scale from 0 to 4 +; faint reaction designated ± .
[b]JMV tumor suspensions prepared from tumorous spleens of 8 to 10-day-old line 7_2 of 7 x 100 chicks inoculated with JMV-S.
[c]Cells were from 3-week-old normal line 7_2 chickens.
[d]Low percent due to poor cell preparation.

indicate a B cell surface antigen, the staining with anti-T serum could not be ignored and therefore, the data appear to be inconclusive at this time.

Alloantigenic markers on JMV tumor cells. In an attempt to determine whether JMV tumor cells were of donor or host origin, antisera directed against B_2 and B_{21} B blood group (major histocompatibility) antigens were used. The analysis of tumors produced in $\underline{B}^{21}/\underline{B}^{21}$ line N and $\underline{B}^{2}/\underline{B}^{2}$ line 7_2 hosts are presented in Table 14. Both B_2 and B_{21} antisera reacted normally with their homologous small lymphocytes present in the JMV tumor preparations, as well as the homologous normal control chicks; however, the staining reaction of tumor cells in both lines differed from that of normal host lymphocytes. The transformed blast cells reacted to both B_2 and B_{21} antisera, but the staining of tumor cells with anti-B_2 serum was weak in comparison to the stronger staining with B_{21} antiserum. The data presented indicate that B blood group alloantigenic markers on JMV lymphoblastic tumor cells differ from those on lymphocytes of the host in which the tumor was produced and that they carry an alloantigen closely related, if not identical, to the B_{21} antigen.

DNA-cRNA hybridization. Table 15 represents preliminary results obtained by Dr. Lucy F. Lee. The data indicate that DNA extracted from 5 of 6 tumor cell suspensions having MATSA positive cells in excess of 40 percent hybridized to a degree with ^{3}H-labelled MDV-cRNA, i.e., the cells were found to contain MDV-DNA sequences. The relative amount of DNA varied from tumor to tumor.

Table 14. Attempts to demonstrate the transplantable nature of JMV-S tumor cells by differences in B blood alloantigenic markers on the surface of JMV tumor cells.

Antiserum	Recepient chicken Genotype	Line	% cells fluorescing (relative intensity)[a] JMV tumor cells: JMV lymphoblasts[b]	JMV tumor cells: JMV lymphocytes[c]	Control cells: Normal lymphocytes[d]
B_2	B^{21}/B^{21}	N	62.7 (±)	0	0
			42.8 (±)	0	0
			55.3 (±)	0	0
	B^2/B^2	7_2	41.1 (±)	40.4 (+)	27.6 (+++)
			61.8 (±)	64.0 (+)	ND
			58.2 (±)	ND	39.6 (+++)
B_{21}	B^{21}/B^{21}	N	52.8 (+)	25.0 (+)	67.2 (+++)
			100.0 (++)	73.3 (++)	88.1 (+++)
			81.2 (+)	66.6 (+)	ND
	B^2/B^2	7_2	59.2 (±)	0	0
			91.5 (++)	0	0

[a]At least 100 cells per sample were evaluated. Relative intensity based on scale of 0 to 4 +.

[b]Large, blast-type cells present in suspensions prepared from tumorous spleens of chicks inoculated with JMV-S

[c]Small, normal appearing lymphocytes present in suspensions prepared from tumorous spleens of chicks inoculated with JMV-S.

[d]Small, normal-appearing lymphocytes present in suspensions prepared from normal control chicks.

Table 15. Detection of MDV genome in JMV tumor cells by hybridization of JMV-DNA with ^{3}H-labelled MDV-cRNA.

DNA source	Pool number	# cells/ pool	% cells positive for MATSA[a]	CPM hybridized[b] rep 1	CPM hybridized[b] rep 2	MDV genome sequences
JMV-S						
spleen cells[c]	1	$10x10^9$	20	260	208	-
	2	$1.4x10^9$	42	410	370	±
	3	$2x10^9$	84	396	388	±
	4	$1x10^9$	70	565	543	+
	5	$2x10^9$	77	570	541	+
	6	$1x10^9$	74	600	574	+
Normal						
spleen cells[c]	1	$4x10^9$	0	312	320	-
	2	$1x10^9$	0	312	306	-
MDV-CEF[d]	1			2280	2200	+
Normal CEF	1			184	124	-
Calf thymus	1			250	212	-

Abbreviations:

MATSA = Marek's disease associated tumor specific antigen.

CPM = Counts per minute

MDV = Marek's disease virus

CEF = Chick embryo fibroblasts

Rep. = Replicate

[a]Indirect membrane immunofluorescence done with MATSA positive JMV antiserum.

[b]Replicate 10 ug samples of DNA were hybridized on filters with ^{3}H-labelled MDV-cRNA. Counts 20 percent or more above those of normal spleen cell DNA preparations were considered indicative of homology.

[c]Single cell suspensions from spleens pooled from 3 to 13 donor chickens.

[d]Chick embryo fibroblasts infected with the GA isolate of MDV was used as the positive control.

Electron microscopy. The cell suspensions prepared from portions of the spleen sections given to Dr. Keyvan Nazerian are described in Table 16. Although each preparation contained MATSA positive cells, there was no evidence of herpesvirus or any other virus (Figure 8).

Table 16. Description of JMV tumor cells analyzed by the electron microscope.

Agent	Donor	% MATSA positive lymphoblasts	EM
JMV-S	1	83	-
	2	50	-
	3	77	-
	4	81	-
	5	40	-
JMV-x55	1	37	-
	2	76	-
	3	65	-
	4	50	-
	5	70	-

Abbreviation:

EM = Electron microscope.

Figure 8. Electron micrograph of a JMV lymphoblast from the tumorous spleen of a chicken inoculated with JMV-S. X24,000

