MAREK'S DISEASE

DEVELOPMENTS IN VETERINARY VIROLOGY

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Payne, L.N. (ed.) *Marek's Disease* (1985)

MAREK'S DISEASE

Scientific Basis and Methods of Control

edited by

L. N. Payne
Houghton Poultry Research Station
England

Martinus Nijhoff Publishing
a member of the Kluwer Academic Publishers Group
Boston/Dordrecht/Lancaster

Distributors for North America:
Kluwer Academic Publishers
190 Old Derby Street
Hingham, MA 02043

Distributors outside North America:
Kluwer Academic Publishers Group
Distribution Centre
P.O. Box 322
3300 AH Dordrecht
The Netherlands

The figure on the cover is from Purchase, H.G., "Clinical Disease and its Economic Impact." The figure appears on page 27 of this book.

Library of Congress Cataloging in Publication Data
Main entry under title:

Marek's Disease.

(Developments in veterinary virology)
Includes index.
1. Marek's disease. I. Payne, L. N. II. Series.
SF995.6.M33M37 1985 636.089'692 85-7234
ISBN-13:978-1-4612-9635-5 e-ISBN-13:978-1-4613-2613-7
DOI: 10.1007/978-1-4613-2613-7

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Softcover reprint of the hardcover 1st edition 1985

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Preface to the series Developments in Veterinary Virology

It is my pleasure to introduce the first volume in our new series, Developments in Veterinary Virology. Since virus diseases in domestic animals and fowl are a major threat to the economy of all nations, veterinary measures used to curb the appearance of epizootics in animals are of major importance. With this series, we hope to expand the knowledge on virus diseases which have a disastrous effect on agricultural economics, and which are the cause of concern to veterinary services all over the world.

Developments in Veterinary Virology will devote a volume to each of the major virus diseases of animals and poultry, presenting up-to-date knowledge in each area. Each volume will deal with the veterinary aspects of a disease, and its causative virus from the biological as well as the genetic engineering viewpoints, and will include material on the pathology and epidemiology of virus vaccines and genetic resistance. The latest basic and practical scientific developments will be included, so that the series should be of special interest to practicing veterinarians and farmers.

Forthcoming volumes will deal with avian leukosis (G. de Boer, ed.), bovine leukosis (A. Burny and M. Mammerickx, eds.), classical swine fever and related infections (B. Liess, ed.), foot-and-mouth-disease (F. Brown, ed.),and African swine fever (Y. Becker, ed.).

I hope that the present series will be instrumental in providing a better understanding of current knowledge on virus diseases and the practices used in their control.

I would like to express my appreciation to the editors and authors of this and future volumes for their valuable contributions.

Yechiel Becker
Jerusalem

PREFACE

Take a disease of complex pathology with inflammatory and neoplastic features, which affects lymphoid and neural tissues, belonging to a disease group which killed one chicken in five, and which defied efforts to understand and control it for more than 50 years, and one can begin to appreciate the interest Marek's disease has received. Compound these characteristics with the finding of the causal herpesvirus, its recognition as the neoplasm first discovered to be so caused, and its prevention by vaccination, and the special place of Marek's disease in veterinary medicine and comparative oncology becomes clear.

This book sets out to provide an authoritative and comprehensive account of knowledge of Marek's disease and its control. I hope that it will be of value to veterinary research workers, teachers and students who need information about the disease, to veterinarians, poultrymen and vaccine manufacturers who have to diagnose and control it, and to oncologists in other fields interested in comparative aspects. Other reviews of the disease exist, of course, but this is the first multi-authored book devoted to the subject.

I have been fortunate in persuading many of the leading research workers who provided much of our knowledge of Marek's disease to contribute to this book, and I am grateful for their participation. It is also a pleasure to thank colleagues and friends at Houghton and elsewhere for their help in various ways. My thanks are due particularly to Helen Tiddy, who mastered the word processor and produced the final typescript for publication.

L.N. Payne
Houghton

MAREK'S DISEASE

1. HISTORICAL REVIEW

L.N. PAYNE

1. INTRODUCTION

The purpose of this Chapter is to provide an introduction to the main studies and concepts concerning the nature and cause of Marek's disease (MD) published between 1907, when the disease was first described by Marek (1), and the early 1960's, when the transmissibility of MD was clearly established and rapid progress began to be made in its understanding. During this period there was great argument about the pathological nature and cause of MD, its relationship to other leukotic diseases, and its mode of natural transmission. The extensive literature published (see reviews 2-5) stands witness to this long debate and to the failure of the numerous studies to provide satisfactory answers to these questions. In retrospect, this was due to inadequate virological knowledge and techniques, lack of defined chickens for experimental reproduction of the disease, and inadequate facilities for properly controlled transmission studies.

2. JÓZSEF MAREK AND THE FIRST DESCRIPTION OF MAREK'S DISEASE

Dr. József Marek (1868-1952) (Fig. 1) was Professor and Head of the Department of Veterinary Medicine at the Royal Hungarian Veterinary School in Budapest from 1901 to 1935. He was an outstanding scientist and clinician, wrote extensively on veterinary medicine and pathology, and was co-author, with Hutyra and Manninger, of the three volume classic, "Special Pathology and Therapeutics of the Diseases of Domestic Animals" (6) which became a standard textbook in many countries. Marek was a member of the Hungarian Academy of Sciences and received several high decorations and orders, and a number of honorary Doctorates. On the basis of his outstanding contributions he is established as a figure

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FIGURE 1. József Marek.

of major historical importance in veterinary medicine (7,8) but, although he will be so remembered, it is a quirk of fate that his name seems likely to be perpetuated most obviously in eponymous association with the neuropathic disease of chickens he described, and which later was termed Marek's disease.

In 1907, Marek (1) described, in a paper entitled "Multiple Nervenentzündung (Polyneuritis) bei Hühnern" and published in Deutsche Tierärztliche Wochenschrift (Fig. 2), a paralytic disease affecting the legs and wings of four cocks, and characterized, in the one case studied thoroughly, by thickening of the sacral plexi and spinal nerve roots, loss of nerve fibres, and infiltration of affected nerves by mononuclear cells. Marek considered the disease to be a "neuritis interstitialis" or a "polyneuritis". He could not ascertain the cause.

Deutsche
Tierärztliche Wochenschrift

herausgegeben von

Prof. Dr. **Dammann**, Geheimer Regierungs- und Medizinalrat, Direktor der Tierärztlichen Hochschule in Hannover.

Prof. **Röckl**, Geheimer Regierungsrat in Berlin.

Unter Mitwirkung von

Landestierarzt Prof. Dr. **Edelmann**, Medizinalrat in Dresden, Landestierarzt **Feist**, Regierungsrat in Strassburg i. E., Veterinärrat Dr. **Garth** in Darmstadt, Bezirkstierarzt Dr. **Görig** in Buchen, Oberamtstierarzt **E. Theurer** in Ludwigsburg und Prof. Dr. **Vogel** in Stuttgart

redigiert von Prof. Dr. **Malkmus** in Hannover.

Die „Deutsche Tierärztliche Wochenschrift" erscheint jeden Sonnabend. **Bezugspreis vierteljährlich Mk. 4.**— durch die Verlagsbuchhandlung von **M. & H. Schaper** in **Hannover** (bei direkter portofreier Zusendung), sowie durch alle Buchhandlungen und Postanstalten. **Anzeigenpreis** für die viergespaltene Petitzeile oder deren Raum **25 Pfg.** Schluss der Anzeigen-Annahme Donnerstag Morgen.
Sämtliche Zuschriften und redaktionelle Anfragen werden an Professor Dr. **Malkmus** in **Hannover** erbeten; Korrekturen und Anzeigen an die Verlagsbuchhandlung von **M. & H. Schaper** in **Hannover**.

№ 30. **Ausgegeben am 27. Juli 1907.** **15. Jahrgang.**

Multiple Nervenentzündung (Polyneuritis) bei Hühnern.

(Aus der medizinischen Klinik der tierärztlichen Hochschule in Budapest.)

Von Prof. Dr. J. **Marek**.

(Mit 2 Abbildungen.)

Unsere Kenntnisse bezüglich der nervösen Erkrankungen des Geflügels sind derzeit noch recht bescheiden. In der Literatur findet man nämlich nur in sehr spärlicher Zahl von Nervenkrankheiten bei Vögeln beschrieben, deren anatomische Grundlage zudem gewöhnlich nicht näher untersucht wurde. Eine Ausnahme hiervon bildet die Beri Beri-ähnliche Polyneuritis der Hühner in Niederländisch-Indien, welche von Eykmann[1]) beschrieben und hin-

Unter dem Einfluss des von Eykmann supponierten Giftes entsteht nun in den peripherischen Nerven eine Degeneration, welche allmählich zum Zerfall der Markscheide der Nervenfasern führt, infolgedessen dann die Schwannsche Scheide eine Art Emulsion von Myelin-Kügelchen enthält und schliesslich die ganze Nervenfaser in einen dünnen, marklosen Faden sich verwandelt. Dabei verfallen die zugehörigen Muskeln der einfachen Atrophie.

Klinisch kennzeichnet sich die Krankheit durch sowohl an In- als an Extensität rasch zunehmende Lähmungserscheinungen seitens der Beine, so dass die letzteren alsbald gespreizt und im Knie- und Mittelfussgelenk gebeugt gehalten werden und das Tier beim Laufen öfters sinkt oder auch umfällt. Schliesslich wird das Belasten der Füsse überhaupt unmöglich und in diesem Stadium

FIGURE 2. Title page of the first publication on Marek's disease.

3. CLASSICAL STUDIES, 1920 - 1939

The next published observations on "paralysis of the domestic fowl" were those of Kaupp (9), who first saw the disease in the USA in 1914. Clinical features were similar to those described by Marek, and Kaupp noted perivascular round cell infiltration in the spinal cord and regarded the disease as a "transverse myelitis". Fowl paralysis was observed again as an epizootic disease in the Netherlands in 1921 by Van der Walle and Winkler-Junius (10), and they suggested the term "neuro-myelitis gallinarum". Other early reports of the disease in the USA were those of May et al. (11) and Doyle (12). Kaupp and Doyle also observed that blindness, due to iritis, sometimes accompanied fowl paralysis. These early authors attempted to transmit the disease, with results which were either negative or unconvincing; nevertheless, all groups of investigators thought the disease to be infectious.

The seminal study of fowl paralysis is that of Pappenheimer and his colleagues (13-15), who described in detail the clinical and epizootiological features, gross and microscopic pathology, attempts at bacteriological study, and transmission experiments. Particularly noteworthy is their recognition that visceral lymphomas, especially affecting the ovary, occurred with the neural disease, their introduction of the term "neuro-lymphomatosis gallinarum" for the disease, and their evidence for successful transmission by inoculation of chickens with tissues from a paralysed bird. The authors comment on the results of their transmission experiment VII (Table 1): "There can be no question that in this experiment there is a significantly higher incidence of infiltrations in all parts of the nervous system, and particularly in the peripheral nerves, in the inoculated group. One cannot escape the conclusion that the inoculation of the supposedly infective material is in some way correlated with this higher incidence, inasmuch as the inoculation was the only known variable in the experiment". The results of their experiments epitomize, however, those of so many of the classical investigators: while fowl paralysis occurred in the inoculated birds, it also did so, albeit often in lower frequency and severity, in uninoculated controls. In retrospect, it seems likely that transmission was indeed achieved in these and many similar investigations, but while cases occurred in controls the possibility could not be excluded that inocula-

TABLE 1. Summary of transmission experiment VII from Pappenheimer *et al.* (13), showing incidence and severity of nervous lesions in inoculated and control chicks.

	Brain			Cords			Nerves		
	Marked	Moderate or slight	Neg.	Marked	Moderate or slight	Neg.	Marked	Moderate or slight	Neg.
Inoculated	0*	24	7	2	23	6	3	14	14
Controls	0	8	24	1	15	16	0	5	27

*Number affected.

tion merely exacerbated a pre-existing condition.

During the years that followed, fowl paralysis (range paralysis) was reported in Germany in 1927 (16) in South Africa in 1928 (17), in England in 1929 (18), in Japan in 1930 (19), and subsequently in many other countries, accompanying the growth of the poultry industry. Efforts continued, particularly in the USA, to transmit fowl paralysis experimentally and to understand its cause and pathogenesis, major investigators being Patterson et al. (20), Gibbs (21), Johnson (22), Furth (23), Jungherr (24,25) and Durant and McDougle (26) in the USA, Warrack and Dalling (27) and Blakemore (28) in England, and Lerche and Fritzsche (29,30) in Germany. The results of these investigations were reported in great detail but none gave conclusive evidence of transmission, although some were highly suggestive. Thus Furth (23) reported induction of neurolymphomatosis following mainly intravenous or intraneural inoculation of blood, nerve or tumour cellular material from affected birds in 61/110 birds in passages of his strain 5 material and in 35/66 birds in passages of strain 6. Furth (23) remarked that the "transmitting agent circulates in the blood of the paralyzed chicken during the entire period of manifest disease", and observed that freezing or drying blood or nerve inocula almost invariably inactivated the agent and that cell-free plasma had little if any potency. Gibbs (21) and Durant and McDougle (26) also recorded successful transmission when viable nerve tumour cells or fresh blood were used as inocula. The latter authors induced paralysis in 91/527 birds inoculated with blood, compared with an incidence of 18/507 in the controls. Much later, the requirement for live cells in inocula for successful transmission was confirmed (31), and explained by the closely cell-associated nature of the herpesvirus responsible for MD (32,33).

3.1. Relationship of neurolymphomatosis to other leukotic diseases

Apart from the problem of fowl paralysis in control birds, as discussed above, interpretation of fowl paralysis transmission experiments was often made difficult by the occurrence, in inoculated and control birds, of other leukotic and neoplastic diseases. Pappenheimer and his co-workers (13-15) recognized the presence of visceral lymphomatosis, especially in the ovary, in some chickens with neurolymphomatosis, and they regarded it as an expression of fowl

paralysis, as did later workers (23,27,30,34).

Many workers (21,23,25), although not all (20,22,34), considered the transmissible lymphoid, erythroid and myeloid leukoses described by Ellerman (35) to be aetiologically and pathologically separate from the neural and visceral manifestations of fowl paralysis. Pappenheimer and his colleagues (13-15) believed neurolymphomatosis was distinct from the true leukaemias but were uncertain whether an aleukaemic lymphoid leukaemia, with lymphoid infiltrations in the viscera, as described by Ellerman (35), existed as an entity apart from the visceral lymphomas associated with the neural disease. Thomas and Hamilton (34), however, in 8139 autopsies, observed 100 birds with lymphoma but without macroscopic or microscopic evidence of nerve infiltration, and Jungherr (25) distinguished between lymphomatosis associated with fowl paralysis and "big liver" disease. Gibbs (21), on the other hand, stated that (pathologically) neurolymphomatosis and visceral lymphomatosis (with or without neurolymphomomatosis) seemed to be closely associated, and not readily differentiated from "lympholeukosis". Patterson (20,36) and Johnson (22) thought neurolymphomatosis and the various types of leukosis to be different manifestations of infection by the same agent. Johnson, for example, stated "evidence has been obtained which must be interpreted as supporting the view that the so-called neuro-lymphomatosis of fowls (Pappenheimer and others) and the erythroid and myeloid leucosis (Furth) are different expressions in response to a common filterable agent". Furth (23), on the other hand, clearly distinguished, both aetiologically and pathologically, between two types of transmissible lymphomatosis. One virus (strain 2) produced a lymphomatous neoplasm, and also endotheliomas and myelocytomatosis, and nerve infiltration but without clinical paralysis. Other viruses (strains 5 and 6) produced extensive infiltration of nerves and clinical paralysis, but rarely leukaemia and never endotheliomas or myelocytomas. The nature of the lymphoma produced by strain 2 is unclear: Furth believed it to differ from "big liver" disease (hepatolymphomatosis) - now termed lymphoid leukosis.

4. THE MIDDLE PERIOD, 1940 - 1959

During the next two decades, work continued in attempts to unravel

the complexities of fowl paralysis and possibly related neoplastic diseases. In the USA a major organisational advance was the establishment in 1939 by the US Department of Agriculture of the Regional Poultry Research Laboratory (RPRL) at East Lansing, Michigan, to improve the viability of poultry and, specifically, to determine the cause of fowl paralysis, and to develop measures for its prevention and control (5, 37). This laboratory acted as a centre for collaboration with many of the state agricultural experiment stations at which much of the earlier work had been done.

In 1940, Jungherr, Doyle and Johnson, acting as a committee for the RPRL, proposed a tentative pathological nomenclature for the avian leukosis complex and other tumours (38), as shown in Table 2. This nomenclature "had the object of facilitating uniformity in terminology and interpretation of data" and was widely adopted in the USA. It did not imply classification on the basis of aetiology.

Effort at the RPRL was directed at the transmission of "lymphomatosis" and, in a series of admirable studies, Burmester, Cottral and Waters, and their colleagues, clearly established the viral aetiology, pathology, and routes of transmission, of "visceral lymphomatosis" (39-42). But as these studies progressed, it became apparent that the visceral lymphomatosis produced - the so-called "big liver disease" or hepatolymphomatosis - was not accompanied by an increase in neural lymphomatosis. Waters (43) concluded "that the four types of lymphomatosis i.e. visceral, neural, ocular and osteopetrotic, represent four distinct disease entities and are caused by four distinct and different agents". Later it became clear that the visceral lymphomatosis studied by the RPRL group was retrovirus-induced lymphoid leukosis, and unrelated to neurolymphomatosis (Marek's disease). Davis and Doyle (44,45) similarly transmitted visceral lymphomatosis and concluded that it was distinct from other forms of leukosis.

Meanwhile, others had continued to investigate the transmissibility of fowl paralysis. Durant and McDougle (46) induced a 76% incidence of fowl paralysis in 398 young chicks by inoculation with blood from a flock affected with ocular and neural forms of fowl paralysis; 34% of 398 control chicks reared in contact with the inoculated chicks developed fowl paralysis. Unfortunately isolated control chicks were not

TABLE 2. Classifications of the avian leukosis complex. (After Biggs (56) by kind permission.)

Common names	Classifications: 1941 Jungherr *et al*. (38)	1952 Cottral (50)	1957 Chubb and Gordon (3)	1961 World Veterinary Poultry Association (55,56)
Marble bone, or Big bone disease	Lymphomatosis: Osteopetrotic	Osteopetrotic lymphomatosis, or Osteopetrosis	Osteopetrosis	Osteopetrosis
Grey eye, or Pearly eye disease	Lymphomatosis: Ocular	Ocular lymphomatosis	Ocular → Lymphomatosis	Marek's disease: Ocular
Fowl paralysis, or Range paralysis	Lymphomatosis: Neural	Neural lymphomatosis	Neural (Fowl paralysis) → Lymphomatosis	Marek's disease: Neural (Fowl paralysis)
Big liver disease	Lymphomatosis: Visceral	Visceral lymphomatosis	Visceral → Lymphomatosis; Diffuse, Discrete → Lymphoid leukosis → Leukosis	Marek's disease: Visceral; Leukosis: Lymphoid leukosis (Diffuse, Nodular)
Fowl leukaemia or leukosis	Leukosis: Erythroblastic; Granuloblastic	Erythroblastosis; Myeloblastosis or Granuloblastosis	Erythroleukosis → Leukosis; Diffuse → Myeloid leukosis → Leukosis	Leukosis: Erythroleukosis; Myeloid leukosis (Diffuse)
White tumours	Other tumours: Myelocytoma, Sarcoma, Etc.	Myelocytomatosis	Discrete → Myeloid leukosis	Leukosis: Myeloid leukosis (Nodular)
				Conditions interrelated with the leukoses: Sarcomas, Endotheliomas, Renal tumours

kept. Blakemore (47) continued his transmission studies and following serial passage of his agent produced mortality within a few days, accompanied by inflammatory lesions in the liver and heart. These were at first thought to represent an acute form of fowl paralysis but later work particularly by F.D. Asplin suggested mycoplasmal contamination to be responsible (5).

During this period Hutt and Cole (48,49) continued their classic studies, started in 1935, on selection of strains of fowl genetically susceptible or resistant to "leucosis" following natural exposure. They demonstrated that the disease could be greatly reduced by breeding resistant stock, or by rearing susceptible stock in isolation from adult birds. In their studies, neurolymphomatosis was much more prevalent than visceral or ocular lymphomatosis. They too recognized (48) that there might be "more than one virus, and that one virus may cause mostly paralysis, another the visceral form of the complex", and, percipiently "that the particular viruses causing neural and visceral lymphomatosis must frequently exist together on the same premises and in the same bird". This reflection was later shown to be correct and explains much of the confusion of the earlier years. Thus the work of Burmester and his colleagues, and of Hutt and Cole, provided firmer foundations for the conviction of many workers, particularly in the UK, that lymphoid leukosis and fowl paralysis were two distinct diseases. This view also explained the long debate, often forcefully expressed, about whether lymphomatosis was egg transmitted, as claimed for visceral lymphomatosis by the East Lansing workers, but not for leukosis by Hutt and Cole.

In spite of these views, workers in the USA still preferred to use the generalized term "avian lymphomatosis", which was divided into visceral, neural and ocular forms, as in Cottral's (50) classification (Table 2). On the other hand, Campbell (51,52) in the UK, had long advocated that lymphoid leukosis and fowl paralysis were distinct conditions with different aetiology and mode of transmission, but that visceral lymphoid lesions occurred in both conditions. In the American classifications (Table 2), these two types of visceral lesions were covered by the single term visceral lymphomatosis. In 1957, Chubb and Gordon (3) proposed a new classification (Table 2) to emphasize this distinction. Biggs (53) and Campbell (54) considered that the term

lymphomatosis was the cause of confusion and that it should be abandoned. Accordingly, another classification (Table 2) (55,56) was proposed, and adopted by the World Veterinary Poultry Association in 1961, in which the term "Marek's disease" was used to cover neural lymphomatosis and the visceral and ocular lesions associated with it, and "lymphoid leucosis" used for the visceral lymphomatosis of Burmester. This new classification has been widely used since. A later classification proposed by Sevoian (57), in which MD was termed Type II lymphoid leukosis, has not found favour.

4.1. Is fowl paralysis neoplastic?

For many years, workers argued about whether fowl paralysis (MD) is an inflammatory or neoplastic disease. Terms used by workers who believed the nerve lesion in fowl paralysis to be inflammatory included "neuritis interstitialis" and "polyneuritis" (1), "transverse myelitis" (9), "neuritis" (12), and "neurogranulomatosis infectiosa gallinarum (Marek)" (30). Campbell (52) considered the nerve lesions to be inflammatory but recognized that these could rarely become neoplastic. Initally he believed that the visceral lymphoid proliferation occurring in MD were not true neoplasms, but termed them "lymphogranulomas" (52); later he acknowledged that a proportion could become neoplastic, particularly in acute MD (58).

Pappenheimer and co-workers (13,14) originated the opposing viewpoint that the neural lesions were neoplastic and, as already mentioned, proposed the term "neuro-lymphomatosis gallinarum". They also recognized the occurrence of visceral lymphomata in a proportion of cases and believed these to be a manifestation of the disease. These concepts and terms were subsequently widely adopted, as discussed in Section 3.

These arguments depended largely on the most conspicuous lesions seen in various circumstances and on the supposed relationship between them. To a considerable extent they have now been resolved by experimental work on MD carried out since the early 1960's. Proponents of the inflammatory and neoplastic concepts have both been proved to be partly correct! (see Chapter 3).

5. EVENTS SINCE 1960

The modern era of research on MD dates from 1960 and was initiated

by the successful transmission experiments of Sevoian and his colleagues in the USA (59,60) and by Biggs and Payne in the UK (31,61). Sevoian's group was able to transmit the neural, visceral and ocular forms of "avian lymphomatosis" by inoculation of day-old susceptible chicks with tumour cell suspensions, blood and other tissues; from these transmissions the widely studied JM isolate was derived.

In the UK in 1959, in the face of continuing concern about lack of understanding of MD, a Leukosis Experimental Unit was established at Houghton Poultry Research Station by the Agricultural Research Council. Here, Biggs and Payne (31,61) transmitted the neural and visceral forms of MD, their success being dependent on the use of whole blood inocula from MD-affected birds and young susceptible chicks as recipients, and of strict isolation facilities, which enabled uninoculated control chicks to be kept free from adventitious infection. They compared the pathology of experimentally-induced MD with that of lymphoid leukosis, and the properties of the MD agent with leukosis virus, and provided further evidence for dissociating the two diseases. Subsequently Biggs and his colleagues (62) transmitted a virulent form of lymphomatosis that appeared in the UK in 1965. They showed that it was an acute form of MD and apparently identical to a disease which was becoming increasingly prevalent in broilers in the USA (63,64), where it was termed "visceral lymphomatosis" or "acute leukosis".

These findings, and the increasing severity of acute MD in many countries, greatly stimulated research on MD. By 1967 a herpesvirus had been identified as the cause by Churchill and Biggs (32) and Nazerian and his colleagues (33) and by 1970 live virus vaccines against MD had been developed (65,66) and were available commercially. The 25 years since 1960 have been marked by the growth of a large body of knowledge on MD, much of which comprises the rest of this book.

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2. CLINICAL DISEASE AND ITS ECONOMIC IMPACT

H.G. PURCHASE

1. INTRODUCTION

Marek's disease (MD) is the most common clinical neoplastic condition of any animal, including man, on the globe. However, the clinical disease represents the "tip of the iceberg" in that many more birds are infected with the virus than show clinical signs. Clinical signs are also very varied.

The disease was first recognized as a clinical entity by Marek in 1907 (1). The form known as "classical" MD was a chronic, neuropathic condition: it was reported sporadically from many countries during the first half of the century. A more highly pathogenic, acute form of MD was first reported in Delaware in the 1950's (2) and subsequently spread throughout the USA. The acute form was first recognized in the UK in 1965 (3). Following the advent of the vaccine against MD and its use worldwide in the early 1970's (4), the incidence of clinical disease declined substantially. However, there is now a resurgence of reports of outbreaks of disease in laying hens and condemnations in broilers which have been vaccinated. These "vaccine failures" may be due to the appearance of exceptionally virulent viruses against which the vaccine fails to protect (5-7). These new "variant strains" are causing peracute disease in vaccinated chickens resulting in significant losses.

2. OCCURRENCE OF INFECTION AND DISEASE

2.1. Prevalence

2.1.1. Global prevalence. MD probably occurs in poultry-producing countries throughout the world. In developed countries with advanced poultry-producing technology, the disease is well recognized and most flocks of laying chickens are vaccinated against it. It is likely that

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every flock of chickens raised under an intensive production system suffers some loss from MD; however, detection and reporting systems vary greatly. For example, international disease reports indicate that MD has not been recorded in Yugoslavia (8,9), though it is highly unlikely that any country is completely free of the disease (Table 1).

In countries with developing market economies, MD could easily occur without being recognized. In some of these countries there is a great shortage of veterinary personnel and transportation, so diagnostic services are lacking. Where there are diagnostic services, uncontrolled acute diseases such as fowl typhoid and Pullorum disease are so prevalent that they mask the occurrence of MD (10). MD is sometimes confused with lymphoid leukosis and is recorded as such and is therefore not reported as MD. It is possible that isolated flocks in field situations may occasionally be free of the disease though it is unlikely that entire countries, like Cuba and Turkey (8,9), are free of the disease (Table 2).

2.1.2. Prevalence under different production practices. Pastoral, extensive husbandry practices probably delay the time of first exposure of the chick to MD virus (MDV) and reduce the virus dose. Chickens with early signs of disease may succumb to predation or may not be recognized as ill because of infrequent observation. Thus the prevalence of MD is likely to be lower under these extensive husbandry conditions.

Under intensive husbandry there is often a considerable residue of waste, dust, and feathers left in the growing house between crops of birds, resulting in the early infection of young chicks. Chicks are in close proximity to one another, facilitating contact spread. Also, there is a limited free exchange of air, particularly in the winter, resulting in a high concentration of MDV-infected dander in the air. These conditions lead to an earlier infection with a larger dose of virus than under extensive conditions. The greater transmission of virus from chicken to chicken in the winter is reflected in higher condemnations from MD among broilers during those months (see Section 2.2.2.).

Particularly in the USA, certain areas with intensive poultry production have higher condemnations than others. For example, Delaware has for many years had a consistently higher condemnation rate than Georgia (Table 3). The reasons for this difference are not clear.

TABLE 1. Presence and incidence of disease in developed countries. (From Biggs (8) by kind permission.)

	Avian encephalomyelitis	Fowl plague	Fowl pox	Infectious bronchitis	Infectious laryngo-tracheitis	Leukosis	Marek's disease	Newcastle disease	Ornithosis	Mycoplasmosis	Fowl cholera	Infectious coryza	Pullorum disease	Spirochaetosis	Tuberculosis	Coccidiosis
Canada	(+)	-		++	+	++	(+)	(+)	-	+	+	(+)	(+)	-	+	++
U.S.A.	(+)	-	+	++	+	+	+	+	-	+	+	+	(+)	(+)	+	++
Austria	+	-	(+)	+	(+)	+	++	(+)	(+)	+	-	+	(+)	-	+	
Belgium	-	-	(+)	++	+	+	(+)	(+)	-	++	-		(+)	-	(+)	+
Bulgaria	-	-	(+)	-	-	+	++	-	-	+	+	-	+	+	+	++
Czechoslovakia	(+)	-	+	(+)	(+)	+	++	+	(+)	(+)	(+)	(+)	+	-	+	++
Denmark	+	-	-	+	-	+	+	-	-	(+)	-	+	-	-	+	+++
East Germany	(+)	-	+	(+)	-	++	(+)	(+)	(+)	(+)	(+)	(+)	+	-	+	+++
France	+	-	++	++	-	++	++	-	+	++	-	++	+	-	+	++
Greece	+	-	+	+	+	+	++	+	-	++	+	+	+	+	+	++
Hungary	+	-	+	++	-	+	+	-	-	++	++	-	+	-	+	++
Italy	+	-	+	+	-	+	+	++	(+)	+	(+)	+	+	-	(+)	++
Netherlands	(+)	-		++	-	++	+	+	+	+	+	-	-	-	+	+
Poland	(+)	-	-	(+)	-	(+)	+	-	-	+	+	-	+	-	+	++
Portugal	+	-	+	+	-	++	++	++	-	++	+	-	++	-	+	++
Romania	+	-	+	(+)	(+)	++	+	(+)	-	+	+	+	(+)	-	-	++
Spain	(+)	-	-	+	-	++	+	+	-	+	(+)	(+)	(+)	(+)	(+)	++
Sweden	(+)	-	-	+	-	+++	+	-	-	+	-	(+)	-	-	+	+++
United Kingdom	+	-	+	++	+	++	+	-	-	+	+	+	(+)	-	+	++
West Germany	+	-	+	(+)	(+)	+	+	+	+	+	(+)	+	+	-	+	++
Yugoslavia	-	-	-	-	-		-	++	-	(+)	+	-	+	-	+	
U.S.S.R.	-	-	-	+	+	+	+	+	(+)	+	(+)	-	+	+	(+)	+
Israel	(+)	-	+	+	+	+	++	+	-	++	++	++	-	(+)	-	++
Japan	++	-	+	+	+	+	++	+	-	++	-	++	+	-	-	++
Australia	++	-	++	++	++	++	++	-	-	++	+	++	+	+	(+)	++
South Africa	+	-	++	+	+	++	+	+	-	+	(+)	+	+	+	+	++

INCIDENCE
- Not recorded
(+) Rare
+ Low sporadic
++ Moderate
+++ High

Data from FAO, WHO, OIE Animal Health Yearbook 1979
Countries and diseases have been grouped alphabetically within geographical areas and according to type of causative organism respectively.

TABLE 2. Presence and incidence of disease in developing countries. (From Biggs (8) by kind permission.)

	Avian encephalomyelitis	Fowl plague	Fowl pox	Infectious bronchitis	Infectious laryngo-tracheitis	Leukosis	Marek's disease	Newcastle disease	Ornithosis	Mycoplasmosis	Fowl cholera	Infectious coryza	Pullorum disease	Spirochaetosis	Tuberculosis	Coccidiosis
Argentina	+	-	++	++	++	+	+	++	-	+++	++	++	++	-	+	++
Brazil	(+)	-	++	(+)	(+)	++	++	++		++	+	++	+	(+)	+	++
Columbia	+	-	+	(+)	+	+	+	++	-	++	+	+	+	-		+
Peru	-	-	++	++	++	++	++	++	-	++	++	++	++	-	-	+++
Venezuela	++	-	++	+	-	++	++	+++	-	+++	++	+++		-	(+)	+++
Cuba	-	-	+	-	-	+	-	+	-	+	+	++	+	-	-	++
Mexico	++	-	++	++	++	++	++	+++	-	++	(+)	++	++	-	+	+++
Egypt	-	-	++	(+)	-	++	+	+++	(+)	++	++	++	+++	++	+	+++
Iran	(+)	-	++	+++	+++	++	+++	+++	+	++	++	+	(+)	++		++
Turkey	-	-	+	-	-	+	-	++	-	+	-	-	+	-	(+)	(+)
China								++			+					
India	-	-	+	++	++	+++	+++	++	+++		+	++	+	++	+	+++
Indonesia	+	-	+	-	-	(+)	+	+++	-		-			-	(+)	++
Korea	+	-	+		-	++	++	++	-	++	-	+	+	-	-	++
Malaysia	+	-	++	++	+	++	+	++	-	++	+	++	+	-	+	++
Philippines	(+)	-	++		(+)	(+)	+	++	-	+++	++	+++	++	-	-	++
Thailand	-	-	+	+	(+)	+	(+)	+	-	(+)	+	(+)	+	-		+
Vietnam	-	-	+++	+	+	+	+	+++			++	+	+	+		++
Nigeria	+	-	++	+	+	++	+++	+++	+	++	++	+	+	++	+	+++

INCIDENCE
- \- Not recorded
- (+) Rare
- \+ Low sporadic
- ++ Moderate
- +++ High

Data from AO, WHO, OIE Animal Health Yearbook 1979
Countries and diseases have been grouped alphabetically within geographical areas and according to type of causative organism respectively.

Chickens from many of the same genetic stocks are used in both locations. Husbandry practices differ only slightly due to the slightly lower average year-round temperature in Delaware than in Georgia. It is possible that the major poultry-producing farms are closer together in Delaware than in Georgia though this trend is not obvious. Another possibility is that more highly virulent strains of MDV occur in Delaware than in Georgia.

TABLE 3. Leukosis condemnations in young chickens

Year	USA	Georgia	Delaware
61	0.11%	0.08%	0.28%
62	0.13	0.09	0.47
63	0.22	0.22	0.75
64	0.43	0.50	1.10
65	0.52	0.54	1.52
66	0.92	0.89	2.36
67	1.27	1.75	3.06
68	1.54	2.71	2.85
69	1.48	2.54	2.29
70	1.57	2.89	1.96
71	1.42	2.26	1.71
72	0.85	0.90	1.40
73	0.45	0.26	0.98
74	0.28	0.15	0.57
75	0.15	0.15	0.27
76	0.17	0.17	0.16
77	0.16	0.08	0.29
78	0.10	0.03	0.23
79	0.11	0.03	0.22
80	0.08	0.02	0.25
81	0.08	0.02	0.29
82	0.08	0.02	0.46
%Reduction	94.9%	99.3%	76.5%
1970-1982	20-fold	144-fold	4-fold

Data are from the Statistical Reporting Service, United States Department of Agriculture (Source R.L. Witter)

2.1.3. Prevalence among different avian species. Signs compatible with MD have been observed in many different species of birds (11). However, there is little evidence that the signs were actually caused by MDV, whether or not the virus was isolated from diseased birds. Thus Kenzy and Cho (12) isolated MDV from a mature quail that had ocular lesions. However, they did not show that the virus so isolated induced ocular lesions in recipient pathogen-free quail. Successful experimental transmission has been accomplished in some species; however, typical signs of MD in recipient birds are either very rare or are lacking. For example, inoculation of turkeys with MDV may lead to tumours after a long latent period but very rarely (in one of four experiments) results in the classical nerve involvement seen with MD in chickens (13).

2.2. Incidence

2.2.1. Rate of occurrence. Prior to the use of vaccines, morbidity and mortality could range in egg-laying flocks from almost 0 to as high as 60%. Losses of up to 30% were common. Among meat-type birds the incidence was usually lower because they were slaughtered at an early age, 7 weeks for broilers in the USA. In other countries, and in roasters in the USA which are grown for 10 to 12 weeks, morbidity and mortality could reach up to 30%. In extensive farming systems and countries with developing marked economies, where vaccines against MD are not available, current losses may reach these levels.

Since the use of vaccines against MD, the incidence has decreased dramatically. Only occasionally, for example when vaccines have become inactivated due to mishandling, do losses similar to those described above occur. Almost all (95 to 100%) egg-type chickens are currently vaccinated against MD (Table 4). In most countries less than 5% of the birds started or hatched die of MD. Italy appears to be an exception where 18% die of MD. Less than 2% are condemned from MD at slaughter.

Fewer meat-type chickens than egg-type chickens are vaccinated except in Italy. Whereas in Canada, and the USA 75% or more are vaccinated (Table 5), many countries do not vaccinate meat-type chickens against MD. Losses during the growing period are usually 0.1% or less, except in Israel where they are 0.5%, and in Australia where they are 1.5%. In Italy, the USA, and Israel, condemnations among slaughtered birds are 0.2% or above.

TABLE 4. Economic importance of Marek's disease[a] in egg-laying chickens in selected countries[b].

	United States	Japan	France	United Kingdom	Italy	Netherlands	Canada	Germany	Australia	South Africa	Greece	Sweden	Israel	Denmark	Norway	Switzerland
Hen egg production (metric tons per year X 10^{-3})	4073	2004	827	811	635	504	321	320	195	154	120	114	100	76	43	43
Hen egg production per capita (kilograms per capita per year)	18.4	17.2	15.4	14.5	11.1	35.8	13.4	19.1	13.6	5.3	12.6	13.8	26.0	14.8	10.6	6.8
Percent of chickens vaccinated against Marek's disease	100	100	100	100	100	100	100	100	100	100	100	98	100	97	100	95
Age at peak loss from Marek's disease (weeks)	18	23	24	25	18	10	18	20	18	20	17	25	20	?[c]	24	?
Approximate percent loss of productivity from Marek's disease (i.e., loss of weight gain or eggs produced)	1.5	?	5.0	?	?	0	?	?	1.5	5.0	?	5.0	?	0.5	3.5	?
Average age at slaughter (weeks)	90	?	71	74	76	80	72	77	69	70	76	80	80	78	78	80
Percent of total birds started (or hatched) that die of Marek's disease	3	?	0.05	?	18	0.5	3	2	1.5	3	5	3	1	0.5	1.5	?
Percent of total birds started (or hatched) that die of all causes other than Marek's disease	15	?	7	3	10	11	11	12	10	8	18	15	20	13	12	?
Percent of total birds slaughtered that are condemned for Marek's disease (example: weight of whole birds and parts condemned divided by weight of all birds slaughtered X 100)	0.1	?	0.07	1.5	1	0	?	?	0.1	0	?	2	0.5	0	?	?
Are these figures based on actual counts on actual weights, or are they estimates?	E	E	A	E	E	A	E	E	E	E	E	E	E	A	E	E

[a]Marek's disease must be distinguished from lymphoid leukosis as far as possible. This request is for losses from Marek's disease

[b]Data for rows 1 and 2 of Tables 4 and 5 are from the 1981 Food Balance Sheet File produced by the Food and Agricultural Organization and are averages for the four years 1978 through 1981

Data for the remainder of Tables 4 and 5 and for Table 6 are from questionnaires sent to the corresponding secretaries of the World Veterinary Poultry Association for each country. They do not necessarily represent official data from those countries

[c]? = No loss recognized or impossible to estimate

TABLE 5. Economic importance of Marek's disease[a] in meat chickens in selected countries.

	United States	Japan	France	Italy	United Kingdom	Canada	Netherlands	Australia	South Africa	Israel	Germany DR	Greece	Denmark	Sweden	Switzerland	Norway
Chicken meat produced (metric tons per year X 10^{-3})	5424	978	833	775	627	399	355	275	221	154	147	136	90	40	22	10
Chicken meat produced (kilograms per capita per year)	24.5	8.0	15.5	13.6	11.2	16.7	25.2	19.1	7.7	40.1	8.8	14.2	17.7	4.9	3.4	2.5
Percent of chickens vaccinated against Marek's disease	99	40	0	100	3	75	0	0	0	50	0	10	0	0	0	0
Age at peak loss from Marek's disease (days)	56	45	?[b]	?	?	45	?	?	0	8	?	50	?	30	?	?
Approximate percent loss of productivity from Marek's disease (i.e. loss of weight gain or eggs produced)	0.5	?	0	?	?	?	0	?	0	?	?	?	0	1	?	?
Average age at slaughter (days)	56	60	67	55	50	45	44	42	47	57	36	55	42	36	38	40
Percent of total birds started (or hatched) that die of Marek's disease	0.1	?	0	0	0	0	0	1.5	0	0.5	?	0.1	0	?	?	?
Percent of total birds started (or hatched) that die of all causes other than Marek's disease	4.0	?	3.0	8.0	5.5	5.0	3.5	5.0	7.0	7.0	3.5	4.0	3.3	3.0	?	3.5
Percent of total birds slaughtered that are condemned for Marek's disease (example: weight of whole birds and parts condemned divided by weight of all birds slaughtered X 100)	0.2	?	0.04	2.0	0.01	0.1	0	0	1.0	0.25	0	?	0	0	?	?
Are these figures based on actual counts or actual weights, or are they estimates?	E	E	A	E	E	E	A	E	E	E	A	E	A	E	E	E

[a] Marek's disease must be distinguished from lymphoid leukosis as far as possible. This request is for losses from Marek's disease

[b] ? = No loss recognized or impossible to evaluate

2.2.2. Seasonal incidence. Among meat-type chickens which are slaughtered when young, there is a distinct seasonal incidence. Thus the peak of condemnations in the USA occurs during the winter and early spring months from February to April (Fig. 1). The incidence is lowest in the summer from July to September and highest in the winter from February to April. The trend is remarkably consistent from year to year. The incidence is probably higher in the winter months because the circulation of air is reduced in order to maintain a suitable growing temperature and because the virus survives longer in the low temperatures of winter than in the high temperatures of summer.

Among egg-type chickens there does not appear to be a clear seasonal variation in incidence of MD.

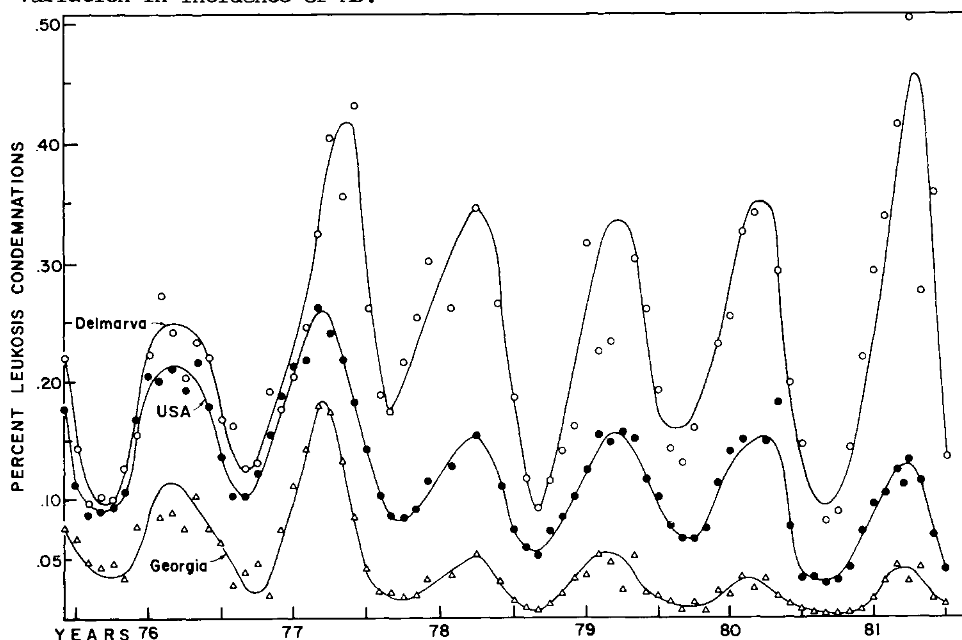

FIGURE 1. Recent trends in leukosis condemnations in broilers

Percent condemnations from MD in the USA and the States of Georgia and Delaware, Maryland and Virginia (Delmarva). Data are for leukosis condemnations of young chickens, all of which are from MD, from the Statistical Reporting Service, United States Department of Agriculture. (Source R.L. Witter).

2.2.3. Incidence under different husbandry practices. Prior to the use of the MD vaccine, the only known methods of control were increasing the genetic resistance of the chickens and reducing the opportunities of exposure by careful attention to hygiene. Even though the trends were not always consistent, it appeared that operations with the lowest losses had "all-in all-out" systems and were cleaned out between every batch, particularly after batches which had a high incidence of MD. Occasionally severe losses occurred in unexpected situations such as in new houses. These were explained on the basis that a highly virulent strain of MDV entered the flock and spread before a mild or nonpathogenic strain.

Since the extensive use of the vaccine, husbandry practices have become lax. In the USA conditions were aggravated by the energy crisis which occurred in the mid 1970's. Houses were cleaned less frequently and less thoroughly. Limited-area brooding was used to reduce the amount of space that needed heating. In this brooding practice, chicks are confined to a limited area of the house, usually one end, which is separated from the remainder of the house by a curtain. This places chickens in close proximity to one another, particularly when they are young and most susceptible to infection. In order to conserve energy further, some poultry farmers tried brooding at one end of the house when older chickens were at the other end. The older chickens would provide some heat for the younger chickens. Also the older chickens were an excellent source of virus infection and some very severe losses occurred.

2.2.4. Recent trends. Condemnations from MD in young chickens have declined significantly in the USA since 1970, when the MD vaccine was introduced (Fig. 2). National condemnations have decreased from a maximum of 1.57% in 1970 to 0.08% in 1982, a decrease of 94.9% or 20-fold (Table 3).

In Georgia the decrease was even more dramatic from 2.89% in 1970 to 0.02% in 1982, a decrease of 99.3% or 144-fold. However, in Delaware condemnations have been consistently higher than in Georgia or the average for the USA. Even before the advent of the vaccine, from the initiation of the inspection of chickens in 1961, condemnation from MD in Delaware has exceeded that in Georgia or the average for the USA. It

reached the maximum of 3.06% in 1967 and declined to 0.16% in 1976. However, between 1976 and 1982 it has increased to 0.46%. The reason for this increase is not clear, but it may be due to the highly virulent variants of MDV enzootic in this growing area.

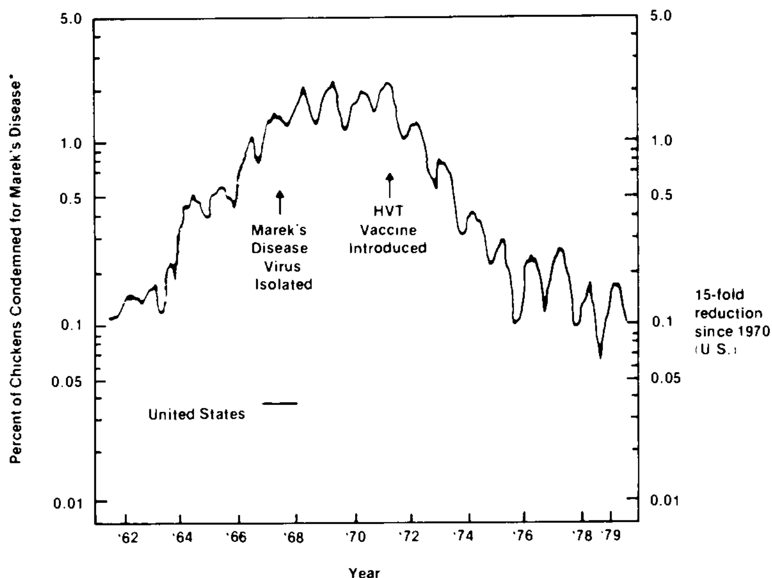

FIGURE 2. Control of MD in broiler chickens by vaccination with turkey herpesvirus (HVT). (Data from Statistical Reporting Service USDA).

In October 1983, a bivalent vaccine against MD, containing the herpesvirus of turkeys and the SB-1 strain of MDV, was licensed in the USA. This vaccine appears to be highly effective and preliminary reports indicate that there is a dramatic reduction in condemnations in the problem States in the USA, such as Delaware (Fig. 1), where it has been used (14).

2.3. Incubation period

2.3.1. Experimental. Susceptible chicks inoculated at 1 day of age generally do not show clinical signs until between the 3rd and 4th week (11). In some birds, clinical signs may not appear until they are several months old.

2.3.2. Field. Under field conditions, when the time of infection is unknown, clinical signs sometimes appear as early as 3 weeks.

However, most flocks show few signs until the 7th or 8th week which, in the USA, is just prior to slaughter of broiler chickens. In egg-type chickens, clinical signs are often first noted just prior to sexual maturity at 16 to 20 weeks of age. Particularly in recent years in vaccinated flocks, the disease has first become apparent at 24 to 30 weeks, though this is unusual.

A transient paralytic syndrome referred to as temporary paralysis (15,16) or transient paralysis (17,18) has been described. Clinical signs may appear in a very few or up to 50% of a flock at about 4 weeks of age. In individual birds the signs rarely last more than 2 to 3 days before the birds recover. However, recovered birds often succumb a few weeks later to clinical signs of MD.

3. THE DISEASE DURING PRODUCTION

3.1. Visible signs

The most common clinical sign, particularly in the classical form of MD, is some form of interference with nerve function. It usually takes the form of a progressive paresis, spastic or flaccid, eventually culminating in complete paralysis (Fig. 3). Almost any nerve in the body may be involved unilaterally or bilaterally. Most commonly, one or more extremities are involved. An uneven gait, lameness, favouring of one leg over the other, and paralysis of one leg, are most common. Drooping of one or both wings also occurs. Involvement of the motor nerves to the neck muscles may result in the head being held low and torticollis. Involvement of the eyelid muscles results in ptosis or closed eyelids. Involvement of the vagal nerve can result in paralysis and dilation of the crop leading to a pendulous crop and involvement of the intercostal nerves can result in gasping. A very common characteristic attitude in advanced disease is vertical or lateral recumbency with one leg stretched forward the other backward. This attitude may be a result of a spastic rather than a flaccid paralysis.

In an affected flock, birds appear unthrifty, have delayed feathering, have ruffled feathers, and are stunted.

The effects of tumour masses are sometimes seen. Occasionally uni- or bilateral exophthalmia is seen caused by a retrobulbar tumour. Sometimes tumours can be seen at the base of the comb and on the featherless

parts of the hock and shank resulting in roughening of the scale-covered skin. Occasionally lumps occur between the toes. On palpation, feather enlarged follicles or, occasionally, tumours may be felt on the feathered parts of the body.

FIGURE 3. Paralysis of the legs in Marek's disease.

Sometimes birds affected with particularly acute strains of virus or those becoming ill during the height of mortality are reluctant to move and die without showing any of the signs described above (19).

When tumour cells infiltrate the iris (an iridocyclitis), it may turn from reddish brown to gray, a condition often referred to as gray eye, ocular leukosis or the ocular form of MD. Such irises are usually unresponsive to light. Sometimes so much tumourous material invades the iris that the tumour obliterates the pupil.

3.2. Clinical biochemistry

3.2.1. General. The clinical chemistry has not been studied in detail, probably because changes are not specific or diagnostic for MD. Chickens may be dehydrated, resulting in an increased haematocrit or, less commonly, anaemic, resulting in a decreased haematocrit. In

advanced cases of disease, erythrocytes appear more fragile and haemolysis is often noted.

Birds with MD may have a very severe immunosuppression. The immunosuppression often exacerbates concurrent or secondary infections such as coccidiosis, septicaemia and air-sacculitis, so it is not unusual to have clinical signs of one of these diseases mask the signs of MD.

3.2.2. Specific effects on nerves. In chickens affected with MD, it is likely that nerve function tests would reveal dysfunctions before clinical signs appear or in the absence of clinical signs.

3.2.3. Nonspecific effects of tumours. Tumours can interfere with functions of almost any organ. Thus tumours of the liver would result in liver dysfunction and tumours of the gonad would interfere with the reproductive function. Tumours in the intestine can result in diarrhoea, ulceration and haemorrhage.

3.3. Sub-clinical effects

3.3.1. Broiler production. There is no doubt that in chickens with MD the growth rate and feed conversion are adversely affected. Until the advent of the vaccine against MD, it was impossible to measure the effect of the disease on production. However, assuming that the vaccine is completely effective in preventing the adverse effects of MD (which it is not), it is possible to estimate the subclinical effects of MD on production. These are therefore conservative estimates.

An average reduction in feed conversion by MD was estimated to be 0.5%. Thus Eidson et al. (20) estimated, on the basis of trials involving approximately 3 million chickens, that vaccination of broilers with one-half dose or less of vaccine increased body weight of vaccinated birds 0.1 pounds and that vaccination with a full dose increased body weight of vaccinated birds 0.2 pounds. In other experiments (M.A. Norcross, unpublished data) involving 4,000 birds, feed conversion in vaccinated birds was better than in control birds by between 0.7 and 1.5%. Other losses ascribable to MD include 1.6% condemnation from MD, 1.9% condemnation from other causes, 5.0% broiler mortality, and a 20% loss of breeders (21).

3.3.2. Egg production. Increase in egg production on a hen-day basis in different field trials was between 4 and 9.3% when the vaccine was used (22-24). The number of eggs produced per chicken in the USA

reached a plateau in the years from 1967 through 1970, but in 1971 the number was approximately 2% greater and from 1972 to 1973 it was 4% greater than the plateau level (21). In some studies (22), vaccinated birds required 0.12 pounds more feed per dozen eggs than unvaccinated birds; whereas, in others (23), unvaccinated birds required 0.4 pounds more feed per dozen eggs than vaccinated birds. In none of the trials was there any significant effect on egg quality. Although there may be some adverse effects of MD on feed conversion, they cannot be considered significant. Thus, a decrease in feed efficiency is not concurrent with the decrease in egg production from MD.

4. THE DISEASE AT SLAUGHTER

4.1. Criteria for condemnation from MD.

4.1.1. Clinical criteria. In the USA and many other countries, ante-mortem inspection of poultry on the day of slaughter is required. The inspection may occur in a broiler house crowded with chickens or in crates on the back of a truck. During inspection under these conditions it is unlikely that the early signs of MD would be detected. For example, ruffled feathers, lameness, or droopiness of a few chickens would not be recognized. However, the obvious signs of more advanced disease would be easily recognized. For example, paralysis, blindness or visible tumours on the nonfeathered part of the body can be readily seen whether or not these signs are caused by MD. However, it is most likely that chickens exhibiting these signs would be condemned as unfit for human consumption. The clinical signs of MD are so varied and non-specific that it is almost impossible to make a definitive diagnosis based on clinical signs alone. Nevertheless, birds exhibiting such clinical signs would be considered unhealthy and therefore unwholesome.

4.1.2. Postmortem criteria. Postmortem inspection of slaughtered poultry usually begins after stunning, bleeding and plucking. It usually occurs after evisceration but before the viscera are separated from the carcass.

Birds with extensive tumours pose no problems of disposition. Even though there is no evidence of harmful effects of eating tumour tissue from birds, whether cooked or not, the tumour tissue is not considered aesthetically wholesome. Thus birds with extensive tumours are not con-

sidered suitable for human consumption. However, occasionally in rural settings in developed countries, and often in developing countries, slaughtered birds with extensive tumours are eaten. There are no known harmful effects of such a practice.

In developed countries and where poultry are reared intensively there are two possible dispositions of chickens with just one organ such as the liver, gonad, muscle or skin, affected with MD tumour.

One action is to condemn the whole carcass as unfit for human consumption when any evidence of a tumourous condition due to MD is present. The rationale is that MD is a malignant and systemic condition and therefore present in all parts of the body. Aesthetically this elimination of all chickens with MD from human consumption is pleasing to the lay consumer and reassuring that the poultry being eaten do not contain tumour. However, significantly more chicken meat is condemned than if the tumourous portion of the organ is removed and the remainder passed for human consumption. A single lesion in a carcass may be an indicator of widespread systemic disease. Thus chickens with visible tumours in one portion of the body may have microscopic tumours elsewhere. Frankly neoplastic lymphocytes may be present in the circulation or lymphoid tissues. However, because a large percentage of birds at slaughter are infected with virus and show no signs, virus infected chickens would be passed for human consumption. There is no known harmful effect on humans of eating virus-infected chickens although some concerns have been expressed. There may be a difference between a carcass containing a virus and a carcass containing a tumour because some factor, in addition to the virus, has to be present for the tumour to occur. However, no such factors which could be harmful to the consumer have been identified.

An alternate action is to remove only the organ or part of the organ which contains the visible tumour. Aesthetically this action is less pleasing to the lay consumer than the condemnation of the whole carcass because of the known ability of "cancer" (and MD is a cancer) to spread to other parts of the body (metastasize). Therefore tumours could occur in edible parts in which no overt tumours are seen. Scientifically this disposition of tumourous tissue is more defensible than the condemnation of the whole carcass because most of the chickens slaughtered, whether

they have tumours or not, are infected with virus and neither the virus nor the incipient tumours are harmful to the humans that consume them. Much less of the poultry processed is condemned and thus more is available for human consumption.

Very small tumours that are only just visible with the unaided eye pose an additional problem in meat inspection. The correct identification of such lesions is particularly important where the whole carcass must be condemned if the lesion is shown to be MD. For example, in the USA, one enlarged feather follicle with the characteristic whitish translucent appearance of lymphoid infiltration of MD will result in condemnation of the whole carcass. Histological examination of such follicles has confirmed the ability of experienced meat inspectors to recognize the early lesions of MD. Gaining experience in recognition of these small MD lesions and development of uniform regulations for disposition of affected chickens and implementation of such regulations was at least partially responsible for the increase in the rate of condemnation from MD in the USA between the time that meat inspection was first instituted in 1961 and the peak of MD losses in 1967 to 1970 (Table 3).

4.1.3. Criteria for distinguishing MD from other conditions. At ante-mortem inspection, postmortem inspection, examination in diagnostic laboratories and pathologic examination, it is important to distinguish between MD and other diseases which affect the same organ systems as MD.

Clinical signs of idiopathic polyneuritis (25) are similar to those of MD. Clinical signs of several diseases of the central nervous system can be readily confused with MD. Examples are virus diseases such as Newcastle disease, avian encephalomyelitis, equine encephalitides; bacterial diseases such as botulism and fowl cholera; fungal diseases such as aspergillosis; and nutritional diseases such as encephalomalacia. Similarly, diseases of the musculoskeletal system can be confused with MD, such as infectious stunting, infectious synovitis, perosis, rickets, bumblefoot, osteopetrosis, arthritis of various causes and injuries. Blindness from physical injury and opacity of the cornea from infection, such as infectious coryza, should not be confused with ocular lesions of MD.

Other tumours and localized inflammatory conditions may be confused with MD tumours. Examples are tumours such as lymphoid leukosis and

sarcomas; and infections such as Hjarre's disease or colibacillosis, tuberculosis and salmonellosis. Diseases of the skin which can be confused with MD include tumours of the skin, fowl pox, scaly leg mites, and injuries.

4.2. Inspection requirements in different countries

4.2.1. Nature of inspection. In the USA, Canada and UK regulations for red meat inspection preceded those for poultry inspection. However, most developed countries now require that all poultry in commercial channels in the Nation must be inspected for wholesomeness (Table 6). The USA regulations follow (26): "A postmortem inspection shall be made on a bird-by-bird basis on all poultry eviscerated in an official establishment. No viscera or any part thereof shall be removed from any poultry processed in any official establishment, except at the time of post mortem inspection unless their identity with the rest of the carcass is maintained in a manner satisfactory to the inspector until such inspection is made. Each carcass to be eviscerated shall be opened so as to expose the organs and the body cavity for proper examination by the inspector and shall be prepared immediately after inspection as "ready-to-cook poultry"."

Many countries have laws which require inspection but for economic reasons they are not enforced. The degree of enforcement is impossible to assess from the written regulations. Some indications of the extent of the regulations were obtained from a questionnaire sent to corresponding secretaries of the World Veterinary Poultry Association (Table 6). All countries responding except Australia, Israel, and Switzerland had poultry inspection and in all countries, except Australia and Switzerland, the inspection was under veterinary supervision. It is interesting to note that whether or not birds were inspected had no relationship to the amount of poultry produced per capita or per country.

In the USA the examination of individual chickens has proved expensive. During the last several years, in order to adapt to a growing and changing industry, the efficiency and effectivness of poultry inspection have been significantly increased. The Food Safety and Inspection Service of the U.S. Department of Agriculture, the agency responsible for poultry inspection, has designed, tested and is now evaluating a new

TABLE 6. Meat inspection requirements in selected countries.

	United States	Japan	France	Italy	United Kingdom	Canada	Netherlands	Australia	South Africa	Israel	Germany DR	Denmark	Sweden	Switzerland	Norway
Chicken meat producted (metric tons per year X 10^{-3})	5424	978	833	775	627	399	355	275	221	154	147	90	40	22	10
Chicken meat produced (kilograms per capita per year)	24.5	8.4	15.5	13.6	11.2	16.7	25.2	19.1	7.7	40.1	8.8	17.7	4.9	3.4	2.5
Are all poultry inspected for wholesomeness in processing plants?[a]	Y[b]	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y	N	Y
Is inspection under veterinary supervision?	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	N	Y
Is the presence of leukosis or Marek's disease specifically identified as a reason for condemnation?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y
Are entire birds with localized signs of Marek's disease (e.g., tumor of gonad) condemned?	Y	N	Y	Y	Y	Y	N	N	N	N	Y	Y	Y	N	Y
Are only affected parts from birds with Marek's disease tumors removed and condemned?	N[b]	Y	N	N	N	N	Y	Y	Y	N	N	N	N	Y	N

[a]These questions refer only to meat-type birds slaughtered for human consumption. They refer to current practices within the country not to poultry for export. Regulations that are not enforced should not be considered.

[b]Y = Yes, N = No

inspection procedure for broilers which will greatly increase production line speeds and also permit improved productivity for the agency. The procedure places the responsibility for trimming carcasses on the plant, without direct Federal supervision. A federal inspector verifies that the trimming was properly peformed.

The agency is exploring the possibility of flock testing as a time-saving supplement to poultry inspection. It is studying the feasibility of predicting the level of disease conditions likely to occur at slaughter based on characteristics of the flock and its environment. Such an approach might enable the agency to adjust the intensity of post mortem inspection to the needs of a particular flock of birds (27).

