MAREK'S DISEASE: VIRUS - HOST CELL RELATIONSHIPS IN VITRO AND IN VIVO, AND BIOLOGICAL MARKERS FOR CLONED PREPARATIONS OF THE VIRUS AND A HERPESVIRUS OF TURKEYS

Thesis for the Degree of Ph. D.

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HARVEY GRAHAM PURCHASE

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THESIS

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ABSTRACT

MAREK'S DISEASE: VIRUS-HOST CELL RELATIONSHIPS IN VITRO AND IN VIVO, AND BIOLOGICAL MARKERS FOR CLONED PREPARATIONS OF THE VIRUS AND A HERPESVIRUS OF TURKEYS

By

Harvey Graham Purchase

A specific, indirect fluorescent antibody test was developed for the detection of Marek's disease (MD) virus induced antigen in cell culture and antibody in the serum of chickens. The test has limited application for the detection of antigen. It is 10 to more than 320 times more sensitive than the agar gel precipitation test for the detection of antibody. Maternal antibody could be detected in the sera of 1 to 11 day old chicks from MD exposed dams after antibody was no longer detectable by the precipitation test. Actively acquired antibody in contact exposed chickens was detected earlier than by the precipitation test. Some sera that had a high titer of antibody in the fluorescent antibody test did not produce precipitation in agar. Eight isolates of MD virus could not be distinguished from one another by the indirect fluorescent antibody test which indicated that either the strains were antigentically identical or contained a common antigen or contaminant.

Cloned preparations of MD virus differed from one another in pathogenicity for chickens, antigenicity and effect on cell cultures. These three properties were independent of one another. All avian cells tested were susceptible both to the MD virus and to the herpesvirus of turkeys.

Antigens induced by MD virus were detected in the feather follicle epithelium, the lung, bursa of Fabricius, thymus, spleen and caecal tonsil of MD infected birds by both the direct fluorescent antibody and agar gel precipitation tests. However the antigens could not be detected in the tumors themselves by either test, although MD was readily transmitted with intact tumor cells. There was a direct relationship between the presence of antigen and the cells undergoing degeneration and necrosis. Intranuclear inclusion bodies were in the feather follicle epithelium where similar necrobiotic changes were taking place. Filtrable virus was found only in skin extracts and originated from the feather follicle epithelial cells where virus maturation and envelopement were completed. This epithelium is probably the portal of exit of the virus from the host and accounts for the highly infectious nature of MD.

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By

Harvey Graham Purchase

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LIST OF ABBREVIATIONS

AGP	=	agar gel precipitation
B14	=	P.M. Biggs' 14th strain of MDV which is of classical type from the HPRS
C_1	=	California one isolate is an acute strain of MDV from R. Bankowski, University of California, Davis, California
Cal-1	=	C_1
CAM	=	chorioallantoic membrane
CEF	=	chick embryo fibroblasts
CK	=	chick kidney
Conn A	=	Connecticut A isolate is a classical MDV from R. Luginbuhl, University of Connecticut, Storrs, Connecticut
Cornell S	=	Cornell Line of chickens susceptible to MD and bred by R. Cole, Cornell University, Ithaca, New York.
CR64	=	an acute isolate of MDV passed in chickens by W. Staples, Cobb Breeding Corporation, Connecticut in 1964 and isolated in cell culture by H. G. Purchase
DEF	=	duck embryo fibroblasts
DFA	=	direct fluorescent antibody
DK	=	duck kidney
DNA	=	deoxyribonucleic acid
FA	=	fluorescent antibody
FAPP	=	filtered air positive pressure

FC50	=	an acute isolate of MDV from field case 50 from Port Huron, Michigan by H. G. Purchase
GEF	=	goose embryo fibroblasts
HMD	=	high passage MDV i.e. the JM strain of MDV which had been passed over 40 times in CEF and DEF = JMHP
HPRS	=	Houghton Poultry Research Station, Houghton, Huntingdon, England
HPRS 16	=	acute
HPRS 17	=	classical
HPRS 18	=	acute
HPRS 20	=	acute

} isolates of MDV made by H. G. Purchase while on secondment to the HPRS

IBA	=	infectious bursal agent
IF	=	immunofluorescent
IFA	=	indirect fluorescent antibody
ILTV	=	infectious laryngotracheitis virus
JM	=	an acute isolate of MDV from M. Sevoian, University of Massachusetts, Amherst, Massachusetts
JM19,JM30,JM31, JM32,JM34,JM35,JM36	=	different cloned preparation of the JM strain of MDV
JMHP	=	HMD
JMN	=	a line of chickens bred for resistance to JM MD by three generations of selection by R. Cole, Cornell University, Ithaca, New York
JMP	=	a line of chickens bred for susceptibility to JM MD by three generations of selection by R. Cole, Cornell University, Ithaca, New York

JQEF	=	Japanese quail embryo fibroblasts
LMD	=	low passage MDV, i.e. MDV which had been passed less than 17 times in CEF and DEF. Included are GA, RPL39 and cloned preparation of JM MDV.
MD	=	Marek's disease
MDV	=	Marek's disease virus
MSD_1	=	Merck, Sharpe and Dohme one violate of MDV from T. Maag, Rahway, New Jersey
nm	=	nanometers
PBS	=	phosphate buffered saline
PhEF	=	pheasant embryo fibroblasts
PiEF	=	pigeon embryo fibroblasts
RPRL line 6	=	an RPRL inbred line of chickens resistant to MD and lymphoid leukosis tumor development but not resistant to virus infection
RPRL line 7	=	an RPRL inbred line of chickens susceptible to MD and resistant to subgroup A lymphoid leukosis virus infection. Bred by L. B. Crittenden and N. F. Waters
RPL	=	Regional Poultry Laboratory
RPRL	=	Regional Poultry Research Laboratory = RPL
RPL 39	=	RPL 39th identification, an acute isolate of MDV from a flock in Georgia, by H. G. Purchase
RSV	=	Rous sarcoma virus
TEF	=	turkey embryo fibroblasts
USDA	=	United States Department of Agriculture

INTRODUCTION

Marek's disease (MD) is a neoplastic disease of the hymphoid system of chickens of great economic importance. Infiltration and proliferation of lymphoid cells occurs in the nerves and visceral organs. The etiologic agent can be readily transmitted from affected birds to genetically susceptible chicks using whole blood or intact tumor cells. The agent is highly cell associated and treatments which destroy the viability of the cells also eliminate infectivity. However, in nature, MD is highly infectious and nearly all mature flocks are infected.

MD virus (MDV) produces characteristic cytopathic effects in avian epithelioid or fibroblastic cell cultures. When MDV is propagated in cell culture, antigens are produced which react in the agar gel precipitation (AGP) test with sera from birds that have been exposed to MDV. The AGP test can be used to detect antibody in sera from experimental birds and field flocks. As described in this thesis, the indirect fluorescent antibody (IFA) test was developed as an alternative method for detecting MDV antigens and antibody (Purchase, 1969, Article I; Chen and Purchase, 1970; Nazerian and Purchase, 1970; Purchase and

Burgoyne, 1970, Article II). This IFA test, however, failed to reveal comparative serologic differences between isolates of MDV.

At this stage in the understanding of MD, the author formally proposed that, since different isolates of MDV vary in their biological activity, distinguishable herpesviruses could be isolated both from clones derived from a single inoculum and from clones derived from different inocula. As reported in this thesis (Purchase et al., 1970a, Article IV; Purchase et al., 1970b, Article V) cloned preparations of MDV differed in pathogenicity, antigenicity and cytopathic effect in different cell types.

Concurrently, the author applied the direct fluorescent antibody (DFA) test to the detection of antigen in vivo. Antigen was found, among other locations, in the epithelial cells lining the feather follicle (Purchase, 1970a, Article III). This was the only location in the living host in which a significant amount of virus matured to its fully infectious form. Enveloped infectious virions, and both precipitating and immunofluorescent antigens could be found at this site, which is the portal of exit of MDV from the chicken. The discovery of this "missing link" in the life cycle of MDV infection established the herpesvirus as the etiologic agent of MD.

In the thesis below the important historical and current literature on MD is reviewed with emphasis on work that is relevant to the thesis research. The results of preliminary research are presented in the form of three published papers (Articles I, II, and III). The experimental efforts to substantiate the aforementioned formal proposal are described in two pre-publication manuscripts (Articles IV and V). Finally, the composite findings are discussed and drawn together as generalized conclusions.

LITERATURE REVIEW

Marek's disease (MD) is a common lymphoproliferative disease of chickens which affects the peripheral nerves and visceral organs. The syndrome was first described by Marek (1907) who, thinking it to be an inflammatory disease, called it "polyneuritis". More recent studies (Payne and Biggs, 1967) indicated that "Marek's disease is characterized by a neoplastic-like proliferation of lymphoid cells in the nerves and in other organs, notably the ovary".

Incidence and Distribution of MD

Marek's disease is of immense economic significance to the poultry industry and causes losses among chickens throughout the world, particularly in areas of intensive poultry production (Churchill and Biggs, 1967; Biggs et al., 1968a; Nazerian et al., 1968; Solomon et al., 1968). In the United States, annual losses attributable to "leukosis" (most of which now is considered to be MD) are estimated to be in excess of 150 million dollars (Calnek, 1967). Whereas the number of chickens slaughtered and inspected by the United States Department of Agriculture

(USDA) did not change significantly between 1961 and 1968, the number condemned from "leukosis" per 10 thousand inspected increased from 10 to nearly 200 (Goldstein, 1968). In 1968 leukosis replaced airsacculitis as the single most important cause of condemnation in young chickens inspected by the USDA. Marek's disease is not confined to young broilers. Severe losses have occurred in started pullets and in layers (Purchase et al., 1966; Purchase et al., 1969) where it has been confused with lymphoid leukosis (Purchase, 1965; Burmester and Witter, 1966; Siccardi and Burmester, 1970).

Etiologic Agent

Since 1926, attempts to transmit MD have met with varying degrees of success (Pappenheimer et al., 1926; Pappenheimer et al., 1929; Blakemore, 1939; Durant and McDougle, 1939; Durant and McDougle, 1945). An important contribution to the understanding of MD came from Biggs and Payne (1963 and 1967), who succeeded in serially transmitting the HPRS-B14 strain of MD virus (MDV). Purchase and Biggs (1967) then isolated and characterized four "acute" and one "classical" strains of MDV. The discovery in England (Churchill and Biggs, 1967) and in the U.S.A. (Solomon et al., 1968; Nazerian et al., 1968) of a herpesvirus closely associated with MD was a significant breakthrough. A large amount of data subsequently was accumulated, which circumstantially linked the virus to MD (Biggs et al.,

1968; Witter et al., 1969a). The discovery of the site of maturation of the virus (Calnek et al., 1970a; Nazeran and Witter, 1970; Purchase, 1970a, Article III) and transmission of the disease with cell-free virus preparations has left little doubt that the herpesvirus is the cause of MD.

MDV (Burmester et al., 1969; Witter et al., 1969a) belongs to the cytomegalovirus or B group of cell-associated herpesviruses (Lee et al., 1969). Enveloped virions from the feather follicles are 200-400 nm in diameter (Calnek et al., 1970a; Nazerian and Witter, 1970). The capsid has icosahedral symmetry with 162 hollow cylindrical capsomeres approximately 85-100 nm in diameter (Ahmed and Schidlovsky, 1968; Epstein et al., 1968; Nazerian et al., 1968; Calnek et al., 1970a). Complete particles have a centrally located, dense nucleoid approximately 65 nm in diameter. Virions are rarely observed in tumors (Schidlovsky et al., 1969; Calnek et al., 1970b). However, naked particles are sometimes in the nuclei of cells in the bursa of Fabricius (Calnek et al., 1970b) and occasionally in the nuclei of Schwann cells (Ubertini and Calnek, 1970). Complete, enveloped particles are frequently present in the nuclei and in cytoplasmic inclusions in cells of the feather follicle epithelium (Calnek, 1970a; Nazerian and Witter, 1970).

The deoxyribonucleic acid (DNA) of MD herpesvirus contains 56 to 57 moles percent of guanine and cytosine (Lee et al., 1969). The composition of the DNA and its lack of infectiousness in cell-free preparations of cell cultures also suggests that the virus belongs to the herpesvirus group B (Melnick et al., 1964; Wilner, 1969).

Strains of MD

Many different strains of MD have been described such as JM (Sevoian et al., 1962; Witter and Burmester, 1967), C_1 (Bankowski et al., 1969), GA (Eidson and Schmittle, 1968), Conn A (Chomiak et al., 1967), CR 64, RPL 39, FC 50, MSD_1 (Purchase, 1969), HPRS B14 (Biggs and Payne, 1963; Biggs and Payne, 1967), HPRS 16, HPRS 17, HPRS 18, HPRS 19, and HPRS 20 (Biggs et al., 1965; Purchase and Biggs, 1967). Herpesviruses have been identified in most of these strains and they do not appear to differ from one another serologically as measured by the AGP or IFA tests (Chubb and Churchill, 1968; Purchase, 1969). They do, however, differ greatly in the pathologic manifestations they induce in chickens. Some induce mainly visceral lesions, whereas others affect predominantly the nerves (Purchase and Biggs, 1967).

Host Range in Vivo and in Vitro

Chickens are the natural hosts but infection has been artificially transmitted to turkeys (Sevoian et al., 1963b; Witter et al., 1970a), pheasants (Harris, 1939; Johnson,

1941) and quail (Kenzy and Cho, 1969; Witter, 1970). However MDV could not be re-isolated from turkeys with MD tumors (Witter et al., 1970a). Lesions similar to those described for MD have been reported as occurring naturally in the duck (Cottral and Winton, 1953), goose, canary, budgerigar and swan (Wight, 1963). In one experiment sparrows were apparently refractory (Kenzy and Cho, 1969). Natural antibody to MDV could not be detected in pigeons, starlings, yellow hammer, sparrows, and pheasants but MDV could be re-isolated from ducks after artificial infection (Baxendale, 1969). The few attempts that have been made to infect mammals have been unsuccessful (Churchill, 1968; Calnek, 1970; Purchase, 1970c).

The MDV propagates well in chick kidney (CK) cells (Churchill and Biggs, 1967; Churchill, 1968) and duck embryo fibroblasts (DEF) (Solomon et al., 1968) and under certain circumstances in chicken embryo fibroblasts (CEF) where it may (Vindel, 1964; Kottaridis et al., 1968; Nazerian, 1968; Nazerian, 1970) or may not produce cytopathic effects (Witter et al., 1968a). Procedures for assaying MDV in CK and DEF have been thoroughly studied (Churchill, 1968; Calnek and Madin, 1969; Witter et al., 1969b). The MDV has also been reported to grow in pheasant embryo fibroblast cells (Baxendale, 1969). A thorough attempt to propagate this virus in a wide variety of mammalian cells was unsuccessful (Calnek et al., 1969).

Gross Pathology

The mildest or "classical" form of MD is characterised by paralysis of one or more of the extremities of 12 to 14 week old chickens. The motor function of any nerve may be affected so the symptoms vary. Incoordination is followed by paralysis of the leg which may result in a characteristic attitude in which one leg is stretched forward and the other backward. The wings, tail, neck or eyelids may droop and birds sometimes have respiratory difficulty. Morbidity and mortality are usually low and birds that die or are killed have enlarged gray nerves. In the most acute form, the disease may affect birds as early as 6 to 8 weeks of age and the only antemortem signs may be depression and anorexia. Morbidity and mortality may exceed 50% of the flock and birds that die have lymphoid tumors which affect most commonly the gonad, liver, lung and skin. The disease may also occur in older birds.

All degrees of severity of the disease may be encountered in the laboratory and in the field. The gross pathologic picture, morbidity, mortality and incubation period are all influenced by the particular isolate of the MD agent, the degree of exposure, the age at the time of exposure and the genetic constitution of the host. Examples of "classical" isolates are HPRS B14 (Biggs and Payne, 1963; Biggs and Payne, 1967), HPRS 17 (Purchase and Biggs, 1967) and Conn A (Chomiak et al., 1967) and

examples of "acute" isolates are HPRS 16, HPRS 18, HPRS 19, HPRS 20 (Purchase and Biggs, 1967) and GA (Eidson and Schmittle, 1968). Examples of genetically susceptible chickens are Cornell S and JMP lines (Cole, 1968), Regional Poultry Research Laboratory (RPRL) Line 7 (Crittenden, 1968) and Houghton Poultry Research Station (HPRS) Rhode Island Reds (Biggs and Payne, 1967; Purchase and Biggs, 1967). Examples of relatively resistant chickens are RPRL Line 6 (Crittenden, 1968), HPRS Brown Leghorns (Biggs and Payne, 1967; Purchase and Biggs, 1967) and the JMN line (Cole, 1968). Susceptibility to MD declines with age (Sevoian and Chamberlain, 1963; Biggs and Payne, 1967). Females are more susceptible to both the classical and acute forms of MD than males (Biggs and Payne, 1967; Purchase and Biggs, 1967).

Microscopic Pathology

The most characteristic lesions of MD occur in the nerves where there is an infiltration with lymphoid cells. Initially, the lesion resembles neuritis but later the size of the lesion and anaplasia of the cells are characteristic of a neoplastic-like process. Lesions of type I (Wight, 1962) or A type (Payne and Biggs, 1967) consist of small, medium and large lymphocytes, a few plasma cells and large dark staining cells which are thought to be degenerating lymphoblasts and are referred to as "MD cells". In lesions that appear neoplastic, large lymphocytes may

predominate but there is still considerable pleomorphism. Edema, myelin degeneration, and Schwann cell proliferation may also occur. Type II (Wight, 1962), B type (Payne and Biggs, 1967) or edematous type lesions contrast with the type I, A type, or proliferative lesions described above. They occur in older birds, or in long standing cases, where the majority of the cellular infiltration is replaced by edema and there may only be a few small lymphocytes and plasma cells. Payne and Biggs (1967) described another, C type, or mild lesion in which there was only a light infiltration by plasma cells and small lymphocytes. Lesions of this type occur in clinically normal older birds.

In the brain, the lesion is a nonpurulent encephalitis with perivascular cuffing with lymphocytes and occasional, small areas of gliosis and endotheliosis. In the eye, lesions are often demonstrable only by histologic examination. The most constant change is a mononuclear infiltration of the iris but infiltration of the eye muscles has also been found.

The lymphoid tumors of the visceral organs and skin are all composed of masses of pleomorphic lymphocytes, much like those in the A type neural lesions. In the liver, lesions consisting of collections of lymphoid cells begin around the portal tracts. The lesions then enlarge and eventually large areas of the liver are replaced by lymphoid tissue. In the heart and muscles, lymphoid infiltration

may be extensive causing degeneration and necrosis of the muscle fibers. Skin lesions begin as enlarged lymphoid foci in the subcutis but they progress until all layers beneath the stratum basale are infiltrated with masses of lymphocytes.

Changes in the bursa of Fabricius and thymus are either degenerative or proliferative. Degenerative changes in the bursa consist of cortical and medullary atrophy, necrosis and cyst formation, or replacement of the cortex and medulla of the follicles with reticular tissue. In the thymus, there may be a complete absence of the cortex and a lack of thymocytes in the medulla and Hassel's corpuscles may become necrobiotic. Proliferative changes in the bursa consist of an interfollicular lymphoid infiltration with characteristic pleomorphic lymphoid cells. Eventually the cells may penetrate the follicles and obliterate them. A similar lymphoid proliferation occurs in some cases in the thymus.

Alterations are present in the epithelium lining the feather follicles. There are no changes in the basilar layer but degenerative changes commence in the intermediate and transitional layers. Cells filled with a clear vacuole, which either displaces the nucleus into a crescent on one side of the cell or which surrounds the nucleus, are more frequently present in infected birds than in uninoculated controls. Many of the nuclei in the transition layer may

contain characteristic Cowdry type A inclusions resembling those produced by MDV in cell culture. Sometimes the inclusion bodies are unusually dense and contracted, reflecting the variable amounts of cell degeneration. The cytoplasm of the cells in this layer is slightly eosinophilic and granular. In the outer layers, in the position of the stratum corneum, the nuclei of the cells are either basophilic and about the size of inclusion bodies or have degenerated completely and are not visible. Instead of becoming flattened, the cytoplasm of the cells is filled with indistinct, highly eosinophilic granules. The most superficial layers disintegrate into fragments and finely granular material. These alterations are largely confined to the ephithelium in the deeper two-thirds of the follicle and are present in only a small proportion of follicles of an infected bird.

Pathogenesis

There is a difference of opinion as to whether MD is primarily an inflammatory process or a true neoplasm. Necrobiotic lesions have been described in the bursa (Purchase and Biggs, 1967; Jakowski et al., 1969; Purchase, 1970a), thymus (Purchase and Biggs, 1967), hematopoietic organs (Jakowski et al., 1970) and the feather follicle epithelium (Calnek et al., 1970b; Nazerian and Witter, 1970; Purchase, 1970a). Herpes virions have been reported in the nerves of birds with MD (Ubertini and Calnek, 1970). It is

possible that the virus also causes some destructive changes in the nerves which can not be observed histopathologically but which account for the predilection for nerve tissue of the infiltrating lymphocytes.

The destructive nature of the disease is exemplified in the immunologic deficiencies in diseased birds (Purchase et al., 1968) and their inability to develop immunity to coccidiosis (Biggs et al., 1968b; Kenzy et al., 1970). These observations agree with Marek's early interpretations and those of Campbell (1956) and Wight (1962) who consider the disease to be primarily inflammatory.

Several early workers considered the basic response to be neoplastic with the inflammatory and degenerative changes being secondary (Pappenheimer et al., 1926; Furth, 1935). Payne and Biggs (1967) came to the same conclusions as follows:

> Undoubtedly, the pathological changes in birds which died with A type nerve lesions and visceral lymphoid tumors fulfill many criteria of neoplasia, such as 1.) progressive proliferation, 2.) qualitative differences from, and excessive increases over, lymphoid hyperplasia produced by many infections of the fowl, 3.) multifocal and diffuse origin, and 4.) possible abnormal cells . . . . On this basis, we believe Marek's disease should be classified as a neoplastic condition.

Wight and Siller (1965), while comparing birds with MD to those in which they had induced an experimental, allergic encephalomyelitis, and Vindel (1965) from histopatholigic studies, speculated that MD might be an autoimmune disease. Support for the involvement of an autoimmune

phenomenon comes from observations that birds with MD have increased levels of circulating globulin (Howard et al., 1967; Ringen and Akhtar, 1968; Samadieh et al., 1969), that there is a remission of clinical signs following treatment with cortisone or 6-mercaptopurine, both immunosuppresive agents (Foster and Moll, 1968), or stress from Mycoplasma gallisepticum infection (Katzen et al., 1969). However, in spite of earlier references to the contrary (Payne and Biggs, 1967), bursectomy does not reduce the incidence of the disease in experimentally infected chickens (Payne and Rennie, 1970a). This contrasts with the dramatic effect of bursectomy on lymphoid leukosis (Peterson et al., 1964; Peterson et al., 1966). It seems possible that the MDV may initiate the primary change, but subsequent pathologic manifestations may have an immunologic basis.

Payne and Biggs (1967) considered the earliest microscopically visible changes to involve cells of the lymphoid series. However, Sevoian and Chamberlain (1964) thought the nerve changes resulted from proliferation of neurilemmal cells followed by differentiation to lymphoid cells. These observations do not resolve whether MD is primarily a disease of the nervous or lymphoid systems.

Specimens of Choice for Virus Isolation

Virus can be readily isolated from diseased chickens and from many normal appearing chickens once infection is established in a flock (Witter et al., 1970b). Infection

persists in some birds for long periods, possibly for the remainder of their lives (Witter, 1970b). Although congenital infection has been reported (Sevoian, 1968a) it probably occurs very infrequently and is of no practical importance since embryos and chicks from infected chickens are free of virus (Solomon et al., 1970). Tumor cells, kidney cells and leukocytes from the spleen or peripheral blood are the specimens of choice for isolation of the virus (Witter et al., 1969b). Since MDV is highly cell-associated, whole cells must be used as inoculum. Storage of specimens for virus isolation should be under conditions which preserve the viability of the cells, i.e., addition of dimethyl-sulfoxide, slow freezing and storage at -196°C (Spencer and Calnek, 1967). Recently, a method has been described so that cell-free virus can be extracted from cell cultures in reasonable quantity and lyophilized (Calnek et al., 1970). If repeatable, this technique offers many advantages for the storage of MDV.

Contaminated dust and dander, oral and nasal washings, feces and litter are infectious even after prolonged storage but they are not good sources of the virus (Kenzy and Biggs, 1967; Witter and Burmester, 1967; Witter et al., 1968b; Beasley et al., 1970). Enveloped and filterable virus has been recovered from the feather follicles which may be the only place where complete virus is produced in infected birds. Virus from this source is probably

responsible for the infectivity of dander, litter, and poultry house dust (Calnek et al., 1970a; Nazerian and Witter, 1970a). Viral preparations from feather follicles or feather tips remain infectious even after dessication and storage at room temperature.