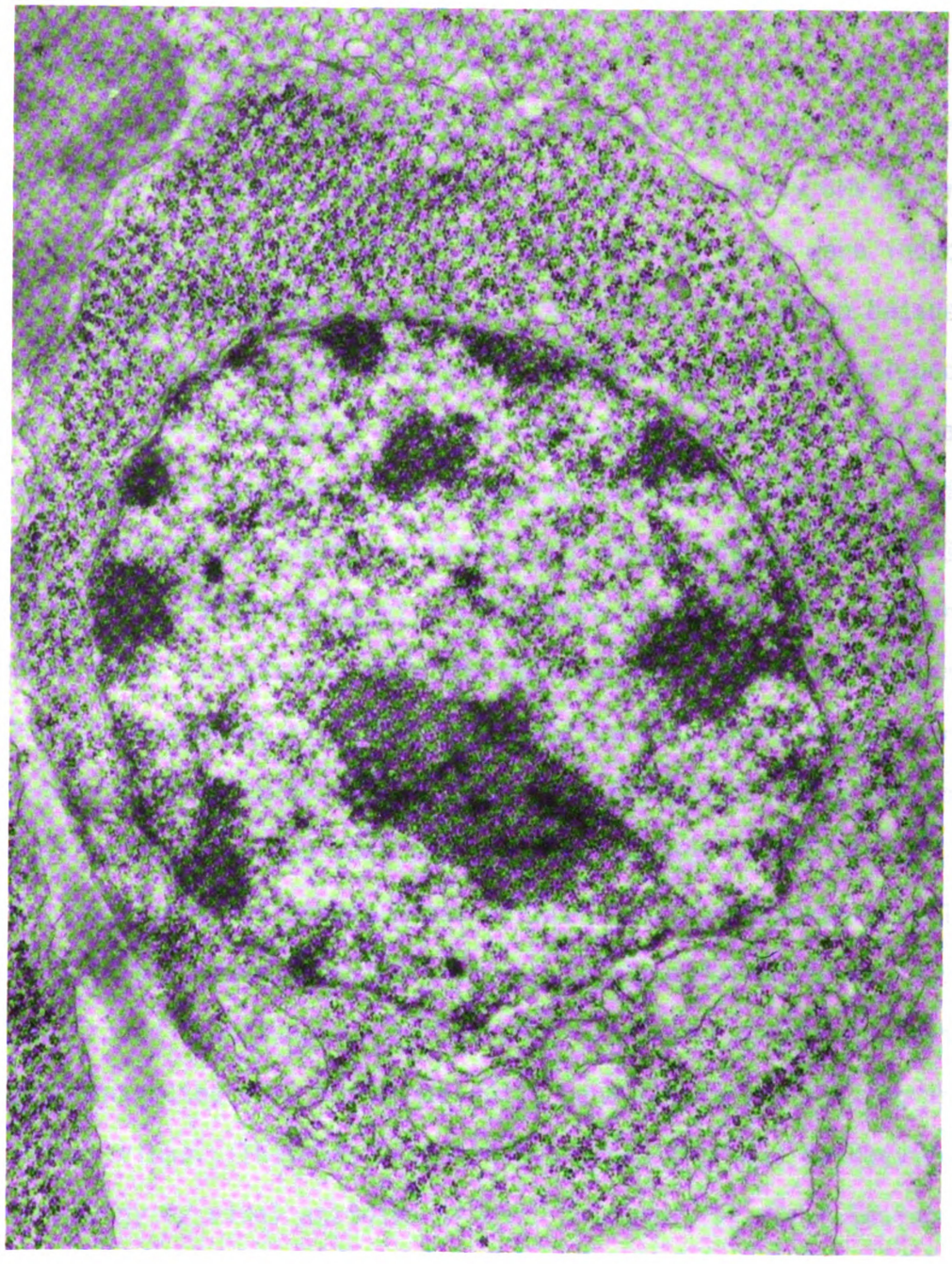


Figure 8

DISCUSSION

The major purpose of this research was to biologically characterize JMV and to determine whether lesions induced by JMV tumor cells were due to transplantation of the tumor cells or were induced by MDV released from the tumor cells. Confusion has arisen because MDV is a highly cell-associated virus and Sevoian (1964) developed JMV by repeatedly passaging in birds tumor cells that were originally induced by the JM isolate of MD. Yoon and Kenyon (1975) and Sevoian and others (Shieh and Sevoian, 1974, 1975; Hamdy and Sevoian, 1973; Kenyon et al., 1969; Kim et al., 1972) have considered that a highly virulent virus is released from JMV cells which induces the lesions observed. Spencer et al. (1973a) postulated that gross lesions prior to 14 days post inoculation were not virus induced but were due to cell transplantation and that survival was related to the host's ability to reject the transplanted cells. Lesions observed at a later stage (within 3 weeks post inoculation) were considered to be induced by MDV derived from inoculum cells. The results presented in this study demonstrated that JMV tumor cells were apparently lacking in infectious virus. This was based on the absence of (a) herpesvirus cytopathology or viral antigens in cell culture, (b) virus lesions in the CAM and (c) the production of lesions or immune response in susceptible chickens.

Probably the most sensitive method for establishing that JMV was free of MDV was the repeated inoculation of chickens with JMV cells in increasing doses and the subsequent analysis of the resulting sera for evidence of herpesvirus infection. Since it has been previously stated that 10^2 to 10^5 tumor cells are necessary to induce microplaques in cell culture (Churchill and Biggs, 1967), the number of JMV tumor cells assayed in either chickens or cell cultures certainly would seem sufficient to detect a herpesvirus. The data established that JMV cells must be intact in order to produce lesions. Furthermore no virus was isolated from feather follicle epithelium of JMV inoculated birds; whereas, virus was isolated from this site when birds were inoculated with the JM isolate of MDV. Jones et al. (1969) also reported that passage of JMV cell-free material was unsuccessful.

An association between JMV and MDV has been indicated by immunization studies that have demonstrated an antigenic relationship between JMV, HVT, and MDV. Sevoian (1967) demonstrated that offspring of dams immunized with JMV tumor cells had a higher degree of resistance to JMV and MDV and that blood from MD infected chickens adsorbed protective antibodies from anti-JMV serum. Mason and Jensen (1971) were able to demonstrate that HVT protected chicks inoculated 7 to 9 days later with the lethal JMV agent and suggested that the protection was a reflection of antigens being shared between JMV and HVT. Sevoian and Weston (1972) also reported that HVT vaccination induced protection against JMV challenge. By means of complement fixation, serum neutralization and neutralization kinetics, Hamdy and Sevoian (1973) reported that JMV appeared closely related to JM virus, but that HVT was

less antigenically related to JM and JMV. Their results indicated that the complement fixation titer of JMV serum with JM virus-specific antigen was comparable with the homologous JM reaction and that the serum neutralization titer of JMV serum with JM virus exceeded the homologous reaction. Spencer et al. (1973b) reported that vaccination with HVT 1 week prior to challenge with JMV significantly suppressed the development of wing web tumors. The inoculation of JMV tumor cells has also been shown to make chickens refractory to further challenge with higher doses of JMV tumor cells or virulent MDV (Sevoian, 1967; Kenyon et al., 1969; Hong and Sevoian, 1974; Shieh and Sevoian, 1974). The data presented here appears to corroborate these findings in that birds given an initial sub-lethal dose were able to survive increased doses of JMV cells. Analysis of the JMV sera indicated that there was no reaction with MDV infected cells by immunofluorescence or serum neutralization, nor with MD specific antigen in the immunoprecipitin test. In addition hyperimmune sera, produced by a series of increasing doses of JMV cells, appeared to react by membrane immunofluorescence to cell surface antigens on MSB-1 cells. The data also indicates that the titer of individual sera against MSB-1 cells seemed to relate to the ability of the sera to protect chicks against 50 CLD_{50} of JMV tumor cells. Although the numbers of chickens included in the protection tests were not sufficient to establish statistical significance, there did seem to be a trend that would warrant additional study. The limiting factor in the present study was the amount of immune sera available. The results on passive immunization against JMV challenge supports the work of Kenyon et al. (1969),

Sevoian (1967) and Hong and Sevoian (1974) who observed a neutralizing effect in serum of birds which had survived JMV. The nature of the neutralizing effect was indicated by an increased latent period and reduced mortality. The bi-phasic mortality associated with JMV reported by Spencer et al. (1973a) poses a question. In light of reports that inoculation of JMV cells appears to protect chicks against challenge with MDV, it is interesting that chicks did not appear to be protected by the initial JMV cell inoculation and were diagnosed with MD at the 5th week after inoculation. The possibility of adventitious infection of inoculum cells seems likely.