4.2.2. Regulations concerning disposition of carcasses affected with MD. All countries with regulations requiring inspection of poultry identify MD (often by different names) as a reason for condemnation (Table 4). In Switzerland the meat inspection instructions indicate "attention must be given to leukosis".

Examples of the regulations in countries in which the whole bird must be condemned if a diagnosis of MD is made are the USA, UK and Israel. The USA regulation reads: "Carcasses of poultry affected with any one or more of the several forms of the avian leukosis complex shall be condemned." The UK regulation reads "indication of unfitness for human consumption:.... (o) malignant or multiple tumours, (p) avian leukosis complex, (q) Marek's disease" (28). The regulation for Israel reads "where in any fowl there is revealed.... (b) leukosis of all forms--condemnation shall be complete" (29). Regulations in Israel apply only to poultry products for export. An example of a regulation where only the affected parts of the carcass need to be condemned is that of South Africa which reads: "Total or partial condemnation shall take place if poultry upon inspection is found to be suffering from leukosis, Marek's disease, neoplasms....the entire bird shall be condemned if--(a) the disease is accompanied by emaciation, and/or dehydration; (b) advanced pathological changes are observed; or (c) the condition has so spread that affected portions or organs cannot easily be separated (30)."

5. ECONOMIC LOSS FROM DISEASE

5.1. Vaccination.

5.1.1. Proportion vaccinated. The herpesvirus of turkeys (HVT) is the most widely used vaccine. Recently a bivalent vaccine combining HVT and apathogenic MD has been licensed for use in the USA. All chicks are vaccinated in the hatchery at 1 day of age. When the HVT vaccine first became available in the early 1970's, manual vaccination procedures were used and only 500 chicks could be vaccinated per hour. Quality control of the vaccination procedure was poor and some chicks probably failed to get vaccinated. Chicks still have to be handled manually but the injection is done automatically and chicks can be vaccinated at over 5,000 per hour. Quality control of the administration has improved greatly. For example, many companies add a blue dye to the vaccine and then carefully examine a portion of the vaccinated chicks to ensure that the vaccine is delivered parenterally. Very few, if any, chicks are missed.

Sometimes antibiotics or other vaccines are combined with the MD vaccine for administration. Great care should be taken to ensure that materials added to the vaccine do not interfere with the potency of the vaccine. Potency control of the vaccine and quality control of the vaccination procedure are extremely important. All vaccine used in the USA is cell-associated and is stored in liquid nitrogen. Some vaccine used in other countries is lyophilized.

5.1.2. Cost of vaccine. When the vaccine first became available it often cost between 35c and 50c per dose and the application cost over 5c per chick. With the advent of mass application procedures the cost of the vaccine plus the cost of administration is often less than 1c per chick. To reduce cost, vaccine for some broiler chicks is diluted. Batches of vaccine are often carefully titrated by the purchaser and then a dilution is selected that will allow a minimum of 1,000 plaque-forming units per chick. Because USA regulations forbid the sale of extra diluent with the vaccine, the purchaser must prepare his own diluent.

5.2. Initial benefit from vaccination

5.2.1. Reduction of disease losses. Previous studies have identified the components of loss from MD that were reduced by vaccination

(21). Thus in the year 1974 benefits from the reduction of broiler condemnation in the USA were $27.3 million, from other condemnations $6.3 million, from broiler mortality $5.6 million, from feed utilization $3.1 million, from broiler breeder mortality $5.0 million, from egg-type chicken mortality $15.5 million, from condemnation of egg-type chickens $0.1 million and from increased egg production $105.5 million. The total benefits from reduced losses from MD were $168.5 million. Benefits continue to this day at these or higher levels.

5.2.2. Cost of research. The total estimated budget for research on MD for the Agricultural Research Service (ARS), ARS extramural support, Cooperative State Research Service, and other Federal agencies in the USA, e.g., the National Institutes of Health, and State agencies, is $17.1 million (21). Excluded from these figures are the cost of construction of facilities and the support of research prior to 1964. The sum of the cost of research from 1965 to 1974 was $17.1 million and the cost compounded at 10% per annum to 1974 was $24.7 million.

5.2.3. Economic return from investment. The returns from research are estimated at $460.0 million from 1965 to 1974 or $2.30 per person in the USA. Annual returns are $168.5 million or about 84c per person in the USA. The flow of return (benefit minus research costs) extends from 1971 into perpetuity.

Because benefits had reached a maximum by 1974, it was assumed that after 1974 research only maintained the level of productivity and did not increase it further. This assumption is rather conservative and should result in a conservative estimate of the rate of return. The external rate of return, if we use a 10% discount rate, is 442.8%. This can be interpreted to mean that the average dollar invested in research on MD (public and private) in the USA has returned 10% annually to society from the date of investment to 1974 and is now paying off at a rate of 442.8% per year. The benefit-cost ratio calculated from this is 44.3, which means that the average dollar spent on MD research will return $44 in economic benefits.

When annual saving to broiler production are divided by the weight of broilers produced, the benefits from MD research amount to 0.56c per pound. Similarly, the MD vaccine reduced the cost of producing eggs by 2.22c per dozen.

5.3. Current economic loss from MD.

5.3.1. Cost of vaccine and application. If it is estimated that it costs approximately 1c for the vaccine and its application and that all broilers and layers in the USA are vaccinated, then the cost of vaccinating 4,150 million broilers is $41.5 million and of vaccinating 287 million layers is $2.9 million. Thus the total cost of vaccination is $44.4 million.

5.3.2. Mortality. A mortality of 0.1% (Table 5) which generally occurs just before slaughter would decrease the total income from broilers from $4,703 million (31) by $4.7 million. Similarly a mortality of 3% of egg-laying chickens would decrease the gross income of $123 million (31) by $3.7 million. Thus total losses from mortality from MD are $8.4 million.

5.3.3. Condemnations. Losses from broiler condemnations are 0.08% (Table 3). This would reduce the gross income from $4,703 million by $3.8 million.

5.3.4. Loss of egg production. If one assumes a loss of egg production of 3% due to egg-type chicken mortality just before or just after sexual maturity and to decreased production by affected chickens, then income of $3,457 million from egg production is reduced by $103.7 million.

5.3.5. Total cost of MD. In the USA the total cost of MD is approximately $160.2 million. In broilers this represents the cost of vaccinations and the loss from mortality and condemnation of $50 million. In layers this represents a cost of vaccination and loss from mortality and loss of egg production of $110.3 million.

If one assumes that the cost of vaccination and loss from MD is constant per metric ton of eggs for laying chickens and per metric ton of meat for broilers throughout the world, then one can extrapolate the cost of MD worldwide. Using these assumptions the cost of MD in broilers is $217 million and in layers $726 million. Thus total world cost of MD is $943 million. This is probably a very conservative estimate.

6. CONCLUSIONS

In spite of the development of a very effective vaccine against MD,

MDV infection is still ubiquitous. Losses from MD, though much smaller than they were before the vaccine was developed, still occur worldwide. Losses from MD are likely to be higher in developing countries where vaccines are not as readily available as in developed countries.

In certain parts of the USA and possibly other parts of the world, there appears to be a resurgence in losses from MD. The development of new, more effective vaccines such as bivalent and trivalent vaccines, and of new methods of application, promises to control these increased losses. There is little or no possibility of ever eradicating MD from a country. It is hoped that continued research will keep pace with the appearance of new, more virulent strains of MD and thus keep losses from MD worldwide to minimum.

ACKNOWLEDGEMENTS

The author wishes to thank and acknowledge the help of Dr. R.L. Witter, in providing summarized data on poultry condemnations for Table 3 and the figures; Marge Stanton for performing the statistical manipulation of FAO data; and corresponding secretaries of the World Veterinary Poultry Association from different countries and colleagues in industry in the USA for completing the questionnaire.

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3. PATHOLOGY

L.N. PAYNE

1. INTRODUCTION

Observations made more than 50 years ago on naturally occurring cases provided the foundations of knowledge of the main gross and microscopic features of Marek's disease (MD): these include enlargement of peripheral nerves as a result of infiltration by inflammatory or neoplastic lymphoid cells, and lymphoma formation in various sites (1,2). For many years there was much speculation on the nature and interrelationship of these lesions, but significant progress in understanding the pathology and pathogenesis of the disease was made only after experimental transmission became possible and the viral agent of the disease was isolated, during the 1960's. Since then the main pathological events that occur during the development of MD have been elucidated (3), usually from studies on young genetically-susceptible chickens infected experimentally with pathogenic strains of MD virus (MDV). These events are described in this Chapter.

2. GROSS APPEARANCES

The gross changes commonly present in classical and acute MD are enlargement of peripheral nerves, lymphoma formation in various organs and tissues, and atrophy of the bursa of Fabricius and thymus. Ocular changes are sometimes observed. In the uncommon syndrome termed transient paralysis that may follow infection with MDV, no macroscopic lesions occur.

2.1. Peripheral nerves

Enlarged peripheral nerves are present in almost all chickens with clinical signs of classical MD and in many chickens with the acute form of the disease (Fig. 1). Affected nerves are usually two to three times

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FIGURE 1. Enlarged brachial and sciatic nerves, and an ovarian lymphoma (arrow), in a 5-week-old chicken with acute MD.

their normal thickness, and occasionally much more. The enlargement may affect a nerve uniformly along its length or it may be more limited or nodular. Enlarged nerves lose their normal glistening cross-striations and have a grey or yellow discolouration; they may be oedematous and have a rubbery consistency. Dorsal root ganglia may be enlarged. The distribution of affected nerves varies from case to case. The nerves of the lumbosacral and brachial plexuses, and the sciatic and brachial nerves, are often enlarged, and a high incidence of involvement of autonomic nerves, notably the coeliac plexus, is observed. In a survey of 502 macroscopically affected natural cases of neural MD, Goodchild (4) detected 99% by examination of the following groups of nerves: coeliac plexus, cranial mesenteric plexus, nerve of Remak, greater splanchnic nerves, brachial plexuses, and middle lumbo-sacral plexuses. Examination of the coeliac plexus alone detected 78% of cases.

2.2. Lymphoma formation

Lymphomas occur in one or several organs or tissues in a minority of cases of classical MD. In a survey of 131 field cases, Fujimoto et al. (5) recorded a diminishing frequency of gross tumourous involvement in the following sites: proventriculus, lungs, serosa, liver, ovary, kidneys, thymus, spleen, skeletal muscles, intestines, heart, pancreas and adrenals.

In acute MD, multiple lymphomatous involvement of various tissues is usual (Fig. 1). These authors (5) observed a diminishing frequency of gross lymphomas in the following locations in 50 field cases of acute MD: ovary, liver, spleen, proventriculus, lungs, adrenals, kidneys, intestines, thymus, serosa, skeletal muscles and heart.

Other sites not mentioned by these authors, but in which lymphomas occur in acute MD, are the skin, usually around feather follicles ("skin leukosis"), and the bursa of Fabricius. Lymphomatous enlargement of the liver and spleen seems to be more common in adult than in juvenile birds.

Experimental transmission studies have revealed lymphoma occurrence and tissue distribution similar to those in field cases, but with minor variations depending on strain of virus and chicken.

2.3. Other changes

In classical MD, muscular wasting is not uncommon. Gross atherosclerotic lesions in the coronary arteries, aorta and aortic branches have been associated with experimental MD (6), and this infection may be involved in the atherosclerotic lesions which can be observed naturally (7). An iridocyclitis may occur in field cases, sometimes as the sole gross lesions, and is characterised by depigmentation of the iris ("grey eye"), loss of accommodation in response to light, and pupillary distortion (2). In acute MD, atrophy of the bursa and thymus often occurs. A scabby, exudative dermatitis may occur in experimental cases.

3. HISTOPATHOLOGICAL AND ULTRASTRUCTURAL APPEARANCES

An overall view of the pathogenesis of MD, as exemplified by the disease induced in young maternal-antibody-free susceptible chicks infected with pathogenic MDV, is provided in Fig. 2. Understanding of the changes that occur is aided by recognition of the different types of virus-cell interactions that pertain in MD (see also Chapter 4). These are characteristic for many members of the family Herpesviridae, and are as follows: 1) Fully productive infection, characterized by the production of fully infectious virions, accompanied by cell death, and exemplified in MD solely by the infection of feather follicle epithelium cells (see Section 3.2). 2) Semi-productive infection (restrictive or abortive infection), in which viral antigens and naked nuclear virions are formed but in which infectivity remains cell-associated. This type of infection is exemplifed by the infection which occurs in lymphoid and parenchymatous tissues, and leads to cell death (see Sections 3.1 and 3.9). 3) Non-productive neoplastic infection, in which viral genome persists in lymphoid cells, with limited antigenic expression but including the appearance of the MD tumour-associated surface antigen (MATSA) (see Section 3.6. and Chapter 4) resulting in immortalization of cells. Neoplastic lymphoma cells (Section 3.6), transplantable lymphomas (Section 5), and lymphoma-derived lymphoid cell lines (Section 4) typify this type of non-productive infection. 4) Non-productive latent infection, in which viral genome persists in lymphoid cells which express no viral or tumour antigens but from which virus can be rescued by inoculation into chickens or onto cultured cells. This type of

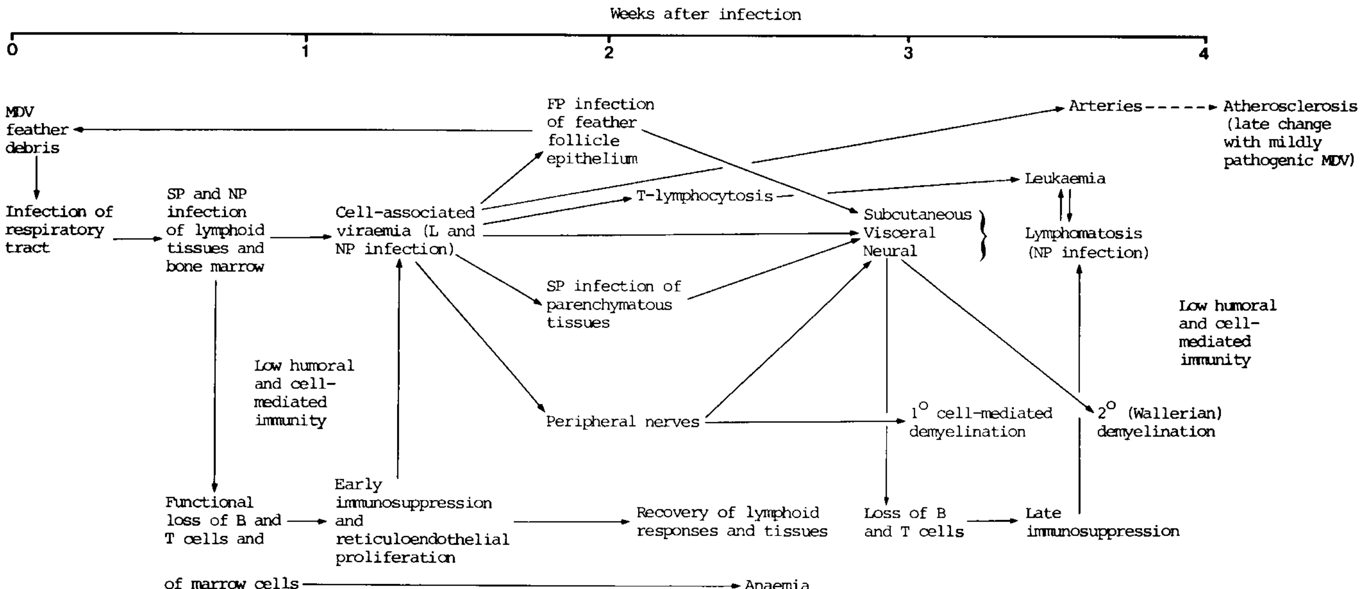

FIGURE 2. Representation of pathogenesis of MD induced in maternal-antibody-free susceptible chicks infected with pathogenic MDV. Types of infection: FP, fully productive; SP, semiproductive; L, latent; NP, non-productive (From Payne (90) by kind permission.)

infection is observed in some blood and spleen lymphocytes and lymphoma cells in MD.

3.1. Lymphoid tissues

Primary infection by MDV, which occurs naturally usually via the respiratory tract, is followed within 3 days by a severe acute cytolytic infection of lymphoid tissue in the bursa of Fabricius, thymus, spleen and elsewhere (8-10) (Fig. 3). The infection occurs in reticulum cells, macrophages and lymphocytes, is of the semi-productive type, and is characterized by the production of abundant viral antigens, as detected by fluorescent antibody techniques or agar gel precipitin reactions, immature intranuclear virions, and cell-associated infectivity. Intranuclear inclusion bodies can be seen in infected cells (Figs. 3 & 4). It has been observed that this early lytic infection of lymphoid organs can be eliminated by bursectomy in ovo (11); this finding suggested that B-cells are the initial target cells for cytolytic MDV infection and this has been confirmed by their direct identification in in vivo infections, even in the thymus, and in in vitro infections (12,13). Neonatal splenectomy delays the acute infection of bursa and thymus, suggesting that cells in the spleen are important in the early replication and dissemination of virus (14).

The acute lymphoid infection reaches a peak at 5 to 7 days after infection: the abundant viral replication is accompanied by cytolysis of lymphocytes and other cells, a hyperplasia of reticulum cells, accumulations of macrophages and infiltration by granulocytes. Cells bearing the tumour-associated antigen MATSA are also present, first appearing in the spleen at 5 days and in smaller numbers in the bursa and thymus at 7 days (15). In the thymus, the cytolytic infection and hyperplasia of reticulum cells occur mainly in the medulla, and the cortex is markedly regressed (Fig. 5). Similar changes occur in the bursa, with destruction of the normal follicular architecture (Fig. 6). These changes result in marked loss of weight of the thymus and bursa. The cytolytic infection and reticulum cell hyperplasia also occur in the spleen, but lymphoid regression is not a factor in this organ, and the response results in a weight increase.

These inflammatory changes have been classified as an acute lymphoreticulitis (3) and termed the stage of reticuloendothelial

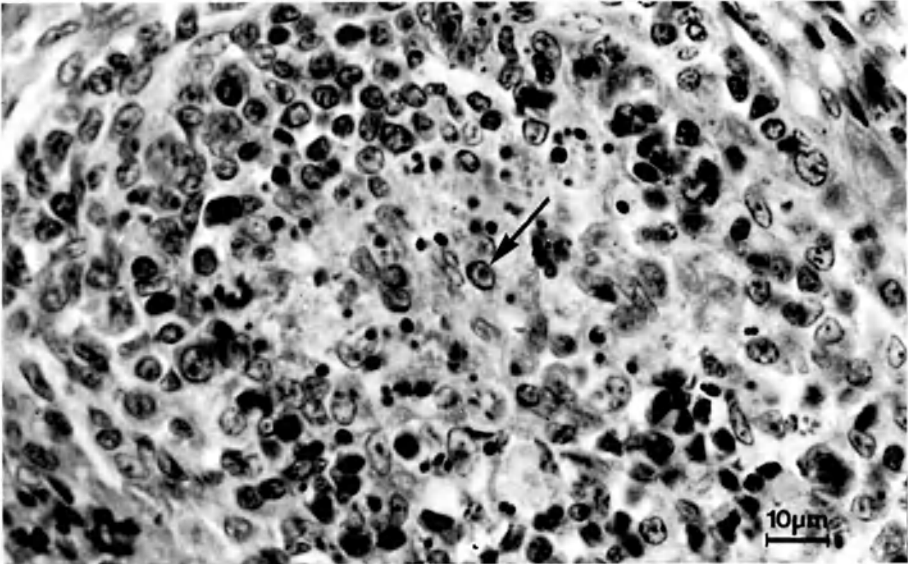

FIGURE 3. Acute cytolytic infection by MDV in lymphoid follicle in bursa of Fabricius, 1 week after infection of 1-day-old chick. An intranuclear inclusion body (arrow) is present.

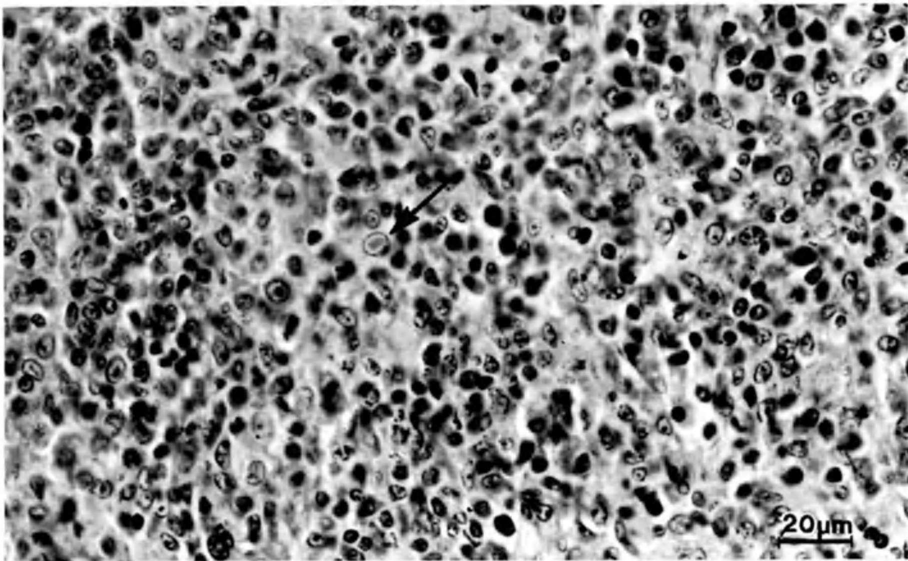

FIGURE 4. Intranuclear inclusion body (arrow) in a cell in the thymic medulla; same case as Figure 3.

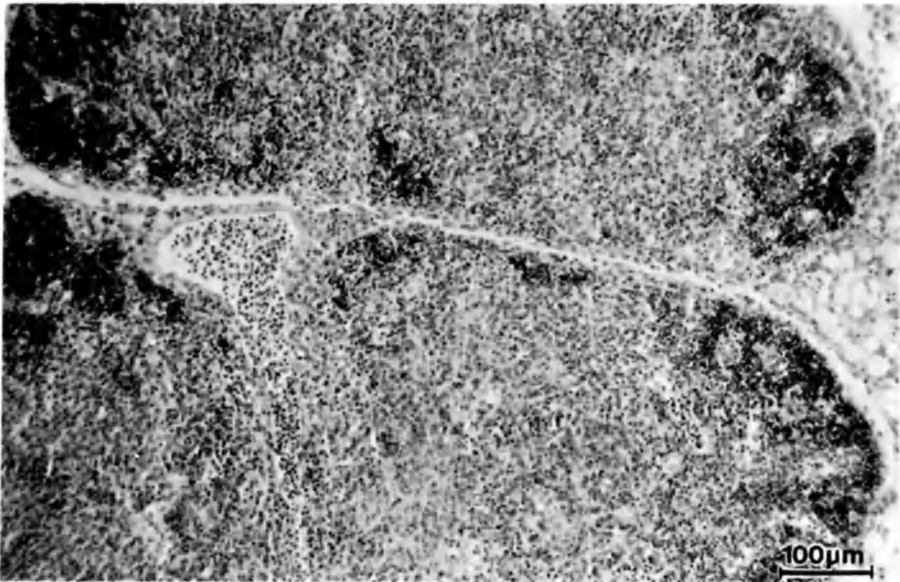

FIGURE 5. Regression of cortex of thymus; same case as Figure 3.

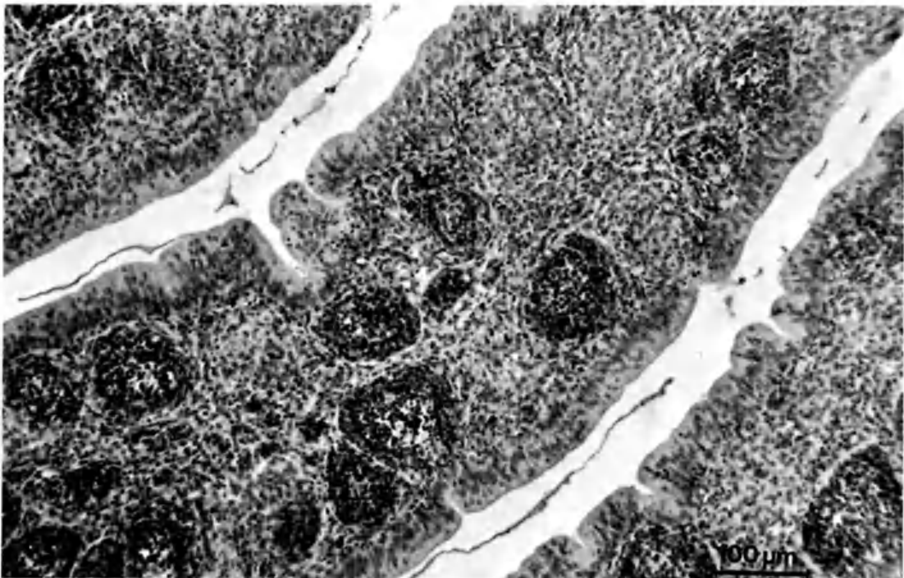

FIGURE 6. Regression of lymphoid follicles in bursa of Fabricius; same case as Figure 3.

proliferation (16). They resolve after 7 days and by 14 days the normal architecture of the bursa, thymus and spleen is largely restored. The areas of reticulum cell hyperplasia in the spleen become replaced by areas of lymphoid hyperplasia. The lymphoid atrophy which occurs during the first week may be at least partly responsible for the transient immunosuppression which is seen at this time (see Chapter 7).

A persistent cell-associated viraemia can be detected in the blood from about 5 days after infection, apparently in lymphocytes and as a latent non-productive infection, reaching an initial peak at about 8 days after infection and a second peak at about 4 weeks (8,17). This viraemia serves to distribute MDV throughout the body.

After the recovery phase, a second wave of semi-productive virus infection, cytolysis and lymphoid atrophy can be observed in the bursa, associated with the development of the lymphomatous stage of the disease.

Very highly oncogenic (variant) strains of MDV have recently been isolated (e.g. Md/5 and Md/11) that induce severe early bursa and thymus atrophy, with lymphoid cell necrosis, and early death in the absence of lymphomas or nerve enlargement (18).

3.2. Feather follicles

The feather follicle epithelium provides the single location in the chicken in which a fully-productive infection by MDV occurs, with release in the feather debris of cell-free infectious virus (19). The associated pathological lesion has two main components: 1) infection and degeneration of epithelial cells of the feather follicle, and 2) accumulations of lymphoproliferative tissue around blood vessels in the dermis and around the infected follicles (20,21).

Viral antigen may be detected in feather follicle epithelium as early as 5 days after infection (22), but the majority of birds do not show follicular antigen until about 14 days post-infection, at which time intranuclear inclusion bodies appear (8,9,21,22). Subsequently viral antigen, inclusion bodies and infectious virus can be detected in feather follicle epithelium for many weeks. Cytopathic changes occur in cells of the transitional and corneous layers of the epithelium and include cloudy swelling and hydropic changes, margination of chromatin and the development of intranuclear and intracytoplasmic inclusion

bodies (Fig. 7). Naked and occasionally enveloped virus particles are present in the nucleus, and cytoplasmic inclusion bodies containing enveloped virions develop.

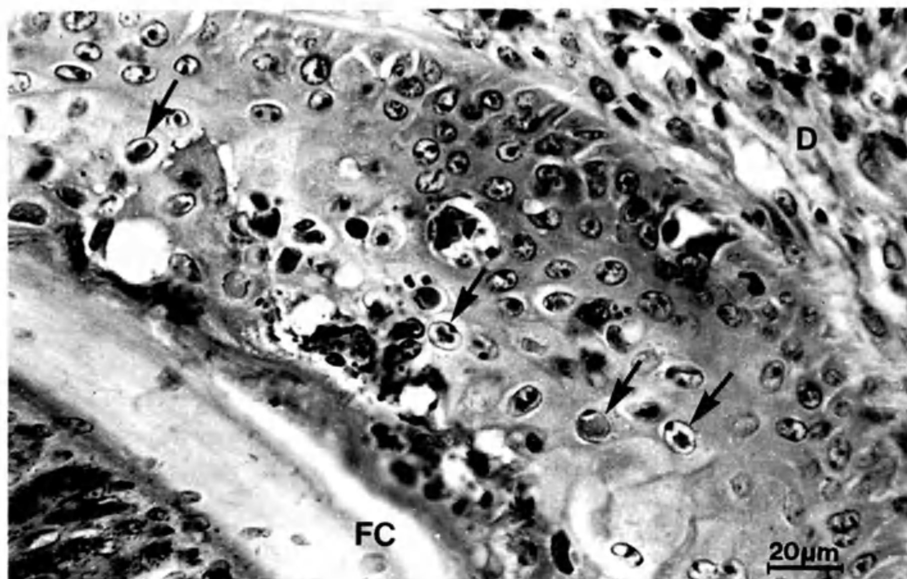

FIGURE 7. Intranuclear inclusion bodies (arrows) in cells of the transitional layer of feather follicle epithelium, 4 weeks after infection of 1-day-old chick with MDV. Also present are the dermis (D), follicular cavity (FC), and feather (F).

The earliest changes in dermal lymphoid tissue are at 5 to 7 days post-infection, when perivascular lymphoid accumulations occur. These increase in size and coallesce and may infiltrate dermal adipose tissue, pilomotor smooth muscle, and subcutaneous nerves, and give rise in some birds to lymphomatous tumours ("skin leukosis"). There is a correlation between the presence of lymphoid proliferation and viral antigen in the feather follicles , but the sequential relationship is still uncertain. It is possible that the earliest perivascular lymphoid accumulations convey the virus to the dermal area, since viral antigen can be seen by 5 days post-infection in the lymphoid cell aggregates (9), with

subsequent infection of the feather follicle epithelium. The viral antigen in the epithelium may then stimulate the further lymphoid accumulations that occur around the feather follicles. Occasionally lymphoid cells may infiltrate the feather follicle epithelium, the epidermis of the skin, and the pulp of the feather.

3.3. Haematology

The bone marrow becomes infected by MDV by 5 days after infection (8), and this may lead to a depression in red blood cell count (23). In chicks lacking maternal antibody to MDV, infection leads to marrow aplasia and a marked anaemia (24). Quantitative studies on the bone marrow in MD have not been made.

In a sequential study of the leukocytic response in the blood in MD, Payne and Rennie (25) observed an increase in absolute numbers of B-cells, T-cells, total lymphocytes, and heterophils, and a decrease in numbers of monocytes and eosinophils, during the early stage of acute infection of lymphoid tissues. During the later lymphoproliferative phase, T-cells and total lymphocytes increased, resulting in a leukaemia in some birds, and B-cells, monocytes, heterophils and basophils decreased. In leukaemic birds, most of the increase in lymphocytes was accounted for by a T-cell increase, and blast cells and atypical lymphoid cells were also increased.

3.4. Peripheral nerves

Two main pathological processes occur in the peripheral nerves in MD: 1) a neoplastic lymphoproliferation, similar to that which gives rise to lymphomas in other tissues, and 2) a primary, segmental, cell-mediated demyelination. Both changes usually occur, within the same bird and even in the same nerve, when genetically susceptible birds are infected with virulent MDV. In infections of relatively resistant birds with virulent virus, or infections of susceptible birds with low virulence virus, the demyelinative form often occurs alone.

A variety of types of nerve lesions, as seen under the light microscope, have been described (5,26,27), and have given rise to several classifications (Table 1). It seems likely, although not yet certain, that these different types of lesion are explicable in terms of the lymphoproliferative and demyelinative processes mentioned above, with variation dependent on the occurrence, severity and stage of the two

processes. Payne and Biggs (27) provided a simple classification of nerve lesions based on nerves collected from two strains of chickens in MD transmission experiments. Three lesion types were described: 1) A-type lesion, characterized by a lymphoproliferative infiltration and demyelination (Fig. 8). 2) B-type lesion, characterized by inter-neuritic oedema, a lighter, inflammatory infiltration by mainly small lymphocytes and plasma cells, and demyelination (Fig. 9). 3) C-type lesion, characterized by a light infiltration by small lymphocytes and plasma cells. This type is considered to be a mild inflammatory lesion. This classification has been used by us and other workers to classify neural lesions induced by other virus strains in various strains of fowl, and also lesions in naturally occurring cases, and has been found adequate for classification of at least the greater majority of cases. Nevertheless, further investigations of the variety and nature of neural lesions in MD would be of value.

TABLE 1. Classifications of Marek's disease neuropathy. (From Payne *et al*. (3) by kind permission.)

Predominant lesion	Classification of		
	Wight (26)	Payne and Biggs (27)	Fujimoto *et al*. (57)
Infiltration by small lymphocytes	Type I	-	T_I-type
Infiltration by mixed lymphocytes	Type I	A-type	T_{II}-type
Infiltration by lymphocytes	Type III	-	-
Infiltration by reticular or undifferentiated mesenchymal cells	-	-	T_{III}-type
Sparse infiltration by small lymphocytes and plasma cells	-	C-type	-
Interneuritic oedema, with infiltration by small lymphocytes and plasma cells	Type II	B-type	R-type

Following infection the earliest changes seen in the nerves are, under the electron microscope, an interneuritic infiltration by mainly macrophages and some lymphocytes at 5 days, and under the light micro-

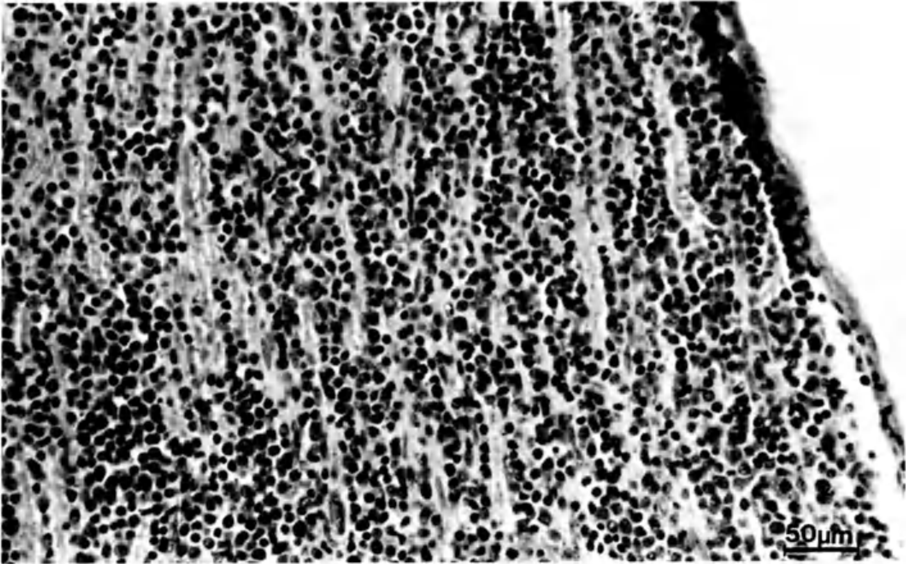

FIGURE 8. A-type nerve lesion, 5 weeks after infection of 1-day-old chick with virulent MDV. Note severe lymphoproliferative infiltration.

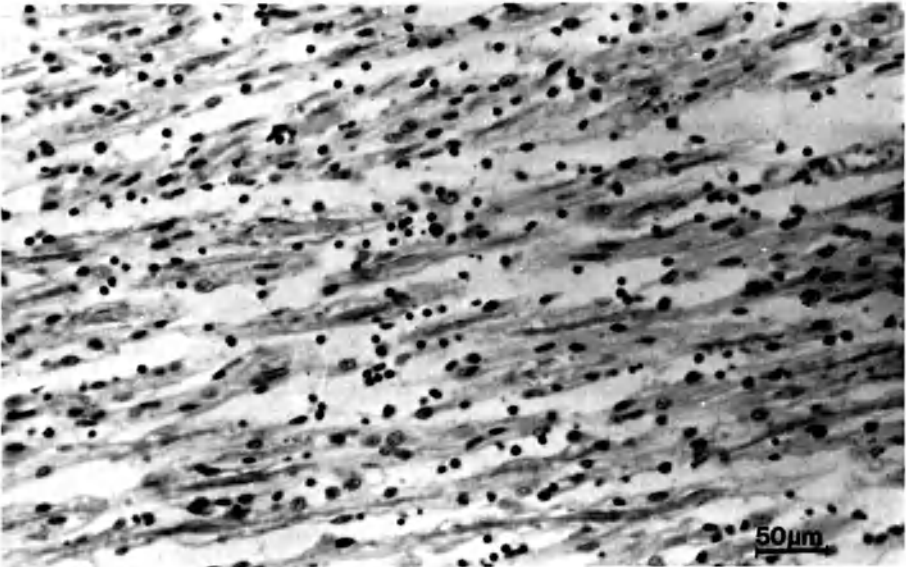

FIGURE 9. B-type nerve lesion, 10 weeks after infection of 1-day-old chick with virulent MDV. Note interneuritic oedema and sparse cell infiltration.

scope, infiltration by mainly lymphocytes at about 10 days (9,28). An accumulation and proliferation of lymphoid cells and macrophages proceeds during the 2nd and 3rd weeks after infection, to give rise to the marked lymphoproliferative infiltration which characterizes the A- type lesion, comprised of a mixed population of small, medium and large (blastic) lymphocytes, activated reticulum cells and macrophages. Sometimes so-called "Marek's disease (MD) cells" are present amongst the other cell types. These have been identified as degenerating lymphoblastoid cells in which immature herpesvirions have been observed in the nuclei, and more rarely coated herpesvirions in the nuclear membrane (29). Virus particles, usually intranuclear, are rare in other lymphoid cells in the proliferative lesions. They are not observed at any stage of the neuropathic process in other infiltrating cells, nor in Schwann cells or neural fibroblasts. Although herpesvirions are rarely seen in the neural lesion, in situ molecular hybridization studies have revealed that the majority of infiltrating lymphoid cells in A-type nerves contain viral genome (30). It is evident that the infection of lymphoid cells is of the non-productive type, either neoplastic or latent.

About 75% of lymphocytes from A-type nerves are T-cells and most of the others B-cells (31,32). About 9% carry the membrane antigen MATSA (P.C. Powell and M. Rennie, unpublished results), which may be a marker for neoplastically-transformed cells. Ultrastructurally the lymphoid cells show no features which delineate them qualitatively from normal lymphoid cells. Depending on their differentiation, they have the structure of small, medium or large (blastic) lymphocytes (33,34). Nuclear projections are not uncommon in the more immature lymphoid cells, but they are also occasionally observed in normal lymphoid tissue. The demyelination of peripheral nerves in MD has been studied with the light microscope (26,35,36) and electron microscope (28,33,37-39). There has been disagreement about the relationship between the demyelination and the lymphoid proliferation. Some investigators have suggested that demyelination precedes proliferation, resulting either from primary Schwann cell damage leading to demyelination and a cellular response (33) or from a primary autoimmune cell-mediated demyelination (37). Infection of Schwann cells by MDV has been considered as a possible initiating cause of the demyelination, but evidence for this is

equivocal. Virions, viral antigens, and viral genome have rarely been detected in these cells while in the nerve (8,9,28-30). Some workers (40,41) however, have reported virions and antigens in satellite cells and non-myelinating Schwann cells after organ culture of explants although virions were not reactivated in myelinating Schwann cells.

Other investigators believe that the demyelination follows cellular infiltration and proliferation. In a sequential study, Lawn and Payne (28) observed minimal demyelination during the 2nd and 3rd weeks after infection, even when severe cellular infiltration was present. Severe demyelination commenced during the 4th and 5th weeks, at the time of onset of neurological signs, and was characterized by a migration of lymphocytes and macrophages within the basement lamina of the Schwann cell, with destruction of myelin but usually sparing of axons (Fig. 10). This lesion was classified as a primary, segmented, cell-mediated demyelination. In some birds this process of lymphoproliferation and active demyelination was progressive, whereas in others cellular infiltration was more sparse, oedema was present, and demyelination occurred, corresponding to the B-type nerve lesion. This study thus indicated an association between the initial tumour-like lymphoproliferation and the subsequent demyelination. The reasons for the initial migration of macrophages and lymphocytes into the nerves, commencing as early as 5 days after infection, and for the later infiltration of these cells into the axon, accompanied by demyelination, have not been elucidated.

According to Hoffmann-Fezer and Hoffman (32), B- and T-lymphocytes, and plasma cells, are numerous in B-type nerves. In the mild, C-type lesion, there is a preponderance of T-lymphocytes.

Several studies indicate that the primary demyelination is mediated immunologically. Skin hypersensitivity reactions to normal myelin, and the presence of tissue-bound and serum antibodies to myelin, have been detected in MD (40-42). Furthermore, primary demyelination in MD closely resembles ultrastructurally that in experimental allergic neuritis in the fowl (39,40,43,44). Recently, a spontaneous peripheral neuritis, termed idiopathic polyneuritis, which occurred in a flock free of MDV and other neurotropic viruses, and which showed close pathological similarity to the B-type lesion of MD, was described by Biggs *et al*. (45). It was suggested that idiopathic polyneuritis is a natural

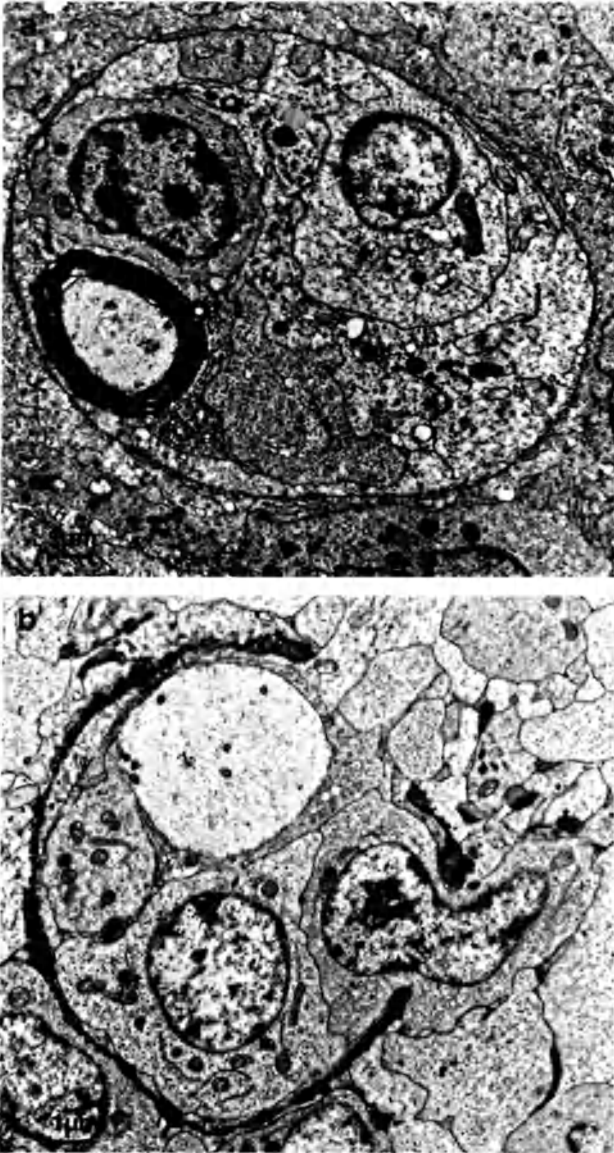

FIGURE 10. a) Invasion by macrophages and lymphocytes of nerve fibre of peripheral nerve of MDV-infected chicken, leading to b) Demyelination of axon (AX). (From Payne (90), by kind permission.)

autoimmune disease of chickens. The pathological similarities between MD neuropathy, experimental allergic neuritis, and the Landry-Guillain-Barré-Strohl syndrome (idiopathic or acute infective polyneuritis) of man have been discussed by a number of investigators (37,39,40,46).

In addition to the primary demyelinative process, some workers have observed secondary (Wallerian) degeneration of axons and myelin, particularly in heavily infiltrated nerves (33,37,39) suggesting damage caused by compression or toxic effects.

3.5. Central nervous system

The central nervous system is often histologically normal in MD, and when lesions do occur they are usually minimal (47). The most frequent abnormality is perivascular cuffing usually by mature lymphocytes and macrophages which lie in the perivascular space of Virchow-Robin. In naturally-occurring cases of MD, Wight (47) observed minimal to mild cuffing in 64%, moderate in 4%, and severe in 7%, with 25% showing no cuffing. The cuffing occurs in the white and grey matter of the brain and cord. Microgliosis and astrocytosis sometimes occur, particularly adjacent to severe cuffing. Neuronal degeneration is rare. Primary demyelination does not occur, but secondary demyelination may be caused by severe cuffing or mechanical distortion. In a survey of field cases, Fujimoto et al. (5) recorded cuffing in 76%, lymphoreticular cell proliferation in 13%, and astrocytic proliferation in 33%. In experimentally produced cases cuffing is observed at 1 to 2 weeks after infection, reaching a peak of severity at 5 weeks after infection (9,23,48).

Ultrastructural changes have been studied by Lawn and Watson (48). In mild lesions a few isolated lymphocytes or macrophages were confined between the basement lamina of the vascular endothelium and the basement lamina of the nervous tissue. These cells occurred as compact masses in more severe lesions, with a structure indistinguishable from that of dense lymphoid tissue elsewhere. In the most severe lesions the lymphocytes and macrophages passed through breaks in the basement lamina of the nerve tissue and wandered amongst glial cells and nerve cells often at considerable distances from the blood vessels. Rarely plasma cell and basophils (or mast cells) were found in the nervous tissue.

The cause of cuffing in MD is not understood. Although cell-associated virus (49) and viral precipitins (8) can be detected in the

brain, attempts to localize virus by immunofluorescence have been unsuccessful (8,9). Lawn and Watson (48) favoured the hypothesis that in MD T-lymphocytes become more motile and have an abnormal migratory behaviour, which enables them to penetrate the endothelial barrier in the central nervous system. In the Virchow-Robin space they secrete lymphokines which attract more lymphocytes and macrophages from the blood. A similar mechanism could explain the initial migration of these cells into the peripheral nervous system.

Although lesions in the central nervous system are unimportant in the common neural and visceral forms of MD, the uncommon manifestation of infection by MDV termed transient paralysis (50) is accountable to a brain disorder. The features of transient paralysis are a variable perivascular lymphocyte cuffing in the central nervous system, mild lymphocytic infiltration of the meninges, and mild focal or diffuse microgliosis (51). The cerebellum and brain stem are reported to be the most affected, with other regions of the brain, and the spinal cord, less frequently involved. Neuronolysis, axon degeneration and demyelination are rarely seen (51,52). Mild C-type lymphocytic infiltration of peripheral nerves occurs and mild and variable lesions in other organs have been reported (51,53,54). Recent studies by Kornegay et al. (55, 56) indicate the essential lesion in transient paralysis is oedema (vacuolation) of the grey and white matter of the brain possibly resulting from reversible inflammatory changes in the wall of cerebral blood vessels.

Acute ataxia and paralysis, with encephalitis, similar to transient paralysis, were also features of infection by the very highly oncogenic (variant) viruses (e.g. Md/5 and Md/11 reported by Witter et al. (18)).

3.6. Lymphomas

MD lymphomas consist of a mixture of small and medium lymphocytes, lymphoblasts, primitive and activated reticulum cells, macrophages, and in some instances degenerative lymphoblasts termed MD cells (27,57) (Figs. 11 & 12). Lymphocytes predominate, with the degree of differentiation varying between different cases from mainly small lymphocytes to mainly lymphoblasts. A fine reticulin network is present between the tumour cells. Ultrastructurally the lymphoid cells show no qualitative features that differentiate them from normal lymphoid cells (3,58). The

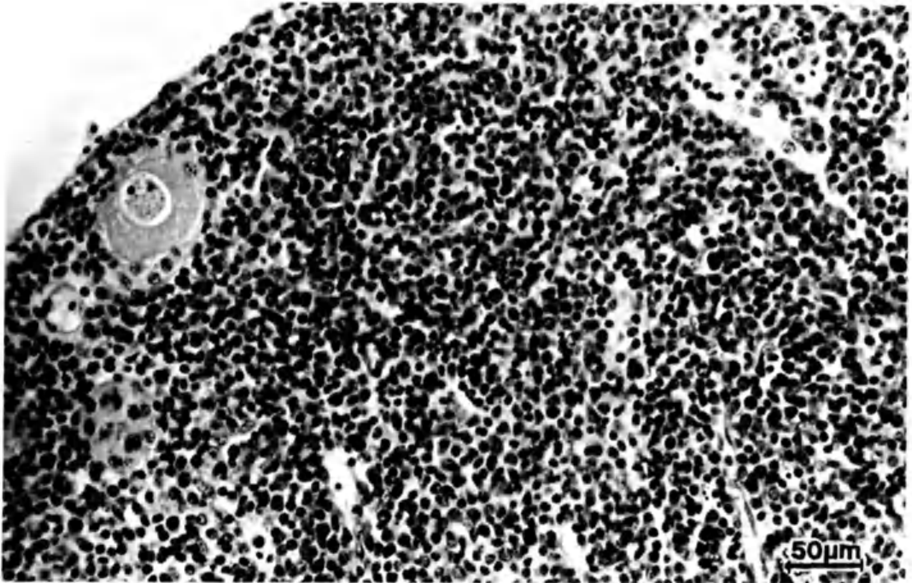

FIGURE 11. Ovarian lymphoma; same case as Figure 8.

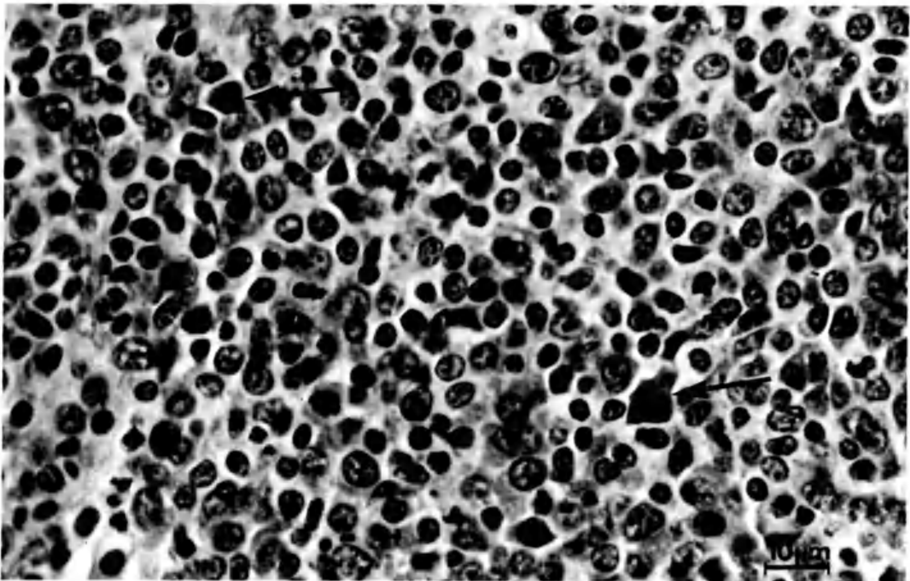

FIGURE 12. Ovarian lymphoma showing varying morphology of lymphoid cells, including so-called MD cells (arrows); same case as Figure 11.

lymphoid tumour cells are pleomorphic, with abundant cytoplasm containing few organelles other than mitochondria and ribosomes. There is little endoplasmic reticulum and few lysosomes or structures resembling the Golgi apparatus. The nuclei are fairly large, irregular in shape and nucleoli may be seen. Nuclear projections, rarely seen in normal lymphoid cells are frequently present in tumour cells. Rarely, intranuclear herpesvirus particles, mainly unenveloped, are seen in lymphoma cells.

Multifocal lymphomatous foci are first seen at about 7 days after infection with pathogenic MDV, mainly in the gonads, liver, proventriculus and peripheral nerves. They become visible macroscopically as small greyish foci at 14 days, and develop into frank tumours from 21 days after infection.

The majority (60% to 80%) of lymphoma cells are T-cells, and most of the other cells (10% to 20%) are B-cells, with small proportions of null cells and non-lymphoid cells such as macrophages (31). A variable minority of lymphoma cells, from less than 1% to more than 35% in different birds, express the membrane antigen MATSA (59,60). Although viral antigens and virions are rare in lymphomas, viral genome can be detected in a majority (60% to 70%) of cells by _in situ_ nucleic acid hybridization (30). Thus the MD lymphoma is cytologically complex. The T-cell component is considered to include neoplastically transformed cells, because lymphoblastoid line cells and transplantable lymphoma cells derived from primary MD lymphomas are all T-cells, carry MATSA, and contain multiple copies of MDV genome (Sections 4 and 5). Many investigators believe that MATSA is a specific marker for transformed cells. Among the lymphoma T-cells, many do not carry MATSA, but are latently infected and carry viral genome and rescuable virus (61). These cells form part of the non-neoplastic component of the lymphoma together with B-cells and non-lymphoid cells and may be involved in immunosurveillance against transformed cells. Macrophages and natural killer cells from lymphomas have been shown to be cytotoxic against transformed cells (62).

3.7. Atherosclerosis

According to Paterson and Cottral (7), sclerotic changes in the coronary and other arteries are extremely common in adult fowl, and they

suggested an aetiological relationship between coronary sclerosis and lymphomatosis (Marek's disease). This possible association has been investigated in detail by Fabricant and her colleagues (6,63,64), who observed that although mild atherosclerotic arterial lesions were present in chickens uninfected by MDV, the presence of infection by mildly pathogenic MDV greatly increased the severity of the lesions, especially in birds given supplemental cholesterol. The lesions, which were often occlusive, were found in the coronary arteries, aorta and major aortic branches, and were classified as: 1) fatty, 2) proliferative, or 3) fatty-proliferative.

In these studies, MDV antigens were detected by immunofluorescence in the medial layer of affected arteries, apparently in smooth muscle cells, suggesting that virus-induced medial necrosis is an initial change in the development of atherosclerosis. Interestingly, vaccination with the herpesvirus of turkeys protected chickens against MDV-induced atherosclerosis. Pathologically, atherosclerosis in chickens shows similarities to the disease in man, and the involvement of a herpesvirus in the avian disease is of considerable comparative interest.

3.8. <u>Ocular changes</u>

The histopathology of so-called ocular lymphomatosis, considerd by many to be a form of MD, has been described by many workers (2,5,65,66).

The common findings are lymphocytic, plasma cell and sometimes heterophil infiltration of the iris, ciliary body, and conjunctiva, and less commonly of the choroid membrane, pecten and retina. Although earlier studies were made on naturally-occurring cases, with consequent uncertainty about their aetiology, essentially similar changes have been observed following experimental infection with MDV (67,68). Simpson (69,70) studied the ultrastructural changes in natural cases of the ocular disease, and observed numerous mature and budding retrovirus-like particles in a variety of cells, raising the possibility that agents other than MDV may also be involved.

3.9. <u>Other tissues</u>

Tissues other than those described above do not normally appear to be particularly susceptible to MDV infection or to show lesions. In experiments in which birds died 10 to 17 days after MDV infection,

apparently from an overwhelming infection, Calnek (71) observed focal necrosis of renal tubular epithelium, with intranuclear inclusion bodies, glomerulitis or glomerular necrosis, and focal or generalized necrosis of pancreas, proventriculus, liver and heart. Intranuclear inclusion bodies may occur in adrenal cortical cells (9). Focal necrosis of liver was observed by Witter _et al_. (18) in the early-mortality syndrome induced by variant MDV isolates.

4. LYMPHOMA-DERIVED CELL LINES

Lymphoid tumour cell lines can be established from a proportion of MD lymphomas cultured _in vitro_. The success rate was at first low, with only 10% or fewer lymphomas yielding cell lines (59,72), but in more recent studies up to 50% have given rise to lines (73-75). Factors helpful in the initiation of lines include culture at 41°C, use of special media, including 2-mercaptoethanol and chicken serum, and derivation from transplantable lymphomas. In total, more than 80 cell lines from various MDV-host strain combinations are now available. The lymphoid cell lines can be initiated either in liquid medium, in which case lymphoblastoid cell lines develop, or in soft agar medium, in which case the cell lines are at first lymphocytoid (75) but become lymphoblastoid after transfer to liquid medium (Fig. 13). The relationship between these two forms is not yet clear. The lymphocytoid lines grow better at 41°C than at 37°C, are relatively slowly growing and are density- dependent. The lymphoblastoid lines grow more rapidly, better at 37°C than at 41°C, and lose their density dependency. It seems probable that the lymphoblastoid state represents a progression of the transformed cell towards greater malignancy.

Morphologically the lymphocytoid line cells resemble medium to large peripheral blood lymphocytes in smears stained with May-Grunwald-Giemsa stain, and measure 8 to 16 μm in diameter. The lymphoblastoid line cells have a more basophilic cytoplasm, a large nucleus and nucleolus, a fine chromatin pattern, and range from 13 to 34 μm across. Ultrastructurally the lymphocytoid cells show a greater resemblance to normal lymphocytes than do the lymphoblastoid cells. Intranuclear immature herpesvirus particles are only rarely present in the lymphocytoid and lymphoblastoid cell lines (75,76).

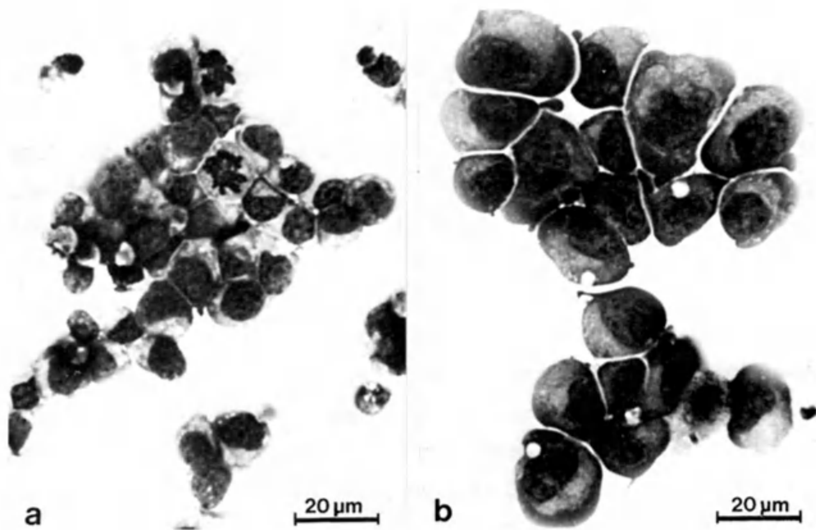

FIGURE 13. a) Lymphocytoid cell line, and b) Lymphoblastoid cell line, derived from MD ovarian lymphoma. (From Payne et al. (75), by kind permission.)

The lymphoid tumour cell lines, both lymphocytoid and lymphoblastoid are T-cells and all carry the tumour-associated antigen, MATSA (59,77). An embryonic antigen (chicken foetal antigen) is present in a minority (2 to 16%) of cells in the lymphocytoid state, and in almost all lymphoblastoid cells (75,78). Other antigenic determinants present on lymphoblastoid line cells include Hanganutziu and Deicher-type heterophile antigen (79), Forssman antigen (80), Ia-like antigen (81) and major histocompatibility complex antigens (82,83). The cells are negative for Fc receptors and surface IgM (81). The cell lines are essentially diploid but chromosomal abnormalities may be present, none

of which appears to be specific for transformation (84). Most cells in a line are non-productively infected but MDV can be rescued from most lines by inoculating them into chicks or onto cultured cells (74,75,85). A small proportion of cells spontaneously express viral antigens and immature virions, and this can be increased by treatment with 5-iodo-2-deoxyuridine (IUdR) and 5-bromodeoxyuridine (BUdR). The RPL-1 lymphoblastoid line, derived from the transplantable JMV lymphoma, is a true non-producer line from which MDV cannot be rescued (86), as were apparently a number of lines produced by Payne _et al_. (75). The lymphoblastoid cells contain multiple copies of the MDV genome; the state of the genome in cell lines is discussed in Chapter 5.

Recently lymphoblastoid cell lines have also been developed from tumours from MDV-infected turkeys. In the studies of Nazerian _et al_. (87) the cell lines, unexpectedly, had the properties of B-lymphocytes; cell lines similarly produced by Powell _et al_. (88), however, were T-cells, as in the chicken. The basis for this discrepancy is not yet understood.

The development of cell lines from MD lymphomas was important conceptually in showing the presence in lymphomas of neoplastically transformed T-cells. Subsequently these lines have been used to characterize further the transformed cell and to study the nature of the virus-cell interaction (see Chapter 5). The cell lines have also been of value as target cells in _in vitro_ assays for the detection of cytotoxic lymphocytes, macrophages and natural killer cells (see Chapter 7).

5. TRANSPLANTABLE LYMPHOMAS

Several transplantable lymphomas have been derived from MDV-induced lymphomas by rapid serial passage in chicks. The first transplantable tumour developed, designated JMV, produces an acute lethal lymphoblastoid leukaemia which was originally thought to be caused by the presence of virulent MDV, but was later shown to result from a transplant (89). Some substrains of JMV did apparently carry MDV, but others had no rescuable virus, although part of the viral genome was present and the cells expressed MATSA. Subsequently, several other transplantable lymphomas have been developed (90). They grow more slowly than JMV in chicks, produce local tumours, are normally dependent on the

histocompatibility of the host for progressive growth, and release MDV. These various transplantable lymphomas have been used principally to study mechanisms of vaccinal immunity (see Chapter 8) and the effect of the genetic constitution of the host on tumour growth (see Chapter 11).

6. FACTORS AFFECTING PATHOGENESIS

6.1. Virus strain and dose

Isolates (strains) of MDV may be classified into five groups (see Chapter 4) according to their oncogenicity 1) very highly oncogenic (variant) strains, which are oncogenic in HVT-vaccinated birds and genetically resistant strains, and which have a marked ability to cause acute lethal cytolytic infections in MD-susceptible chicks, 2) highly oncogenic (acute) strains, which cause a high incidence of visceral and neural lymphomatosis in susceptible chicks, 3) moderately (classic) to mildly oncogenic strains, causing mainly neural MD, often at low incidence, 4) minimally oncogenic strains, causing minimal lesions often only in very susceptible chickens, and 5) non-oncogenic strains. As discussed in Chapter 4, the four oncogenic pathotypes fall into serotype 1 and non-oncogenic MDV into serotype 2. HVT falls into serotype 3.

The virus properties responsible for differences in oncogenicity are not fully understood. Strains of low oncogenicity grow to lower levels in chickens (49), and cause less damage to the lymphoid system than do highly oncogenic strains (91), suggesting that virus-induced immunosuppression may determine oncogenicity. Furthermore, some strains (e.g. Cu-1 and Cu-2) of MDV of low oncogenicity induce lymphomas in immunosuppressed hosts (92), and the very highly oncogenic strains (e.g. Md/5 and Md/11) cause marked atrophy of lymphoid organs (18). Other strains of low oncogenicity (e.g. SB-1 and HVT) are not oncogenic in immunosuppressed hosts, suggesting that they lack transforming ability. The lack of damage to the lymphoid organs by non-pathogenic viruses such as SB-1 and HVT is related to the absence of, or the very limited, viral replication in these sites (91,93). HVT can induce mild foci of lymphoblastoid cells in the nerves of turkeys and chickens, but not gross lesions, and MATSA-bearing lymphoid cells are induced in chickens by HVT (91,93). Whether these changes are indicative of a limited neoplastic transformation is still debatable: induction of lymphoblastoid cell

lines by HVT alone has not been reported.