Isolation, Cultivation, and Identification Methods

The presence of MDV may be established by the inoculation of susceptible chicks, cell cultures, and embryonating chicken eggs with specimens suspected of containing MDV. Pathogenic strains of MDV produce symptoms and lesions in genetically susceptible chicks, such as line 7 or Cornell S, 18 to 21 days after chicks are inoculated at one day of age. Gross or microscopic lesions in the nerves and/or viscera, virus isolation in cell culture, specific antigen in the feather follicles, or the presence of antibody in serum are all suitable criteria of infection. Experiments should be of at least 10 weeks duration for maximum sensitivity (Biggs and Payne, 1967; Payne and Biggs, 1967; Purchase and Biggs, 1967; Witter et al., 1969a; Witter et al., 1969b). Birds for these bioassays should be kept in strict isolation to prevent cross contamination.

The MDV produces characteristic cytopathic changes in DEF and CK cell cultures (Churchill and Biggs, 1967; Solomon et al., 1968). Cytopathic areas which are produced in 6 to 14 days consist of rounded and fusiform, refractile

cells and polykaryocytes which have Cowdry type A, DNA containing intranuclear inclusions. The most sensitive test for virus is the direct cultivation of kidney cells from test chickens. Virus present in the kidneys produces a characteristic cytopathic effect in the cultures. Inoculation of CK cultures from uninfected chicks with tumor, spleen, kidney, buffy coat cells or whole blood from test chickens is also suitable. Cell culture is from 10 to more than 1000 fold less sensitive than chickens for isolation of virus (Witter et al., 1969b).

The virus produces pocks on the chorioallantoic membrane (CAM) of embryonating chicken eggs inoculated via the yolk sac or CAM at 4 to 6 or 10 to 11 days of incubation, respectively. If viable, competent lymphocytes are in the inoculum administered by the CAM route, the response is complicated by the graft-versus-host reaction which produces non-specific pocks. This confusion is reduced when the yolk sac route is used (Bülow, 1968; Bülow, 1969).

Identification of the virus may be based on both the in vivo response and cell culture changes. Present or past MDV infection in a bird is confirmed by virus isolation, demonstration of antibody, or by examination of the feather follicles for immunofluorescent or precipitating antigen or for herpes virions by electron microscopy. In cell culture, the characteristic cytopathic

response may be prevented by inhibitors of synthesis of viral DNA. Also, infected cells contain characteristic inclusions and both nuclear and cytoplasmic immunofluorescent antigen. Naked and occasionally enveloped herpes virions are in the nucleus of infected cells and sometimes in the cytoplasm. The DNA extracted from the virus has a high guanine-cytosine content similar to that of cytomegaloviruses (Lee et al., 1969).

Serology

Antibody can be demonstrated in the sera of infected or recovered birds by the AGP (Chubb and Churchill, 1968), IFA (Purchase, 1969; Purchase and Burgoyne, 1970) or passive hemagglutination tests (Eidson and Schmittle, 1969). Antigen for the AGP test can be produced in CK or DEF cultures. Cultures with confluent cytopathic areas induced by low passage MDV are harvested in a small quantity of cultural fluid and the cells are disrupted. This extract is placed in a well in an agar layer containing 8% NaCl (Okazaki et al., 1970b) and the antibody (serum) in an adjacent well. After a suitable incubation period, as many as six different lines of precipitates may form between a serum with antibody and MDV antigen. The major line has been referred to as the A line and the others have been lettered alphabetically (Churchill et al., 1969a).

Antigen for the IFA test is usually produced in infected CK cells on coverslips. After fixation of the cells in acetone, the diluted chicken serum is added. The serum and cells are allowed to react then the excess serum is washed off. The cells are then stained with fluorescein conjugated anti-chicken globulin and examined with a fluorescence microscope. If antibody is present, the antigens in both the nucleus and cytoplasm of the infected cells fluoresce (Purchase, 1969, Article I).

In the passive hemagglutination test, antigen similar to that of the AGP test is added to tanned chicken erythrocytes, incubated for a period and then thoroughly washed. The erythrocytes are mixed with dilutions of serum. If serum diluted 1/16 or greater causes agglutination of the erythrocytes, the test is considered positive for antibody (Eidson and Schmittle, 1969).

Agglutinins have been described in chickens with MD but their specificity is in question (Zacharia and Sevoian, 1969; Payne and Rennie, 1970b; Zacharia and Sevoian, 1970).

The AGP test is most widely used for detection of antibody to MDV and for distinguishing between the virulent and attenuated MDV (Churchill et al., 1969a; Purchase et al., 1970a). The IFA test is used to detect antibody to MDV and to distinguish between antibody to MDV and to HVT (Witter et al., 1970b).

Most birds and almost all flocks of chickens have antibody to MDV by the time they reach sexual maturity whether or not the flock has apparent losses from MD. Thus, inapparent infection is common. The presence of antibody is only an indication of past infection and is of no value in determining the cause of death.

Epizootiology

Although ovarian transmission of MDV has been described (Sevoian, 1968), considerable evidence has accumulated against this route of infection (Rispens et al., 1969; Solomon et al., 1970; Witter, 1970a). The observation that large groups of commercial chickens reared in isolation have no evidence of MDV infection indicates that if egg transmission occurs at all it is a rare phenomenon (Drury et al., 1969; Rispens et al., 1969; Witter, 1970a).

The natural environment of chickens may be contaminated with MDV. Virus has been detected in saliva (Kenzy and Biggs, 1967; Witter and Burmester, 1967), feces (Witter and Burmester, 1967) and dander (Beasley et al., 1970) from infected chickens. Air (Sevoian et al., 1963a), litter and droppings (Witter et al., 1968b) from cages containing infected birds, and even air from such a cage after the chickens have been removed (Colwell and Schmittle, 1968) are infectious. Also certain beatles, Alphitobius diaperinus, mechanically transmit infection (Eidson et al., 1966).

The natural route by which chickens become infected has not been determined although infection via the respiratory tract appears to be the most logical. In a detailed epizootiological study in broiler flocks conducted recently in Georgia infection could be detected in chicks one to two weeks after they had been placed in a contaminated poultry house (Witter et al., 1970b). Once established, the incidence of infection increased rapidly until at eight weeks, MDV could be isolated from almost all birds and there was a parallel increase in microscopic lesions. The presence of maternal antibody in hatched chicks does not prevent infection but it may slightly delay the subsequent development of lesions (Chubb and Churchill, 1969). Virus and antibody may persist for at least 18 months but the levels may fluctuate. Although nearly all chicks become infected before they reach sexual maturity, clinical disease is the exception rather than the rule. The existence of viral infection in the absence of clinical or pathological evidence of disease has been well documented (Chubb and Churchill, 1868; Witter et al., 1969a). The conditions necessary for the development of the disease are poorly understood.

Approximately one week after infection, chickens begin to shed MDV (Kinzy and Biggs, 1967) into the environment in the form of enveloped virus associated with, or originating from, the feather follicle (Calnek and Hitchner,

1969; Calnek et al., 1970a; Nazerian and Witter, 1970; Purchase, 1970a, Article III). Desquamated cells and feather sheath cells are abundant components of chicken house dust and dander which adheres to all objects within the house or which enter or leave the house. Excretion of virus in this volatile form provides a means for airborne transmission.

Control

Conventional methods for controlling infectious diseases have been largely unsuccessful in reducing the incidence of MD (Chute et al., 1964). Some success has been obtained in controlled environment houses which employ biologically filtered air under positive pressure (FAPP) with strict environmental sanitation, and rearing in isolation (Drury et al., 1969).

Different genetic stocks of chickens are highly variable in their susceptibility to MD (Hutt and Cole, 1947; Biggs and Payne, 1963; Purchase and Biggs, 1967; Crittenden, 1968; Sevoian, 1968b). The mode of inheritance is complex but resistance appears to be dominant (Cole, 1970). There is no correlation between genetic resistance to MD and the genetic control of various production traits or genetic resistance to lymphoid leukosis. However, there is a significant relationship between the susceptibility of chickens to experimental inoculation and to natural

exposure (Biggs et al., 1968c). Also, sufficient genetic heterogeneity is present in most commercial chickens for a worth-while selective breeding program directed towards resistance to MD. Rapid progress can be made in this direction in a few generations (Biggs et al., 1968c; Cole, 1968).

Maternal antibody passed from a hen to her offspring delays the onset and reduces the incidence of disease in chicks challenged at one day of age by a natural route, but it has little effect when birds are challenged by intra-abdominal inoculation (Chubb and Churchill, 1969). The protective effect is lost by the time the birds are three weeks of age. Hyperimmunization of dams with a virulent strain of MD has conferred some degree of immunity on the progeny (Eidson et al., 1968). Since field exposure may not occur until after birds are three weeks old, this procedure does not appear to have any practical application.

Adaptive transfer of resistance has been claimed (Feldbush and Maag, 1969) but undoubtedly MDV was transmitted with the immune spleen cells. There was no way of assessing what portion of the resistance was due to the immune competence of the cells themselves.

The development of live virus vaccines against MD has been reviewed (Purchase et al., 1970b). There have been at least four strains of virus used for this purpose: namely two attenuated and one apathogenic MDV and an apathogenic HVT.

An attenuated MDV vaccine has been developed (Churchill et al., 1969a and 1969b). When a strain of "acute" MDV was passed serially in CK cell culture it produced larger cytopathic areas (macroplaques) than those produced by the parent strain and it was nonpathogenic for chickens. This strain had also lost the "A" precipitating antigen present in cultures infected with low passage MDV. Chicks inoculated with the attenuated strain were protected against the disease when subsequently challenged with a virulent strain of virus. The vaccine is also effective in the field (Biggs et al., 1970).

A nonpathogenic herpesvirus, HVT, isolated from a group of turkeys (Witter et al., 1970), had some antigens in common with MDV. In CK or DEF cultures, it produced macroplaques which were distinguishable from those produced by the attenuated high passage and the virulent low passage strains of MDV. In addition, antibody to HVT could be distinguished from antibody to MDV by the IFA test. The HVT protected chicks from MD when they were subsequently challenged with virulent MDV. The vaccine virus only rarely spread from vaccinated to unvaccinated birds in contact with them (Okazaki et al., 1970). This vaccine is also highly effective in field trials (Purchase et al., 1970c).

A strain of MDV which had undergone very few passages in CEF and produced no detectable cytopathic effects, reduced the incidence of MD in challenged chickens (Kottaridis and Luginbuhl, 1969). Some of the vaccinated birds died of MD and the designs of the experiments were not adequate to determine whether this was due to the pathogenicity of the vaccine or to its partial ineffectiveness as an immunizing agent.

A naturally avirulent strain of MDV isolated from a normal flock was also able to produce protection (Rispens et al., 1969). The virus had undergone approximately 20 passages in cell culture. A virus of this description would probably be serologically and virologically indistinguishable from the virulent virus and would be expected to spread rapidly from bird to bird.

MATERIALS AND METHODS

The details of materials and methods are presented in each of the five articles. A convenient reference index is given in Table 1.

TABLE 1.--Index of Materials and Methods.

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RESULTS AND DETAILED DISCUSSIONS

Experimental results and detailed discussions are presented in each of the five articles. A convenient reference index of results is given in Table 2.

TABLE 2.--Index of Results.

TABLE 2.--Continued.

ARTICLE I

IMMUNOFLUORESCENCE IN THE STUDE OF MAREK's DISEASE. I. DETECTION OF ANTIGEN IN CELL CULTURE AND AN ANTIGENIC COMPARISON OF EIGHT ISOLATES

By

H. G. Purchase

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Immunofluorescence in the Study of Marek's Disease

I. Detection of Antigen in Cell Culture and an Antigenic Comparison of Eight Isolates[1]

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The indirect fluorescent-antibody (FA) test was applied to the detection of Marek's disease (MD) antigen in cell culture and antibody in the serum of birds. For the detection of antigen, sera were obtained from birds hyperimmunized with the JM strain of MD. MD antigen could be detected in the nucleus and in the cytoplasm of duck and chick embryo fibroblasts and in those of chick kidney cells infected with material known to contain the MD virus. Uninoculated cultures of chicken cells were always free of MD antigen. When chick kidney cells were infected with a stock cellular preparation of MD virus, infected cells could be detected after 24 hr with the FA test. At this time no cytopathological areas were seen by conventional light microscopy. By 7 days after infection, the same number of infected areas were detected by both methods, and the fluorescent areas coincided with the cytopathological areas. This indicates that the fluorescent areas and the areas with cytopathology are caused by the same agent. A straight-line relationship between the dilution of inoculum and the number of fluorescent or morphological foci obtained indicates that one infectious unit produced one fluorescent or morphological focus. In addition, this time sequence study confirmed the cell association of the virus and demonstrated the cell-to-cell spread of infection. Cell cultures inoculated with eight different isolates of MD were tested in all combinations with sera prepared against the same isolates. The antigens were indistinguishable from one another, indicating that either the strains are antigenically identical or there is a common antigen or contaminant in all of them so that they stained equally well. The FA test can detect MD antigen before cytopathological areas develop in cell culture; however, the small size of the area usually examined precludes its use in initial isolations in which only a small number of infectious units are present in the inoculum. MD-infected cells contain a heat-stable antigen similar to that found in herpes simplex-infected cells.

A wealth of circumstantial evidence has accumulated which incriminates a highly cell-associated herpesvirus as the etiological agent of Marek's disease (MD) (4, 12, 15, 18). The agent produces characteristic cytopathic changes in cultures of chick kidney cells, duck embryo fibroblasts, and, under special conditions, in chick embryo fibroblasts (11). Although some attempts to obtain cell-free virus have been successful, most efforts have failed. It has been impossible to obtain sufficient cell-free virus to perform neutralization tests for the identification of the virus or the detection of antibody in sera of birds. Recently an antigen has been detected in infected-cell cultures which produces a line of precipitation in an agar-gel when diffused against serum from infected or recovered birds (3). This antigen can be used in the agar-gel precipitin test for the detection of antibody in birds which have been exposed to MD.

Kottaridis and Luginbuhl (10) have described a direct fluorescent-antibody (FA) test for MD antigen in cell culture. They used sera obtained from rabbits which had been hyperimmunized with extracts of infectious blood. This paper describes the application of the indirect FA test to the detection of MD antigen in cell culture and antibody in the serum of birds.

[1] Preliminary results were reported at the 105th annual meeting of the American Veterinary Medical Association in Boston, Mass., July 1968.

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MATERIALS AND METHODS

Chickens and eggs. The inbred lines maintained at the Regional Poultry Research Laboratory were used throughout. Line 7 chickens and those produced by the cross between line 15 males and line 7 females are highly susceptible to MD, line 15 and line 15I are intermediate, and line 6 chickens are resistant to MD (5). Except where otherwise indicated the parent lines are maintained in conventional chicken houses and the flock is known to harbor MD viruses. Progeny from these chickens reared in modified Horsfall-Bauer isolators are usually free of all signs of infection.

Sources of viruses. The origin of the JM isolate of MD has been described (17). MSD 1 was obtained from T. Maag, Merck & Co., Inc., Rahway, N.J.; GA from S. Schmittle, University of Georgia (8); CONN A from R. Luginbuhl, University of Connecticut (2); CR 64 from W. Staples, Cobb Breeding Corp., Connecticut; C1 from R. Bankowski, University of California (1); RPL 39 from a field outbreak of MD in Georgia; and FC 50 from an outbreak of MD in Michigan. These isolates had been passaged 40, 1, 19, 0, 5, 1, 2, and 1 times, respectively, in chickens from the Regional Poultry Research Laboratory.

Two strains of laryngotracheitis virus were kindly supplied by R. Luginbuhl. Bryan's high titer strain of Rous sarcoma virus with Rous-associated virus$_1$ as helper [BH-RSV (RAV_1)] and BH-RSV (RAV_2) were obtained from P. K. Vogt (9).

Antigen. Kidney cultures were prepared as described by Churchill and Biggs (4) from a bird which had been inoculated with MD-infected blood and which had clinical signs and gross lesions of MD. When cytopathological areas appeared (4, 19), the cells were trypsinized and plated on fresh confluent monolayers of kidney cells prepared from uninfected birds. This procedure was repeated until an extensive cytopathological effect (CPE) was obtained, whereupon the cells were trypsinized and frozen in dimethyl sulfoxide and stored in liquid nitrogen (7).

Primary chick kidney cultures were prepared and grown on cover slips (11 by 22 mm) placed in 60-mm plastic disposable petri dishes and they were infected with various sources of MD virus. At intervals or when a clearly visible CPE was present, cover slips were removed, rinsed in phosphate-buffered saline (PBS), and fixed by immersion in acetone at 4 C for 2 min. The cover slips were dried under an air blower and stored at 4 C until used within the next few days.

Primary chick embryo and duck embryo fibroblast cultures were prepared as described previously (15, 16). Chick embryo fibroblasts infected with the JM strain of MD were obtained from K. Nazerian.

Antisera. Line 6 chickens were reared in modified Horsfall-Bauer isolators. At 8 weeks of age, a blood sample was obtained to confirm the absence of antibody. They were then inoculated intraperitoneally with 0.5 ml of fresh whole blood obtained from a chicken with clinical and gross signs of MD. Simultaneously, 0.5 ml of complete Freund's adjuvant was inoculated into the breast muscle. Birds were bled 2 weeks later for the cross-fluorescence studies in Table 1. In other instances in which a high titer of antibody was required, the above procedure was repeated 3 times at 2-week intervals and birds were exsanguinated 2 weeks after the last inoculation. All sera were heat-inactivated at 56 C for 30 min and clarified by centrifugation at approximately $1,000 \times g$ for 5 min before dilution and application in the FA test.

Fluorescein-conjugated antichicken globulin. Conjugates from various commercial sources were tested by the following procedure. Spleen sections from freshly killed, 6- to 10-week-old chicks from any source available were cut at a 6 μm-thickness on a cryostat (Lab-tek; B. C. Ames Co., Waltham, Mass.). They were immediately fixed in acetone at -10 C for 2 min and then air dried. After moistening with PBS, the cover slips were flooded with twofold dilutions of various commercial fluorescein-labelled antichicken globulins and allowed to react for 30 min at room temperature and then washed in PBS for 15 min. The sections were then permanently mounted (6) and examined under a Leitz fluorescence microscope with a BG 12 excitor filter and an OG 1 barrier filter. Dilutions of the conjugate which stained the globulin-producing cells in the spleen with the least amount of nonspecific fluorescence of the surrounding tissue were selected for use in the indirect FA test. Conjugates from various commercial and laboratory sources were found to differ greatly in quality. The same dilution of the best conjugate was found to give the most satisfactory results in the indirect test by using MD virus-infected cell cultures as antigen and reacting this antigen with globulin from recovered serum and then with the antiglobulin as described below.

Staining procedure. Cover slips were divided into 1 to 4 areas with water-repellent ink and were attached horizontally to the top of rubber stoppers with adhesive tape. They were flooded with PBS for a few seconds, and then dilutions of serum were placed on the different areas of the cover slip. The stoppers were carefully placed around the periphery of a plastic beaker so that the cover slips pointed toward the center. The tightly covered beaker contained sufficient PBS, which was stirred continuously on a magnetic stirrer to keep the atmosphere humidified. After incubation at room temperature for 30 min, they were submerged in PBS and rinsed by gentle stirring for 15 min. The PBS was removed from the beaker with a vacuum device, and the cover slips were covered with an appropriate dilution of fluorescein-labelled antichicken gamma globulin and allowed to react for 30 minutes. The cover slips were again submerged for 15 min in PBS, removed from the stoppers, dipped in distilled water, and mounted on glass slides in 90% glycerol and 10% PBS, in Elvanol (13) or in Unimount (6).

Terminology. Cytopathological areas observed under conventional light microscopy are referred to as morphological foci, whereas those observed after FA staining are referred to as fluorescent foci.

RESULTS

Development of antigen. Monolayers of chick kidney cells on cover slips were infected with a stock preparation of JM-infected chick kidney cells. At 1, 3, 5, and 7 days after infection, cover slips were removed, fixed, and stained with the indirect FA technique in which sera prepared against the JM isolate were used, and they were examined under the fluorescence microscope. Uninfected cultures were similarly treated.

On the 1st day after infection, with conventional light microscopy, many rounded refractile cells could be seen attached to the monolayer, but they could not be recognized as morphological foci. Upon FA staining, however, some of the cells fluoresced very brightly. Many of them were spherical (Fig. 1), others had thin processes extending from them, and a few were flattened and resembled the surrounding kidney cells which were normal in shape but contained antigen (Fig. 2). There were many groups of cells which were morphologically indistinguishable from the surrounding cells but contained brightly staining antigens (Fig. 3 and 5). These areas could not have been recognized as cytopathological areas by conventional light microscopy.

By 5 and 7 days postinoculation, progressively more morphological foci were visible than at 3 days, and, on close examination, some larger refractile cells could be seen. Some fluorescent foci consisted mainly of rounded refractile cells (Fig. 6), and others contained one or more polykaryocytes (Fig. 7). Among different foci, the proportion of rounded cells to polykaryocytes varied. By the 7th day postinoculation, nearly all the fluorescent foci also contained cells with cytopathology (i.e., they coincided with morphological foci).

Both cytoplasmic and nuclear staining was observed, and it was not possible to determine which antigen appeared first. The staining in the nucleus was usually diffuse (Fig. 2 and 3) and did not obscure the unstained nucleolus. There was usually a nonstaining halo around the brightly stained "intranuclear inclusion" (Fig. 3 and 9). A diffuse staining was most common in the cytoplasm, although some cells also contained brightly staining, irregular granules (Fig. 9). The rounded cells in the center of the focus stained the most brightly and the intensity decreased centrifugally (Fig. 6).

At no time during these experiments did uninfected chick kidney cultures stain (Fig. 8).

In another experiment similar to that described above, chick kidney cultures were prepared directly from JM-infected birds showing clinical signs of MD. Antigen was first detected on the 2nd day after preparation of the cultures. The development of staining and cytopathology progressed as described above.

Staining of MD antigen in duck and chick embryo fibroblasts. Antigen in duck embryo fibroblasts infected with the JM isolate of MD stained brightly. There was a diffuse nuclear antigen and a diffuse and irregularly granular cytoplasmic antigen. In addition, many cells in both the infected and normal duck embryo fibroblast cultures contained small, uniform, spherical cytoplasmic granules which tended to obscure the specific stain in the infected cultures.

Chick embryo fibroblasts infected with MD contained a diffuse nuclear and cytoplasmic antigen, and granular cytoplasmic antigen could be easily detected in infected cells (Fig. 4).

Staining of heterologous antigens. Chick kidney cultures on cover slips were infected with two strains of infectious laryngotracheitis virus, BH-RSV (RAV_1), BH-RSV (RAV_2), and JM isolate of MD, and chick embryo fibroblasts on cover slips were infected with BH-RSV (RAV_1) and BH-RSV (RAV_2). They were fixed and stained in the indirect FA test with a JM antiserum. There was no fluorescent staining in any of these cultures, except in those infected with the JM isolate of MD which showed bright specific fluorescence.

Controls within the indirect FA test. When saline or serum from uninfected birds replaced the anti-MD serum in the first step of the indirect test, no staining was obtained

The specificity of the indirect test was also examined by absorbing a positive serum with MD antigen. Antigen was prepared from JM-infected duck embryo fibroblasts and chick kidney cells by a method similar to that described by Chubb and Churchill (3). A similar batch of antigen was prepared from normal duck embryo fibroblasts and chick kidney cells. A 1:10 dilution of a positive serum was added to an equal quantity of MD cell antigen, normal cell antigen, and saline in separate tubes. The tubes were incubated at room temperature with intermittent agitation for 2 hr and centrifuged at 3,000 $\times$ g for 30 min; the supernatant fluid was used in the indirect FA test to stain positive JM antigen grown on cover slips. The brightness of staining was scored from 0 to 4 plus. Both MD-cell antigens absorbed out the MD antibody from the serum, and only a 1 plus staining was obtained, whereas the normal cell antigens and the saline left the antibody which gave a 4 plus fluorescence of the MD antigen.

Comparison of sensitivity of FA and TC tests for antigen. The supernatant medium from confluent chick kidney cultures growing on cover slips was replaced with 5 ml of 1/3 log dilutions of a stock of JM-infected kidney cells. After 1, 3, 5,

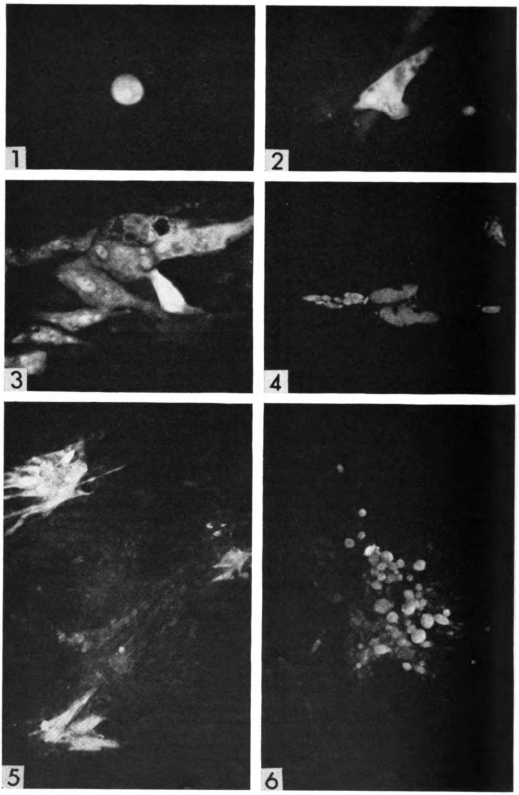

FIG. 1–6

and 7 days of incubation, cover slips were removed and stained by the indirect FA technique with antisera prepared against the JM isolate. Replicate plates without cover slips were examined with an inverted conventional light microscope for cytopathological areas (morphological foci). The numbers of foci per 100 mm^2 of surface area as observed by each method were plotted (Fig. 11).