The mechanim by which passive immunization protects against JMV has not been established. However, the results presented in this research make clear that the effect was not due to neutralization of infectious MDV as implied by Shieh and Sevoian (1974). It must be pointed out from their data that the JMV preparation used in their study was quite different from the JMV tumor preparations used in this study. More than likely the antibody reported on here is directed against tumor specific antigens or histocompatibility antigens on the tumor cells or both.

Because there was no evidence that infectious MDV was associated with JMV, the possibility that JMV cells were transformed by an avian RNA tumor virus was considered. JMV cells were examined for exogenous avian tumor viruses of the A through D subgroups and for REV. Tests for these viruses proved negative for any evidence of infection. The possibility of endogenous tumor viruses was considered but since nearly all avian cells contain or express the ubiquitous

endogenous avian tumor virus genome, the value of such tests in determining the course of the original transformation of JMV cells was questionable. Also negative results were obtained from tests for an unspecified replicating agent that would replicate in cell culture and induce an immune response in chickens. These findings added to the other evidence that JMV cells were totally free of replicating virus.

The data tend to support the hypothesis of Spencer and Calnek (1967) that JMV tumors resulted from transplantation of inoculum cells. Their hypothesis was based on the sudden onset of the disease, tumors at the site of inoculation, and enlarged leukotic livers and spleens. These observations were confirmed in the present study. In order to lend further support to the hypothesis, the membrane immunofluorescence technique was used to demonstrate B blood group alloantigenic markers on JMV cells in an attempt to establish the transplantability of the cells. The B gene is concerned with histocompatibility (Schierman and Nordskog, 1961) and can be expressed through the function of the lymphocytes (Schierman and Nordskog, 1962). The antigens of the B and C system are common to lymphocytes and erythrocytes, while A, D, and L antigens are erythrocyte specific (Schierman and Nordskog, 1962). B antisera have been shown to react specifically with their B erythrocyte antigen, but not with other known alloantigenic markers (Pazderka et al., 1975). Based on the intensity of staining, JMV-S tumor cells appeared to carry antigens more closely related to B_{21}. The data suggest that JMV is a transplant and carries B blood group surface antigen detectable with anti-B_{21} serum. The

$\underline{B}^{21}$ allele has been associated with resistance to Marek's disease (Pazderka et al., 1975), a finding which makes the detection of the $\underline{B}^{21}$ allele on JMV cells important in tumor immunology and the study of regression. In this regard it is interesting that line N is homozygous for $\underline{B}^{21}$ and is resistant to MDV, but preliminary observations (unpublished data) indicate that line N is highly susceptible to JMV tumor induction.

The observation that JMV-S hyperimmune serum reacted with JMV and MSB-1 tumor cells, both derived from individual MD lymphomas, was considered evidence for the existence of a common tumor antigen since the antigen appeared unique to the tumor cell. This common antigen demonstrated on two classes of MD lymphomas in this study has now been called MATSA, an acronym for Marek's associated tumor specific antigen (Witter et al., 1975). The possibility that the common antigen was merely a histocompatibility antigen was considered; however, the reaction was confined to the lymphoblastoid cells and was not found on normal lymphocytes in the same JMV preparation. In addition the observation that normal spleen cells did not fluoresce with JMV or MSB-1 antiserum was considered evidence for the specificity of the reaction.

Additional work has been done at the Regional Poultry Research Laboratory on the specificity of this tumor antigen (Witter et al., 1975). As shown by the work presented here and by Witter et al. (1975) the lack of antibodies to viral antigens in MATSA sera may be related to the absence of MDV in JMV cells. It was also reported that MATSA

did not appear related to embryonic antigens contained in DEF and CEF since adsorption of JMV sera with normal DEF and CEF failed to reduce the MATSA activity of the sera. The specificity of the MATSA sera produced in chickens with JMV and rabbits with MSB-1 cells was shown by reaction with MD tumor cells of three classes; the MSB-1 cell line, MD lymphoma cells induced by the GA isolate, and JMV tumor cells. The sera did not react with REV tumor cells, RNA avian tumor virus transplant RPL-16 or with the transplantable lymphoid tumor (TLT) cell line (Nazerian et al., 1976). There were antigenic differences noted between MATSA of JMV and MSB-1 cells. From the titration of antisera on homologous and heterologous cells and by adsorption tests with homologous and heterologous antisera it was evident that a common antigenic determinant existed but that these antigens were not completely identical.

A similar type of cell surface antigen was reported by Powell et al. (1974) on cells from HPRS line 1 and MD lymphoma cells by using specific antisera raised in rabbits against suspensions of MD lymphoma cells. The high proportion of lymphoma cells and HRPS-1 cells staining with the anti-tumor serum also supports the finding of a tumor specific antigen.

The MATSA reported in this study and by Witter et al. (1975) and the tumor specific antigen reported by Powell et al. (1974) may explain the protection against JMV by another MD related herpesvirus, HVT, and MDV protection by JMV. HVT is currently used as a vaccine against Marek's disease (Okazaki et al., 1970) and its effectiveness appears to involve activation of host cell-mediated immunity (Purchase

and Sharma, 1974). The appearance of a common tumor-specific antigen on both JMV and MD tumor cells suggests a possible cell mediated response to antigens on the tumor cells and may imply a possible mechanism for HVT effectiveness.

The immunologic response demonstrated in the protection tests and the ability of anti-JMV serum to detect tumor specific antigen is indicative that the chicken is able to recognize the tumor antigen and illicit an immune response. The importance of humoral immunity in tumor regression is not known but it was observed that immunologically competent birds (4 to 6 weeks of age) could withstand increasing doses of JMV cells. If humoral antibodies were induced by the first injection of cells, these may have blocked the tumor antigenic sites on cells, thereby, preventing a cytotoxic or cell-mediated immune response from occurring. The occurrence of blocking antibodies has been previously reported (Hellström and Hellström, 1973).