The effect of dose of MDV on disease incidence appears to depend on host factors. Low doses of MDV retard the time course but not the final incidence in susceptible chickens, whereas in more resistant chickens time course and final incidence may be depressed with low virus doses (94).

6.2. Host genotype, age and sex

Differences in genetic susceptibility to MD depend on lymphoid responses. Two types of genetic resistance have been recognized: 1) that exemplified by line 6 chickens and controlled by genes at the *Ly-4* locus responsible for an antigen present on lymphocytes, and 2) a second type exemplified by N-line chickens and associated with the *B-21* allele at the major histocompatibility complex of genes (*Ea-B* locus) (see Chapter 11).

The resistance of line 6 is present at hatching and is believed to depend essentially on lowered susceptibility of T-lymphocytes for transformation (95,96) and, compared with the susceptible line 7, is characterized by lower viraemia, lower titres of virus in the lymphoid organs, a milder early degenerative change in these organs, and a low incidence of lymphomas and nerve lesions. Virus-neutralizing antibody levels and anti-tumour-cell cell-mediated immunity are higher, probably as a consequence of the milder immunosuppression (97). In contrast, the resistance of N-line is believed to be mediated primarily by the immunological responses to the infection (98). It develops during the first few weeks of life, and is thought to be similar to age-resistance by Calnek (99) because N-line chicks, if free of maternal antibody, are susceptible to MD if infected at 1 day of age, but develop resistance over a period of several weeks. Irrespective of age, the initial semi-productive infection of lymphoid tissues occurs in line N chickens, but they fail to develop lymphomas. Compared with P-line, N-line has, late in the infection, lowered levels of viraemia, higher virus-neutralizing antibodies, and a decrease in MATSA-bearing (possibly transformed) cells. These characteristics suggest that the *B-21*-associated resistance of the N-line is mediated by a superior ability to reject transformed lymphoid cells.

Sharma *et al* (100) concluded from studies on 12-week-old line 15 x 7

chickens that their resistance to MD was expressed by regression of microscopic and gross neural lesions.

Sex influences the distribution and incidence of MD lesions, the ovary being particularly prone to lymphoma formation and females more prone to the disease than males (3). The basis of this effect is not known.

6.3. Immune status of host

Apart from natural, genetically-controlled differences in immune responses to MDV, discussed in the previous section, acquired immunity can greatly influence pathogenesis. Such immunity can be acquired passively from maternal antibody transferred to the chick via the yolk sac, or actively as a consequence of natural infection by MDV of low pathogenicity or of infection by vaccinal strains of MDV or HVT (see Chapter 7).

Passively acquired antibody for MDV, which persists for about 3 weeks, has four main effects: 1) it suppresses the stage of acute cytolytic infection of lymphoid tissues, with decreased viral antigen formation and decreased bursal and thymic atrophy, 2) reduces lymphoma formation, 3) delays disease onset and period to death, and 4) lowers MD mortality (9,101).

Infection by HVT or nononcogenic MDV (SB-1 strain) is followed within 1 week by localization, but limited replication, of virus in the bursa, thymus and spleen, unaccompanied by the acute cytolytic changes which occur with virulent virus. MATSA-bearing cells appear in these organs at about the same time and may persist for many weeks (91). HVT-vaccinated chicks develop a persistent cell-associated viraemia and mild, transient lymphoproliferative lesions in the nerves and gonads, possibly the result of a limited transformational event (16). Following challenge of vaccinated birds with virulent MDV, the early cytolytic infection of lymphoid tissue, immunosuppression, MDV viraemia and lymphoproliferative responses seen in unvaccinated birds, are suppressed (16).

6.4. Stress

Chickens selected for high plasma corticosterone levels in response to social stress were more susceptible to MD than were birds selected for low levels (102), and feeding of dichlorodiphenyl-dichloroethane

(DDD) or metyrapone, which block the production of corticosterone by the adrenals, lowered the incidence of MD (103,104). The higher susceptibility to MD of the high responder line was associated with lowered immunological responsiveness. These findings suggest that high corticosterone levels occurring as a response to stress cause immunodepression and consequently increased susceptiblity to MD, and lend credence to the anecdotal association between certain stresses that occur in commercial poultry practice and outbreaks of the disease.

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4. CHARACTERISTICS OF THE VIRUS

K.A. SCHAT

1. INTRODUCTION

The isolation of a herpesvirus from Marek's disease (MD) tumour cells and the subsequent reproduction of the disease with tissue culture propagated virus during the late 1960's clearly established the aetiology of MD. The isolation of MD herpesvirus (MDV) also ended the dispute on the aetiological relationship between MD and avian lymphoid leukosis, which is caused by a retrovirus. The key to the successful isolation of MDV was the use of intact tumour cells or blood lymphocytes as inoculum onto chick kidney cell (CKC) cultures (1) or duck embryo fibroblasts (DEF) (2,3), respectively. Shortly after the first isolations of MDV were reported, a herpesvirus was isolated from healthy turkeys (herpesvirus of turkeys, HVT), which was antigenically related to MDV (4,5). The existence of a third, related group of viruses was demonstrated by Biggs and Milne (6), when they reported the isolation of nonpathogenic MDV strains from healthy chickens. These viruses could be differentiated in cell culture from the oncogenic viruses by a slightly different plaque morphology. It is now generally accepted that MDV and HVT isolates can be divided into three serotypes, which will be discussed in Section 2. A vast amount of information on these viruses has been generated over the last 15 years. In this chapter, the following characteristics of MDV will be reviewed: virus strains; the morphological, physical and chemical characterization of the virus; cultivation and replication of the virus; and the antigens expressed during the infection. Where appropriate, the characteristics of different serotypes will be compared.

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2. VIRUS STRAINS

MDV and HVT are widespread geographically and virus isolations have been reported from numerous countries. A comprehensive list of isolates has been compiled by Purchase (7). Since the first isolation of MDV, it has been demonstrated that all strains isolated from chickens can be divided into two major, serologically related but distinct groups. All oncogenic viruses form serotype 1, while the nononcogenic viruses belong to serotype 2. HVT isolates are classified as serotype 3 (Table 1). This classification was first proposed by von Bülow and Biggs (8,9) and subsequently confirmed by Schat and Calnek (10) using agar gel precipitin (AGP) and indirect fluorescent antibody (IFA) assays. The existence of three serotypes was further demonstrated by virus-neutralization (VN) tests (11), comparison of the two-dimensional (2D) gel patterns of virus-specific polypeptides (12) and recently by the use of monoclonal antibodies specific for the three serological groups (13). In addition, Ross (see Chapter 5) demonstrated differences in viral DNA obtained from viruses from the three serogroups by restriction enzyme analysis.

TABLE 1. Related herpesviruses from chickens and turkeys

Virus type	Serotype	Oncogenicity[1]	Representative isolates	Reference
MDV	1	+++	Md/5,Ala-8,RB-1B,Md/11	(14,15,16)
		++	GA,HPRS-16,JM	(17,18,19)
		+	HPRS-17,Conn-A	(18,20)
		±	Cu-2,CVI 988	(21,22)
	2	-	HPRS-24,SB-1,HN-1	(6,10)
HVT	3	-	FC 126,WTHV-1,HPRS-26	(4,5,6)

[1]+++ = very high, causes MD in vaccinated, genetically susceptible and non-vaccinated, genetically resistant strains of chickens.

++ = high, causes high incidence of MD in susceptible strains and none or low incidence in resistant strains of chickens.

\+ = causes moderate to low incidence of MD in genetically susceptible chickens.

± = causes minimal lesions, often only in very susceptible strains of chickens.

A large diversity exists within serogroup 1 in regard to oncogenic potential. Some of the more recently isolated strains such as MD-5, Ala-8, and RB-1B were obtained from flocks of HVT-vaccinated chickens experiencing MD breaks. These viruses can cause a high incidence of MD in genetically resistant, nonvaccinated birds or genetically susceptible, HVT-vaccinated birds (14-16). Other virus strains like GA (17), HPRS-16 (18) and JM (19) can cause a high incidence of MD in genetically susceptible but not in resistant birds. Even less virulent viruses can induce tumours in only a minority of susceptible chickens; examples are HPRS-17 (18) and Conn-A (20). The least oncogenic of serotype-1 viruses are strains like Cu-2 (21) which may cause tumours only rarely or CVI 988 (22), which may cause only nerve lesions in highly susceptible breeds of chickens (23). Some of the viruses of each of these pathotypes have been listed in Table 1. It is of considerable interest to note that, so far, these pathotypes have not been distinguished by conventional or monoclonal antibodies. Van Zaane et al. (12) compared the virus-induced polypeptides of the GA-5, K, Cu-2 and CVI 988 strains by 2D gel analysis and they were unable to find differences with the possible exception of GA-5, for which the presence of glycoprotein (gp 4) could not be demonstrated. Yet, their technique was sensitive enough to allow them to differentiate the HVT isolates into two subgroups. The absence of detectable differences in polypeptides among MDV's with varying degrees of oncogenic potential is important in relation to the question of the mechanism of transformation. Several explanations can be offered, some of which relate to features of MD unrelated to the virus itself. Of concern here are those which might be virus-related. It could be that oncogenic viruses have a promoter sequence to induce the transcription of cellular onc genes and that these are more efficient with the more oncogenic strains than with less oncogenic strains. Or, they could possess more, or more efficient, viral onc genes directly involved in transformation. The presence of promoter sequences, although not generally accepted, has been demonstrated for at least one other oncogenic herpesvirus, Epstein-Barr virus (EBV) (24). On the other hand, it could be that the more oncogenic viruses are so because they are more immunosuppressive than the less oncogenic strains. This possibility is supported by the observation

that inoculation of the low oncogenicity Cu-2 strain in neonatally thymectomized chicks or after in ovo inoculation will indeed result in an increased incidence of MD (25). Further support for this hypothesis was provided by Witter et al (26) with the finding that the highly oncogenic or very virulent strains cause more severe immunodepression than the less oncogenic viruses.

Serotype 2 viruses, which can be frequently isolated (28), seem to be nonpathogenic or nononcogenic in nature (10,27). The term non-oncogenic might be more precise than nonpathogenic, because under certain conditions, such as in ovo inoculation at the 8th day of incubation, serotype 2 strain SB-1 can cause a lytic infection of the lymphoid tissue resulting in severe immunosuppression and subsequently B-type (29) lesions in the nerves. Tumours, however, have never been observed even in immunosuppressed chickens (20,27, J. Pol, personal communication).

Repeated passage in cell culture of serotype-1 viruses will lead to attenuation resulting in loss of oncogenicity (30,31). One possible explanation is that infectivity and replication patterns are altered. This will be discussed later (see Section 4.2). Alternatively, it might be that the changes observed in restriction enzyme patterns of viral DNA coincident with attenuation are related to the intrinsic capacity of the virus to transform (32). In addition to the loss of oncogenicity, attenuated viruses normally do not spread horizontally in chickens and they often lose the capability to produce A antigen (see Section 6) (30). The latter might not be absolute, however, since 2D gel patterns of virus-specific polypeptides from HPRS-16/att demonstrated the presence of A antigen (12), while AGP tests were negative. Regardless, the loss of A antigen is not a prerequisite for attenuation; Purchase et al. (33) described A antigen-negative, pathogenic clones obtained from the JM strain.

Attenuation has also been described for HVT, where it resulted in the inability to replicate in vivo (34,35). The nature of the attenuation for HVT is unknown. Thus far, attenuation of serotype 2 viruses has not been reported, but this may simply reflect the fact that these viruses have been less extensively studied than the other two groups.

3. PHYSICAL AND CHEMICAL PROPERTIES

3.1 Virus morphology and morphogenesis.

Most studies on morphology have been conducted with serotype 1 and 3 viruses. Qualitative differences were not detected between these two groups (36,37). The structure of MDV and HVT is typical of the herpes-virus groups; the nucleocapsid measures between 95 and 100 nm and has 162 hollow-centred capsomeres (38). Electron microscopic examination of infected cells demonstrated the presence of small particles of approximately 35 nm in the nucleus (37-39), which start to appear at 8 hours post-infection (39). The particles were positive for MDV antigen in immunoferritin labelling studies (40) and could not be labelled with ^{3}H-thymidine (41) suggesting that these might be protein in nature rather than DNA. Recently, Okada et al. (41) suggested that six of these small nuclear particles fuse into a cylindrical mass in a capsid. DNA consisting of strands of 2 nm in diameter forms a band which spools repeatedly around the cylindrical mass (43). The nucleoid measures 50 to 60 nm and has a toroidal structure which can cause a cross-shaped appearance (Fig. 1) (44). Occasionally, crystalline arrays are formed in MDV-infected cells and more frequently in the case of HVT infection (36,37). The first nucleocapsids can be observed 10 hours after infection and the first enveloped virions appear at 18 hours (39). This process occurs at the inner nuclear membrane and enveloped particles of 150 to 170 nm can be found free in the nucleoplasm or in nuclear vesicles. Sometimes, naked and enveloped virions can be detected in the cytoplasm and rarely in the extracellular spaces. If present these particles are larger than the mature particles in the nucleus (190 to 230 nm versus 150 to 170 nm) and peripheral spikes can be detected (39,41). Large numbers of cytoplasmic enveloped virus particles can be found in the feather follicle epithelium. In negative-stained preparations, these paricles have large envelopes measuring from 270 to 400 nm and often appear as irregular amorphous structures (Fig. 2) (45). MD-derived lymphoblastoid cell lines and tumour cells occasionally contain naked particles which are essentially similar to the virions observed in infected cell cultures. However, an additional particle of about 70 nm can be observed in the lymphoblastoid cell lines, which is believed to

be an aberrant form of DNA (46). Serotype 2 viruses have not been studied in detail for their morphological appearance. However, typical herpesvirus particles were observed in infected chicken embryo fibroblasts (CEF) (10).

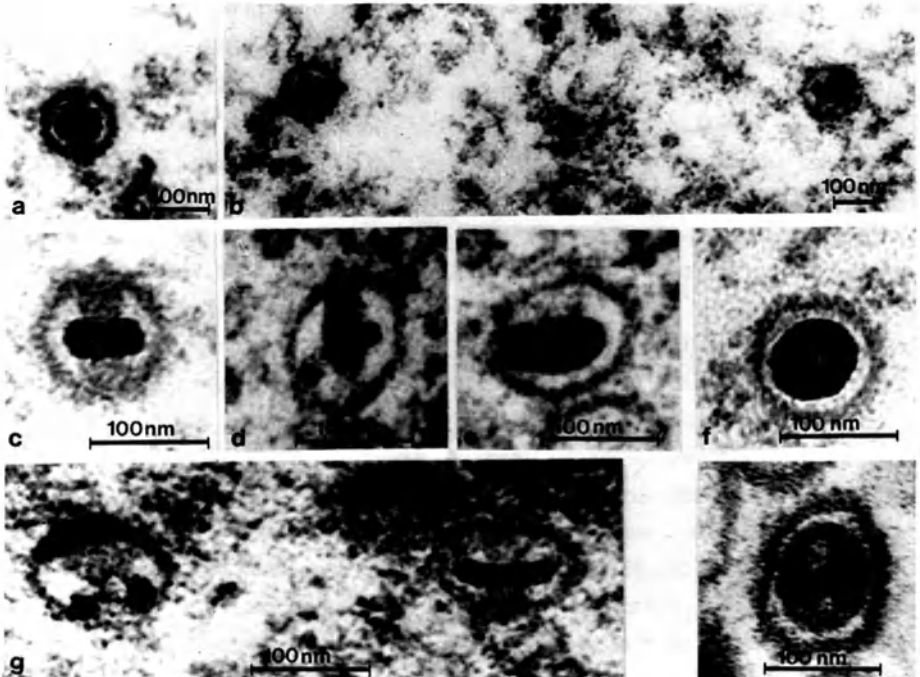

FIGURE 1. Selected electron micrographs of MDV. All are from thin sections stained with uranyl acetate and lead citrate. Cores in these virions are sectioned at different angles and show the position of the torus within the capsid. As seen in this figure, the electron-opaque torus is usually central and perpendicular to the axis of a central, less opaque mass (a,b,c,e). It can also be seen shifted to the sides (g) or be oblique to the central axis (d). The usual doughnut shape of the core is seen in f). The torus is less clear in enveloped virions (h), but the central filamentous mass is quite clear. (From Nazerian (44) by kind permission.)

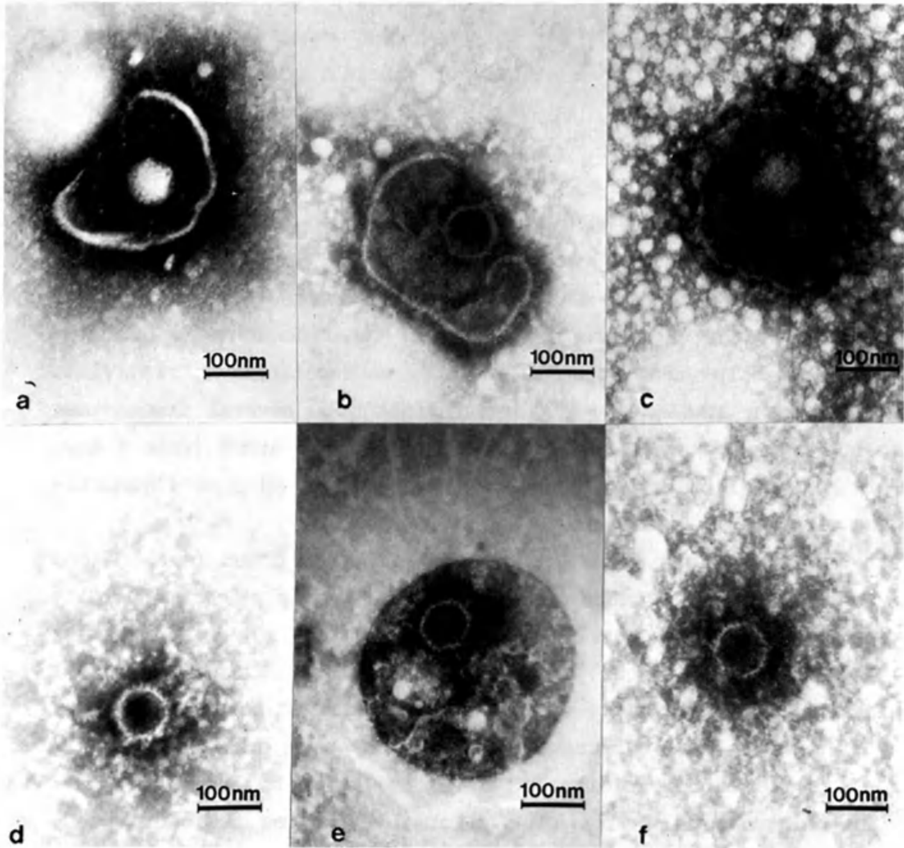

FIGURE 2. Negatively stained preparations of herpesvirus-like particles in lysates of chicken feather follicle epithelium. a-c) are representative of the enveloped virus particles observed. The capsids in a) and c) are "full", whereas that in b) has been penetrated by the stain. d-f) are illustrations of naked virus particles. The capsids are hexagonal in outline, and the capsomeres appear as tubular projections. (From Calnek _et al_. (45) by kind permission.)

3.2 Physical and chemical properties

MDV, and to a slightly lesser degree HVT, are strongly cell-associated *in vitro*. It was not until the discovery that infectious, cell-free virus could be extracted from feather follicle epithelium (45) that the physical characteristics of MDV could be studied. An additional helpful factor was the finding by Calnek *et al*. (47) that cell-free virus could be obtained from infected cell cultures by ultrasonic treatment in the presence of a stabilizer consisting of sucrose, phosphate, albumen and (but this is not essential) sodium glutamate. The stabilizer also allowed the lyophilization of cell-free virus with little or no loss in titre.

Calnek and Adldinger (48) used crude cell-free preparations of JM virus to study resistance against various physical treatments. Infectious virus passed through a 300 nm but not a 220 nm Millipore membrane filter. Much of the virus was sedimented by centrifugation at 5000 x g for 1 hour suggesting that it was associated with cellular debris. Virus preparations were stable at -65°C and resistant to several freeze-thaw cycles, but infectivity was lost rapidly at 20°C and above (from 4 days at 22 to 25°C to 10 minutes at 60°C). Treatment at pH 3 or 11 inactivated the virus in 10 minutes.

Cell-free infectious virus can be obtained from dried feathers and poultry dust for 8 to 12 months, although increases in relative humidity will decrease the virus survival time (49,50). At 4°C, dried feathers still had infectious virus after 10 years (B.W. Calnek, personal communication). The stability of virus in dust and feathers presents an important problem for effective disinfection especially at poultry farms. Attempts to use acidity (pH of 2) to lower the level of infectivity in chicken pens failed (51). Formaldehyde gas fumigation destroyed most, but not all, infectivity associated with dust samples and feathers. Iodine bound to organic carriers was effective in disinfection of dust and feathers, while chlorine components were only active in the latter case (49,52). Cell-free virus found in nature is probably mostly bound to cellular debris, thus allowing filters with a dust spot rating of over 93% to remove MDV efficiently from the air (49,53).

The resistance of cell-free HVT to ultra-violet (UV) light has been investigated. It was found that HVT was relatively resistant, and that

CEF cultures unexposed to UV light could reactivate exposed and partially inactivated virus (54,55). Similar experiments have not been reported for serotype 1 and 2 MDV's. However, MDV can be isolated from cells from lymphoblastoid cell lines after exposure to ultra-violet light (K.A. Schat, unpublished data).

4. ISOLATION AND CULTIVATION

4.1. Isolation.

Cell-associated MDV of both serotypes and HVT can be readily isolated from viable lymphocytes and, in the case of serotype 1 viruses, from lymphoma cells. Kidney cells have been used for virus isolation by direct cultivation. Although Calnek and Hitchner (56) described the presence of viral antigens in these and other epithelial tissues, it is not clear that lymphocytes were excluded from these inocula and these could contribute to the infectivity of a cell suspension. Serotype 1 MDV is commonly isolated by inoculation of DEF and CKC cultures, while CEF are preferred for the isolation of serotype 2 MDV and HVT.

In addition, cell-free material obtained from the feather follicle epithelium of chickens and turkeys infected with MDV and HVT, respectively, can be used for virus isolation. The use of 10 mM EDTA will increase the efficacy of the isolation of cell-free MDV, but will not influence the isolation of HVT (57) or SB-1 (serotype 2 MDV) (K.A. Schat, unpublished). The EDTA apparently aids the penetration of the virus into the cell, possibly by chelating the Mg++ ions (57). The methods for virus isolation will be discussed in detail in Chapter 6.

4.2. Cell culture propagation.

MDV and HVT can be propagated in a wide variety of avian cell cultures after the initial isolation. Purchase *et al.* (58) were able to propagate MDV and HVT in primary fibroblast cultures obtained from turkey, goose, pigeon, pheasant, bobwhite and Japanese quail embryos, although the degree of replication and the type of cytopathic effect (CPE) varied. In addition, it was found that primary cultures of thyroid gland cells were susceptible and could also be used for primary isolation (59). MDV, and to a lesser degree HVT, are strictly cell-associated *in vitro*, consequently little or no infectious cell-free virus is released in the medium (5,59-61). In an attempt to mimic, *in*

vitro, the in vivo production of cell-free virus, Prasad and Spradbrow (62) infected chicken embryo skin epithelial cell cultures. Both HVT and MDV replicated well in these cultures, but cell-free virus was not released into the supernatant fluid. The cultures consisted mainly of keratinocytes, but keratinization did not occur. It would be of interest to repeat these experiments while inducing keratinization with hydrocortisone (63).

Recently, another avian cell culture system has been used for the propagation of MDV. Calnek et al. (64,65) described the propagation of MDV in spleen cell suspension cultures. Infection was established by exposure of freshly prepared spleen lymphocytes to cell-free virus, infected CKC cultures or lymphocytes obtained from infected birds. At 48-hour intervals passages were made by the addition of fresh spleen cells. Viral antigen expression, virus isolation and hybridization techniques were used to monitor the level of infection. It was found that most of the cells with active virus infection were bursa-derived lymphocytes, similar to the observations made by Shek et al. (66) that B-cells are a frequent target for cytolytic infection of lymphoid organs in vivo. It was subsequently found that T-cells also become infected in vitro, but less frequently than B-cells (65). So far, latent or transforming infections in vitro have not been described. A report that lymphocytes were transformed in vitro by MDV (67, 71), has not been confirmed and was possibly an error because the transformed cells were found to be positive for reticuloendotheliosis virus, and free of rescuable MDV and MDV antigens (B.W. Calnek, personal communication). In contrast to lymphocytes, macrophages were resistant to in vitro infection with MDV and HVT (68).

Chicken embryo kidney (CEK) cells had very limited susceptibility to infection with serotype 1 MDV (B.W. Calnek unpublished, quoted in 69) and serotype 2 SB-1 strain (K.A. Schat, unpublished). Attenuated MDV can replicate in CEK, albeit to much lower titres than in CKC (K.A. Schat, unpublished). On the other hand, HVT replicates equally well in CKC and CEK (69). The reasons for these and other differences in susceptibility of avian cells to infection of the three serotypes are unknown. It could well be that some differences among CEK, CKC, CEF and other avian cells related to cellular DNA replication pathways play a role in deter-

mining susceptibility to virus infection. Ware et al. (55) demonstrated that HVT requires intact host cell DNA for its replication in CEF. It is hard to relate the differences to putative herpesvirus receptors when cell-associated virus inocula are used, unless viral membrane antigens (see Section 5.4) are also recognized by such receptors and attract virus-containing cytoplasmic fragments (see below).

Several attempts have been made to infect mammalian cells. Both primary cultures and established cell lines were inoculated with cell-associated and cell-free MDV and HVT. Most investigators (59,70-72) were unable to detect evidence of virus replication. In general, six to ten blind passages were made and the cultures were examined for direct evidence of infection such as CPE development or presence of viral antigens (FA assays) and indirect evidence by bird inoculation. Hložánek and Sovova (73) found mammalian cells refractory to infection with MDV and HVT even after inducing artificial cell hybridization with inactivated Sendai virus. However, evidence that chicken-mammalian cell hybrids were induced was not presented. Earlier, Hložánek (74) had used this technique to increase the levels of MDV infection in CKC cultures. In contrast to these negative findings are the reports by Bedigian and Sevoian (75-78) and Elliott et al (79) that HVT and MDV can replicate in primary hamster and guinea pig kidney cell cultures and in hamster urogenital cells. However, Witter and Sharma (80) demonstrated that the presence of replicating virus after inoculation with cell-associated virus could be caused by surviving avian cells and they were unable to infect primary hamster and kidney cells with cell-free HVT and MDV using the same virus strains reported to initiate infection by others (75-79). These contradictory results reported for cell-free virus are hard to reconcile, but contamination with other herpesviruses or small differences in the virus strains were offered as possible explanations (80).

The absence of viral replication in newborn hamsters (60,73), immunosuppressed rhesus monkeys (22) and marmoset monkeys (81) are in support of the majority of the data that show mammalian cells to be refractory to infection with MDV and HVT.

4.3. In vitro replication.

Discrete foci will develop between 2 and 7 days post-infection depending on the virus strain, the number of passages in vitro and the

type of cell culture. In general, the foci consist of refractile rounded or fusiform cells. Syncytia are often formed and Cowdry type A intranuclear inclusion bodies can be seen. The three serotypes can be differentiated in CKC and CEF. Serotype 1 viruses cause only very small plaques in CEF and these cells are much less susceptible to infection than CKC. The reverse is true for serotype 2 viruses, since they form small plaques in CKC compared to CEF and the latter are the preferred cells for virus isolation and replication (6,10,82,83). HVT replicates well in both CEF and CKC and causes relatively large plaques in both cell types. Oncogenic viruses can be adapted to CEF by cocultivation of infected CKC or DEF with CEF. Virus attenuation will occur after a variable number of passages (usually 30 to 40 or more) in CKC or CEF, but apparently does not occur in lymphocytes. Ross _et al_. (84) rescued virus from high passage levels of the MD tumour cell line MDCC-HP1 and found it to be oncogenic and to produce A antigen. Schat _et al_. (submitted for publication) found the same for virus passaged in cultured spleen lymphocytes. The ability of attenuated virus to replicate in lymphocytes _in vitro_ and _in vivo_ was found to be absent or diminished. Examples of CPE induced by various viruses are shown in Fig. 3. The genetic source of the chickens from which the CEF or CKC cultures are derived is not an influencing factor in the replication of serotype 1 MDV (85), serotype 2 MDV (K.A. Schat and R.E. Pitts, unpublished data), or HVT (69).

In addition to differences in plaque morphology, Cho (86) reported that HVT could be differentiated from either serotype 1 or 2 MDV by the ability of only the former to replicate in QT35 cells, a chemically transformed quail fibroblast cell line.

Other changes can be noted in inoculated cultures; infection by HVT, as of many other viruses, results in an increased glucose uptake and production of lactic acid causing a change in pH (55). The induction of interferon has been reported after infection of CEF and CKC cultures with oncogenic MDV's, HVT and some apathogenic MDV's (87-89). The latter were not characterized for serotype. Infection of cultures with one serotype can interfere with the replication of virus of another serotype (89,90).

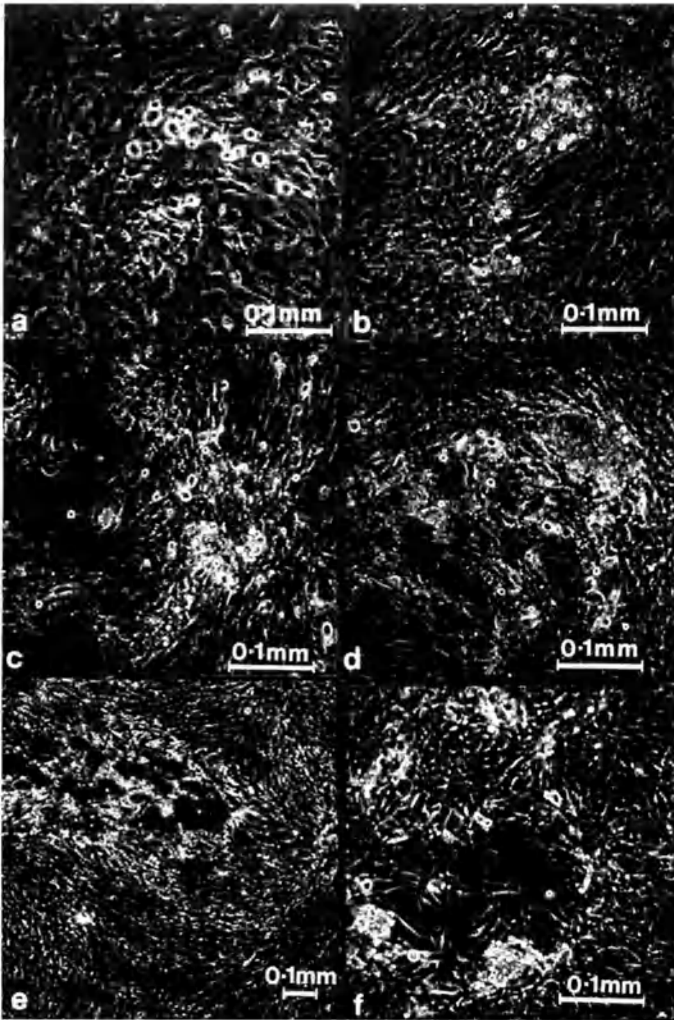

FIGURE 3. Cytopathic effects of MDV serotype 1 (JM-16 and attenuated JM-16) and 2 (SB-1) and HVT in chicken embryo fibroblasts (CEF) and chick kidney cells (CKC). a) A medium plaque of SB-1 in CEF, 4 days post-infection (dpi). b) A small plaque of SB-1 in CKC, 6 dpi. c) Small plaque of JM-16 in CEF, 7 dpi. d) Medium plaque of JM-16 in CKC, 7 dpi. e) Large plaque of attenuated JM-16 in CEF, 3 dpi. f) Large plaque of HVT in CEF 3 dpi. All photographs were made with phase-contrast.

Fabricant et al. (91) used chicken arterial smooth muscle cell cultures to study the effect of infection with cell-free MDV and HVT on the lipid metabolism of smooth muscle cells. Infection with MDV caused a marked increase in the number of cells positive for lipid using the oil red O staining technique. Chemical analysis of the lipids in MDV-infected cells indicated that these differed qualitatively and quantitatively from the lipids present in control or HVT-infected smooth muscle cells. It is not clear if the alterations are the result of an abortive infection, or that virus is replicated.

One of the remarkable features of MDV and HVT is their highly cell-associated character. With the exception of the feather follicle epithelium, virus replication in vivo and in vitro is cell-associated. Infectivity of tumour cells, infected lymphocytes or cell cultures can be readily destroyed by disruption of the cells by freezing and thawing, suspension in water or ultrasonic disintegration (59,60). On the other hand, Cook and Sears (92) obtained cell-free MDV by the disruption of cells in distilled water and Witter et al. (5) extracted cell-free HVT by ultrasonic treatment. Davis and Sharma (93) found evidence that MDV did not accumulate in cytoplasmic vacuoles, and MD virions in the cytoplasm underwent degradation. Cell-free, but always naked, particles were only seen when the infected cells had undergone cytoplasmic degeneration. They speculated that the destruction of virions was caused by excessive lysosomal activity in the cytoplasm of infected cells. The degenerative processes, however, do not prevent the transfer of virus from cell to cell. It was speculated that virus spread from infected cells to neighbouring cells occurred by intracellular bridge formation, because addition of antisera to the medium did not prevent virus spread to adjacent cells and secondary foci did not develop until infected cells became detached (60). Additional support came form the observation that treatment of CKC with inactivated Sendai virus increased the level of infectivity (74). In an elegant experiment using timelapse photography and labelled antibodies, Kaleta (89) demonstrated that virus-containing cytoplasmic fragments became detached from infected cells and were taken up by uninfected cells. The addition of cytochalasin B to the cell culture medium, which inhibits cell mobility, was found to prevent the formation of plaques (94).

The transfer of cell-associated virus from infected to uninfected cells is very rapid, being more than 50% complete within 30 minutes. The adsorption of cell-free virus is also a fast process, most of the virus being adsorbed in 30 minutes, and the process is normally completed after 2 hours (57). Penetration of cell-free HVT requires up to 3 hours (69). It had been noted when MDV and HVT were first isolated that virus replication could be inhibited by the addition of 5-iodo-2-deoxyuridine and 5-bromo-2-deoxyuridine (IUdR and BUdR, respectively) (1,5). Since then, many antiviral drugs have been developed and some have been tested against MDV and HVT _in vitro_ and _in vivo_. The importance of these studies is that the drugs can be used to investigate the viral replication. Phosphonoacetate (PA) and phosphonoformate inhibited virus replication in CEF and DEF, but not in chickens. The effect of these drugs is most likely by the inhibition of virus-induced polymerase (95,96). Interestingly, PA had no influence on the growth of MSB-1, a MD lymphoblastoid cell line. However, a decrease was found in the number of viral genome copies during cultivation in the presence of PA. Withdrawal of PA from the medium resulted in an increase of genome copies (97). A similar dichotomy was recently found with the newly synthesized drug FMAU, (1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-methyluracil), which was highly effective in preventing the replication of MDV and HVT in CKC, CEF, and lymphocytes, but did not seem to influence the presence of MDV in two cell lines (MDCC-CU12 and-CU36) (98). In addition to these drugs which are specific against herpesviruses, one other antiviral drug (Virazole = 1-D-ribofuranosyl-1,2,4 triazole-3-carboxamide) has shown to be effective _in vitro_ but not _in vivo_ against MDV (99).

4.4. Embryonated eggs.

Although largely superseded by the use of cell culture, embryonated eggs have been used for the isolation and propagation of MDV and HVT. Inoculation of the chorioallantoic membrane, yolk sac, allantoic cavity or by the intravenous route (100-103) will result in the formation of plaques on the chorioallantoic membrane and splenomegaly. The value of embryonated eggs for the isolation of virus is limited due to a graft-versus-host response, which can be induced by immunocompetent cells present in some inocula. Isolation of an agent therefore has to be

followed by a positive identification, e.g. FA test (104). Maternal antibody-free embryos have to be used to obtain optimal results (100, 101). The sensitivity of these assays is roughly comparable to that of cell culture assays, although cell culture attenuated virus does not replicate well in embryonated eggs (103).

Longenecker et al. (102) reported that embryos from genetically susceptible chickens were producing significantly more lesions than those obtained from resistant chickens (P-line versus N-line, line 7 versus line 6 and S strain versus K strain). This finding is of interest because differences are not detectable in CKC cultures from different genetic backgrounds (85). In addition, the early pathogenesis in birds is not different for genetically susceptible P-line and resistant N-line birds (105).

5. VIRUS REPLICATION

5.1. Introduction.

Two types of virus-cell interaction are important for an understanding of the replication cycle of MDV and HVT. These are similar to interactions described for other oncogenic herpesviruses (106). The first one is the productive infection in which virus replication occurs and invariably causes the death of the cell. This type of infection can be further divided into fully productive and productive-restrictive infections. The former is characterized by the production of enveloped, fully infectious cell-free virus, while the latter type does not result in the release of cell-free virus and intracellular virions are mostly nonenveloped. The second type of interaction is the nonproductive infection, in which there is only limited or no expression of the virus genome. Limited expression characterizes transformed T-cells while the absence of recognizable expression is considered typical for latent infection.

5.2. Productive infection.

Fully productive infection with the subsequent release of cell-free virus occurs for all three serotypes in the feather follicle epithelium (10,45,107). A possible exception was reported for the Dutch vaccine strain CVI 988, where virus replication could not be demonstrated in the feather follicle even though the virus is capable of bird to bird

spread. An alternate site for fully productive replication was not identified (108), so the difference may be quantitative rather than qualitative. In vivo, productive-restrictive infections can be found in lymphoid organs, epithelial cells and occasionally in tumours (109,110). Thus far, all reports on in vitro replication described the productive-restrictive type of infection. This type of infection is most likely similar in vivo and in vitro. Payne et al. (111) reviewed in detail the morphological changes in infected cells (see also Section 3.1 for the morphogenesis of the virus). At least three factors have been identified which play an important role in the actual replication of the virus, two of which are virus-induced. Productive and productive-restrictive virus replication is dependent on the presence of a viral-induced polymerase (112), while the replication of the resident viral DNA in tumour cells can use cellular polymerase (97). Apparently the same is true for the thymidine kinase. It was recently found that FMAU is a potent inhibitor of MDV and HVT replication, but it did not inhibit the multiplication of MDV cell lines (98). FMAU inhibits viral replication by the virus-induced thymidine kinase pathway (113). Another crucial factor for the replication of virus is the presence of arginine. In arginine-deprived cultures the viral DNA will not replicate, but some early antigens are produced (114) (see also Section 6.1).

5.3 Latent infection.

Thus far, the in vitro establishment of latent infection or transformation has not been reported. Consequently, the mechanism(s) of inducing and maintaining latency have not been studied in detail. It has been well established that both serotypes of MDV and HVT can persist in latent form in lymphocytes for the lifetime of the bird. Viral antigens or virions cannot be detected in the latently infected lymphocytes. The presence of MDV viral genome can be demonstrated, however, by cocultivation and by short-term culturing of the lymphocytes, which will cause the production of virions (115) and viral antigens (116). The induction of viral antigens was used to identify the populations of latently infected lymphocytes. Dual fluorescence techniques were applied for the detection of lymphocyte surface markers with monoclonal antibodies and rhodamine-labelled conjugate, and MDV antigens with a fluorescein-labelled anti-MDV serum. The majority of the latently

infected cells were characterized as Ia-bearing T-lymphocytes and a minority were identified as B-cells (66,117).

The identity of lymphocytes latently infected with either HVT or SB-1 has not been firmly established. Shek et al. (118) were only able to identify the cells as non-B-cell and non-macrophage. The problem, especially with HVT, but also with SB-1 (116), is the absence or low frequency of the in vitro development of a cytolytic infection during incubation of latently infected lymphocytes, identifiable with the FA test. Positive identification with a dual FA test is therefore impossible.

One of the important questions in the biology of herpesvirus is how latency is induced and maintained. Latency was originally defined as the presence of the virus genome without detectable expression in the form of virions or viral antigens. Lately, it has been questioned if this is indeed the case. In a recent review (119) on EBV it was mentioned that at least two antigens are expressed in latently infected, but not transformed, lymphocytes. One is LYDMA, a lymphocyte dependent membrane antigen detectable with sensitized T-lymphocytes, and the other is EBNA (EB-nuclear antigen). There is also evidence of extensive transcription of viral DNA. It can be argued that it might be difficult to distinguish between these latently EBV-infected lymphocytes and truly transformed lymphocytes. However, there is also evidence with other herpesviruses (e.g. herpes simplex virus) that transcription of viral DNA occurs during latency (120). The possibility of genome expression in lymphocytes latently infected with MDV or HVT has not been investigated in great detail. It was recently found that a MD cell line MDCC-BO1 (T), contained RNA that could be hybridized to DNA from both HVT and MDV (121). This cell line was established from spleen cells obtained from an HVT-vaccinated chicken. However, it has not been established if MDV or HVT or perhaps both are responsible for the maintenance of the transformation (122). Until that question has been resolved it will be difficult to evaluate this model for the study of genome expression during latency. Another finding that might be relevant for the study of latency has been the observation that lymphocytes expressing MATSA appear as early as 5 days post-infection in birds infected with either serotype of MDV or HVT (123,124). Moreover, MATSA-bearing cells can be

detected over long periods in HVT-infected chickens (125). MATSA had been originally described as a Marek's disease tumour-associated surface antigen present on lymphoblastoid cell lines and tumour cells (126,127). The induction of MATSA by non-oncogenic strains of virus is therefore of considerable interest and the question was asked if MATSA could be part of a limited genome expression needed to induce and maintain latency or transformation. However, Calnek *et al.* and Sharma (116,128) were unable to identify MATSA on latently infected cells and the role of MATSA during infection needs further study.

5.4. Transformation.

The development of neoplastic cells is the ultimate consequence of infection with serotype 1 MDV. The target cells for transformation are T-cells, even though transformed cells lack the major T-cell antigen (84). More recently, using monoclonal and allo- antibodies, tumour cells have been shown to be activated T-cells, expressing Ia antigen (129). Studies with monoclonal antibodies will be very useful to further characterize the tumour cells. It has already been found that a thymocyte antigen is lacking but an antigen present on circulating T-cells can be found (K.A. Schat, A.A. Benedict and C-LH. Chen, unpublished data).

The establishment of cell lines was once a difficult procedure, but recently several groups have been successful and numerous new cell lines have been reported (see Chapter 3). Most cell lines carry the complete genomic information and virus production can be turned on either *in vitro* or *in vivo*. A number of cell lines are called nonproducers (e.g. TKT-1, 130), but on inoculation into chickens, virus production is turned on and these cell lines are therefore not truly nonproducers. An interesting exception are the cell lines established from the JMV transplantable tumour. It has been speculated that the MDV genome is incomplete in these cells (131,132). Other true nonproducer cell lines have been reported, but information on the presence of complete or incomplete viral genome is lacking (133,134).

Transformation of the lymphocyte results in limited expression of the viral genome and subtle changes in the expression of cellular genes. All tumour cell lines express MATSA, but the antigenic composition of MATSA seems to vary between cell lines (135-137). There is also an

enhanced expression of embryonic antigens (134,138,139), Forssman antigen (140), and Hanganutziu-Deicher type heterophile antigen (141); the importance of these antigens is not clear. Most cell lines express viral membrane antigens (VMA) and viral internal antigens (VIA) in a small percentage of the cells. Quite often these antigen-positive cells will contain virus particles in the nucleus. VIA expression can be enhanced by treatment with IUdR and BUdR (142,143), and the tumour promoter 12-0-tetradecanoylphorbol-13 acetate (TPA) (144). Calnek et al. (143) compared 31 cell lines for differences in VIA and VMA expression. It was found that the cell lines established from tumours induced by the more oncogenic virus strains (GA-5, RB-1B) had, in general, a lower level of viral antigen expression than those established with the less oncogenic JM-10 virus. Perhaps, the higher levels of expression indicate a less stringent control of the virus genome resulting in an easier turn-on and subsequent death of the cell. If this explanation is correct it might help explain the differences in oncogenicity. In combination with the finding that MDV-DNA polymerase can be detected in MSB-1 cells cultured for 6 days (Leinbach, quoted in 112), it could be speculated that the degree of oncogenic potential might be related to the degree of repression of the viral polymerase gene.

5.5 Cellular interactions.

Three possible consequences of infection have been discussed: 1) two types of productive infection, 2) latent infection and 3) transformation. Calnek (145) mentioned that the consequences of infection could be determined in part by the cell type and he speculated that different (sub)populations of lymphocytes might be involved as targets for the productive-restrictive infection, the latent infection and the transforming infection. Recent work has shown that this hypothesis might be basically correct. It was mentioned before that the main target cell for the productive-restriction infection is the μ-bearing B-lymphocyte (66), although a small percentage of T-cells can be involved (117). Soon after the cytolytic phase the latent infection is already present mainly in Ia-bearing T-cells. It was then hypothesized that the majority of the T-cells become infected after activation. The activation of the T-cell could be a response to the early lytic infection of the B-cell. Perhaps Ia antigen, which plays an important role in cell-to-

cell communication, is important for the transfer of virus. The finding that tumour cells are activated Ia-bearing T-cells would be consistent with the idea that either transformation occurs directly after the infection of the activated cell or that a latent phase precede the transformation. Virus replication has to be inhibited in order for transformation to occur, but a limited genome expression might be expected. It is not clear if this control of virus genome expression is regulated intrinsically or extrinsically. The former would be cell-type dependent while the cell-type would be unimportant for the latter. Perhaps small differences in cellular DNA replication between B- and T-cells are responsible for the absence of transformation of B-cells.

6. VIRAL ANTIGENS

6.1. Molecular aspects.

In vivo and *in vitro* infection with MDV and HVT results in the production of structural and nonstructural antigens. Most of these antigens have been poorly characterized due to the cell-associated nature of the virus.

Chubb and Churchill (146), using an AGP test, described the presence of up to six antigens in extracts of infected CKC cultures. The three most common antigens were named A, B and C. A antigen can be detected in supernatant fluids of cell cultures in contrast to the B and C antigens, which are strictly associated with cellular material. A soluble antigen can be extracted from feather follicle epithelium, which seems to be identical to the A antigen (149,150). Attenuation of serotype 1 MDV will cause a marked reduction in the production of A antigen (see Section 2), but the presence of A antigen is not a marker for oncogenicity as originally proposed (30).

The A antigen has been studied extensively by several groups and was identified as a glycoprotein (gp) (148-151) and was tentatively identified as gp 5. Estimates on the molecular weight (mol. wt.) have varied to a large degree, but recently three independent groups have reported the mol. wt. to be between 60,000 and 65,000 daltons (151-153). The presence of an antigen similar to the A antigen has also been described for HVT (5) and for serotype 2 viruses (12). Van Zaane *et al.* (12) found that gp5 was more alkaline for HVT and SB-1 (serotype 2 MDV) than

for their reference strain CVI 988. In addition, the gp5 of SB-1 had a lower mol. wt. than that of the two other serotypes.

The B antigen has been less extensively studied than the A antigen. It is an antigen commonly found in cells infected with serotype 1 MDV and HVT (154,155). Although not formally proven, it is likely also to be present in serotype 2 viruses (9). In contrast to A antigen, it is produced in tumour cell lines together with the C antigen (84). The B antigen is a glycoprotein that shares a number of characteristics with the A antigen such as its resistance to pH 2 and treatment with 1 M urea-0.05% Brij 35. It differs from A antigen in its slightly higher mol. wt. and lower isoelectric point.

Chen et al. (156) detected eight viral proteins by analysis of partially purified virions by polyacrylamide gel electrophoresis (PAGE). Later studies by Ikuta et al. (152) demonstrated the presence of at least 46 polypeptides in productive-restrictive infection, by immuno-precipitation with antibodies purified by affinity chromatography followed by PAGE. It was suggested with this technique that the polypeptides of MDV and HVT had more cross-reactive determinants than the glycoproteins (157). Van Zaane et al. (12,151) reported 35 polypeptides, of which 18 were identified as glycoproteins. They mentioned that, in addition to gp5 (tentative A antigen), p4/p5/p6 complex, gp3 and gp8/gp9 complex were different for the three serotypes. For further details see Chapter 5.

FA techniques (56,158,159) can be used to detect viral antigens in the nucleus and cytoplasm of fixed cells. This technique does not allow a clear differentiation of the antigens unless monoclonal or mono-specific antibodies are used as reagents. Most often the antigens detected by this technique are referred to as viral internal antigens (VIA). The recent development of monoclonal antibodies against MDV and HVT will be important for the characterization of the different viral polypeptides (13,160). It has already been demonstrated that a number of monoclonal antibodies detected cross-reactive antigenic determinants on MDV and HVT polypeptides. These cross-reactive determinants could be important for a better understanding of the protective immunity by HVT and SB-1, especially in view of the very limited amount of DNA homology between the different serotypes (see Chapter 5).

The use of the FA technique on live, infected CKC demonstrated the presence of a membrane antigen, referred to as VMA (viral membrane antigen) (161). It was proposed that this VMA was related to the A antigen, because virus attenuation resulted in the loss of both antigens (162). The production of VMA is often seen in conjunction with the production of viral particles and it was suggested that VMA represents a structural viral protein (163,164). Mikami et al. (165), however, described the presence of early and late VMA. The former could be detected in arginine-deficient medium, suggesting that it was produced prior to DNA replication, while the latter required DNA replication. Later studies demonstrated that early and late VMA could also be detected on HVT-infected cells (166). Antigenic differences were demonstrated beween HVT-induced intracellular and membrane antigens (167). Recently, it was reported that A antigen and the late VMA of HVT and MDV had at least cross-reactive determinants detectable with monoclonal antibodies (160). It can be expected that monoclonal antibodies will become very important for the further study of the relationship between VIA's and VMA's.

Thus far, two virally induced enzymes have been reported. Kit et al. (168) described a thymidine kinase induced by HVT, which differed little from the chick mitochondrial thymidine kinase except for a larger sedimentation coefficient. Recently, Schat et al. (98) demonstrated an increase in thymidine kinase activity in CKC infected with HVT and JM-16, indicating the presence of a virally induced thymidine kinase. A DNA polymerase induced by MDV (161) and HVT (95) has been described. The mol. wt. of this polymerase was estimated to be 100,000 daltons and it can be distinguished from cellular A and B polymerases by its chromatographic behaviour on phosphocellulose, sedimentation coefficient and catalytic properties (see Chapter 5).

In addition to the antigens detectable in productively infected cells, an antigen has been observed on the surface of transformed cells (MATSA, see also Sections 5.2 and 5.3 and Chapter 5). This antigen has been characterized as a polypeptide with a mol. wt. of 40,000 daltons (170). It is not clear if MATSA is a viral-coded protein, a modified major histocompatibility (MHC) antigen or a derepressed cellular antigen unrelated to MHC antigens. Coleman and Schierman (171) were unable to demonstrate co-capping of MATSA with MHC antigens, which would suggest

that it is not a MHC antigen. Recently, monoclonal antibodies have been developed against MATSA (172,173) which will enable further biochemical characterization studies.

6.2. Biological characterization.

Most studies on the biological characterization of viral antigens and MATSA have been done with crude preparations. The experiments generally were studies on protection by inoculation of inactivated virus-infected cells, subcellular preparations or tumour cells. Most of these studies will be reviewed in Chapter 7 dealing with immunity. A few comments have to be made in connection with the viral antigens. It has been suggested that the A antigen of HVT is expressed on the viral membrane and antibodies prepared against it will neutralize the virus (174). Kaaden and Dietzschold (175,176) demonstrated the presence of at least two viral proteins on plasma membranes obtained from HVT or MDV-infected cells. It was suggested that the plasma membrane contained B and C antigens. These preparations were indeed immunogenic and could protect against challenge. Similarly prepared preparations from MSB-1, a tumour cell line, were only weakly immunogenic. Lesnik and Ross (177) reported basically similar data using soluble and insoluble antigens. Some HVT-induced glycoproteins which were partially purified were able to induce neutralizing and precipitating antibodies. Challenge experiments demonstrated at least partial protection (178). Numerous other groups have used inactivated tumour or virus-infected cells and no definitive conclusions can be drawn in regard to specific immunogenic polypeptides.

6.3. Cross-reactions with other herpesviruses.

It has been mentioned above that MDV and HVT share a large number of antigenic determinants, but that they can be differentiated into different serotypes. The three serotypes of MDV do not cross-react with at least 12 other avian herpesviruses in plaque reduction tests using monospecific antisera. The following viruses were included: duck enteritis virus, infectious laryngotracheitis virus and a number of viruses isolated from pigeons, falcons, owls and other birds (179). The presence of a cross-reactive antigen in MDV and EBV has been suggested by indirect FA and AGP tests (180,181). There was a suggestion that the titres against EBV and MDV were higher in people working with chickens than in office workers, but this could have been caused by exposure to

dust and dander containing MDV antigens (182). Stevens et al. (183) were unable to confirm these data, however. They found a cross-reaction between MDV-positive sera and an antigen present in an EBV-negative, lymphoblastoid cell line. The presence of a group-specific antigen in nucleocapsid preparations was described for Lucké frog virus, herpes simplex virus, cytomegalovirus and MDV (184). Similar types of results were obtained for MDV, herpes simplex virus and pseudorabies virus (185) and for EBV, MDV and bovine infectious rhinotracheitis virus. The importance of these cross-reactive antigens is of course limited, because MDV does not replicate in mammalian species (see Section 4.2).

7. CONCLUDING REMARKS

Remarkable progress has been made during the last 15 years in our understanding of the virology of MDV and HVT. Future research on the exact nature of replication and viral antigens will be greatly facilitated by the new techniques of molecular cloning and monoclonal antibodies. It will be important to elucidate the mechanism(s) which govern the productive-restrictive, latent and transforming types of infection.

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5. MOLECULAR BIOLOGY OF THE VIRUS

L.J.N. ROSS

1. INTRODUCTION

The study of the molecular biology of Marek's disease virus (MDV) started more than a decade ago when Lee et al. (1,2) characterized its DNA. However, progress in the analysis of virus structural proteins has been hampered by the unusual cell-associated properties of MDV and the lack of enveloped infectious particles in cultured cells. For these reasons and because of the shortage of conditional lethal mutants, genetic analysis of virus functions by classical approaches has not been possible. The recent progress in molecular cloning of MDV (3,4) and the development of monoclonal antibody to virus-specific epitopes (5,6) have provided alternative approaches which hold great promise for furthering our understanding of oncogenesis and for defining virus structural components and their functions.

In this Chapter, attention will be focused on viral DNA and proteins with emphasis on the differences between strains. This is followed by sections on virus replication and inhibitors of virus growth. In the final section, the structure and expression of viral DNA in transformed cells is examined.

2. VIRAL DEOXYRIBONUCLEIC ACID

2.1. General properties

The DNA of MDV is a linear, double-stranded molecule approximately 175 kilobase pairs (Kbp) long. It is similar in size to Epstein-Barr virus (EBV) DNA but slightly larger than the DNA of herpes simplex virus (HSV) and of herpesvirus of turkeys (HVT). In neutral sucrose gradients, DNA of the JM strain of MDV has a sedimentation coefficient of 56S corresponding to a molecular weight of 120 x 10^6 daltons (2). Molecular

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weights of 110 x 10^6 daltons were reported for the GA and C2 strains (7). These differences are probably not significant since estimation of the contour length of viral DNA by electron microscopy (8) has indicated that the genomes of GA, JM, Cal-1 and CVI 988 strains were similar in size and corresponded to the mol. wts. estimated by sedimentation analysis (120 x 10^6 daltons). However, DNA of strains that have been attenuated by serial passage is larger by 2 to 3 Kbp (see Section 2.5). In alkaline sucrose gradients, MDV DNA sedimented as a heterogeneous population indicating the presence of "nicks" or single-stranded gaps, a property shared with other herpesviruses (9).

The density of MDV DNA in neutral CsCl is 1.705 g/ml, close to that of chick cell DNA (2). Assuming that the bases are unmodified, the density reported corresponds to 46% C + G. No difference in the density of the DNA of the GA, JM and C2 strains was found (7). However, changes in buoyant density during serial passage of virus in a cell-associated form have been reported by Tanaka _et al_., (10). They demonstrated that DNA isolated from plaque-purified virus banded as a homogeneous peak (density 1.705 g/ml) until six passages _in vitro_, whereas DNA obtained after 15 passages was heterogeneous in density and had a major peak of density 1.700 g/ml. It is not known whether these changes are the result of intramolecular rearrangements in which DNA's of lower density displaces DNA of higher density in other parts of the genome as has been reported for pseudorabies virus (11) and HSV (12), or of recombination with cell DNA. Although changes both in density and in restriction enzyme patterns occur rapidly during serial passage (see Section 2.5), it is not clear whether these are causally related since the restriction enzyme patterns of viral DNA of high and low density are reported to be similar (13).

The DNA of HVT (mol.wt. 103 x 10^6 daltons) is smaller in size but is of higher density (1.707 g/ml) than MDV DNA. It is also linear, double-stranded and contains "nicks" as found for MDV DNA (7,14). DNA of a highly passaged HVT (HVT-HP) (7) which had lost its capacity to confer protective immunity to MD appeared to be similar in size and in buoyant density to the DNA of a vaccine strain (HVT-01) that had been passed 20 times. Moreover the restriction enzyme patterns of HVT-01 were identical to those of HVT-HP. It is possible that minor differences in DNA

sequence not detectable by restriction enzyme analysis could account for the differences in the biological properties of HVT-HP and HVT-01. Further studies are required to resolve this question.

MDV DNA has been shown to be infectious both in vitro and in vivo (15). The specific infectivity was 10 pfu/μg in chick embryo fibroblasts (CEF). The latent period of tumour development after intra-abdominal inoculation was 6 weeks. These are interesting observations since they open up the possibility of investigating gene functions by transfection experiments using modified viral DNA.

2.2. Purification

Purification of MDV DNA is complicated by the fact that its density is similar to that of chick cell DNA. Furthermore, because of the cell-associated nature of the virus which limits the proportion of cells that can be infected, the yield of virus particles is usually poor. Several methods have been described which provide viral DNA suitable for restriction enzyme analysis and cloning. The method described by Kaschka-Dierich et al. (16) involves the use of particles released in the medium and should in theory result in virus less contaminated with cell DNA than virus derived from disrupted cells. After treatment with DNase, virus particles were purified by centrifugation in sucrose gradients prepared in D_2O and centrifuged to equilibrium. Purified virus was lysed with sarcosyl and treated with pronase. The viral DNA was further purified by centrifugation to equilibirum in CsCl and the yield was approximately 8 μg/litre of medium. The DNA obtained does not appear to be contaminated with cell DNA since RNA prepared using the DNA as template does not hybridize to cell DNA.

Other methods of purification involve extraction of virus nucleocapsids from the cytoplasm of infected cells with Triton or NP 40 followed by rate zonal centrifugation in sucrose gradients (17) or centrifugation to equilibrium in CsCl (3). High mol. wt. viral DNA is released by treatment with 1% sarcosyl and pronase at 37°C overnight. The DNA obtained is suitable for restriction enzyme analysis but is not suitable for making probes because of cell DNA contaminants, mainly ribosomal DNA. Further purification by rate zonal centrifugation in 10 to 30% glycerol gradients containing sarcosyl yields 56S viral DNA substantially free from cell DNA (7,17). Usually 5 to 10 μg of high mol.

wt. viral DNA are obtained from 10^9 cells.

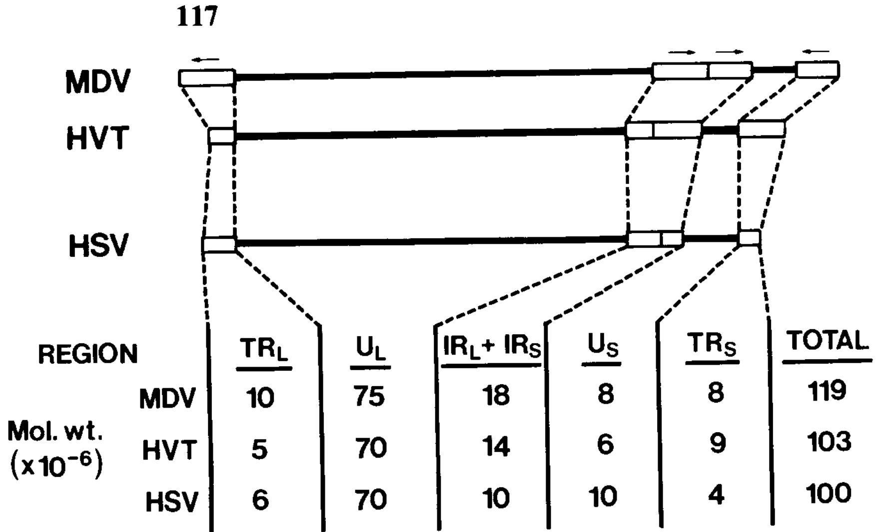
2.3. Structure

The structure of MDV DNA closely resembles the structure of HSV and not that of the other lymphotropic oncogenic viruses EBV and herpesvirus saimiri. Electron microscopy of partially denatured DNA (8) showed that both MDV and HVT DNA's contain long and short regions of unique nucleotide sequences (U_L and U_S respectively) each enclosed by inverted repeat sequences (TR_L, IR_L) and (TR_S,IR_S) as shown in Fig. 1. The total extent of inverted repeats is greater in MDV (36 x 10^6 daltons or 54 Kbp) compared to HVT (28 x 10^6 daltons or 42 Kbp). The U_S and possibly also U_L regions of MDV are also longer. The structure of several strains examined were similar. Single-stranded tails observed at the junction of inverted repeats in a proportion of the molecules of partially denatured MDV DNA probably indicate occasional heterogeneity as has been found in the terminal repeats of HSV (18). The structure noted for MDV and HVT predicts that recombination between the ends of the molecule and the internal inverted repeats could occur, resulting in inversion of U_L and U_S. Unfortunately, it has not been possible to demonstrate inversion by restriction enzyme analysis because the enzymes used so far have cleavage sites within the repeat regions and do not therefore generate 0.25M fragments which are characteristic of inversion of both U_L and U_S.