All infected viable cells could be detected by the FA technique on the day after infecting the culture. However, they were easier to count on the 3rd day after infection when the fluorescent foci were larger. It was not until the 7th day that most fluorescent foci had developed cytopathology which could be seen under the fluorescence microscope as a rounding and retracting of cells and as the presence of polykaryocytes. At this time, there were individual, rounded, fluorescent cells attached to the monolayer between the large fluorescent foci. They were similar to the cells seen at 1 day after infection and were probably cells which had been washed off the foci and were initiating secondary foci.

Morphological foci were first detectable on the 3rd day after infection when they consisted of small groups of six or more rounded, refractile cells, often with adjacent, fusiform, refractile cells. The foci increased in size and numbers until they reached a maximum at about the 7th day (Fig. 12).

There is a linear relationship between the fluorescent foci and the dilution of inoculum and a similar relationship between the morphological foci and the dilution of inoculum (Fig. 11). The lines for the fluorescent foci and for the morphological foci seen at 3, 5, and 7 days after infection are parallel.

Detection of MD herpesvirus in field samples. Blood samples were obtained from different field flocks and used to inoculate replicate plates of chick kidney cells. One plate contained a cover slip which was removed between the 4th and 7th day after inoculation and stained by the indirect FA test with JM antiserum. The other plate was examined for morphological foci between the 10th and 21st day postinoculation. MD herpesvirus was detected in 21 (72.4%) of the 29 samples. Of these, 7 (33.3%) produced morphological and fluorescent foci, whereas 14 (66.6%) produced only morphological foci; no fluorescent foci were detected on the cover slip. There were no samples which produced only fluorescent foci, and eight samples (27.6%) were negative by both tests.

In order to increase the number of infectious units per culture, and thus increase the likelihood of a focus occurring on a cover slip, cultures were passaged to fresh confluent monolayers of kidney cells 7 days after inoculation with blood. An additional 43 field samples were examined by this method. Only 15 (34.9%) contained MD herpesvirus. Of these, eight (53.3%) produced morphological and fluorescent foci, whereas seven (46.7%) produced only morphological foci. Twenty-seven samples (62.8%) were negative by both tests, and one sample (2.5%) had fluorescent foci on the cover slip but no morphological foci on the petri dish. In this instance, a replicate sample of blood was inoculated into line 15 × 7 chickens and lesions of MD were produced. None of the 10 samples obtained from isolated control birds and examined by this method produced either morphological or fluorescent foci in cell culture.

Antigenic relationship between isolates of MD. Antigen and antibody for the indirect FA test were prepared from the same inoculum source. Serum from each immunized bird was used in the indirect FA test on each of the antigens. The brightness of fluorescence was scored from 0 to 4 plus by two observers, and the average score for each group of antisera was referred to as the staining index (Table 1).

Birds inoculated with the GA isolate failed to produce antibody and died of MD shortly after being bled for antibody. The staining index of the antibody against homologous antigen was

FIG. 1. *Chick kidney monolayer one day after inoculation with a stock of MD-infected chick kidney cells. Single, spherical cell stains. Ca. × 380.*

FIG. 2. *Chick kidney monolayer one day after inoculation with a stock of MD-infected CK cells. Single, flattened cell with an intranuclear inclusion stains. Ca. × 380.*

FIG. 3. *Chick kidney monolayer one day after inoculation with a stock of MD-infected CK cells. A group of flattened cells with diffuse and granular cytoplasmic antigen and intranuclear inclusions. Ca. × 380.*

FIG. 4. *Chick embryo fibroblast cultures infected with JM strain of MD virus. The diffuse nuclear antigen and the irregular cytoplasmic granules stain brightly. Ca. × 250.*

FIG. 5. *Chick kidney monolayer at 1 day after inoculation with a stock of MD-infected CK cells (same monolayer as Fig. 1–3). Three fluorescent foci can be clearly seen. Ca. × 130.*

FIG. 6. *Chick kidney monolayer 5 days after infection with a stock of MD-infected CK cells. A fluorescent (and morphological) focus composed of rounded cells. Ca. × 130.*

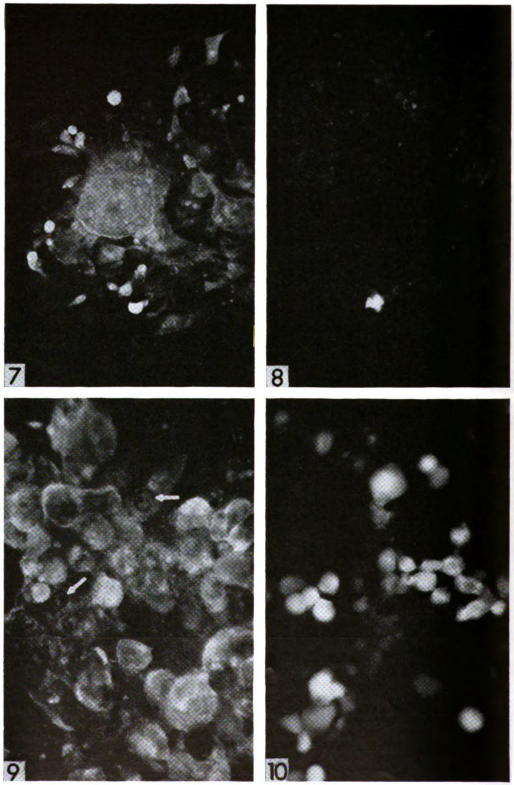

FIG. 7–10

TABLE 1. *Antigenic relationship among eight isolates of MD*

Antibody produced to	No. of Chickens per group	Antigens								$\bar{X}^a$	$\bar{\bar{X}}^b$
		JM	MSD_1	GA	CONN A	CR64	C_1	RPL39	FC50		
JM	4	**3.0**[c]	2.0	2.8	1.3	1.5	2.3	2.0	2.1	2.1	1.9
MSD_1	3	1.5	**0.5**	1.8	0.7	1.0	1.0	0.8	1.2	1.1	1.6
GA	2	0.0	0.0	**0.0**	0.0	0.0	0.0	0.0	0.0	0.0	2.0
CONN A	4	1.5	1.3	1.9	**1.3**	1.0	1.8	1.5	1.4	1.5	1.6
CR64	4	3.8	2.8	3.3	3.3	**2.0**	3.5	3.6	2.8	3.1	1.3
C_1	4	2.0	2.1	1.8	2.3	1.4	**1.6**	2.1	1.6	1.9	1.7
RPL39	3	2.3	2.3	2.5	2.2	1.8	1.8	**2.2**	1.5	2.1	1.7
FC50	4	1.4	1.8	1.9	1.6	1.4	1.4	1.4	**1.0**	1.5	1.5

[a] Mean staining index of antibody, i.e. mean of the scores for one group of antisera against all eight antigens. Sera from eight control birds had a mean index of 0.0.

[b] Mean brightness of antigen, i.e. mean of the scores for all groups of antisera against one antigen. Control antigen had a mean brightness of 0.0.

[c] Values in boldface are average scores (staining indexes) for each group of antisera; the brightness of fluorescence was scored from 0 to 4 plus by two observers.

higher than the mean staining index against all antigens in two instances (JM and RPL 39), but was lower than the mean staining index in the others. The mean brightness of the antigen was similar in each instance.

Agreement between observers. In the above test, two observers examined each preparation and scored the brightness of fluorescence from 0 to 4 plus, independently (Table 2). Of the 224 observations, 143 (63.8%) were in full agreement, 79 (35.2%) deviated by a score of 1 plus and 2 (0.9%) deviated by a score of 2 plus. There was full agreement among the 64 observations with sera from control birds and among the 28 observations with control antigen. There was a 98.4% agreement among positives and negatives.

Heat stability of MD antigen. Two cover slips on which JM-infected chick kidney cells had been grown were fixed as described above. One was placed in distilled water and boiled for 90 min. They were then stained in the indirect FA test.

There was bright nuclear and cytoplasmic staining in the unboiled cover slip (Fig. 9). After being boiled for 90 min, the cells shrank slightly but there was no decrease in the intensity of staining (Fig. 10).

DISCUSSION

When chick kidney cells were infected with a stock cellular preparation of the JM isolate, fluorescent cells could be detected after 24 hr, but at this time no morphological foci were seen. By 7 days after infection, the same number of infected areas were detected by both methods and the fluorescent foci coincided with the morphological foci. A straight-line relationship between the dilution of inoculum and the number of fluorescent or cytopathic areas obtained indicates that one infectious unit produced one fluorescent or morphological focus. Thus the antigens detected in cell culture by the indirect FA test are induced by the virus which causes the characteristic cytopathology.

The indirect FA test as described here is highly specific since MD antisera did not stain cultures infected with other poultry pathogens. In addition the antibody could be absorbed from the serum by MD-infected chick or duck cells but not by uninfected cells. Control uninfected cultures developed neither cytopathic areas nor fluorescent-staining foci.

Antigen could be detected in both the nucleus and the cytoplasm of duck and chicken cells and its morphology and distribution were similar to those described for herpes simplex although no small nuclear granules were seen (14).

The origin of the FA-staining cytoplasmic granules in duck embryo fibroblast cultures is

FIG. 7. *Chick kidney monolayer 7 days after infection with a stock of MD-infected CK cells. A fluorescent (and morphological) focus composed mainly of polykaryocytes. Ca.* × *130.*

FIG. 8. *Uninfected chick kidney monolayer fixed and stained at the same time as that in Fig. 7. Note areas of rounded epithelioid cells at top of picture which do not stain. The fluorescing artifact can be easily distinguished from MD antigen. Ca.* × *130.*

FIG. 9. *Chick kidney monolayer with a large proportion of MD-virus-infected cells. Arrows show diffuse and irregularly granular nuclear staining. Ca.* × *320.*

FIG. 10. *Chick kidney monolayer identical to that in Fig. 9 but boiled for 90 min in distilled water. Ca.* × *320.*

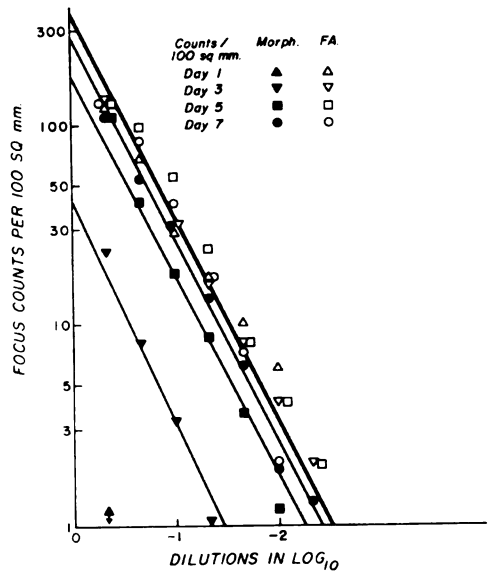

FIG. 11. *Numbers of foci detected morphologically and by FA in cultures at various times after inoculation with dilutions of MD-infected cells.*

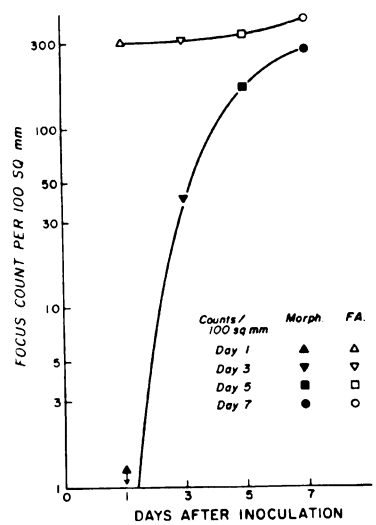

FIG. 12. *Numbers of foci detected morphologically and by FA in cultures at various times after inoculation.*

unknown, but they could be easily distinguished from the MD antigen by their morphology and distribution. Since they were present in all cultures examined, duck embryo fibroblasts were only rarely used for antigen.

TABLE 2. *Number of sera examined by each observer which had the brightness indicated*

Score 1	Score 2				
	0[a]	1	2	3	4
0	**54**[b]	3			
1	2	**24**	14		
2		9	**24**	17	
3			11	**34**	6
4			2	17	**7**

[a] Brightness of fluorescence was scored from 0 to 4 plus.

[b] The 64 observations with sera from control birds and 28 observations with control antigen were in full agreement and have been omitted from this table. Values in boldface are in agreement.

MD herpesvirus-infected cells possess an antigen which is not destroyed by boiling for 90 min. In this respect MD herpesvirus is similar to herpes simplex (14).

Cell culture antigens appeared about 3 days before the CPE could be detected, but eventually nearly all the fluorescent foci developed into morphological foci. In this respect, the FA test is as sensitive as that depending on morphological foci for detecting infectious virus; however, it suffers the disadvantage that only a small area (in this case 100 mm^2) is usually examined. Morphological foci may occur on the petri dish and not on the cover slip. This possibility accounts for the observations in which morphological foci but not fluorescent foci were detected. In an attempt to increase the sensitivity of the FA test, I passaged cultures to fresh kidney cells. This increased the efficiency of recovery from 33.3 to 53.3%. Larger cover slips could be used, but the time required to scan them for fluorescent foci is much greater than is required to examine a petri dish for morphological foci. This precludes the use of the FA test in initial isolations in which only a small number of infectious units are present in the inocula.

Approximately the same number of fluorescent foci were detected at 1 day after infection of chick kidney cultures as were detected at 7 days after infection. Since all cultures were maintained under liquid media, this indicates that there was very little, if any, spread of virus through the media. The slight increase in the number of foci at 5 and 7 days after infection is probably due to secondary foci originating from infected cells which had drifted loose from cytopathological areas. These individual cells were clearly visible when stained with FA.

The fluorescence on day 1 probably reflects the

presence of infected cells in the inoculum. It is possible that the foci originated from division of the infected cells added to the culture rather than by infection of surrounding cells. This is unlikely since infected cells do not propagate in continuous culture and slough off from the monolayer and die as the culture gets older. Also the morphology of the foci produced is characteristic of the recipient monolayer and not of the donor cells (19). In mature foci, there was a gradation of staining from very bright staining in the center of a focus to just detectable staining at the periphery. If the entire focus had originated by division of an infected cell, one would expect a focus of cells containing approximately the same amount of antigen as was seen at 1 day after infection (Fig. 3 and 5). The gradation of staining from the center outward indicates that the infection is spreading from cell to cell in a centrifugal direction (Fig. 6). These findings confirm the highly cell-associated nature of this virus and demonstrate that infection is transmitted from infected to adjacent cells. Convincing proof of cell to cell transmission awaits studies on the mechanism of focus formation.

Of the eight isolates studied in these experiments, seven could not be distinguished from one another by the indirect FA test. No conclusions can be drawn with regard to the eighth isolate since the chickens that were inoculated did not produce antibody to this isolate. This may have been because the chickens succumbed to MD before they could produce antibody. In another experiment (*unpublished data*) chickens produced antibody to this isolate and it stained JM antigen well. These results indicate that either the eight isolates are antigenically identical or that there is a common antigen or contaminant in all stocks of the isolates.

The indirect FA test can be considered to be fairly objective. Both observers had considerable experience with the indirect FA test and the observations were made independently; there was excellent agreement between them.

ACKNOWLEDGMENTS

The author wishes to acknowledge the skilled technical assistance of C. A. Hunt and P. A. Frank.

ADDENDUM IN PROOF

Recent electron microscopic studies have demonstrated that all cells containing antigen demonstrable by the FA test also contain herpesvirus and that cells which do not contain antigen do not contain herpesvirus particles (K. Nazerian and H. G. Purchase, *manuscript in preparation*).

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Article II

IMMUNOFLUORESCENCE IN THE STUDY OF MAREK'S DISEASE: DETECTION OF ANTIBODY

By

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Immunofluorescence in the Study of Marek's Disease: Detection of Antibody

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SUMMARY

Indirect fluorescent antibody (FA) and agar gel precipitin (AGP) tests were used to detect antibody in serums of chickens which were exposed to Marek's disease (MD) virus. There was a 92% agreement between the results of the FA and those of the AGP tests on 418 serums from various sources. By the FA test, maternal antibody in young chickens from MD-exposed dams was detected after antibody was no longer detectable by the AGP test, and acquired antibody in contact-exposed chickens was detected earlier than by the AGP test. The FA test was 10 to more than 320 times more sensitive than the AGP test; also, some serums which had a high titer of antibody demonstrable by the FA test did not produce precipitation in the AGP test.

Because of the highly cell-associated nature of the herpesvirus which is implicated as the cause of MD, it has not been possible to obtain sufficient cell-free virus for serologic studies.[1,3,4,6] However, Chubb and Churchill[2] described an AGP test which can be used to detect antibody to the MD virus in the serums of affected chickens or of chickens in the recovery stage. The purposes in the present report are to describe the application of the indirect FA test to the detection of MD antibody in the serums of affected and recovered chickens and to compare the FA test results with the AGP test results. The application of the FA test to the detection of antigen in cell culture has been previously described.[5]

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Materials and Methods

The chickens and embryos used were of lines 6, 7, 15, and 15I and of 15 × 7 cross kept at this laboratory.[6] All chickens were necropsied at the termination of the experiments, and when necessary the diagnosis was confirmed histopathologically. The viruses, JM, GA, and CR64 strains of MD, have been described previously.[6]

Antigen for the indirect FA test was made as follows: Chicken kidney (CK) cells from JM virus-infected chickens with clinical and gross lesions of MD were cultured by the usual procedure.[5,6] When cytopathologic alterations developed, the cells were

Purchased by the U. S. Department of Agriculture for official use

trypsinized and placed on additional CK cell cultures prepared from JM-affected chickens. This was repeated 2 to 5 times until there were more than 20 cytopathologic areas per 100 sq. mm. of the petri dish surface.

The cells were then passaged on CK cells prepared as described, but grown on 11- by 22-mm. coverslips in plastic petri dishes. When the cytopathologic changes were well developed, the coverslips were removed, rinsed in phosphate-buffered saline solution (PBS), fixed in acetone at 4 C. for 2 minutes, and air dried under a blower. The coverslips were stored dry in a petri dish at −70 C. and used over several months, during which time they did not lose their antigenicity. Before the coverslips were used, they were divided into 5 areas with waterproof ink. This division allowed 4 serum samples to be tested and the 5th area to be used as a "handle" by which the coverslip could be attached with adhesive tape to a rubber stopper.

All serums were heated to 56 C. for 30 minutes and were centrifuged at 1,000 g for 5 minutes before they were used. A 1/20 dilution of each serum in PBS was made in a 96-cup plastic tray.[a] The diluted serum was stirred and transferred to a section of a marked coverslip with a capillary tube. The staining procedure for the FA test was the same as that previously described.[5] The brightness of staining in the FA test was scored negative, 1+, 2+, 3+, and 4+ (brightest). Titers are expressed as reciprocals of the highest dilutions of serums which gave fluorescence.

Precipitating antigen was prepared from cultured CK cells from JM virus-infected chickens as described by Chubb and Churchill,[3] except that the antigen used in the present study originally contained 4×10^7 cells/ml. Antigen was prepared in a similar manner from JM virus-infected duck embryo fibroblasts (DEF).[6] The technique of double diffusion in agar gel containing 8% sodium chloride was similar to that described by Woernle,[7] except that 1% agar was used. Titers are expressed as reciprocals of the highest dilutions of serums which gave a line of precipitation.

Experiment 1.—Serums from chickens given different treatments were used to determine whether a correlation existed between results of FA and AGP tests and previous exposure of chickens to MD virus. Eleven chickens were inoculated intra-abdominally with JM, GA, or CR64 strains of MD virus when they were 1 or 42 days of age. Blood samples were collected and the chickens were killed 6 weeks later, at which time 7 of 11 chickens (63.7%) had lesions of MD. Another group of 10 chickens was kept in direct contact from 1 day of age with the JM virus-inoculated chickens. Blood samples were collected, and chickens were killed at 6 weeks of age. All 10 chickens had lesions of MD. Two chickens were inoculated intra-abdominally with 0.2 ml. of normal blood when they were 1 day old. Blood samples were collected and chickens were killed 6 weeks later. Lesions of MD were not detected in either chicken. Serum was prepared from blood collected from 7 noninoculated chickens which were reared in Horsfall-Bauer isolators adjacent to the inoculated chickens and from 20 specific-pathogen-free chickens more than 6 months old that were kept in plastic isolators during their entire lifetime. None of the control chickens had lesions of MD at necropsy.

Experiment 2.—Serums from chickens given different treatments were used for comparative titration in FA and AGP tests (Table 2). Serums 1, 2, 3, 10, 11, 13, and 14 were from 6-week-old line 15 × 7 chickens inoculated when they were 1 day old with blood from JM virus-infected chickens, and serums 15, 16, 17, and 18 were from similar noninoculated controls. Serums 7, 8, 9, and 12 were from 6-month-old line 15 × 7 chickens which survived contact exposure to JM virus-inoculated chickens and serums 19, 20, 21, and 22 were from line 7 chickens reared under specific-pathogen-free conditions. Serums 4, 5, and 6 were obtained from 8-week-old line 6 chickens from which blood samples were collected 2 weeks after they were given a series of 3 inoculations with blood from JM virus-infected chickens as described previously[4] and serums 23, 24, 25, and 26 were from similar noninoculated controls.

Experiment 3.—Serums from chicken flocks in the field were used to compare FA and AGP tests. Blood samples were collected on the processing line directly from hearts of 100 broilers, 8 to 9 weeks old, from 8 different farms, each of which held 3,800

[a] Joseph E. Frankle Company, Philadelphia, Pa.

TABLE 1—Agreement Between Results of Indirect Fluorescent Antibody (FA) and Agar Gel Precipitin (AGP) Tests on Certain Serums

Experiment No.	No. of chickens	Exposure to Marek's disease virus	Agreements: Pos. to FA test and pos. to AGP test	Agreements: Neg. to FA test and neg. to AGP test	Disagreements: Pos. to FA test and neg. to AGP test	Disagreements: Neg. to FA test and pos. to AGP test
1	11	Inoculated with MD virus	9	1	0	1
	10	Exposed by contact to MD virus-inoculated chickens	10	0	0	0
	2	Inoculated with normal blood	0	2	0	0
	27	Kept in isolation (nonexposed)	0	27	0	0
2	10	Inoculated with MD virus infected blood	7	0	3	0
	4	Exposed by contact to MD virus-inoculated chickens	4	0	0	0
	12	Kept in isolation	0	12	0	0
3	100	Exposed to MD under field conditions	32	59	4	5
	74	Field isolated	0	74	0	0
4	24	Chickens under 3 weeks of age*	8	4	12	0
	24	Inoculated with MD virus	20	3	1	0
	40	Exposed by contact to MD virus-inoculated chickens	24	10	5	1
	80	Kept in isolation (nonexposed)	0	80	0	0
Total	418		114	272	25	7
(Per cent)			(92.3)		(6.0)	(1.7)

* The test-positive reactions indicate maternal antibody. Pos. = positive; Neg. = negative.

to 6,600 chickens. Total percentage of chickens condemned because of "leukosis" ranged from 0.5% to 32.3% of the chickens processed, and approximately 50% of the blood samples came from these chickens. In addition, 74 serums were prepared from blood collected from 5 isolated breeder flocks which were selected because they did not

TABLE 2—Comparison of Titers of Certain Serums by the Indirect Fluorescent Antibody (FA) and Agar Gel Precipitin (AGP) Tests with Chicken Kidney and Duck Embryo Fibroblast Antigens (Experiment 2)

Serum No.	FA titer*: CK	FA titer*: DEF	AGP titer*: CK	AGP titer*: DEF	FA/AGP**: CK	FA/AGP**: DEF
1	640	640	32	32	20	20
2	640	80	8	8	80	10
3	320	320	8	8	40	40
4	320	320	2	4	160	80
5	320	320	Neg.	Neg.	>320	>320
6	320	320	Neg.	Neg.	>320	>320
7	320	160	4	4	80	40
8	320	160	2	2	160	80
9	320	160	1	2	320	80
10	320	80	16	4	20	20
11	320	80	4	4	80	80
12	320	80	2	2	160	40
13	160	80	4	8	40	10
14	160	80	Neg.	Neg.	>160	>80
Av. titer of serums 1–14	342.9	205.7	5.9	5.6		
15–26	Neg.	Neg.	Neg.	Neg.	N.A.	N.A.

* Titers are expressed as the reciprocal of the highest dilution of serum which produced fluorescence or a precipitin line. ** The relative sensitivity of the FA test over the AGP test is indicated by dividing the FA titer by the AGP titer.
N.A. = Not applicable.