Attempts to classify JMV tumor cells on the basis of B or T cell markers were not conclusive. The predominance of T cell antigenic markers on MD tumors and cell lines has been well documented (Rouse et al., 1973; Nazerian and Sharma, 1975; Powell et al., 1974; Powell and Rennie, 1974; Payne and Roskowski, 1972) and suggests that the transformed cells in MD tumors are T lymphocytes. In the three trials presented in this study, the intensity of staining on JMV cells with anti-B serum was consistently stronger than with anti-T serum on JMV cells, and compared with the homologous staining of anti-B serum and bursa lymphocytes. The weaker staining of JMV cells with anti-T serum could not be explained and differed from the homologous anti-T

serum with thymus cells. The variation from trial to trial in the percent of cells staining may have been due to differences in the conditions of the cells in the preparations. Staining of JMV cells was especially influenced by the stage of tumor development. For example there was a poor cell yield from necrotic spleens of birds near death. Because the data challenges the theory that only T cells are transformed in MD tumors, it will be necessary to conduct additional experiments before a conclusion may be drawn. Development of a JMV cell line maintained *in vitro* would provide a more homogeneous population of cells as opposed to the *in vivo* system. The *in vivo* system is confounded by many cell types in the lymphoma and both B and T cells may be present in tumor cells containing viral genome. Another explanation may be that the B cells are transformed and the T cells are present as part of the host's immune response.

The specificity of the MATSA on JMV cells was further substantiated by the contribution of Dr. Lucy F. Lee. The existence of MDV-DNA sequences in JMV cells was established by hybridization between DNA extracted from those JMV-S tumor preparations having greater than 40 percent MATSA positive cells and ^{3}H-labelled MDV-cRNA. The apparent lack of rescuable virus from JMV tumor cells may possibly indicate that JMV only has DNA sequences in common with MDV sequences that control induction of cell transformation. The application of BUDR or IUDR to JMV tumor cells may induce virus production as in the Raji cell line or MSB-1 cell line, in which case it could be assumed that the JMV tumor cells possessed complete genome. Although DNA hybridization studies demonstrated that at least part of the MD genome is in

JMV tumor cells, the use of gamma irradiation at 5000 r, which is sufficient to inhibit cellular proliferation, was not able to induce rescuable virus from the tumor cells.

The presence of MDV-like DNA sequences in JMV tumor cells and the lack of rescuable virus indicates a similarity with the non-producer Raji cell line, a human lymphoblastoid cell line derived from a Burkitt lymphoma (BL). The Raji cell line does not express Epstein-Barr virus (EBV) or viral antigens, viral capsid antigen or early antigen by the conventional methods of cell culture, egg inoculation or laboratory animal inoculation (Klein, 1973).

Thin section of BL tumor biopsy examined by electron microscopy is negative for virus (Klein, 1973). Similarly thin section of JMV spleen tumors expressing MATSA were also negative for virus particles. Complete viral genome was demonstrated by annealing Raji cell DNA with purified radioactive fragments of EBV-DNA (zur Hausen and Schulte-holthausen, 1972). Raji cells were made resistant to BUDR by incorporation of 100 ug of the drug into the culture medium for 6 months. After removal of the drug, repressed EBV particles were detected (Hampar et al., 1972). Incorporation of IUDR and BUDR into MSB-1 cells resulted in a higher number of cells actively producing virus, which indicated a close relationship between virus and host-cell DNA (Nazerian, 1976). Possibly the use of such drugs would induce JMV tumor cells to release complete virus.

The importance of this research has been in the biological characterization of the JMV sources used in this study and has pointed out that not all preparations of JMV may be as described in this study.

Two controversial issues have been discussed and clarified. First the claims that infectious virus was responsible for JMV lesions have been refuted by the various techniques used to detect virus or viral antigens in the three preparations of JMV. Second, evidence that JMV is an MD tumor cell appears to be established because JMV and MD tumor cells have a common MATSA and have MDV-DNA sequences in common. No virus could be recovered from JMV cells by *in vivo* or *in vitro* procedures possibly because JMV tumor cells lack complete MDV genome; whereas, virus could be recovered from MD tumor cells. Work with *B* blood group alleles has provided support that JMV is a transplant. It appears from these data that JMV is a non-productively infected transplantable cell lacking any rescuable virus. The establishment of a JMV tumor cell line in continuous culture would be useful in further studies on neoplastic transformation and immunity to Marek's disease.

SUMMARY

This paper deals with the characterization of the lymphoblastic cells of JMV Marek's disease tumor and their inter-relationship with the host cell. An experimental model using B blood group surface alloantigens to detect differences between tumor cells and host lymphocytes suggests the probable non-host origin (transplantibility) of the tumor. JMV tumor cells appear to be devoid of replicating herpesvirus and viral antigens, but possess MDV-DNA sequences and carry Marek's disease tumor-associated surface antigen (MATSA). These transformed cells possess or appear to possess B cell surface antigenic markers, although conclusive evidence is lacking. The appearance of B cell surface markers is in contrast to other MD transformed lymphoblastoid cell lines which have T cell surface antigenic markers. The importance of this work lies in three major areas. First, claims of infectious virus associated with JMV lesions have been refuted, but evidence for JMV as a class of MD tumor cell has been firmly established; these points have been highly controversial up to this time. Second, the finding of B cell markers on JMV cells may, if confirmed, constitute evidence contrary to a central theory of MDV-induced oncogenesis that only T cells can be transformed by MDV. Third, the characteristics established for this unique tumor cell now permit its use as a tool in further studies on MD.

RECOMMENDATIONS

Further investigations into the transformation of bursa-derived cells would substantiate the findings of this study. If indeed JMV tumor cells are transformed B cells, they would provide another tool for further study of neoplastic transformation.

The development of a JMV tumor cell line in continuous culture would provide a homogeneous population of cells from which more conclusive data could be obtained concerning the B or T cell origin of the tumor cells. Treatment of such cells with BUDR or IUDR would possibly induce the expression of virus particles, which would further establish the association of MDV with JMV tumor cells.

Further investigation into malignant transformation in the absence of complete virus should be done using JMV as a tool.

LITERATURE CITED

LITERATURE CITED

Agricultural Research Service, USDA; An Evaluation of Research on Lymphoid Leukosis and Marek's Disease, June. 1975.

Ahmed, M. and G. Schidlovsky: Electron Microscopic Localization of Herpes Virus Type Particles in Marek's Disease. J. Virol. 2: 1443-1457, 1968.

Akiyama, Y. and S. Kato: Two Cell Lines from Lymphomas of Marek's Disease. Biken J. 17: 105-116, 1974.

Akiyama, Y., S. Kato, and N. Iwa: Continuous Cell Culture from Lymphoma of Marek's Disease. Biken J. 16: 177-179, 1973A.

Akiyama, Y., M. Naito and S. Kato: Cell Surface Antigen and Viral Antigen of Marek's Disease Virus in Lymphoid Cells of the Spleen from Chickens Inoculated with Virulent Strains. Biken J. 16: 91-94, 1973 B.