2.4. Molecular cloning and mapping of restriction enzyme sites

A partial library of EcoR1 fragments of HPRS-16/att, cloned in the plasmid pBR322, has been obtained (3) and has been shown to comprise 85% of the viral genome (19). Most of the fragments that have not yet been cloned are located in the repeat regions flanking the long unique sequence U_L. A number of cloned fragments were similar in size but were unrelated since they produced different patterns upon subsequent digestion with other enzymes (19). It now seems likely that the multimolar fragments seen in EcoR1 digests of HPRS-16/att DNA are not due to multiple copies of identical fragments (indicative of defective DNA's) but are the result of co-migration of unrelated fragments of similar size.

Clones of Bam H1 fragments of the GA strain have been obtained using various cloning strategies (4) involving the use of the plasmids pACYC



REGION		TR_L	U_L	$IR_L + IR_S$	U_S	TR_S	TOTAL
Mol. wt. ($\times 10^{-6}$)	MDV	10	75	18	8	8	119
	HVT	5	70	14	6	9	103
	HSV	6	70	10	10	4	100

FIGURE 1. Comparison of the genome structures of MDV, HVT and HSV determined by electron microcopy. U_L and U_S refer to the long and short regions of unique sequences. TR_L and TR_S are the terminal repeats and IR_L and IR_S are internal repeats. (From Cebrian et al. (8) by kind permission.)

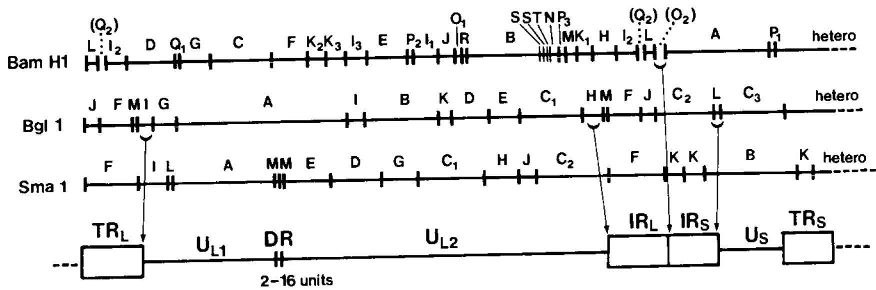


FIGURE 2. Physical maps of the GA strain of MDV DNA. Note presence of direct repeats (DR) which separate the long unique sequence U_L into U_{L1} and U_{L2}. (From Fukuchi et al. (4) by kind permission.)

184, and pBR 322 and the cosmid vector pHC 79. Most of the restriction fragments were cloned except for Bam H1 O_2 which spans the IR_L/IR_S junction and Bam H1 Q_2 which maps within IR_L. As has been found for the EcoR1 clones, many of the Bam H1 clones are of similar size but have unique sequences. Thus there are three K clones, three I clones, three P clones, two Q clones, two O clones and two S clones (Fig. 2).

Early work on mapping of restriction enzyme sites was hampered by the shortage of MDV DNA and the results were incomplete (20). However, as the structure of MDV DNA became known and clones of viral DNA became available, mapping has become possible. Fig. 2 shows maps of Bam H1, Sma 1 and Bgl 1 restriction sites of the GA strain which have been constructed by Fukuchi _et al_. (4). The inverted repeats flanking the long unique region U_L contain the Bam H1 restriction fragments L, Q_2, I_2 and part of H and D. The short unique region (U_S) is bounded by another set of inverted repeats which comprises part of Bam H1 fragment A and a heterogeneous terminal fragment. Three features of MDV DNA are of interest: 1) The presence of single repeats (2 to 16 units long), reminiscent of direct repeats in EBV DNA, which divides the U_L region into two regions U_{L1} and U_{L2}, 2) The presence of heterogeneous terminal fragments which are not found internally, and 3) The presence of sequences homologous to Bgl 1 M fragment (located near the junction IR_L/U_{L2} and TR_L/U_{L1}) throughout the viral genome.

2.5. Restriction enzyme analysis of strains of serotype 1

The restriction enzyme digestion patterns of several strains of serotype 1 are very similar (7,13,16,21,22) as shown in Fig. 3. Minor differences have been observed between GA,CVI-988,JM and Cal-1 strains (16) and between GA, HPRS-16, RB-1B and Ala-8 (23). Some of the differences noted are probably strain-specific since they occur in regions of viral DNA that do not change with serial passage. However, differences in the mobility of the fragments EcoR1 F (6.8 Kbp), Bam H1 H (5.4 Kbp) and Bam H1 D (11.8 Kbp) cannot be used to differentiate between strains since these fragments increase in size during continuous growth of the virus (Fig. 3). It is now known that changes in Bam H1 H and D fragments, first reported by Hirai _et al_. (13) for the BC, GA, JM and C2 strains, also occur during passage of HPRS-16. It is known further that the changes are located in the repeat regions flanking the long unique

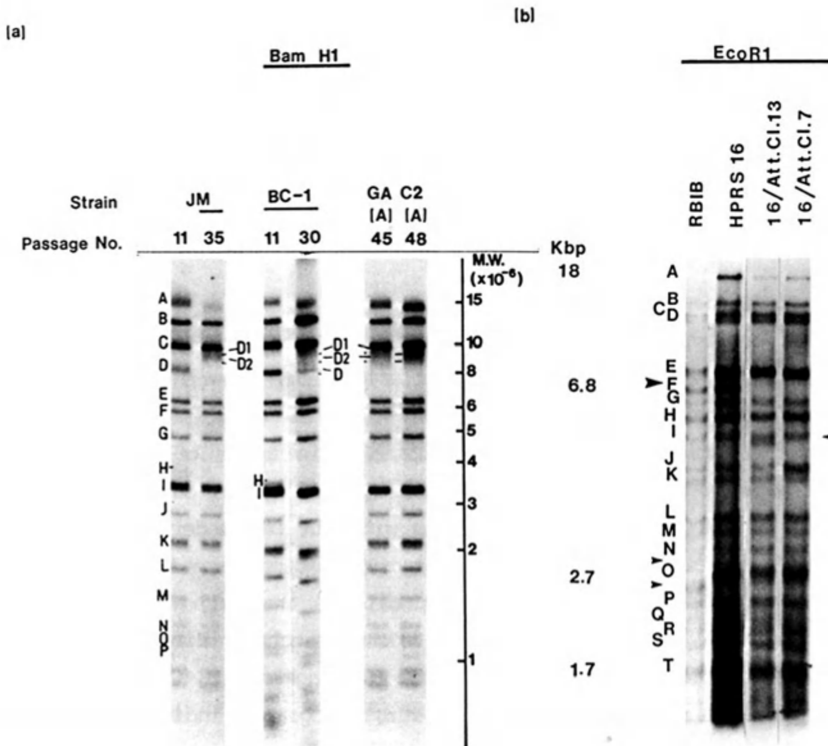


FIGURE 3. Restriction endonuclease patterns of ^{32}P-labelled DNA from strains of serotype 1. Data adapted from Hirai et al. (13) (a) and from Ross et al. (23) (b) by kind permission. The patterns obtained with different strains are similar except for the fragments Bam H1 H, Bam H1 D and EcoR1 F which increase in size during serial passage. Small arrows in (b) indicate differences which are probably strain-specific.

sequence U_L (22) and that the increase in the size of these fragments is probably due to expansion (tandem repetition) of a 150 bp sequence within the repeat region (24,25). Expansion of the Bam Hl A fragment has also been reported (25). Analysis of the DNA of several plaque-purified isolates of HPRS-16/att has shown that the increase in the size of EcoRl F, Bam Hl H and D fragments is not due to the accumulation of defective molecules during passage since virus with altered fragment sizes is infectious (23). It is probable therefore that attenuation arises by selection of infectious particles containing alterations in regions of viral DNA that are not essential for infectivity but which could be important for pathogenicity. However, it is not known whether the changes that occur in the repeat regions are responsible for the loss of virulence. Definite evidence could probably be obtained by conversion of attenuated virus to the virulent genotype by recombination with the DNA fragments containing the putative genes associated with virulence. This may now be possible since clones of MDV DNA are available.

2.6. Homology between serotypes

Viruses of serotype 1, 2 and 3 each have a unique restriction enzyme pattern (7,16,22) (Fig. 4). There is little homology between the three serotypes as determined by reassociation kinetics experiments under stringent hybridization conditions (7,16,22,26), in spite of antigenic similarities between them. In Southern blot hybridization, only the Bam Hl J fragment of HVT formed stable hybrids with MDV DNA under stringent conditions (27). It has been estimated that the most conserved sequences are 400 bp long. Using less stringent conditions that allow detection of homology between sequences that are 30% mismatched, Hirai et al. (27) noted weak homology between HVT and Bam Hl fragments B, C, D, E, F, G, I and K of MDV. Densitometry of the autoradiographs indicated that the overall degree of homology between MDV and HVT under these relaxed conditions of hybridization was approximately 5% when compared to the homologous reaction. When hybridization was carried out under relaxed conditions in the presence of dextran sulphate, bands of comparable intensities were observed in homologous and heterologous reactions. The overall degree of homology between MDV and HVT was estimated to be approximately 70% under these conditions (28). It could be argued

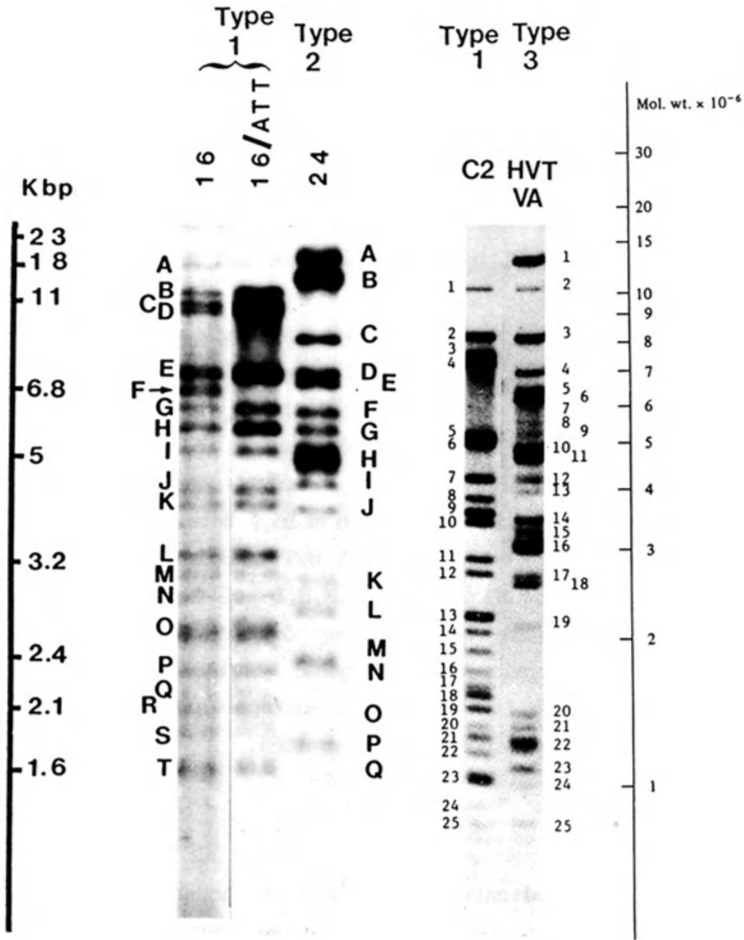


FIGURE 4. EcoR1 cleavage patterns of ^{32}P-labelled viral DNA. Virus strains of serotype 1, 2 and 3 each have a unique restriction enzyme pattern. (Data from Ross et al. (22) and Hirai et al. (7) by kind permission.)

however, that Southern blot hybridization in the presence of dextran sulphate overestimates the degree of homology because the DNA fragments are large (2 to 23 Kbp) and dextran sulphate promotes the formation of DNA networks which enhances the hybridization signal. Definite conclusions regarding the degree of homology between serotypes must await the results of reassociation kinetics experiments in solution using fragments of DNA 100 to 500 bp in size. Ideally, they should await the results of sequencing experiments.

Results of hybridization between HPRS-24 and HPRS-16 have shown that although several fragments cross-hybridized, under relaxed conditions, the degree of homology judged by the intensity of the bands in the autoradiograms was low compared to the homologous reaction (Fig. 5). This indicates that only a proportion of the sequences in the restriction enzyme fragments formed stable hybrids with labelled heterologous DNA.

In Fig. 6, an attempt has been made to relate the regions of homology between the three serotypes to the physical map of MDV. Sequences located in the Bam Hl B and F fragments mapping in the U_L region appear to be the most conserved ones. There is also some indication of quantitative and qualitative differences in homology between the serotypes, as shown for example by the stronger homology between the Bam Hl fragment E of GA virus and HPRS-24 than with HVT and conversely by the stronger homology between Bam Hl fragments N, H, I_2 of GA virus and HVT. One would expect therefore quantitative differences in the antigenic relatedness of some type common antigens. Evidence for the presence of type common and of type specific epitopes on the same glycoprotein molecules (29) is consistent with this idea.

3. VIRAL PROTEINS

3.1. Structural proteins

Because of the cell-associated nature of MDV, it is not possible to obtain sufficient quantities of purified enveloped particles from cultured cells for biochemical analysis. Attempts to purify mature virus from extracts of feather follicle epithelium have been unsuccessful. Consequently, little is known about the structural proteins of MDV. Early studies in this area lacked sensitivity (30). However,

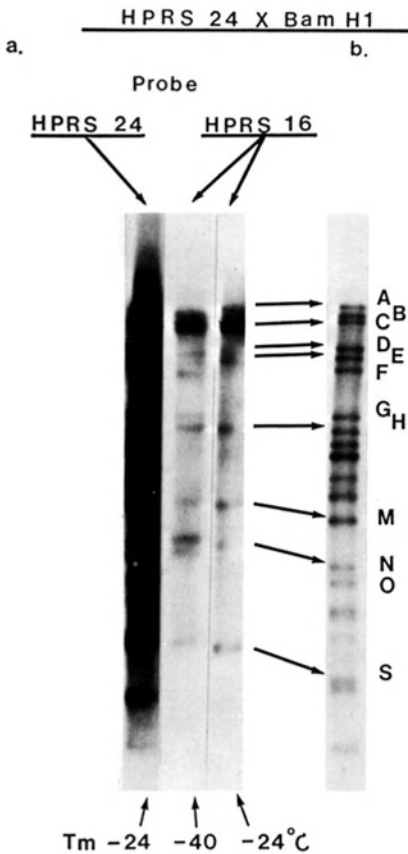


FIGURE 5. Southern blot hybridization showing homology between HPRS-16 and HPRS-24. a) Blots of Bam H1 fragments of HPRS-24 DNA were hybridized to ^{32}P-labelled HPRS-16 DNA and HPRS-24 DNA. Although several fragments of HPRS-24 hybridize to HPRS-16 under stringent conditions (Tm -24°C) and additional ones cross-hybridize under less stringent conditions (Tm -40°C), the degree of homology between HPRS-16 and HPRS-24 is low compared to the homologous reaction judging from the relative intensitiy of the autoradiograms. b) Arrows allow identification of the Bam H1 fragments of HPRS-24 that cross-hybridize with HPRS-16 (From Ross et al. (23) by kind permission.)

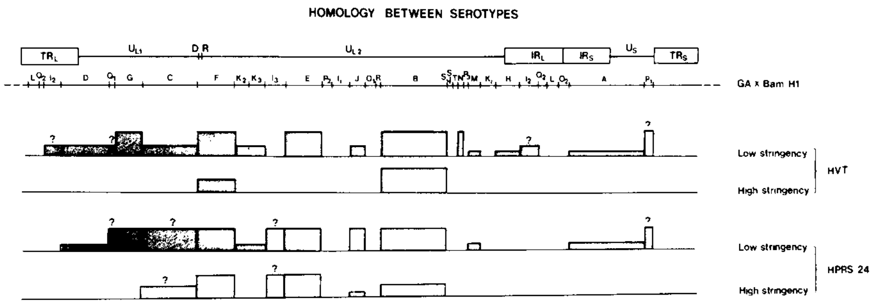


FIGURE 6. Summary of cross-hybridization results showing map positions of homologous sequences among the three serotypes. The linkage map of Bam H1 fragments of the GA strain is that of Fukuchi et al. (4) (by kind permission). The conditions of hybridization were T_m -40°C (low stringency) and T^m -24°C (high stringency). Histograms show relative intensity of hybridization on an arbitrary scale. (?) indicates tentative map positions of some cross-hybridizing sequences. (From Ross et al. (23) by kind permission.)

characterization of the envelope glycoproteins that interact with neutralizing antibody can now be achieved without the need for purified virus particles by immunoprecipitation of labelled cell extracts using monoclonal antibody. Using this approach, three MDV glycoproteins (mol.wt. 115/110,63 and 50) which share epitopes with HVT have been identified (31,32). There is evidence that gp 115/110 is a precursor molecule which is cleaved to produce the mature glycoproteins gp 63 and gp 50 which are probably located in the virus envelope. Nucleocapsids on the other hand can readily be purified and preliminary experiments suggest that there are four major capsid proteins (mol.wt. 140, 50, 35, 32 x 10^3 daltons) as shown by SDS polyacrylamide gel electrophoresis of purified preparations (R. Haynes and L.J.N. Ross, unpublished results). These results need to be confirmed by "Western" blot analysis using labelled antibody.

3.2. Virus-specific enzymes

3.2.1. DNA polymerase. MDV induces increased levels of DNA polymerase activity after infection of CEF. The virus-induced DNA polymerase has been partially purified by chromatography on phosphocellulose and characterized (33). Its mol.wt. was estimated to be 100 x 10^3 daltons by sedimentation analysis. It could be differentiated from cellular polymerase by its mol.wt., by its inability to use either poly A.oligo dT or poly dC.oligo dG as template and by its sensitivity to ammonium sulphate. Since MDV mutants resistant to phosphonoacetate (an inhibitor of DNA polymerase) induce polymerase with altered properties, it is likely that this enzyme is virus-coded (34,35).

3.2.2. Thymidine kinase. Thymidine kinase is induced in CEF following infection with HVT (36). The enzyme could be differentiated from cellular kinase by its location in cytosol but not in mitochondria, by its higher sedimentation coefficient (corresponding to mol.wt. of 87,700 daltons) and by its distinct isoelectric point (pI 6.1) compared with the enzyme of uninfected cells. Increased levels of thymidine kinase have also been found in CEF infected with HPRS-16 but the enzyme activity was only marginally neutralized by MDV antiserum (Table 1). It is likely that MDV codes for thymidine kinase and that the failure of the serum to neutralize its activity completely is due to a deficiency in the serum which was not hyperimmune.

TABLE 1. MDV-induced thymidine kinase

Experiment 1.	Tk activity* ct/min
1. HPRS-16-infected CEF (4 x 10^7 cells/ml; 25% synthesizing MDV antigen)	18750
2. HPRS-16-infected CEF (4 x 10^7 cells/ml; 6% synthesizing MDV antigen)	3900
3. Uninfected CEF (4 x 10^7 cells/ml)	950
4. MDCC-MSB1 (2 x 10^8 cells/ml; 6% synthesizing MDV antigen)	1200
5. MDCC-HP1 (2 x 10^8 cells/ml; 0.1% synthesizing MDV antigen)	1250
6. Chick thymus (4 x 10^8 cells/ml)	1000
Experiment 2.	
1. HPRS-16-infected CEF (same as (2) in Experiment 1.) + buffer.	3100
2. HPRS-16-infected CEF (as above) + SPF serum	3200
3. HPRS-16-infected CEF (as above) + MDV covalescent serum.	2300

*Thymidine kinase activity was estimated by measuring the incorporation of tritiated thymidine into thymidine monophosphate in a standard assay for thymidine kinase at pH 6 for 30 min. (D. Buchan and L.J.N. Ross, unpublished results).
Note that significant kinase activity is found in infected CEF and that MDV antiserum marginally neutralizes its activity compared to specific pathogen free (SPF) serum.

Increased levels of thymidine kinase in cells infected with HVT and the JM strain of MDV have also been noted by Schat et al. (37) who showed in addition that SB-1 does not induce significantly increased levels of kinase. It is not known however whether this reflects at least in part the lower growth rate of SB-1 relative to the other strains or some more profound differences between these strains.

3.3. Viral antigens

3.3.1. A, B and C precipitins. Of the major precipitins described originally by Churchill et al. (38) the A antigen was of special interest since attenuation of HPRS 16 resulted in the loss of the antigen. This observation stimulated studies on the biochemical nature of the A antigen since it appeared to be associated with pathogenicity. Early studies (39) showed that this antigen is a glycoprotein which is heterogeneous in size (mol. wt. 70,000 to 90,000 daltons) and charge (pI 4.5 to 5.5). There have been many reports subsequently on its physicochemical properties with mol. wts. ranging from 20,000 (40) to 44,800 (41). The isoelectric point of a 200-fold purified preparation in the presence of 1 M urea and 0.05% Brij 35 was estimated to be 6.68 (41). These discrepancies reflect the fact that the antigen was studied in its native form and was subject to aggregation. Moreover, as the antiserum used for immunoprecipitation in some studies was a general antiserum, it could have detected other antigens present in culture fluids. These factors, coupled with the existence of precursor forms of the antigen of higher mol. wt., have contributed to the confusing literature on the size of the A antigen.

Recently, Zaane et al. (42), using pulse-chase experiments and 2D gel electrophoresis to analyse immunoprecipitates, identified a heterogeneous glycoprotein (gp 5) 52,000 to 72,000 daltons in mol. wt. which was rapidly excreted in the culture medium and is likely to be the A antigen. These results were confirmed and extended by Ikuta et al., (6). Using a monoclonal antibody they identified a heterogeneous glycoprotein (gp 54/70) in the medium of infected cultures consisting of at least 10 components in the mol. wt. range 54,000 to 70,000 daltons which increased in charge with increasing mol. wt. The isoelectric point of the glycoprotein ranged from pI 4 to pI 5.6. Pulse-chase experiments and the use of tunicamycin and neuraminidase suggested that gp 54/70 probably arises as a 45,000 dalton polypeptide which is glycosylated by addition of N-acetylglucosamine to form two precursor polypeptides, 57,000 and 61,000 daltons in mol.wt. These are then processed at least in part by the addition of sialic acid to form the mature glycoprotein which is rapidly transported to the cell membrane and excreted. There is evidence that gp 54/70 is a late gene product since its synthesis is

blocked by phosphonoacetic acid, an inhibitor of viral DNA synthesis. Isfort et al (43) have recently shown by in vitro translation of selected mRNA's that the non-glycosylated precursor of the A antigen is encoded by sequences within a 6.8 Kbp EcoR1 fragment of MDV DNA. The map position of this EcoR1 fragment is not yet known.

In summary, the data available now suggest that the A antigen (gp 5 of Zaane et al. (42), gp 54/70 of Ikuta et al. (6)) is a heterogeneous glycoprotein of mol. wt. 54,000 to 72,000 daltons and pI 4 to 5.6. Although the antigen does not appear to be a target for virus neutralization (31,44) its presence at the surface of MDV-infected cells suggests that it could have a role in cytotoxic immune reactions. It has been noted that small amounts of A antigen of marginally smaller mol. wt. are produced in cells infected with attenuated virus (6,45,46). The A antigen of HVT which cross-reacts with gp 54/70 of MDV is also marginally smaller. It originates as a 45,000 dalton polypeptide which is processed to a single 54,000 dalton precursor which is finally sialylated to form the mature glycoprotein (gp 50/64).

The B antigen was studied extensively by Velicer et al. (47) and was shown to be a glycoprotein containing α D - mannopyranosyl residues. In the presence of the dissociating agents 1 M urea and Brij 35, the B antigen has an apparent mol. wt. of 58,280 daltons and pI 4.54. Thus, although A and B antigens are physically distinct entities, they cannot be totally separated on the basis of size. However, they can be differentiated on the basis of charge in the presence of urea and detergents (41,47) and the greater resistance of the B antigen to inactivation by trypsin.

Using partially purified B and C antigens, it has been shown that B antigen is common to MDV and HVT and that at least two antigens (including C) are MDV type-specific (46). There is little to be gained in studying biochemical properties of virus-specific antigens detectable in gel diffusion tests in the future, since the antigens have to be extensively purified. More precise characterization of virus-specific antigens is now possible using monoclonal antibodies and SDS polyacrylamide gel electrophoresis of radiolabelled immunoprecipitates.

The antigens referred to as viral internal antigen (48) and the early and late membrane antigens noted on the surface of infected cells

(49) have not been characterized and progress must await the development of monoclonal antibodies that react specifically with these antigens.

3.3.2. Virus-induced polypeptides. At least 35 MDV-specific polypeptides have been identified in extracts of infected cells using 2D gel electrophoresis (Fig. 7) (45) and antibody enriched by affinity chromatography for immunoprecipitation (50). These polypeptides ranged from 160,000 to less than 20,000 daltons in mol. wt. (Table 2). Structural and non-structural proteins have not yet been differentiated (see Section 3.1). Six polypeptides mol. wt. 92, 64, 53, 42, 25 and 24 x 10^3 daltons present in culture fluids of MDV-infected cells cross-react with HVT and 10 polypeptides (mol. wt. 110, 92, 66, 62, 50, 40, 36, 29, 24 and 20 x 10^3 daltons) are phosphorylated as shown by labelling with ^{32}P orthophosphate (50). The relationship between these phosphorylated proteins and the polypeptides characterized by Zaane et al. (42) has not been determined.

Strains of MDV and HVT can be differentiated on the basis of the electrophoretic mobilities of the following polypeptides: p4, p5, p6, gp3, gp5, gp8 and gp9 (45) (see Table 3). Of these, the properties of gp5 (Fig. 8) showed the greatest degree of type-specificity and allowed independent classification of the strains into groups which agreed remarkably well with the serotypes defined originally by von Bülow and Biggs (51).

The majority of ^{35}S methionine-labelled proteins of MDV and HVT cross-react antigenically as shown by immunoprecipitation using heterologous antisera (52) (Fig. 9). This is unexpected in view of the limited homology between these strains (see Section 2.6). However, there is evidence that some glycoproteins are type-specific since only gp 56/64 (A antigen), gp110 and gp42 cross-reacted with HVT. Recent studies by Silva and Lee (29) indicated that five major polypeptides of MDV (p152, p100, p81, p60 and p49) contained antigenic determinants common to all serotypes. Using monoclonal antibodies they showed further that some polypeptides carried multiple epitopes and that some of these were type-common while others were type-specifc. Thus, p79 carried both type-common and type 1-specific determinants whereas gp100, gp80 and gp49 contained antigenic determinants common to type 1 and type 3 which were not shared with type 2. These glycoproteins contained in addition

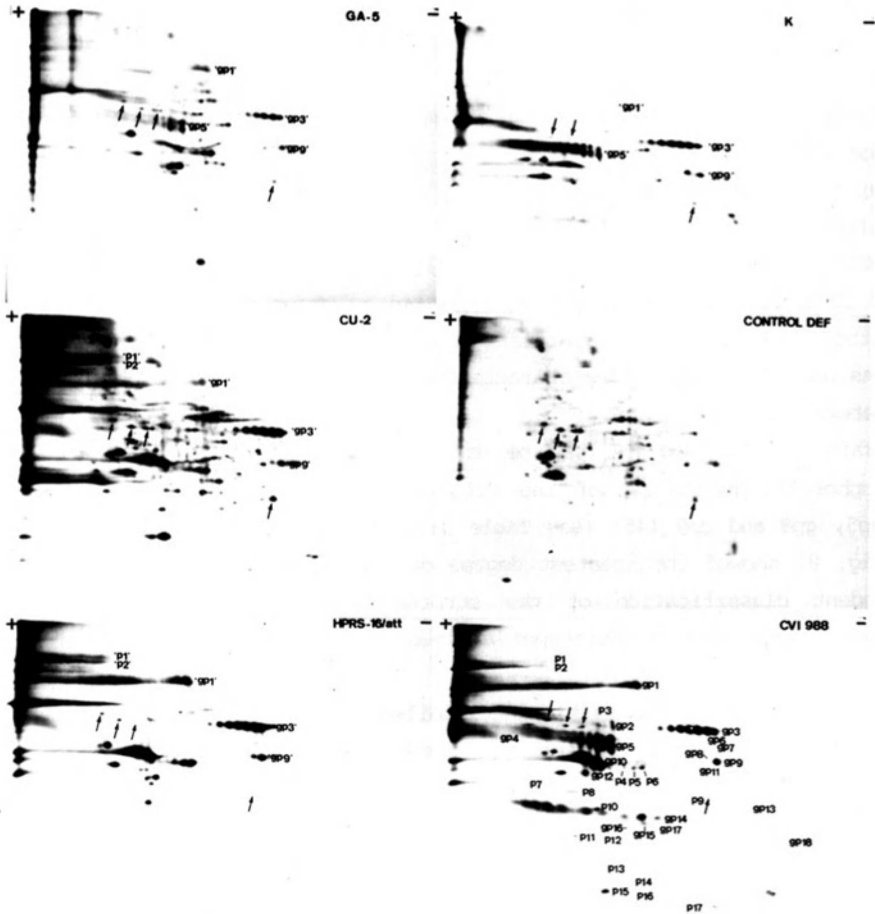


FIGURE 7. Comparison of 2D electrophoresis patterns of virus-specifc polypeptides immunoprecipitated from lysates of infected cells. Small arrows indicate polypeptides which are precipitated non-specifically. Note that strains of serotype 1 (GA,CU2, CVI 988), serotype 2 (SB1, HPRS-24) and serotype 3 (HVT FC126, PB-THV$_1$) have characteristics patterns. Type-specific polypeptides are encircled. (From Zaane et al. (45) by kind permission.)

FIGURE 7. (Continued)

TABLE 2. Virus-specific polypeptides of MDV-CVI 988

Nonglycosylated polypeptide	Estimated MW (kD)	Migration behaviour†	Glycoprotein	Estimated MW (kD)	Migration behaviour†
p1	145-160	A	gp1	115	A-N
p2	145-160	A	gp2	65-85	A-N
p3	87	A	gp3	63-67	B
p4	46	N	gp4	60-80	A
p5	46	N	gp5	52-72	A-N
p6	45	N	gp6	61	B
p7	43	A	gp7	55	B
p8	38	N	gp8	50	B
p9	35	B	gp9	49	B
p10	33	N	gp10	45-55	N
p11	27	N	gp11	45-50	B
p12	25	N	gp12	44	N
p13	21	N	gp13	30-35	B
p14	<20	N	gp14	30-36	A-N-B
p15	<20	N	gp15	30	N
p16	<20	N	gp16	29	N
p17	<20	A	gp17	27	N
			gp18	24	B

Properties of MDV-specific polypeptides synthesized in infected cells.

† Migration relative to the heavy chain of immunoglobulin during electrophoresis in a pH gradient. A (acid), N (neutral) and B (basic). (From Zaane *et al*. (45) by kind permission.)

TABLE 3. Comparison of the migration behaviour of some virus-specific polypeptides of various MDV and HVT strains.

	Virus strain								
Polypeptide	CVI 988	GA-5	K	CU-2	HPRS 16/att	SB-1	HPRS 24	HVT Fc126	PB THV_1
p1,p2	+	+	+	+	+	+	+	+	+
p3	+	+	+	+	+	-	-	+	+
p4/p5/p6	+	+	+	+	+	-	-	B	B
gp1	+	+	+	+	+	+	+	+	+
gp3	+	+	+	+	+	A	A	A	A
gp4	+	-	+	+	+	-	-	+	+
gp5	+	+	+	+	+	LB	LB	B	B
gp6	+	+	+	+	+	+	+	+	+
gp7	+	+	+	+	+	+	+	+	+
gp8/gp9	+	+	+	+	+	+	+	B	B
gp10	+	+	+	+	+	+	+	+	+

Note (+) Migration identical to corresponding polypeptide of CVI 988 A, B, L, H: polypeptide is more acidic, more basic, lower or higher in molecular weight, respectively, relative to the corresponding polypeptide of CVI 988.
(-) Presence of polypeptide not demonstrated or uncertain. More clearly visible after longer exposure of the autoradiogram and in other experiments.
(From Zaane _et al_. (45) by kind permission.)

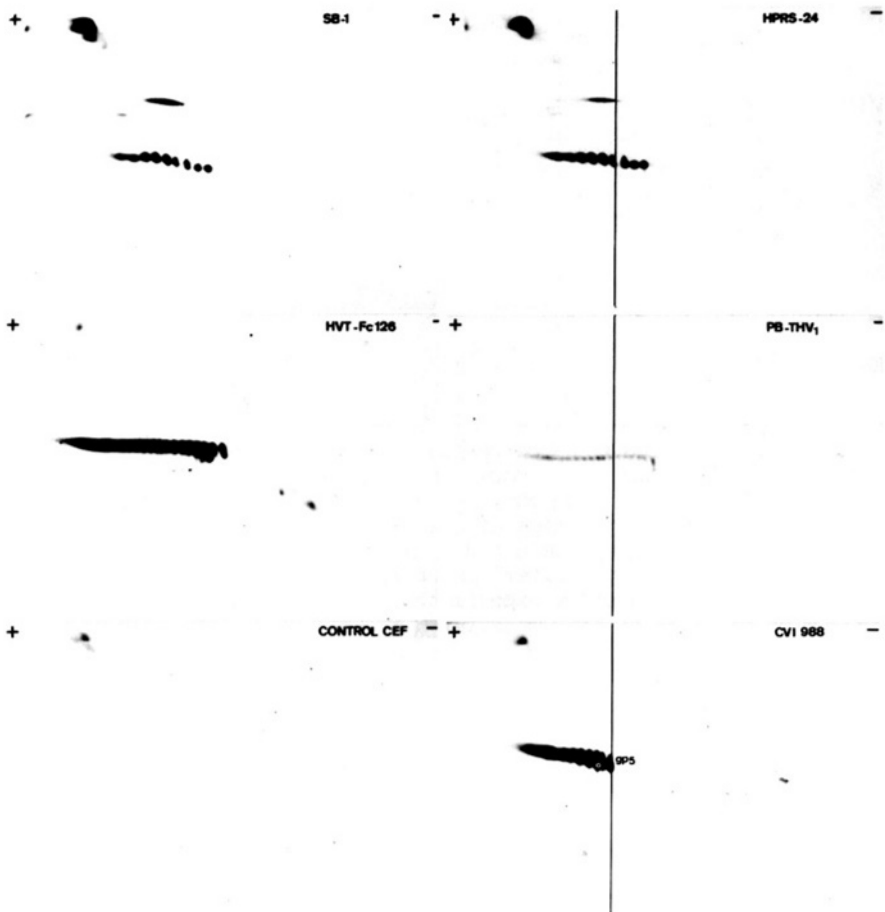


FIGURE 8. Comparison of 2D electrophoresis patterns of the major virus-specific polypeptide (gp 5) present in the culture medium of cells infected with different strains of MDV and HVT. Each serotype has a characteristic pattern. (From Zaane et al. (46) by kind permission.)

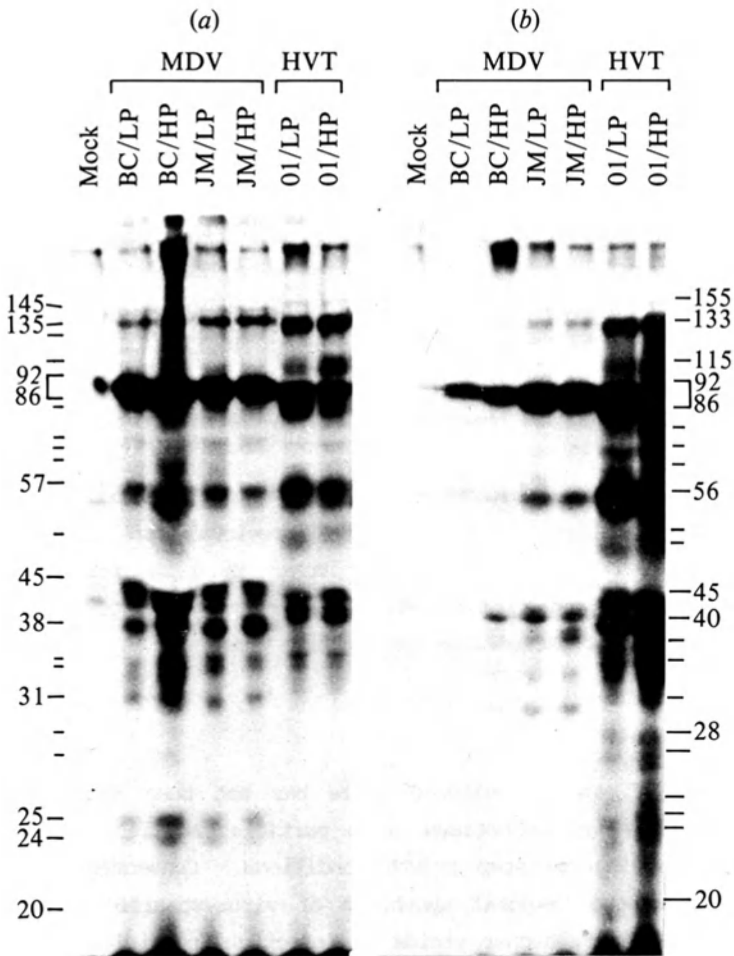


FIGURE 9. Cross-reactions between the proteins of MDV and HVT. Most of the ^{35}S methionine-labelled proteins in extracts of cells infected with MDV and HVT are immunoprecipitated by heterologous antisera. a) MDV antisera, b) HVT antiserum. Mol.wt. x 10^{-3} are indicated (From Ikuta et al. (52) by kind permission.)

a type 3-specific epitope which reacts with neutralizing antibody. Evidence was obtained for (sub-type) GA-specific determinants in the polypeptides p41, p38 and p24.

3.4. Molecular properties of tumour-associated antigen

A polypeptide of mol. wt. 40,000 daltons has been identified in extracts of the MDCC-MSB1 cell line by immunoprecipitation using two independent tumour-specific antisera (53). This polypeptide appears to be specific to MDV-transformed cells since it is distinct from proteins that react with Rous sarcoma virus tumour antiserum and thymus-specific antiserum. It is not immunoprecipitated either from lysates of myeloblasts using MDV tumour-specific antisera. However, since these antisera are heterologous antisera that have been absorbed with normal chicken tissues until specific for tumour cells, the possibility remains that the 40,000 dalton polypeptide could be a histocompatibility antigen. Consequently, its association with MATSA (54,55) is conjectural and definite evidence must await immunoprecipitation with monoclonal antibody.

Since MDV DNA is transcribed in several cell lines (see Section 5.4) and since the immunoprecipitation technique used previously to search for MDV-specific antigens in these cells may have lacked sensitivity (53), the role of virus-coded proteins in the formation of tumour-associated antigens cannot be excluded at the present time.

4. REPLICATION

4.1. Growth cycle

The growth cycle of MDV in cultured cells has not been studied because of the poor titres of infectious virus particles available and the difficulty in achieving one-step growth conditions. Consequently, nothing is known about the temporal synthesis of virus-specific proteins, DNA and RNA. However, higher yields of infectious particles can be obtained with HVT. Using a mini-scale one-step growth experiment, it has been estimated that the growth cycle of HVT is completed in 48 hours at 37°C (D.J. Walker and L.J.N. Ross, unpublished data). There is an eclipse phase of 10 hours followed by a period of exponential growth during which the infectivity increases by 2.5 logs in 30 hours. The growth cycle of MDV is expected to be longer judging from the slower

development of cytopathic effect (see Chapter 4). However, it has been reported that the JM strain of MDV adsorbed rapidly to DEF and that its growth cycle is completed in 24 hours at 37°C (56).

4.2. Effect of temperature on DNA replication

Temperature has a marked effect on the replication of viral DNA. In CEF infected with HVT, Kaschka-Dierich and Thomssen (57) showed by hybridization that the replication of viral DNA was faster at 41°C than at 37°C and reached 1200 genome equivalents per cell in 48 hours at 41°C. Although growth rates were slower at 37°C, the amount of viral DNA/cell attained eventually was the same as at 41°C.

During replication, 45% of HVT DNA became associated with high mol. wt. cellular DNA (57). The nature of the association between viral and host DNA is unknown. Whether this represents true integration or some non-covalent association is not clear. This question can only be resolved by the demonstration by molecular cloning of host-virus DNA junctions in infected cells. It is possible that MDV DNA is also associated with cell DNA in lytically infected cells as suggested by Kaschka-Dierich and Thomssen (57). This would be consistent with the results of in situ hybridization (Fig. 10), which suggested that in infected CEF, MDV DNA was polarized in the nuclei of mitotic cells and probably became distributed with the cell chromosomes into dividing cells in telophase. Using in situ hybridization, it is possible to detect and quantify viral DNA in infected cells (3) (Fig. 10) although the method does not allow detection of fewer than 5 copies/cell at the present time.

4.3. Transcription in lytically infected cells

Early hybridization experiments showed that 45% of MDV DNA was transcribed in infected CEF and that all the viral RNA became associated with polysomes (58). This corresponds to 90% transcription assuming that transcription is asymmetric. Recently, Tanaka et al. (59) showed that labelled cDNA, prepared using RNA from infected CEF as template, hybridized to most Bam H1 fragments of MDV DNA, confirming that most of the viral genome is transcribed in these cells.

Using in situ hybridization and individual cloned DNA's as probes, it is possible to detect both nuclear and cytoplasmic viral RNA in infected CEF (L.J.N. Ross, unpublished observations). The in situ

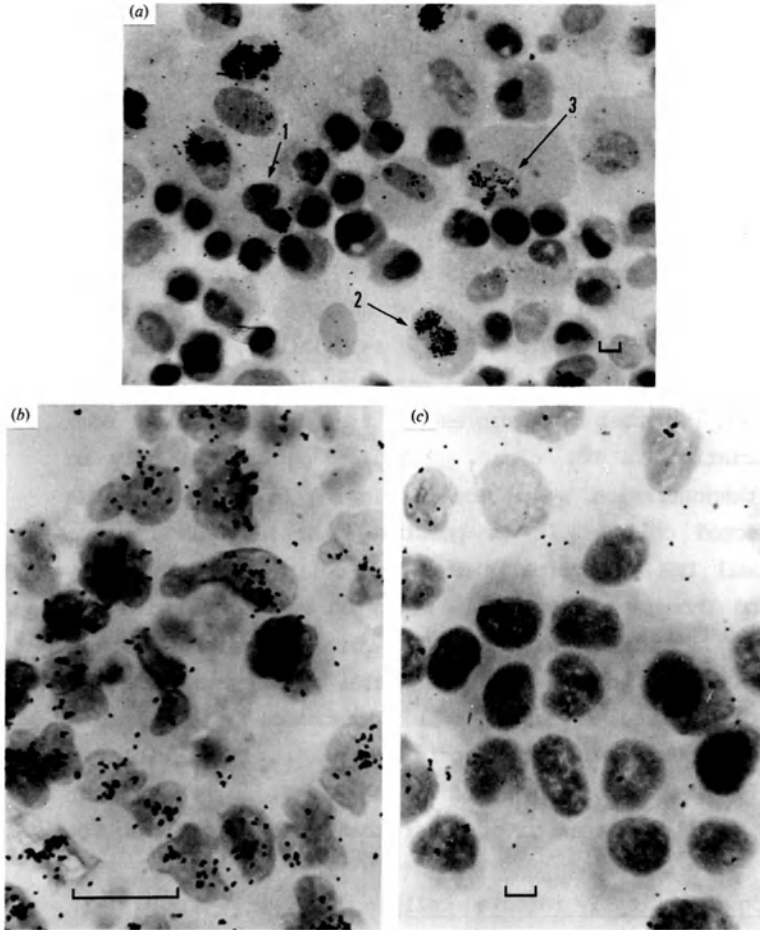


FIGURE 10. In situ hybridization showing MDV DNA. a) Infected CEF 4 days after infection at m.o.i. of 0.001; b) MDCC-MSB 1 cell line; c) uninfected CEF. Autoradiographic exposure was a) 2 days, b) 7 days and c) 24 days. Mean grain-cell in b) is 30 ± 13 (S.D.). It has been estimated that the sensitivity of hybridization is 2 grains/copy/7 day exposure. In a) arrows 1 and 2 show polar, intranuclear distribution of virus DNA in dividing cells. Arrow 3 shows a cell containing approximately 150 copies of viral DNA. Bar markers represent 10 μm. (From Ross et al. (3), by kind permission.)

hybridization results agree in general with the dot blot assays (59). However, one EcoR1 fragment (12 Kbp) does not appear to be transcribed efficiently in infected CEF. Two polyadenylated RNA species have been characterized in MDV-infected CEF (24). One is a 2.5 Kb transcript encoded by the Bam H1 H fragment of MDV DNA. The other is a 2.4 Kb transcript which cross- hybridizes with HVT. In cells infected with attenuated virus, the RNA transcripts complementary to Bam H1 H are heterogeneous (2 to 5 Kb) as expected, since alterations in the repeat regions of the Bam H1 H DNA fragments occurs during attenuation of the virus by serial passage. Nothing is known yet about transcription of viral DNA in lytically-infected B-lymphocytes or in latently infected T-lymphocytes after reactivation of virus replication _in vitro_ (60).

4.4. Inhibitors of virus replication

The replication of MDV is prevented _in vitro_ by phosphonoacetic acid and phosphonoformate which inhibit viral DNA polymerase (34,35) and also by a number of nucleoside analogues such as iododeoxyuridine and cytosine arabinoside which interfere with DNA synthesis. The molar concentration of some of these antiviral compounds for 50% plaque inhibition in chicken kidney cells (Fig. 11) are as follows: iododeoxyuridine (1 μM) trifluorothymidine (5.6 μM), acycloguanosine (12 μM), phosphonoacetic acid (56 μM) and vidarabine (146 μM). It appears therefore that MDV is as sensitive as HSV to iododeoxyuridine and phosphonoacetic acid but is less sensitive to trifluorothymidine and acycloguanosine.

It has recently been shown that a new fluoroarabinosyl pyrimidine nucleoside 1-(2-fluoro-2-deoxy- β -D-arabinofuranosyl)-5-iodocytosine (FIAC) and its uracil and thymine analogues, FIAU and FMAU respectively, inhibited the growth of MDV and HVT in chicken kidney cells (37). However, in CEF, virus growth was inhibited by FMAU and FIAU but not by FIAC. This is attributable to a deficiency in 2-deoxycytidine deaminase in CEF which is needed for conversion of FIAC to the active form FIAU. The molar concentration of FIAU and FIAC for 50% plaque inhibition in chicken kidney cells was 0.059 μM and 0.15 μM respectively, indicating that these compounds are considerably more potent inhibitors of MDV than those tested previously.

It is of interest that infection of lymphocytes _in vitro_ was inhibited by FMAU but not by FIAC (37). It is therefore likely that

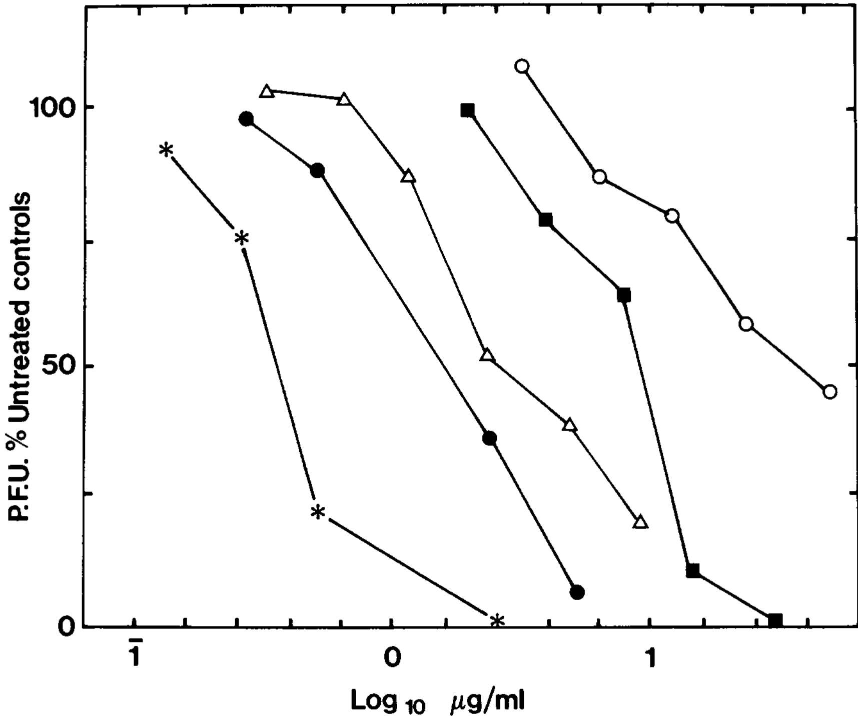


FIGURE 11. MDV plaque inhibition in chicken kidney cells by iododeoxyuridine (*); trifluorothymidine (●); acycloguanosine (△); phosphonoacetic acid (■) and vidarabine (O). The antiviral compounds were added to the monolayers 3 hours after infection with cell-associated HPRS-16 and were maintained until plaques were counted 5 to 6 days later. (L.J.N. Ross and D.J. Bauer, unpublished results.)

lymphocytes also lack deaminase. Neither acycloguanosine (L.J.N. Ross and D.J. Bauer, unpublished observations) nor FIAU (37) inhibited the growth of lymphoblastoid cell lines. Since these compounds require to be phosphorylated by virus-induced thymidine kinase in order to be effective, the lack of effect of these nucleoside analogues on cell lines is not surprising since viral thymidine kinase is not expressed in cell lines (Table 1). These results emphasize the importance of enzymatic differences between tissues on the effectiveness of antiviral compounds and have important implications for chemotherapy.

5. ONCOGENESIS

5.1. Latently infected and transformed lymphocytes

Latently infected T-lymphocytes have been demonstrated in the spleen after the cytolytic phase of infection (60). These lymphocytes contain less than 5 copies of viral DNA/cell (not detectable by *in situ* hybridization) and do not express viral antigens. Upon incubation *in vitro*, synthesis of viral antigens and presumably also of viral DNA occurs within 24 to 48 hours since multiple copies of viral DNA/cell could then be demonstated by *in situ* hybridization. Virus replication and expression are not activated in these cells by treatment with iododeoxyuridine and butyrate (60).

In contrast, transformed cells in established cell lines and in lymphomas contain at least 10 to 20 copies of viral DNA/cell as determined by *in situ* hybridization (3). Expression of viral antigens in these cells can be enhanced by tumour promoters and halogenated pyrimidines *in vitro*. Thus it appears that there are at least two types of latently infected T-cells *in vivo* which differ in the content of viral DNA/cell. Whether the higher number of copies of viral DNA/cell in transformed cells is the cause or the result of transformation is not known.

5.2. State of viral genome in transformed cells

The restriction enzyme patterns of DNA obtained from several cell lines are similar to the patterns of genomic DNA (Fig. 12). This suggests that viral DNA is present in a virtually intact form in cell lines. Similar results have been obtained using DNA derived from lymphomas. Physicochemical characterization of DNA isolated from

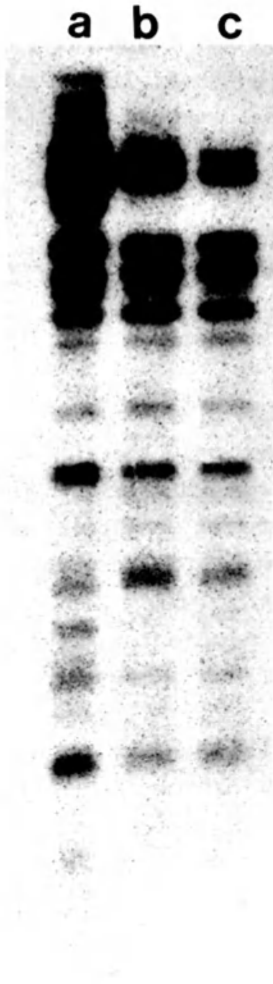


FIGURE 12. Southern blot hybridization showing viral DNA in lymphoid cell lines. Total cell DNA was digested with EcoR1, separated by electrophoresis and hybridized to ^{32}P-labelled DNA from HPRS-16. a) Non-producer MDCC-RPL 1; b) Non-producer MDCC-MKT 1; c) Producer MDCC-MSB 1. Note that most of the restriction enzyme fragments of the viral genome are present in all three cell lines (see Fig. 3). (L.J.N. Ross, unpublished results.)

established cell lines have provided some evidence for the presence of both integrated and episomal DNA's in these cells (61,62). Thus Kaschka-Dierich et al. (61) found that in the non-producer cell line MDCC-HP1, MDV sequences remained associated with cellular DNA upon centrifugation to equilibrium in CsCl under denaturing conditions. No free viral DNA was present in this line. In MDCC-MSB1, cells in contrast, fast sedimenting DNA with properties of free linear DNA (58S) was found as well as integrated DNA. These findings have been confirmed by Rziha and Bauer (63) who showed in addition that both MDCC-MSB1 and HP2 cells contained integrated as well as free viral DNA with properties of circular DNA (100 S). It is of interest that MDCC-RP1 (a non-producer cell line established from a transplantable tumour) contained only integrated DNA and that it has not been possible to rescue virus from this cell line. Tanaka et al. (62) found mainly circular plasmid-like DNA in the non-producer cell line MDCC-MKT1. The differences in the state of the viral genome noted in different cell lines could reflect not only intrinsic differences between the cell lines but also the passage history of the cells since it is known that changes in spontaneous expression of viral genome occur during continuous passage.

By separating chromosomes on sucrose gradients and examining the fractions for the presence of MDV sequences, Hughes et al. (64) found viral DNA associated mainly with chromosome 6 and chromosomes 16 to 19 in the MDCC-MSB1 cell line. However the nature of the association between MDV DNA and cell chromosomes remains uncertain since in other systems (e.g. EBV in Raji cells), viral DNA appears also to be associated with chromosomes although the linkage is alkali-labile and viral DNA is not integrated. Conclusive evidence of integration will require the demonstration of covalent linkage between MDV and host cell DNA by molecular cloning of virus DNA-cell DNA junction fragments.

5.3. Control of viral DNA replication and genome copies in transformed cells

The number of copies of viral DNA/cell in established cell lines ranges from 8 to 15 for the non-producer cell lines MDCC-HPRS 1 (61) and MKT1 (62) to 60 to 108 for the producer cell lines MDCC-MSB1 (61,65). Clearly these differences reflect the fact that extensive virus DNA replication occurs in a proportion of the cells of the producer cell

line MDCC-MSB1. However in the presence of phosphonoacetic acid which inhibits viral DNA polymerase, the copy number/cell in MDCC-MSB 1 approached the levels reported for the non-producer cell lines (66). These results suggest that the replication of viral DNA is partly under the control of the host cell. Support for this has been provided by Lau and Nonoyama (67) who showed that in synchronized MDCC-MKT1 cells the number of viral genomes/cell increased 2-fold just before cell DNA synthesis occurred and rapidly dropped to the normal level after division, indicating that the MDV genome duplicates once during the growth cycle of the cell. Because of this, one can expect some variation in estimates of copy number for a particular cell line depending on the state of the cells at the time of analysis.

Evidence that every cell of several cell lines contains viral DNA has been obtained by in situ hybridization (3) (Fig. 10) and it has been estimated by Southern hybridization using a cloned fragment as a standard that several non-producer cell lines contained approximately 15 copies/cell (3). This is in good agreement with the results obtained using cells cultured in the presence of phosphonoacetic acid and also for lymphomas (68), if the estimates of copy number are recalculated for the lymphoma cells to take into account that only 60 to 70% of the cells contain viral DNA. Thus, the true number of copies of latent genome in transformed cells is probably 5 to 15/cell.

5.4. Transcription in cell lines and tumours

Early studies (69) indicated that selective transcription of viral genes occurred in cell lines since cells of MDCC-HP1 and MDCC-MSB1 which expressed viral antigens spontaneously at the time, synthesized B and C precipitating antigens but not the A antigen. Yet, virus isolated from these cell lines could induce A antigen in CEF.

DNA-RNA hybridization (58) showed that 12 to 14% of viral DNA was transcribed (24 to 28% if asymmetric) in MDCC-MKT1 cells and that 60% approximately of the viral RNAs became associated with polyribosomes. Similar results were obtained in lymphoid tumours of ovary, spleen, liver and kidney. It was shown further that the same species of RNA were transcribed in tumours as in the MDCC-MKT1 cell line (58). In lytically infected cells on the other hand, approximately 90% of the viral genome was transcribed (assuming asymmetric transcription) and all

the viral RNA's became associated with polyribosomes. These results suggest that expression of the viral genome in cell lines and tumours is controlled at both transcriptional and post-transcriptional levels. The block in transcription can be overcome by treatment of the cells with IUDR but transfer of RNA to polyribosomes is only partially increased in the presence of IUDR.

The use of cloned DNA's has allowed rapid progress in identifying regions of viral DNA that are transcribed in cell lines. Using individual cloned DNA's as probes for *in situ* hybridization, it has been shown that sequences present in Bam H1 H and D fragments of viral DNA are among the major ones transcribed in several cell lines (19). Tanaka *et al.* (59) have shown in addition that Bam H1 fragment I_2 is also transcribed abundantly in both producer and non-producer cell lines. Thus the major regions transcribed are located in the repeat regions IR_L, TR_L and in the adjoining regions of U_L. In MDCC-MSB 1 and MDCC-MKT 1 cells treated with IUDR, additional sequences are transcribed. It is of interest that Bam H1 H and D fragments are preserved (22) and are transcribed in cell lines but undergo modification during attenuation of viral by serial passage. The expression of a virus-specific 34K phosphorylated protein in the MDCC-MSB 1 cell line has been reported recently by Hirai *et al.* (24). *In vitro* translation of hybrid selected mRNA has shown that this protein is encoded by a 1.22 Kbp sequence mapping within the inverted repeats of the fragments Bam H1 H and D. It is not known whether genes associated with oncogenicity are located in these regions. Conclusive evidence will require demonstration of the direct involvement of these sequences in transformation.

6. CONCLUDING REMARKS

Much progess has been made in recent years in characterizing the genome of MDV and the virus-specific proteins synthesized in infected cells. Strains of the different serotypes can now be differentiated by restriction enzyme analysis of viral DNA, by the electrophoretic properties of their proteins and by the use of monoclonal antibodies. These techniques and those of hybridization have provided new approaches for studying the epidemiology of the disease and for diagnosis.

However, much remains to be done to determine the precise role of

MDV in transformation and to explain the specificity of the T-cell as the target for transformation in the chicken. Moreover, the origin of the tumour-associated antigen MATSA is another important issue which needs to be addressed using a molecular approach.

As more information accumulates on the genes associated with oncogenicity and immunogenicity, new vaccines might be engineered that offer cheaper and better protection in the future. MD remains one of the most attractive models for studying the pathogenesis and immunoprophylaxis of herpesvirus-induced lymphomatous disease at the molecular level.

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6. LABORATORY DIAGNOSIS

J.M. SHARMA

1. INTRODUCTION

Marek's disease (MD) is one of the most commonly occurring neoplastic diseases of chickens and is endemic in almost all poultry-producing areas of the world. Highly successful vaccination programmes have dramatically reduced natural outbreaks of MD in commercial chickens. However, vaccination has not eradicated MD and outbreaks continue to occur sporadically, hence the need for proper diagnosis of the disease.

Difficulty in diagnosing MD generally arises for two reasons: 1) The disease is often confused with lymphoid leukosis (LL). MD and LL are the two most common naturally occurring neoplastic diseases of chickens. For many years MD was considered a part of the avian leukosis complex, with the implication that there was an aetiological relationship betwen LL and MD (see Chapter 1). This confusion was settled in the late 1960's when a herpesvirus was isolated from cases of MD (1,2) and MD was clearly separated causally from LL and other diseases that are caused by retroviruses (3). Despite this aetiologic distinction, considerable confusion persists in differentiating the pathological manifestations of MD and LL. Both diseases cause lymphoid cell tumours that are often difficult to distinguish on gross examination. Separation of MD from LL is important in laboratory diagnosis of MD and will be discussed further. 2) Marek's disease virus (MDV) is ubiquitous and almost all chickens raised under commercial conditions become exposed to virus in the environment at an early age. Infection with the virus results in persistent MD viraemia. Furthermore, in flocks that have been vaccinated, birds may be persistently viraemic with the vaccine viruses as well as with MDV. All commercial MD vaccines consist of

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live, infectious viruses and they protect against the clinical manifestations of MD and not against infection with MDV. Thus, a disease outbreak in vaccinated chickens cannot be diagnosed as MD merely by establishing that the flock was infected with MDV: additional factors must be considered.

The primary objective of this Chapter is to describe procedures that may be used to diagnose an outbreak of MD. In addition, procedures to diagnose infection of chickens with MDV are also given. Although not necessary for field diagnosis of MD, detection of MDV infection in chickens may be important in research work and in monitoring the disease-free status of specific pathogen-free flocks.

2. DIAGNOSIS OF A FIELD OUTBREAK

Evidence thus far clearly shows that the chicken is the only natural host for MDV. Outbreaks of MD in other avian species have not been reported although antibodies to MDV have been detected in some captive and wild birds (4,5). Lesions suggestive of MD have been reported in several avian species including budgerigar, canary, duck, goose, owl, pheasant, pigeon, quail and swan (6-8). Turkeys, although resistant to environmental exposure, develop typical MD characterized by gross tumours upon experimental inoculation with large doses of virulent MDV, particularly the GA isolate (9-12). The disease in turkeys is of interest because MDV may transform B or T-cells in turkeys (12-14), whereas in chickens, only T-cell transformation has been observed. Further, unlike in the chicken, the turkey herpesvirus (HVT) does not adequately protect turkeys against experimental MD (15). Because natural outbreaks of MD have not been reported in turkeys, field diagnosis of MD in this species is not relevant.

Factors that must be considered in establishing a diagnosis of MD in a chicken flock are discussed below. A schematic approach to diagnosis is shown in Fig. 1.