TABLE 3—Detection of Maternal and Acquired Antibody by Indirect Fluorescent Antibody (FA) and Agar Gel Precipitin* (AGP) Tests (Experiment 4)

Group	Age (wk.) of chickens at time blood samples were collected																	
	0**		1½		3		4		5		6		7		8		12		16	
	FA	AGP	FA	AGP	FA	AGP	FA	AGP	FA	AGP	FA	AGP	FA	AGP	FA	AGP	FA	AGP	FA	AGP
Inoculated	4+	+	3+	Neg.	Neg.	Neg.	2+	+	3+	+	4+	+	4+	+	4+	+	4+	+	3+	+++
	3+	+++	Neg.	Neg.	Neg.	Neg.	2+	Neg.	3+	++	2+	+++	4+	+++	4+	+++	4+	+++	3+	
	3+		1+	Neg.	Neg.	Neg.	2+	+	3+	+	3+	+	4+	+	4+	+	4+	+	4+	
Contact	3+	+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	+	3+	+	3+	+	3+	+	3+	+++++
	3+	+	1+	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	2+	+++	3+	+++	4+	+++	4+	+++	3+	
	3+	+++	1+	Neg.	Neg.	Neg.	1+	Neg.	2+	Neg.	3+	+++	4+	+++	4+	+++	2+	+++	4+	
	3+	+++	1+	Neg.	Neg.	Neg.	2+	Neg.	3+	Neg.	3+	+++	3+	+++	3+	+++	2+	+++	4+	
	3+	++	1+	Neg.	Neg.	Neg.	Neg.	Neg.	1+	Neg.	2+	++	3+	++	3+	++	2+	++	4+	
Control 1	N.D.	N.D.	6/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Control 2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0/8	N.D.	0/8	N.D.	0/8	N.D.	0/8	N.D.	0/8	N.D.	0/8	N.D.	0/8	0/8
Control 3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0/8	N.D.	0/8	N.D.	0/8	0/8

* The AGP test was performed in duplicate with chicken kidney and duck embryo fibroblast antigen with identical results. ** Blood samples were collected when chickens were 1 day old; these are not the same chickens from which subsequent blood samples were collected.

Brightness of fluorescence in the FA test was scored from Neg. to 4+; results of the AGP test are scored as + or Neg.; numerator = No. of samples test-positive; denominator = total No. samples tested; N.D. = not determined.

have history of losses from MD and because none of the serums reacted in the AGP test.

Experiment 4.—To study the pathogenesis of MD, 55 line 15 × 7 chicks were divided among 4 Horsfall-Bauer isolators as follows. Ten chicks were placed in the 1st isolator and were inoculated intra-abdominally with a dilution of JM virus-infected blood which contained approximately 100 minimal infectious units of virus per dose, and 15 noninoculated chicks were placed in the same isolator to be exposed by contact. Three groups of 10 chickens each were kept in the other 3 isolators and were used as noninoculated controls. Samples of blood were collected from chicks at 11 days and 2, 4, 5, 6, 7, 8, 12, and 16 weeks after they hatched. Consecutive serum samples from 8 of the exposed and 24 of the control chickens were tested serologically (Table 3). In addition, a random sample of 8 chicks from the same hatch and source as those placed in isolators were exsanguinated when they were 1 day old and serum prepared from the blood was also tested.

Results

Agreement Between Results of FA and AGP Tests.—There was 92% agreement between the results of the FA and the AGP tests on a total of 418 serums (Table 1). Of these serums, 199 were from exposed chickens, and there was 87% agreement between the tests on them. All 193 controls were free of antibody by both tests. There were 25 serums in which results of the FA test were positive and results of the AGP test were negative and 7 serums where the reverse occurred. A large proportion of the discrepancies were in experiment 4 (Table 3) where the FA test detected maternal antibody in 11-day-old chicks and the AGP test did not. Similarly, in the chickens exposed to MD infection by contact, the FA test detected acquired antibody before it was detected by the AGP test. The 3 serums in experiment 2 that reacted in the FA test, but not in the AGP test, will be described later.

None of the control serums from chickens which had been maintained under different degrees of isolation reacted in either the FA test or the AGP test.

A nonspecific patchy fluorescent staining of the nuclear membrane of all cells in the monolayer occurred with 8 of 12 serums from the line 7 specific-pathogen-free chickens in experiment 1. In contrast, the serums from 2 chickens inoculated with normal blood did not react in either the FA test or the AGP test.

Comparative Titration of Serums.—Serums from 14 chickens which had been exposed to MD virus and 12 nonexposed chickens were titrated by both FA and AGP tests to determine which of the tests was the more sensitive and to determine whether there was a relationship between the titers obtained with the 2 tests. Titrations were performed with both CK and DEF antigens to determine whether there were qualitative or quantitative differences between the antigens.

The serums were ranked according to the FA titers (Table 2). The serum with the highest titer in the FA tests also had the highest titer in the AGP test, but beyond this there was very little relationship between the titers of individual serums. Serum titers in the FA test were from 10 to more than 320 times higher than those obtained in the AGP test, indicating that less antibody can be detected in serum by the FA test than by the AGP test. Three samples which had a titer of between 80 and 320 in the FA test did not produce a precipitin line in the AGP test.

With both the FA and AGP tests, there was very little difference between the titers of the different serums obtained with the CK and DEF antigens, indicating that there was probably no qualitative difference between the antigens. In the FA test, however, serum titers were higher when measured with CK antigen than when measured with DEF antigen, and this is reflected in the average titer of the test-positive serums which was 1/343 with CK antigen and 1/206 with DEF antigen. There was no quantitative difference between the precipitin antigens.

Serums from 12 normal nonexposed chickens were test-negative in both the FA and AGP tests.

Detection of Maternal and Acquired Antibody.—Maternal antibody was detected in all chickens at 1 day of age (Table 3) by both tests. At 11 days of age, maternal antibody was demonstrated in 12 of 16 chickens (75%) by the FA test, but antibody was not detected by the AGP test. The staining of serums from 11-day-old chickens was not as bright as that obtained with serums from 1-day-old chickens. By 3 weeks of age, chickens did not have antibody; however, at 4 weeks of age, all 3 inoculated chickens and 2 of 5 contact-exposed chickens had antibody demonstrable by the FA test, but antibody was detected in only 2 of the inoculated chickens by the AGP test. At 5 weeks, all the inoculated chickens had antibody detectable by both tests; however, 3 of the contact-exposed chickens were test-positive by the FA test only. From the 6th week on, antibody was demonstrated in exposed chickens by both tests, except for 1 chicken which was test-negative by the FA test on the 6th week. The brightness of staining in the FA test seemed to increase with time. None of the control chickens had antibody detectable by either method after 3 weeks of age.

All inoculated chickens and 8 of 14 contact-exposed chickens (57%) either died of the disease or had lesions at necropsy. Of the 30 control chickens, 1 had a minor microscopic lesion indistinguishable from that of MD; however, other lesions were not seen in this chicken and antibody could not be demonstrated in its serum. Lesions of MD were not detected in any of the other 29 control chickens.

Discussion

Agreement Between Tests.—Results of the FA and the AGP tests were in close agreement; however, in most samples, antibody was detected by the FA test and not by the AGP test rather than vice versa. This was especially evident when there were low levels of antibody in the serum just before maternal antibody was

lost and early in the development of acquired antibody. In these circumstances, the FA test seemed to detect antibody which did not react in the AGP test. There was no relationship between the titers obtained by the 2 tests; thus, some serums had high titers in the FA test but had negative results in the AGP test. The discrepancies between the 2 tests indicate that different spectrums of antigen-antibody reactions were being observed in the 2 tests. The FA test would be expected to be more sensitive than the AGP test, since it would detect both the antigen-antibody combinations that produce a visible precipitate in addition to other antibody-antigen combinations which did not form a lattice large enough to produce a grossly detectable precipitate in the AGP test. These factors explain the instances in which the FA test seemed more sensitive than the AGP test.

There were very few instances in which a precipitin line was observed in the AGP test and there was no staining in the FA test. These discrepancies could have been due to subjective differences in the reading of the tests, particularly the FA test.

The specificity of these reactions is supported by the following observations.

Only young chickens from dams which were known to be exposed to MD virus and older chickens with lesions of MD or known to be exposed to the MD virus had antibody. Isolated controls always remained free of antibody which could be detected by either the FA or the AGP test after they were 3 weeks old. Thus, even though the 2 tests detect antibodies directed against different spectrums of antigens, both are specific for MD virus-induced antibody. In addition, serums which had positive results in the indirect FA test stained only the cytopathologic areas in infected CK cultures. This provides evidence that the agent which induces the cytopathologic effect in cell culture is the same as that which produces MD in chickens. Since the cytopathologic features are typical of those induced by a herpesvirus and a herpesvirus has been seen in similar infected cultures,[1,3,4,6] this supports the view that this herpesvirus is the etiologic agent of MD.

The nonspecific nuclear staining obtained with 8 serums from the line 7 specific-pathogen-free flock and the nonspecific precipitin lines obtained from 2 serums from the line 7 and 2 serums from the line 15I specific-pathogen-free flocks cannot be explained. Since the serum from chickens inoculated with normal blood did not react in either test, it was unlikely that isoantigens were involved.

Sensitivity of the Tests.—The serum titers obtained with the FA test were 10 to 320 times higher than those obtained in the AGP test. Even when serums are used in the FA test at a 1/20 dilution, the test is usually more sensitive than the AGP test with nondiluted serums.

Comparison of Antigens of Chicken Kidney- and Duck Embryo Fibroblast-Origin.—Although noninfected DEF cultures contained granules which stained in the FA test, the fluorescence of these granules could be distinguished by their morphologic features and distribution from the fluorescence obtained in MD virus-infected cells. However, DEF antigen was not as sensitive as CK antigen, probably because there was a more circumscribed cytopathologic effect in the CK cultures than in the DEF cultures, and this afforded a better contrast in staining between the antigen-containing cells and the background of noninfected cells. Also, the fluorescence of "normal" granules in DEF cultures may have affected the interpretation of the endpoint. Thus, CK antigen was considered superior to DEF antigen for the FA test.

There was very little difference between the serum titers obtained with the 2 antigens in the AGP test. This is also reflected in the results of the pathogenesis study (experiment 4) where there was a full agreement between positives and negatives obtained with the 2 antigens. However, antigen pools vary in quality, and only single pools of CK or DEF antigen were compared.

Pathogenesis of Marek's Disease.—Ma-

ternal antibody was detected by both tests in chickens shortly after they hatched, but it was detected only by the FA test at 11 days after chickens hatched. Chubb and Churchill[2] were able to detect maternal antibody in 12 of 18 2-week-old chickens in one experiment and 3 of 21 in another experiment. The failure to detect antibody by the AGP test in 11-day-old chickens in the present experiments may be due to the line of chickens used or to a difference in the antigen used. Antibody could be detected in chickens 4 weeks after they were inoculated at 1 day of age. Some chickens in contact with inoculated chickens developed antibody which could be detected by the FA test at the same time as the inoculated chickens, but most chickens did not produce precipitating antibody until they were 6 weeks old. Chickens which acquired antibody continued to have antibody in their serums until they were at least 16 weeks old.

Advantages of the FA Test Over the AGP Test.—In our experience, the FA antigen is easier to prepare than the AGP antigen for the following reasons. In the FA test, the brightness of staining of individual cytopathologic areas is a measure of the amount of antibody in the serum. Thus, the brightness of staining is relatively independent of the number of cytopathologic areas on a coverslip. Also, coverslips can be fixed at any time after cytopathologic features appear when there are cells containing large amounts of antigen. In the AGP test, in contrast, the amount of precipitating antigen is highly dependent on both the amount of cytopathologic features in a culture and the stage in the cycle of the virus when the antigen is harvested. Thus, the conditions necessary for preparing antigen must be closely controlled, and each batch of antigen should be standardized. In the FA test, the cytopathologic areas can be examined morphologically, and contaminating agents which do not produce the characteristic cytopathologic features can be detected. These agents could conceivably produce false-positive reactions in the AGP test.

The FA test requires more manipulations than the AGP test, since serums must be centrifuged and diluted before use; however, the tests are almost comparable in ease of performance. The FA test is considerably more sensitive than the AGP test, but where serums contain very low levels of antibody, reading of the results is more subjective and requires more experience in the former than in the latter. At certain stages in the pathogenesis of MD, antibody can be detected by the FA test but not by the AGP test.

The indirect FA test for antibody is a readily performed, sensitive test which is highly specific for MD virus-induced antibody. It should be considered in immunologic, epizootiologic, and pathogenicity studies of MD, both in the laboratory and in the field.

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Article III

VIRUS-SPECIFIC IMMUNOFLUORESCENT AND PRECIPITIN ANTIGENS AND CELL-FREE VIRUS IN THE TISSUES OF BIRDS INFECTED WITH MAREK'S DISEASE

By

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Virus-specific Immunofluorescent and Precipitin Antigens and Cell-free Virus in the Tissues of Birds Infected with Marek's Disease

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SUMMARY

A variety of organs from chickens inoculated with Marek's disease virus and from control uninoculated chickens were examined for immunofluorescent antigen with the direct fluorescent antibody test with Marek's disease hyperimmune chicken globulin, for precipitin antigen with selected sera in the agar gel microprecipitin test, and for filtrable virus infectious for chick kidney cell cultures. A parallel histopathological examination of the organs was also made.

Immunofluorescent antigen was found in superficial cells of the epithelium of the feather follicles, lungs, follicles of the bursa of Fabricius, thymus, spleen, and cecal tonsil. It was not present in tumors of any organ. Precipitin antigen, which may have been identical to immunofluorescent antigen, was detected in all these organs except the cecal tonsil. Filtrable infectious virus was recovered from extracts of skin, but not from extracts of lungs, bursas, or thymuses. Histopathological examination revealed a close association between the antigens in these organs and cells undergoing degeneration and necrosis. There were intranuclear inclusion bodies in the cells of the epithelium of the feather follicles where similar necrobiotic changes were taking place.

INTRODUCTION

MD[1] is a lymphoproliferative disease which is considered to be caused by a highly cell-associated Group B herpesvirus (3, 17, 24, 30). In chick kidney or duck embryo fibroblast cultures, the virus produces a characteristic area of syncytial cytopathology (7, 24, 31) in which herpes virions and virus-induced antigens are detectable by the indirect fluorescent antibody test (16, 18, 20). Heavily infected cultures also contain MD-specific antigens demonstrable by the agar gel precipitin test (6, 21).

Kottaridis and Luginbuhl (12) described an antigen in bone marrow smears which reacted in the indirect fluorescent antibody test with rabbit antiserum. However, no antigen was reported in other organs. Chubb and Churchill (6) were unable to demonstrate antigen which would react in the agar gel precipitin test in homogenates of MD tumors.

The objective of the present studies was to examine various organs with and without MD tumors for the presence of IF and precipitin antigens specific for MD and to correlate the presence of antigen with histopathological changes. While the work was in progress, Spencer and Calnek (25), Calnek and Hitchner (5), and von Bülow and Payne (27) reported finding IF antigen in various organs, including the bursa of Fabricius, thymus, and kidneys. Calnek and Hitchner (5) demonstrated antigen in the feather follicle epithelium and Calnek *et al.* (4) recovered infectious cell-free virus from the feather shaft. Nazerian and Witter (19) demonstrated ultrastructural changes, including intranuclear inclusion bodies and enveloped virus feather follicle epithelium. The present communication confirms some of the above findings and extends the histopathological and immunological observations.

MATERIALS AND METHODS

Source of Chicken. Inbred lines 6 and 7 and the cross between line 15 males and line 7 females maintained at the Regional Poultry Research Laboratory were used throughout these experiments (20).

Source of Viruses. The JM and RPL 39 isolates of MD have been described (20). The RPL 39 isolate produces many more visceral lesions than the JM isolate (H. G. Purchase, unpublished data). Stocks of blood from birds with clinical signs and gross lesions of MD or cultured duck embryo fibroblasts with characteristic cytopathological areas were preserved with 10% dimethyl sulfoxide and stored in liquid nitrogen (10). Infectious bursal agent (2), as a homogenate infected bursa, was obtained from Dr. Roland Winterfield, Purdue University, Lafayette, Ind.

Antisera. Groups of 6- to 8-week-old line 6 chickens were inoculated intraabdominally 3 times at 2-week intervals with 0.5 ml of heparinized whole blood from birds with clinical and gross signs of MD and simultaneously inoculated i.m. with Freund's complete adjuvant (0.5 ml) (20). The birds were exsanguinated 2 weeks after the last inoculation, and serums prepared from the blood were tested for antibody by the indirect fluorescent antibody and agar gel precipitin tests. Both chick kidney and duck embryo fibroblast antigens were used in the agar gel precipitin test (6, 21).

Preparation of Fluorescein-conjugated Globulin. The

[1] The abbreviations used are: MD, Marek's disease; IF, immunofluorescent.

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globulin fraction of selected serums was precipitated 3 times with 18, 14, and 14% sodium sulfate, as described by Wier (28). The final precipitate was resuspended in borate buffer, pH 7.4, to one-half the original serum volume. It was then passed through a Sephadex G-25 column (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) equilibrated with phosphate-buffered saline, pH 7.2 (FTA hemagglutination buffer, Baltimore Biological Laboratories, Baltimore, Md.). The total bed volume of the column was at least 3 times the volume of the globulin. A solution of Blue Dextran 2000 (0.1% w/v) (Pharmacia) was placed on the column ahead of the globulin fraction to indicate when the void volume had been eluted. A volume of eluant was collected equal to 20% more than the volume of globulin placed on the column. The protein content was determined by the method of Lowry *et al.* (14), with bovine serum albumin as a standard. Immediately prior to conjugation, the pH of the solution was adjusted to 9.0 with 0.1 N sodium hydroxide and fluorescein isothiocyanate adsorbed onto Celite (Calbiochem, Los Angeles, Calif.) was added at a rate of 0.5 mg/mg protein. Conjugation was allowed to proceed for 30 min at 4°, during which time the reaction mixture was agitated continuously and maintained at a pH of 9.0 by adding sodium hydroxide. The mixture was centrifuged at 1500 × *g* for 3 min and the supernatant was placed on a Sephadex G-25 column equilibrated with phosphate-buffered saline. Once again a volume of eluant was collected equal to 20% more than the volume of conjugate placed on the column. Aliquots (1 ml) sealed in glass vials and stored frozen were thawed when needed and diluted in phosphate-buffered saline to a concentration determined by experience with that conjugate (1:80 or 1:160 for the conjugate used for most of the work reported here). Merthiolate (Eli Lilly and Co., Indianapolis, Ind.) was added to a final concentration of 1:10,000, w:v, and the solution was stored at 4° and used over a period of 2 to 3 months.

Preparation and Staining of Tissue Sections. Sections of various organs from freshly killed birds were embedded in OCT matrix, frozen, and cut at 6 μ thickness on a cryostat (Ames Lab-Tek, Westmont, Ill.), fixed immediately in acetone at 4° for approximately 2 min, and then rapidly air dried. They were stained, mounted in Elvanol (22), and examined as described previously (20), except that for direct fluorescent antibody staining only 1 incubation at room temperature with the dilution of conjugated serum was required. Whenever possible, duplicate samples of tissue were fixed in formol sublimate, dehydrated, embedded in wax, sectioned, and stained with hematoxylin and eosin.

Assay for Filtrable Virus. Portions of the blocks used for frozen sections and stored in the cryostat were removed and thawed and the excess embedding matrix was removed. A 10% extract prepared in cold phosphate-buffered saline by mincing with scissors was sonicated for 30 sec with the small probe of a Bronwill Biosonik oscillator (Will Scientific, Inc., Rochester, N. Y.) at 70% of maximal intensity. The extract was centrifuged at 1500 × *g* for 5 min, and the supernatant fluid was removed. The supernatant, after addition of 2 drops of a 3-times-washed 24-hr culture of *Serratia marcessens*, was then passed through a 0.45-μ filter (Swinnex 25 unit, Millipore Filter Corp., Bedford, Mass.), pretreated with bovine fetal serum (26). The first and last few drops of filtrate were placed in tryptose phosphate broth and incubated aerobically at 37° for 3 days, during which time bacterial growth was not detected. Duplicate 0.2-ml samples of the filtrate were assayed for virus on chick kidney cells (31).

Agar Gel Precipitin Test. Organs were treated as described above, except that a more concentrated (25%) extract was prepared. Some samples were homogenized with a TenBroeck grinder, in place of sonic extraction. The microprecipitin test was performed as previously described, with agar containing 8% NaCl (6).

Experimental Design. In the 1st experiment, 15 3-week-old chickens were inoculated intraabdominally with 0.2 ml of the stock of JM MD blood, and another group of 15 birds was inoculated by the same route with 1×10^7 JM MD-infected duck embryo fibroblast culture cells. A 3rd group of 15 birds was maintained uninoculated to serve as controls. The 3 groups were placed in separate adjacent Horsfall-Bauer isolators. Portions of liver, spleen, kidney, adrenal, gonad, bone marrow, lung, bursa of Fabricius, thymus, cecal tonsil, pancreas, proventriculus, brain, brachial, sciatic and celiac plexuses, and skin from which the feathers had been plucked were removed for examination from 1 bird from each group at 5, 9, 14, 19, 23, 28, 35, and 42 days postinoculation and the survivors were discarded at 42 days postinoculation.

In the 2nd experiment, 15 1-day-old chicks were inoculated intraabdominally with JM MD blood. A 2nd group of 15 chicks was similarly inoculated with RPL 39 MD blood and a 3rd group of 9 chicks served as uninoculated controls. Birds (2 or 3 from each group) were killed and portions of the skin, lung, bursa of Fabricius, and thymus were removed for examination at 5, 8, 12, 15, and 19 days postinoculation. Survivors were discarded at 19 days postinoculation.

In the 3rd experiment, 1 drop of a 1:10 dilution of stock infectious bursal agent virus was placed in each eye of 16 3-week-old chicks. A 2nd similar group was inoculated intraabdominally with RPL 39 blood and a 3rd group served as uninoculated controls. Pieces of the lung, bursa of Fabricius, and thymus of 4 of each exposed and control group were removed for examination at 1, 2, 4, and 8 days postinoculation.

RESULTS

Distribution of IF antigen. IF antigen was first detected in the feather follicle, lung, and bursa (Table 1) at 5 days after inoculation of 1-day-old or 3-week-old chicks. Antigen occurred sporadically thereafter in these organs and in the thymus, spleen, and cecal tonsils until 42 days postinoculation, which was the longest time tested. Many of the birds inoculated with either the JM or RPL39 strains of MD virus had antigen detectable in several different organs.

Initially, IF antigen was diffusely distributed in the cytoplasm of a few round cells in the affected organ (Fig. 5) but in more advanced cases, some cells contained brightly staining granules of irregular size and shape (Fig. 6).

Occasionally, the nuclei of cells were filled with a homogeneous IF antigen which stained very brightly.

Antigen was most commonly seen in cells in the superficial layers of the corneous portion of the feather follicle epithelium which lies adjacent to the coreum produced by the growing feather (Fig. 1). It varied from a small amount of diffuse cytoplasmic antigen in a few individual cells to large amounts of diffuse and granular cytoplasmic and diffuse nuclear antigen in a band of cells about 4 deep extending from the basal end of the follicle to the interfollicular skin. It usually appeared to be most concentrated in the deeper 2/3 of the follicle wall, and it never extended to the interfollicular skin. When sections of skin including follicles were stained with hematoxylin and eosin, alterations were seen in the epithelium lining the feather follicles in 4 birds (Table 1 and Fig. 2). All 4 birds had IF antigen in their follicles. No changes could be seen in the basilar layer; however, there were degenerative changes in cells in the intermediate and transitional layers [The basal, intermediate, and transitional layers together form the *stratum germinativum* (15)]. Cells filled with a clear vacuole which either displaced the nuclei into a crescent on 1 side of the cell or surrounded the nucleus were more frequently seen in infected birds than in uninoculated controls. In 1 bird with IF antigen, many of the nuclei in the transitional layer contained characteristic inclusion bodies (Fig. 2) resembling those produced by MD virus in cell culture (31). Sometimes the inclusion bodies were unusually dense and contracted, reflecting the variable amounts of cell degeneration. The cytoplasm of cells in this layer was slightly eosinophilic and granular. In the outer layer, in the position of the *stratum corneum*, the nuclei of the cells were either basophilic and about the size of inclusion bodies or had degenerated completely and were not visible. The cytoplasm of the cells was filled with indistinct highly eosinophilic granules. The most superficial layers were disintegrating into fragments and finely granular material. In follicles from which the feathers were not removed, the feather sheaths and the feathers themselves appeared normal. Even in preparations in which many of the feather follicles showed the above changes, there were some follicles which appeared normal. Since, in the few follicles sectioned longitudinally, the alterations were largely confined to the epithelium in the deeper 2/3 of the follicle, the level at which the follicles were sectioned transversely may have determined whether alterations were seen. In 4 birds, there were large accumulations of lymphoid cells in the nerves and in the connective tissue of the subcutis. There was no IF antigen in these areas; however, 2 of these had IF antigen in their feather follicles.

IF antigen also occurred in the lungs in cells located between the epithelial linings of adjacent air capillaries and sometimes in the epithelial cells themselves (Fig. 3). Occasionally, fluid in the intermediate cavities and lumens of tertiary bronchi also stained with the conjugate. Most of the lungs appeared normal in histological sections. In some there were diffuse infiltrations of lymphoid cells between the epithelial linings of the air capillaries (Fig. 4). In the lungs of 5 of the birds examined, there were large areas of pleomorphic lymphoid cells characteristic of MD lymphoid tumors, but none of these contained IF antigen.