Bankowski, R. A., J. E. Moulton, and T. Mikami: Characterization of the Cal-1 Strain of Acute Marek's Disease Agent. Amer. J. Vet. Res. 30: 1667-1676, 1969.

Beasley, J. N., L. T. Patterson, and D. H. McWade: Transmission of Marek's Disease by Poultry House Dust and Chicken Dander. Am. J. Vet. Res. 31: 339-344, 1970.

Benton, W. J. and M. S. Cover: The Increased Incidence of Visceral Lymphomatosis in Broiler and Replacement Birds. Avian Dis. 1: 320-327, 1957.

Biggs, P. M.: Marek's Disease. In The Herpesviruses. edit. A. S. Kaplan, Academic Press. pp. 557-594, N. Y. and London, 1973.

_____, A. E. Churchill, D. G. Rootes, and R. C. Chubb. The Etiology of Marek's Disease-An Oncogenic Herpes-Type Virus. Perspectives in Virol. 6: 211-237, 1968A.

_____ and B. S. Milne: Use of the Embryonating Egg in Studies on Marek's Disease. Am. J. Vet. Res. 32: 1795-1809, 1971.

_____ and B. S. Milne: Biological Properties of a Number of Marek's Disease Virus Isolates. In: Oncogenesis and Herpesviruses. Lyon, France, International Agency for Research on Cancer, Scientific Publications, No. 2, pp. 88-94, 1972.

_____ and L. N. Payne: Transmission Experiments with Marek's Disease (Fowl Paralysis). Vet. Rec. 75: 177-179, 1963.

_____ and _____: Transmission Experiments with Marek's Disease (Fowl Paralysis). Vet. Rec. 75: 177-179, 1963.

_____ and _____: Studies on Marek's Disease: I Experimental Transmission. J. Nat'l Cancer Inst. 39: 267-280, 1967.

_____, _____, B. S. Milne, A. E. Churchill, R. C. Chubb, D. G. Powell, A. H. Harris: Field Trials with an Attenuated Cell Associated Vaccine for Marek's Disease. Vet. Rec. 87: 704-709, 1970.

_____, H. G. Purchase, B. R. Bee and P. J. Dalton: Preliminary Report of Acute Marek's Disease (Fowl Paralysis) in Great Britain. Vet. Rec. 77: 1339-1340, 1965.

_____, R. J. Thorpe and L. N. Payne: Studies on Genetic Resistance to Marek's Disease in the Domestic Chicken. Brit. Poultry Sci. 9: 37-52, 1968B.

Calnek, B. W., H. K. Adldinger and D. E. Kahn: Feather Follicle Epithelium: A Source of Enveloped and Infectious Cell-Free Herpesvirus from Marek's Disease. Avian Dis. 14: 219-233, 1970A.

_____ and S. B. Hitchner: Localization of Viral Antigens in Chickens Infected with Marek's Disease Herpesvirus. J. Nat'l Cancer Inst. 43: 935-949, 1969.

_____ and S. H. Madin: Characteristics of In Vitro Infection of Chicken Kidney Cell Culture with a Herpesvirus from Marek's Disease. Am. J. Vet. Res. 30: 1389-1402, 1969.

_____, T. Ubertini and H. K. Adldinger: Viral Antigen, Virus Particles and Infectivity of Tissues from Chickens with Marek's Disease. J. Nat'l Cancer Inst. 45: 341-351, 1970B.

_____ and R. L. Witter: Marek's Disease. In: Disease of Poultry, M. S. Hofstad, ed., Iowa State Univ. Press, pp. 470-502, 1972.

Chen, J. H. and H. G. Purchase: Surface Antigen on Chick Kidney Cells Infected with the Herpesvirus of Marek's Disease. Virol. 40: 410-412, 1970.

Chomiak, T. W., R. E. Luginbuhl, C. F. Helmboldt and S. D. Kottaridis: Marek's Disease. I. Propagation of the Connecticut A (Conn-A) Isolate in Chicks. Avian Dis. 11: 646-653, 1967.

Chubb, R. C. and A. E. Churchill: Precipitating Antibodies Associated with Marek's Disease. Vet. Rec. 83: 4-7, 1968.

Churchill, A. E.: Herpes-type Virus Isolated in Cell Culture from Tumors of Chickens with Marek's Disease. I. Studies in Cell Culture. J. Nat'l Cancer Inst. 41: 939-950, 1968.

_____ and P. M. Biggs: Agent of Marek's Disease in Tissue Culture. Nature, Lond. 215: 528-530, 1967.

_____ and _____: Herpes-type Virus Isolated in Cell Culture from Tumors of Chickens with Marek's Disease. II. Studies In Vivo. J. Nat'l Cancer Inst. 41: 951-956, 1968.

_____, R. C. Chubb and W. Baxendale: The Attenuation with Loss of Oncogenicity of the Herpes-type Virus of Marek's Disease (Strain HPRS-16) on Passage in Cell Culture. J. Gen Virol. 4: 557-564, 1969A.

_____, L. N. Payne and R. C. Chubb: Immunization Against Marek's Disease Using a Live Attenuated Virus. Nature, 221: 744-747, 1969B.

Cole, R. K.: Studies on Genetic Resistance to Marek's Disease. Avian Dis. 12: 9-28, 1968.

_____: Proceedings of the Midwest Poultry Health and Management Symposium held at Purdue University, Lafayette, Ind., L. Arrington, ed., pp. 9-16. Compiled by Michigan State Univ. 1970.

Colwell, W. M. and S. C. Schmittle: Studies on Acute Marek's Disease. VII. Airborne Transmission of the GA Isolate. Avian Dis. 12: 724-729, 1968.

Drury, L. N., W. C. Patterson and C. W. Beard: Ventilating Poultry Houses with Filtered Air under Positive Pressure to Prevent Airborne Diseases. Poultry Sci., 48: 1640-1646, 1969.

Eidson, C. S. and S. C. Schmittle: Studies on Acute Marek's Disease. I. Characterization of Isolate GA in Chickens. Avian Dis. 12: 467-476, 1968A.

Eidson, C. S. and S. C. Schmittle: Studies on Acute Marek's Disease. V. Attempted Transmission of Isolate GA with Feces and Nasal Washings. Avian Dis. 12: 549-553, 1968B.

_____, D. J. Richey and S. C. Schmittle: Studies on Acute Marek's Disease. XI. Propagation of the GA Isolate in Tissue Culture. Avian Dis. 13: 636-653, 1969.

Gavora, J., J. L. Spencer, A. A. Grunder, A. Robertson and G. W. Speckmann: Genetic Resistance to Marek's Disease (MD): Response of Strains and Inbred Lines to Challenge with JM-V Tumor Cells or the BC-1 Isolate of MD Virus. XV World Poultry Congress, pp. 255-256, 1974.