2.1. History of the flock

Vaccination programmes, age of the flock and hygiene practices are among the most important items that should be learnt from the history. Ordinarily, an injection of MD vaccine at hatching results in lifelong protection against MD (16). Thus, a vaccination programme that ensures

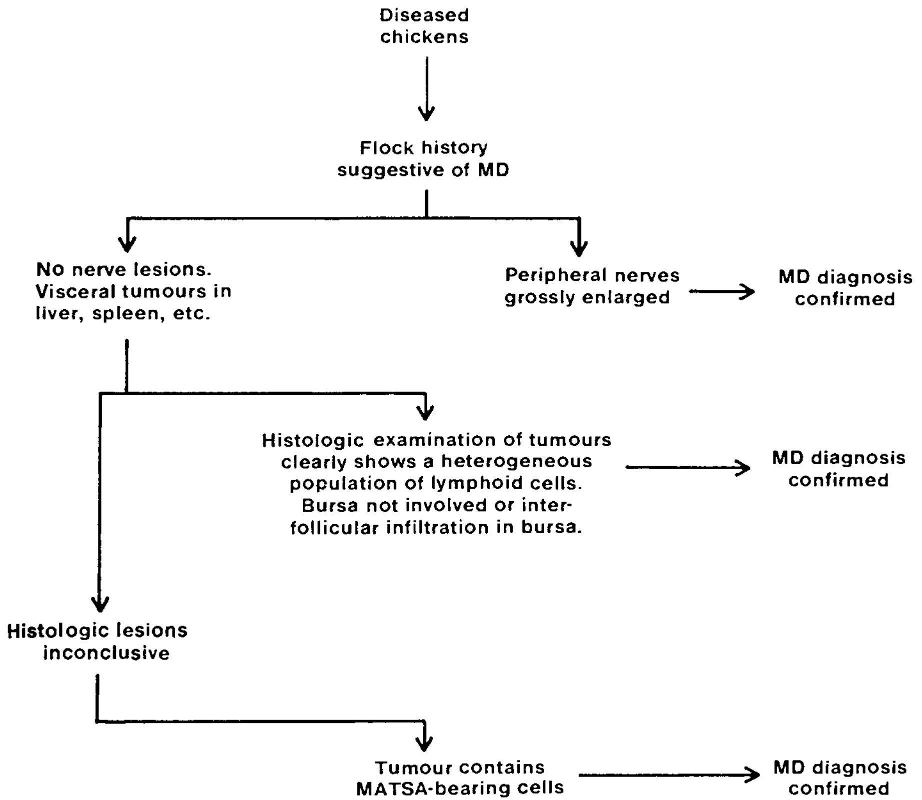

FIGURE 1. Scheme of field diagnosis of Marek's disease.

proper vaccination minimizes the chances of MD outbreaks. However, lack of vaccination or the use of faulty vaccine, or improper vaccine administration that does not result in parenteral introduction of the vaccine virus into each chick, may provide inadequate protection against environmental challenge with virulent MDV. The selection of the vaccine is also important. Several types of MD vaccines are in use commercially although HVT is used most widely. Recently, excessive MD losses in certain flocks vaccinated with HVT have been partly attributed to highly virulent isolates of MDV against which HVT did not provide adequate protection under laboratory conditions. A polyvalent vaccine containing HVT and vaccine strains of MDV may be required to provide adequate protection (17). Thus, HVT may not be the best choice for vaccinating chickens that are likely to be exposed to such highly virulent MDV isolates.

Poor management practices resulting in relaxed hygienic measures may make the best of vaccines ineffective. Improper cleaning and disinfection between hatches may leave an unusually high concentration of virulent MDV in the environment within a house. This virus may provide an immediate, massive challenge to chicks when they are placed in a house. Under commercial conditions, chickens are injected with MD vaccines at hatching and usually within hours of vaccination the chicks are placed in brooder houses. Because vaccinal immunity may take several days to reach protective levels, massive immediate challenge with the virulent environmental virus may result in disease outbreak. Immunologic stress resulting from infection with extraneous disease agents or enviromental factors may also reduce vaccine efficacy (18-21)

The age of the chicken flock when clinical disease appears may be important in determining if MD is involved. Whereas MD may occur at any age, gross LL tumours almost never occur in birds younger than 16 weeks of age and are most common in chickens that have reached sexual maturity. If tumourous disease is detected in chickens younger than 16 weeks of age, MD may be strongly suspected. Leukotic lesions in broilers are almost always due to MD. Gross tumourous lesions in chickens older than 16 weeks may be due to LL or MD and require further examination for definitive differential diagnosis.

2.2. Clinical signs and lesions

MD is characterized by neoplastic proliferation of lymphoid cells in

various tissues and organs (22). The accumulation of proliferating cells is particularly common in peripheral nerves. This lesion results in malfunction of the nervous system and clinical signs may depend upon the nerves that are affected. The most common clinical sign of MD is partial or complete paralysis of legs and wings. In acute outbreaks of MD, it is common to find a proportion of chickens with ruffled feathers showing signs of severe depression and ataxia. Death usually occurs from starvation and dehydration because of the inability of sick chickens to reach food and water. Some birds may die without displaying obvious clinical signs.

A description of lesions of MD is provided in Chapter 3. Briefly, the most characteristic gross lesion in MD is the thickening of peripheral nerves. The affected nerves appear oedematous, grayish in colour and lose cross-striations. In a suspect flock, the diagnosis of MD may be confirmed if necropsy examination of sick chickens reveals gross lesions in peripheral nerves. Any number of peripheral nerves may be involved, although on routine necropsy, diagnosis may be based on examination of vagi, brachial and sciatic plexuses and their trunks. In addition to changes in the peripheral nerves, solid lymphoid cell tumours may be commonly found in a variety of organs. In some chickens, visceral tumours may be present without detectable involvement of peripheral nerves. Gonads, particularly ovaries, are most often grossly tumorous although other organs such as liver, spleen, kidney , heart, proventriculus and intestine may also show nodular or diffuse enlargement due to accumulation of lymphoid cells (Fig. 2). In commercial broilers that are routinely inspected at processing at 7 to 8 weeks of age in the USA, nodular tumours on the skin are often attributed to MD. The spectrum of lesions may vary with the strain of MDV and the genetic background of the chickens.

Histologically, a typical MD lesion in the peripheral nerves or the visceral organs consists of an accumulation of morphologically heterogeneous populations of lymphoid cells (Fig. 3). Small, medium and large lymphocytes, plasma cells and lymphoblasts in various stages of mitosis or degeneration may be present. The heterogeneity of the lymphoid cell population is an important characteristic that differentiates MD lesions from an LL lesion, in which most lymphoid cells constituting a lesion

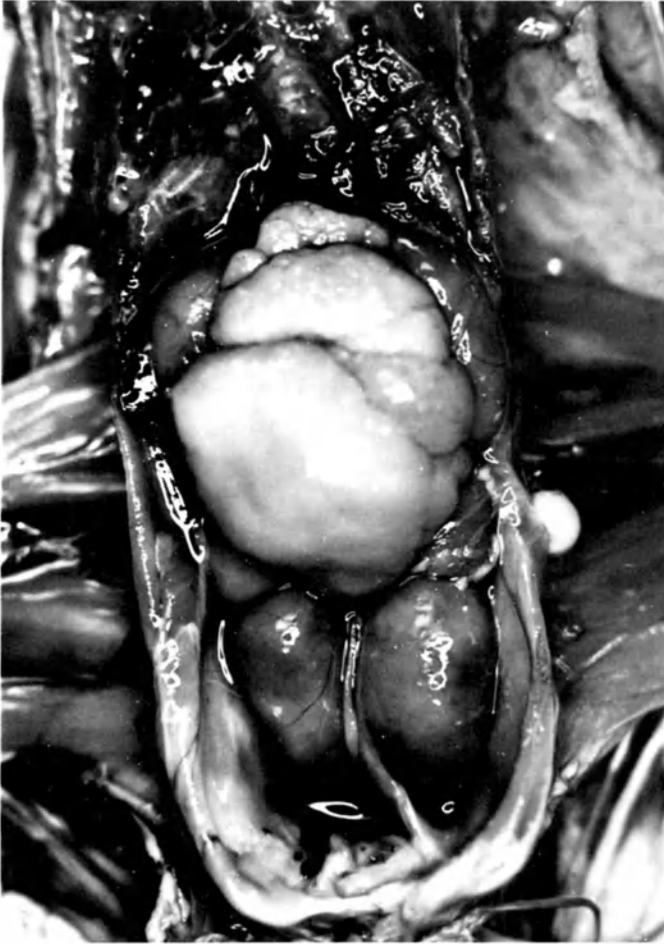

FIGURE 2. Gross Marek's disease tumour in the ovary and kidney of a chicken.

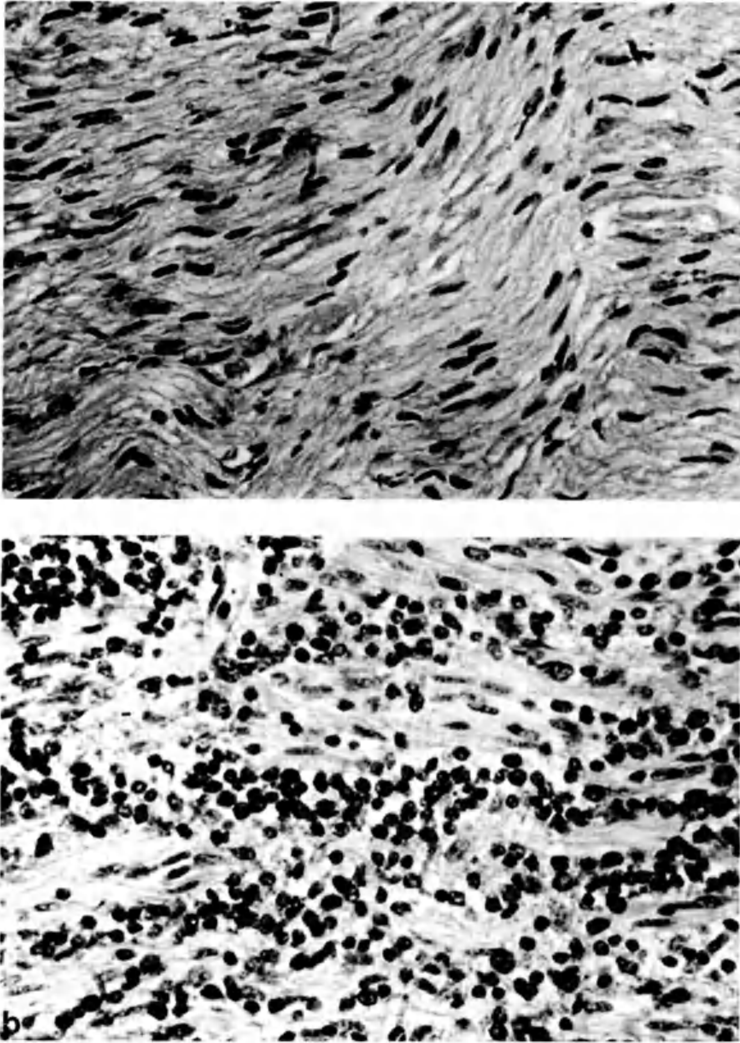

FIGURE 3. Histologic appearance of vagus nerve. a) normal nerve; b) nerve with Marek's disease lesion. (H & E stain)

are morphologically uniform blast cells (Fig. 4). Some peripheral nerves in MD may exhibit an inflammatory reaction comprised of inter-neuritic oedema and minor infiltration with small lymphocytes and plasma cells.

An important distinguishing feature between LL and MD is that in LL, the bursa of Fabricius is almost always tumourous, whereas in MD, lesions in the bursa are rare. Further, lymphoid cell infiltration in the bursa is intrafollicular in LL and interfollicular in MD. The bursal involvement is pathognomonic for LL. Thus, if in birds of over 16 weeks of age, gross visceral tumours are consistently accompanied by gross lesions in the bursa, MD may be safely excluded. The disease is most likely LL.

Histologic neural lesions somewhat similar to those in MD may be induced by experimental inoculation with reticuloendotheliosis virus (23), but because reticuloendotheliosis is not a naturally occurring disease in chickens, a field outbreak in which tumours have a hetero-geneous lymphoid cell accumulation can be attributed to MD. Caution should be exercised, however, if accidental inoculation of chickens with reticuloendotheliosis virus is suspected, as by injecting vaccines con-taminated with the reticuloendotheliosis virus. Such infected chickens will possess anti-reticuloendotheliosis virus antibodies. Experimental inoculation of chickens with the reticuloendotheliosis virus may also induce tumours similar to those of LL after a long latent period.

2.3. Detection of MATSA on tumour cells

If suspect chickens lack characteristic gross or histologic lesions, the diagnosis of MD can be confirmed by demonstrating the presence of Marek's disease tumour associated surface antigen (MATSA) on tumour cells (see Chapters 3 and 4). The proportion of MATSA-bearing cells in MD tumours is variable and may range from 2 to more than 50% (Fig. 5). Because MATSA has not been detected on cells of other tumours, including LL tumours, the presence of this antigen on even a small proportion of tumour cell suspension may be used as confirmatory evidence that the tumour is that of MD.

To date, immunofluorescence (IF) is the only procedure used for detecting MATSA-bearing cells. Antibodies against MATSA may be prepared by hyperimmunizing mammalian hosts such as rabbits by repeated

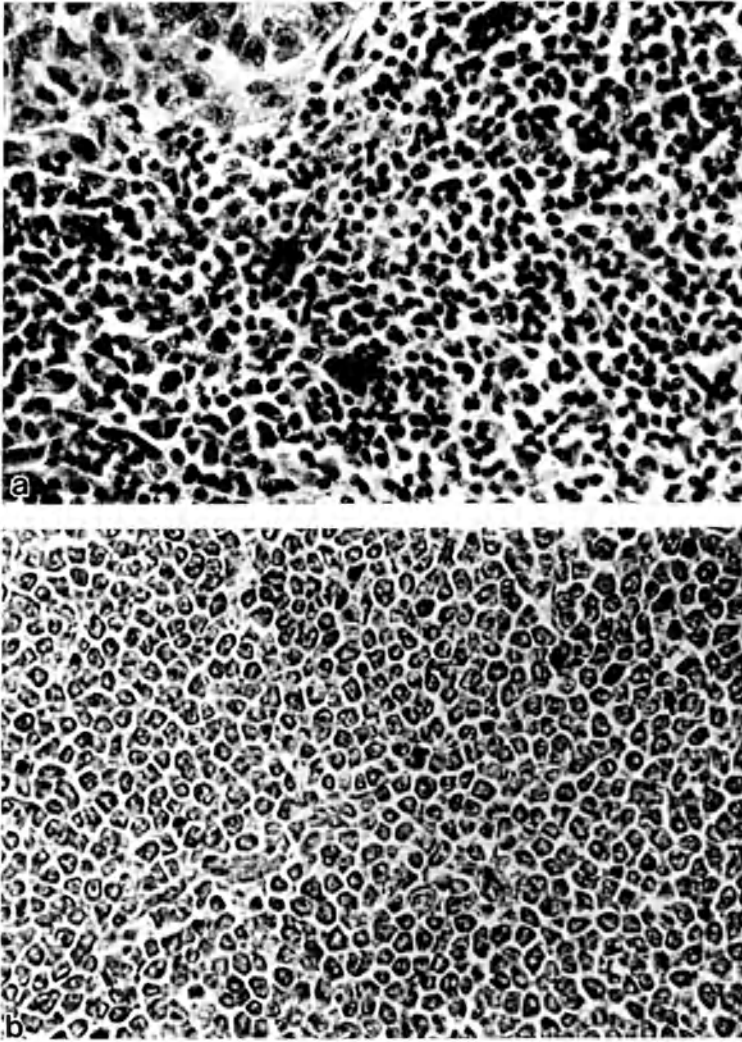

FIGURE 4. Relative morphological appearance of tumour cells in Marek's disease and lymphoid leukosis. a) section of a Marek's disease tumour in the testis; b) section of a lymphoid leukosis tumour in the bursa. (H & E stain)

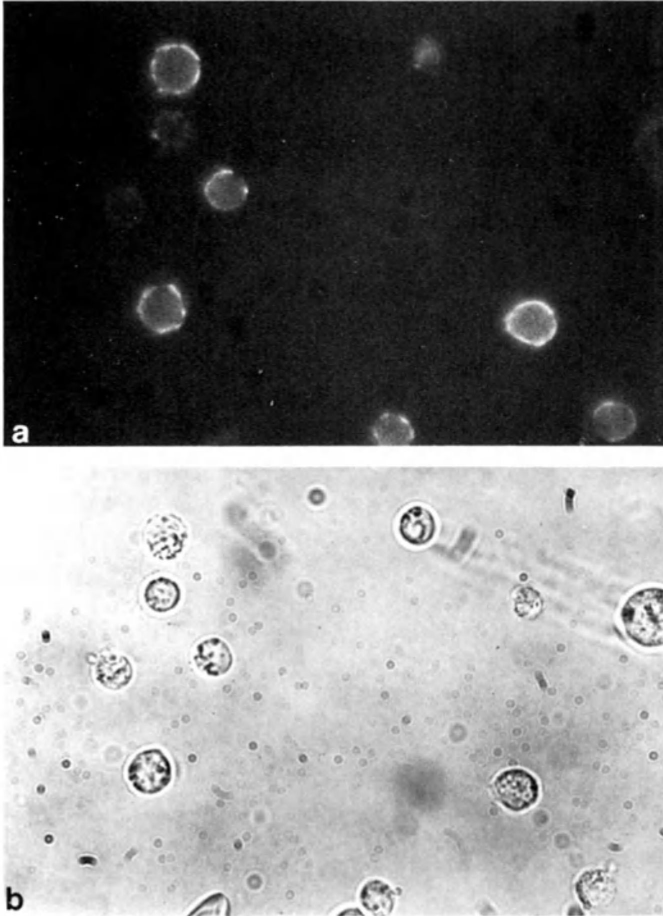

FIGURE 5. Marek's disease tumour cell suspension stained by immune fluorescence with anti-MATSA serum. a) a field of cells examined under fluorescent light; b) same field of cells as in a), examined with ordinary light. Note presence of MATSA on some cells in a).

injections with MD tumour line cells (24). Hyperimmune serum should then be extensively absorbed with normal chicken lymphocytes obtained from spleen, thymus and bursa to remove non-specific activity. Fully absorbed serum should stain a high proportion of the MD tumour line cells but not normal chicken lymphocytes. The need for extensive absorption can be obviated by preparing anti-MATSA serum in chickens using a syngeneic system (25). Adult chickens are given repeated injections of syngeneic MD tumour line cells. Hyperimmune serum produced in this manner can be used without further absorption although reactivity of the serum is generally lower than that of serum prepared in mammals. Recently, monoclonal antibody against MATSA has been developed in mouse cell hybridomas (26). This highly specific, high titre antibody has recently been used to diagnose a field outbreak of MD (27) and should prove to be of value as a diagnostic reagent.

To examine a tumour for MATSA, fresh tumour tissue should be prepared into a single cell suspension in cold phosphate buffered saline (PBS). The cells are reacted with unconjugated anti-MATSA antibody, washed, and then reacted with the appropriate fluorescein isothiocyanate-conjugated anti-immunoglobulin preparation. Spleen from a known MD-free chicken and MD lymphoma line cells should be included as negative and positive controls, respectively. After staining, the cells should be examined under a dark field microscope and the percentage showing surface fluorescence should be determined. If the proportion of fluorescent cells in the tumour cell suspension is significantly greater than the background staining in the normal spleen cell suspension, the tumour may be considered to be caused by MDV.

2.4. Differential diagnosis of LL and MD

Viruses of LL and MD are ubiquitous among chickens and outbreaks of neoplastic disease in chickens over 16 weeks of age may be caused by either virus. Table 1 shows criteria that may be considered to differentiate between LL and MD. Differential diagnosis of MD and LL has also been discussed elsewhere (28,29). Recent work has shown that of the criteria shown, the recognition by serologic means of specific cell surface antigenic markers on tumour cells is the best way to establish a definitive diagnosis. If tumour cells contain a proportion of cells that express MATSA, a diagnosis of MD is confirmed. In a tumour that

lacks MATSA-bearing cells but contains a preponderance of cells (usually over 85%) with surface IgM, a diagnosis of LL is firmly established. Cell-surface IgM expression can be detected by indirect IF staining of viable cells by the use of anti-chicken IgM antibody and relevant fluorescein-conjugated immunoglobulin. Cell surface markers have been successfully used in establishing definitive diagnoses of field cases that could not be readily diagnosed on the basis of clinical signs and gross lesions (27,30,31).

TABLE 1. Differential diagnosis of Marek's disese and lymphoid leukosis

Criteria	Marek's disease	Lymphoid leukosis
MATSA-bearing cells in tumours	Present	Absent
Proportion of IgM-bearing cells in tumours	Usually less than 10%	Over 85%
Age when clinical disease appears	Over 4 weeks of age	Over 16 weeks of age
Tumours in bursa	Usually absent. If present, cellular infiltration inter-follicular	Present in 90% chickens. Cellular infiltration intra-follicular
Enlargement of peripheral nerves	Usually present	Absent
Morphology of lymphoid cells constituting tumours	Heterogeneous population containing blast cells, small and medium lymphocytes and plasma cells	Uniform lymphoblasts
Tumours in skin	May be present	Absent
Usual distribution of visceral tumours	Gonads, liver, spleen	Bursa, liver, spleen
Typical clinical signs	Paralysis, depression, ruffled feathers	Depression, ruffled feathers. No paralysis.

Other criteria are also helpful in reaching a diagnosis, especially if facilities and reagents necessary for conducting cell surface marker tests are not available. All neoplastic disease outbreaks occurring in chickens younger than 16 weeks of age are likely to be those of MD. Presence of gross lesions in peripheral nerves in MD, and in the bursa of Fabricius in LL, are helpful in reaching tentative diagnoses. Such diagnoses should be followed up by histologic examination of tumourous lesions. Presence of a heterogeneous population of lymphocytes containing blast cells, small, medium and large lymphocytes, and plasma cells indicates MD, whereas presence of a uniform poulation of lymphoblasts indicates LL.

3. DIAGNOSIS OF INFECTION

Chickens may be infected with MDV without showing signs of MD. Such chickens are permanent carriers of the virus and a source of virus infection to other chickens raised in the same environment, as they continuously shed MDV in dander.

The presence of MDV infection in a flock may be established by isolating MDV or anti-MDV antibody from a proportion of the birds in the flock.

3.1. Virus isolation

There are several well-established procedures for isolating and identifying MDV. In a well-equipped virology laboratory, isolation of MDV in cell cultures is probably most convenient although the virus may also be isolated in embryonated chicken eggs or in susceptible chickens.

3.1.1. Selection of test samples for virus isolation. MDV is widespread in the tissues of infected chickens, thus a variety of tissues may be used for virus isolation. Because MDV is highly cell-associated, it is important that the test sample consists of viable cells. Virus is difficult to recover from chickens that have undergone postmortem degeneration.

Blood and cellular suspensions of tumour tissue or spleen are commonly used as test samples. Blood is generally preferred because repeat samples may be obtained from the same chicken. In chickens exposed to MDV at hatch, peak virus titres appear in blood and other tissues at about 3 to 4 weeks of age; subsequently the virus levels drop

but the virus remains persistently detectable in the blood and tissues (32).

3.1.2. Preparation of test samples. Freshly drawn heparinized blood, or heparinized blood stored on wet ice for up to 24 hours, may be used for virus isolation. Thus, blood samples from field cases may be shipped to a distant laboratory for testing. Because viral infectivity is associated with the white cell fraction in the blood, the infectivity of the blood sample may be enhanced by using the buffy coat fraction for virus isolation. White blood cells may also be concentrated by slow speed centrifugation or by centrifugation on density gradients such as Ficoll-hypaque or bovine serum albumen (33,34).

Tumourous tissue or spleen are removed aseptically from chickens immediately after euthanasia and suspended in ice cold PBS and processed immediately. The tissues should be minced into small pieces with scissors, washed two or three times with PBS to remove erythrocytes, and then trypsinized to obtain a single cell suspension. The trypsinized cells are washed, pelleted and suspended in tissue culture medium or PBS.

Cell-free MDV may also be isolated from feather tips or skin of infected chickens (35,36). Large feathers are pulled from the body and a 3 to 5 mm length of each tip containing pulp is cut off with scissors and saved for virus isolation. Alternatively, feathers are clipped off at the surface of major feather tracts and skin strips are removed and minced into small pieces. Feather tips or minced skin are suspended 1:5 or 1:10 (w/v) in a buffer containing sucrose, phosphate, glutamine, albumen and sodium ethylenediaminetetraacetate (SPGA-EDTA). The homogenate is sonicated for 2 to 3 minutes with an ultrasonic ossilator (e.g., Biosonic II, Brownell Scientific, Rochester, NY, with the needle probe set on intensity of 70). The resulting suspension is centrifuged and the supernatant is filtered through a 0.45 μm millipore filter prior to testing for the presence of cell-free MDV.

3.1.3. Isolation in cell cultures. Monolayer cultures of avian cells may be used. Extensive testing has revealed that mammalian cells are not suitable for MDV isolation (37-39). Cell cultures prepared from chicken kidney (CK) or 10- to 12-day-old duck embryos are most suitable. Chicken embryo fibroblasts (CEF) are less susceptible to primary isola-

tion of most field isolates of serotype 1 MDV than are duck embryo fibroblasts (DEF) or CK.

The single cell suspension of fractionated or unfractionated blood cells or of tissue cells is inoculated onto preformed monolayers of permissive cells. Generally, a 0.2 ml suspension containing 1 to 10 x 10^6 cells is used for each of the duplicate monolayer cultures pre-grown in 15 x 60 mm plastic cell culture dishes. When whole blood is used, a 0.1 ml inoculum per cultured dish is adequate. The inoculated and uninoculated control cultures are incubated at 37ºC in a humidified incubator containing 2 to 5% CO_2. The culture medium is renewed on alternate days. The areas of cytopathic effects (CPE) of MDV, termed plaques, appear within 3 to 4 days, but are best enumerated 7 to 10 days after inoculation.

For cell-free virus isolation from skin or feather tips of test chickens, CK cells are most suitable. A 0.2 ml portion of sonicated feather tips or skin homogenate is inoculated onto drained CK cell monolayers. After 30 minutes of adsorption at 37ºC the medium is added to the cultures. Subsequently, medium is renewed on alternate days. Plaques may be enumerated at 5 to 7 days after inoculation. Because of the chelating agent EDTA present in the inoculum, the monolayer cells tend to detach from the plastic substrate shortly after inoculation with the test sample. Upon addition of the growth medium, the cells resettle and form monolayers.

Occasionally MDV is isolated by preparing monolayer cell cultures of kidney tissue of test chickens. Kidney tissue is minced, trypsinized and a single cell suspension is plated into a tissue culture vessel using appropriate culture medium. The CPE can be visualized from 5 to 7 days after initiation of the cultures.

The areas of CPE or plaques induced by cell-associated MDV on CK cells and DEF or by cell-free MDV on CK cells are generally similar in appearance and consists of clusters of rounded refractile cells. With continued incubation, some cells in the plaques degenerate and slough off into the medium, leaving behind empty spaces or holes. Upon staining with analine dyes, the plaques consist of elongated mononuclear cells mixed with numerous round or irregularly shaped polykaryocytes with some nuclei containing intranuclear eosinophilic inclusion bodies

characteristic of herpesviruses.

Virus isolation attempts from field cases may yield serotype 1 or 2 MDV along with HVT, because most field chickens receive HVT vaccine at hatching. Differences in isolation techniques for serotypes 1 and 2 MDV are discussed in Section 3.3. Like MDV, HVT persists in infected chickens. The tissue culture cells susceptible to MDV are also susceptible to HVT and the CPE induced by the two viruses is similar, although subtle differences in morphologic appearance of the CPE between the two viruses exist and an experienced eye may be able to distinguish between the two viruses (40). The morphologic differences between the CPE of HVT and MDV can be best detected in CK cultures. In contrast to MDV plaques that develop slowly and rarely extend over 0.5 to 1.0 mm in diameter, plaques induced by HVT grow rapidly and may be counted 3 to 5 days after inoculation. By about 7 days after inoculation, HVT plaques reach a diameter of 1.5 to 3.0 mm. The HVT plaques are also characterized by the presence of balloon-shaped refractile cells that are much larger than those seen in MDV plaques. Recently, Cho (41) has reported that HVT, but not MDV, may selectively replicate and produce CPE in monolayer cells of a continuously propagating fibroblast line (QT35) derived from a chemically-induced tumour of quails. The QT35 cells may be useful for separating HVT from MDV. Further separation of HVT and MDV in a sample containing both viruses will be considered in Section 3.1.5.

3.1.4. Isolation in embryonated eggs. A 0.2 ml portion of the test sample containing cellular suspension (10^7 cells per ml) or cell-free virus is inoculated onto the chorioallantoic membrane (CAM) or intravenously in 9- to 11-day-old embryonated chicken eggs and the eggs are incubated at 37°C (42-44). Eggs dying within 24 hours are discarded; those dying subsequently are examined for pocks on the CAM. All surviving eggs are examined for pocks 7 days after inoculation. The presence of pocks on the CAM indicates virus replication (Fig. 6). There are two main disadvantages of virus isolation in eggs. First, viable lymphoid cells may initiate pocks in allogeneic embryos due to a graft-versus-host reaction and these pocks may be morphologically indistinguishable from those produced by MDV. Graft-versus-host pocks are not produced by a cell-free inoculum, such as the suspension made from feather tips or

skin. Graft-versus-host pocks may also be avoided by inoculating cellular inocula into the yolk sac of 4-day-old embryos and examining for pocks 2 weeks later (45). The second disadvantage is that MDV and HVT produce identical pocks on the CAM, thus making it difficult to determine if the test inoculum had MDV, HVT or a mixture of both.

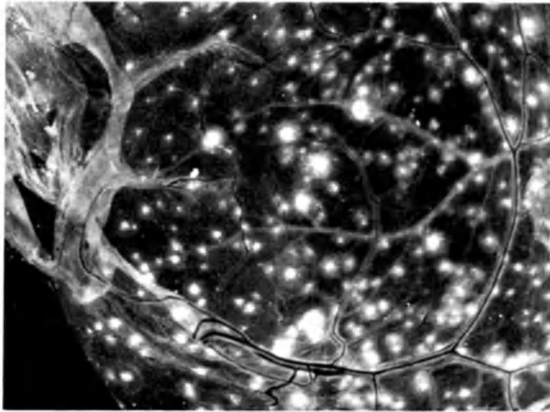

FIGURE 6. Marek's disease virus pocks on the chorioallantoic membrane of embryonating chicken eggs.

3.1.5. Isolation in chickens. Newly-hatched chickens with natural susceptibility to MD and possibly freedom from maternal antibodies to MDV should be selected. The test sample containing cellular or cell-free MDV should be inoculated intra-abdominally in newly-hatched chicks. Three groups of chicks should be used. Group 1 should get the test inoculum and groups 2 and 3 should be left uninoculated. Group 2 should be raised in the same isolator as group 1 to serve as a contact-exposed control. Group 3, serving as a negative control, should be raised in a separate isolator.

The isolators should be supplied with air passed through absolute filters to prevent cross-contamination. Usually, Horsfall-Bauer type cages with internal positive or negative pressure are suitable. MD-specific mortality may begin after 4 to 5 weeks of inoculation, although the test chickens should be held in isolation until about 8 weeks of age. Chickens that die during the observation period should be examined for gross and histologic lesions of MD. At the end of 8 weeks, surviving

chickens should be bled for antibody and necropsied. If gross lesions of MD cannot be found, histologic sections of peripheral nerves (vagi, brachial and sciatic plexuses) and gonads should be fixed in 10% formalin and examined microscopically after staining with haematoxyline and eosin. For positive isolation, chickens of groups 1 and 2 should have antibody and/or lesions of MDV; group 3 should be free of antibody and lesions. If lesions are absent in both groups and antibody is present in group 1, but not in group 2, this result probably indicates isolation of HVT from the inoculum. Because HVT does not readily spread horizontally, the virus induces antibody in inoculated chickens but not in contact-exposed chickens. The antibody to HVT serologically cross-reacts with MDV. In addition to the presence of lesions and antibodies, infection with MDV may also be confirmed by isolating the virus from blood or tissues of test chickens.

Gross tumours appearing in test chickens can be confirmed to be caused by MDV by demonstrating the presence of MATSA as described above.

Chick inoculation is also useful in isolating MDV from samples that may contain MDV and HVT. Such samples may not induce MD in inoculated chickens because of the protective effect of HVT. To identify MDV in samples containing a mixture of the two viruses, virus isolation should be attempted from the contact-exposed group (group 2). The isolated virus is most likely to be MDV because HVT would not be expected to infect the uninoculated contact-exposed group.

3.2. Identity of the isolated virus

MDV has morphologic and biologic characteristics of other herpesviruses. Because MDV is highly cell-associated, the vast majority of the virions recovered from virus-infected tissue culture cells are unenveloped nucleocapsids measuring 90 to 100 nm in diameter, each containing 162 capsomeres arranged in an icosohedral symmetry (46). Enveloped particles found principally in cutaneous epithelial cells appear as irregular amorphous structures and measure 273 to 400 nm in diameter. The DNA of MDV has a buoyant density of 1.705g/cm^3 and an estimated guanine + cytosine content of 46% (47). Cell-free MDV retains infectivity for several months at -60°C. Virus is inactivated in 2 weeks at 4°C, in 4 days at 22° to 25°C, in 18 hours at 37°C, in 30 minutes at 56°C and in 10 minutes at 60°C (36).

The best method for confirming the identity of virus isolated in cell cultures is to stain monolayer cell cultures exhibiting CPE with MDV-specific antibodies. The most commonly used procedure for this purpose is the IF technique. Monolayer cultures with well-developed plaques are fixed in cold acetone and reacted with fluorescein isothiocyanate-conjugated MDV antibody (48).

3.3. Strain variability of MDV

Numerous isolates of MDV with different *in vivo* pathogenicity and *in vitro* growth characteristics have been recovered from chickens. Although there is serological cross-reaction between isolates, the degree of cross-reactivity in IF and agar gel precipitin (AGP) tests was used to classify isolates into two serotypes: serotype 1 and 2 (49,50). Serotype 1 includes pathogenic isolates and the tissue culture attenuated variants of these isolates. Serotype 2 includes isolates of MDV that readily infect chicks but are nonpathogenic. Recently, monoclonal antibodies have been developed using mouse hybridomas that clearly distinguish between the two serotypes of MDV and the serotype 3 herpesvirus, i.e. HVT (51). The monoclonal antibodies are monospecific and antibodies against a given serotype are free of detectable activity against other serotypes.

Both serotypes of MDV may be present in the environment and commercial chickens may acquire infection with either or both serotypes. Recently, the use of polyvalent MD vaccines containing HVT mixed with serotype 2 MDV has resulted in the deliberate introduction of serotype 2 MDV in many concentrated poultry producing areas in the USA and possibly in other countries (17,52-54).

Serotype 2 MDV's, like serotype 1 MDV's, spread horizontally among chickens. Virus isolation procedures for serotype 2 viruses are generally similar to those described above for serotype 1 virus. Serotype 2 viruses replicate well in avian cell cultures and CEF cells that are generally not suitable for primary isolation of serotype 1 virus from chickens work quite well for primary isolation of serotype 2 viruses. The serotype 2 virus CPE, although similar in character to the serotype 1 CPE, appears later and spreads more slowly than that of serotype 1 CPE. Presence of serotype 2 virus may be confirmed by staining CPE-bearing cells with serotype-specific monoclonal antibodies in

the IF test (51). Separating serotypes from cell cultures containing both serotypes of MDV is difficult because both serotypes are cell-associated. Separation of serotypes 1 and 2 may be further complicated if HVT is also present in the test sample. Because of rapid growth, HVT may spread through the culture and mask the CPE induced by MDV. Individual serotypes of MDV may be cloned by passing cells of single, well-isolated plaques from monolayer cultures maintained under agar. A more reliable method would be to use cell-free virus from skin of infected chickens for cloning in cell cultures. Cloned virus should be carefully examined for purity by staining infected cell cultures with specific monoclonal antibodies.

The isolated viruses of the two serotypes may be further distinguished by inoculating susceptible chicks with cloned virus preparations. The unattenuated serotype 1 virus would cause MD in chickens, whereas serotype 2 virus would be nonpathogenic but immunogenetic for chickens.

3.4. Detection of antibody

Because MDV persistently replicates in chickens, anti-MDV antibodies also continue to be produced and can be detected through the life of the infected birds. A number of serological techniques have been used to detect antibody. The most commonly used techniques include the AGP, the IF and the virus-neutralization tests. Recent attempts on the use of the enzyme-linked immunosorbent assay (ELISA) have also produced promising results (55). It is best to inactivate serum by heating it at 56°C for 1/2 hour prior to testing for antibody.

In the AGP,the serum is reacted with MDV antigen in an agar substrate (56). The antigen for this test may consist of disrupted (by sonication or repeated freezing and thawing) tissue culture cells with advanced MDV CPE. Alternatively, skin extract or feather pulp obtained from large feathers of the major feather tracts of infected chickens may also be used as antigen (57,58). A 1% suspension of Nobel agar in PBS containing 8% NaCl is solidified on glass slides or in petri dishes. After the agar solidifies, one central and six peripheral equidistant wells, each 3 mm in diameter and about 3.5 mm apart, are cut with a template. The central well is filled with antigen. The peripheral wells in numbers 1 and 4 (top and bottom) positions, are filled with

a known MD positive serum and the remaining wells are filled with various test sera. The reagents are diffused for 24 to 72 hours at room temperature or at 37°C. In a specific antigen-antibody reaction, the precipitin band should form a line of identity with the band produced by the known positive serum. Certain sera may produce multiple precipitin bands. The most prominent band is attributed to the A antigen of MDV that is released into the medium of infected cells, whereas other minor bands are due to other antigens that may be cell-associated.

For detecting antibody by the IF test, the antigen, which consists of acetone-fixed monolayer cell cultures with MDV plaques, is reacted with serum antibody (59). Both direct or indirect IF tests have been used. In a direct IF test, each test serum must be conjugated with fluorescein isothiocyanate dye, whereas in an indirect test, the antigen is first reacted with unconjugated test serum and then with dye-conjugated anti-chicken immunoglobulin usually of mammalian origin. Because a common source of conjugated reagent can be used in an indirect test, this test is preferred when multiple test sera need to be screened for antibody. The fluorescence is also brighter in the indirect test than in the direct test because of the multiple layer effect.

The virus neutralization test (36) is not commonly used for routine diagnosis because cell-free MDV required for the test is difficult to obtain. The cell-free MDV generally obtained from skin extracts of infected chickens should have a titre of about 10^3 plaque-forming units per ml or higher. Workable quantitites of cell-free virus may also be obtained by disrupting cell cultures infected with attenuated serotype 1 MDV (L.F Lee, unpublished results). One part of 2- or 10-fold dilutions of test serum and 4 parts of virus suspended in SPGA-EDTA buffer are reacted for 30 minutes at 37°C or at room temperature. Subsequently, 0.2 ml of the mixture is inoculated onto preformed chicken kidney cell monolayers. Plaques are enumerated 6 to 8 days later. The titre of serum is the reciprocal of the serum dilution that reduces the virus titre by 50%. Appropriate known positive and negative serum controls should be included in the test.

3.5. Distinguishing MDV and HVT antibodies

Most vaccinated commercial chickens possess antibodies to both HVT and MDV. Because of cross-reaction, it is quite difficult to distinguish

between the two types of antibodies. The following points may be of assistance: 1) In an indirect IF test, HVT-infected cells show nuclear staining when reacted with MDV antibodies but nuclear and cytoplasmic staining when reacted with the homologous antibody. In the HVT homologus reaction, the cytoplasmic staining appears granular. When MDV infected cells are reacted with anti-MDV or anti-HVT antisera, the staining is diffused through the entire cell although the homologous reaction is more prominent than the heterologous reaction, 2) The serum containing HVT antibody alone will have a higher virus neutralization titre against homologous virus than against the heterologous virus. A serum containing antibodies against both MDV and HVT is unlikely to show preferential neutralizing activity against either of the two viruses.

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7. IMMUNITY

P.C. POWELL

1. INTRODUCTION

Immune responses in Marek's disease (MD) are complicated by antagonism between immunity on the one hand and immunosuppression on the other. Lymphocytes are responsible for mounting immune responses, some of which may contribute to protection while others may be involved in the pathology of the disease, and it is the same cell type that is the primary target for infection with Marek's disease virus (MDV). Infection may be lytic, resulting in cell death, or it may lead to malignant transformation; both types of infection appear to be associated with immunosuppression. It is assumed that an efficient host immune response limits the extent and spread of virus replication, and also contributes to the regression and rejection of tumour cells. At the same time virus-induced immunosuppression will be acting to limit the effectiveness of these responses. The outcome of infection therefore depends upon a balance between the immune responses provoked by the virus and tumour antigens and the immunosuppression caused by the replication of the virus in the lymphoid organs. Nevertheless, the importance of the immune system in ensuring that this internecine struggle results in a satisfactory outcome from the chicken's point of view has been amply demonstrated by the success of a variety of live virus vaccines against MD (1).

2. EXPRESSION OF IMMUNITY

Infection of a chicken with MDV does not necessarily result in disease and a number of factors affect oncogenesis. These can be classified as properties of the infecting virus, of the infected chicken or of the environment (2). Thus the dose of infecting virus, and more

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particularly, the virus strain have an important influence. Some virus strains appear to be totally non-oncogenic, even when given in very large doses, possible because they lack the genes conferring transforming potential (3). Host characteristics contributing to resistance to the development of disease include genotype, sex, age and passively or actively acquired immunity. Environmental factors are also important; outbreaks of MD in commercial poultry are often associated with management practices assumed to cause stress, such as handling and movement of birds, vaccination against other diseases, debeaking and dietary changes (4). It is assumed that these stressors operate by reducing the efficacy of immunosurveillance mechanisms.

The properties of the host that determine the outcome of infection may be classified as innate, passively acquired or actively acquired.

2.1. Innate resistance

2.1.1. Genotype. The genotype of the host greatly influences the outcome of infection by MDV, affecting mortality, the distribution of virions and the type of lesions (see Chapter 11). These differences can be observed within commercial strains of fowl and between inbred lines. It has been known for many years that resistant and susceptible strains of chickens could be produced by artificial selection based on sib and progeny testing. For example, in four generations a resistant line with 6.5% susceptiblility and a susceptible line with 94.4% susceptibility were derived from random bred stock with 51.1% susceptibility (5,6). Cole and Hutt developed the resistant strains K and C and the susceptible S strain (7). After 20 years of breeding the mortality of these three strains after experimental exposure to MDV was 0.8%, 1.5% and 38.3% respectively. Similarly, Waters (8) developed the resistant strain line 6 and the susceptible strain line 7.

Two distinct genetic loci have been implicated in controlling genetic resistance (4). An association exists between resistance to MD and genes of the erythrocyte antigen group B (Ea-B) locus, the major histocompatibility locus of the fowl. The B^{21} allele is strongly associated with resistance, and this allele is widely distributed in poultry populations including the progenitor of domestic poultry, the Red Jungle Fowl, suggesting that it has survival value for the species. Other alleles at the B locus (B^2, B^6) also confer some resistance. In

spite of this, information about the second locus controlling resistance has come from the two inbred chicken lines 6 and 7 (respectively highly resistant and highly susceptible) which are both homozygous for the B^2 allele. In these birds the Ly-4 locus which controls T-lymphocyte antigens has been identified. The characteristic allele of line 6 is $Ly\text{-}4^a$, and that of line 7 is $Ly\text{-}4^b$. An association between the presence of these alleles and response to MD was found, although their influence was confounded by other alleles.

2.1.2. Sex. Sex influences the distribution of MD lesions and the incidence of gross lesions and mortality (2). The ovary is more likely than the testis to be involved in lymphoma formation, and females are more susceptible to clinical disease than males, by up to two times. The sex effect is not always seen, and it appears that sex affects mortality more than total gross lesions, indicating that males survive longer than females. The expression of the sex effect depends upon the strain of virus involved (9).

2.2. Passively acquired resistance

Chickens that are infected with MDV, but do not succumb to the disease, carry the virus for the remainder of their lives. This results in a relatively high antibody response to the virus, and the transfer of maternal antibody to the chicken *via* the yolk sac (10). The effects of maternally-derived antibody may be mimicked by the passive administration of serum (11). Four main effects of maternal antibody on the development of MD have been observed: 1) lower mortality, 2) delayed onset and increased latent period to death, 3) a reduction in tumour formation, and 4) suppression of the acute destructive lesions of the lymphoid and haematopoietic tissues that result from the acute cytolytic infection with MDV which occurs in the absence of maternal antibody. The protection provided by maternally derived antibody can be enhanced by repeated immunization of breeding hens with MDV (12). Maternal antibody to both MDV and to HVT may interfere with onset of vaccinal immunity (4).

2.3. Actively acquired resistance

2.3.1. Vaccination. Since the development of the first vaccine by attenuation of a virulent strain of MDV in tissue culture (13), vaccination has become widely practised (see Chapter 8). Chicks are usually

inoculated with live vaccine at 1 day of age. Protection against challenge develops quickly but maximal protection depends upon an interval of 1 to 2 weeks between vaccination and exposure to virulent virus. This is sometimes difficult to achieve on poultry farms, particularly on multi-age sites, and premature challenge accounts for at least some instances of vaccine failure.

There are three types of live vaccine against MD: 1) attenuated MDV, 2) apathogenic MDV and 3) herpesvirus of turkeys (HVT) (2,4). These vaccines behave in inoculated chicks free of maternal antibodies in a manner similar to pathogenic MDV in terms of virus localization. Degenerative and regressive lesions are, however, minimal and viral antigens are rarely seen (14) despite the presence of latently infected cells. None of the vaccines against MDV prevent super infection with virulent virus, but they do prevent the early cytolytic infection, and virulent virus viraemia and virus shedding from the feather follicles are reduced (15). Vaccinated chickens that are exposed to virulent MDV become chronically infected with both the vaccine virus and the challenge virus. The presence of the virulent virus in a latent state implies that should surveillance be lowered, clinical disease may result.

2.3.2. Age. A second type of acquired resistance is "age resistance". Older chickens are often more resistant to MD than young birds (16). There appears to be a relationship between age resistance and genetic resistance since genetically resistant strains free of maternal antibody may be susceptible when infected at hatching, but resistant later in life; conversely, genetically susceptible strains develop disease irrespective of the age at which they are infected (17).

3. IMMUNE RESPONSES TO MAREK'S DISEASE-ASSOCIATED ANTIGENS

In the context of the present discussion of immunity, MD-associated antigens are important because of the immune responses that they stimulate. They are described in detail in Chapter 4. For the present it is sufficient to say that they can be divided into two categories, viral antigens and tumour antigens. Virus-related antigens may be detected in permissive cells infected in vitro, and in the feather follicles, bursa, thymus, spleen and other tissues of chickens infected

with MDV (2,4). The relationship between antigens detected by immunological techniques, structural components of virus particles and viral polypeptides has not been defined.

Lymphoblastoid cells transformed by MDV express a membrane antigen which is believed to be a marker for neoplastic transformation (18). This "Marek's disease tumour associated surface antigen" (MATSA) (19) is most easily seen on lymphoblastoid cell lines grown *in vitro*, but it is also carried by a proportion of the cells in MD lymphomas, although it is induced not only by virulent strains of MDV but also by non-oncogenic (20) and attenuated MDV (21), and by HVT (20,21). It is distinct from the viral antigens of productively or abortively infected cells. MATSA is probably related to normal cellular antigens, and it may be a virus-modified histocompatibility antigen (22). MD lymphoma cells and cultured lymphoblastoid line cells also express other antigens including a chicken foetal antigen (23), and an Ia-like antigen (24) as well as heterophile antigens.

3.1. Antibodies

Antibodies against virus antigens can be detected in sera from infected birds by precipitation in agar gel (10), immunofluorescence (25), virus neutralization (26,27), indirect haemagglutination (28), complement fixation (29) and enzyme-linked immunosorbent assay (30). These antibodies are directed against internal antigens, membrane antigens and virus envelope. Precipitating antibodies may be detected between 7 and 14 days after inoculation with MDV (31). There is no relationship between the development of precipitins and recovery from infection (26). Fluorescent IgM antibody was observed between 5 and 12 days after inoculation with MDV (31). This corresponded to the initial detection of neutralizing antibody. IgG antibodies first appeared after 7 or 8 days and then gradually increased, particularly in resistant birds, paralleling the increase in neutralizing antibody. Neutralizing antibodies peaked between 6 and 12 days, followed by a drop, and then a gradual rise in titres for several weeks. The levels were generally higher in resistant birds, and a correlation between neutralizing antibody level and survival in genetically resistant birds has been observed (26).

There is little evidence for a significant humoral response against

MD tumour cells. Antibodies against MATSA can be raised in chickens by the inoculation of lymphoma or of MD lympoblastoid line cells (19,32) but they have not been demonstrated in convalescent MDV or HVT-infected chickens. Sera from MDV or HVT-infected birds were not cytotoxic to MSB-1 lymphoblastoid line cells in complement-dependent cytotoxicity or ADCC tests, suggesting absence of anti-MATSA activity (33,34). Humoral cytotoxic activity has been demonstrated in sera from chickens immunized with inactivated MSB-1 cells but this is unlikely to have been specific for MATSA. Despite this, it has been observed that the passive administration of serum from convalescent birds hastened the regression of transplantable MD tumours (35), presumably through the activity of cytophilic antibodies acting alone or in combination with lymphoid cells.

3.2. Cell-mediated responses

The first direct evidence for a cell-mediated immune response against viral antigens in MD came from delayed hypersensitivity reactions to various MD-associated antigens in chickens with naturally occurring infections (36). Stronger reactions were elicited with cellular antigens, probably B and C antigens, than with a soluble antigen (A antigen) and the extent of the response was correlated with the presence of gross lymphomatous lesions. The inhibition of leukocyte migration *in vitro* has also been reported (37).

Activity against cell-free virus or against infected cells has been demonstrated *in vitro*. B-cells were thought to be involved in the inactivation of cell-free MDV by peripheral blood lymphocytes (38), and B-cells with anti-viral immunity were found in the spleens of infected chickens (39). Ross, however, demonstrated the presence of effector T-cells in a plaque inhibition test (40). Lymphocytes from birds immunized with attenuated MDV inhibited plaque formation in cells infected with virulent MDV. Plaque inhibition was mediated by sensitized T-cells which interacted with virus-specific antigens at the surface of infected leukocytes or kidney cells. Interestingly, this inhibitory activity was not restricted by major histocompatibility complex antigens; inhibition occurred whether the infected target cells were syngeneic or allogeneic with the sensitized T-cells. However, the activity is relatively specific for either MDV or HVT; lymphocytes from birds vaccinated with HVT had limited activity against MDV-infected

target cells, and similarly, those from MDV-inoculated birds had limited activity against HVT-infected targets. This specificity, however, was lost when either anti-MDV or anti-HVT antisera were added to the system, so that, for example, anti-HVT antiserum could act in conjunction with normal or with HVT-sensitized lymphocytes against MDV-infected target cells (41,42). Cell-mediated cytotoxicity against MD lymphoblastoid line cells has been demonstrated in vitro (43-46). Spleen cells or peripheral blood lymphocytes from chickens infected with various strains of MDV or HVT are cytotoxic against target cells labelled with (^{51}Cr)-sodium chromate (14,47), (^{3}H)-proline (48) or (^{35}S)-methionine (34). Activity appears soon after infection with pathogenic, apathogenic or attenuated strains of virus, and seems to peak at about 8 days, coinciding with the appearance of MATSA in the spleen (49), although the reported association between cytotoxic activity and the stage of infection is extremely variable. The effector cell involved has been shown to be a T-cell (50). The degree of specific isotope release was low in all reports, but it may be increased by treatment of the lymphoblastoid targets with neuraminidase (50), presumably by enhanced exposure of the relevant tumour-associated antigens. The antigens involved in this immune attack are unknown. An obvious candidate is MATSA, but Schat and Murthy (51) showed that MATSA could be removed from target lymphoblastoid cells by treatment with papain without influencing the results of the chromium release assay, and blocking MATSA with specific antiserum also failed to alter specific release. Another possibility would be a LYDMA-like antigen on MD tumour cells. The significance of the results of the in vitro isotope release assays is in question, however. It may be that the observed cytotoxicity is an artefact because most reports are of reactions between allogeneic effector cells and target cells. Where the major histocompatibility antigen was shared between target cells and effector cells very little or no specific release was observed (52,53). The minor histocompatibility antigens also have some influence on the degree of specific release (53). In addition, it is not clear that these observations indicate the presence of an effective anti-tumour immune response as Dambrine et al. (48) found cytotoxicity only with effector cells from tumour-bearing donors. Other workers were unable to associate the cytotoxic activity of leukocytes from individual

chickens with the outcome of infection (46,54) although, on a group basis, chickens showing lower mortality or incidence of gross lesions because of genetic or age resistance did exhibit higher cytotoxicity.

Sensitized T-cells from convalescent chickens or from those vaccinated with attenuated MDV were found to be cytotoxic not only to chicken kidney cells infected with MDV _in vitro_ but also to latently infected lymphocytes isolated from infected birds(40,55). This activity was thought to be mediated through an antigen common to abortively infected, non-lymphoid cells and non-productively infected lymphocytes, although such an antigen has not been demonstrated serologically. Such an immune response would be capable of eliminating infected, non-transformed lymphocytes and could account for the fall in titres of infected cells in the spleens and other tissues of resistant birds after the first week following infection. Most of these cells express no virus antigens detectable by immunofluorescence, and of those with internal antigens soon after infection, very few have virus membrane antigens: it is therefore unlikely that this immune response is directed against virus-specific antigens.

The relationship between the lymphocyte-determined membrane antigen and the antigens on transformed cells is not clear. As immunization with inactivated tumour cells had no effect on the behaviour of the virus after infection, it seems probable that different antigens are involved. In contrast, the successful immunization against the MD transplant JMV, which lacks virus-specific antigen, with inactivated infected chicken kidney cells (56) raises the possibility of shared antigens between abortively infected epithelial cells and transformed lymphoblasts.

3.3. _Antibody-dependent cell-mediated cytotoxicity (ADCC)._

It is likely that both direct cell-mediated cytotoxicity and ADCC directed against virus-infected cells are important in controlling the spread of cell-associated virus in infected chickens. ADCC has been demonstrated with antiserum from MDV- or HVT-infected birds, which, in conjunction with normal splenic or blood lymphocytes is cytotoxic to cells infected with either virus (41,42). ADCC activity against lymphoblastoid line cells has not been found in serum from infected birds, but was in serum from chickens immunized with the lymphoblastoid cells them-

selves (43).

3.4 Macrophages

Macrophages, or their reticulum cell precursors, appear to be a target cell for MDV infection although attempts to infect them *in vitro* have been unsuccessful. They may have a role in immunity to MD. Treatment with silica particles caused a delay in the appearance of clinical signs in susceptible birds, and also reduced the amounts of fluorescing virus antigen in the thymus and bursa at 5 days after infection (57). Contrary to expectation, however, the silica treatment was found to increase the number of glass adherent cells in the peripheral blood by up to 10-fold, and it was suggested that silica treatment induced macrophage proliferation, and consequently restricted virus replication or spread. In contrast, inoculation of antimacrophage serum or trypan blue suppressed macrophage function *in vivo* and was associated with elevated virus titres and increased tumour incidence, supporting an immunosurveillant role for macrophages (58).

Macrophages from infected chickens have been shown to restrict MDV replication *in vitro*, and also to influence the proliferation of lymphoma cells in the spleens of infected chickens (39). Macrophages also inhibited the DNA synthesis of MD lymphoblastoid line cells *in vitro* (59). The antiviral effect appeared to be mediated through a cooperative action of macrophages and antibody (39,60). It has also been shown that macrophages can inactivate cell-free MDV in cooperation with B-cells (38). At least some of the activity of macrophages may be attributable to their regulatory function with regard to T-cells. This could explain their activity in suppressing the mitogenic response of spleen cells at 1 week after virus infection (59). This effect, and the restriction of lymphoblastoid cell line growth, is seen both with macrophages from infected birds and with normal macrophages when present in large numbers (61), suggesting that the effect observed may result from the amplification of a normal phenomenon.

3.5. Interferon

Interferon may be produced as a response to infection with some strains of MDV and HVT (62,63), and levels were found to be higher in resistant than in susceptible birds (64). Interferon had a protective effect against the JMV transplantable tumour (65). It might play some

role in the initial host response to MDV; however, an effect would be expected soon after infection, yet as a general rule, early pathogenesis is unaffected by resistance factors such as age or genetic resistance. Additionally, not all strains of virus induce interferon, so that it is unlikely to be essential to MD resistance.

3.6. Natural killer cells

Apart from the activity of normal macrophages against MD tumour cells, spleen cells from certain strains of chickens were found to be cytotoxic to MD lymphoblastoid cell lines *in vitro* (66). It was possible to transfer protection against the JMV transplantable tumour by injecting non-macrophage, non-T, non-B cells from 8-week-old chickens into younger birds suggesting a role for natural killer cells in age resistance to this transplant (67). They may also be involved in genetic and vaccinal resistance. Natural killer cell activity was found to be inhibited in susceptible chickens during the development of MD, but activity was enhanced in genetically resistant or vaccinated chickens following infection with MDV (68).

4. CONTRIBUTION OF IMMUNE RESPONSES TO THE EXPRESSION OF IMMUNITY

The preceding section of this chapter has dealt with the immune responses that have been documented in MD. The mere demonstration of a particular immune response does not imply that it is important in conferring resistance. It is now necessary to consider the contribution made by the different immune reactions to the various types of innate, passively or actively acquired resistance described earlier.

4.1. Innate resistance

4.1.1. Genetic resistance. As mentioned previously, two distinct genetic loci are associated with genetic resistance. It now appears that the two types of genetic resistance are mediated by distinct mechanisms, although in neither case does resistance reside at the level of cellular susceptibility to infection as cells from different genetic strains replicate MDV equally well *in vitro*. Resistance associated with the B^{21} allele is believed to depend upon differences in immunosurveillance and perhaps therefore should be properly regarded as a form of acquired rather than innate resistance. In contrast, the line 6 type of resistance which is associated with the *Ly-4* locus is present at

hatching and is believed to depend primarily on differences in susceptibility of the presumed target cell for MDV, the T-lymphocyte. Compared with chickens of the susceptible line 7, line 6 chickens develop low titres of virus in the blood and the lymphoid organs, and this difference is seen in situations where the immune system would not be expected to have any influence, for example, immediately after infection and in chick embryos (69). Significant differences were also noted in the size and cellular composition of the lymphoid organs of the two lines (70). Direct evidence for a target cell difference between the lines was provided by two experimental approaches. The transplantation of line 7 thymuses into thymectomized line 6 chickens conferred susceptibility on line recipients, although the reciprocal procedure did not make line 7 chickens resistant (70,71). Line 7 lymphocytes were also shown to be more susceptible to infection by MDV in vitro (71). Nevertheless, immunosurveillance appears to have some part in line 6 resistance because thymectomy increased susceptibility in this line, implicating cell-mediated immunity. Bursectomy, however, did not influence the expression of resistance (72), showing that antibodies are not essential, although neutralizing antibody titres are lower in line 7 than in line 6 chickens (27), presumably owing to immunosuppression. Similarly, macrophages are thought not to be involved in this type of genetic resistance (73). Higher antiviral and anti-tumour cell-mediated immunity has been observed in line 6 compared with line 7 chickens (46,69), accompanied by the disappearance of MATSA-bearing cells in the spleen of the resistant chickens. These results suggest that immune mechanisms are effective in line 6 chickens because of the innately lower levels of lymphocyte infection (and hence of immunosuppression) and of transformation.

Resistance associated with the B^{21} allele has some characteristics in common with resistance seen in line 6 chickens. Thus resistance is not abolished by bursectomy (74) although resistant birds have higher virus neutralizing antibody titres (26). The lower titres seen in susceptible birds are due to the immunosuppressive effects of MDV infection rather than to an inherent inability to produce antibodies because susceptible and resistant strains develop similar levels when infected with non-pathogenic strains of MDV (31,75). Lymphoma incidence

in resistant chickens is increased by thymectomy (76), as in line 6. It is noteworthy that thymectomy decreased lymphoma formation in line 7 and other susceptible chickens (77), presumably by diminishing the number of target cells available for transformation. The differential effect of thymectomy on lymphoma formation in susceptible and resistant lines might be predicted as T-cells provide both targets for transformation and effectors for the elimination of virus and of transformed cells (2).

In other respects, resistance associated with the $\underline{B^{21}}$ allele differs from that described for line 6 chickens. The early virological events are similar in resistant and susceptible birds (78) but a significant difference in the behaviour of MDV occurs between 7 and 10 days after the period of cytolytic infection. There is an apparent restriction of infection in resistant birds, shown by a fall in virus titres in the blood and spleen (78), which coincides with the development of humoral antibodies and of cell-mediated immunity. MATSA-bearing cells also appear simultaneously in the two types of bird, but disappear in resistant birds (79). The transfer of the thymus from susceptible chickens to thymectomized resistant chickens did not influence lymphoma formation; in contrast, the reverse procedure, transferring thymus from resistant to susceptible chickens rendered the recipients more resistant (76). These results sugest that resistance linked to the $\underline{B^{21}}$ allele is mediated by a superior immune response to MD-associated antigens, resulting in the suppression of virus replication and rejection of transformed cells. Natural killer cells may also be involved in this type of genetic resistance (68).

4.1.2. Sex. The effect of sex has not been widely studied and the mechanism involved is unknown. Treatment of chickens with androgenic or oestrogenic hormones did not influence the outcome of infection with MDV.

4.2. Passively acquired immunity

The protective effects of maternal antibody are attributable to the observed reduction in virus replication shown by lowered levels of leukocyte-associated viraemia and reduction in virus antigen production in the lymphoid organs. Passive antibody reduces the number of tissues with viral antigen and the amount of antigen in positive tissues is lowered, and fewer infected cells can be detected (11,80). It prevents bursal atrophy and lengthens the latent period for antigen and cell-free virus production in the feather follicle epithelium (81). The initial response to infection is a proliferation of lymphoid tissue, containing only a minor reticulum cell element with no intranuclear inclusion bodies. The presence of antibody was found to delay the disappearance of the dense granular lymphocytes first seen in the lymphoid organs at 3 days after infection and it tended to inhibit regressive changes and the appearance of cell debris, pale reticulum cells and macrophages (82). Virions were not seen.

As most MDV is present in an infected chicken in an intracellular, cell-associated form, virus neutralization itself presumable does not occur. Complement-dependent cytotoxicity and ADCC may reduce the number of cells carrying virus. Burgoyne and Witter (81) inoculated cell-associated MDV into passively immunized chickens and described neutralization in vivo whose effect, however, was greater against cell-free virus. A similar effect was noted when MDV-infected cells were injected into the antibody-containing yolk of embryonated eggs (83), or when MDV-infected cells were mixed with immune serum and inoculated onto chick kidney cell cultures (84). Some strains of MDV, e.g. GA strain, may spread through the animal, at least partly, as cell-free virus in the plasma, and would be directly available to neutralizing antibody.