The earliest signs of antigen in the bursa of Fabricius, 5 days after inoculation of day-old chicks, consisted of scattered individual cells in the medulla of the follicles (Fig. 5) which increased until the whole medulla stained very brightly (Figs. 6 and 7). Granular cytoplasmic antigen in cells also scattered through the cortex and sometimes between the follicles (Fig. 6) could be distinguished from the occasional granules of fluorescent precipitate in the serum by their morphology and plane of focus (Fig. 13). After hematoxylin and eosin staining, the bursas with few scattered cells containing IF antigen had follicles which were uneven in size and in some the medulla was atrophied. There was usually some evidence of necrosis, *i.e.*, pyknosis and karyorrhexis. Many birds with atrophic follicles without signs of necrosis contained no cells with IF antigen. In 3 birds where the medulla of some follicles contained masses of antigen, there was massive necrosis with lysis and karyorrhexis of the lymphoid cells (Fig. 8). In the bursas of birds which had recovered from the necrotic process, there were often cysts containing cellular debris and in some cases the medullas were filled with reticular cells and fibrous tissue (Fig. 9). Occasionally, in older birds, there were large groups of heterophils in the cortex of, or between, the follicles. IF antigen was not detected in the bursas with these regenerative changes, although antigen was often present in other organs of the same bird. Bursas with massive extrafollicular infiltration of pleomorphic lymphocytes (tumors) (Fig. 11) and follicular atrophy did not contain IF antigen.

In the thymus, IF antigen was usually confined to isolated

Table 1

Fluorescent antibody and hematoxylin and eosin staining of tissues from MD-infected and control birds.

	Fluorescent antibody		Hematoxylin and eosin	
	Inoculated	Control	Inoculated	Control
Feather follicle	19/29[a]	0/9	4/18[b]	0/8
Lung	12/53	0/20	5/48	0/17
Bursa	8/53	0/20	24/48[c]	0/17
Thymus	7/50	0/19	5/48[c]	0/17
Spleen	2/23	0/11	0/18	0/8
Cecal tonsil	1/19	0/9	1/16	0/7
Nerve	0/20	0/9	28/48	0/17
Brain	0/7	0/2	10/18	0/8
Gonad	0/19	0/9	9/18	0/7
Liver	0/11	0/4	1/18	0/8
Kidney	0/11	0/4	4/18	0/8
Pancreas	0/7	0/2	0/17	0/8
Proventriculus	0/7	0/2	2/18	0/8
Total positive	23/59	0/21	37/48	0/17

[a]Number positive over number examined. Birds were examined at various times between 5 and 42 days postinoculation.

[b]Lymphocytic infiltrations in the subcutis. 4/18 had vacuolization of nucleus and cytoplasm and disruption of cells of the feather follicle epithelium. Of these, 1 also had intranuclear inclusion bodies.

[c]Includes atrophic and necrotic lesions in addition to the lymphocytic infiltrations or tumors which are characteristic lesions of MD in other organs.

cells in the medulla. In some birds, particularly those with advanced regenerative changes in the bursa, there were large groups of cells in the thymus which contained antigen (Fig. 10). After hematoxylin and eosin staining, the thymuses were severely atrophied (Figs. 15 and 16) and groups of cells in the medulla exhibited necrosis and karyorrhexis (Figs. 17 and 18).

In the spleens and cecal tonsils, IF antigen was present in a variable number of round cells. In 1 bird, the cecal tonsil and surrounding musculature of the intestine were tumorous, but there was no IF antigen in this organ.

Massive infiltrations of pleomorphic lymphoid cells occurred in the nerves, gonad, liver, kidney, or proventriculus. No IF antigen was detected in any of these tumors.

Cells containing granules which stained very brightly (Fig. 12) were seen in the bone marrow smears of both normal and infected birds. This staining was not considered specific.

Demonstration of Specificity of Staining. Two criteria were used in the initial evaluation of the specificity of the staining. Firstly, specific staining was very much brighter than the background staining obtained in other areas of the section and in sections of tissues from other birds. Secondly, specific staining was absent from the tissues of control birds. Staining was observed in bone marrow and skin (epidermis) of both infected and control birds. Further evidence that the staining in the epidermis was nonspecific was that it could be eliminated without affecting the staining in the feather follicles by further dilution of the conjugate; however, this procedure did not reduce staining of the bone marrow cells. Similar results were obtained by von Bülow and Payne (27). This nonspecific staining was omitted from the tables of results.

Serums from 3 different chickens hyperimmunized with blood from birds infected with the JM strain of MD and conjugated with fluorescein isothiocyanate stained similar areas in the bursa and lung equally well, whereas fluorescein-conjugated serum from a bird which was hyperimmunized with normal duck embryo fibroblast cultures and did not contain MD antibody detectable by the indirect fluorescent antibody or agar gel precipitin tests did not stain this antigen. Fluorescein-conjugated anti-rabbit globulin and anti-human globulin and rhodamine-conjugated human albumin did not stain IF antigen in similar sections.

The staining of IF antigen could be completely inhibited by pretreatment of the tissue section with unconjugated serum before staining with the conjugated serum. When conjugated serum was doubly absorbed with MD chick kidney precipitin antigen, it no longer stained tissue culture antigen and the intensity of staining of IF antigen in the bursa was considerably reduced. Control chick kidney antigen prepared in a manner similar to the above precipitin antigen did not alter the staining ability of the serum.

Sections of the bursa from one 16-week-old bird with a grossly visible lymphoid leukosis tumor follicle induced by a Subgroup A virus (RPL 12) and from ten 3-week-old birds between 2 and 8 days after inoculation with infectious bursal agent, showing characteristic degenerative and necrotic changes, were free of IF antigen.

Distribution of Precipitin Antigen. Homogenates of various organs (Table 2) were examined for precipitin antigen with the fluorescein-conjugated MD hyperimmune serum used in the above IF studies (but undiluted), serum from a bird which had recovered from JM MD, sera from 3 birds which had been inoculated at 1 day of age with MD-infected duck embryo fibroblasts, sera from 2 adult birds which had been challenged at 10 weeks of age by intraabdominal inoculation of blood from a bird infected with MD, and sera from 4 naturally exposed birds from a commercial flock. A common precipitin line was obtained with all these sera, but not with serum from a 16-week-old bird reared in isolation. When tested beside MD duck embryo fibroblast and chick kidney antigens, there was a continuation between the precipitin line obtained with the bursa and skin antigens and the strongest line obtained with the cell culture antigens, which was probably the A antigen line (9) (Fig. 14).

The Presence of Filtrable Virus. Six samples of skin, lung,

Table 2

Distribution of precipitin antigen among various organs which had been previously examined for IF antigen

Organ homogenate	Source	No. of birds examined	IF antigen status	No. of organs with precipitin antigen
Skin	MD inoculated	3	Positive	1
	MD inoculated	7	Negative	2
	Control	7	Negative	0
Lung	MD inoculated	10	Positive	2
	MD inoculated	26	Negative	0
	Control	9	Negative	0
Bursa	MD inoculated	12	Positive	3
	MD inoculated	52	Negative	0
	Control	18	Negative	0
Thymus	MD inoculated	8	Positive	2
	MD inoculated	28	Negative	0
	Control	9	Negative	0
Spleen	MD inoculated	2	Positive	2
Cecal tonsil	MD inoculated	1	Positive	0

thymus, and bursa which contained IF antigen were examined for filtrable virus. None of the lung, thymus, or bursa samples contained virus demonstrable by the procedures used; however, 4 skin specimens yielded 10, 7, 1, and 1 focus-forming units/0.2 ml extract, respectively. When these cultures were stained with the fluorescein-conjugated chicken globulin by a modified procedure (16), IF antigen was detected in the cytopathological areas. The distribution of antigen was identical to that previously described (20).

DISCUSSION

Three different types of virus-host cell interactions occurred *in vivo*. Firstly, there were processes in which neither antigen nor infectious cell-free virus could be demonstrated. Thus, tumors of the visceral organs and nerve lesions did not contain IF antigen, precipitin antigen, or infectious cell-free virus (8) and, except in rare instances (K. Nazerian, unpublished, and Ref. 23), have not been reported to have virus particles. Since small numbers of intact tumor cells will induce MD when inoculated into susceptible disease-free chickens (31), the viral genome must be present within these cells.

Secondly, antigen may be present, but not infectious virus, and a cytolytic process may occur. Both IF and agar gel precipitin antigens were detected in the bursa of Fabricius, where they were associated with necrosis of the medullas of the follicles. The IF antigen and the cytolytic process were described by Spencer and Calnek (25) and Calnek and Hitchner (5). A degenerative process was also seen in the thymuses which contained IF antigen. Thus it appears that production of antigen, particularly in these organs, is associated with a cytolytic process in the cells. The IF antigen in the lung could have been infection from inhaled material from other infected birds in the same isolator. Many of the birds with IF antigen in their lungs also had antigen in other organs, suggesting that the antigen was being produced at several sites simultaneously. No infectious cell-free virus was found in the bursa or lung, indicating that replication of the viral genome and production of antigen occurred without formation of complete infectious virions, although incomplete virions may have been produced at these sites. The same relationship probably existed in other organs which contained IF antigen, but which were not examined for infectious virus.

Lastly, in the feather follicle epithelium, both antigen and infectious cell-free virus were detected. The virus was infectious even after storage at -15° for 2 months. Similar results have been obtained by Calnek *et al.* (4). Both Calnek *et al.* (4) and Nazerian and Witter (19) detected virus in the base of the feather shaft, but did not examine skin homogenates. In both instances the origin of the virus was most likely the feather follicle epithelium. This is the only place where infectious cell-free virus has been detected *in vivo*. Cells in this location are continuously being sloughed off in normal birds. In infected birds, an additional degenerative process is involved, and the cells fragment and release their contents. These cells have been found to contain large numbers of enveloped virions (K. Nazerian, unpublished).

Instances have been reported in which cultured cells were found to be infectious when inoculated *in vivo* and yet there was no evidence of cytopathology in the cultures (13, 29, 30). This situation was similar to the 1st type of virus-host cell interaction described above. Usually, however, infected susceptible cells in culture contained IF antigen, underwent degeneration, and eventually became part of a cytopathic area. Very few complete herpes virions were seen by electron microscopy in these cultures and only small amounts of virus can be recovered from them (16, 18). Thus, there is a parallel between this situation and that which exists in the bursa of Fabricius of infected birds, *i.e.*, the 2nd virus-host cell interaction described above.

The techniques used were not accurate enough to identify with certainty each cell containing antigen; however, it appeared that antigen did occur in lymphocytes in the bursa, thymus, and lung, and only in the latter organ may some of the epithelioid cells themselves have been involved. In the feather follicles the interrelationships were unique, and the only cells affected were the keratinizing cells of the stratified squamous epithelium. No IF antigen was detected in any of the kidneys, gonads, or nerves examined, whereas Spencer and Calnek (25) and Calnek and Hitchner (5) have observed fluorescence in these organs. On the other hand, they were unable to observe fluorescence in the lungs, whereas this was a common site of antigen detection in the present studies. These discrepancies could have been due to differences in the strains of chicken, in the virus used, or in the specificity of the conjugates. Satisfactory frozen sections of feather follicles were difficult to obtain in this laboratory. This may have been responsible for the lower proportion of feather follicles with IF antigen in this work than was reported by Calnek and Hitchner (5), and it could account for the 2 instances in which precipitin antigen was detected, but no IF antigen was detected.

Nuclear inclusion bodies in the superficial layers of the epithelium of the feather follicle were observed by Nazerian and Witter (19), who also found enveloped particles in cytoplasmic inclusion bodies. However, they were not described by Calnek and Hitchner (5).

Both the IF and agar gel precipitin antigens in cell culture and *in vivo* are virus-induced and appear specific for MD virus, since they are found only in cells or birds infected with MD virus and not in cells or birds infected with other agents, such as lymphoid leukosis or infectious bursal agent. Antigens produced by the JM and RPL 39 strains of virus were indistinguishable. It was not possible to determine whether the same antigen was being detected by the fluorescent antibody and agar gel precipitin tests *in vivo* and in cell culture. Since the organ extracts and cell culture extracts gave a "line of identity" in the agar gel precipitin test, they must both contain at least 1 antigen in common. Since the line of identity between these antigens was obtained in the agar gel precipitin test with the fluorescein-tagged antiserum, it was likely that a similar common antigen was being detected by both the fluorescent antibody and agar gel precipitin tests. Here, as in the test for antibody (21), it appears as if the fluorescent antibody test is more sensitive than the precipitin test.

IF and precipitin antigens were detected in the feather follicles or skin extracts as early as 5 days and as late as 42 days postinoculation, which was the longest time tested. It is probable that virus is also produced throughout most of this period, since the cycle of replication of the virus is completed in these cells and since birds have been shown to be infectious for most of this time (11). The infected feather follicle cells are probably eased out of the follicle as the feather grows and they form part of the dander which is infectious (1) and which has also been shown to contain infectious cell-free virus (J. N. Beasley, personal communication). Inasmuch as large amounts of dander are produced by chickens, this is the most likely source of virus for dissemination to the surroundings.

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Fig. 1. IF antigen (*small filled arrow*) in the superficial layers of the epithelium of the feather follicle. The antigen stains bright green, whereas the feather (*open arrow*) reflected blue light of lower intensity. *Large and small filled arrows*, positions of the basilar and corneous layers, respectively, in Figs. 1 and 2. Fluorescent antibody stain, X 120.

Fig. 2. Inclusion bodies (*open arrow*) in the nuclei of cells in the transitional layer of the epithelium lining the feather follicle 19 days after inoculation of MD virus into a 3-week-old chick. The epithelium is convex instead of concave because the follicle collapsed after the feather was removed. It is unusually deep because of the oblique angle of the section. The basal layer (*large arrow*) and intermediate layers in this section are not affected, but the transitional (*open arrow*) and corneous layer (*small arrow*) show necrobiosis. H & E, X 300.

Fig. 3. IF antigen in cells between the air capillaries in the lung in a 28-day-old chicken inoculated with MD virus at 1 day of age. Both diffuse bright fluorescence throughout the whole cell, and granular cytoplasmic fluorescence are visible. For orientation, compare with Fig. 4. Fluorescent antibody stain, X 300.

Fig. 4. Cellular infiltration in the lung of a 19-day-old bird which also contained IF antigen. *pb*, tertiary bronchus or parabronchus; *a*, atrium; *i*, intermediate space; *c*, air capillaries. H & E, X 300.

Fig. 5. Immunofluorescent antigen in the medulla of a follicle of the bursa of Fabricius 9 days after inoculation of MD virus into a 3-week-old chicken. The cytoplasm of many small round cells and 1 large round cell stain brightly. Fluorescent antibody stain, X 480.

Fig. 6. Large amounts of IF antigen in the medulla of a follicle in the bursa of Fabricius of a bird the same age and treatment as that in Fig. 5. Granular cytoplasmic antigen is present in many cells in the cortex. This bursa also had precipitin antigen. Fluorescent antibody stain, X 400.

Fig. 7. IF antigen in the medulla of another follicle from the bursa of the same bird as Fig. 6. *Arrow*, periphery of the cortex of the follicle. Fluorescent antibody stain, X 120.

Fig. 8. Necrosis of the cells of the medulla of a follicle from the bursa of the same bird as Figs. 6 and 7. H & E, X 120.

Fig. 9. Replacement of the medullas of follicles of the bursa of Fabricius by reticular cells in a chicken 42 days after inoculation at 3 weeks of age with MD virus. This bursa did not contain IF or precipitin antigen, although IF antigen was detected in the lung, thymus (same bird as Fig. 10), and feather follicle. H & E, X 120.

Fig. 10. IF antigen in the thymus 42 days after inoculation at 3 weeks of age with MD virus. For orientation and location of antigen compare with Fig. 18. This thymus also contained precipitin antigen. Fluorescent antibody stain, X 300.

Fig. 11. Massive interfollicular infiltration of the bursa of Fabricius with pleomorphic lymphocytes 19 days after inoculation at 1 day of age with the RPL 39 strain of MD virus. This bursa did not contain IF antigen. H & E, X 120.

Fig. 12. Nonspecific staining of granules in cells of a bone marrow smear. Fluorescent antibody stain, X 300.

Fig. 13. Bursa of Fabricius from an uninfected bird stained with the fluorescein-conjugated chicken globulin used throughout these studies. The granules which are precipitated from the serum can be distinguished from specific stain by their morphology and by the plane at which they come into focus. Fluorescent antibody stain, X 120.

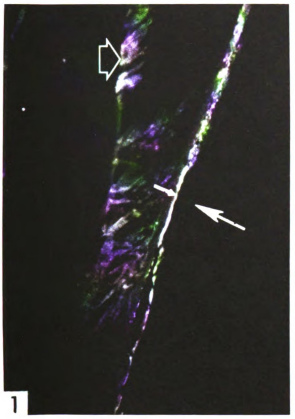
Fig. 14. Agar gel precipitin test. *Well 1* contains antigen prepared from normal skin, *Well 2* and *5* contain chick kidney cell culture precipitin antigen, *Wells 3* and *6* contain duck embryo fibroblast culture precipitin antigen, and *Well 4* contains antigen prepared from the skin of an infected bird with IF antigen in the feather follicles. The *center well* contains the fluorescein-conjugated chicken globulin, which was also used in the fluorescent antibody tests. One line, probably the A antigen line, is common between the different antigens. X 2.

Fig. 15. A normal thymus from a bird 56 days old. H & E, X 30.

Fig. 16. Severe atrophy of the thymus of a chicken 42 days after inoculation with MD virus at 1 day of age. This thymus contained IF antigen, but not precipitin antigen. H & E, X 30.

Fig. 17. Higher magnification of the medulla of the same thymus as in Fig. 15. h, Hassall's corpuscle. H & E, X 300.

Fig. 18. Higher magnification of the same thymus as in Fig. 16. Note the Hassall's corpuscle (h) and the area of degenerating cells (*arrow*), which is thought to correspond with the areas which contain IF antigen, as in Fig. 10. H & E, X 300.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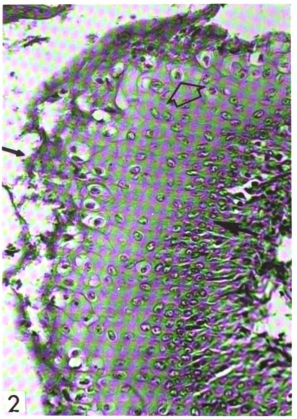
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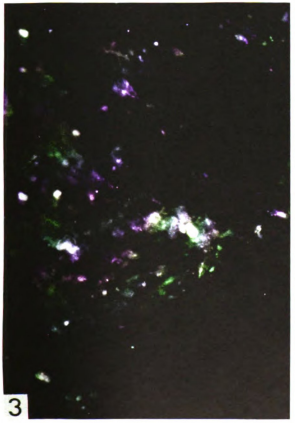
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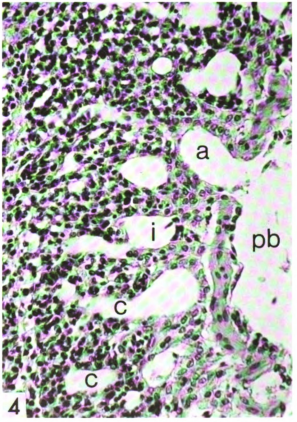
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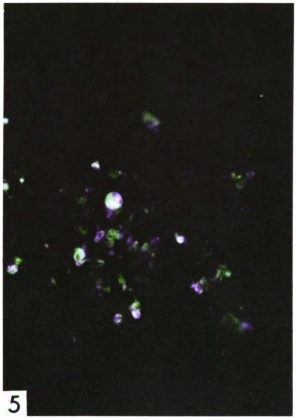

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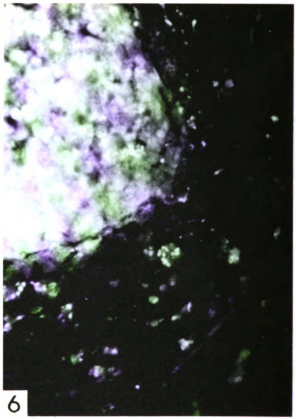

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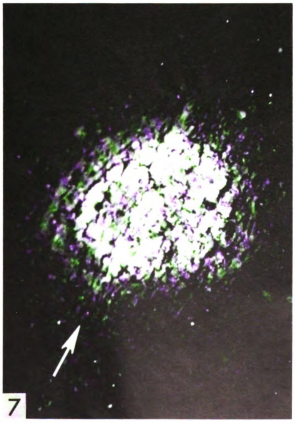

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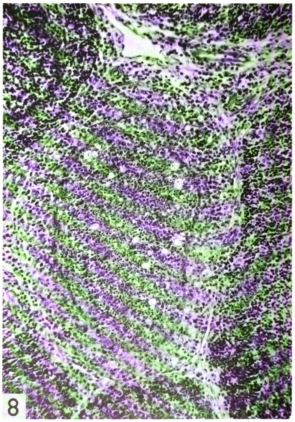

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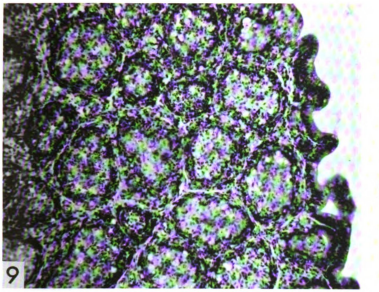
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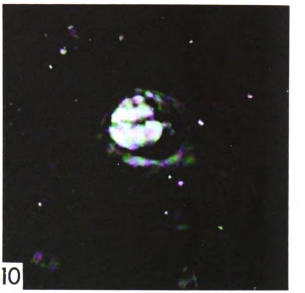
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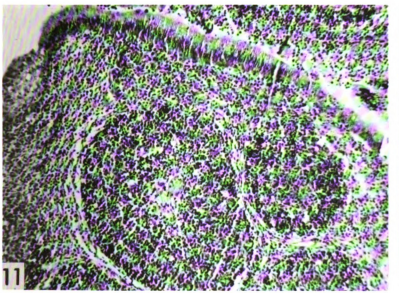
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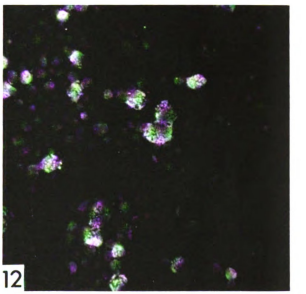
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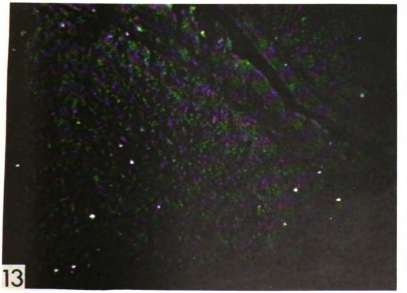
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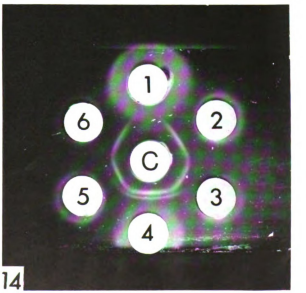
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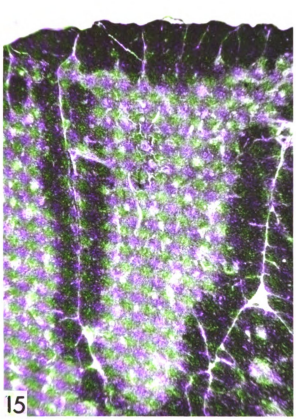
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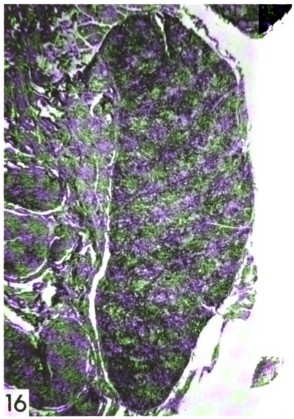
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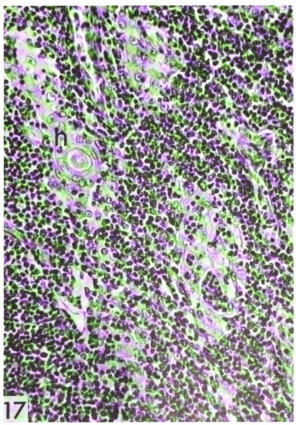
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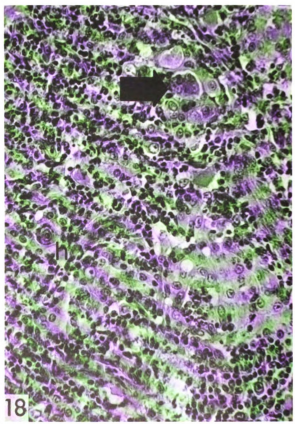
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Article IV

PATHOGENICITY AND ANTIGENICITY OF CLONES FROM STRAINS OF MAREK'S DISEASE VIRUS AND THE HERPESVIRUS OF TURKEYS

By

H. G. Purchase, B. R. Burmester and C. H. Cunningham

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PATHOGENICITY AND ANTIGENICITY OF CLONES FROM STRAINS OF MAREK'S DISEASE VIRUS AND THE HERPES VIRUS OF TURKEYS[a]

H. G. Purchase[b,c], B. R. Burmester[b,c], and C. H. Cunningham[c]

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a This report is a portion of a thesis submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree.