Hamdy, F. and M. Sevoian: Immunologic Relationship between JM and JMV Leukosis Strains and Herpesvirus of Turkeys. Avian Dis. 17: 476-485, 1973.

Hampar, B., J. G. Derge, L. M. Martos and J. L. Walker: Synthesis of Epstein-Barr Virus after Activation of the Viral Genome in a "Virus-Negative" Human Lymphoblastoid Cell (Raji) Made Resistant to 5-Bromodeoxyuridine. Proc. Nat'l Acad. Sci. 69: 78-82, 1972.

Hellström, K. E. and I. Hellström: I. Lymphocyte Mediated Cytotoxicity and Blocking Serum Activity to Tumor Antigens. Advanc. Immunology. 18: 209-277, 1973.

Hong, C. C. and M. Sevoian: The Comparative Influence of Passive (Maternal) Antibodies in Early Exposure to JM Virus of Progeny Chicks of Dams Vaccinated with JM-V Leukosis Strain and Herpesvirus of Turkey. Avian Dis. 18: 305-317, 1974.

Hudson, L. and L. N. Payne: Analysis of T and B Cells of Lymphomas in Chickens. Nature New Biol. 241: 52-53, 1973.

Hutt, F. B. and R. K. Cole: The Development of Strains Genetically Resistant to Avian Lymphomatosis. Proc. 8th World's Cong. Copenhagen. pp. 719-725, 1948.

Ishikawa, T., M. Naito, S. Osafune and S. Kato: Cell Surface Antigen of Quail Cells Infected with Herpesvirus of Turkey or Marek's Disease. Biken J. 15: 215-222, 1972.

Ishizaki, R. and P. K. Vogt: Immunological Relationship Among Envelope Antigens of Avian Tumor Viruses. Virol. 30: 375-387, 1966.

Jakowski, R. M., T. N. Fredrickson, L. W. Schierman and R. A. McBride: A Transplantable Lymphoma Induced with Marek's Disease Virus. J. Nat'l Cancer Inst. 53: 783-789, 1974.

Jones, N. D., A. J. Kenyon, C. F. Helmboldt and M. Sevoian: Lymphoproliferative Diseases of Fowl: High LDH Levels Associated with Lymphoblastic Leukemia (JMV). Avian Dis. 13: 579-584, 1969.

Kaupp, B. F.: Paralysis of the Domestic Fowl. Am. Assoc. Instr. and Invest. Poultry Husbandry J. 7: 25-31, 1921.

Kenyon, A. J., M. Sevoian, M. Horwitz, N. D. Jones and C. F. Helmboldt: Lymphoproliferative Diseases of Fowl: Immunologic Factors Associated with Passage of a Lymphoblastic Leukemia (JMV). Avian Dis. 13: 585-595, 1969.

Kenzy, S. G., G. McLean, W. J. Mathey and H. C. Lee: Preliminary Observations of Game-Fowl Neurolymphomatosis. Nat'l Cancer Inst. Monograph 17: 121-130, 1964.

_____ and P. M. Biggs: Excretion of the Marek's Disease Agent by Infected Chickens. Vet. Rec. 80: 565-568, 1967.

Kim, S. N., J. W. Yoon, J. Olmsted and A. J. Kenyon: Chromosome Studies of Lymphoblastic Leukemia of Domestic Fowl (JMV). Fed. Proc. Abstract 31: 260, 1972.

Klein, G.: The Epstein-Barr Virus. In: The Herpesviruses. A. S. Kaplan, ed. Acad. Press. N. Y. and Lond. pp. 521-555, 1973.

Lee, L. F., E. D. Kieff, S. L. Bachenheimer, B. Roizman, P. G. Spear, B. R. Burmester and K. Nazerian: Size and Composition of Marek's Disease Virus Deoxyribonucleic Acid. J. Virol. 7: 289-294, 1971.

_____, K. Nazerian and J. A. Boezi: Marek's Disease Virus DNA in a Chicken Lymphoblastoid Cell Line (MSB-1) and in Virus Induced Tumors. In: Oncogenesis and Herpesviruses. Vol. 2, International Agency on Research for Cancer, Lyon, France. pp. 199-204, 1975.

_____, B. Roizman, P. G. Spear, E. D. Kieff, B. R. Burmester and K. Nazerian: Marek's Disease Virus: A Cytomegalovirus? Proc. Nat'l Acad. Sci. 64: 952-956, 1969.

Marek, J.: Multiple Nervenetzundung (Polyneuritis) bei Huhnern. Deut. tierarztl. Wochschr. 15: 417-421, 1907.

Mason, R. J. and K. E. Jensen: Marek's Disease: Resistance of Turkey Herpesvirus-Infected Chicks Against Lethal JMV Agent. Am. J. Vet. Res. 32: 1625-1627, 1971.

Möller, G.: Demonstration of Mouse Isoantigens at the Cellular Level by the Fluorescent Antibody Technique. J. Expt. Med. 114: 415-432, 1961.

Nazerian, K.: Further Studies on the Replication of Marek's Disease Virus in the Chickens and in Cell Culture. J. Nat'l Cancer Inst. 47: 207-217, 1971.

_____: Oncogenesis of Marek's Disease. Cancer Res. 33: 1427-1430, 1973A.

Nazerian, K.: Studies on Intracellular and Membrane Antigens Induced by Marek's Disease Virus. J. Gen Virol. 21: 193-195, 1973B.

_____: Induction of an Early Antigen of Marek's Disease Virus in a Lymphoblastoid Cell Line. In: Oncogenesis and Herpesviruses. Vol. 2. International Agency on Research for Cancer. Lyon, France. pp. 345-350, 1975.

_____: Interaction of Marek's Disease Virus with Host Cell. Proc. Int. Comparative Leukemia Symposium. Copenhagen. In Press, 1976.

_____ and B. R. Burmester: Electron Microscopy of a Herpes Virus Associated with the Agent of Marek's Disease in Cell Culture. Cancer Res. 28: 2454-2462, 1968.

_____ and L. F. Lee: Deoxyribonucleic Acid of Marek's Disease Virus in a Lymphoblastoid Cell Line from Marek's Disease Tumors. J. Gen Virol. 25: 317-321, 1974.

_____, _____ and J. M. Sharma: The Role of Herpesvirus in Marek's Disease Lymphoma of Chickens. Prog. in Med. Virol. In Press, 1975.

_____, T. Lindahl, G. Klein and L. F. Lee: Deoxyribonucleic Acid of Marek's Disease Virus in Virus-Induced Tumors. J. Virol. 12: 841-846, 1973.

_____ and H. G. Purchase: Combined Fluorescent Antibody and Electron Microscopy Study of Marek's Disease Virus-Infected Cell Culture. J. Virol. 5: 79-90, 1970.