The effect of maternal antibody on the amount of MDV in the tissues would probably have a direct effect on the development of lymphomas, for it is known that there is a correlation between the levels of leukocyte-associated infectivity and subsequent tumour formation (27,85,86). An early sparing of the lymphoid tissues by maternal antibody could protect the immune system from damage, maintain surveillance and reduce the level of infection and viraemia, thus reducing the chances of interaction between virus and potentially transformed target cells. There is

also the possibility of a direct effect of antibody on the expression of the virus genome in transformed cells, for virus particles have not been seen in lymphoma cells from antibody-positive chicks (82). Antibodies capable of mediating MD tumour cell aggression may, in part, account for the influence of maternally derived antibody on the development of the disease (35).

4.3. Actively acquired resistance

4.3.1. Vaccination. Vaccinal resistance is probably mediated by a conventional immunological process. Other possible explanations, such as virus interference, are not consistent with successful immunization using inactivated antigens (87). It has also been shown that HVT and MDV genomes can co-exist in individual cells of a T-lymphoblastoid cell line (88). The importance of the immune system is also indicated by the development of various immune responses against viral and tumour antigens in vaccinated birds, and the abrogation of vaccinal immunity by treatment with the immunosuppressive drug cyclophosphamide (89).

Immunity induced by vaccination seems to have anti-viral and anti-tumour components. Anti-viral effects are to be expected in view of the antigens common to vaccine and pathogenic strains, and neutralizing antibodies and antiviral cytotoxicity develop in vaccinated chickens. Both HVT and attenuated MDV have been shown to protect against the early cytolytic infection caused by the virulent virus, and the normally observed immunosuppression is prevented by prior vaccination (15). The vaccine viruses also provoke immunity against tumour antigens, presumably as a response to MATSA or other tumour-associated antigens that they induce in vaccinated birds. Vaccine viruses, therefore may have a limited transforming potential, sufficient at least to stimulate an immune response. Unchallenged HVT-infected birds develop a persistent viraemia and mild, transient lymphoproliferative lesions in the nerves and gonads, suggesting a limited transformational event (15). Cytotoxicity against MD cell lines can be assayed in vitro, and HVT, attenuated or non-oncogenic MDV (SB-1) can all immunize chickens against a variety of MD transplantable tumours (90,91), including the transplant JMV which does not possess viral antigens and lacks rescuable MDV but which expresses MATSA. These viruses all stimulate the appearance of MATSA-bearing cells and of lymphocytes cytotoxic to tumour cells: however,

inactivated preparations of the viruses are unable to immunize against the JMV transplant (91,92), emphasizing the importance of tumour antigens in this type of protection.

The absence of anti-tumour immunity may also account for the failure of HVT to protect turkeys against the oncogenic effects of MDV (93), as HVT infects turkeys but does not induce the appearance of MATSA in this species (94). In contrast, immunization with an attenuated strain of JMV was found to protect against challenge with low-passage tumorigenic JMV but not against primary tumours caused by MDV (95).

These findings are consistent with the "two-step" mechanism of resistance originally suggested by Payne et al. (2). This envisages an initial resistance to virus replication and spread; the reduced level of virus activity would result in a lowered incidence of malignant transformation and at the same time the lymphoid system would be spared the immunosuppressive effects. The second step would be an immunological rejection of transformed cells, independent of the earlier virological events, although influenced by them to the extent that immunosuppression and lymphocyte transformation occur. Virus and tumour-specific antigens respectively would stimulate these responses. This hypothesis has gained support from the reports of successful immunization against MD using inactivated viral or tumour antigens; in these experiments it was possible to dissociate the immune repsonses to the two types of antigen. Inactivated soluble or insoluble proteins extracted from infected cultured cells, or inactivated whole infected cells, were found to protect against MD by inducing immunity against virus antigens (96,97). Similarly, inactivated lymphoblastoid line cells were partially protective by virtue of an immune response against transformed cells (87,98, 99). Both types of inactivated vaccine inhibited the appearance of MATSA-bearing cells (i.e. transformed cells) and prevented lymphomas developing but apparently by different mechanisms. The immunity induced by viral antigens was characterized by the development of neutralizing antibodies, and there was a lowering of MDV viraemia, inhibition of virus replication and the early degenerative changes in lymphoid organs, but absence of lymphocytes cytotoxic against tumour cells. Nevertheless, lymphomas did not develop, presumably because of the combined effects of the sparing of the immune system and the limited transforma-

tion, both consequences of the reduced levels of virus activity. Immunization with tumour cells did not influence the early virological events, but lymphocytes cytotoxic to lymphoblastoid line cells were detected and this anti-tumour immune response was presumably responsible for mediating tumour cell rejection.

Attempts have been made to implicate a single resistance mechanism as all-important in determining vaccinal resistance to MD and the available evidence suggests a dominant role for T-cell mediated immunity (100). Thus full protection was observed in agammaglobulinaemic, bursectomized birds vaccinated with attenuated MDV (101). HVT, however, was found to be less effective in bursectomized chickens than in normal chickens indicating some role for humoral immunity in HVT vaccination (102). Nevertheless, thymectomy was found to reduce protection provided by HVT, and the impairement of HVT-induced vaccinal immunity by cyclophosphamide (89) was found to be transient, development of protection apparently being related to recovery of the thymic system (103), although partial recovery of the bursal system cannot be excluded. It is probable that normally all branches of the immune system are involved in resistance to MD and it may be that there is no absolute requirement for any one form of immune response in order to confer resistance on an individual. Thus, under appropriate experimental conditions, a degree of resistance might be provided by cell-mediated immunity, antibodies, macrophages or natural killer cells alone. Some support for this view comes from the results of experiments involving vaccination of immunosuppressed chickens with inactivated vaccines, and the adoptive transfer of protection by spleen cell transfer within an inbred strain of chickens (104).

4.3.2. Age resistance. The gradual development of resistance with age also seems to depend upon immune mechanisms for it develops in parallel with the acquisition of immune competence (17), and it is abolished by thymectomy (105), but not by bursectomy (74). The observation of the regression of lymphoproliferative lesions has caused the rejection of transformed cells to be implicated as the basis for age resistance (106). The early pathogenesis of MDV infection is similar in susceptible and resistant birds, and in resistant birds irrespective of age (78), so that resistance must operate at a stage later than the

abortive lytic infection of the lymphoid organs. Age resistance seems to depend upon genetic resistance of the B^{21}-linked type, since genetically resistant strains free of passive antibody may be nearly as susceptible at hatching as genetically susceptible strains, but the former develop resistance over a period of several weeks (17).

5. IMMUNOSUPPRESSION

Immunosuppression is a prominent feature of MD and the degree of immunosuppression may be important in determining the likelihood of subsequent neoplastic disease. The same factors that influence the development of lymphomas (virus strain, age, genetic constitution etc.) also affect the degree of immunosuppression. Both antibody and cell-mediated immunity are affected (2).

Depression of the *in vitro* responses of lymphocytes to the lectin mitogens phytohaemagglutinin and concanavalin A seems to be a feature of early infection. This is seen at 7 days after infection with virulent MDV, coinciding with the stage of acute cytolytic infection of lymphoid tissue (107-109). The same effect, however, is seen with non-oncogenic MDV and HVT. The initial depression is transient, and responses are regained, although variably so, in both MD-susceptible and MD-resistant chickens. Susceptible birds that succumb to the disease show depression later, but responses are normal or enhanced in genetically-resistant birds or in those infected with non-oncogenic MDV or HVT. Vaccinated chickens when challenged with oncogenic MDV were protected against both the initial and secondary depression of mitogen responsiveness (107).

The secondary depression coincides with impaired humoral and cell-mediated immune responses. Primary and secondary antibody responses and delayed hypersensitivity responses are all depressed and allograft rejection is delayed (110-114). Infected chickens are more susceptible to other diseases such as coccidiosis (115), and MD-affected chickens were also found to be deficient in their ability to regress Rous sarcomas (116).

5.1. Mechanisms of immunosuppression

Cytolytic infection and attendant destruction of the lymphoid tissue, although severe in the case of infection with oncogenic MDV, is unlikely to account entirely for the early depression of mitogen res-

ponsiveness as non-oncogenic MDV and HVT, which do not cause these changes, are similarly depressive (14). Nevertheless, these viruses localize in the lymphoid tissue and may bring about functional alterations without obvious histological signs. An alternative explanation is the presence of suppressor macrophages (59,107). Macrophages taken from spleen cell cultures with depressed responses could in turn depress the responses of normal spleen cells to which they were added. Similar cells may be present in the spleens of normal chickens (61). However, primary immunodepression is a feature of many virus diseases, and a variety of infectious or non-infectious virus particles can inhibit the mitogen responses of chicken lymphocytes (117).

Immunosuppression during the lymphoproliferative phase of MD could have a number of explanations. There could be depletion of normal responsive cells as a result of lymphoid atrophy or replacement of normal cells by non-reactive lymphoma cells (1). Alternatively there could be suppressor influences. Soluble suppressor factors, but not suppressor cells, were found in the spleens of immunosuppressed chickens (118,119). Subpopulations of suppressor cells were also demonstrated in chickens bearing the JMV transplantable lymphoma. It has been shown recently that suppressor factors are produced in the supernatants of cultured cell lines, suggesting that MD lymphoma cells may themselves be transformed suppressor T-cells (L.N. Payne, personal communication). This would provide a direct mechanism for MD lymphoma cells causing immunosuppression, and would explain the association of immunosuppression with lymphoproliferation and clinical disease rather than with infection alone.

Whatever the mechanisms involved, the experimental data are consistent with the view that the secondary immunosuppression seen in MD may be a consequence rather than a cause of lymphoma formation. The primary immunosuppression may contribute to the development of lymphomas, but its occurrence in chickens infected with non-oncogenic viruses makes its role uncertain.

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8. PRINCIPLES OF VACCINATION

R.L. WITTER

1. INTRODUCTION

Vaccines are a natural focal point for Marek's disease (MD) as they pose challenging questions regarding their mechanism of action and constitute the main means by which the disease can be controlled. The first practical vaccine for MD was developed by Churchill *et al.* (1) and consisted of an attenuated serotype 1 MD virus (MDV). This development followed shortly the isolation of the causative herpesvirus of MD and its propagation in tissue culture. Several other vaccines were subsequently developed. Probably turkey herpesvirus, a serotype 3 virus, is the most widely used of the vaccine types at present.

Vaccination is by far the preferred control procedure for MD and is in world-wide use for broiler breeder and layer chickens. In addition, commercial broilers are vaccinated routinely in the USA and in certain other countries.

Excessive MD losses have been noted sporadically among vaccinated broiler and layer flocks in several countries. Commonly termed vaccination failures or vaccine breaks, these losses include those due to excessive exposure to virulent MD strains and to misdiagnosis in addition to those due to true failure of the vaccines to induce immunity against MD. Nonetheless, such losses have been a potent incentive for continued effort on the development of improved vaccines.

Vaccines have been prepared from viruses of serotype 1, 2 and 3 (see Chapter 4 for details of serotype analysis). Classification of vaccine virus strains by serotype has recently become routine following the development of type-specific monoclonal antibodies (2). Within serotype 1, the oncogenic MDV's, mildly virulent, virulent, and very virulent pathotypes have been recognized. Vaccines have been prepared from each

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pathotype following appropriate attenuation in tissue culture. Serotype 2 and 3 viruses are naturally nononcogenic strains derived from chickens or turkeys, respectively, and are used as vaccines without modification. Although viruses of the several serotypes are antigenically distinguishable, all share abundant common antigenicity. Presumably one or more of the common antigens is responsible for the cross-protection by serotype 2 or 3 vaccines against disease induced by virulent serotype 1 MDV. Types of MDV, including vaccine strains, are listed in Table 1.

TABLE 1. Classification and nomenclature of Marek's disease virus types.

Name	Serotype	Pathotype	Attenuation	Used as vaccine
Oncogenic MDV	1	very virulent[1]	no	no
		virulent	no	no
		mildly virulent	no	rarely
		very virulent	yes	yes
		virulent	yes	yes
		mildly virulent	yes	yes
Nononcogenic MDV	2	avirulent	no	yes
Turkey herpesvirus (HVT)	3	avirulent	no	yes

[1]Poorly protected against by serotype 3 vaccine

2. TYPES OF VACCINE

2.1. Attenuated virulent serotype 1

As noted, Churchill *et al*. (1) described a vaccine prepared from an attenuated virulent serotype 1 MDV (strain HPRS-16). A number of other virulent MDV's were subsequently attenuated (3 - 7) but commercial application was limited. This type of vaccine is normally used in the cell-associated form since little cell-free virus can be extracted from infected cells. The attenuated HPRS-16 vaccine was used extensively in Europe following its development and was considered effective, but now

has been largely superseded by other products. Reversion to virulence and spread by contact have not been reported.

2.2. Attenuated mildly virulent serotype 1

Rispens *et al*. (8,9) described a vaccine from strain CVI 988, a mildly virulent serotype 1 isolate further attenuated by 20 tissue culture passages. This cell-associated vaccine has been widely used in the Netherlands and sporadically in other countries. Mild pathogenicity for chickens of high genetic susceptibility has been noted (10), probably indicating incomplete attenuation. This virus spreads readily by contact; however, contact spread does not occur rapidly enough to induce the early immunity needed to counteract exposure to virulent virus in the first weeks of life (9,11). Therefore, as with other vaccines, this type of vaccine is administered by inoculation of every chicken at hatching. This vaccine is reported to be highly effective (12,13). Other mildly virulent strains (14,15), despite some inherent or residual pathogenicity, also provided significant protection against MD.

2.3. Attenuated very virulent serotype 1

An experimental vaccine prepared from an attenuated very virulent strain (Md11/75C) was described by Witter (16). This vaccine was particularly effective against challenge with very virulent MD viral strains in chickens free of maternal antibodies. However, it was highly neutralized *in vivo* and was relatively ineffective in chickens with either homologous or heterologous maternal antibodies (17). Upon backpassage, this virus developed partial virulence. The pathogenicity of the derived virus was mild and was stable upon further backpassage. Furthermore, the derived virus replicated to a higher titre in inoculated chickens than the parent strain. It cannot be determined whether this derived virus is a true revertant or was an unattenuated contaminant in the original stock. This type of vaccine has been used as a component of experimental polyvalent vaccines (16,18) but has not been produced commercially.

2.4. Serotype 2

The protective efficacy of serotype 2 viruses was first observed by Zander *et al*. (19) who vaccinated chickens with chicken blood later found to contain the HN-1 strain of MDV (20), and by Biggs *et al*.

(21,22) who found flocks first exposed to serotype 2 viruses in the field experienced fewer MD losses than other flocks. Jackson et al. (6) used the naturally apathogenic MDV-19 strain as a vaccine. Schat and Calnek (23) isolated the SB-1 strain and characterized it as non-oncogenic since no proliferative lesions were observed even in immuno-suppressed chickens; however, some pathogenicity for chicken embryos was noted. Serotype 2 vaccines are cell-associated products since very little cell-free virus can be derived from infected cells. The SB-1 strain spreads readily by contact, is considered efficacious against challenge with most virulent MD viral strains, and has recently been marketed commercially. Its major use is in combination with serotype 3 virus as a bivalent vaccine to which it provides an additive or synergistic effect.

2.5. Serotype 3

Kawamura et al. (24) first isolated turkey herpesvirus (HVT) from tissue cultures of normal turkeys. Witter et al. (25) isolated the FC126 strain from a commercial turkey flock and characterized the virus as nonpathogenic and antigenically related to MDV. The virus is ubiquitous among turkeys where it is highly contagious (26). However, among chickens its spread by contact is poor and probably only occurs at certain time intervals after infection of chicks with large doses of virus (27 - 29). Okazaki et al. (30) and Purchase et al. (31,32) described the efficacy of the FC126 strain as a vaccine. Several other serotype 3 strains have been isolated (33,34,35) and appear equally effective as vaccines. Although the vaccine is commonly used as a cell-associated product, substantial amounts of cell-free virus can be extracted from infected cells (25) which can be preserved by lyophilization (36), thus forming the basis of a cell-free vaccine. Both cell-associated and cell-free vaccines have been widely used in virtually all countries of the world. This type of vaccine has been considered highly effective and is the prototype of MD vaccine viruses.

2.6. Polyvalent

The concept of polyvalent vaccines derives from the observation by Witter (16) that different vaccine viruses have a partial specificity for challenge viruses of certain serotype 1 pathotypes. A trivalent vaccine composed of viruses representing attenuated serotype 1, serotype

2 and serotype 3 was found to be highly effective in protecting chickens against challenge with very virulent MD viral strains even in the presence of homologous maternal antibodies (16,17). The efficacy of this trivalent vaccine was significantly greater than that of serotype 3 or any other single viral component used alone. Through laboratory and field trials it was ultimately determined that the attenuated serotype 1 component was not necessary (17,18). Thus a bivalent vaccine consisting of the SB-1 strain of serotype 2 MDV and the FC126 strain of serotype 3 was found to be superior to serotype 3 alone in field trials on commercial broiler and layer flocks (18,37). This bivalent vaccine was licensed in the USA for commercial production in October 1983 and is in current use in flocks and geographical regions where HVT vaccine has not previously provided desired levels of protection. The bivalent vaccine is administered as a cell-associated product where the two component viruses are mixed just prior to inoculation of chicks.

2.7. Vaccines lacking infectious virus

Several experimental vaccines lacking in infectious virus have been studied. One such vaccine consists of suspensions of live, non-producer transplantable MD tumour cells of the JMV strain. These cells lack rescuable MDV but possess the MD tumour-asociated surface antigen (38). The tumourigenicity of this transplant was reduced by serial passage in embryos (39) or by serial cultivation of the tumour cells *in vitro* (40). Although this type of vaccine was reported to be useful for immunization for chickens (40), evaluation in other laboratories has shown protection to be minimal (41 - 43).

Significant protection has been induced by inactivated, intact cells productively infected with serotype 3 (44) or serotype 1 MDV (45 - 47). Subunit vaccines prepared from such preparations are also effective. Membrane fractions from cells productively infected with serotype 3 were even more effective than vaccines made from inactivated whole cells (44). Antigens solubilized with detergents from MD virus- infected cells (48) and polysomes (49) or high molecular weight glycoproteins (50) extracted from serotype 3-infected cells have also induced significant protective immunity. Lesnik *et al.* (51) described protection by an extract from the skin of MDV-infected chickens. Inactivated lymphoblastoid cell lines expressing tumour-associated antigens have also induced

significant protective immunity (45 - 47). Protective responses induced by such inactivated vaccines have at best been of modest magnitude. Protection induced by vaccines prepared from productively infected cells have ranged from 50 to 80%; Kaaden et al. (44) claimed 94% protection with a vaccine prepared from purified membranes of serotype 3-infected cells. Protection by inactivated lymphoblastoid cell lines has been generally poorer, i.e. 40 to 50%. Although none of these vaccines have been considered from commercial application, they have helped to elucidate basic mechanisms of vaccinal immunity and may be the forerunners of a future generation of genetically engineered vaccines for MD.

3. RESPONSES TO VACCINATION

In practice, vaccines are usually administered to newly-hatched chicks by parenteral inoculation, usually the subcutaneous or intramuscular routes, at doses of 1,000 to 2,000 plaque forming units (pfu) up to 10,000 pfu or more. As all commercial vaccines contain live infectious virus in cell-associated or cell-free form, infection ensues following vaccination and a variety of responses are induced.

3.1. Virologic responses

Most vaccine viruses rapidly induce a cell-associated viraemia, i.e. virus can be rescued from blood lymphocytes by co-cultivation in chick embyro fibroblast cultures. Virus has been recovered as early as 2 days post-vaccination for serotype 3 (17,52), 5 to 6 days for serotype 2 (17, 53), and 4 days for attenuated virulent serotype 1 (54). Peak titres are reached in 8 to 12 days for serotype 3 (17,55), subsequently decline, and remain at a consistent, low level for long periods (55,56). When serotype 3 vaccine is administered to 18-day-old embryos (57), virus is detected earlier and reaches higher titres than in chickens inoculated at hatch (58). For serotype 2, peak titres occur from 6 to 21 days post- vaccination (17,53). Peak titres have not been as carefully documented for attenuated serotype 1, but occurred in one case on day 11 (17) and in another case at the 5th week (35). Infection with vaccine viruses, as for virulent serotype 1 (59), is probably lifelong. Purchase et al. (32) isolated serotype 3 from 58% of day-old-vaccinated chickens at 75 weeks post-vaccination. However, the frequency with which virus can be reisolated decreases with increasing age.

Comparative data on maximum viraemia titres with different vaccine viruses are minimal. Titres for serotype 3 range up to 1 - 3 x 10^2 pfu per 10^6 peripheral blood leukocytes (17,53) but tend to be 2- to 10-fold lower for serotype 2 under comparable conditions. Titres for attenuated serotype 1 are very low by comparison (17,35,60) and it seems clear that these viruses, following attenuation, partly lose their ability to replicate *in vivo* (17,35,54,60,61). Virus titres are also influenced by the presence of maternal antibodies (17,33,55), strain of chicken (62), viral dose (33,55,56), and immunosuppression (52,55,63).

In chickens free of maternal antibody, one bird infectious dose is approximately 1 to 5 pfu for serotype 3 (64 - 66). However, Thornton *et al.* (61) found for one nonprotective serotype 3 variant virus that 220 to 380 pfu were required to induce viraemia in 50% of inoculated chickens.

Infection with vaccine viruses of serotypes 2 and 3 is first detected in the lung and various lymphoid tissues (53,54), as is also the case for virulent serotype 1 MDV . Indeed, lymphoid cells from the peripheral blood or spleen constitute the preferred source inoculum for the re-isolation of vaccine viruses. Serotype 2 and 3 viruses do not induce productive-restrictive infections in lymphocytes characterized by cytolysis and antigen production as do virulent serotype 1 viruses (53, 55,67). Furthermore, the target cell for infection is probably neither a B-cell nor a macrophage since most infected cells lacked surface Ia and IgM and were not depleted by carbonyl iron treatment (68). However, a role for B-cells in serotype 2 infection was suggested by Calnek *et al.* (69) who reduced the infectivity of lymphocyte preparations with both anti-B and anti-T serum by complement lysis. Upon *in vitro* culture, lymphocytes latently infected with serotype 2 expressed viral antigen whereas no antigen was detected in cultured lymphocytes latently infected with serotype 3 (69); this suggests that the nature of the latent infections with serotypes 2 and 3 may differ.

The possibility that vaccine viruses induce transformation of lymphocytes is discussed in the next section.

Virus shedding, presumably through productive infection of the feather follicle epithelium as occurs for virulent serotype 1 virus, occurs readily with the CVI 988 strain of attenuated mildly virulent

serotype 1 virus and with serotype 2 strains. Sporadic shedding has been reported for serotype 3 (25,32,70). Unlike the readily spreading viruses, serotype 3 replicates only transiently in the feather follicle epithelium and is most easily demonstrated between 14 and 21 days (27, 28). Attenuated virulent and very virulent serotype 1 strains thus far tested have not spread by contact (17,54,71).

Embryo transmission of vaccine strains has not been reported. Progeny of vaccinated breeders will remain free of infection if reared in a pathogen-free environment (R.L. Witter, unpublished data).

No interference was noted *in vivo* between serotype 3 and attenuated very virulent serotype 1 viruses (17). Prior vaccination will significantly reduce titres of virulent serotype 1 challenge virus (17,55) but this is probably due to immunity rather than to viral interference. Clearly, different virulent and avirulent strains may coexist in the same chicken (72,73). This situation is prevalent in the field and must be considered when viruses are isolated.

3.2. Pathologic responses

No commercial vaccine strain is overtly pathogenic for chickens. In the USA, master seed virus stocks must not induce MD mortality or gross lesions and must not depress body weights through a 120 day period when inoculated into susceptible chickens at 10 times the normal field dose (Code of Federal Regulations 9:113:165).

In general, no clinical disease or depression of body weight has been noted with MD vaccines in laboratory or field experiments (1,8,17, 23,25,32). One strain of attenuated mildly virulent serotype 1 virus that has been widely used as a commercial vaccine produced gross nerve enlargements and MD mortality in up to 28% of a susceptible strain of chickens (10), yet in practice this type of vaccine has been found both safe and effective (12). Indeed, most if not all vaccine viruses induce pathologic responses of some magnitude under certain circumstances. Dobos-Kovacs *et al*. (74) reported endophthalmitis in flocks repeatedly vaccinated for MD but failed to establish MD vaccines as the primary cause of the lesion.

Of peripheral interest is the possibility that the inoculation process by which MD vaccines are administered to chicks may serve to disseminate by needle contamination other virus infections that may be

present in the newly-hatched chick (75,76), thereby contributing to disease problems of other types.

Although cytolytic lesions, i.e. organ atrophy, necrosis and viral antigen production, are commonly induced in lymphoid tissues by virulent serotype 1 viruses, few if any such changes are induced in intact, immunologically uncompromised chicks by vaccine strains. The bursa and thymus show no change in size or histological character (23,53,55,67,77) but minor ultrastructural changes consisting of invaginations of the nuclear and plasma membranes, and an increased number of lysosomes have been reported (52). A transient splenomegaly has been reported with serotype 2 and 3 viruses (53) but is not always apparent (67). Cytolytic lesions are more easily demonstrated in chicks following chemical or surgical immunosuppression, or in chicks inoculated _in ovo_. Although no significant cytolytic lesions were induced by serotype 3 virus in cyclophosphamide-treated (55) or thymectomized (63) chickens, _in ovo_ inoculation during early stages of embryonation resulted in pock formation on the chorioallantoic membrane (25) and in viral antigen-containing necrotic lesions in the heart and liver of embryos (78). However, only one of 70 chicks infected with serotype 3 virus _in ovo_ developed bursal or thymic atrophy (79). Under similar conditions serotype 2 virus appears somewhat more pathogenic. Occasional cytolytic lesions were observed in bursectomized or thymectomized chickens inoculated with the SB-1 strain (23) but in chicks infected _in ovo_ such lesions were relatively frequent and were accompanied by high mortality in some cases (23,79).

Lymphoproliferative lesions have also been induced by vaccine strains, although it is not clear whether these lesions are neoplastic or inflammatory in character. Gross lymphomatous lesions have not been reported in intact chickens (as previously stated) or in immunosuppressed (23,79) chickens following vaccination. However, minor histologic lesions consisting of lymphoid cell infiltrations have been observed in the peripheral nerves (53,55,63) of chickens inoculated with serotype 2 and 3 viruses. Although some pleomorphic cells typical of those in MD lymphomas occur in such lesions (55), the lesions do not progress. Calnek _et al_. (53) considered most of these lesions of the inflammatory type. These lesions were slightly delayed, but were no

more severe in cyclophosphamide-treated (55) and thymectomized (63) chickens following inoculation with serotype 3 virus. However, 4 to 8% of chickens infected early *in ovo* with serotype 2 or 3 viruses developed grossly enlarged nerves and up to 30% were clinically paralyzed (79). Histologic nerve lesions were much more frequent in chickens inoculated *in ovo* with serotype 2 compared to those inoculated with serotype 3 viruses (79). The MD tumour-associated surface antigen has been detected on a small proportion of lymphocytes from chickens vaccinated with serotype 2 and 3 viruses (53,80 - 82) but thus far has not been localized on cells comprising the lymphoproliferative nerve lesions. These several observations are consistent with a hypothesis (55) that following vaccination, a limited transformational event may occur, the progression of which is prevented by the host immune response. The inflammatory cells occasionaly observed in such lesions may be generated by this immune response. More data is needed to resolve whether or not a true neoplastic transformation is induced by vaccine strains.

3.3. Immunosuppression

In accord with the lack of morphologic changes in lymphoid organs, there is no evidence that vaccine strains of serotypes 1, 2 or 3 suppress or interfere with cellular or humoral immune responses to other antigens (17,83 - 85), even when inoculated into embryos (86). However, a transient depression of the response of T-cells to the mitogens, phytohaemagglutinin or concanavalin A has been noted about 7 days after vaccination (87 - 89) after which time the response recovers to normal levels or is even enhanced (87,90).

3.4. Immune responses

In general, vaccine strains induce similar types of immune responses as virulent strains (see Chapter 7). These responses are both humoral and cellular, and are directed towards both viral and tumour-associated antigens.

Antibodies reactive with viral antigens are induced by infection with all known vaccine strains (8,16,25,91). Any of several types of tests may be used for the detection of antibodies; however, antibodies against attenuated serotype 1 viruses that have lost the "A" antigen cannot be demonstrated by the agar gel precipitin test and other procedures, e.g. immunofluorescence or virus neutralization, must be used.

Virus neutralizing antibodies are usually considered most relevant to disease resistance (92). Although antibody titres are generally higher against antigens of the homologous serotype compared to heterologous antigens (93,94), tests to identify antibodies of one serotype in a mixture of antibodies to other serotypes are not yet available. An immunofluorescent method for distinguishing serotype 3 antibodies in mixtures has been described (25) but interpretations are subjective. Antibodies against tumour-associated antigens have not been detected in vaccinated chickens, and are induced only following hyperimmunization with MD tumour cells (95,96). Anti-viral antibodies are induced within 1 or 2 weeks following vaccination (86,91) and persist for life at relatively stable titres (16), but these relationships have not been well studied, perhaps because vaccine antibodies are normally obscured following infection with virulent field strains. Active antibody titres are lower following vaccination of chickens with maternal antibodies than in chickens without maternal antibodies (97). Functionally, the role of antiviral antibodies induced by vaccine strains may be to neutralize cell-free virulent virus (98), to cooperate with normal host lymphocytes to cause the lysis of productively-infected cells (99), and, when passively transferred to progeny chicks, to interfere with the efficacy of vaccines (100).

Cell-mediated immune responses are also induced in chickens by vaccine strains of all serotypes. Responses directed against viral antigens include delayed hypersensitivity (101), *in vitro* cytotoxicity as measured by plaque reduction (102,103) and antibody dependent cellular cytotoxicity (99,103). A number of workers have described cytotoxicity of effector T-cells from chickens vaccinated with serotype 2 or 3 viruses against cultured MD tumour cells bearing tumour-associated antigens on their surface (53,104 - 109). However, these responses are not always strong (110) and part of this response may be directed against alloantigens since reactions are much stronger in allogeneic compared to syngeneic systems (111,112). A delayed hypersensitivity reaction to fixed MD tumour cells induced by serotype 3 vaccine has also been described (113).

Rejection of MD tumour transplants can be considered another type of cell-mediated immune response induced by vaccination. All serotypes of

vaccine virus induce resistance to challenge with the JMV strain of transplantable MD tumour cells (41,114 - 117). This phenomenon appeared to be an immune response directed primarily against tumour-associated antigens because the JMV cell expresses tumour-associated but not viral antigens on its surface (38). The protection was ameliorated by thymectomy (117). However, vaccinated chicks were not resistant to certain other MD tumour transplants, especially when tested in a syngeneic host (96,117,118). Furthermore, resistance against JMV cells was not induced by inactivated fibroblasts infected with serotype 1, 2 or 3 vaccine viruses (117,119), but was induced by inactivated cells infected with virulent serotype 1 virus (120). This system, although widely studied, is still poorly understood.

Macrophages from chickens infected with serotype 3 virus inhibited colony formation by MD lymphoblastoid cell cultures (121), and natural killer cell activity is elevated following vaccination of chicks (122) or embryos (86) with serotype 3 vaccine, indicating further types of immune response to vaccination. Functionally, these cellular responses may constitute an immune surveillance system to eliminate cells bearing viral or tumour-associated antigens on their surface.

4. MECHANISMS OF PROTECTION

4.1. Characteristics of protection

Protection against virulent MD viral challenge can be detected a few days after vaccination and is considered fully effective by 1 to 2 weeks of age. When challenge is given simultaneously with vaccination, little protection occurs (123,124). Basarab and Hall (125) demonstrated protection when chickens were challenged at 24 hours, but not at 12 hours post-vaccination. The onset of immunity is also affected by the genetic strain of chickens (14) and the type of vaccine (43). Protection can be induced earlier; however, when vaccine virus is administered to 18-day-old embryos, chicks hatched from such embryos are significantly more resistant to early challenge than chicks immunized at hatching (57). The persistence of vaccinal immunity was demonstrated through at least 40 weeks (56). Thus, reinforcement of this immunity by early exposure to field virus is probably not essential, although possibly beneficial. Although some workers found that maintenance of a detectable vaccine

viraemia was correlated with maintenance of vaccinal immunity and freedom from MD in individual chickens (126 - 128), Witter and Offenbecker (56) demonstrated that vaccinated chickens from which vaccine virus could no longer be easily isolated were adequately protected against challenge with virulent MDV. Protection against tumour induction, even under optimal conditions, is rarely absolute; however, a protective efficacy of 85 to 95% is commonly achieved both in the laboratory and in commercial practice.

Vaccinated chicks are partially protected against the replication of virulent serotype 1 MD viral strains. In sharp contrast to unvaccinated chickens, little if any cytolytic infection of lymphoid tissues is induced by virulent MDV in vaccinated chickens (53,55) and there is no significant depression of immune response (129). Indeed, this sparing effect on the immune system may play a key role in the mechanism of protection. Titres of cell-associated virulent MD virus are significantly lower and develop later in vaccinated chickens than in non-vaccinated controls (35,55). The protective efficacy of vaccines against viraemia of virulent MDV is often greater than against tumour induction (17). However, sufficient virulent MD virus is shed from the feather follicle epithelium of vaccinated, challenged chickens (130,131) so that vaccination is little deterrent to the perpetuation of virulent MD viral strains in the field.

Virulent MD viraemia levels are clearly related to lesion formation, i.e. chickens with high virus titres are the most likely to develop lesions and subsequently die of MD (59). MD tumours consist of both transformed cells and nontransformed reactive cells, most of which possess T-cell markers (132). The infectivity of tumour-derived cells is not strictly correlated with cell size or the presence of tumour-asociated antigens (122). Since some latently infected lymphocytes in the peripheral blood that are detected in viraemia assays may thus be derived from tumours or be generated in response to tumours, viraemia may be both a consequence and a cause of tumour formation. Nonetheless, viraemia appears to be an excellent prognostic criterion for MD and may be valuable in evaluation of vaccine efficacy.

4.2. Specificity and synergism

Cross-protection between MD viral antigens is illustrated by the

efficacy of serotype 2 and 3 vaccines against virulent serotype 1 MDV and is the basis for the use of such vaccines in the field. Furthermore, vaccines of all three serotypes have been effective in the field. Thus, it might be assumed that all vaccines are equally effective against challenge with all strains of virulent serotype 1 virus. Evidence to the contrary was provided by studies on an experimental attenuated very virulent serotype 1 vaccine, Md11/75C (16). In chickens lacking maternal antibodies, Md11/75C vaccine was significantly more effective than serotype 3 vaccine against challenge with very virulent serotype 1 virus. Conversely, serotype 3 vaccine was more effective than Md11/75C against challenge with certain virulent serotype 1 strains. These data suggested at least a limited degree of specificity of vaccinal immunity for certain challenge virus strains.

This finding prompted experiments with polyvalent vaccines which led to the recognition of protective synergism among vaccine viruses (16). The efficacy of serotype 3 vaccine was enhanced by the addition of as little as 4% (by pfu content) of serotype 2 vaccine, and even fractional doses of serotypes 2 and 3 together, e.g. 400 pfu of each, were superior to full doses (2,000 pfu) of either vaccine alone (17). Although synergism has been best studied between serotypes 2 and 3 since this combination appears most suitable for use in the field (18,37,133), synergism has been detected among vaccine viruses of all three serotypes; in most cases mixtures of two vaccine viruses provided better protection than either vaccine virus singly and in some cases mixtures of two vaccines could even be improved by the additon of a third virus (17).

The mechanism of this specificity and synergism is not understood. Among the possibilities are unrecognized antigenic differences between viral strains, the ability of certain viral proteins to act as adjuvants for proteins of another viral strain, and the ability of different vaccine viruses to infect and thereby stimulate immunity through different subpopulations of lymphocytes. Different cell tropisms for serotype 2 and 3 viruses is suggested by the greater virus rescue from lymphocytes following culture (69) and the greater resistance of latently-infected lymphocytes to cytolysis by anti-Ia serum (68) of serotype 2 virus compared to serotype 3.

4.3. Immune mechanisms

The evidence that protection against MD lymphomas following vaccination is due to specific immune responses has been reviewed by Payne et al. (134), Payne (135) and many others. The salient points are the abrogation of vaccinal immunity by chemical immunosuppression (136, 137), induction of immune responses against viral and tumour-associated antigens as previously discussed, and induction of protective immunity with inactivated vaccines, thereby ruling out interference phenomena. As originally proposed by Payne et al. (134), immunity is directed against both viral and tumour-associated antigens. Both humoral and cell-mediated immune mechanisms appear to be involved. The relative roles of these factors are gradually becoming more clear.

T-cell-mediated immune responses appear relatively more important than humoral responses since immunity was abrogated by surgical thymectomy (138) or temporarily abrogated by cyclophosphamide treatment (136, 137) but was not affected (138,139) or only slightly affected (140) by bursectomy. Immunity to viral antigens may be more readily induced and thus more important than immunity to tumour antigens since chickens inoculated with inactivated preparations of fibroblasts productively-infected with virulent serotype 1 virus were better protected against MD lymphomas than chickens inoculated with inactivated lymphoma or line cells bearing tumour-associated antigens but few or no viral antigens (46,47). Moreover, anti-viral immunity constitutes an earlier line of defence against MD than does anti-tumour immunity. Thus, one may speculate that cell-mediated responses against viral antigens may be a singularly important component of vaccinal immunity.

Humoral immunity against viral antigens may also play an important role in vaccinal immunity. Virus neutralizing antibody titres correlated with MD lymphoma resistance among different genetic lines of chickens, and although this may have been the consequence of greater immunosuppression in the susceptible line, no comparable suppression of precipitin antibodies was noted (92). Bursectomy was partially effective in reducing the protective efficacy of serotype 3 vaccine in one trial (140). Antibodies induced by vaccine viruses are active in antibody-dependent cellular cytotoxicity reactions (99,103). Powell et al. (141) found immunity induced by inactivated attenuated virulent

serotype 1 antigens could be transferred to syngeneic recipient chickens by inoculation of immune spleen cells, but not if the spleen cells were derived from immunized, bursectomized chickens. Thus anti-viral humoral immunity, although not requisite for protection against MD lymphomas, clearly contributes to this end result.

Cell-mediated immune repsonses directed against tumour-associated antigens are also an important component in protection induced by vaccine viruses. Infection with vaccine viruses induces lymphocytes cytotoxic to target cells from MD lymphoblastoid cell lines (105,106) and induces resistance to virus-negative MD tumour transplants (114). Vaccines consisting of inactivated preparations of lymphoma cells and lymphoblastoid MD tumour cell lines have provided resistance to tumour induction (45 - 47). The influence of bursectomy on immunity induced by inactivated tumour-associated antigen preparations has not been tested but is probably negligible. The relative contribution of anti- tumour immunity to vaccinal immunity is difficult to assess because it is not easily studied independently of antiviral immunity; however, this must be considered as a significant component of the total immune process.

Humoral immune responses to tumour-associated antigens have not been detected in chickens following vaccination; however, antibodies can be raised following hyperimmunization with lymphoma cells (38,95,96).

Thus, vaccinal immunity appears to be based primarily on cellular responses to viral antigens, supplemented by humoral anti-viral and cellular anti-tumour immune responses. Also contributing are natural immune mechanisms such as natural killer cells (142,143) and macrophages (121,144 - 148). All these responses are undoubtedly augmented following challenge with virulent serotype 1 virus. Probably many of these responses are influenced by immune response genes. The mechanisms of vaccinal immunity, therefore, are highly integrated and unusually complex, and provide continued opportunities for study.

4.4. Protective antigens

Of fundamental interest in the mechanism of vaccinal immunity is the identity of the responsible protein antigens. "A" antigen, a 61,000 dalton glycoprotein (149) excreted into the medium of productively infected fibroblast cultures (150) and which is common to all viral serotypes, may be excluded since A-negative attenuated serotype 1 and

serotype 3 viruses retain their protective ability (1,140) and since chickens immunized with purified A antigen were not protected against MD (151). Of the 40 to 50 remaining virus-specific polypeptides (152, 153), few have been purified or functionally studied. Recently, however, two groups have isolated candidate protective proteins. Ikuta et al. (154) recovered glycoproteins of about 115,000, 63,000 and 50,000 daltons from cells infected with serotype 1 or 3 virus by immunoprecipitation with monoclonal antibodies; two of these proteins induced neutralizing antibodies in chickens and rabbits. Silva and Lee (155) recovered nearly identical glycoproteins of 100,000, 60,000 and 49,000 daltons from cells infected with serotype 1 or 3 virus by immunoprecipitation with a neutralizing monoclonal antibody (2) directed against serotype 3 virus. In both cases, common determinants on all three proteins were identified by a single monoclonal antibody. The immunogenicity of these and other candidate proteins need to be determined. Interest has largely focused on type-common antigens since protection is not serotype specific; however, a role for type-specific antigens as primary immunogens or as inducers of protective synergism should not be excluded.

5. FACTORS AFFECTING VACCINE EFFICACY

In the field, substantial fiscal incentives exist to maximize protection afforded by MD vaccines. Consequently, the poultry industry has developed extremely high expectations on vaccine efficacy, regardless of the degree or types of environmental exposure to virulent serotype 1 MDV's. Much attention has been directed to so-called "vaccine breaks" in recent years with the objective of reducing further the economic loss from MD. To this end, it is valuable to consider various factors known to affect vaccine efficacy since such factors not only represent potential causes of vaccine failures, but also suggest strategies for improving vaccine efficacy.

5.1. Dose

Vaccine doses sufficient to cause infection will result in protection under optimal laboratory conditions (64-66,156). However, to provide an adequate margin of safety, much higher doses are normally used. For example, commercial vaccines in the USA must contain 1,500

pfu per dose. In practice, however, the potency of commercial vaccines varies widely, often exceeding 10,000 pfu per dose. Furthermore, vaccine is occasionally diluted to provide only a fraction of a dose to each chick. Sophisticated buyers will obtain titres of vaccine serials prior to purchase and dilute as needed to provide a specific number of pfu per chick. There is no agreement on the optimal dose. Although protection varies directly with vaccine dose up to 4,000 pfu (157), there seems little justification for the use of very large doses as has been attempted by some poultrymen to increase efficacy. Ball and Lyman (158) found 2,240 and 8,960 pfu of a serotype 3 vaccine were equally effective heavy against natural challenge with an unidentified MD viral strain. Yoshida et al. (159) found doses of 173 and 17,800 pfu were equally effective. Against challenge with very virulent serotype 1 viruses, which by definition are poorly protected against by serotype 3 vaccine, increased doses of 5,650 to 105,000 pfu provided little or no improvement in protection compared to control doses of 695 to 1,375 pfu (157,160), and in no case resulted in acceptable levels of protection. Increased doses, however, may have certain benefits as they also speed the induction of viraemia (157) and decrease the period required for the development of protective immunity (159).

5.2. Maternal antibodies

Homologous maternal antibodies interfere to some extent with the efficacy of both cellular and cell-free serotype 3 vaccines (64,66,161-165) and of cellular serotype 1 and 2 vaccines (16,17). Cell-free serotype 3 vaccine is more susceptible to neutralization by maternal antibodies than cell-associated vaccine (66,166) and, therefore, should probably be used in an appropriately higher dose. Witter and Lee (17) found the adverse effects of homologous maternal antibodies were similar for serotype 2 and 3 vaccines, but were significantly greater for an attenuated very virulent serotype 1 vaccine. A high susceptibility of attenuated virulent serotype 1 vaccine to maternal antibody was also noted by others (6,165). Since the prevalence of infection with serotype 1 and 2 is high in commercial flocks, and since most flocks are immunized with serotype 3 virus, maternal antibody interference is likely to be a consideration with the administration of all vaccine types. Furthermore, neutralizing antibody titres in day-old chicks can

be very high and can vary widely among individuals (167). Solutions proposed for these problems are: (1) use of cell-associated rather than cell-free vaccines, (2) use of higher doses to compensate for neutralization, and (3) avoidance of homologous maternal antibody through selective immunization of breeders. The idea of immunizing breeder flocks and progeny with vaccines of different serotypes to minimize interference by homologous maternal antibodies (6,168) has been practiced in certain countries with considerable apparent success. Since serotype 1 and 2 viruses are ubiquitous in the field (169), this approach is most appropriate when serotype 3 vaccine is used in the generation of prime interest and vaccines of other serotypes are used in their parents.

5.3. Age at vaccination

Vaccines are historically administered at hatching in order to maximize the interval between vaccination and challenge exposure, and thereby to better protect against early exposure. Vaccination at the time of challenge, or following challenge, is ineffective (123). Exposure to virulent serotype 1 virus in the field may occur at any time, varying from 8 weeks or older (21) to 9 days (59). Under present commercial husbandry practices, early exposure is presumed to be common. Although vaccination at older ages is not recommended, older birds respond well to vaccines and are substantially protected in some cases (170,171). Vaccination of 18-day-old embryos is a recent strategy to expand further the interval between vaccination and challenge, and thereby protect more effectively against early exposure (57). By this procedure, significant protective immunity is present at the time of hatching, even in chicks with homologous maternal antibodies (166). Embryo vaccination has been applied successfully to vaccines of all serotypes and to polyvalent vaccines (172). Embryo vaccination appears to have promise for the immunization of broilers, but its commercial potential has not yet been sufficiently evaluated and the instrumentation needed for mass application has not been developed.

5.4. Revaccination

Although Spencer et al. (171) found chickens were better protected with an attenuated serotype 1 vaccine when revaccinated at 23 days, others failed to show any improvement in protection by serotype 3

vaccine from revaccination (127,158,173). There is some evidence that repeated administration of vaccine virus results in earlier (174) or higher titre (91) antibody response, but this could be due to increased vaccine dose which is known to produce similar effects (157). Zander (175) found progeny of dams hyperimmunized with virus-containing blood were no more resistant to MD than progeny of once-immunized dams.

5.5. Age at challenge

Vaccinal immunity can be overwhelmed by early exposure. Protection is unsatisfactory when challenge occurs simultaneously with vaccination (57,123) or at the 2nd (17) or 3rd (57) day post-vaccination. However, good protection is obtained from vaccines of all three serotypes when challenge with virulent serotype 1 virus occurs 5 to 8 days post-vaccination, even in chickens with homologous maternal antibodies (17, 57,123,166,172) and may be maximal by the 7th to 8th day since no better protection was noted when challenge was delayed further (17,123). Even against challenge with very virulent serotype 1 virus, protective immunity induced by serotype 3 is no better at 21 (172) or 26 (176) days than at 7 days post-vaccination. Since chickens vaccinated as 18-day-old embryos are protected against contact challenge on the day of hatch or inoculation challenge on the 3rd day of age (57), the speed with which immunity is induced by embryo and after hatch vaccination appears similar. Sanitation is a means by which early exposure can be reduced. The value of thorough cleanout in reducing MD losses in vaccinated broiler chickens has been documented (18,177); even application of brooding paper to cover used litter may be helpful (178). It seems clear that for maximum efficacy, vaccines should be administered as soon as possible after hatching or, possibly, even prior to hatch. Furthermore, reduction of exposure through strict sanitation procedures is a most important adjunct to vaccination programmes.

5.6. Challenge virus strain

For several years, vaccines of all serotypes appeared highly effective in virtually all situations, thus suggesting that the effect of challenge strain was negligible. However, Eidson et al. (157) found protection by serotype 3 vaccine was better against the standard GA strain than against three recent field isolates obtained from broilers in the Delmarva region where unusually high MD condemnations were being

experienced. The existence of very virulent serotype 1 viruses that were poorly protected against by serotype 3 vaccine was soon confirmed by others (133,160,179,180). In contrast to earlier conclusions (160), these very virulent serotype 1 strains appeared clearly more pathogenic than standard virulent strains; they caused greater depression in body and bursal weights, induced more deaths through the early mortality syndrome and induced more lymphomas in both susceptible and resistant chickens (169). Protection by serotype 3 vaccine against challenge with these very virulent strains was significant (52 to 77%), but was less than that against standard virulent strains under comparable conditions (169). The very virulent strains are not easily distinguished from virulent strains since time-consuming pathogenicity and protection trials are required and no standard criteria exist. It is likely that the serotype 1 viruses represent a continuum of virulence with the very virulent isolates representing one extreme. Because they are widely distributed, are more prevalent now than previously, and are circumstantially associated with vaccine failures in the field (169), very virulent serotype 1 strains represent an important factor affecting vaccine efficacy. Although protection by serotype 2 and 3 vaccines is suboptimal, polyvalent vaccines offer reasonably good protection against challenge with very virulent strains (16,18,37,133).

5.7. Stress

Stress from infectious disease and other causes may influence the efficacy of vaccination, presumably through immunodepression of cell-mediated immune responses. Infectious bursal disease virus (IBDV), a virus known to induce long-lasting B-cell immunodepression and transient T-cell immunodepression, has been suggested as a likely cause of MD vaccine failures in the field. This possibility was supported by the finding of Giambrone *et al*. (181) that chickens naturally exposed from hatch to IBDV were less well protected by serotype 3 vaccine than chickens not exposed to IBDV (63% versus 83% protection) against challenge with virulent serotype 1 virus at 2 weeks of age. Jen and Cho (182) found no interference by IBDV with levels of serotype 3 viraemia, but noted some depression in neutralizing antibody response. However, Sharma (58) found IBDV interference with MD vaccinal immunity only when virulent IBDV and serotype 3 vaccines were given at hatch to chickens

without maternal IBDV antibody and challenged at 7 days with virulent serotype 1 virus; no interference was seen if serotype 3 vaccine preceded IBDV, if MD challenge was delayed, if maternal IBDV antibodies were present, or if less pathogenic IBDV strains were used. Thus, IBDV should only rarely cause problems with MD vaccines in the field. Contamination of MD vaccines with reticuloendotheliosis virus has been shown to interfere with induction of antibodies to serotype 3 (183) and induction of protection by serotype 3 against challenge with virulent serotype 1 virus (184). Simultaneous vaccination of chickens with serotype 3 and attenuated reovirus vaccines resulted in poor protection against natural MD exposure in the field compared to vaccination with serotype 3 alone (185). Although Landgraf _et al_. (186) found cold stress or deprivation of food and water during the first few days after hatching had no adverse influence on immunity induced by serotype 3 vaccine, physiological and environmental stresses may be important, particularly as a cause of decreased immunity after 20 weeks of age. This possibility requires further study.

5.8. Genetic strain and sex

Susceptible strains of chickens are still more susceptible to MD challenge after vaccination than are resistant strains after vaccination (171,173,187). Thus, the resistance conferred by genetic constitution and vaccination appears to be additive. Although both males and female are protected by vaccines, the greater MD susceptibility of females compared to males in nonvaccinated chickens is evident also after vaccination (97). Probably there is little effect of genetic strain or sex on vaccine efficacy when measured by percent protection. However, in one study, females seemed more responsive to vaccination than males (97). In contrast to studies on virulent serotype 1 viruses (92,188), antibody titres induced by serotype 3 vaccine were not affected by genetic strain (97). However, serotype 3 viraemia titre and persistence may vary among chicken strains (62). In practice, commercial chicken strains can be expected to vary in MD susceptibility, general immune response and response to stress (187,189). Less desirable strains that experience high losses from MD even after vaccination have been recognized, and are usually quickly replaced by more competitive strains. Increased disease resistance and immune responsiveness through genetic selection

programmes is clearly a powerful adjunct to vaccination for MD control.

5.9. Method of administration

Both cell-associated and cell-free vaccines are usually administered by parenteral inoculation. The subcutaneous route is more widely used, but intramuscular and intraabdominal routes are also effective. Oral, intranasal, and intraocular routes, however, are ineffective (8,190). The choice of route is largely determined by convenience and available inoculation equipment. Vaccines can also be administered to 18-day-old embryos by a route that results in placement of the inoculum directly in the embryo or in the amnionic sac; either placement is effective (57). Mass vaccination methods have been disappointing. Spread of attenuated mildly virulent serotype 1 or serotype 2 vaccines by seeder chicks has been attempted with some success (8,19) but immunity is slow to develop and the practice is now rarely used unless accompanied by concurrent inoculation with serotype 3 vaccine. Aerosol administration of cell-free serotype 3 vaccine has been proposed (191 - 194) but viraemia and antibodies were delayed and protection was inferior compared to chickens vaccinated by parenteral inoculation, especially in chickens with maternal antibodies (195,196).

5.10 Reconstitution

Cell-free and cell-associated serotype 3 vaccines are relatively labile after reconstitution and should be used as soon as possible, usually within 30 minutes, to prevent unnecessary loss of titre. Factors affecting the rate of titre loss include diluent (197,198), source of virus (199,200) and holding temperature (198,201). The importance of thawing speed, thawing temperature, diluent temperature, vial rinsing, and other factors, on the potency of cell-associated serotype 3 vaccines has been discussed by Halvorson and Mitchell (202). Such considerations, although of trivial scientific interest, are crucial if each chick is to receive a full dose of vaccine.

5.11 Additives

Dyes, antibiotics, and other viruses occasionally have been added to reconstituted MD vaccines prior to administration. This practice entails serious risks and is discouraged unless the additive has specifically been shown not to reduce vaccine potency or to interfere with immunity development. Although a number of antibiotics have been used

successfully in MD vaccines (193,203 - 205), problems have been experienced with others (198,203,205). MD vaccine has also been supplemented with fowl pox vaccine (206 - 208), Newcastle disease vaccine (209,210), infectious bursal disease vaccine (211), and reovirus vaccine (185); detrimental effects on MD immunity were reported only in the case of reovirus vaccine. Vegetable dyes have been used with apparent success (18,205) to monitor the efficacy of subcutaneous inoculation procedures.

5.12 Environmental vaccination

Protective viral strains naturally present in the environment of the chicken include not only serotype 2 but also unattenuated mildly virulent serotype 1 viruses. Despite significant pathogenicity for genetically susceptible chickens, these mildly virulent strains offer substantial protection against inoculation or contact challenge with virulent serotype 1 strains in more resistant, commercial chickens (15). Such naturally occurring protective strains will protect unvaccinated chickens against subsequent exposure with more virulent strains (21,22, 212), and may add to the protective efficacy of vaccines administered at hatching through the mechanism of protective synergism. Dissemination of serotype 2 virus through a seeder chick programme has apparently improved MD control in chicks vaccinated with serotype 3 virus at hatching (175). Therefore, it seems likely that the efficacy of vaccines may vary on different farms depending on whether protective viral strains are present in the environment and when exposure to such strains occurs in relation to exposure to virulent strains.

6. INVESTIGATION OF VACCINE FAILURES

Both in the field and in the research laboratory, much effort has been committed to the retrospective determination of why MD losses are excessive in certain vaccinated flocks (126-128,213). In some cases it is desirable to monitor the vaccination and immune status of flocks prospectively, to determine problems before they are reflected in increased MD losses. These have been difficult and usually unrewarding tasks, even for the most sophisticated laboratories. It may be useful to discuss which questions are most relevant, which can be studied profitably, and how such studies might be accomplished. Some of the

necessary procedures are described in Chapter 6.

It is possible to determine if excessive losses are due to excessive exposure or to vaccine failure by comparing the response of vaccinated chickens to that of unvaccinated controls. If nonspreading vaccine viruses are used, the groups should be intermingled. One such field study in Maryland, a region noted for excessive MD losses in vaccinated broilers, showed that broilers vaccinated with serotype 3 virus experienced 0.5% condemnations from MD - an unacceptably high level - but compared to unvaccinated controls in the same house were protected at a 97% level (176); in this case vaccine efficacy was good but challenge exposure was excessive.

To determine if chicks were uniformly infected with vaccine virus, peripheral blood lymphocytes from chicks at 7 to 21 days of age may be assayed for virus in chicken embryo fibroblast cultures and isolated viruses identified by plaque morphology or type-specific monoclonal antibodies. Tests on relatively few samples wil determine if a viable vaccine was administered. It is usually not feasible to determine if a small percent of the chickens were "missed" during vaccination because a prohibitively large number of chicks must be tested and because vaccine virus will not be recovered from every infected chick. However, inspection of the inoculation site after addition of dye to the vaccine may be helpful.

It is possible to determine the pfu dose of vaccine administered through in vitro assays on replicate ampoules or on the residual reconstituted vaccine obtained from the hatchery. Ready access to a suitable laboratory is needed. The variation among laboratories in the sensitivity of virus assays is well known and hinders the establishment of absolute vaccine titres. This procedure is probably best used prior to purchase of vaccines.

The magnitude and variation of maternal antibody titres can be determined by quantitative virus neutralization assays on serum from newly hatched chickens (167). As homologous antibodies are considered most detrimental, a cell-free virus of the same serotype as the vaccine is used as antigen.

The susceptibility of chickens to MD can be evaluated by comparison with other strains; no absolute standard exists. For best results, the

chicks of several strains hatched at the same time should be intermingled, exposed to MDV and observed. Vaccinated chickens may also be compared, as the ranking of the susceptibility of strains is similar in vaccinated and unvaccinated chickens (171), but larger lot sizes may be required.

The type of vaccine used may be confirmed by isolating the virus from the vaccine stock or from the vaccinated chickens and typing the isolate by plaque morphology and type-specific monoclonal antibodies (2). With the advent of vaccines containing multiple viral serotypes, this question may become more relevant.

The age when chickens are exposed to virulent serotype 1 virus can be determined by several procedures. Virus isolation and serotyping at regular intervals from a proportion of the flock is probably the best procedure. The assay of feather tips for precipitating antigen has been used (A. Zanella and R. Marchi, personal communication) but this assay is not totally specific for serotype 1 viruses. Likewise, assay of serum antibodies reactive in the agar gel precipitin test against a serotype 1 virus antigen had been used with some success in chickens vaccinated with attenuated virulent serotype 1 virus (163,214). A sample of chickens can be removed periodically from the flock and placed in an isolation cage for several weeks; a positive test is indicated by the appearance of MD lesions in the isolator (59). Ideally, nonvaccinated birds should be used, but vaccinated birds may be tested by including unvaccinated susceptible contact controls in each isolator. This procedure is sensitive and provides a more accurate and direct estimate of time of infection than the other alternatives, but requires specialized facilities.

Very virulent serotype 1 isolates are detected in a flock with difficulty. The serotype 1 virus must be isolated, purified from viruses of other serotypes, propagated into suitable stocks, tested for pathogenicity by chicken inoculation, and, finally, used as a challenge virus in serotype 3 vaccinated and unvaccinated chickens. Known very virulent and virulent serotype 1 strains are useful as controls in the protection test. Isolates poorly protected against by serotype 3 vaccine are designated as very virulent strains (16) but specific guidelines are lacking. This procedure is expensive, time-consuming,

and is used only as a research tool at present. Furthermore, very virulent strains, when present at low frequency, may not be detected by this procedure unless a large number of isolates are classified.

It is not possible to determine whether a sufficient and appropriate immune response has been stimulated by vaccination, as no suitable *in vitro* correlate of vaccinal immunity has been identified. A sample of chicks can be separated from the flock at 7 days and separately challenged, providing unvaccinated controls are also challenged and no prior exposure to serotype 1 virus has occurred. However, the chance that useful information will be obtained by such a procedure is not high.

Lesions may be confirmed as MD by gross and microscopic examination (215) but are best identified by the presence of tumour-associated antigen on the surface of living tumour cells (216,217). The importance of an accurate diagnosis to an evaluation of vaccine efficacy is obvious. Problems are most often encountered in chickens over 16 weeks of age with visceral lymphomas but without involvement of the bursa or the peripheral nerves.

From the foregoing, it is clear that productive investigation of excessive losses in vaccinated flocks is difficult at best. However, with appropriate resources, some progress can be expected. This inability to identify specific factors contributing to increased MD losses has been a major hindrance to the development of rational solutions, and has prompted instead a variety of empirical approaches. However, if specific causes can be identified, solutions may become evident. A list of types of vaccine failures, their causes, methods of confirming the causes, and possible solutions are listed in Table 2.

7. COMPARATIVE EFFICACY OF VACCINES

The increasing number of available types and combinations of vaccines for MD has created a greater need to conduct critical comparisons of efficacy. As protection is a complex response influenced by many variables, many of which are difficult to control, precise estimates are inherently difficult to obtain. This section is designed to briefly present some general principles of efficacy evaluation and some of the available comparative efficacy data.

TABLE 2. Types of Marek's disease vaccine failures

Type	Cause	Confirmation method	Solution
True failure[1]	Full vaccine dose not received by all chicks	Assay chick viraemia frequency, vaccine titre; dye check	Improve administration and handling of vaccine
	Early challenge with virulent MDV	Sequential assay of chick viraemia (serotype 1)	Sanitation; Embryo vaccination
	Challenge with very virulent MDV	Isolate, determine pathogenicity for vaccinated chicks	Polyvalent vaccination
	Interference by homologous maternal antibody	Assay neutralizing antibody in dams and chicks	Alternate generation vaccination; Cell-associated vaccine
	Suboptimal genetic resistance	Compare MD susceptiblity of test and control strains	Breeder selection for genetic resistance
	Stress; Immunodepression	Normally cannot confirm; serology for immunodepressive viruses	Good management and disease control
	Wrong serotype of vaccine virus	Isolate and identify with type-specific antibodies	Replace vaccine
Apparent failure[2]	Wrong diagnosis	Gross and histopathology; Tumour-associated antigens	Not applicable
	Excessive exposure to virulent MDV	Demonstrate satisfactory protection by comparison of vaccinated and nonvaccinated groups	Sanitation and good hygiene

[1]Percent protection suboptimal due to fault of vaccine, vaccination procedure, environment, or strain of chicken.
[2]Percent protection satisfactory.

7.1. Efficacy tests

The efficacy of vaccines is typically measured by comparing the frequency of MD lesions in vaccinated and unvaccinated chickens, each challenged with virulent serotype 1 MDV; the results are often expressed as percent protection. When two vaccines are compared, it may be sufficient to determine the frequency of MD lesions in groups receiving different vaccines but similarly challenged with virulent MDV. Such tests are usually done in the laboratory where experimental variables, especially the spread of vaccine viruses among treatment groups, can be carefully controlled. Field tests, although considered a definitive test of efficacy for single vaccines, are difficult to apply for a comparison of vaccines unless none of the vaccines spread by contact and the different treatment groups can be intermingled in a common environment. Otherwise, large numbers of replicate farms may be required (12,18).

The use of chickens with maternal antibodies of all three serotypes may be desirable since field use requires vaccines to be effective in the presence of homologous maternal antibodies and since not all vaccine viruses are equally neutralized by homologous antibody (17). The challenge virus should be representative of the most virulent strain expected to be encountered in the field. Mixtures of challenge viruses may be useful since no interference has been noted (R.L. Witter, unpublished data) and since vaccines exhibit a specificity of protection for certain virulent serotype 1 strains (16). It may also be valuable to administer the challenge exposure early enough so that protection afforded by vaccines is in the range of 30 to 70%, thus facilitating comparisons. Replicate lots can be used to control the variations between different isolation cages. The value of conducting preliminary trials to standardize the biological system should be obvious.