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ABSTRACT

Virus was extracted by filtration from chicken embryo fibroblast cultures infected with the JM, high passage JM(JMHP), GA, and RPL39 strains of Marek's disease virus (MDV) and from the herpesvirus of turkeys (HVT) and purified by cloning. The plaques produced by clones of HVT, JMHP and other MDV differed in morphology from one another. Clones of MDV varied greatly in pathogenicity for chickens but JMHP and HVT were non-pathogenic. Two pathogenic clones of JM virus and a clone of JMHP virus lacked the A precipitin antigen present in all other clones tested. All clones had at least one B antigen in common. HVT and MDV clones with and without the A precipitin antigen could be distinguished from each other by the indirect fluorescent antibody test. Changes in virus-host cell relationships, loss of pathogenicity and loss of the A antigens were independent events.

INTRODUCTION

Marek's disease herpesvirus (MDV) antigen and antibody have been detected by the agar gel precipitin test (4, 16) and by both the direct and indirect fluorescent antibody tests (17, 18, 20, 22). Initial examination of several laboratory and field isolates of MDV failed to reveal any antigenic differences between them. However, there was no indication of the purity of the strains used, and a common contaminant could have accounted for the serologic cross-reactions.

An antigenic change has been described in MDV that had been passaged many times in chicken kidney (CK) cell culture (7). During the 20th to 30th passages in cell culture, the virus lost an antigen which was usually found in the supernatant fluids of cultures infected with the original strain. Futhermore, the subcultured virus had become apathogenic for chickens, its growth characteristics in cell culture had become altered, and larger plaques containing larger syncytia occurred in a shorter time than with the original virus. A similar attenuation and morphologic change of the cytopathologic areas (plaques) occurred on passage of the JM strain of MDV (13).

Witter, et.al. (25) described a non-pathogenic herpesvirus isolated from turkeys (HVT). The HVT produced larger plaques than MDV and was antigenically similar, but could be distinguished from it by the indirect fluorescent antibody test. Vaccination with HVT protected chickens against subsequent challenge with MDV (15). A similar herpes-

virus isolated from turkeys (10) has given similar protection (D.P. Anderson, unpublished data).

Variants of herpes simplex virus could be distinguished from one another by the cytopathic effects they produced (9, 14, 21), the distribution and intensity of fluorescent staining in cell culture (11), and the size of the pocks produced on chorioallantoic membranes of chicken embryos (12). Similar differences in cytopathic effect were described for variants of B virus (Herpesvirus simiae), and there was a relationship between plaque size and virulence for rabbits (2). This virus could be distinguished from herpes simplex by the direct or indirect fluorescent anyibody tests (1). Passage of pseudorabies virus in cell culture altered the size of plaques produced and decreased the pathogenicity for rabbits (23). From the above, it appeared likely ;that variants of MDV might exist and that characteristics used to detect the variants might be useful in distinguishing MDV from HVT.

The present paper describes the purification by cloning of three strains of MDV and one of HVT and their pathogenicity in chickens and antigenic differences by the indirect fluorescent antibody and agar gel precipitin tests. A description of the plaques produced by the clones and of host range studies will be presented in another communication.

MATERIALS AND METHODS

Source of Viruses. Blood or tumor cells from 3 to 6 week-old chicks infected at one day of age with the JM, RPL39 and GA strains of MDV were used (17). A strain of JM virus (JMHP) which had been passaged more than 45 times in CEF and duck embryo fibroblast (DEF) culture was kindly supplied by Dr. K. Nazerian (13). The FC126 isolate of HVT used in these studies has been described (25).

Chickens and Embryos. Line 15 x 7 chickens (8) and line 1900 embryos were from the Single Comb White Leghorn flocks reared at the Regional Poultry Research Laboratory. Line 1900 are commercial chickens of C/O phenotype reared under pathogen free conditions. They are free of lymphoid leukosis viruses and MDV (H. A. Stone, unpublished data). Embryonated duck eggs were obtained from Truslow Farms Inc., Chestertown, Maryland 21620.

Cell Cultures. Primary CEF and DEF cultures were prepared from 10 and 14 day old embryos respectively as previously described (26) and grown in medium F10 and 199 (Grand Island Biological Co., Grand Island, N.Y.) with 5% calf serum (Colorado Serum Co., Denver, Colorado). The CK cells were prepared from birds under 4 weeks of age and were cultured in Eagle's basal medium with 5% bovine fetal serum (Grand Island Biological Co.) (26).

Propagation and Cloning Procedures. Secondary DEF cultures were inoculated with blood or tumor cells from birds infected with the JM, RPL39 and GA strains of MDV. The cells were passaged 1 to 3 times until extensive cytopathologic effects had developed. Because CEF cultures were shown to produce more cell-free virus than DEF cultures (13) the viruses were adapted to grow in CEFs. They were first propagated in a mixture of equal numbers of CEFs and DEFs in culture. When extensive cytopathologic effects had developed, usually 2 to 6 passages, the cells were passed onto pure CEF cultures. At each subsequent passage on CEF cultures an attempt was made to extract filterable virus as described below.

The JMHP and HVT produced cytopathologic changes in CEFs and filterable virus was extracted from them.

Filtration and cloning. The growth medium on heavily infected CEF cultures was replaced with antibiotic free medium 24 hours before extraction. When nearly all the cells in the cultures had become morphologically altered and there were large numbers of rounded cells floating in the medium, but before holes appeared in the monolayer, the cells were scraped off the plate into the supernatant medium. This suspension was kept on ice and sonicated with the Bronwell Biosonic oscillator (Will Scientific Inc., Rochester, N.Y.) using the small needle probe at 70% of maximal intensity for 10 seconds, a procedure which will disrupt over 99% of the cells. The extract was then centrifuged at approximately 2000 x g for 5 minutes. The supernatant fluid was decanted into a fresh tube and a few drops of a turbid,

3 times washed 24-hour old culture of Seratia marcessens was added. The fluid was then filtered through a 0.45 μm membrane filter (Swinnex, Millipore Corporation, Bedford, Mass.) which had been pretreated with bovine fetal serum (24).

The integrity of the filter was ascertained by bacteriological sterility tests of the first few drops after the void volume of the filter had been expressed and the last few drops after filtration. The filtrate, 1 ml/plate, was used to inoculate CEF, DEF and CK cultures grown in 60 mm plastic petri dishes (Falcon Plastics, Los Angeles, California). Growth medium was added to the cultures four hours after inoculation and it was changed every 2 days thereafter. Cultures were examined daily for cytopathologic changes.

When plaques consisting of about 10 or more rounded refractile cells had appeared, the cultures were overlaid with growth medium containing 1% agar. Holes, approximately 2 mm in diameter, were cut with a sterile cork borer through the agar above the plaques. These areas and a few surrounding cells were removed from the petri dish with a few drops of 0.05% trypsin and placed in 0.5 ml of calf serum until they were used to inoculate DEF cultures. These clones were propagated in DEF cultures until a sufficient stock was obtained, usually 2 to 3 passages, at which time the cells were preserved in liquid nitrogen.

Pathogenicity of clones. Dilutions of virus-infected cells containing 1,000 plaque forming units (PFU) per 0.2 ml were inoculated intra-abdominally into one-day-old line 15 x 7 chicks which were then reared in modified, stainless steel Horsfall-Bauer isolators. All chickens dying during the experiment were necropsied. All survivors were exsanguinated and necropsied at 10 weeks of age. When a diagnosis could not be made on gross examination, portions of the left and right brachial and sciatic plexuses, the celiac nerve and a gonad were removed for histopathologic examination.

Fluorescent antibody and agar gel precipitin tests. The proceddres used for the indirect fluorescent antibody test have been described (17). Cell monolayers infected with each clone were prepared by inoculating confluent CK cultures on 11 x 22 mm coverslips in petri dishes with approximately 100 PFU per coverslip. The coverslips were removed and fixed when early cytopathologic changes were visible, i.e., at 1 to 3 days after inoculation.

Reagents for the agar gel precipitin tests were prepared from the stocks of cloned viruses after two additional passages in DEF cultures (20). When there were advanced cytopathologic changes the supernatant fluid and cells were harvested. The supernatant medium was centrifuged at 1500 x g for 10 minutes to remove most of the cells and then concentrated approximately 50 times by precipitation twice with 80% and then 60% saturated ammonium sulfate. The final precipitate, resuspended in distilled water, is referred to as supernatant reagent.

Cells were scraped off the petri dish and suspended in phosphate buffer saline, pH 7.4, at a concentration of approximately $2x10^7$ cells per ml. They were placed on ice and sonicated for 30 seconds with the small probe of a Biosonic oscillator at 70% of maximum intensity. This material is referred to as cell reagent. Both the supernatant and cell reagents were stored at -20°C until just before use.

Agar gel precipitin tests were performed as previously described (16, 20) with the following modifications. A 25x75 mm glass slide was painted with a thin layer of a 0.5% agar containing 0.05% glycerine and dried rapidly on a warm (70-90°) surface. A second slide was placed above the coated slide and separated from it by 2 layers of electrical tape and agar (0.5%, 1% or 2% in phosphate buffered 8% NaCl solution) was poured between them. After the agar had solidified, the uncoated slide was removed and a plexiglas template (Bolab Incorporated, Reading, Mass.) with a flat polished lower surface lightly coated with silicone grease was placed on the agar. The holes in the template were then filled with 50 µm antigen or serum. Incubation was at room temperature for 72 hours.

Sera used in both the indirect fluorescent antibody and agar gel precipitin tests were from the survivors of the pathogenicity tests. The hyperimmune sera were the same as those used previously (17).

RESULTS

Filtration and cloning of viruses. Of the 50 attempts to filter the viruses, only 18 (36%) were successful (Table 1). The JMHP and HVT were filtrable in 4 of 5 (80%) attempts whereas the low passage JM, RPL39 and GA strains yielded filtrable virus in 14 of 45 (32%) attempts. There was less virus (1.3-14.7PFU/ml) produced by the low passage strains than by the JMHP (56.5PFU/ml) and HVT (250PFU/ml). Of the 8 filtration experiments performed with MDV which had been passed between 4 and 10 times in cell culture none were successful. However, sporadic successes were obtained with viruses between the 11th and 18th passages.

None of the uninfected control cells had cytopathic changes or yielded filtrable virus.

Morphology of plaques in CK cells. The appearance of plaques produced in CK cultures by all the clones except JMHP and HVT was similar to that previously described (5, 6, 7, 26). Infected cells became rounded and highly refractile and were sometimes multilayered. In each plaque the number of spherical refractile cells increased with time so that it resembled a "bunch of grapes". However, the plaques rarely exceed 1 mm in diameter even in cultures 14 days postinoculation.

The plaques produced by JMHP were similar to those described (7, 13). They appeared 1 to 2 days earlier, were larger than those produced by low passage virus, and contained larger spheroidal, highly refractile syncytia. Cells lysed and/or became detached so that plaques developed a hole in the center. This occurred more frequently with plaques induced by JMHP than with those induced by low passage virus.

Plaques produced by HVT could be detected as early as 2 to 3 days after inoculation (25). After 10 days, the plaques were 1.5 to 2 mm and occasionally 3 mm in diameter. In their earlier stages of development, plaques consisted of a few polygonal refractile cells. Later the cells in the center were lysed and cells to the periphery became more rounded and refractile. Syncytia spread out over the surface of the petri dish and were flattened. The syncytia and the rounded cells were less refractile than those of the JMHP or low passage viruses.

Pathogenicity of cloned viruses.

In order to compare the pathogenicity of different clones from a single strain of MDV, the 7 clones from the JM strain were selected. In addition, single clones from the JMHP, GA, RPL39 and HVT strains were also used. The different clones derived from the JM strain killed 80% (JM 19) to 0% (JMHP) of the chickens and induced from 90% to 0% lesions respectively (Table 2). The clones of the GA and RPL39 strains produced an intermediate level of mortality and proportion of lesions.

However, these strains produced many more visceral lesions than any of the clones of JM virus. In addition to tumors of the gonad, over half the tumors produced by the GA and RPL39 strains occurred in the liver, lung, kidney, heart and muscle, whereas those produced by the clones by the JM strain occurred almost exclusively in the gonad. The median latent period to death was not directly related to the degree of mortality or gross or microscopic lesions. The HVT was nonpathogenic in these tests.

Antigenic analysis by the agar gel precipitin test.

Antigen preparations were the supernatant and the cell reagents from each clone and from uninfected cells, calf serum and tryptose phosphate broth. They were tested in the agar gel precipitin test against sera from two birds inoculated with each clone, against selected hyperimmune sera and against sera from uninoculated control birds of the same source and age. Reagents from uninfected cells, calf serum and tryptose phosphate broth did not react with any of the sera and the sera from uninoculated birds did not react with any antigen. Clone reagents tested against positive sera produced as many as 6 different precipitin lines. The strongest line of precipitation produced by most supernatant reagents represented an antigen referred to by Churchill et al (7) as antigen A. This antigen was not present in high passage viruses and was also absent from the JMHP supernatant and cell reagents. In addition this line of precipitation did not occur with JM30 and JM31 but was present

in HVT reagents. All clones including the HVT appeared to have at least one other antigen in common, the B antigen which was probably similar to that described by Churchill (7). Although there were often multiple lines of precipitation in this region no one line could be consistently identified, so they were referred to as B antigen lines to distinguish them from the A antigen lines.

All supernatant reagents produced strong A antigen lines. However, using selected sera the B and other antigens could also be detected in most of these reagents. The cell reagents were more variable. Some (e.g. JM19 and GA) had A, B and additional antigens whereas others (e.g. JM34 and JM35) lacked the A antigen.

In order to confirm and extend the above observations the following reagents and sera were selected for use in subsequent tests:

1) The A antigens (supernatant reagents from JM19 and GA) gave strong A lines and very weak B and other lines.

2) The B antigen (JMHP cell reagent) gave no A precipitin line with any antiserum.

3) The AB antigens (cell reagents from RPL39, GA and JM32) gave multiple strong precipitin lines.

4) The A antisera (sera from birds infected with JM19 and JM32) gave strong A lines when reacted with an A antigen and weak or no B lines when reacted with a B antigen.

5) The B antisera (sera from birds infected with JM30 and JM31) gave no reaction with A antigen, strong reactions with B antigens and were prepared against clones which lacked the A antigen.

All clones were examined for the presence of the A and B antigens. RPL39, JM19, GA, JM32, JM34 had A antigens and JMHP, JM30 and JM31 lacked these antigens (Figures 1 and 2). Sometimes the A antigen produced two lines of precipitation both of which were absent from the clones lacking the A antigen (Figure 1). The HVT had one antigen in common with the other clones but another was absent or differed (Figures 1, 7 and 8). All clones had at least one B antigen in common (Figures 3 and 4). All MDV clones which had A antigen were serologically indistinguishable.

In order to confirm that JM30 and JM31 lacked the A antigen, sera from birds inoculated with these clones were examined for antibody to the A antigen (Figures 5 and 6). None of the serums had antibody to the A antigen.

The HVT supernatant and cell reagents were examined in more detail for identity of their antigens with the A and B antigens of the MDV. In both supernatant and cell preparations, one of the two A antigens gave a line of identity and the second a reaction of partial identity as there was a spur on the HVT antigen side (Figures 7 and 8). The HVT cell reagent (Figure 7) had three antigens at least one of which was in common with one of the B antigens of MDV. The supernatant preparation reacted much more weakly with the B antiserum and only one precipitin line could be identified (Figure 8).

The A antigen appeared to be of lower molecular weight than the precipitating globulin in the antiserum since the line was concave towards the serum well. In addition, it formed as equally strong line in a supporting medium containing 2% or 0.5% agar indicating that it diffused readily through these matrices. The B antigen had a molecular weight similar to that of the precipitating antibody since it usually produced a straight line. It reacted more strongly in 0.5% agar than in 2% agar.

The A antigen line was nearly always closer to the serum well than the other lines.

Antigenic analysis by the indirect fluorescent antibody test. Uninfected cells and cells infected with each clone were analyzed by the indirect fluorescent antibody test with the same sera as were used above. Uninfected control cells did not become stained with any serum and control sera did not stain any of the cell preparations. A good cross reaction was obtained between cells infected with all MDV clones and sera from chickens which survived inoculation with the clones. When JM30, JM31, and JMHP infected cells were reacted with homologous antisera the rounded, refractile cells in the plaques stained intensely whereas the surrounding flattened cells adjacent to them stained very poorly or not at all (Figure 9). When cells infected with other clones were treated with homologous antisera which stained antigen intensely, the rounded cells in the plaques were surrounded by morphologically normal cells whose cytoplasm contained a bright diffuse or very finely granular stain (Figure 10). The number of flattened cells staining in this way varied in different plaques and cell preparations

because of the different stages of development of the plaques. The flattened cells could not be easily identified using sera which stained the rounded cells less brightly. When MDV-infected cells were treated with antiserum to HVT, the morphologically altered cells stained weakly and it was not possible to distinguish between the MDV clones. When HVT-infected cells were treated with homologous antiserum, antigen could be demonstrated in both the nucleus and the cytoplasm in the morphologically altered cells and in a broad band of morphologically normal cells surrounding them. There was frequently a perinuclear ring of brightly stained coarse spherical particles and/or a diffuse, very fine granular antigen-containing particles. When similar cells were reacted with antiserum to any of the MDV clones only the nuclear antigen stained, and then at a much lower intensity than with homologous antiserum. When the nuclear staining was particularly bright, a faint diffuse cytoplasmic antigen could also be detected in the morphologically altered cells but usually not in the broad band of cells surrounding them in which the nuclei were very prominent. Thus, antibody against MDV could be readily distinguished from antibody against HVT.

DISCUSSION AND CONCLUSIONS

Infectious cell-free virus could be obtained inconsistently from low passage MDV-infected CEF cultures. Although there were minor variations in culture conditions, in stage of development of cytopathologic alterations at the time of harvest and in the method of harvest, none of them could be directly linked to the success or failure of a particular filtration experiment. It appeared to be necessary to have optimal conditions so that there was a maximum yield of infectious virus to offset the inefficiency of the extraction and filtration procedures. As reported previously (13, 25) the JMHP and HVT generally yielded virus in larger amounts than the low passage strains.

Extracted virus was filtered through a 0.45 μm filter in order to remove clumps of virus and to increase the probability that each cytopathologic area would develop from a single infectious particle. The average pore diameter was only slightly greater than the diameter of an enveloped virion. MDV is entirely cell associated (6), thus contamination of a clone by released virus is highly unlikely though there is a danger from floating infected cells. To reduce the danger clones of low passage MDV were selected as soon after infection as possible from plates containing 11 plaques or less. The cloning procedure was not repeated because additional passages of MDV in cell culture would have further modified the virus clones. The clones prepared from JM virus were different from one another indicating that

a selection had taken place. Thus it is likely that in most instances the procedure was sufficiently exact to ensure isolation of the progeny of a single infectious particle.

The cloning procedure for JMHP and HVT were less rigorous since clones were selected from plates with a larger number of plaques. However conclusions derived from the comparison of these viruses with the low passage MDV are not as dependent on success of the cloning procedure.

Some of the characteristics of the original strains were present in the cloned preparations. Thus, the RPL39 and GA clones produced a large proportion and wide distribution of visceral tumors characteristic of these strains whereas the pathogenic JM clones produced a low incidence of visceral tumors almost exclusively of the gonad. The JMHP and HVT clones remained non-pathogenic.

The pathogenicity of different clones of the JM strain of MDV varied. The most pathogenic was JM19 which induced lesions in 90% of the birds and the least pathogenic was the JMHP which did not induce any lesions. There are two possible explanations for this variation, namely that the original stock of JM virus was a mixture of many genetically different viruses which varied in pathogenicity or that a change of pathogenicity occurred during passage of the virus. Strains that vary widely in pathogenicity have been observed (19), and it is quite possible that unpurified stocks would contain more than one virus. The variable responses obtained with different unpurified stocks of the same strain and the variation in pathogenicity with passage from chicken to chicken which has been well documented (3) could be explained by an alteration in the relative amounts of the more pathogenic and less pathogenic strains in a given stock. However, loss of pathogenicity may also occur during passage in cell culture. The contribution of cell culture passage to the lack of pathogenicity of some of the clones can not be estimated.

The viruses studied could be divided into three groups on the basis of their antigenic properties. Firstly, there are those with the A and other antigens namely JM19, JM32, JM34, JM35, JM36 RPL39 and GA. These clones were serologically indistinguishable. Secondly, there are those which lacked the A antigen such as clones JM30, JM31, and JMHP. It is not known whether viruses of this group occur naturally or whether they are products of cell culture passage. Thirdly, HVT has an antigen in common with at least one component of the A antigen of the first group of MDV's. Because of the close proximity of the precipitin lines produced by the two components of the A antigen they often appeared as a single line. The HVT had at least one B component in common with the MDV. It is possible that reagents could be developed which would distinguish between HVT and the other viruses in the agar gel precipitin tests.

JMHP produced cytopathologic changes in a shorter time than the low-passage clones of MDV, and the plaques were of different morhpology. It is unlikely that virus similar to JMHP could have been present in the original stock since it would easily have been recognized and since none of the low-passage clones produced plaques of this type. Thus the change in morphologic appearance and rate of growth of the plaques can be attributed to passage in cell culture.

Loss of pathogenicity during passage in cell culture or innate lack of pathogenicity are unrelated to the presence or absence of the A antigen. Thus relatively pathogenic clones with (JM19) and without (JM31) the A antigen and relatively non-pathogenic clones of the same strain with (JM32) and without (JMHP) the A antigens have been isolated. Similarly the change in appearance of the plaques and increase in rate of growth characteristic of JMHP are not related to loss of the A antigen since JM30 and JM31 were morphologically indistinguishable from the other low passage clones and yet lacked the A antigen. Thus the above observations indicate that although passage in cell culture may be responsible for loss of pathogenicity, loss of the A antigen and change in virus-host cell relationship, these three events are independent of one another. Also, only in the case of the change in virus-host cell relationship can the alteration be attributed entirely to passage in cell culture.

Differences between viruses with and without the A antigen could also be observed using the indirect fluorescent antibody test. However, it was not possible to differentiate between these viruses consistently because of variations in the stages of development of the plaques in different preparations and in the amount of antibody in the sera. In plaques produced by clones with the A antigen, a diffuse antigen in the cytoplasm of infected morphologically normal cells surrounding the plaques stained with homologous antiserum.

Cells in close proximity to plaques induced by viruses without the A antigen did not stain with their homologous antisera and in these plaques only the rounded refractile cells stained. Since the virus infection spreads centrifugally by cell to cell contact, the cells around the periphery of a plaque would be the most recently infected. Therefore, it appears that the A antigen is produced earlier in the cycle of infection than the other antigens, an observation confirmed by sequential harvesting of antigen for the agar gel precipitin test (Okazaki and Purchase, unpublished observation).

As has been reported elsewhere (15, 25) HVT can be distinguished from MDV by the intensity and distribution of staining antigen in HVT and MDV infected cells. The pattern of staining was consistent for the sera prepared against all clones of MDV studied. A similar difference in the appearance of nuclear and cytoplasmic antigen of Herpesvirus hominis types 1 and 2-infected cells stained in the direct fluorescent antibody test was reported by Nahmias et al (11). They could use the test to distinguish between the two strains of virus.

Figure 1. The A antigen of different clones of MDV and HVT. The center well contains the A antiserum (against JM32) and wells 1 to 6 supernatant reagents from RPL39, JM19, GA, JM31, JM19, HVT. (1% Agar).

Figure 2. The A antigen of different clones of MDV. The center well contains the A antiserum (against JM32) and wells 1 to 6 supernatant reagents from JM19, JM30, JM32, JM34, JM35, JM36. (1% Agar)

Figure 3. The B antigen of different clones of MDV and HVT. The center well contains the B antiserum (against JM30) and wells 1 to 6 cell reagents from RPL39, JM19, GA, JM31, JM19, HVT. (0.5% Agar).

Figure 4. The B antigen of different clones of MDV and HVT. The center well contains the B antiserum (against JM30) and wells 1 to 6 cell reagents from RPL39, JMHP, HVT, GA, JM32, HVT. (0.5% Agar).

Figure 5. The absence of anti-A antibody among birds inoculated
and 6. with JM30 and JM31. The center well contains the AB antigen (RPL 39 cell reagent), wells 1, 3, and 5 A antiserum (against JM32) and wells 2, 4, and 6 sera from birds inoculated with JM30 (Figure 5) and JM31 (Figure 6).

Figure 7 and 8. Relationship between HVT antigens and MDV antigens. The center well contains HVT cell reagent (Figure 7) and HVT supernatant reagent (Figure 8), well 1 A antigen (GA supernatant reagent), well 2 A antiserum (against JM19), well 3 B antiserum (against JM30), well 4 B antigen (JMHP cell reagent) well 5 B antiserum from a different bird than well 3 (against JM30) and well 6 A antiserum (against JM32). (0.5% Agar).