_____ and J. M. Sharma: Brief Communication: Detection of T-Cell Surface Antigens in a Marek's Disease Lymphoblastoid Cell Line. J. Nat'l Cancer Inst. 54: 277-279, 1975.

_____, J. J. Solomon, R. L. Witter and B. R. Burmester: Studies on the Etiology of Marek's Disease. II. Finding of a Herpesvirus in Cell Culture. Proc. Soc. Expt. Biol. Med. 127: 177-182, 1968.

_____, B. J. Sprandel and H. G. Purchase: Localization of Marek's Disease Herpesvirus and its Antigens by Electron Microscopy and Fluorescent Antibody Techniques. Proc. Elect. Micro. Soc. Am. 27: 230-231, 1969.

_____ and R. L. Witter: Cell-Free Transmission and In Vivo Replication of Marek's Disease Virus. J. Virol. 5: 388-397, 1970.

Nazerian, K. and R. L. Witter: Properties of a Chicken Lymphoblastoid Cell Line from Marek's Disease Tumor. J. Nat'l Cancer Inst. 54: 453-458, 1975.

Okazaki, W., H. G. Purchase and B. R. Burmester: Protection against Marek's Disease by Vaccination with a Herpesvirus of Turkeys. Avian Dis. 6: 413-429, 1970.

_____, H. G. Purchase and B. R. Burmester: Phenotypic Mixing Test to Detect and Assay Avian Leukosis Viruses. Avian Dis. 19: 311-317, 1975.

Olmsted, J. and A. J. Kenyon: Lymphoproliferative Diseases of Fowl: Radiologic Evidence of Transplantable Nature of Lymphoblastic Leukemia (JMV). Avian Dis. 15: 672-679, 1971.

Pappenheimer, A. M., L. C. Dunn and V. Cone: Studies on Fowl Paralysis (Neurolymphomatosis gallinarium). I. Clinical Features and Pathology. J. Expt. Med. 49: 63-86, 1929.

Payne, L. N.: In: Oncogenesis and Herpesviruses. Pathogenesis of Marek's Disease--A Review. International Agency for Research on Cancer, Lyon, France. pp. 21-37, 1972.

_____, P. C. Powell and M. Rennie: Response of B and T Lymphocytes and other Blood Leukocytes in Chickens with Marek's Disease. Cold Spring Harbor Symposium on Quant. Biol. 39: 817-826, 1974.

_____ and J. Roszkowski: The Presence of Immunologically Uncommitted Bursa and Thymus Dependent Lymphoid Cells in the Lymphomas of Marek's Disease. Avian Path. 1: 27-34, 1972.

Pazderka, F., B. M. Longenecker, G. R. J. Law, H. A. Stone and R. F. Ruth. Brief Communication: Histocompatibility of Chicken Populations Selected for Resistance to Marek's Disease. Immunogenetics 2: 93-100, 1975.

Powell, P. C., L. N. Payne, J. A. Frazier and M. Rennie: T Lymphoblastoid Cell Lines from Marek's Disease Lymphomas. Nature 251: 79-80, 1974.

Purchase, H. G.: Marek's Disease Virus and the Herpesvirus of Turkeys. Prog. Med. Virol. J. L. Melnick, ed., Houston, Texas. S. Karger; Basel, Switzerland. Vol. 18, pp. 178-197, 1974.

Purchase, H. G.: Virus-Specific Immunofluorescent and Precipitin Antigens and Cell-Free Virus in the Tissues of Birds Infected with Marek's Disease. Cancer Res., 30: 1898-1908, 1970.

Purchase, H. G. and P. M. Biggs: Characterization of Five Isolates of Marek's Disease. Res. Vet. Sci. 8: 440-449, 1967.

_____ and G. H. Burgoyne: Immunofluorescence in the Study of Marek's Disease: Detection of Antibody. Am. J. Vet. Res. 31: 117-123, 1970.

_____, W. Okazaki and B. R. Burmester: Field Trials with the Herpesvirus of Turkeys (HVT) Strain FC 126 as a Vaccine against Marek's Disease. Poultry Sci. 3: 775-783, 1971.

_____, W. Okazaki and B. R. Burmester: Long-Term Field Trials with the Herpesvirus of Turkeys Vaccine against Marek's Disease. Avian Dis. 16: 34-44, 1972.

_____ and J. M. Sharma: Amelioration of Marek's Disease and Absence of Vaccine Protection in Immunologically Deficient Chickens. Nature, Lond. 248: 419-421, 1974.

Reed, L. J. and H. Muench.: A Simple Method of Estimating Fifty Percent Endpoints. Am. J. Hygiene 27: 493-497, 1938.

Rispens, B. H., F. Van Vloten and H. F. L. Maas: Some Virological and Serological Observations on Marek's Disease. Brit. Vet. J. 125: 445-453, 1969.

_____, H. Van Vloten, N. Mastenbroeck and H. J. L. Maas: Control of Marek's Disease Virus (Strain CV 1988) and its Use in Laboratory Vaccination Trials. Avian Dis. 16: 108-125, 1972.

Rouse, B. T., R. J. H. Wells and H. L. Warner: Proportion of T and B Lymphocytes in Lesions of Marek's Disease: Theoretical Implications for Pathogenesis. J. Immunol. 110: 534-539, 1973.

Schidlovsky, G., M. Ahmed and K. E. Jensen: Herpesvirus in Marek's Disease Tumors. Sci. 164: 959-961, 1969.

Schierman, L. W. and A. W. Nordskog: Relationship of Blood Type to Histocompatibility in Chickens. Sci. 134: 1008, 1961.

_____ and _____: Relationship of Erythrocyte Antigens in Chickens. Sci. 137: 3530, 1962.

Sevoian, M.: Immunity Studies in Chickens Inoculated with a Virulent Type II Avian Leukosis Strain (JMV). 39th Ann. Mtg. Northeastern Conf. Avian Dis. State Univ. N. Y., Long Island, 1967.

_____: Egg Transmission Studies of Type II Leukosis Infection (Marek's Disease). Poultry Sci. 47: 1644-1646, 1968.

Sevoian, M., D. M. Chamberlain and F. Counter: Avian Lymphomatosis. I. Experimental Reproduction of the Neural and Visceral Forms. Vet. Med. 57: 500-501, 1962.

_____, D. M. Chamberlain and R. N. Larose: Avian Lymphomatosis. V. Air-Borne Transmission. Avian Dis. 7: 102-105, 1963.

_____, R. H. Larose and D. M. Chamberlain: Avian Lymphomatosis. Increased Pathogenicity of JM Virus. Proc. 101st Ann. Mtg. Am. Vet. Med. Assoc. pp. 342, 1964.