Quantitative assays to determine the number of pfu of vaccine virus needed to protect 50% of chickens appear to be relatively inaccurate as indicated by wide 95% confidence intervals and variable slopes of response curves (66,156,218). The value of this method for the comparative evaluation of vaccines appears limited.

Although MD mortality or lesions are the response criteria most frequently used, alternate criteria to shorten, simplify or otherwise

improve the procedure have been proposed. Since vaccination markedly reduces viraemia with serotype 1 challenge virus, serotype 1 viraemia has been used as a criterion to evaluate vaccine efficacy (17). The correlation between viraemia and lesion response appears strong and viraemia titres offer a quantitative method for measuring efficacy; however, the considerable variation in viraemia titres between similarly treated chickens has complicated the detection of statistical differences among vaccines where differences in protection against lesion development were obvious (17). "A" antigen production in the feather follicle epithelium is considered a correlate of serotype 1 viraemia and has been used as a criterion of vaccine efficacy (A. Zanella and R. Marchi, personal communication) but vaccine strains of all serotypes can induce the same response, at least to some degree. Rejection of the MD tumour transplant, JMV (114 - 116) is another criterion of vaccine efficacy. This procedure is much shorter than the conventional protection test but interpretation of the results is difficult since JMV rejection is a measure of anti-tumour immunity but not of the more important anti-viral immunity, and since rejection is also influenced by histocompatibility antigens. Use of any of these alternate response criteria, therefore, may require appropriate caution.

7.2. Comparisons

An extensive body of literature has developed on the comparative efficacy of different commercial and experimental vaccines. Differences are subtle, and there is no agreement on which vaccine is superior. Because this issue has large potential economic impact and has been extensively debated, it seems appropriate to present briefly some of the considerations and literature relevant to the choice of an optimal vaccine. The principle comparisons are between cellular and cell-free serotype 3 vaccines, among the several vaccine virus strains, and between monovalent and polyvalent vaccines.

Cellular and cell-free serotype 3 vaccines have been repeatedly compared. In some studies, the two vaccines have had similar efficacy (7, 16,125,165,173,219 - 226), whereas in others cellular vaccine has been superior (66,157,162,164,168,227,228). There are also a few reports where cell-free vaccine appeared more efficious than cellular vaccine (229 - 231). There seems little doubt that homologous serotype 3

maternal antibodies have a greater detrimental effect on cell-free vaccine than on cellular vaccine (66), but this may be compensated for by adjustment of the vaccine dose. Overall, there seems to be less risk associated with the use of cellular vaccine, but both types of vaccine provide adequate protection in most situations.

Different strains of serotype 1 and 2 vaccine virus have also been compared, mainly against serotype 3 vaccine. Of a number of attenuated virulent serotype 1 vaccine strains evaluated, most were inferior compared to serotype 3 (6,7,165,224,229,232,233) but a few appeared comparable to serotype 3 (4,222). Unfortunately, the only strain in this class to be widely used in the field, the attenuated HPRS-16 strain (150), has not been similarly evaluated. None of these attenuated virulent strains iswidely used at present, but this more likely reflects higher production costs than lack of efficacy.

The efficacy of attenuated mildy virulent CVI 988 strain of serotype 1 virus is generally similar to that of serotype 3 vaccine (12,121), even over long period of use in the field (12). Some workers (8,234, 235) found some advantage of CVI 988 strain compared to serotype 3 vaccine, but others (43) found immunity was induced more slowly by CVI 988 and Picault _et al_. (231) reported one strain of chicken where CVI 988 was less effective compared to serotype 3.

An experimental attenuated very virulent serotype 1 vaccine, Md11/75C, was less effective than serotype 3 in chickens with maternal antibodies of all three serotypes, although this virus was actually more effective than serotype 3 in antibody-free chickens challenged with very virulent serotype 1 strains (16,17).

Comparisons of serotype 2 and 3 vaccines have given mixed results. King _et al_. (168) found the SB-1 strain of serotype 2 virus was less effective in the presence of serotype 2 maternal antibodies, but more effective in the presence of serotype 3 maternal antibodies than was serotype 3 vaccine. Schat _et al_. (133) found that vaccination with SB-1 induced lower levels of protection in three strains of chickens than vaccination with serotype 3 virus. Compared to serotype 3 vaccine, Witter (16) found the efficacy of SB-1 lower, similar and higher in various trials, and in a later study (17) the two vaccines were similar in nearly every comparison. Another naturally avirulent strain, presum-

ably of serotype 2, had roughly similar efficacy compared to serotype 3 vaccine (6).

Polyvalent vaccines, i.e. vaccines composed of multiple virus strains representing different serotypes, appear clearly superior to serotype 3 or other monovalent vaccines in protecting chickens against challenge with very virulent serotype 1 viruses (16,133) and in the field (18,37). This advantage is apparent even in chickens with maternal antibodies of all serotypes. Little difference was noted between the efficacy of a bivalent (serotype 2 and 3) and a trivalent (serotype 1, 2 and 3) vaccine (18) in maternal antibody-positive chickens.

8. STRATEGIES AND PERSPECTIVES

8.1. Current strategies

There are generally three types of vaccination strategies, monovalent, alternate generation, and polyvalent. Monovalent vaccination involves the selection and use of a single vaccine virus. This approach has been used from the initial discovery of vaccines and is highly effective in most situations. Probably any of the commercial vaccine strains can be used to advantage.

Alternate generation vaccination is a strategy that requires the use of vaccines of different serotypes in parents and progeny. Most commonly, parents are vaccinated with a serotype 1 or 2 vaccine and progeny are vaccinated with serotype 3. By this strategy, serotype 3 vaccine is administered to chickens lacking maternal antibody of the homologous serotype and, therefore, should be more fully effective. Full protection of parent stock may be more difficult, however, since interference by homologous maternal antibody will invariably occur, regardless whether serotype 1 or 2 vaccines are used.

Polyvalent vaccination is a third strategy that utilizes the principal of synergism between vaccine viruses to achieve improved protection. A mixture of two or more vaccine viruses, carefully selected for optimum synergism, is used. Normally the need for polyvalent vaccines is restricted to those farms and areas where other vaccination strategies have not been adequately effective. Such vaccines may be given to both parent and progeny flocks, since interference by homologous maternal antibodies, although measurable, does not negate their effectiveness.

The possibility of combining strategies 2 and 3 by vaccinating parent flocks with polyvalent vaccines composed of serotype 1 and 2 viruses, and vaccinating progeny with polyvalent vaccines composed of serotype 3 plus additional viruses of serotypes 1 or 2 should be considered.

8.2. Benefits derived from vaccination

Without question, the use of MD vaccines has produced substantial economic benfits to the poultry industry. Condemnation of broiler chickens in the USA from MD has decreased by about 95% from peak levels reached in 1970 just prior to the advent of vaccination (Fig. 1). A variety of other benefits in both broiler and layer flocks have been noted. These include: 1) reduced condemnations of broilers for causes other than MD, 2) reduced broiler mortality, 3) reduced broiler breeder mortality, 4) reduced layer mortality, 5) reduced condemnation of layer hens, 6) improved feed utilization, and 7) increased egg production. Purchase and Schultz (236) estimated the annual benefit in the USA to be $168 million, largely from increased egg production. These benefits are also discussed in Chapter 2. The overall efficacy of MD vaccines in the field is probably 90% or more, a level superior to that of most other vaccines in any species.

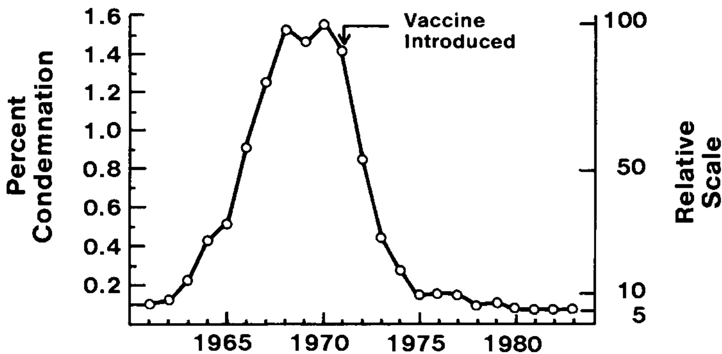

FIGURE 1. Broiler condemnations for Marek's disease in the USA.

8.3. Future challenges

Despite the success achieved thus far, interest in advancing technology in MD immunization remains high. Problems and research oppor-

tunities are abundant. Ways to determine the cause of vaccine failures in a flock are urgently needed so that the most appropriate corrective strategies may be implemented. Embryo vaccination should be studied not only as a means of providing early protection against MD, but also as a means of reducing vaccination costs and administering other biologic products. Additional combinations of vaccine viruses should be evaluated to determine those mixtures with optimal synergism for use in improved polyvalent vaccines. Development of polyvalent vaccines of serotype 1 and 2 viruses for use in breeder flocks may be desirable. The relevant immunogenic proteins and their respective viral genes need to be determined. Knowledge of the mechanisms of vaccinal immunity is still incomplete; the role of lymphocyte subpopulations may be particularly important. Likewise, the mechanism of protective synergism should be better understood. Application of the techniques of recombinant DNA technology to the development of subunit or recombinant live virus vaccines will certainly further knowledge on basic mechanisms of immunity, and in the long run may provide improved vaccines for commercial use.

8.4. Conclusion

It seems clear that vaccines will continue to be the dominant control method for MD for years to come. Eradication on an industry-wide level will not be practical unless a vaccine or other procedure is developed to prevent infection with or shedding of virulent serotype 1 MD viral strains. New vaccines may be necessitated by the continued mutation of virulent viruses. However, improvement of vaccines will be difficult because of the extremely high efficacy and low cost of the existing products. Even with improved vaccines, further gains in MD control may be minimal unless we develop more immunologically competent and genetically resistant chicken strains which will respond better to vaccines, and better husbandry and sanitation procedures which will lower MD exposure and reduce other immunodepressive stress.

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9. PRODUCTION OF VACCINES

A.E. CHURCHILL

1. INTRODUCTION

Experimental vaccines against Marek's disease (MD) were developed in 1969 (1,2) and after extensive field trials began to become commercially available in 1970. In Chapter 8 attention is drawn to the types of vaccine strain available in commercial products and the two forms of presentation, i.e. cell-associated or cell-free (lyophilized). The cell-associated form of the vaccine was the first to be developed and set a precedent for the pharmaceutical industry. The production, storage and distribution of the product that had to be preserved at each stage in liquid nitrogen seemed a formidable undertaking but it was surprising that the problems were so rapidly overcome that worldwide distribution of the product developed in a matter of months. The development of a freeze-dried product was soon to follow (3), but even up to the present day this has only proved to be effective in the case of the turkey herpesvirus (HVT), Marek's disease virus (MDV) being too unstable in the cell-free form for effective lyophilization.

Like other live virus vaccines for use in poultry the product is supplied as a small multidose container of preserved virus. A second component consisting of a suitable sterile fluid is supplied for diluting the virus prior to its injection.

2. PRODUCTION METHODS

2.1. Substrates for virus propagation

The first reported laboratory cultivations of MDV were in one case in cultures of primary chicken kidney cells (4) and in another in duck embryo fibroblasts (5). Virus has also been propagated in the fertile egg (6). The subsequently adopted substrates for commercial virus

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production have been primary chick embryo fibroblasts (CEF) derived from specific pathogen-free (SPF) flocks and duck embryo fibroblasts. No continuous cell line that would be suitable for vaccine production has yet been found but if it were it would offer considerable advantages over the existing systems, avoiding the need for a continuous supply of fertile eggs from SPF flocks.

The essential steps in producing cell culture for virus propagation are described below. Variations in procedure do occur from one commercial company to another, but all of such information is not published or freely available. Therefore much of the information given below is drawn from the author's own experience. The steps are:

1) SPF eggs are fumigated on receipt at the laboratory.
2) Eggs are incubated for 10 to 12 days.
3) After removal of dead and infertile eggs, the fertile eggs are transferred to a laminar flow cabinet where the shell is sterilized, opened and the embryo removed. The methods of opening the eggs may vary from the use of mechanical shell cutters to manual opening using scissors and forceps. The batch size being handled will determine the usefulness of adopting mechanical means. Commercial production batches may use from about 100 to 2000 fertile eggs.
4) Groups of decapitated embryos are pooled together in groups of up to 100. The tissue is then reduced to small fragments by a mechanical means of chopping. This procedure can be carried out manually using sterile scissors in a laminar flow cabinet, the embryos being collected in a large petri-dish. The cells of the fragmented tissue are then dispersed by conventional trypsinizing procedures using 0.05% trypsin in Dulbecco's phosphate buffered saline part A (7). If the cell suspension after trypsinizing contains large cell clumps, these may be removed by passing the suspension through a stainless steel sieve with a mesh size of between 100 and 200μm. When the vaccine is to be cell- associated, it is important to establish cultures containing a minimum of large cell clumps. This facilitates the optimal use of seed virus and aids the harvesting of well-dispersed cell suspensions. The recovery of large cell clumps at this stage

introduces the need for a further sieving procedure before filling the product into the final container, with a consequent loss of infected tissue and a lowering of the final infectivity titre.

5) After sieving, the cells are deposited from the trypsin suspension by low speed centrifugation at 1000 to 2000g. The cell deposit may then be resuspended in a suitable volume of medium for making a cell count. A yield of between 1×10^8 and 2×10^8 cells per embryo can be expected. A suitable dilution of the cells in growth medium can then be made to ensure the correct seeding rate for the chosen type of culture container. Such containers may be flat-bottomed vessels for stationary incubation or cylindrical vessels for rolled incubation. Examples of suitable containers either in disposable plastic or in glass for washing and re-use are shown in Fig. 1. A loaded roller apparatus of a type in common use is shown in Fig. 2.
   Once a routine procedure has been established cell counting may not be found necessary, and an empirical ratio of trypsinised embryos to containers can be adopted. Suitable media for cell growth may be based on LAH medium (8), Eagle's minimum essential medium (9) or medium 199 (10), in each case buffered with sodium bicarbonate and supplemented with 5% calf serum. The trypsinized cells are suspended in the chosen growth medium at a concentration of about 2 to 4 million cells per ml and the appropriate volume transferred to the growth containers. As an example, 200 ml of cell suspension is suitable for a roller bottle 40 cm. long with a diameter of 10 cm having a cell growth surface area of 1,250 cm^2. Thus, the cell seeding rate is of the order of 3×10^5 cells per cm^2.

6) The incubation temperature adopted may be 38° to 39°C. Under these conditions, the time taken for a monolayer to develop suitable for infection is 2 to 3 days. In rolled cultures, a roller apparatus speed giving 5 to 10 revolutions per hour is suitable.

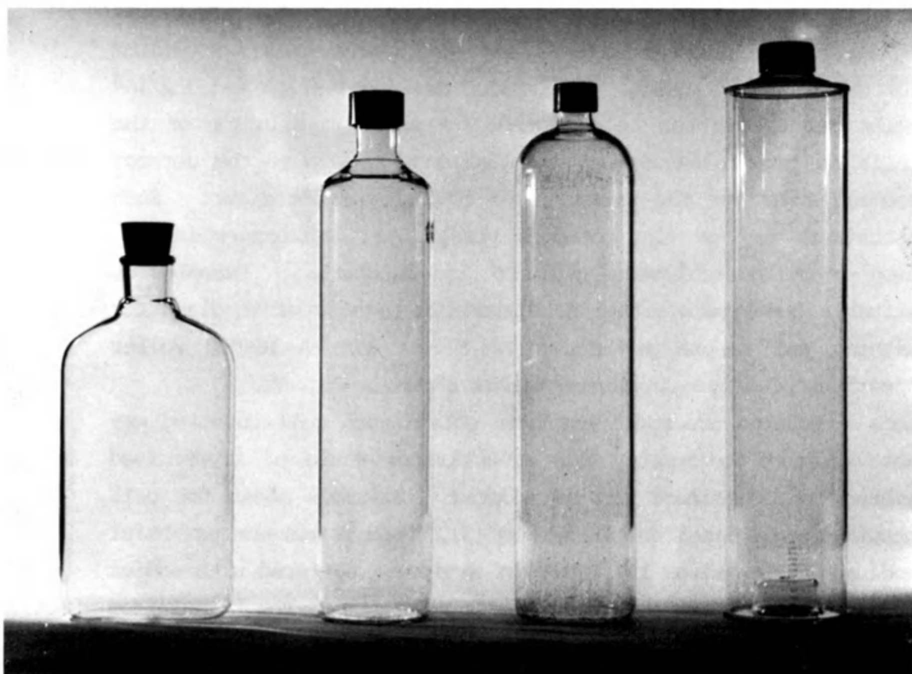


FIGURE 1. Types of culture vessel.
Left to right, 1) Thompson bottle. Flat sided for stationary culture with monolayer surface area of 364 cm^2. 2) Wide necked glass roller bottle with culture area of 1200 cm^2. 3) Narrow necked glass roller bottle with culture area of 1350 cm^2. 4) Disposable plastic roller bottle with area of 1690 cm^2.

FIGURE 2. Adjustable speed roller apparatus loaded with culture bottles.

2.2. Infection of cell cultures

Cultures are infected from a preserved preparation of "working" seed virus. This will usually be in the form of cell-associated virus, whether HVT or MDV. The working seed will be one or two passages ("primary" or "secondary" seed) beyond the master seed stock. The ability to introduce two cycles of propagation beyond the master seed passage level to make working seed ensures that the master seed stock will last virtually indefinitely, thus the continuing uniformity of quality of the finished product is better assured.

The exact procedure at infection will vary from one manufacturer to another. In some cases a change from growth medium to a maintenance medium with lower calf serum content (e.g. 1%) will be made while in other cases the infection may be made introducing the seed virus into the original growth medium. Where the latter method is adopted, a pH adjustment at this stage by the introduction of additional sodium bicarbonate may be found necessary. Working seed virus in the form of infected cells are taken from the liquid nitrogen storage and rapidly thawed by dropping the containers into a water bath at 37°C. This procedure should be carried out behind some form of shield because occasional containers will explode due to the sudden vapourisation of liquid nitrogen that has entered the container through an incomplete seal. The thawed seed is then introduced into the cell culture vessels at the appropriate rate. Infection rates of the order of 10^3 plaque forming units (pfu) per cm^2 of monolayer are suitable. Higher rates than this may be used by some producers. Continued incubation after infection is for 48 hours where cell-associated vaccine is to be prepared and for 72 hours for cell-free vaccine. However, these intervals cannot be regarded as invariable as each producer will determine the optimum incubation period that applies to his own particular conditions and type of end-product. The rate of multiplication of different vaccine strains will vary and will be affected by differences in incubation temperature, resulting in variations in the optimum harvesting time for maximum yield.

A modification of the above procedure was described by Garrido et al. (11) in which a further addition of freshly trypsinized embryo cells was made to the infected cultures 1 to 3 days after infection.

Increased virus yields were obtained when the cultures were harvested after further incubation. Some manufacturers have adopted this "multi-layer" technique for commercial vaccine production, but in the author's experience the yields claimed for this method can be obtained with the simple monolayer technique.

2.3. Harvesting, filling and preservation of cell-associated vaccine

The methods used in these stages are similar, whether the product contains attenuated MDV, HVT or blends of these. Sterility is maintained by carrying out all procedures in laminar flow cabinets.

2.3.1. Harvesting. The cells of the infected monolayer are removed from the culture vessel using a phosphate buffered solution of 0.02% EDTA (pH 7.2) to which 0.05% trypsin has been added. A suitable procedure is to discard the cell culture medium, wash the cell sheet once rapidly with the EDTA/Trypsin (E.T.) solution to remove traces of calf serum inhibition to trypsin, and then allow a fresh volume of E.T. solution to act upon the cell sheet for a further brief period. This latter period is sufficient to allow the cells to commence detaching from the vessel. At this point the bottle is drained, sealed and returned to the hot room until the cells have detached. The cells are then resuspended in the freezing mixture and transferred to a bulk harvest container ready for filling out into ampoules. Alternatively the second addition of E.T. solution may be left in the culture container until the cells have detached.

It is then necessary to pellet the cells by centrifugation before they can be resuspended in the freezing medium. It is desirable to subject cells to the minimum possible trauma during harvesting as they are of increased fragility due to the developing cytopathology, and therefore it is preferable to avoid the latter method requiring a centrifugation procedure.

The freezing mixture may be composed of cell growth medium with an addition of 7.5 to 15% of dimethyl sulphoxide (DMSO). This latter substance is toxic to cells and therefore the holding of cells for long periods at ambient temperatures after its addition should be avoided.

Once cells are suspended in the freezing mixture, they should be held at 4°C or alternatively filled out immediately. Where the cell resuspension and bulking procedure is liable to be protracted the DMSO

may be omitted from the medium used and only added just before filling out into ampoules begins.

2.3.2. Filling out the final container. In the production of this vaccine it is not possible to assay the bulked harvest before filling out the final containers because the bulk cannot be held while awaiting the results of assays. Thus, the infectivity titre placed in each container cannot be accurately predetermined. The chosen resuspension volume and the volume filled into each container is determined empirically, being based upon previous experience. The label applied to the finished product giving the number of doses in a container can only be chosen after the results of assays on the finished product are known. It is necessary therefore for the producer to have a range of product marketed of different dose numbers to avoid large losses from wastage. A suitable range to give satisfactory flexibility is from 250 doses to 2000 doses per container.

This problem is compounded when the finished product is to be a combined vaccine where the components are to be blended in suitable proportions before filling out. Decisions on the proportions of the blend must be made based on the expected titres of the components rather than on the assay results of samples of the actual harvest. As the number of components in a multiple vaccine increases the chances of such empirical blending producing the required infectivity proportions in the finished product are reduced. A preferable method is to fill each component separately and combine them in the necessary proportions (at the dilution stage) just prior to administration.

The numbers of doses per container should be varied by adjusting the dilution of the harvest rather than by altering the volume filled into the final container. Different volumes in the final container from batch to batch would cause variations in the freezing characteristics of the batches, thus precluding the use of standard conditions for freezing.

Glass sealed ampoules are the most suitable type of container for product to be stored in liquid nitrogen. The efficiency of flame sealing must be high to prevent the possibility of liquid nitrogen entering any containers. The comprehensive labelling of such containers is only possible by means of tags or by labelling a further outer

container as the full specification for the label, in particular the number of doses, is not determined until after the product is put into liquid nitrogen.

2.3.3. Preservation. It is useful to establish standard conditions for cooling the product through all batches produced. As long as the numbers of containers and the volume filled remain the same, very simple procedures can be adopted for the freezing process that will give uniformity of titre from container to container and minimal drop in titre from that harvested to that recovered when the final container is thawed for use.

Cell-associated vaccine is normally stored and transported in liquid nitrogen at -196°C. The maintenance of the viability of the cells in the vaccine is essential. This is achieved by the use of the appropriate protective agent (DMSO), a steady slow rate of freezing and rapid thawing when required for use. Optimal rates of freezing lie within the range of 1°C to 5°C per minute drop in temperature (11). This controlled rate of freezing can be achieved with sophisticated electronically controlled programmable cryopreservation equipment but can also be satisfactorily achieved by the use of simple insulated boxes placed in a low temperature refrigerator. The automatic cryopreservation equipment constantly monitors the product temperature through a thermocouple and adjusts the rate of cooling by means of controlled bursts of liquid nitrogen injected into the cooling chamber. It has the advantage that at the point of change of state an increase in the supply of coolant will be made to overcome the effect of the latent heat of fusion, thus ensuring a smooth cooling curve. An alternative, less sophisticated but nevertheless quite effective method is to put the batch of ampoules in an expanded polystyrene box and place this either in an electric -70°C refrigerator or in the gas phase of a liquid nitrogen vivostat. Such a box should have sides that are much thicker than the bottom to ensure that the cooling takes place through the bottom thus avoiding the possibility that the containers at the side cool much more rapidly than those in the centre. A metal plate placed inside the container upon which the ampoules are placed will, through improved conductivity, help to ensure that all containers cool through the bottom at the same rate. A fine thermocouple (connected to a recorder) placed in one container in

the centre of the batch and one at the edge will allow a comparison of the two cooling curves to confirm the uniformity of conditions at the two points in the box. This latter method does not compensate for the production of latent heat at the change of state and a blip in the cooling curve will occur at this point. Also, when the temperature of the product approaches that of the environment the rate of cooling will slow as the temperature gradient decreases across the thickness of the bottom of the box. If the freezing is carried out in an electrical refrigerator the product may be transferred to liquid nitrogen storage once the temperature has reached -60°C.

2.4. Harvesting, filling and preservation of lyophilized cell-free virus.

The work of Calnek *et al*. (3) made the development of a commercial freeze-dried vaccine possible. However a suitable product of this type can only be made from HVT, as a method of stabilizing adequate titres of MDV through a lyophilization procedure has yet to be found.

2.4.1. Harvesting. The cells of heavily infected monolayers showing extensive cytopathic effects are first removed from the vessel. Extensive cell disruption should be avoided prior to resuspending the cells in stabilizer. Cells may be detached using the method previously described for cell-associated vaccine, or alternatively may be removed mechanically by scraping, using a sterilized rubber bung attached to a stainless steel rod (Fig. 3). Once practice is achieved, the latter method is slightly less time consuming and gives similar yields. The cells are removed from the containers in a small volume of growth medium, deposited by centrifugation and resuspended in a suitable volume of stabilizer for virus release by ultrasonic disruption of the cells. Other mechanical methods of cell disintegration may be suitable, but the author has no experience of these and there is no published data on the efficiency of such methods. The basis of the stabilizer is a buffered solution of 8% sucrose. The resuspension of the cells in this preparation will retain the infectivity of HVT when released from cells by sonication. The suspension of the cells in the complete stabilizer (3) including the proteinaceous component (bovine albumen or N.Z amine) is not necessary at this stage and indeed is a disadvantage as the inclusion of protein results in excessive frothing during sonication.

The absence of protein during sonication does not adversely affect the titre of infectious virus obtained (Fig. 4). The optimum conditions for virus release by sonication must be determined by the manufacturer and will depend upon the type of equipment used and the volumes being handled. Effective sonication may be carried out on relatively small volumes of concentrated cell suspension, which are later diluted in more stabilizing solution with added protein before filling out. For optimum yields it will be found necessary to control the temperature of the suspension during sonication. A suitable means of doing this is to have the cell suspension in a stainless steel container in an ice bath. A plot is made of sonication time against virus yielded at a given amplitude of the ultrasonic vibrations. The temperature is also monitored during such an experiment.

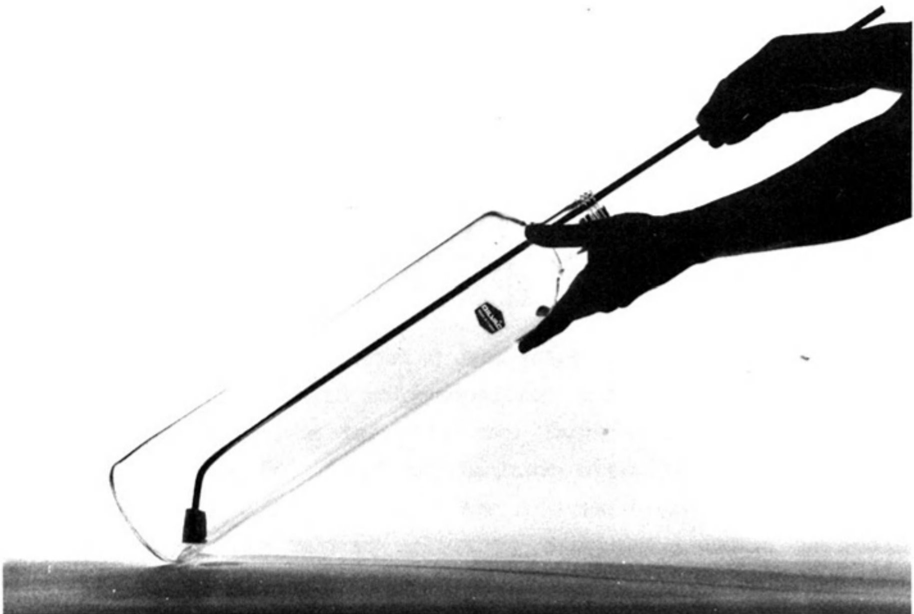
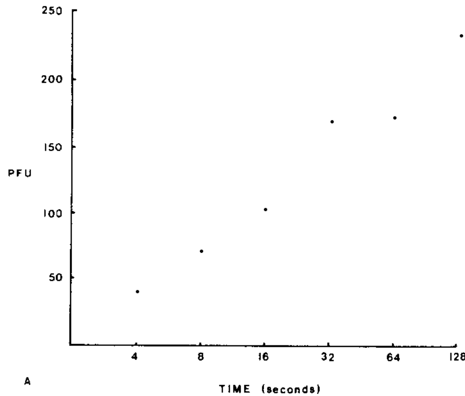


FIGURE 3. Illustration of the method of monolayer removal at harvesting by scraping.



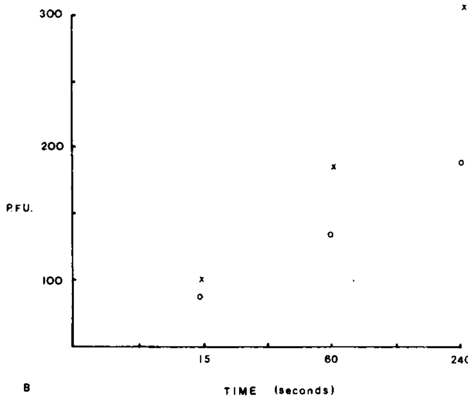


FIGURE 4. a) Graph of the relationship between sonication time and cell-free virus titre in pfu. Disintegration carried out in a glass container in an ice-bath. Final temperature of suspension after 128 seconds was 30°C. Final titre obtained was 9.3×10^8 pfu per roller bottle. Approximate yield per cell was 3 pfu. b) Relationships as in a) (different harvest) but with sonication carried out in stainless steel container in an ice-bath. o———o = curve obtained with bovine albumen included in stabilizer. x———x = curve obtained with bovine albumen excluded from stabilizer. Final temperature of suspension after 240 seconds sonication was 24°C.

The plot obtained from two such experiments using an MSE ultrasonic machine with a 10 mm probe operating at an amplitude of 14 microns is given in Fig. 4. Provided the suspension was precooled in the ice bath, even after 4 minutes continuous sonication the temperature did not rise above 24°C. Even after the maximum period of 4 minutes the titre of cell-free virus was still rising. The slope of the curve showed an approximate 2-fold titre increase for a 4-fold increase in sonication time. Where no attempt is made to control temperature, the curve will flatten out much earlier. After sonication any remaining cell debris that has not been disintegrated may be removed by coarse filtration or low speed centrifugation.

2.4.2. Filling. For maximum efficiency in the freeze-drying process the volume to be filled in the final container should be kept to the minimum that is compatible with accuracy. This may be in the region of 0.2 to 0.5 ml according to the filling apparatus used. However, some companies choose for commercial reasons to make the fill larger than this so that the customer can see better what he is paying for!

An appropriate dilution is made of the disintegrated cell suspension using complete stabilizer with the proteinaceous component included. As in the case of the cell-associated product, the infectivity titre filled into each container cannot be predetermined, so the dilution factor applied to the sonicate is based on previous experience and the final number of doses required per container.

2.4.3. Preservation. Standard freeze-drying techniques are used for the lyophilization of this product. The relatively high sucrose content of the stabilizer makes drying to a final moisture content below 1% difficult. A final moisture content of between 1 and 2% gives a stable product, which may be given a shelf life of 12 months at 2° to 8°C. During such a storage period a reduction in titre of less than 0.5 $\log_{10}$ should be found.

2.4.4. Diluent. A volume of diluting fluid is supplied with both the cell-associated and freeze-dried product. The formulation of this diluent is different for each product. There are advantages in keeping the volume of the inoculum per animal to a minimum that is compatible with the accuracy of the automatic injection apparatus in use. The advantages are, less tissue trauma to the one-day-old chick and less

mechanical work is necessary to deliver the inoculum reducing the possible damaging effect on cell viability in cell-associated vaccine by pressure changes. However, some forms of apparatus cannot be relied upon to deliver volumes of less than 0.5 ml without a high percentage of error. Good quality syringes can deliver 0.2 ml accurately and the supply of volumes of dilutent based on this inoculum afford considerable economies in storage space and transport costs.

A variety of formulae for the diluting fluid of cell-associated vaccine are suitable. Basically the requirement is for a fluid that will maintain cell viability with a minimal drop in infectivity over the period that diluted vaccine is likely to be in use at the hatchery or poultry farm. Suitable formulae range from complete cell culture medium to a simple buffered saline with a proteinaceous additive such as lact-albumen hydrolysate. The latter type of combination has the advantage that it can be heat sterilized, whereas the former would be sterilized by filtration. Such diluting fluids are quite unsuitable for lyophil-ized vaccine where a stabilizer for cell-free virus is necessary. A buffered solution of sucrose serves this purpose. The sucrose con-centration is kept down to the minimum that is compatible with adequate virus stability . A solution of 2% sucrose will achieve this, buffered with phosphate to a pH of 7.0 to 7.2 (13). Higher concentrations of sucrose may cause injection apparatus to stick.

3. CONCLUSION

The recommendation of vaccination equipment and methods is also part of the producer's responsibility, however, these matters are covered in Chapter 13. Likewise, the quality control of the product is an integral part of the production process. The methods adopted are influenced by statutory requirements and a discussion of these will be found in Chapter 10. Therefore, this chapter has been confined essentially to the practical considerations of the production process.

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10. QUALITY CONTROL AND STANDARDIZATION OF VACCINES

DENISE H. THORNTON

1. INTRODUCTION

Vaccines against Marek's disease (MD) have been generally the most successful immunological products introduced to the poultry industry in recent years, but some vaccines have also been disastrous, being ineffective or contaminated and causing serious financial losses. In order to avoid such problems, it is essential that care is taken to ensure the product is prepared in accordance with good manufacturing practice. Quality control must be exercised at each stage of production from starting materials through to the final product to ensure each batch is safe and efficacious.

In this Chapter, a discussion of control procedures is followed in Section 6 by recommended test methods.

2. SAFETY

2.1. Criteria for vaccine strains

There are those who would question the wisdom of allowing the use of any live herpesvirus vaccine because of the possibility of latency and reactivation, leading to disease. However, no long term ill effects have been noted with MD vaccines.

The vaccine strain should be safe for the target species and it should not pose a hazard to wildlife or to man during manufacture, administration or by spreading from vaccinates. No such hazard has been identified, but care should be taken to avoid unnecessary exposure: for instance, administration by spray is not to be recommended (1).

Consideration should be given to the desirability of cloning seed viruses for live vaccines. The more conventional means of developing vaccine strains, attenuation by passage, has been likened to genetic

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roulette (2). The final viral population resulting from repeated passage in embryonated eggs or cell cultures is genetically very heterogeneous and may contain virulent components that could be reselected by reverse passage. On the other hand, cloning may lead to a selected population ineffective in some situations. In addition, the stability of cloned populations is being questioned now that sensitive methods are available to select and detect variants; herpesviruses, however, being double-stranded DNA viruses, are probably among the more stable (3).

A desirable feature of vaccine strains is the presence of a genetic marker, because of its value for virus identification during vaccine manufacture. However, its main use is in facilitating field investigations where vaccine safety or performance is suspect (4). At present, methods of distinguishing field isolates of Marek's disease virus (MDV) from vaccine strains are limited to serological and pathogenic features which do not give a clear distinction and which are not universally applicable (5,6). For instance, naturally apathogenic strains used as vaccines have many of the characteristics of field viruses. Such strains are also able to spread from bird to bird. With the current lack of knowledge about the recombination potential of MDV's, particularly as field virus continues to infect and to replicate in vaccinated birds, caution should be exercised when deciding whether to accept such vaccine strains (7). In the future it is possible that reactions with monoclonal antibodies (8) or molecular characteristics (9) will provide satisfactory distinctions between vaccine and field strains.

Exotic strains should be introduced with care: if they are not essential for controlling disease, they may only prove to be a source of new genetic material which increases the variability of field viruses.

Vaccines against MD are prepared using a seed lot system whereby a stock of master seed virus is produced and all batches of vaccine are prepared from this within a limited number of passages. It is therefore possible to carry out extensive testing of the master seed virus, or a representative derivative, to establish that it is free from pathogenic and oncogenic effects, and to carry out more limited testing on each vaccine batch.

2.2. Safety of seed virus

The strain should be extensively examined to ensure it does not cause gross lesions. The extent of any damage to neural and visceral tissue should be assessed histologically. Although vaccine strains may produce microscopic lesions in very susceptible birds, they should be no more than minor and transient. Such lesions have been seen with strains of the herpesvirus of turkeys (HVT) (10). However, the CVI 988 strain of MDV used as a vaccine strain was shown to be capable of producing gross neural lesions and death (11).

The strain should be stable in its avirulence. No problems have been found with HVT vaccines or with attenuated non-spreading strains of MDV which have lost the A antigen. No increase in virulence was noted when the CVI 988 strain was passaged through chickens (12). Genetic markers should remain present throughout chick passages.

Finally, when the vaccine has been deemed safe by in-house trials, the safety of the strain is confirmed by field use in large numbers of birds of various breeds.

2.3. Safety of final product

The extent of safety testing of the final product can be limited to a test to detect errors in manufacture. Each batch of diluent should also be tested either alone or when used to reconstitute the vaccine. A simple error in the buffer composition of diluent has been known to have an adverse effect in chicks (D.H. Thornton, unpublished results). Chemical tests and pH measurement can be used to check for such errors.

3. EFFICACY

3.1. Criteria for seed strains

Factors to be taken into account in respect of efficacy are the genetic susceptibility of commercial chickens, the virulence and serotype of prevalent field viruses and the level and specificities of maternal antibodies. Suitable strains may be selected for particular situations on the basis of information given in Chapter 8.

3.2. Efficacy of seed virus and vaccine

Extensive tests should be done on material derived from the seed virus at the maximum end of the passage range that will be used for vaccine production.

The extent of passaging after initial isolation should be as res-

tricted as possible. Some viruses that have been extensively passaged _in vitro_ do not provide adequate protection (13,14). Characters such as plaque morphology (15) and growth at normal temperatures (13,16) may change during passage and a careful examination should be made of these features at all passages within the range used. Any change noted would cast suspicion on the acceptability of the seed virus. The plaque morphology of a non-protective isolate of HVT (D.H. Thornton, unpublished results) is shown in Fig. 1. The range of passages for vaccine is limited in the US regulations to no more than five from the master seed virus (17).

FIGURE 1. Plaque morphology of a non-protective turkey herpesvirus vaccine.

When both cell-free and cell-associated vaccines are to be prepared from the same strain, each should be tested. The tests must include a demonstration of protection in a situation that parallels the most severe challenge in the field, using genetically susceptible birds and

early exposure. The influence of maternal antibody should also be assessed. It is not possible at this stage to rely on tests other than those that demonstrate protection against challenge.

The batch potency tests, including virus content assay, which should be used on all batches of vaccine, should be carried out on the material used in the protection trial. The values obtained are then used to fix the minimum pass level for these tests. For instance, it is unacceptable to reduce the virus content of batches to a level below that shown to confer protection.

Finally, the efficacy of the vaccine should be confirmed in the field using various breeds of birds with various levels of maternal antibody, including antibody to the vaccine strain produced in response to double vaccination of parents. Other influences, such as interference by other medicaments, may become apparent in the field situation although some of these can be examined during in-house trials. Birds should be monitored throughout their life to confirm that adequate immunity persists, as the disease may take a long time to manifest itself, particularly in partially immune birds.

3.2.1. Protection tests. For evaluation of vaccine strains and particularly for comparison of vaccines, a standardized test method is desirable, and a reference preparation should be included in order to reduce test-to-test variation. The challenge should be done as early as possible, certainly within a week of vaccination. Rapid development of protection is an essential feature, for chicks in the field are exposed to infection almost immediately. Late challenge in the experimental situation may fail to detect an inadequate product. A vaccine which proved to confer inadequate protection in the field was shown to provide no protection in a laboratory test when challenge was carried out immediately after vaccination (18) or at 5 days, but at 28 days protection was afforded (D.H. Thornton, unpublished results). This vaccine was thought to consist of a mixed population, and the delay probably allowed replication of the protective constituent sufficiently to confer protection (D.H. Thornton, unpublished results). Other experimental vaccines that failed to confer adequate protection were detected by challenge at 9, 14 or 22 days (13,15). An additional point in favour of early challenge is the development of age-related resistance in the bird (see

Chapters 7 and 11). This makes it difficult to obtain a sufficiently high proportion of affected birds in the unvaccinated control group. Protection should be based on prevention of clinical signs, death and gross lesions. Attempts to assess microscopic lesions are complicated by the effects produced by the vaccine itself and in any case such lesions alone are probably of little significance to the bird.

Two types of test have been described, the protection index test (19), where a single dose level of vaccine is used to compare the incidence of disease in vaccinates with that in controls, and the PD_{50} test (19,20). The PD_{50} test has been criticized on the grounds of variability (21) but the protection index test is less sensitive (19). The results obtained with an unsatisfactory product (18) show that both types of test would detect such a vaccine. Typical vaccines give protection indices in excess of 80 (22) but this is influenced by very virulent challenge strains (23). PD_{50} values of 4 plaque forming units (pfu) have been obtained for HVT (24) and 16 to 75 pfu for CVI 988 virus (21). It has been proposed that a higher PD_{50} content is required for cell-free vaccines because these are more inhibited in the presence of maternal antibody (25).

3.2.2. Compatibility. MD vaccines are given at the same time as many other medications. The immunity conferred by a typical batch of vaccines should be shown not to be reduced in such circumstances, nor should the vaccine impair the performance of other products. Products that have been examined for compatibility include antibiotics (26), and vaccines against reovirus (27), Newcastle disease (28) and fowl pox (29). Interference by infectious bursal disease vaccine has been discussed (30).

3.2.3. Virus content assay. The specifications for acceptable virus content should be based on the quantity required for effective protection. Virus content tests can only be used as a measure of batch to batch consistency: they cannot replace protection tests. Some vaccines shown to have adequate virus content failed to stimulate satisfactory responses *in vivo* (13,15,16,18). Many vaccine strains replicate better at 37°C than at the normal bird temperature (31), and temperature-sensitive strains have been shown not to protect (13) or infect chickens (16).

Although in ideal circumstances very small quantities of suitable virus (approximately 1 pfu) will infect and eventually protect antibody-free birds, the vaccine should stand up to the toughest field situations, and some excess should be included to allow for a certain amount of misuse. In most cases, a figure of 1000 pfu per dose has been settled upon, although suggestions for a higher value for cell-free vaccines have been made. The test is very subject to technical variation and it is advisable (19) to use a standard vaccine tested in parallel. Such a standard has been shown to reduce assay variation (32) and it may be used either to determine the validity of the assay (33) or to express the results of the vaccine in terms of the standard (34). Important features of the test are adequately drained culture plates, a small inoculum volume for cell-free viruses, and the appropriate incubation temperature. The plaques must be counted before secondary plaques have formed (35): the formation of secondary plaques can alternatively be avoided by using a solid overlay. For vaccines that form macroscopic plaques, plates can conveniently be examined after staining using a microfilm document reader. The reliability of the assay depends upon the number of plates and dilutions used and the range of plaques per plate which it is considered valid to include (36). It is important to randomize the cell cultures and to count plaques without reference to the expected answer. Retest limits for borderline results have been suggested (34).

3.2.4. Viraemia assay. The viraemia response is a reliable indicator of protection against challenge. No instances have yet been reported of vaccines which produce rapid viraemia but fail to protect against challenge by homologous strains, whereas several vaccines that fail to protect also fail to stimulate viraemia promptly (13,15,18,37). The viraemia assay may be of value in screening potential vaccines.

3.2.5. Antibody responses. The antibody response following vaccination is not considered to be a major factor contributing to protection against challenge, so such a test can only be used to assess batch-to-batch consistency. For this purpose, a virus-content assay is quicker, cheaper and less variable, and it avoids the use of animals.

3.2.6. Cell-mediated immune responses. These responses are more likely to reflect protection against challenge. Some test methods have

been described (38) but much more work needs to be done to correlate the results of such tests with ability to confer protection.

3.2.7. Stability. To establish the shelf life of a product, batches of the vaccine should be stored at the recommended temperature and titrated at intervals until beyond the proposed shelf life. The titrations should be done in comparison with a standard vaccine or, for freeze-dried vaccine, with samples stored at -20°C or below. Accelerated degradation tests on freeze-dried products, that is titration after incubation for short periods at high temperatures, cannot be used to predict long-term stability. However, those products that show little loss after incubation at 37°C for 7 days are likely to be adequately stable at 4°C. It has been suggested that vaccines should contain at least 500 pfu per dose after incubation at 37°C for 7 days (39). Such tests are of value in screening stabilizers and other variables that affect stability. They also indicate whether the product will withstand a certain degree of incorrect storage. For freeze-dried products, vacuum and residual moisture checks during storage are of value. Such checks should also be done after withdrawal from storage in the deep freeze if the freeze-dried product is stored in the freezer, as the stopper may be affected, leading to moisture ingress and product deterioration (D.H. Thornton, unpublished results). A survey revealed that some products on the market may be insufficiently stable (40).

Stability after reconstitution is also important, as the vaccine may be left for some time at hatchery temperature. The ingredients in the diluent are of importance; sucrose is essential (41). The product containing sucrose, phosphate, glutamate and albumin (SPGA) (42) is one of the best substances but various omissions and amendments may be made to the formula without unacceptable deterioration (43).

4. PURITY

The purity of the final product depends on the purity of the starting materials and on protection against contamination during manufacture. The principle is that rather than relying on testing the final product, care should be taken to ensure that contamination does not occur by using ingredients that have likewise been shielded from contamination. The three main areas of concern are the substrate used

to grow the vaccine, the seed virus and materials of animal origin. However, other ingredients should be effectively sterilized before use: bacterial contamination of a vaccine resulted from the use of dimethyl sulphoxide that had not been sterilized (D.H. Thornton, unpublished results). However, the final product should also be carefully examined: any contaminant will have had the opportunity to replicate during production and may be more readily detected. The final product is also the only material normally available for independent scrutiny by the consumer or national control authority. Detection of contaminants which the manufacturer fails to find is an indication of inadequate testing procedures.

4.1. Substrate

At present, all avian cell lines are overtly transformed and hence are not considered suitable for vaccine production. The potential for growth on mammalian cell lines has not been extensively explored. MD vaccines are therefore prepared in primary avian cell cultures. This has the advantage that contaminants typical of cell lines passaged in the laboratory (44), such as cell-culture adapted mycoplasmas, contamination by foreign cell lines and induction of endogenous C type viruses, are not usually a problem. However, it does mean that validation of a master cell stock is not possible, so each batch of cells represents a risk. The first and major step is to use a cell substrate obtained from specific-pathogen-free (SPF) flocks. These are closed flocks which are regularly demonstrated to be free of avian pathogens. Recommendations for the housing and management of such flocks have been published (19). Great care should be taken at the onset to ensure that each bird is free from vertically transmitted agents and that the flock is free from horizontally transmitted agents. Serological tests should be supported by virus isolation attempts where necessary. The extent of further testing may depend on the risk of introduction of the agent, its rate of spread and the sensitivity of its detection. The screening regime should be organized so that the results of samples taken before and after collection of eggs for vaccine production are known to be satisfactory, to ensure active infection was not present at the crucial time.

Normally, chick embryo fibroblasts are used for vaccine production. In some cases, chick kidney cells may be required. In this case, these

must be from chicks derived from SPF embryos hatched and reared in isolation. Some viruses have been shown to grow better in duck cells than in chicken cells. However, insufficient is known about agents infecting ducks and their possible effect on chickens to recommend a testing regime - but enough is known to advise against the use of heterologous cells. Egg drop syndrome 76 virus, for instance, may have been introduced into the chicken population in a vaccine grown in duck cells (45) and reticuloendotheliosis virus, found in MD vaccines in Australia (46) and Japan (47), is known to infect ducks and may have been introduced by use of duck cells.

The second area of control is to prepare uninoculated cell cultures from each production batch of cells and to carry out purity tests on these. Such tests may prove more sensitive than flock screening for certain agents such as reovirus and adenovirus.

4.2. Substances of animal origin

Substances of animal origin used in MD vaccines include serum, trypsin and bovine serum albumen. These substances may be contaminated with mycoplasmas and bacteria and their associates such as toxins and phages. Bovine (44) and porcine (48) viruses may be present too. Although such viruses are not native to the chicken, many viruses replicate readily in chick embryo cells, so they could be present in significant numbers in the final product. They may represent a risk to vaccinates - the potential for interspecies infection has not been investigated at all thoroughly. The risk to humans during manufacture and administration must also be considered. Risks in both these areas also apply to large animals: cross-contamination may occur during vaccine manufacture or administration. The acceptability of such a risk must be carefully considered when defining the precautions to be taken. The problem can be approached in several ways (49). The substances may be derived from a SPF source. It may be decided that indigenous conventional animals are sufficient to avoid the risk of introduction of exotic viruses: however, most products are sold in more than one country. Alternatively the substances may be tested for freedom from contaminants or they may be treated in a manner that will destroy them. Tests should include culture on cells of the species of origin with observation for cytopathic effects, haemadsorption and specific tests

for agents of particular concern (17). Irradiation with gamma rays (2.5 megarads) is suitable for inactivation and trypsin can be treated at pH 1 (50). Bovine serum albumen used as a stabilizer for vaccine and diluent can be omitted or replaced with autoclavable substances.

The substances should in any case be tested for freedom from bacteria, fungi and mycoplasma and, if used for cell growth, should be used in the control cell cultures.

4.3. Seed virus

It is possible that the vaccine manufacturer may not obtain the vaccine seed until it has been attenuated or otherwise treated during development stages and therefore has had no control over environment, substrates or substances of animal origin already used. Hence the virus may need to be purified and appropriately tested for purity before preparing a master seed virus.

The seed should be demonstrated to be free from the list of agents applied to SPF flock testing. If the strain is of turkey origin, in addition, the absence of lymphoproliferative disease and haemorrhagic enteritis viruses should be ascertained.

Cell cultures used for passage may have contributed contaminants; if duck cells have been used then duck hepatitis viruses types I and II, duck virus enteritis, Pasteurella anatipestifer and Chlamydia should be shown to be absent.

The virus should be tested in suitable cell cultures for viruses likely to be present in serum and trypsin. It should be tested for laboratory acquired contaminants, including other vaccine strains, and for bacteria, fungi and mycoplasma. A vaccine seed contaminated with Mycoplasma hominis was found to give rise to several batches of contaminated vaccine (D.H. Thornton, unpublished results).

4.4. The final product

4.4.1. Freedom from bacteria, fungi and mycoplasma. Such tests on final container samples serve solely as a check for gross contamination, and are no substitute for good manufacturing procedures.

The tests for bacteria and fungi should be based on a random selection of containers taken from each stage exposed to a different contamination risk, for instance, each filling and freeze-drying lot. Mycoplasma tests should be done on a representative sample of each

batch. Diluents should be tested for mycoplasma if they are not terminally sterilized.

The quality and freshness of the media are of the utmost importance particularly for detecting salmonella (51) and mycoplasma (20). One of the recommended media for mycoplasma detection (17) has been shown not to support the growth of a mycoplasma present in a vaccine (52). Recently isolated low passage strains should be used to test each batch of medium for its ability to initiate and maintain the growth of a range of organisms in the presence and absence of the test material. The ability of the media to support growth is more important than their composition; however, several media have been suggested (17, 53-55). Inhibitory substances present in the vaccine or diluent should be removed, neutralized or diluted out. Membrane filtration can be used and this technique is recommended for testing diluents. The media must of course be checked before and during use to ensure they are sterile and the tests must be conducted in a suitable environment. Retest criteria must be decided to ensure that extraneous contamination is recognised as such.

An *in vivo* test may be included for detection of *Salmonella pullorum*, *Mycoplasma gallisepticum* and *M. synoviae*. A comparison (56) of *in vitro* and *in vivo* tests for *M. gallisepticum* and *M. synoviae* as suggested by the European Pharmacopoeia (53) showed similar sensitivity; unfortunately the most effective route of inoculation, the intratracheal route, has been omitted from the later version of the European Pharmacopoeia (54).

4.4.2. *Freedom from extraneous viruses*. Several instances of contaminated MD vaccines have been recorded. The rapid turnover of poultry and the widespread use of vaccine can mean that a contaminant has become widely disseminated before its presence and implications are realised. Some contaminated vaccines have led to serious financial loss. A reovirus was found in a MD vaccine in Germany (57) and reticuloendotheliosis virus in vaccines in Japan (47) and Australia (46). Egg drop syndrome 76 was possibly introduced via a MD vaccine (45). A case of Aujeszky's disease in chicks following MD vaccination was due to the presence of Aujeszky's disease vaccine, although it is not clear at which stage this was introduced (58). A lentogenic strain of Newcastle

disease virus found in a MD vaccine is thought to have occurred following inadequate separation during manufacture (D.H. Thornton, unpublished results). The day-old chick has poor defence against administration of a pathogen by injection: it is not fully immunocompetent, its natural secretory defences are by-passed and it has rapidly replicating tissue favoured by many viruses. These features may help to explain why MD vaccines cause more problems in the field than other vaccines; no other product is given in this way on such a scale. One of the other main reasons is the use of seed virus and cells derived from heterologous sources or those that were not SPF. Care must be taken in respect of extraneous agents right up to the stage of vaccination; losses through Pseudomonas aeruginosa contamination of inoculation equipment have been reported (59). Needle transmission of avian leukosis virus has been demonstrated experimentally during MD vaccination (60) and it has been suggested as a means of spreading egg drop syndrome 76 virus (61).

Tests for extraneous viruses on the final product should include the complete range. In all tests where cell cultures or embryonated eggs are used the test material should be passaged; a test recommended for detecting Newcastle disease virus without passage (17) is not sufficiently sensitive.

Inoculation of neutralized virus in chick kidney, chick embryo kidney or chick embryo liver cells followed by examination for cytopathic effect can reveal reoviruses and adenoviruses. Haemagglutination and haemadsorption tests on the cultures may demonstrate the virus of egg drop syndrome 76. Passage in suitable genetically susceptible chick embryo fibroblast cell cultures followed by the COFAL test, phenotypic mixing test or ELISA should be done for detection of leukosis virus. Chick embryo fibroblast cultures can also be used to examine for reticuloendotheliosis virus by the fluorescent antibody test (62). Inoculation of embryonated eggs onto the chorioallantoic membrane should detect infectious laryngotracheitis and fowl pox viruses. Inoculation into the allantoic cavity followed by haemagglutination tests will reveal Newcastle disease and influenza viruses. Staining of cells harvested from the allantoic fluid by the fluorescent antibody test (63) was shown to be the most sensitive method of detecting infectious bronchitis virus

(64). Infectious avian encephalomyelitis virus can be detected by hatching inoculated embryos or inoculating day-old chicks intracerebrally and observing the chicks for clinical signs. The fluorescent antibody test in chick embryo brain cells may prove to be a suitable alternative (D.H. Thornton, unpublished results). Vaccine strains of MDV, HVT and infectious bursal disease may be detected by examination for cytopathic effects or lesions in the cell culture and embryo tests described above. Field strains of these agents are unlikely to be present in the final product as they are not egg transmitted. However, if it is considered desirable to include a test for such strains, the most sensitive way to detect them is probably an in vivo test which includes observation of neural, visceral and bursal tissue for lesions. Careful clinical observations should also be made. Serological tests can also be done on these birds for a complete range of agents. Although for the more likely vaccine contaminants bird inoculation may be the least sensitive test method, it is the only one where it is possible to avoid the use of neutralizing serum to prevent lesions caused by the vaccine virus and it may lead to the detection of previously unrecognized agents.

5. STANDARD PREPARATIONS

The inclusion of a reference vaccine in some tests, particularly the tests for potency and virus content, should be done wherever possible. The preparation of reference vaccines for HVT and CVI 988 strains was recommended by the International Association of Biological Standardization (19). Other standardized reagents are needed for tests such as control strains of micro-organisms (20,53,55) and viruses for testing for sterility and extraneous agents. Seed strains and samples of some reagents may be obtainable from national control authorities (17,55).

6. RECOMMENDED TESTS

The tests suggested here are based on those recommended by the Ministry of Agriculture, Fisheries and Food (MAFF) (55), the British Pharmacopoeia (BP) (20), the European Pharmacopoeia (EP) (53,54), the United States Code of Federal Regulations (CFR) (17), the International

Association of Biological Standardization (IABS) (19) and independent publications referred to previously. The guidelines of other national authorities have also been consulted. The status of these tests varies; those of MAFF and CFR are specifications for manufacturers, whereas the Pharmacopoeias are for independent analysts assessing the final product. No monograph for MD yet appears in the EP: however, it does include tests for sterility and absence of extraneous micro-organisms and viruses, though strictly speaking these only apply to products for which a monograph exists. The tests of the IABS are international recommendations. Detailed methods for some of the tests are included in these publications and the USA issues Supplemental Assay Methods for some tests (17).

The tests, and their stages of application, are listed in Table 1.

TABLE 1. Recommended test schedules

Test	6.1	6.2	6.3.1	6.3.2	6.3.3	6.4	6.5	6.6	6.7.1	6.7.2	6.8	6.9
Stage												
Flock	x											
Seed virus		x	x		x			x	x		x	x
Control cells								x				
Vaccine harvest			x	x		x						
Final vaccine		x	x		x	x	x	x		x		
Diluent			x		x		x			x		

6.1. Specific-pathogen-free flocks

Flocks used to produce cultures and embryos for vaccine production and testing should be free from the agents listed in Table 2. Methods for detecting these agents are also suggested.

TABLE 2. Recommended testing of specific-pathogen-free flocks

Agent	Egg transmission	Source for virus isolation	Serological or virological examination		
			Test[a]	Specificity	Sensitivity[b]
Adenovirus	+	Faeces Cultured cells	SN FA ELISA AGP	Type specific Type specific Group specific Group specific	H H ? L
Avian encephalomyelitis virus	+	Faeces	ES	Group specific	H
Infectious bronchitis virus	+	Faeces Trachea	SN HI AGP	Type specific Type variation Group specific	H M L
Infectious laryngotracheitis virus	-	Trachea	SN	Type specific	M
Influenza A virus	-		AGP	Group specific	H
Newcastle disease virus	(+)	Trachea	HI	Group specific	H
Fowl pox virus	-	Lesions	AGP	Group specific	L
Bursal disease virus	-	Faeces	AGP	Group specific	H
Marek's disease virus	-		AGP	Group specific	H
Leukosis virus	+	Vagina	SN PM COFAL ELISA	Type specific Group specific Group specific Group specific	H H H H
Reticuloendotheliosis virus	+		FA AGP	Group specific Group specific	H M
Reovirus	+	Faeces Cultured cells	SN AGP	Type specific Group specific	H L
Egg drop syndrome '76 virus			HI SN	Group specific Group specific	H H
Salmonella pullorum	+	Faeces	SPA	Species specific	H
Mycoplasma	+		SPA HI	Species specific Species specific	H H

[a]AGP Agar gel precipitin
COFAL Complement fixation for avian leukosis
ELISA Enzyme linked immunosorbent assay
ES Embryo susceptibility
FA Fluorescent antibody
HI Haemagglutination inhibition
PM Phenotypic mixing
SN Serum neutralization
SPA Serum plate agglutination

[b]H High
M Medium
L Low

6.2. Identity

The purposes of this test are to help establish the identity of the seed virus and to check that the final product is of correct specificity. Monospecific neutralizing serum is raised in SPF chickens using a virus strain that is serologically identical to the vaccine strain but of independent origin. The serum is checked for freedom from neutralizing capacity for other viruses and toxicity for cell cultures.

The test is based on the demonstration that the virus is no longer able to cause specific cytopathic effects in susceptible cell cultures after mixing with the serum (20).

6.3. Freedom from bacteria, fungi and mycoplasma

6.3.1. Bacteria and fungi. Suitable media such as soya bean casein digest medium and thioglycollate broth are tested for their ability to support rapid and copious growth of the control organisms in the presence and absence of vaccine and diluent. Samples of the media are inoculated with 100 organisms of *Bacillus subtilis*, *Clostridium sporogenes*, *Staphylococcus aureus* and *Candida albicans*: these are incubated at 30° to 32°C and 20° to 25°C for not more than 7 days.

Representative containers of vaccine and diluent (1% of the batch with a minimum of three and a maximum of 10 containers) are then tested, using membrane filtration if possible. The test cultures are incubated as above for at least 14 days. There should be no evidence of contamination.

6.3.2. Salmonella. Recently isolated strains of salmonella are used to test the ability of the media to initiate and support their growth in the presence and absence of the vaccine material.

This test is done on 10 ml of pooled harvest which is inoculated into selenite F broth and tetrathionate broth. The broths are incubated at 35° to 37°C and 43°C for 48 hours, subculturing at 24 and 48 hours onto desoxycholate citrate, brilliant green and bismuth sulphite agars; these are incubated at 37°C for 48 hours. Any colonies are identified biochemically and serologically. The harvest must be free from salmonella.

6.3.3. Mycoplasma. Suitable media are used such as C medium supplemented with yeast extract and media supplemented with glucose and with arginine. Liquid media contain phenol red. The media are shown to

support the growth of low passage control strains of Acholeplasma laidlawii, Mycoplasma arginini, M. hyorhinis, M. orale and M. synoviae in the presence and absence of the vaccine. Liquid media are inoculated with 20 organisms and solid media with 200. The media are incubated at 35° to 37°C for not more than 14 days in a humid atmosphere aerobically (air plus 5% to 10% carbon dioxide) and anaerobically (nitrogen plus 5% to 10% carbon dioxide).

The vaccine is tested in each medium incubated as above for not less than 28 days, subcultures being made at 3 to 4 day intervals or immediately there is a colour change. All cultures must be examined microscopically. The vaccine must be free from Mycoplasma spp. The absence of avian mycoplasma may be additionally verified in test 6.6.4.

6.4. Virus assay

This method is suitable for cell-free and cell-associated HVT vaccines but may require modification for strains of MDV.

Dilutions of vaccine are prepared in SPGA such that the expected plaque counts fall within the limits of 40 to 140 per plate. Plates (5 cm) of confluent secondary chick embryo fibroblast cell cultures are drained thoroughly without allowing them to dry out and 0.05 ml of each dilution is inoculated onto each of seven plates. The plates are swirled and allowed to adsorb for 1 hour at 38.5°C. Medium (5 ml of Eagle's Minimal Essential Medium) is added and the plates are incubated at 38.5°C for 3 days. Plates are then fixed and stained and plaques are counted.