Figure 9. Antigen prepared from clone JM30 reacted in the indirect fluorescent antibody test with homologous antiserum. Note only the rounded cells stain. X300.

Figure 10. Antigen prepared from clone JM35 reacted in the indirect fluorescent antibody test with serum against RPL39. Note the cells of normal morphology surrounding the rounded refractile cells have a diffuse of finely granular cytoplasmic antigen. X300.

Table 1. Filtration and cloning of JM, high passage JM, RPL39 and GA, MDVs and the HVT

Source	No. Successful / No. Attempted	Average and Range Plaques/ml Filtrate	Max Number a/	Clones: Number Examined	Clones: Final Passage Level
JM	5/17	1.3(1-10)	10	7	11-13
JMHP	3/4	56.5(1-270)	20	3	13 (+41) b/
RPL39	4/16	5.0(7-11)	9	4	15-17
GA	5/12	16.7(1-80)	11	7	15-16
HVT	1/1	250(250)	250	4	3 (+7) c/
Control	0/18	0	-	-	-

a/ Maximum number of plaques on plate from which clones were picked.

b/ Previously passaged 41 times in chick and duck embryo fibroblasts.

c/ Previously passaged 7 times in duck embryo fibroblasts.

Table II. Response of 15 x 7 chickens to JM, high passage JM, GA and RPL39 MDVs and HVT before and after cloning.

	Strain and clone number	PFU Inoculated	Exptl. No.	Percent MD response			Percent with visceral tumors	MLPD[c]	Antigen
				Dead	Gross [a/]	Total [b/]			
Before Cloning	JM[d]		24		96		22		A&B
	GA[d]		16		81		62		A&B
	RPL39		20	85	90	100	85	40	A&B
	JMHP		10	0	0	0	NA[e]	NA	B
	HVT	10,000	20	0	0	0	NA	NA	A&B
After Cloning	JM19	1210	10	80	90	90	44	56	A&B
	GA	460	10	60	70	70	100	49	A&B
	JM31	330	10	60	70	70	33	40	B
	JM30	330	10	50	80	90	44	47	B
	JM35	1050	10	40	70	70	14	27	A&B
	RPL39	1090	9	33	89	89	63	35	A&B
	JM34	920	10	20	70	80	13	45	A&B
	JM36	400	10	0	30	30	33	NA	A&B

Table II, continued

Strain and clone number		PFU Inoculated	Exptl. No.	Percent MD response			Percent with visceral tumors	MLPD[c]	Antigen
				Dead	Gross [a/]	Total [b/]			
After Cloning	JM32	370	10	0	20	20	50	NA	A&B
	JMHP	1020	10	0	0	0	NA	NA	B
	HVT	900	9	0	0	0	NA	NA	A&B
	Control	0	9	0	0	0	NA	NA	None

a/ Includes those birds that died plus those that had gross lesions of MD at termination of the experiment.

b/ As above, but also includes those with histologic lesions.

c/ MLPD = Median latent period to death.

d/ Birds were killed at 6 weeks post-inoculation and examined for visceral lesions. These data kindly supplied by R. L. Witter.

e/ NA = Not applicable.

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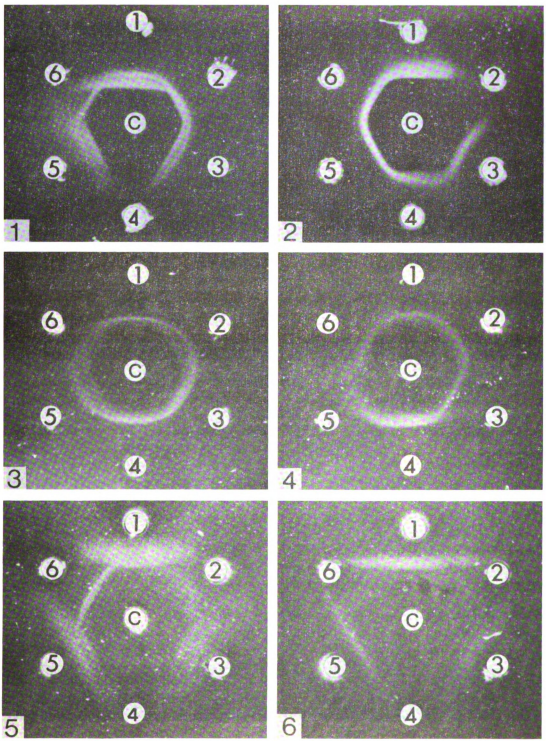
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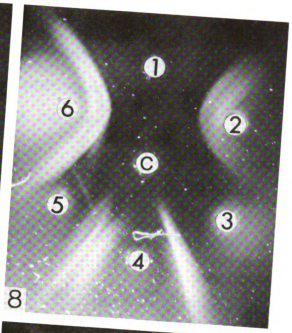

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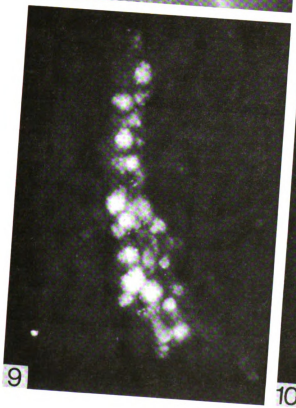
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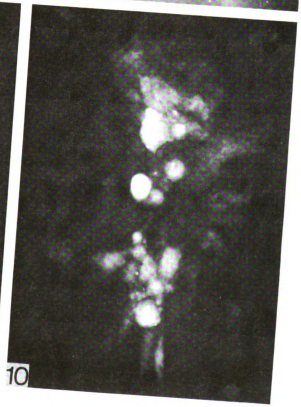
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Article V

RESPONSES OF CELL CULTURES FROM VARIOUS AVIAN SPECIES TO MAREK'S DISEASE VIRUS AND THE HERPESVIRUS OF TURKEYS

By

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SUMMARY

Cells were cultured from chicken, duck, bobwhite, and Japanese quail, turkey, pheasant, pigeon, and goose embryos. These avian cell cultures, HeLa cells and the chorioallantoic membranes of chicken embryos were tested for susceptibility to cloned preparations of Marek's disease virus recently isolated in cell culture (LMD), to MD virus passed many times in cell culture (HMD) and to the herpesvirus of turkeys (HVT). Avian cells were susceptible to all of the viruses but the cultures varied considerably in degree of susceptibility and in cytopathological response. Morphological changes produced by LMD, HMD, and HVT were characteristic for each virus but could best be differentiated in chick kidney cultures. Chick embryo fibroblasts were much less susceptible than duck embryo fibroblasts to LMD, equally susceptible to HMD and much more susceptible to HVT. HeLa cells were not susceptible to any of the viruses. Cytoplasmic virus-induced antigen was detected by the indirect fluorescent antibody test in HVT-infected cells of all types with HVT antiserum but not with LMD or HMD antiserum. Nuclear antigens fluoresced with all antisera.

INTRODUCTION

Marek's disease (MD) viruses which range in pathogenicity from "acute" to mild or "classical" have been isolated from various sources in different lines of susceptible chickens and in cell cultures (6,12,13, 21,22,24). Chick kidney (CK) (1,4,6,12,20,24) and duck embryo fibroblast (DEF) (21,24) cultures are most commonly used for primary isolation and passage of MD viruses although isolation and propagation in chick embryo fibroblasts (CEF) has been reported (13,14). The MD virus grew in pheasant cell cultures and was re-isolated from ducks after experimental inoculation (3). Antibody could not be demonstrated in pigeons (Columba palambus), starlings (Stuonus vulgaris), sparrows (Passer domesticus), yellow hammer (Emberoza citorinella), pheasants (Phasianus colchicus) and jackdaws (Corvus monedula) (3). The MD virus did not propagate in cultures of mammalian origin (5). The herpesvirus of turkeys (HVT) produced plaques in DEF, CEF, CK, turkey embryo fibroblasts (TEF) and turkey kidney cell cultures (TK) (23).

The studies described herein were initiated to determine whether cell cultures derived from various avian species were susceptible to MD virus and if changes produced could be used as markers to differentiate between viruses, particularly those differing in pathogenicity. It appeared important to be able to differentiate between the pathogenic MD viruses (18), the non-pathogenic MD virus which had been serially passed many times in cell culture (HMD) (15) and HVT since the last two could be used as vaccines to prevent MD (7,8,16,19). Previously cloned MD viruses and HVT, which had been shown to differ greatly in pathogenicity, were employed (18).

MATERIALS AND METHODS

Source of viruses.

The origin of 7 cloned preparations of JM, 4 of RPL 39, and 7 of GA viruses collectively referred to as low passage MD (LMD) have been described (18). They had been passaged 10-17 times during cloning. Three cloned preparations of a strain of JM virus which had been passed over 40 times in cell culture prior to cloning (54 passages in all) and 4 of the HVT strain FC126 (15,18,23) were also employed. Viruses were propagated in DEF and CEF and virus-infected DEF stocks containing 10% dimethyl sulfoxide and 15% calf serum were stored in liquid nitrogen (11).

The cloned viruses, in order of decreasing pathogenicity, were JM19, GA, JM31, JM30, JM35, RPL39, JM34, JM36, and JM32. The HMD and HVT were non-pathogenic (18). Two preparations of JM virus (JM30 and JM31) and the HMD lacked antigens identified as "A" antigens whereas all other viruses produced these antigens (18). The antigens and pathogenicity of 3 cloned preparations of RPL39, 6 of GA and 4 of HVT were not tested.

Chickens and embryos

Line 15 x 7 chickens and line 1900[a] and line 6 embryos were from the Single Comb White Leghorn flocks (Gallus gallus vardomesticus) reared at the Regional Poultry Research Laboratory (9). Embryonated eggs used for cell culture were obtained as follows: bobwhite (Colinus virginianus) and Japanese quail (Coturnix coturnix japonica), turkey (Meleagris gallopavo), pheasant (Phaseanus colchicus) and duck (Anas platyrhynos var domesticus) eggs were from Truslow Farms Inc., Chestertown, Maryland, and pigeon (Columba livia) eggs were from Dr. A. M. Lucas, U.S.D.A, Avian Anatomy Project, East Lansing, Michigan.

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[a] Stone, H. A., unpublished data 1970.

Cell cultures.

The preparation and propagation of CEF, DEF, and CK cultures has been described (24). Bobwhite quail (BQEF), Japanese quail (JQEF), turkey (TEF), pheasant (PhEF), and pigeon (PiEF) embryo fibroblast cultures were prepared from embryos at the midpoint of their incubation period with a procedure similar to that used for DEFs. Duck kidney (DK) cultures were prepared from 2 to 4 week old ducks in a similar manner to CK cultures.

The established line of goose (Anser anser var domesticus) embryo fibroblasts (GEF) was obtained from Dr. L. B. Crittenden, ARS, Beltsville, Maryland and was received frozen. Immediately prior to use an ampule was thawed rapidly, and propagated in a similar manner to CEFs except that it was passaged at 7 to 14 day intervals. HeLa cells obtained from Microbiological Associates, Inc., Bethesda, Maryland were cultured in medium F10 and 199 with 4% calf serum (24) and were passaged at 3 to 4 day intervals.

Primary fibroblasts were plated at 1×10^7 cells on each 160mm plastic petri dish[b], Assays were performed on secondary cultures seeded with 1×10^6 cells (5×10^5 for GEF) in 60mm plastic dishes. Primary kidney cultures, seeded at 5 to 8×10^6 cells per 60mm plastic petri dish, were used for all assays.

Susceptibility of cells to clones of virus.

The susceptibility of CEFs and DEFs was tested with all viruses, however, the susceptibility of all other cell types was screened using the 7 cloned preparations of JM and only one each of GA, RPL 39, HMD and HVT. The culture medium from plates with a confluent monolayer was removed and 0.5ml of each tenfold dilution of inoculum was added to each culture. After incubation at

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[b] Falcon Plastics, Oxnard, California

37° C for one hour the cultures were washed with phosphate buffered saline (PBS) pH7.4 and fresh medium was added. The culture medium was changed every other day thereafter. Cultures were examined daily and cytopathic areas were counted when they were easily visible but before the cell monolayers began to degenerate.

Cytopathic changes were examined in greater detail in preparations stained with hematoxylin and eosin (2) and the presence of virus-induced antigen was demonstrated by the indirect fluorescent antibody test (17). Cultures on 11 x 22mm glass coverslips in plastic petri dishes were infected with tenfold dilutions of virus. Coverslips with clearly visible discrete cytopathic areas were removed from the petri dishes. One set of coverslips was fixed in Zenkers acetic acid and stained with hematoxylin and eosin (2) and another set was rinsed in PBS, fixed in acetone, and stained by the indirect fluorescent antibody technique with antisera specific for some of the cloned viruses (17,18).

Chorioallantoic membrane inoculation

Embryos from line 6 were inoculated with 0.2ml of tenfold dilutions of inoculum on the 10th day of incubation using the false air cell technique (10). Embryos were killed on the 18th day by placing them in a refrigerator for 2 to 18 hours after which time the chorioallantoic membranes were removed and examined.

RESULTS

Morphology of uninfected cells.

Cells from decapitated embryos were morphologically fibroblastic and so are referred to as embryo fibroblasts. The DEF were broad cells (Figs. 7a,b,and c), the BOEF, JQEF, and PhEF were intermediate and CEF (Figs. 2a, b,and c), PiEF and GEF (Fig. 3a) were long and thin. The PiEF monolayers contained many large vacuolated syncytia (Fig. 9a). The GEF cell line grew better in sparse cultures and as they became confluent the cells overlapped one another haphazardly (Fig. 3a and b).

The DK and CK cells were epithelioid in nature. The DK cells formed an even monolayer of flattened polygonal cells (Fig. 4a) whereas the CK cells formed islands of flattened polygonal epithelioid cells separated by cords of fibroblastic cells (Figs. 5a, b,and c). The HeLa cells were characteristically epithelioid.

Cytopathic changes produced by different viruses.

Observations were made of unstained preparations and cells stained with haematoxylin and eosin and the direct fluorescent antibody procedure.

In general, cytopathic areas produced by JM19, JM30, JM31, and JM36 were more easily recognized than those produced by other LMD viruses but could not be individually differentiated so they are described together (Table I). Altered areas were small, developed slowly and contained few small syncytia and rarely was there a hole in the center of them. Cells making up the areas were clustered close together and tended to pile up.

Cytopathic areas produced by HMD and HVT appeared early and developed rapidly and at times looked similar (Figs 2b and c). HMD produced larger, more rounded and refractile syncytia than HVT (Figs 5b,6b,7b,8b). As reported (15) holes in the center of cytopathic areas seemed to develop largely as a result of detachment from the monolayer. The syncytia produced by HVT were angular or

stellate and vacuolated and holes in the center of cytopathic areas looked as if they had formed by lysis of the cells (Figs 5c,6c,7c).

Eosinophilic Cowdrey type A intranuclear inclusion bodies were in plaques produced by all clones of virus in all cell types (Figs. 1a,b, and c). Inclusion bodies were never found in cells not involved in a cytopathic area.

Cytopathic changes in different cell types.

Cell types are described in increasing order of their tendency to develop syncytia (Table I). Changes in embryo fibroblasts, i.e., BQEF, CEF, GEF, JQEF, PhEF, and TEF consisted mainly of small, spherical, highly refractile cells which tended to become multilayered and some detached from the monolayer (Figs 2a,b,c,3a,and b). Interspersed polygonal and fusiform cells were not obvious. Cytopathic areas on BQEF and JQEF tended to have fatter fusiform cells and resembled those in DEF.

In DK cells all viruses produced cytopathic areas with clearly defined, biconcave edges (Figs 4a and b). The diametrically opposite edges merged gradually and irregularly into the surrounding monolayer and often contained fusiform refractile cells. There were occasional small syncytia and holes in the center of cytopathic areas, particularly in those produced by HVT (Fig 1b).

In CK cells cytopathic areas consisted mostly of rounded cells and small syncytia (5a,b,c,6a,b,and c). The LMD produced groups of tightly packed very refractile small spherical cells which formed a small grape-like cluster (Figs. 5a and 6a). In cells infected with HMD there were thick, large, stellate and round refractile syncytia around which were grouped fusiform and refractile cells (Figs. 5b and 6b). Cells had little tendency to pile up and a hole frequently developed in the center of the cytopathic area. The HVT produced thin syncytia with many vacuoles and some rounded or polygonal cells, however all affected cells had less of a tendency to become spherical and refractile than those infected with the other viruses (Figs. 5c and 6c).

Holes invariably developed in the center of the cytopathic areas, probably more due to lysis than detachment of cells. The monolayer around the holes retracted and was thicker than on the remainder of the petri dish (Fig. 6c). The cells immediately surrounding the holes were generally polygonal and vacuolated.

Alterations in DEF cultures took longer to develop but were larger in diameter than those in other embryo fibroblast or CK cells. They consisted of many fusiform refractile cells which piled up. There were few small syncytia in cytopathic areas produced by LMD (Fig 7a and 8a). However, HMD produced characteristic flat brown almost hemispherical syncytia with the numerous nuclei arranged in a circle (Fig. 7b and 8b) and which frequently detached to form large black spheres (Fig 8b). Usually HVT produced large stellate syncytia which were thinner in the early stages than those produced by HMD (Fig. 7c and 8c). They were often attached to the petri dish and to one another by long pseudopods (Fig 7c).

In PiEF, LMD did not produce cytopathic effects however occasional immunofluorescent antigen was detected in some cells. HMD produced rare large flat syncytia which could be distinguished from those in uninfected cells because they contained very few vacuoles and had intra-nuclear inclusion bodies in stained preparations (Fig. 1a). HVT produced enormous thin syncytia with very few vacuoles occupying large areas of the cell culture (Figs. 9b and 9c). They frequently had thick central areas near where detachment and retraction of pseudopods from the petri dish had occurred.

Comparative sensitivity of cells of different types and CAMs to clones of virus.

The susceptibility of all other cells was related to the susceptibility of

DEF since the stocks of virus were prepared in DEFs. The relative ability of a virus to produce cytopathic effects in CEF and DEF is expressed as the ratio of the titer of the virus on CEF to the titer on DEF (Table 2). The viruses fall into 2 groups, namely, LMD viruses such as JM, RPL 39 and GA which gave ratios from 0.08 to 0.14 and HMD and HVT which gave ratios of 3.0 and 21.0 (See also Fig 12)

In a search for biological markers which would differentiate between the viruses, the susceptibility of different cell types to the MD viruses was screened in one or two experiments with each cell type and with CAMs. The viruses did not differ greatly from one another in their ability to produce cytopathic effects on each of the cell types or on CAMs so the data for these viruses have been pooled (Fig 12). Except in a few instances, titers on different cell types did not vary more than 20 fold. In particular the MD viruses produced very few cytopathic areas in PiEF or PhEF and the HVT was impotent in DK cells. However in each case these cells were not more susceptible than DEF to the other viruses. The susceptibility of CEF to HVT has been reported above.

Immunofluorescent antigens in infected cultures

Cultures infected with LMD and HMD viruses were treated with antiserums prepared against these viruses. An area resembling an inclusion body, excluding the nucleolus, stained in the nucleus and fine diffuse and coarse granular particles stained in the cytoplasm of cells involved in the cytopathic areas (Fig. 10a). Sometimes staining was observed in cells of normal appearance surrounding the cytopathic areas.

Staining was observed in both the nucleus and the cytoplasm of cells infected with HVT and treated with homologous antiserum (Figs. 10c and 11c). However, when such cells were treated with serum against LMD or HMD the nuclei stained

much more intensely than the cytoplasm (Figs. 10b and 11b) and the cytoplasm stained diffusely and did not contain the large number of brightly staining coarse granules seen with homologous antiserum. As reported previously (19,23), the difference in staining between antibody to MD viruses and antibody to HVT was most outstanding in HVT-infected CK cells.

HeLa cell cultures were the only cells tested which were not susceptible to any of the viruses used. Inoculated cultures contained scattered areas of fluorescent debris (Fig 11a) which were considered to be remnants of degenerating cells from the inoculum.

If significant amounts of cell-free virus had been produced by some of the viruses in particular cell types, secondary small foci of infection should have been seen. Areas which were not cytopathically involved but contained antigen were not observed.

Uninfected cells did not stain with any anti-serum or negative sera except DEFs which, as previously described (17), contained easily recognisable granules which stained with both positive and negative sera.

DISCUSSION

When using a cellular inoculum to infect test cells of different types, it is possible that the inoculum cells themselves would proliferate and produce cytopathic areas. The presence of cytopathic areas would be interpreted as susceptibility of the test cells. In the studies reported here, growth and morphological change of the inoculum cells were not responsible for the cytopathic areas observed for the following reasons. Very few cells from the inoculum grew without a feeder layer and those that did soon became cytopathically involved, detached and died. Thus once cells were infected they either did not divide or divided so infrequently that they were unable to maintain themselves. HeLa cells are not susceptible to MD virus infection (5). When HeLa cells were inoculated with a large dose of a cellular inoculum and stained by indirect fluorescent antibody procedure only cellular debris stained (Fig IIa). This debris was probably the remnants of the inoculum cells which died rather than forming cytopathic areas. In susceptible cells the appearance of the cytopathic areas was characteristic of the recipient culture and not of the culture in which the virus stocks were prepared, in this case DEF.

The titer of each clone of virus when assayed on a particular cell type was a measure of the relative susceptibility of the cell type to the virus. The titer depended not only on the transfer of infectivity from the inoculum to the recipient cells but also on whether the recipient cells became altered to form clearly distinguishable plaques. Whether recipient cells became altered may have been affected by a large number of factors other than the source species of the cell. For example, extraneous infectious agents could enhance or inhibit the herpesvirus infection or its cytopathic effect. Only the CEF were known to have come from birds extensively tested for and free from known extraneous viruses. There was a large amount of vacuolization and syncytial formation in

the PiEF which could have been due to an infectious agent in these cultures which, in turn, could have inhibited the herpes virus infection. Other factors might act synergistically. Among them are the physiological condition of the cells, the different media in which they were cultured and the time at which they were infected or examined. On the other hand, extraneous agents could not have been solely responsible for the cytopathic effects since specific virus-induced antigens and intranuclear inclusions were present in the cytopathic areas in all cell types. The characteristic cytopathic areas did not occur in uninfected cultures.

The approximately twenty fold increase in susceptibility of CEF to HVT was demonstrated several times (Table 2 and Fig. 1) with similar results. Thus CEF had an advantage in sensitivity for detecting this virus. However it was much more difficult to distinguish between viruses by morphology of the cytopathic areas than when using DEF or CK cultures. Also CEF cultures were not suitable for primary isolation of LMD and in agreement with previous results (15), this virus will only grow in CEFs after several passages in CK or DEF cultures. CEFs are less susceptible to virus passed 11 to 17 times in cell culture (LMD) than to virus passed 54 times (HMD) or to HVT. A brief examination of the susceptibility of other cell types did not reveal any that would be significantly more sensitive to the MD viruses than DEFs or CKs. Also there was no indication that there were cell types other than CEFs in which adequate sensitivity to one virus and resistance to another could be used as a biological marker to differentiate between viruses. Thus none of the cell types examined offered any advantages over CK cultures for distinguishing between LMD, HMD, and HVT. However when working exclusively with HVT and possibly also HMD, CEF cultures were more sensitive.

Since the cultures were maintained under liquid medium cell-free virus would have produced secondary foci of infection by the time the originally infected cells had become morphologically altered. The absence of secondary foci in monolayers stained by the indirect fluorescent antibody technique until after infected cells floated free from the cytopathic areas (4-5 days) was consistant with the hypothesis that none of these cultures released large amounts of infectious virus into the supernatant cultural medium. This is in agreement with the previous observations that the viruses are highly cell-associated (6,7,23).

Using the procedures described it was possible to demonstrate that all avian cells tested and CAMs of chicken embryos were susceptible to the herpesviruses used, however, HeLa cells were not susceptible to these viruses. From this and similar work by Calnek et al (5) it can be concluded that most mammalian cells are not susceptible to MD viruses.

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Table 1. Summary of the morphology of the cytopathic effects produced by clones of MD virus and HVT in cell cultures from different avian species.

Virus clone		Description of CPE	Recipient cell cultures				
			E[A]	DK	CK	DEF	PiEF
LMD[B]	Plaque	Shape	Ovoid	**Biconcave**	**Grapes**	Ovoid	-
		Pile up	++++[C]	+++	+++	++++	-
		Hole	-	-	-	-	-
	Cells	Fusiform	+	++	-	+++	-
	in	Spherical	++++	-	++++	++	-
	Plaque	Refractility	++++	+	++++	++	-
		Syncytia	+	-	+	+	-
HMD	Plaque	Shape	Ovoid	**Biconcave**	Ovoid	Ovoid	**Irregular**
		Pile up	+++	++++	+	++++	-
		Hole	-	-	+++	+	+
	Cells	Fusiform	+	+++	++	+++	-
	in	Spherical	++++	-	+++	++	-
	Plaque	Refractility	++++	++	++++	+++	-
		Syncytia	+	+	+++	+++	+++
HVT	Plaque	Shape	Ovoid	**Bioconcave**	Ovoid	Ovoid	**Irregular**
		Pile up	+++	++	+	+++	-
		Hole	+	+	++++	+	+
	Cells	Fusiform	+	+	-	++	-
	in	Spherical	++++	-	++	++	-
	Plaque	Refractility	+++	+	+	++	-
		Syncytia	+	+	++	+++	++++

A. Bobwhite quail (BQEF), E = embryo fibroblasts from chicken (CEF), goose (GEF), japanese quail (JQEF), pheasant (PhEF) and turkey (TEF); DK = duck kidney; CK = chick kidney, DEF = duck embryo fibroblasts; PiEF = pigeon embryo fibroblasts.