_____ and C. R. Weston: The Effects of JM and JMV Leukosis Strains on Chicks Vaccinated with Herpes Virus of Turkeys (HVT). Poultry Sci. 51: 513-516, 1972.

Shieh, H. K. and M. Sevoian: Antibody Response of Susceptible and Resistant Chickens (Type II Leukosis) Infected with JM and JMV Leukosis Strains and Herpesvirus of Turkeys (HVT). Avian Dis. 18: 318-326, 1974.

Shieh, H. K. and M. Sevoian: Antibody Response of Genetically Susceptible and Resistant Chickens to Cell-Free and Attenuated JMV Leukosis Strain and its Influence on Early Type II (Marek's) Leukosis Infection. Poultry Sci. 54: 69-77, 1975.

Solomon, J. J., P. A. Long and W. Okazaki: Procedures for the In Vitro Assay of Viruses and Antibody of Avian Lymphoid Leukosis and Marek's Disease. A. R. S., U.S.D.A., U. S. Government Printing Office 0-415-9, 1971.

_____, R. L. Witter, K. Nazerian and B. R. Burmester: Studies on the Etiology of Marek's Disease. I. Propagation of the Agent in Cell Culture. Proc. Soc. Expt. Biol. Med. 127: 173-177, 1968.

_____, _____, H. A. Stone and L. R. Champion: Evidence Against Embryo Transmission of Marek's Disease Virus. Avian Dis. 14: 752-762, 1970.

Spencer, J. L. and B. W. Calnek: Storage of Cells Infected with Rous Sarcoma Virus or JM Avian Lymphomatosis Agent. Avian Dis. 11: 274-287, 1967.

_____ and B. W. Calnek: Marek's Disease: Application of Immunofluorescence for Detection of Antigen and Antibody. Am. J. Vet. Res. 31: 345-358, 1970.

_____, J. S. Gavora, A. A. Grunder and A. Robertson: Studies on the Development of JMV Tumors (Marek's Disease) in Non-Vaccinated or Vaccinated Chickens. Vth Cong. World Vet. Poultry Assoc. 510: pp. 200-210, Munchen, 1973A.

Spencer, J. L., J. S. Gavora, A. A. Grunder, A. Robertson and G. W. Speckman: Marek's Disease: Influence of Vaccination and Strain of Chickens on JMV Tumors. Abstract: 110th Ann. AVMA Mtg. Philadelphia, Penn. 1973B.

_____, _____, W. C. D. Hare, A. A. Grunder, A. Robertson and G. W. Speckmann: Studies on Genetic Resistance and On Vaccination-Induced Resistance of Chickens to Lymphoid Tumor Transplants. I. Marek's Disease Tumor Transplant (JMV). Avian Dis. in Press, 1976.

Stone, H. A.: Use of Highly Inbred Chickens in Research. Tech. Bulletin No. 1514. Agr. Res. Ser., U.S.D.A., 1975.

Theis, G. A., L. W. Schierman and R. A. McBride: Transplantation of Marek's Disease Lymphoma in Syngeneic Chickens. J. Immunol. 113: 1710-1715, 1974.

Ubertini, T. and B. W. Calnek: Marek's Disease Herpesvirus in Peripheral Nerve Lesions. J. Nat'l Cancer Inst. 45: 507-514, 1970.

Van der Walle, N. and E. Winkler-Junius: De Neuritis-Episectic bij Kippen to Barnveld in 1921. Tijdschr. Vergelijk. Geneesk. Gezendhleer 10: 34-49, 1924.

von Bülow, V.: Investigations with Embryonated Eggs in Marek's Disease. Berlin Muench. Tieraerzlf, Wochachr. 81: 365-367, 1968.

_____: Diagnosis and Certain Biological Properties of the Virus of Marek's Disease. Am. J. Vet. Res. 32: 1275-1288, 1971.

Wilner, B. I.: A Classification of the Major Groups of Human and Other Animal Viruses. 4th Ed. Burgess Publ. Co., Minn. pp. 1-250, 1969.

Witter, R. L.: Epidemiology of Marek's Disease. A Review. In: Oncogenesis and Herpesviruses. International Agency for Research on Cancer. Lyon, France, pp. 111-122, 1972.

_____, G. H. Burgoyne and B. R. Burmester: Survival of Marek's Disease Agent in Litter and Droppings. Avian Dis. 12: 522-530, 1968A.

_____ and B. R. Burmester: Transmission of Marek's Disease with Oral Washings and Feces from Infected Chickens. Proc. Soc. Expt. Biol. Med. 124: 59-62, 1967.

_____, G. H. Burgoyne and J. J. Solomon: Preliminary Studies on Cell Cultures Infected with Marek's Disease Agent. Avian Dis. 12: 169-185, 1968B.

Witter, R. L., G. H. Burgoyne and J. J. Solomon: Evidence for a Herpesvirus as an Etiologic Agent of Marek's Disease. Avian Dis. 13: 171-184, 1969A.

_____, I. Moulthrop, G. H. Burgoyne and H. C. Connell: Studies on the Epidemiology of Marek's Disease Herpes Virus in Broiler Flocks. Avian Dis. 14: 255-267, 1970A.

_____, K. Nazerian, H. G. Purchase and G. H. Burgoyne: Isolation from Turkeys of a Cell-Associated Herpesvirus Antigenically Related to Marek's Disease Virus. Am. J. Vet. Res. 31: 525-538, 1970B.

_____, H. G. Purchase and G. H. Burgoyne: Peripheral Nerve Lesions Similar to those of Marek's Disease in Chickens Inoculated with Reticuloendotheliosis Virus. J. Nat'l Cancer Inst. 45: 567-577, 1970C.

_____, J. J. Solomon and G. H. Burgoyne: Cell Culture Techniques for Primary Isolation of Marek's Disease Associated Herpesvirus. Avian Dis. 13: 101-118, 1969B.

_____, _____, L. R. Champion and K. Nazerian: Long-Term Studies of Marek's Disease Infection in Individual Chickens. Avian Dis. 15: 346-365, 1971.

_____, E. A. Stephens, J. M. Sharma and K. Nazerian: Demonstration of a Tumor-Associated Surface Antigen in Marek's Disease. J. Immunol. 115: 177-183, 1975.

Yoon, J. W. and A. J. Kenyon: Cytopathogenicity Induced by JMV Lymphoblastic Leukemia Virus. ASM Abstracts pp. 268, 1975.

zur Hausen, H. and H. Schulte-Holthausen: Detection of Epstein-Barr Viral Genome in Human Tumor Cells by Nuclei Acid Hybridization. In: Oncogenesis and Herpesviruses. International Agency for Research on Cancer. Lyon, France. pp. 321-325, 1972.

Typed and Printed in the U.S.A.
Professional Thesis Preparation
Cliff and Paula Haughey
144 Maplewood Drive
East Lansing, Michigan 48823
Telephone (517) 337-1527