B. LMD = low passage Marek's disease (MD) virus; HMD = high passage MD virus; HVT = Herpesvirus of turkeys;

C. The relative frequency or degree of involvement has been scored from ++++ to - thus ++++ means a deep pile of cells, a hole develops in all plaques, there are many fusiform or spherical cells or syncytia and the cells are very refractile; - means little or no piling up, holes do not develop in plaques, there are rare or no fusiform or spherical cells or syncytia and the cells are not refractile. Characteristic shapes are given in bold type (underlined). The size and ease with which syncytia can be observed increases toward the bottom and right of the table.

TABLE 2 Relative titers on CEF and DEF of cloned preparations of MDHV and HVT.

Source of virus A/	No. of preparations examined	Mean of ratios of titers CEF / DEF B/	Standard deviation
RPL39	4	0.08	0.012
GA	7	0.14	0.032
JM	7	0.14	0.069
HMD	3	3.00	1.35
HVT	4	21.00	5.75

A. RPL39, GA, and JM are low passage MD (LMD) viruses which have been passed 11-17 times in cell culture. HMD is a strain of MD which has been serially passaged 54 times in cell culture. HVT is FC126 herpesvirus of turkeys.

B. Monolayers of chick embryo fibroblasts (CEF) and duck embryo fibroblasts (DEF) were infected in parallel with tenfold dilutions of virus. Figures are means of ratios of titers (plaque forming units per ml) of each virus on CEF divided by the titers on DEF. A high figure indicates CEF are more susceptible to the virus than DEF.

Legends for Figures

Fig. 1. Cowdrey type A intranuclear inclusion bodies: (a) A single large syncytium produced by HMD in PiEF. H&E x 480. (b) A syncytium with vacuoles, a hole in the center of the cytopathic area and several adjoining cells with inclusion bodies in HVT infected DK cells. H&E x 480. (c) A syncytium and individual cells with inclusion bodies in GEF infected with HVT. H&E x 525.

Fig. 2. Cytopathic areas in CEF induced by: (a) LMD, (b) HMD and (c) HVT. Viewed unstained with an inverted microscope cytopathic areas consist mainly of spherical, highly refractile cells which are sometimes multilayered. x30.

Fig. 3. Cytopathic area induced by HMD in GEF: (a) Unstained x30. (b) Two syncytia beside the cytopathic area consisting of dark fusiform and spherical cells overlapping in a haphazard fashion. H&E x 120.

Fig. 4. Cytopathic areas in DK cells produced by HMD: (a) Two unstained areas with characteristic biconcave appearance. x30. (b) One cytopathic area stained with homologous antiserum by indirect fluorescent antibody. x300.

Fig. 5 & 6. Cytopathic areas in CK cells infected with: (a) LMD, (b) HMD and (c) HVT. For detailed description see text. Fig. 5 Unstained. x30. Fig. 6. H&E x120.

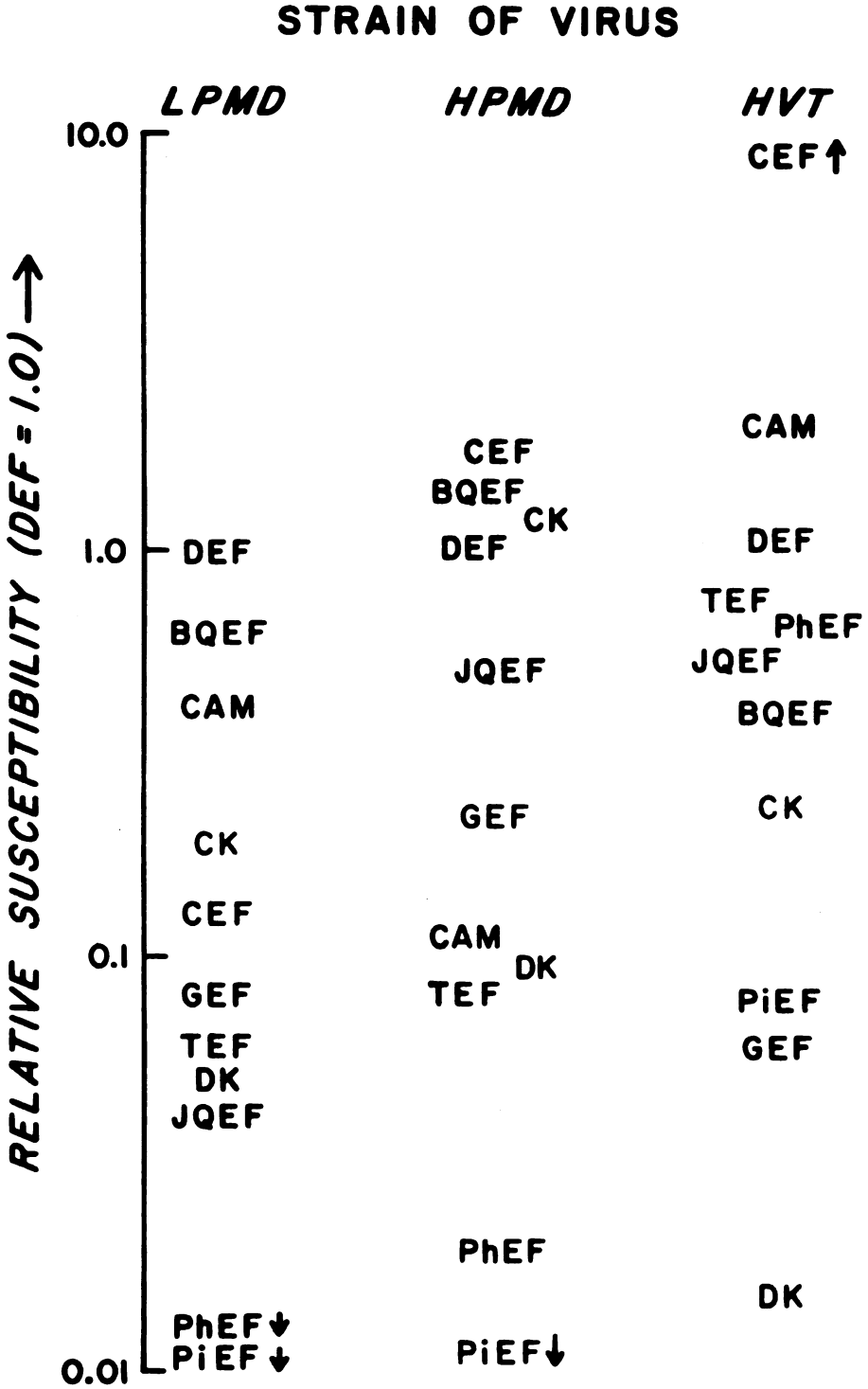
Fig. 7 & 8. Cytopathic areas in DEFs infected with: (a) LMD, (b) HMD and (c) HVT. For detailed description see text. Fig. 7, unstained x30. Fig. 8, H&E x90.

Fig. 9. (a) Uninfected PiEF. A vacuolated syncytium can be seen beside a hole in the monolayer. H&E x90. (b) PiEF infected with HVT. Enormous syncytia with very few vacuoles are visible. H&E x90. (c) PiEF infected with HVT and stained with homologous serum by indirect fluorescent antibody. x300.

Fig. 10. GEF stained by indirect fluorescent antibody: (a) Infected with LMD and stained with homologous antiserum. There is both nuclear and diffuse cytoplasmic staining. x300. (b) Infected with HVT and stained with LMD antiserum. The nuclear staining predominates. x300. (c) Infected with HVT and stained with homologous antiserum. Clusters of granules are stained in the cytoplasm. x300.

Fig. 11. (a) HeLa cells infected with a very high dose of LMD and stained by indirect fluorescent antibody with homologous antiserum. Some fluorescent debris is staining. x300. (b) DK cells infected with HVT and stained by indirect fluorescent antibody with LMD antiserum. Many nuclei are staining in the cells bordering the hole in the cell culture. x120. (c) DK cells infected with HVT and stained by indirect fluorescent antibody with homologous antiserum. The nuclei do not stain and appear like shadows but there is granular and diffuse stain in the cytoplasm. x300.

Fig. 12. Relative susceptibility of cell cultures of various types and chorio-allantoic membranes (CAM) of chicken embryos to cloned preparations of LMD, HMD and HVT. The susceptibility of duck embryo fibroblasts (DEF) is 1.0. Embryo fibroblasts were from bobwhite quail (BQEF), chicken (CEF), goose (GEF), Japanese quail (JQEF), pheasant (PhEF), pigeon (PiEF) and turkey (TEF). Kidney cultures were prepared from chickens (CK) and ducks (DK). HeLa cells were not susceptible to any of the viruses. Relative susceptibility of CEF to HVT = 18, of PhEF to LMD = 0.002, and of PiEF to LMD and HMD = <0.001.


STRAIN OF VIRUS
LPMD
HPMD
HVT
RELATIVE SUSCEPTIBILITY (DEF = 1.0) →
10.0
1.0
0.1
0.01
CEF ↑
CAM
CEF
BQEF
CK
DEF
DEF
DEF
TEF
PhEF
BQEF
JQEF
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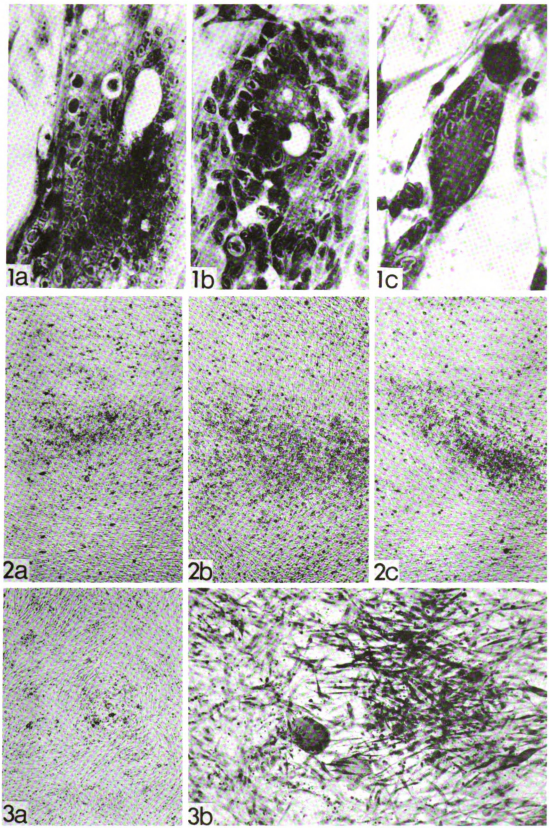
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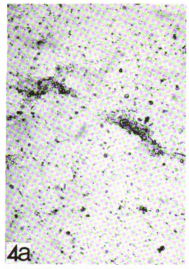

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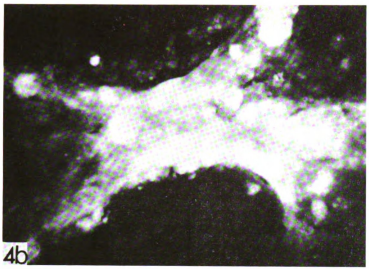

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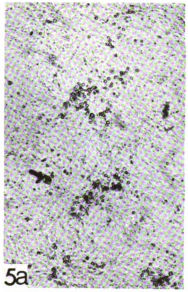

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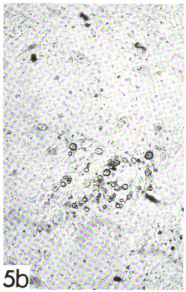

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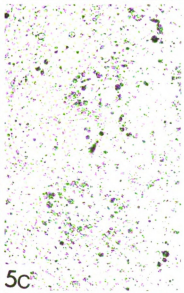

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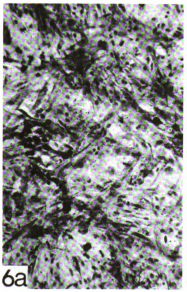

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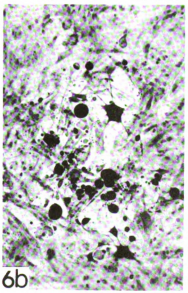

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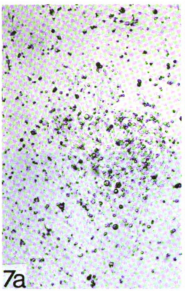
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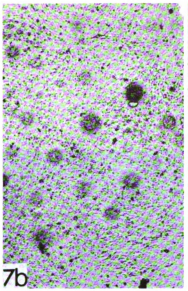
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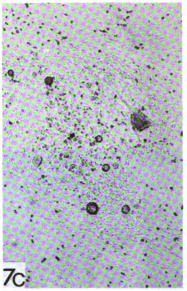
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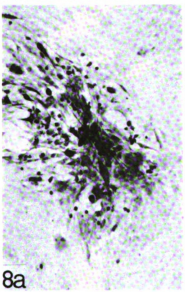
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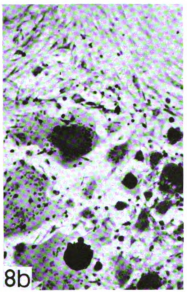
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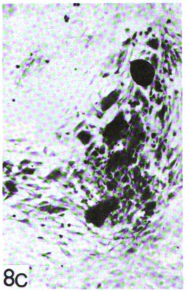
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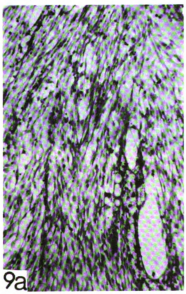
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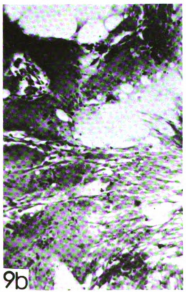
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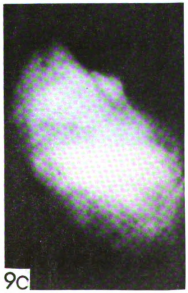
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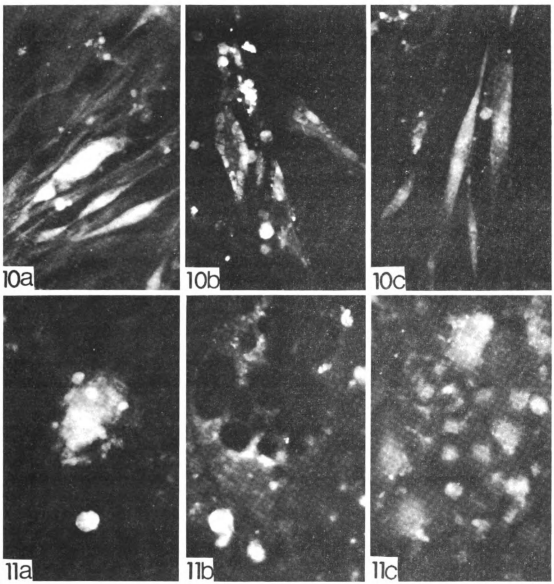
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GENERAL DISCUSSION AND CONCLUSIONS

Techniques for Detection of Antigen and Antibody in Vitro and in Vivo

A specific IFA test for the detection of MD antigen in cell culture has been described (Purchase, 1969, Article I). There was a direct relationship between the number of fluorescent foci and the dilution of the inoculum used to infect the cells indicating a one hit phenomenon. Thus, one infectious unit of virus produced one fluorescent focus or one morphological focus (cytopathic area) in cell culture. Approximately the same number of fluorescent foci were detected at one day after infection of CK cultures as were detected at seven days after infection. The peak in the number of morphological foci was only reached at seven days post-infection. The IFA test has limited application in virus isolation studies because of the small area of the culture which can be examined and the time and effort required to perform the test. When examined by this test, all eight isolates of MD were antigentically identical. An alternative explanation was that there was a common antigen or contaminant in all stocks of the isolates.

The IFA and the AGP tests were compared in detail (Purchase and Burgoyne, 1970, Article II). There was a 92% agreement between the results of the IFA and those of the AGP tests on 418 serums from various sources. The IFA test was 10 to 320 times more sensitive than the AGP test; some serums which had a high titer of antibody demonstrable by the IFA test did not produce precipitation in agar. This observation has been confirmed by Witter (1970). Thus, even though the two tests detect antibody which reacts in different ways, both are specific for MDV-induced antibody. The IFA test would be expected to be more sensitive than the AGP test since it would detect the antigen-antibody combinations that produce a precipitate in addition to other antigen-antibody combinations which did not form a lattice large enough to produce a grossly visible precipitate.

A method is described for preparing fluorescein conjugated anti-MDV chicken globulin for use in the direct fluorescent antibody (DFA) test (Purchase, 1970a, Article III). This globulin was used to detect MDV-induced antigen in the feather follicles, lung, bursa of Fabricius, thymus, spleen, and cecal tonsil of MD-infected birds. Precipitating antigen was also demonstrated in most of these sites. Filtrable virus, however, was only found in the skin, and presumably originated from the feather follicle epithelium.

Differences Between Strains of MDV and HVT

Even though eight isolates of virus previously examined by the IFA test were found to be identical, cloned preparations from these isolates differed greatly in their pathogenicity. Two pathogenic JM viruses and the HMD virus lacked antigens identified as the A antigens in the agar gel precipitin test. The HMD produced larger plaques in a shorter time than the other MD viruses. Either there was more than one virus in the original stocks or the virus became altered during cloning. Unfortunately, because of its highly cell-associated nature, 11 to 17 passages in cell culture were required to clone purify the virus and changes may have occurred in the virus during passage. The change in morphological appearance of the cytopathic areas must have occurred during passage in cell culture since large cytopathic areas would most certainly have been present in the early passages. Since the absence of an antigen or lack of pathogenicity of a small proportion of virus in the original stock would not have been detected, it is not possible to determine whether the differences in antigens and pathogenicity were due to passage in culture or were present in the initial stocks of virus.

The HVT produced large cytopathic areas and was non-pathogenic for chickens. This virus and MDV shared two A antigens and at least one B antigen. These viruses

could not be distinguished from one another by the AGP test. However, MDV and HVT could be distinguished from one another, as could antibody to each virus, by the intracellular location of antigen stained by the IFA test.

Growth of Clones of Virus in Cultures of Various Types

Bobwhite quail, chicken, duck, goose, Japanese quail, pheasant, pigeon, and turkey embryo fibroblasts, chick and duck kidney cells were all susceptible to LMD virus, HMD virus and the HVT. The cytopathic areas produced in cells of different types varied greatly. Clones of these three types of virus could best be distinguished from one another by the cytopathic changes they produced in CK cultures. The LMD virus did not grow as well in CEF as in DEF however the reverse was true for HMD and HVT (Purchase et al., 1970b, Article V).

Virus-Host Cell Relationships in Vitro

The MDV is highly cell-associated and spreads from cell to cell by direct contact. The areas containing antigen which become the cytopathic areas or plaques enlarge peripherally by infection of neighboring cells. Even though cultures are maintained under liquid medium, secondary fluorescent foci of infection are not initiated until rounded cells float free from the initial cytopathic areas and settle on uninfected cells. This process was well demonstrated by the IFA test (Purchase, 1969, Article

I). On clone isolation or passage in cell culture, MDVs become altered in pathogenicity, in the appearance of cytopathic areas and presence or absence of the A antigen. It appears that change in any one of these three properties may occur independently of change in any other.

Virus-Host Cell Relationships in Vivo

The loss of maternal anitbody in chicks and the subsequent acquisition of antibody after infection were adequately demonstrated using the IFA test (Purchase and Burgoyne, 1970, Article II). However, by the IFA test, maternal antibody in chicks from MD-exposed dams was detected after antibody was no longer detectable in the AGP test. Also, acquired antibody in contact-exposed chickens was detected earlier than by the AGP test. The sera of some older birds had a high titer of antibody demonstrable by the IFA test but did not produce precipitation in agar. In addition to being more sensitive, the IFA test will detect antibody of a type different than that by the precipitation test.

Three different types of virus-host cell interactions occurred in vivo. Firstly, there was a situation where neither antigen nor infectious cell-free virus could be demonstrated. This occurred in tumors of the visceral organs and nerve lesions. Secondly, antigen was present but not infectious virus and a cytolytic process occurred.

This is exemplified by changes which occur in the bursa of Fabricius of infected chickens. Naked viral particles have been observed in the nuclei of cells undergoing this type of degeneration (Calnek, et al., 1970c). Lastly, in the feather follicle epithelium, both antigen and infectious cell-free virus can be detected. The virus was infectious even after storage under conditions in which cells would not retain their viability. Degenerative and necrobiotic changes are continuously occurring in the superficial layers of the stratified squamous epithelium and intra-nuclear inclusion bodies could be seen in these cells. Furthermore, enveloped virions were observed in the nucleus and in cytoplasmic inclusions (Nazerian and Witter, 1970). This is the only location in which virus maturation and envelopement is completed. It is probably the portal of exit of MDV from infected birds.

The Etiology of MD

At the time that the IFA test was developed, there was still some uncertainity that the herpes-virus was the cause of MD. Demonstration of a reaction between sera from diseased birds and the cytopathic areas which contained inclusion bodies and virions (Nizerian, 1968; Nazerian et al., 1968) added evidence that the herpesvirus was the cause of MD. Demonstration by Calnek and Hitchner (1969) of immunofluorescent antigen in the epithelium of the feather follicles led to the discovery of enveloped virus

infections for cell cultures and chickens at this site (Calnek et al., 1970a; Nazerian and Witter, 1970). Work reported in this dissertation confirmed these findings. Demonstration of the maturation and envelopement of herpesvirus in the feather follicle epithelium and the reproduction of the disease with virus from this site removed the last shadows of doubt about the etiology in agent of MD. A herpesvirus has now been firmly established to be the cause of MD. This is the first time that a herpesvirus has been definitely incriminated as the cause of a neoplastic disease.

SUMMARY

1. The IFA test was applied to the detection of MDV antigen in cell culture and MDV antibody in the serum of birds.

2. Nuclear and cytoplasmic antigen could be detected as early as 24 hours after infection at which time no cytopathic changes could be detected by conventional light microscopy. By seven days after infection, the same number of infected areas were detected by both methods.

3. There was a linear relationship between the concentration of inoculum and the number of fluorescent foci or cytopathic areas indicating that one infections unit produced one fluorescent focus or one cytopathic area.

4. The time sequence study confirmed the cell association of the virus and that there was cell-to-cell spread of infection.

5. When examined by the IFA test, eight isolates of MD were indistinguishable indicating that either the strains were antigentically identical or there was a common antigen or contaminant in all stocks.

6. Cells infected with MDV contain a heat stable antigen similar to that found in herpes simplex-infected cells.

7. There was 92% agreement between the results of the IFA and AGP tests for antibody.

8. By the IFA test, maternal antibody in young chickens from MD exposed dams was detected after antibody was no longer detectable by the AGP test and acquired antibody in contact exposed chickens was detected earlier than by the AGP test.

9. The IFA test was 10 to more than 320 times more sensitive than the AGP test. Some serums with high titers of immunofluorescent antibody did not produce precipitation in agar gel.

10. Using the DFA and micro AGP tests, antigen was found in superficial cells of the feather follicle epithelium, lung, follicles of the bursa of Fabricius, thymus, spleen and cecal tonsil.

11. Filtrable, infectious virus was recovered from extracts of skin but not from extracts of lung, thymus or bursa of MD-infected chickens.

12. Histopathologic examination revealed a close association between antigens in these organs and cells undergoing degeneration and necrosis.

13. There were intranuclear inclusion bodies in cells of the feather follicle epithelium.

14. Different cloned preparations of virus varied greatly in pathogenicity.

15. Some viruses had lost the A precipitin antigen during passage in cell culture.

16. HMD produced larger plaques in a shorter time than other MDVs.

17. Loss of pathogenicity, loss of the A antigen, and change in virus-host cell interrelationships are independent events.

18. The MDV and HVT shared at least two A antigens and one B antigen. These two viruses could not be readily distinguished from one another by the AGP test.

19. The MDV and HVT could be distinguished from one another, as could antibody to each virus, by the intracellular location of antigen by the IFA test.

20. Cultures of fibroblasts from chicken, duck, bobwhite and Japanese quail, turkey, pheasant, pigeon and goose embryos, cultures of chicken and duck kidney cells and the chorioallantoic membranes of embryonating chicken eggs were all susceptible to low and high cell culture passaged MDV and the HVT.

21. HeLa cells were not susceptible to MDVs or HVT.

22. CEF were much less susceptible to LMD, equally susceptible to HMD, and more susceptible to the HVT than were DEF.

23. The three viruses could best be differentiated by the morphological appearance of cytopathic areas in CK cultures.

24. A cytoplasmic virus-induced antigen could be detected by the IFA test in cells of all types infected with the HVT using homologous antiserum but not by using MDV antiserum. Nuclear antigens were detected by both sera.

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