The vaccine should contain not less than 1000 pfu per dose, throughout its shelf life, or more if this has been shown to be necessary in protection tests.

6.5 Stability after reconstitution

The vaccine is reconstituted if necessary and diluted to field strength with the recommended diluent and held at 20°C. Titrations are carried out immediately and at intervals. After 2 hours, the suspension should contain not less than 50% of its initial content of live virus and not less than 1000 pfu per dose.

6.6. Extraneous viruses

Embryos, cell cultures and chicks used in these tests should be derived from SPF flocks. Each cell culture should have an approximate

area of 30 cm^2.

6.6.1. Tests in embryos. Embryonated eggs, 9 to 11 days of age, are inoculated with 10 doses of neutralized vaccine, 10 onto the chorio-allantoic membrane and 10 into the allantoic sac. A further passage is carried out after 7 days using separate pools from live and dead embryos. After a further 7 days, the embryos and membranes are examined for abnormalities, the allantoic fluid is tested for haemagglutinins, and cells centrifuged from the allantoic fluid are tested by the fluorescent antibody test for infectious bronchitis virus.

6.6.2. Tests in chick embryo fibroblasts. For the detection of reticuloendotheliosis virus, five cultures of chick embryo fibroblasts are each inoculated with 10 doses of neutralized vaccine. The cultures are passaged twice at 3 to 4 day intervals. Cultures from the final passage are tested, in conjunction with positive and negative control cultures, by the fluorescent antibody test. For the detection of leukosis viruses, fibroblasts susceptible to subgroups A and B are used which do not produce virus or group-specific antigen of subgroup E. Ten doses of neutralized vaccine are each inoculated into 10 cultures and these are subcultured at 3 to 4 day intervals for at least 14 days. A test for leukosis virus is done on each passage using either an ELISA, the COFAL test or phenotypic mixing test.

6.6.3. Tests in chick kidney cells. Five cultures of chick kidney, chick embryo kidney or chick embryo liver cells are each inoculated with 10 doses of neutralized vaccine. After allowing adsorption for 1 hour, the cultures are incubated for a total of 20 days, subculturing at 4 to 5 day intervals. The cultures are examined for cytopathic effects and the cells and fluids tested for haemadsorption and haemagglutination. There should be no evidence of contamination.

6.6.4. Test in chicks. A group of 20 chicks, 2 weeks of age, are each given 10 field doses of vaccine by eye drop, intramuscular, intra-tracheal and foot pad routes. The inoculations are repeated after 3 weeks. Serum samples obtained from each bird before and 3 and 5 weeks after the initial inoculation are tested for freedom from antibodies to the list of agents specified for SPF flocks. There should be no evidence of contamination.

6.6.5. Intracerebral test in chicks. At least 1 field dose of

vaccine is inoculated intracerebrally into 10 chicks, 1 day of age. The chicks are observed for 3 weeks for signs of ataxia. The test is invalid if more than two chicks die from non-specific causes. There should be no evidence of contamination.

6.7. Safety

6.7.1. Seed lot verification. Three groups of genetically susceptible, antibody-free, day-old chicks are used. Fifty chicks acting as positive controls are given virulent MDV sufficient to cause lesions in 70% of the birds in 70 days. A further 50 are given the equivalent of 10 field doses of vaccine and 50 remain as uninoculated controls. The groups are retained in isolation for 17 weeks, birds which die being examined for gross lesions. For vaccine strains which do not spread, or where spread is transient, samples of feather follicle epithelium are obtained at 6 weeks of age: these are tested by the single radial diffusion test (see Chapter 6) for the presence of antigen. Serum samples are obtained and examined by gel diffusion test. At the end of the observation period, each bird is weighed, killed and examined for gross lesions of MD. For the test to be valid, not more than five birds should die from non-specific causes during the 1st week of the test, at least 40 negative control birds should survive the 17 week observation period, no gross lesions, antigen or antibody being detected, and at least 70% of the positive controls should be affected. For the vaccine strain to be considered satisfactory, the average body weight of the vaccinated group should not be significantly less than that of the control group, and the variation in weight should be similar. The number of survivors in the vaccinated group should be not less than 90% of that of the control group and all must be free from gross lesions. The antigen and antibody responses should be characteristic of the vaccine strain and distinct from those of field strains.

6.7.2. Batch safety test. Ten doses of vaccine or two doses of diluent are given to a group of 10 SPF chicks, 1 day of age, which are observed for 14 days. The product fails the test if there are any adverse local or systemic reactions.

6.8. Reversion

The equivalent of 10 doses of vaccine virus is given to each of 10 genetically susceptible, antibody-free chicks, 1 day of age. After 3

weeks, a pool is made of 2 ml of heparinized blood from each chick and this is inoculated into a further group of similar chicks by the intraperitoneal route. Five further passages are carried out in a similar manner. The final passage is carried out in a group of 50 chicks which are examined as in test 6.7.1. Material at each passage is inoculated onto cell cultures to check that the virus is being passaged. The vaccine strain is unsatisfactory if there is any indication of an increase in virulence.

6.9. Potency

6.9.1. Protection index test. Two groups of genetically susceptible, antibody-free chicks, 1 day of age, are used, 30 being given the equivalent of one field dose of vaccine virus and 30 remaining as unvaccinated controls. All chicks are challenged not more than 8 days later and observed for at least 12 weeks or until at least 70% of the controls have gross lesions of MD. Birds which die, and all birds at the conclusion of the test period, are examined for gross lesions. The vaccine strain is unsuitable if the number of vaccinated birds showing lesions is not reduced by at least 80% compared with the controls.

6.9.2. PD_{50} test. Five groups of 20 genetically susceptible, antibody-free chicks, 1 day of age, are used. Graded doses of virus such as the equivalent of 10-fold dilutions from 1:10 to 1:10,000 of a field dose, are given to four of the groups and the other group is kept unvaccinated. After not more than 8 days, the birds are challenged and observed for at least 12 weeks, when at least 80% of the controls have gross lesions of MD. The vaccine strain is unsuitable if there are less than 100 PD_{50} in one field dose.

6.9.3. Viraemia. The equivalent of one field dose of vaccine is given to each of 10 SPF chicks. Five days later, heparinized blood samples are obtained from each chick and the presence of vaccine virus is determined by assay on cell cultures. The vaccine strain is unsuitable if any chick is found not to be viraemic.

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11. GENETIC RESISTANCE

B.W. CALNEK

1. SIGNIFICANCE AND HISTORICAL ASPECTS OF GENETIC RESISTANCE

Genetically controlled differences in susceptibility to Marek's disease (MD) were first reported over 50 years ago, when it was noted that the incidence of the disease differed amongst families (1-4). In 1935, Hutt and Cole (5) initiated their classic studies which were to prove that selection for genetic resistance could constitute a practical method for the control of disease in poultry flocks. Two resistant strains, K and C, and one susceptible strain, S, were developed by selection based on mortality after natural exposure. Emphasis was on the avian leukosis complex and other neoplasms. Ultimately, marked differences were observed consistently in consecutive generations (6). This was accomplished without interference with concurrent and successful selection for important production traits (5). Other workers reported similar success in selection for resistance (7,8), and these early studies prompted some poultry breeders to develop their own resistant strains (9).

Although the inspiration for studies on genetic selection came from the need for resistant stock, it is ironic that the use of susceptible strains in research may have been of even greater importance through discoveries regarding the aetiology, pathogenesis, and immunological control of the disease. The first consistently successful transmission studies (10,11) made use of the Cornell S-strain, and the Houghton Poultry Research Station Rhode Island Red strain, both of which were highly susceptible to MD virus (MDV), indeed, the ability to induce MD regularly by inoculation of young chicks with infectious materials led in turn to much more efficient methods for genetic selection wherein

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high levels of resistance could be obtained within two generations (12). For a period of several years, selection for genetic resistance by commercial poultry breeders, along with isolation rearing to delay and/ or reduce virus exposure levels, constituted the only defence against MD.

The establishment of strains resistant or susceptible to MD also provided comparative tools for investigating aspects of pathogenesis and immunity in this disease (see reviews 13-16). Additionally, they led to the discovery of an association between resistance and certain erythrocyte antigen groups B (Ea-B) or lymphocyte antigen (Ly-4, Th-1) alleles (17,18). This, in turn, opened the possibility of selection for resistance based on blood group or lymphocyte antigens.

There are both practical and academic reasons for continued interest in genetic control of resistance to MD or MDV infection. Most poultry breeders stopped or reduced emphasis on selection for MD resistance as a control method once effective vaccines became widely available after 1969. However, it was soon determined that vaccinal immunity was greater in resistant than in susceptible stock (19,20). This became very important with the recent emergence of MDV strains against which neither vaccine nor genetic resistance alone can protect adequately against challenge, but the combination of the two prevents high losses from MD (21). For these reasons, resistance to MD is, or should be, once again included as one of the traits considered in genetic selection by many poultry breeders.

The importance of host genotype on the outcome of neoplastic and other diseases has been known for a long time. MD, as a representative herpesvirus infection and a neoplastic disease, provides an elegant model for the study of both the phenomenon itself and the mechanism by which resistance is effected. There will undoubtedly be continued strong interest in genetic resistance to MD for its potential contribution to the field of comparative medicine.

2. EXPRESSION OF RESISTANCE

2.1. General features of genetic resistance

2.1.1. Methods and criteria for demonstrating resistance. In general, clinical signs and mortality, coupled with characteristic MD

lesions, have constituted the criteria for determining the incidence of the disease (5, 22-30). The test period has varied from a few weeks to over 1 year, depending on the challenge method and the virulence of the challenge virus. Similarly, there has been variation in the proportion of survivors examined at the termination of the test period, and the thoroughness of the search for lesions in those birds which were examined. Peripheral nerves, gonads and major visceral organs have most frequently been the tissues examined. Microscopic examination of equivocal gross lesions, or sometimes even tissues from chickens free of gross lesions, has been done by some investigators but many relied only on gross examinations.

Morris _et al_. (24) studied the lesion distribution in MDV-infected chickens and found the pattern to vary according to the genetic strain. They concluded that nerves and gonads were the most important tissues to examine, and that relative MD resistance could be determined by observing only those organs plus the spleen and liver. Cole (12) proposed that mortality alone is sufficient to rank strains or sires providing other diseases are not present to confound the evaluation. In addition to differences in incidence and distribution of lesions, the mean latent period to death may be longer with resistant than susceptible strains (12, 22).

The worry of selecting for the wrong type of resistance by using an experimental inoculation procedure was allayed when it was learned that there was a positive correlation between the responses from inoculation and from field exposure challenge in various strains of chickens (12,22, 23,25,26). Cole (12) found a high rank correlation coefficient (r_s) of +0.82 in 10 commercial strains infected by inoculation or natural field exposure. Similar results (r_s = +0.66 to +0.80) were subsequently reported by Hartmann and Sanz (31) and Grunder _et al_. (26), although von Krosigk _et al_. (30) found a correlation of only +0.38 between inoculation and contact exposure. Payne (14) concluded that selection based on injection of MDV will lead to resistance to natural exposure but that the two methods for selection may not measure the same type of resistance. He pointed out that contact exposure should correlate most highly with field exposure.

Similarly concern over the choice of virus strain for experimental

challenge lessened when it was learned that various MDV isolates could be used without altering the ranking of resistance among genetic strains (22,25,32,33). However, because some virus isolates are so virulent that they can overwhelm even moderately high levels of genetic resistance, caution in choice of challenge virus was urged by Schat et al. (34). An ideal virus strain would give a large spread in MD incidence among groups (strains, sire families, etc.) being compared.

Tests of siblings and progeny have both been used effectively. Cole (12) showed good correlation (+0.87) between the responses of male and female progeny (females are generally more susceptible), and this permits the use of otherwise unneeded male chicks in progeny tests.

Other methods suggested for demonstrating resistance to MD have been less correlative to results from field exposure or experimental infection with MDV. Initial studies by Spencer, Gavora and their co-workers (28,35,36), using strains 4,5, K and S, suggested good agreement between resistance to challenge with a MD tumour transplant (JMV) and resistance to MD. Fabricant et al. (37) were unable to confirm this with other strains, and Gavora and Spencer (38) subsequently concluded that the correlation was too inconsistent to be of value. It has also been proposed that the response to vaccine viruses, like turkey herpesvirus (HVT), might be considered as an alternative means of assessing relative resistance to MD (38). This was based on the failure of vaccination to affect ranking of resistance to MDV challenge among strains (19,39) and on differences of HVT viraemia levels in various strains of chickens (40). The usefulness of this as an approach to resistance selection is not established, however.

2.1.2. Variability within and among genetic strains. High levels of heterogeneity have been found among strains, inbred lines and sire families (3,5,12,21-26,29,32,33,41-43). Differences can be very substantial within tests, as illustrated in studies by Cole (12) who found susceptibility of 33 strains to range from 18 to 96% and by Schmittle and Eidson (23) who observed susceptibility levels of 4 to 92% among 12 strains or crosses. Biggs et al. (22), in three trials each involving 20 to 54 sire families, found MD incidences to vary among families from 1 to 22, 0 to 23 and 41 to 100%. Similarly Grunder et al. (42) reported MD incidences in sire families to range from 0 to 46%, and in

experiments comparing inbred lines, the extent of variation ranged from a low of 2 to 21% to as high a spread as 89%. They were unable to correlate inbreeding coefficients with MD incidence and noted that inbred lines derived from high egg production lines had at least as much variation in MD resistance as did sire families of production lines.

2.1.3. Effects of selection. Using progeny or sib testing with artificial exposure to MDV, it has been possible to effect a large increase in resistance to MD in a relatively short time. This was dependent on a reasonable degree of heterogeneity in the strain and on selection directed solely at MD resistance. In their early studies, dependent on the whims of natural exposure, Hutt and Cole (5) gradually reduced tumour mortality over a period of 10 years from about 15% in unselected SCWL stock to 4 to 5% in two selected strains, K and C. A parallel selection for susceptibility (S-strain) resulted in a level of 38% mortality from neoplasms (Fig. 1a). In marked contrast to that slow, albeit steady and impressive progress, was the very rapid and effective selection method later tested by Cole (12) (Fig. 1b). He used virus inoculation of progeny in an 8-week test period to select breeders from the Cornell randombred SCWL breeding stock with a 51.1% level of susceptibility. Within two generations he achieved susceptibility levels of 90.7% in the derived susceptible P-line and 12.9% in the derived resistant N-line. Further progress (94.4 and 7.3% MD for P-line and N-line, respectively) was made in a third generation (44). By comparison, the susceptibility of the K-, C-, and S-strains to the same virus challenge used to select N- and P-lines was found to be 38, 71 and 98% after over 3 decades of selection by natural exposure. It was later learned (see Section 2.1.5) that Cole had unknowingly selected birds homozygous for the $\underline{B}^{21}$ allele in the N-line, an allele associated with resistance. The presence of the $\underline{B}^{21}$ allele in the randombred stock which was the source of N- and P-lines may have been fortuitous and permitted an ultimately higher level of resistance than could be attained in the K- and C-strains which do not carry the $\underline{B}^{21}$ allele (45).

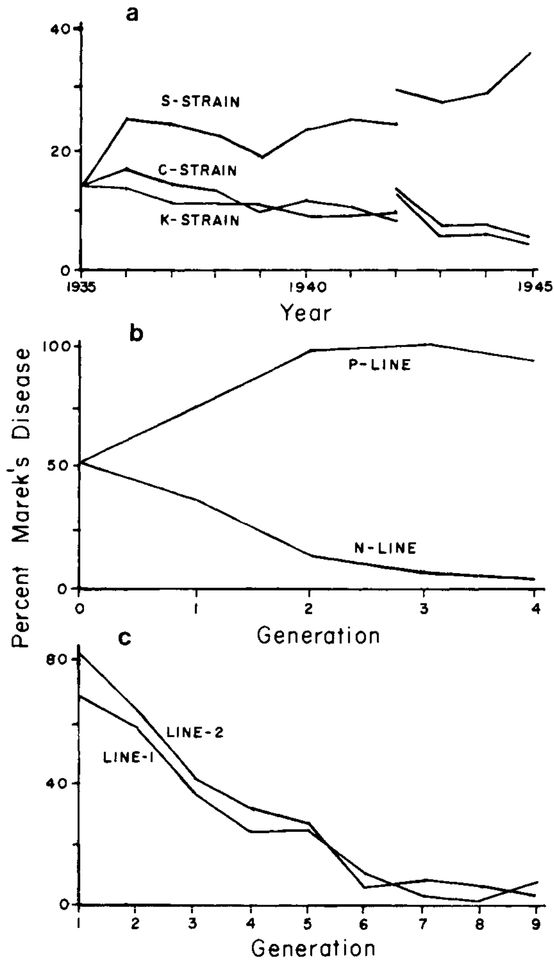

FIGURE 1. Effects of genetic selection for susceptibility or resistance to Marek's disease: a) (top) Selection based on mortality after natural exposure to MDV. From Hutt and Cole (5), b) (middle) Selection of breeders based on an 8-week test of progeny by MDV inoculation. From Cole (12,49), c) (bottom) Selection by breeding from survivors of heavy MDV natural exposure. From Maas _et al_. (27).

Others have had results similar to those of Cole, although less dramatic. Morris et al. (46) obtained resistant and susceptible lines after selection for two generations with respective susceptibilities to MD of 58 and 96%, compared to 89% in the unselected population of Columbian Plymouth Rocks. They concluded that selection for resistance resulted in greater progress than selection for susceptibility. Morrisroe (47) compared progeny of selected and unselected Australorp sires and reported a 26% difference in susceptibility (59 versus 33%). Von Krosigk et al. (30) progeny-tested the parents to be used to produce cross-line populations of chickens. Progeny from parents selected only for MD resistance had 17% less mortality than those from unselected parents. A similar reduction in susceptibility (by 14%) was obtained in a single generation with commercial female broiler strain chickens by Friars et al. (48), also using MD resistance as the sole selection criterion. A second generation of selection, which included consideration of other economic traits, was less effective in further reducing susceptibility. Maas et al. (27) followed nine generations of breeding from survivors of heavy MDV challenge (see Fig. 1c). In each of two lines of non-inbred White Plymouth Rocks, there was a gradual reduction in susceptibility for the first six or seven generations, but thereafter no further improvement occurred. Complete resistance was not achieved.

2.1.4. Dominance, heritability and sex-linkage of genes involved in MD resistance. There has been general, although not total, agreement that resistance is a dominant trait (5,23,24,27,29,41,42,47,49-52). The chief contradictory example comes from lines 6 and 7, in which susceptibility was partly or fully dominant over resistance (41). Those lines have the additional distinction of having non-MHC-controlled resistance or susceptibility (see Section 3.3). In most cases, resistance of hybrids has been intermediate to that of the parent strains. Stone (52), who had included the line 6 and line 7 crosses in his study, observed that resistance was dominant only in some crosses and he concluded that it was due to the action of only a few (two to four) genes. The conclusion that only a few genes are involved is supported according to Cole (49), by the facts that: 1) selection for resistance is very effective,

and 2) some inbred lines such as lines 6 and 7 are either very susceptible or very resistant (23,41). At least three genes have been identified, one associated with the Ea-B locus, the others with loci controlling lymphocyte antigens. These will be discussed in Section 3.2.

Cole (49) pointed out that in many reciprocal crosses, the hybrids are more resistant than could be expected from the average of the parent strains, and that this could be due to hybrid vigour (heterosis) or dominance. He cited data from Han *et al*. (43) as evidence for rejecting heterosis *per se* as an explanation. They had found increased susceptiblity in some of their hybrid crosses. Findings similar to Cole's (49) have been reported by Sevoian (51), Schmittle and Eidson (23) and Hartmann (cited in 49). Zeitlan *et al*. (29) studied susceptibility with reciprocal crosses from three strains and concluded that the degree of dominance for resistance varied with the particular crosses involved. Crittenden *et al*. (25) essentially agreed with that conclusion and suggested that the dominance of resistance appeared to depend on both the severity of exposure and the relative susceptibility of the two lines being crossed. They further stated that there may not be a true dominance in the case of MD resistance. Rather, it could be that the severity of exposure may be below the threshold required for mortality.

Probably the strongest argument for the dominance of resistance comes from the studies on the association between the B^{21} allele of the major histocompatibility complex (MHC) and resistance to MD challenge. In virtually all crosses involving that allele, it has conferred resistance in both homozygotes and heterozygotes. MHC-associated resistance will be discussed in detail later (Section 3). In reciprocal crosses both males and females contribute to resistance; often the male contributes more than the female (27,49), even though females may additionally provide protective maternal antibodies. Zeitlan *et al*. (29) believed their data to suggest a sex-linkage of resistance, but Cole (49) determined that neither sex-linked genes nor protective maternal antibodies contributed to the resistance of N-line. Females have generally been more susceptible than males although this feature is variable (reviewed in 13) and, in any case, the responses of male and female progeny are highly correlated ($r_S = +0.87$) (12).

2.1.5. Association of MD with other traits. Cole and Hutt (53), von Krosigk et al. (30), Grunder et al. (42) and Biggs et al. (22) all failed to detect unfavourable associations between MD resistance and egg production traits. In fact, several workers reported significant positive correlations between high egg production rates and resistance (30, 35,54,55). Correlations between MD resistance and reduced growth rate, lower body weight, and lower egg weight, on the other hand, also have been observed in several studies (31,35,37,42,48,54-57). The lower body and egg weights of these reports contrasted with findings of Biggs et al. (22) and also with those of Cole (44) who found them to be slightly higher in resistant N-line than in susceptible P-line. The possibility of a correlation with reduced growth rate is especially important in broiler production, and both Hartmann and Sanz (31) and Gavora and Spencer (35) warned that genetic resistance could be lowered if selection for rapid growth was pursued. Gavora et al. (56) found one strain which was the exception to this general rule and therefore noted that it should be possible to produce rapidly growing resistant stock.

Little has been done to correlate MD resistance with physical traits, aside from blood group or lymphocyte antigens. Somes and Jakowski (58) examined morphological traits in 11 phenotypic pair comparisons and found only one (duplex versus nonduplex combs) to be significant. Miller et al. (59) reported higher total or soluble thiol in livers of resistant compared to susceptible chicken strains, but this association was not clear cut and the differences were marginal at best.

2.1.6. Practical aspects of selective breeding. This section will serve to summarize some of the general considerations which apply to practical breeding programmes based on resistance to virus exposure. The alternate approach of selecting birds possessing certain cell markers associated with the MHC or other loci, which may confer resistance to MD, is essentially straight-forward and will be discussed in Section 3.4.

Until the recent discovery of MHC-linked resistance, response-based selection was the only approach available. Advantages to this method include: 1) it can be applied to any genetic stock regardless of B-alleles present, and therefore it is effective against both MHC- and

non-MHC-linked genetic factors, 2) it very accurately predicts the relative performance (i.e. MD resistance) of strains to field exposure, 3) selection for MD resistance can be carried out in conjunction with selection for other important traits with whatever degree of emphasis is desired, and 4) it is relatively easy to perform. Disadvantages to be considered are: 1) the use of infectious virulent MDV could pose a hazard to other poultry stocks unless isolation is good and, therefore, special facilities may be required, 2) progress may be slow due to the need to preserve other economic traits, 3) the resistance potential of a given genetic stock may be limited, and 4) chickens used for challenge might otherwise have been used in the breeding programme. Experience has taught that none of these disadvantages is great enough to preclude selection for MD resistance by commercial poultry breeders, but some are significant enough to dampen enthusiasm for the programme; in fact, most breeders stopped such selection as soon as MD vaccines were available.

Considerations of importance are listed in Table 1. Whether the approach suggested by Maas et al. (27) is attempted, in which all breeding stock is exposed to provide a natural selection for resistance, or whether the more usual sib or progeny testing is carried out, depends in part on facilities and the degree of effort which can be directed to the project, and in part on the general level of resistance already present in the stock. Challenge of pedigreed chicks should be expected to yield faster results since the best parental lines can be quickly established, but the simplicity of natural selection could be attractive in many circumstances. If a high degree of susceptibility exists, there is the danger of losing a very high percentage of available breeding stock by the Maas method.

Although maternal antibodies have not appeared to interfere greatly with selection, there are two instances in which caution is urged. One would be in comparisons between groups which could be expected to have markedly different antibody levels because of differences in exposure or other conditions in the dams. This could result in susceptibility differences unrelated to genotype. The other potential problem would occur if dams were MDV-free (as in specific-pathogen-free flocks), because genetic resistance may be poorly expressed at hatching in antibody-free chicks. Under either of these circumstances, the problems

are obviated by delaying challenge until the chicks are 2- to 3-weeks-old.

TABLE 1. Points to consider in selection for MD resistance based on response to virus exposure.

A. Groups to be tested
   1. Entire population - selection from survivors
   2. Sibs - selection concurrent with production of breeding stock
   3. Progeny - for selection of dams or sires
B. Age and maternal antibody status when exposed
C. Method of exposure
   1. Natural exposure
   2. Controlled contact exposure
   3. Virus inoculation
      a. freshly prepared blood or tumour cells
      b. frozen stocks of virus
D. Virus strain
E. Time and method of examination

For reasons already noted, the choice of natural _versus_ artificial exposure is probably not critical ultimately, especially if the degree of virus exposure can be controlled by using "seeder" chicks previously infected and shedding virus when placed with groups to be challenged. Inoculation procedures have the distinct advantage of providing a standard challenge which is highly reproducible, especially if pretested batches of virus (stored in liquid nitrogen) are used.

The selection of virus strain is critical. Low virulence strains do not induce a sufficiently high incidence of MD to provide a basis for selection, whereas strains of too high virulence can overwhelm even strong levels of genetic resistance.

The criterion for resistance (mortality, lesion incidence, etc.) does not seem too important providing consistency is exercised. Nor does the length of the observation period appear critical. Mostly, it is important to select conditions relative to virus strain, exposure method, examination criteria, etc., which allow a broad response range to distinguish resistant and susceptible families.

2.2. Factors controlled by the host genotype

2.2.1. Pathogenesis. The most obvious of the effects of host genotype on MD have to do with the final stages in the sequence of events collectively defined as pathogenesis. "Resistant" chickens are, by generally accepted definition, those which fail to develop the characteristic signs and lesions which are described for the disease, and which occur under similar conditions of exposure and rearing in "susceptible" chickens. However, there are some subtle differences in tumour development among those chickens which do ultimately succumb suggesting that resistance is not an all-or-none phenomenon. In addition to the lower overall incidence of neoplasms, MD in resistant strains may be characterized by a longer latent period (46), and a less widespread distribution of lesions (24) than seen in susceptible strains.

Genetic effects are not obvious during the initial phase of infection. Early cytolytic lesions which occur during the first week post-infection (see Chapter 3) were equivalent in susceptible and resistant strains (60,61). A possible exception was reported by Lee *et al*. (62) who found infection levels in the spleen to be lower for resistant line 6 than susceptible line 7 at 3 but not 5 days post-infection. The significance of this is unclear.

The time of appearance and number of lymphocytes which bear MATSA, the putative tumour-associated surface antigen (63,64), were also much the same during the early period when comparisons were made between N- and P-line (65) or between lines 6 and 7 (62). Furthermore, Shek *et al*. (66) and Calnek *et al*. (67) showed that primarily B-cells are involved during the early cytolytic infection in N- and P- lines. A small proportion of T-cells also is cytolytically infected, but relative numbers for resistant *versus* susceptible chickens have not been determined.

After the first week, the patterns in resistant and susceptible chickens differ considerably (13,62,65,66,68-71). Infection levels in peripheral blood lymphocytes and spleen drop rapidly and markedly in resistant chickens by 8 to 10 days post-infection and remain at low levels although infection persists for the life of the host. Likewise, there are few if any cells with viral antigen in lymphoid organs or epithelial tissues after 6 to 7 days post-infection in resistant

chickens, and the number of MATSA-bearing cells drops to low levels. In chickens destined to succumb, all of these aspects of infection either remain at high levels or, after a brief drop, rise to constitute the second part of a biphasic infection which then continues at a high level until the death of the bird.

It might be argued that the presence of MATSA-bearing cells in both resistant and susceptible chickens (65,67), and the finding of transient lymphoproliferative lesions in resistant strains (72), suggest that the pathogenetic events proceed at least to the point of transformation in resistant as well as susceptible genotypes. In fact, Sharma et al. (72) considered lesion regression to be the basis of "age resistance", which is probably genetic resistance expressed only after immunological maturity (60). However, the exact nature of the lymphoproliferative response is unclear and MATSA is not proven to be a marker for transformation; thus, interpretation of any genetic influence or lack thereof on transformation should be guarded.

2.2.2. Immune response, immune competence and immunosuppression. The protective nature of maternally-derived antibodies (73) led Calnek (74) to examine antibody responses in genetically different strains of chickens. The finding of a strong correlation between resistance and the development of virus neutralizing (VN) antibodies provided the first real support of the contention that genetic resistance had an immunological basis. A similar correlation was found in many paired comparisons of resistant and susceptible strains (see 13,68). As it turned out, however, the actual situation is far more complex than was initially envisioned.

First, it was learned that the lack of VN antibodies in susceptible strains was probably due to immunosuppressive effects of MD rather than an inherent deficiency in humoral immunity. VN antibody response in susceptible strains was unimpaired when low virulence strains of MDV were used (75). Furthermore, with high virulence viruses, an early (6 to 12 days post-infection) IgM response with VN activity was the same in susceptible and resistant strains, but thereafter only resistant chickens had persistent IgG class VN antibodies (76,77). In the absence of MDV exposure, susceptible strains had levels of serum immunoglobulins similar (78) or only slightly lower (77,79) than those of resistant

strains. Therefore, there was no primary deficiency of immunoglobulins or inability to respond to MDV antigens to account for susceptibility. In fact, susceptible strains sometimes developed higher levels of precipitating, agglutinating or fluorescing antibodies against MDV than did resistant strains (78,80).

Responses to other antigens or infectious agents have been studied to a limited degree. Carte et al. (81) found a correlation between ability to regress Rous sarcomas and genetic resistance to MD, implying that there might be broad genetic control against tumours, such as one controlled by Ir genes. This also appeared to be the case in a study of B-haplotype-controlled resistance to MD, Rous sarcoma, and lymphoid leukosis tumours reported by Bacon et al. (82). However, MD and RSV resistance are not always correlated (83), and Payne (13) speculated that some strains may lack genes important at the general level of immune responsiveness whereas others lack genes important at the specific level, e.g. to MD or Rous sarcoma. Also of interest in this context is the correlation between response to GAT and MD resistance (84).

Immune responses to HVT and attenuated MDV vaccine strains appear to be under the same genetic control as resistance to MD; i.e., strains genetically resistant to MD are better immunized by the vaccines than are susceptible strains (19,20). Cho (40) found that HVT viraemias were higher and more persistent in susceptible than resistant chickens and speculated that a superior immune response in the latter lowered the infection level.

Innate cell-mediated immunity (CMI) responses have been tested using mitogen stimulation of lymphocytes in vitro. Schat et al. (85) noted an unexplained but significantly higher blastogenic response to concanavalin-A (Con-A) of peripheral blood lymphocytes from susceptible P-line birds compared to resistant N-line birds. Fredericksen and Gilmour (86) found similar differences between susceptible line 7 and resistant line 6 lymphocytes with both Con-A and phytohaemagglutinin (PHA). Lee and Bacon (87) confirmed both studies by comparing all four strains using PHA to induce blastogenesis. A possible correlation between CMI responsiveness and susceptibility to MD became even more apparent when Carpenter and Sevoian (88) reported that T-cell

activation, delayed type hypersensitivity and macrophage activation against Listeria monocytogenes were greatly superior in susceptible S-strains when compared to resistant K-strain chickens. Thompson et al. (89) offered the only contrary evidence. Chickens selected for high plasma corticosteroids had a higher incidence of MD but a lower blastogenic response to PHA or DNP-BSA than those selected for low plasma corticosteroids.

As with humoral immunity, CMI responses also are affected by the immunosuppressive effects of MDV infection. Depressed mitogenic responses (Con-A, PHA) have been observed at 7 days following infection with oncogenic or nononcogenic MDV or HVT regardless of the genetic susceptibility or resistance of the chicken strain (85,90,91). This depression was transient except in genetically susceptible chickens given oncogenic MDV strains; thus the induced immunosuppression pattern seen with VN antibodies included CMI as well. This immunosuppression also affected cytotoxicity tests for antitumour and antiviral immunity. Confer and Adldinger (92) reacted spleen cells with a target of MSB-1 MD tumour cells and found poor levels of cytotoxicity with MDV-infected line 7 donors, but good responses with line 6 donors. Line 7 responsiveness was retained in HVT-vaccinated birds showing that there were no innate deficiencies in that strain. Lee et al. (62) found essentially the same difference between lines 6 and 7 using the MDCC-HP2 tumour cell line as a target. They also observed poor CMI with line 7 but not line 6 spleen cells against MDV-infected chicken kidney cells in a plaque reduction test (antiviral CMI), but in this case the poor response followed an initially good response at 7 days post-infection.

Carpenter and Sevoian (88) also favoured immunosuppression as the explanation for poor anti-MD CMI responses, but considered the mechanism to be other than secondary to virus-induced damage. They tested CMI competence against Listeria monocytogenes in S- and K-strain chickens and concluded that a suppression of the effector stage of responsiveness in the susceptible S-strain occurred immediately following a phase of lymphoproliferation at 3 days post-infection (this was absent in K-strain). Therefore they believed that a population of active suppressor cells is linked with MD susceptibility.

2.3. Factors which influence the expression of genetic resistance.

2.3.1. Viral type. Because MD isolates differ greatly in virulence, and because genetic resistance does not seem to be an all-or-none phenomenon, it is reasonable to expect resistance levels to vary with the challenge virus. This was shown to be the case by Fabricant et al. (61) and Schat et al. (85). High virulence viruses caused higher viraemia levels than were induced by low virulence viruses. This was particularly evident with groups of N-line chickens infected in parallel with moderate (JM-10 isolate), high (GA-5 isolate) or very high (RB-1B isolate) virulence MDV, when total incidences of MD after 70 days were 0, 19 and 75%, respectively (34). The same viruses given to the highly susceptible S-strain caused 100% MD in all three cases, whereas intermediate levels were observed with other genetic strains. It was not determined if the ability of certain viruses like RB-1B to overwhelm genetic resistance is because of greater transforming potential, superior ability to infect key target cells, greater immunosuppressive ability, a combination of two or all of these, or other undetermined reasons. However, highly virulent viruses like RB-1B, Md 5 or Md 11 do cause greater immunosuppression (21,91), than low virulence viruses like CU-2 (75).

2.3.2. Age at exposure. Calnek (60) reported that genetic resistance in N-line and PDRC strain chickens was poorly expressed at hatching but was acquired gradually in parallel with the acquisition of immune competence. Sharma (93), on the other hand, found lines 6 and N to already display considerable resistance at hatching. The discrepancy in results with N-line could have been due to the use of different clones of MDV, or the use of different sublines of N-line chickens. Nevertheless, age resistance is manifested to a marked extent only in genetically resistant strains, and because age resistance is CMI-dependent (94), Witter (95) remarked that genetic resistance may simply be an expression of cellular immune competence, the rate of acquisition of which is under genetic control.

2.3.3. Maternal antibodies. Passively acquired antibodies reduce the severity of the early cytolytic infection, apparently by limiting virus spread (70,96) and consequently probably reduce immunosuppression. They could therefore be expected to enhance genetic resistance and

reduce genetic susceptibility. However, it should be remembered that virtually all of the genetic selection based on sib or progeny testing has been carried out in the presence of maternal antibodies without any apparent interference in the rankings on which selection was based.

2.3.4. Sex. Whereas sex may be influential in determining the incidence of MD tumours (see 13), genetic resistance does not seem to be sex-linked (49).

2.3.5. Immunological impairment. Depletion of T-cells but not B-cells in genetically resistant strains increases their susceptibility to MD (13,94,97-99). However it is necesary to distinguish between a decrease in T-cells causing immunological impairment and the near total elimination of T-cells, since the loss of transformable target cells could decrease the incidence of lymphomas (13,98).

2.3.6. Vaccination. Because vaccination can modify both pathogenesis and tumour incidence in MDV-exposed chickens, it might be expected to affect the expression of genetic resistance or susceptibility. It does, but on a proportional basis which preserves the rank differences when genetic strains, sire families, etc., are compared (19,20). Virus virulence and host genotype can interact to make the vaccinal response differences among strains even more pronounced (21).

3. MECHANISM OF GENETIC RESISTANCE

3.1. Potentially important factors.

Genetic resistance could reside at any one or more of several levels. First, there could be differences between genotypes in the number of specific target cells which are required at any of several stages of the sequential phases of pathogenesis. Second, of those target cells present, there might be genotypic differences in susceptibility to infection (e.g. virus receptors) or ability to replicate the virus (intrinsic factors). Susceptibility to infection could involve events like activation (as occurs with stimulated T- or B-cells), which in themselves are genetically controlled. Third, cells may differ in their response to MDV infection, i.e., some may more frequently enter cytolytic infection with immunosuppression the consequence, whereas others may be more prone to enter latent or transforming infections which would enhance tumour development. Fourth, if transformation

requires integration of viral DNA into the host cell DNA and if the proliferative activity of lymphocytes involved is under genetic control then genotype would be important. Finally, there could be genetically controlled differences in the immune response against infected or transformed cells. Important responses could include such nonspecific functions as interferon induction, NK cell activity, general immune responsiveness, or suppressor cell activity, as well as specific humoral or cell-mediated immunity. There are data pertinent to nearly all of these phenomena which derive from studies on genetic resistance in many laboratories. Indeed, many can be shown to differ when various resistant and susceptible strains are compared (see reviews 13-16, 68).

As already pointed out, there was speculation that multiple genes are involved in the genetic control of resistance to MD (41,49). At least three loci have been identified as being important. These are the MHC or B-complex, the Ly-4 locus, and the Th-1 locus.

3.2. Characteristics and mechanisms of MHC-controlled genetic resistance.

The MHC in the chicken is a group of genes whose function and structure are rapidly being characterized, primarily because of their significance in immunogenetics (100-102) (see Table 2). Several reviews have been published (45,102-106) which illustrate the relationship between the MHC and disease resistance in general or MD resistance in particular. The MHC is associated with a microchromosome (108) and contains three closely linked genetic regions identified as B-F, B-G and B-L, which control cell surface antigens (103). Antigens specified by the B-L and B-F regions are present on lymphocytes and appear to be important in various lymphocyte functions. The immune response (Ir) region of the MHC could be specially important since it contains genes involved in such activities as B- and T-cell cooperation, immunoglobulin production, T-cell helper function, and macrophage cooperation versus pathogens. Pevzner et al. (84) found linkage between genes coding for the immune response to a synthetic amino acid polymer (GAT) and Ea-B genes, thus placing the Ir region within the MHC. Subsequently, Ia-like antigens, which are the Ir gene products important to several of the immunological activities of lymphocytes and macrophages, were associated with the B-L region (101). Ia antigen can be found on B-cells, macro-

phages and activated T-cells (see 102).

According to Briles *et al*. (108), a relationship between the *Ea-B* locus and mortality from "leukosis" was suspected in the late 1950's. Nevertheless, it was not until 1967 that Hansen *et al*. (17) offered the first experimental data to prove the point. Chickens with the B^{21} blood group allele were found to be more resistant to MD than were those with the B^{19} allele. The influence of certain *B* genotypes on MD resistance was confirmed by other workers (see 16), with the most definitive data coming from Briles *et al*. (109) and Longenecker *et al*. (110). Longenecker's data was from experimental crosses between chickens homologous for B^{21}, B^2, B^{14} or B^x. The B^{21} homologus chickens came from two populations, one of which was Cole's N-line (12). Regardless of the source, the inheritance of the B^{21} allele was highly protective against MD. In the Briles *et al*. study (109), N-line (100% B^{21}) and P-line (97% 3% B^{13}) chickens were crossed and their progeny backcrossed with B^{19}, males or females. The backcross progeny homozygous for B^{19} had MD P-line incidences of 58.3 to 80.0% (mean 69.7%) compared to 16.7% (mean 8.6%) for $B^{19}B^{21}$ progeny. Taken altogether, it was amply apparent that there were differences in susceptibility which were associated with different *B* alleles and that B^{21}-linked resistance was inherited as a dominant trait. It is interesting that Hansen *et al*. (17) studied chickens homozygous for B^{21} which had been specifically selected for that allele whereas the N-line developed by Cole (12) was found to be homozygous for B^{21} subsequent to selection for resistance to MDV challenge (45,109). Yet, the association was the same in both cases.

B alleles other than B^{21} have been evaluated (34,82,108,111,112), and certain of them, e.g. B^2 and B^6 (64) may confer an intermediate level of resistance compared to B^{21} and other alleles. Longenecker and Mosmann (113) reviewed various reports and categorized *B*-allele-associated resistance as follows: high resistance, B^{21}; moderate resistance, B^2, B^6, B^{14}; susceptibility, B^1, B^3, B^5, B^{13}, B^{15}, B^{19}, B^{27}. Schat *et al*. (34) have subsequently reported a high level of resistance associated with a new allele, B^Q, and categorized the B^{17} allele as one conferring a moderate degree of resistance. The occurrence of both B^Q (62), and B^{21} (114) in Red Jungle Fowl, the progenitor of the species, did not escape the attention of either research group as a suggestion of

the survival value of these alleles during evolutionary development. The B^{21} has been found in a variety of leghorns, heavy breeds and other breeds (115; see also 16) and thus is of interest to breeders of commercial poultry. B-locus alleles also appear to regulate susceptibility or resistance to transient paralysis, a nontumourous condition sometimes associated with MDV infection (115).

TABLE 2. Subloci of the chicken MHC[a]

Class gene products	Locus	Occurrence on cells	Functions
I	B-F	All nucleated cells	Targets of cell cytotoxicity (graft-versus-host reaction, allograft rejection)
II	B-L	All Ig-positive cells, monocytes, macrophages, some stimulated T-cells	Immune responses (graft-versus-host reaction, allograft rejection, mixed lymphocyte reactions, Ig response, regulation of B-cell/T-cell cooperation, T-cell helper function, macrophage cooperation)
IV	B-G	Erythrocytes	Blood group determination and erythropoiesis

[a]Compiled from Hála et al. (103), Pink et al. (101), and Toivanen and Toivanen (102).

MHC-controlled resistance does not seem to involve deficiencies in the number or susceptibility of targets important in the early phases of infection. Splenic infection of chicken embryos (62,116,117) and *in vitro* infection of spleen lymphocytes (118,119) were both independent of genotype when N- and P-lines were compared. These findings were con-

sistent with in vivo studies in which infections in the lymphoid organs during the first week post-infection were essentially indistinguishable between the two strains (60,61,65,69). An exception to this general rule was reported by Longenecker et al. (117) who found the number of pocks on the chorioallantoic membrane following intravenous inoculation of chicken embryos to be lower with resistant than with susceptible genotypes.

It is the very markedly reduced infection level during the second phase of infection which characterizes the MHC-type resistance. Latent infection of lymphocytes (mostly T-cells) occurs, but at very low levels in N-lines compared to that in P-lines (61,65,66). The same is true of MATSA-bearing cells (65). Also, there is an absence of the second wave of cytolytic infection which occurs in susceptible strain chickens after the second week post-exposure (69,70). The significant question which arises is: why does the infection level drop so profoundly in N-lines but not P-lines? The possibility that N-line birds differ from P-line birds through better immunological responsiveness is attractive (see 13,14,16, 68), and because Ir genes appear to map within the MHC (see 22), this is plausible. Longenecker and his colleagues offered evidence that B^{21}- linked resistance might be mediated through an Ea-B-linked, Ir-gene- controlled immunological mechanism since B^{21} chickens also could reject transplantable lymphomas (114,120). Because GAT response is supposed to be under the control of Ir genes, the higher GAT-response of resistant chickens (101) could be taken as further evidence favouring an immunological basis for MHC-controlled resistance. However, both GAT-high and GAT-low responders were found within each of two populations respectively homozygous for B^{1} and B^{19}; this shows that favourable Ir genes are not necessarily linked only to certain B alleles. Also, there is the very significant recent finding of Briles et al. (121) that MHC-associated resistance to MD is mapped within the B-F region rather than the B-G region where Ir genes are found. This was based on observation with a genetic recombinant with $B\text{-}F^{21}/B\text{-}G^{19}$.

Unfortunately, there are few other data from N- and P-line birds on which to draw; information which is available generally fails to support the contention that N-line birds are innately more responsive immunologically than are P-line birds. Immunoglobulin levels were similar (77),

RSV regression rates were the same (83), and early immune responses against MDV were similar (76) when the two lines were compared. Moreover, when responses of lymphocytes to mitogens, or graft-*versus*-host reactions (GVHR), were tested as criteria of CMI responsiveness, N-line birds or other strains with B^{21} alleles have consistently been less active than P-line birds or other susceptible strains (85-87,122; see also Section 2.2.2). This further suggests that resistance to B^{21} is not the direct result of a superior immune response *per se*.

However, that is not to say that the difference in CMI responsiveness as a genetically controlled trait is not important. On the contrary, there are possible consequences which could make this the most significant point of difference between N- and P-line chickens. Activation of human T-lymphocytes is a requirement for their infection by herpes simplex virus (123). A similar requirement may exist for infection of chicken T-cells by MDV. Schat *et al*. (124) found MD cell tumour lines to all be Ia-bearing T-cells; Ia-antigen on T-cells is characteristic of activation. Similarly, latently infected splenic T-cells were shown to be Ia-bearing (67) as were spleen-derived T-cells infected *in vitro* (119). Calnek *et al*. (119) observed that the number of infected T-cells became significantly greater in terms of actual and proportional numbers in P-line than in N-line spleen cells by 4 to 5 days after *in vitro* infection. This could reflect different rates of activation *in vitro* or different numbers of activated T-cells in the spleens of donor N- and P-line chickens.

At least two potential consequences of enhanced T-cell infection rate in P-line birds over N-line birds would be consistent with an ultimate effect of lower infection level and fewer tumours in resistant N-line stock: 1) Regardless of the intrinsic or extrinsic factors which govern the establishment of latent or transforming infections, a lower number of susceptible T-cells (through less efficient activation) would reduce the level of latent infection in peripheral blood lymphocytes or spleen lymphocytes and also the number of T-cells which could potentially transform and initiate lymphomas. 2) T-cells destined to be immune effector cells would be spared if they did not become infected and undergo cytolytic infection during the early phase. This would preserve immunocompetence leaving the host better able to react against

those tumour cells which do arise.

The possibility that enhanced MDV-induced proliferative responses in susceptible strains such as P-line might result in an overabundance of suppressor T-cells to help account for the immunosuppression should not be overlooked. Carpenter and Sevoian (88) considered that this might account for the poor CMI responses of susceptible S-strain chickens against Listeria monocytogenes, when compared to those of the resistant K- strain. Although the resistance of K-strain is not known to be MHC-related, a search for similar effects in N-line versus P-line could be rewarding. On the other hand, Rouse and Warner (125) hypothesized that resistance might be mediated through the ability to express a strong suppressor regulation of T-cell proliferation in response to viral antigen.

Other differences have been observed between N- and P-line chickens which may be significant. Macrophages in peripheral blood were more abundant in N-line than in P-line chickens (126, B.W. Calnek, unpublished data) and there is evidence that these cells play a role in resistance by one or more of several possible mechanisms, including both antiviral and antitumour activities (127-132). Especially interesting, in view of the above hypothesis regarding activated T-cells, is the fact that macrophages may be very important in suppressing T-cell mitogenic responses (131,132). Also pertinent is the finding that removal of macrophages from N-line lymphocyte preparations restored their mitogen responsiveness to levels comparable to those seen with P-line lymphocytes (K.A. Schat, personal communication). This aspect should receive more attention.

Interferon levels were higher in resistant K-strain than in susceptible S-strain chickens (133), but this aspect of N- and P-line chickens has not been reported. Payne (14) concluded that interferon is probably of only minor significance in any case.

NK cell activity also has differed between N- and P-line chickens suggesting a possible role for these cells in MHC-linked resistance. Sharma (134) reported that after MDV infection, NK cell levels were depressed in susceptible chickens but elevated in resistant chickens.

Thus, MHC-controlled resistance is expressed primarily after the initial cytolytic infection of lymphoid organs, probably with an immuno-

logical basis, but also perhaps being related to a relatively low rate of T-cell infection. The latter two features could be closely associated.

3.3. Characteristics and mechanisms of non-MHC-controlled genetic resistance.

Genes other than those of the MHC must also be involved in resistance or susceptibility to MD. This is exemplified by the profound differences between lines 6 and 7 which are respectively resistant and susceptible to MD (41) and yet are both homozygous for the B^2 allele (45). Three lymphocyte antigens were discovered which differed between these highly inbred lines. Gilmour et al. (135) described Bu-1 and Th-1 loci which respectively determined surface antigen on B and T-cells. Fredericksen et al. (18) discovered another T-cell antigen, coded for by the Ly-4 locus. These three loci are not linked to the MHC, but Th-1 and Ly-4 are thought to be loosely linked, perhaps on a large chromosome (18). Both of the T-cell antigen loci may be involved in resistance in line 6. Lines 6 and 7 respectively carry $Ly\text{-}4^a$, $Th\text{-}1^a$ and $Ly\text{-}4^b$, $Th\text{-}1^b$. The influence of the Ly-4 allele was apparent when comparisons of F_2 and F_3 progeny showed that homozygosity for either allele correlated with resistance or susceptibility as seen in the parental lines (18). However, because the differences between the two allotypes were considerably smaller than those between the parental lines, the investigators concluded that other loci might be involved. Subsequently, Fredericksen et al. (136) showed the Ly-4 and Th-1 loci interact in conferring MD resistance since of the four possible double homozygous Ly-4/Th-1 classes, only one (bb/aa) effected a significant resistance to MD.

In spite of the fact that resistance associated with line 6 versus line 7 must be considered as non-MHC-controlled, there is much in common between features of resistance in line 6 and N-line. Also, there are some significant differences. The similarities relate to immunological responses to infection, whereas the differences have been considered to reside at the level of target cell numbers and susceptibility. The differences may be more quantitative than qualitative.

The significance of immune mechanisms in line 6 resistance was shown when Sharma et al. (94) and Powell et al. (98) were able to reduce resistance by thymectomy and irradiation, and when lesion regression was

determined to characterize line 6 as well as other resistant strains (93). Lee et al. (62) considered cellular antiviral and antitumour responses both to constitute part of the resistance mechanism in line 6. Plaque reduction and cell-mediated cytotoxicity against MD tumour cell lines were strong in those chickens but were absent in susceptible line 7 chickens after MDV exposure. As reported for N- and P-line chickens (65), MATSA-bearing cells could be found at equal levels in both line 6 and 7 chickens during the early phase of infection, but the level subsequently decreased markedly only in the resistant line 6 chickens. The question of whether the differential response in VN antibodies (high in line 6, absent in line 7) (137) represents innate or induced incompetence has not been addressed with these strains, but the latter seems probable since the CMI response measured by mitogen stimulation is high in line 7 chickens before but not after MDV infection and it never recovers after the initial 7 days post-infection depression as it does with line 6 (69).

Target cell differences appear to be of considerable significance with lines 6 and 7. As with other genetic strains (138), resistance at the cellular level was not observed with monolayer cultures of chicken kidney or fibroblasts (139,140). However, there are several lines of evidence to suggest that lymphocytes from resistant line 6 birds are either fewer in number, or less susceptible to MDV, or both, in comparison to those in line 7 chickens. Line 6 spleen and thymus cells adsorb less HVT or MDV than do line 7 cells in vitro (98,140,141). This suggested to Gallatin and Longenecker (140) that in line 6 there were fewer target cells with virus receptors, or the receptors present were of lower affinity, or that there were fewer virus receptors on each of the target cells. The possibility of fewer target cells was supported by Lee et al. (62), who found line 6 to have lighter weight lymphoid organs and fewer splenic or peripheral blood lymphocytes than line 7. The target involved in adsorption, and subsequently in infectious centre assays for HVT, was believed to be a mature T-cell since the difference between the two lines was more pronounced with spleen than with thymus lymphocytes (140). However, none of the studies reported have offered any evidence that virus adsorption involves T-cells alone, or even at all for that matter. B-cells are much more frequently involved in the

early phases of infection with MDV, even in the thymus (66), and the virus adsorption studies may reflect differences in B-cells rather than T-cells.

It would be important to learn if differences between lines 6 and 7 exist *in vivo* during the first week post-infection when B-cells are the predominant target (66,67), and especially if *in vitro* differences in susceptibility of B-cells can be shown following established methods (118,119). Only limited data are available. Lee *et al*. (62) reported virus infectivity to be higher in line 7 than line 6 at 3 days but not at 5 days after infection, and Calnek *et al*. (67), in a single trial, found B-cell infection to be remarkably lower in line 6 than in line 7, N-line or P-line spleen cells exposed to MDV *in vitro*.

Data from experiments involving reciprocal transplantation of thymic or splenic lymphocytes between thymectomized line 6 and 7 chickens do strongly argue that the former have a deficiency of target T-cells for infection and transformation. Gallatin and Longenecker (140,142) and Powell *et al*. (98) found that line 7 spleen cells or thymic lobes conferred susceptibility when given to line 6 chickens, but that the converse was not true; i.e. line 6 cells did not make line 7 chickens resistant. Transplanted embryonic cells were without effect, again suggesting a mature T-cell as the target (140); spleen cells from 1-day-old donors were intermediate in effect. The thymic stroma was thought by Gallatin and Longenecker (142) to provide the conditioning microenvironment to make T-cells ultimately susceptible. Line 6 T-cells would therefore become susceptible by passage through line 7 thymic stroma from the transplanted pieces. However, Powell *et al*. (98) found that even though transplanted line 7 thymic lobes were repopulated by recipient line 6 T-cells, subsequent tumours were often of donor origin.

Gallatin and Longenecker (140) pointed out that other differences between lines 6 and 7 exist as well. For instance, there are more alloreactive cells in line 7 (122), and the cells from susceptible birds may proliferate better than those from resistant birds (117). This may be very important because it provides another feature in common with MHC-linked resistance. Precisely as with N- and P-lines, it has been shown that susceptibility with line 7 correlates with a markedly superior CMI

responsiveness as measured by mitogen stimulation in vitro (86,87). As noted previously, activation of T-cells may enhance their susceptibility to MDV.

The mechanism(s) underlying non-MHC-linked resistance thus appear to be complex and incompletely understood. More than one factor may determine the number of susceptible target cells (total number available and state of susceptibility) and it could be there are deficiencies with both B and T-cells. This contrasts with MHC-linked resistance in which there appears to be no deficiency of any kind with B-cells, and T-cell deficiencies, if they exist in vivo, probably are related only to state of susceptibility rather than available number. These points, however, must be considered only speculative until more information is available. On the other hand, it does appear that immunological responses, probably through antitumour CMI, are involved in all types of genetic resistance. How any of these features of non-MHC-linked resistance relates to the Ly-4 or Th-1 loci is unknown.

3.4. Selection for B-locus or other alleles associated with resistance.

This procedure is obviously of considerable use when a specific allele has been proven to be uniformly associated with resistance. The B^{21} allele coding for blood group antigen has fulfilled this requirement. Providing the genetic stock under selection contains a sufficiently high proportion of chickens with this allele, it should be relatively easy to develop resistant strains without disadvantaging other selection criteria. However, if the allele must be introduced, or if the natural incidence is low, then the selection process is severely hampered by the need to consider other traits in commercial breeding operations. Where B^{21} is involved, selection so that one of the commercial parents (male or female) has a high frequency will result in progeny which are at least heterozygous for that allele.

At present, the only loci, other than B, which appear to be involved in resistance are Ly-4 and Th-1. To date, there is no published information on the alleles associated with these loci in commercial chickens. Virtually all reported work has been done with the inbred lines 6 and 7. Future studies may show these, or other yet undiscovered loci, to be suitable for selection procedures.

ACKNOWLEDGEMENTS

The author appreciates the critical review and the helpful suggestions of Drs. R.K. Cole, K.A. Schat and J. Fabricant during the preparation of this chapter.

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12. SPREAD OF MAREK'S DISEASE

P.M. BIGGS

1. INTRODUCTION

Under natural conditions infection with Marek's disease virus (MDV) occurs primarily in members of the genus Gallus (1), although it has been described rarely in other galliformes such as the turkey and quail (2,3 and see Chapter 2). Infection occurs in jungle fowl, both feral and those in zoological collections (1,4), but it is most important in domestic chickens where it is ubiquitous in poultry populations throughout the world. There is no evidence to suggest that the jungle fowl is a reservoir of infection of importance to commercial poultry. The source of infection for the domestic chicken is other infected domestic chickens. In the absence of vaccination Marek's disease (MD) occurs in commercial poultry populations throughout the world (5) and mortality may reach 80% of a flock. However, it is a characteristic of the disease that its incidence varies greatly in infected flocks, and between houses and pens within a house on a single site (6). These variations can be greater than 10-fold.

2. SPREAD OF INFECTION

2.1. Infection.

Infection can be at any age but usually occurs early in life within the first few weeks after hatching (7,8), although on occasions it may be delayed. Infection spreads rapidly through a flock and persists in some chickens in flocks throughout their normal commercial life (8,9).

MDV spreads readily by direct and indirect contact (10), but the precise route of infection is uncertain. However, a number of observations suggest that infection occurs via the respiratory tract. Infection is readily spread by the airborne route (11,12) and experimentally has

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been shown to occur more effectively via the respiratory tract than via other natural routes (13). In particular the administration of chopped dry feathers or dust by inhalation has been a successful means of transmitting MD (14). In addition material containing virus-specific immunofluorescent antigen was found located adjacent to the pulmonary epithelium in chickens exposed to infection by contact with infected chickens (15) and the lungs appear to be the organ where infection first occurs (16).

Because serotype 1 viruses of varying pathogenicity and serotype 2 apathogenic viruses are ubiquitous in poultry populations and their environments (8,17), it is probable that individual chickens are at times exposed to more than one strain of virus. It is also probable that they can be infected by more than one strain because chickens vaccinated with an attenuated MDV can be superinfected with field virus under natural conditions (18) and chickens have been infected with both acute and mild strains of MDV by exposure to experimentally contaminated litter (19).

2.2. Shedding of infection.

Vertical transmission of MDV through the egg has been claimed (20), but all subsequent studies have not supported this view. Attempts to isolate virus from embryos and chicks from infected flocks have not been successful and progeny of infected flocks reared in isolation have remained free of infection (21,22). It is interesting to note that MD occurred in chickens kept in isolation facilities which were hatched from eggs which had not been disinfected but were incubated and hatched on infected premises (23). It is not clear from this experiment whether infection was transmitted on the surface of the egg or occurred by the introduction of infection into the hatcher from the surroundings.

Horizontal infection between hatched chickens is clearly the method of spread of MDV. Infectivity is present in oral and nasal washings, dander and cloacal swabs, but not faeces, from infected chickens (24-27). The most likely major source of infection is dander which contains desquamated epithelial cells and moulted feathers (27), because the feather follicle epithelium is the only site where enveloped and cell-free infectious virus is formed (14). Litter and droppings have been found to be infective (28) but this is likely to have arisen from

contamination with dander.

Shedding of infectious material occurs about 2 to 4 weeks after infection, prior to the appearance of clinical disease, and can continue throughout the life of the chicken (2,9,24,27). Since infection with MDV is ubiquitous all chickens are a potential source of infection at any time and flocks of chickens at all times. A study which examined the pathogenicity of MDV isolates made throughout the life of a laying flock found that with increasing age pathogenic viruses became less frequent and apathogenic viruses more frequent (8). If this is generally true the serious consequences of exposure to infected chickens would, in general, be reduced as the infected chickens aged.

2.3. Survival.

Oral and nasal washing and dander of infected chickens contain MDV and these are likely to contaminate litter. It is therefore important to know how long, and in what conditions, litter can remain infective. However, because dander is the most important source of infection and it forms a major component of poultry dust, this information is equally important for poultry dust. Poultry dust can remain infective for over 1 year and litter for at least 16 weeks at room temeprature and low humidity (27-29). The infectivity of litter stored for longer than 16 weeks has not been tested. The survival of infectivity in feathers, poultry dust and litter is affected by temperature and humidity and decreases with increasing temperature and moisture (27,28,30). A range of commonly used disinfectants including solutions of chlorine, quaternary ammonium compounds, organic iodine, cresylic acid and synthetic phenol, have been shown to be effective for disinfecting ground-up feathers. Formaldehyde gas was less effective (30). These results suggest that a range of compounds is suitable for use as chemical disinfectants for MDV-infected premises and utensils and that formaldehyde gas could be a useful adjunct to chemical disinfection.

2.4. Mechanisms of spread.

Spread of MDV is horizontal and can be by direct or indirect contact with infected birds (10). However, it is probable that the mechanism of spread, whether between birds in direct or indirect contact with one another, is similar and this is by aerosol. However, because MDV is largely associated with desquamated epithelial cells and probably rarely

cell-free because of a shorter viability in this form, it will be associated with particles (27). Evidence for the air-borne transmission of MDV has been provided by showing that infection passes from infected chicks in one air space to uninfected chicks in another connected air space when air passes from the air space with the infected chicks to the air space containing the uninfected chicks before egress (11). Using a similar design, the efficacy of a number of filters placed in the connecting ducting has indicated that most of the virus is associated with fairly large particles, probably desquamated epithelial cells (30), and that all virus is associated with particles greater than 1 μm in diameter (31). Particles of this nature, for which infectivity can survive for long periods, can be readily passively transported on inanimate objects and by aerosol over long distances. Objects such as clothing, undisinfected eggs, utensils and vehicles should be considered.

Although the darkling beetle (Alphitobius diaperinus) was shown to passively carry MDV (32) it does not appear to be an important method of spread of infection (33). Other possible animate carriers of infection which have been examined for transmission of MDV include mosquitoes, litter mites and coccidial oocysts (28,34-36). In no case was there evidence for transmission.

3. FLOCK INFECTION

Flock infection can occur at any age but because of the ubiquitous nature of the infection and the long survival of MDV outside the body, infection is likely to occur early in life unless stringent precautions are taken. MDV was isolated (7) from a broiler flock as early as about 4½ weeks of age (7) and from a layer flock at 5 weeks of age (8) (see Figs. 1 and 2). However, using exposure to infected susceptible chicks as a method of detection it was found that a sample of chicks from a broiler flock were infected at 9 days of age (7). Only samples of chickens from flocks can be examined and it is likely that if infection is present in a house in which chicks are placed, some chicks will become infected within the first few days of life. Also because of the ubiquitous nature of the infection in most flocks all, or nearly all, 1-day-old chicks have maternally derived antibody to MDV (6,7). There

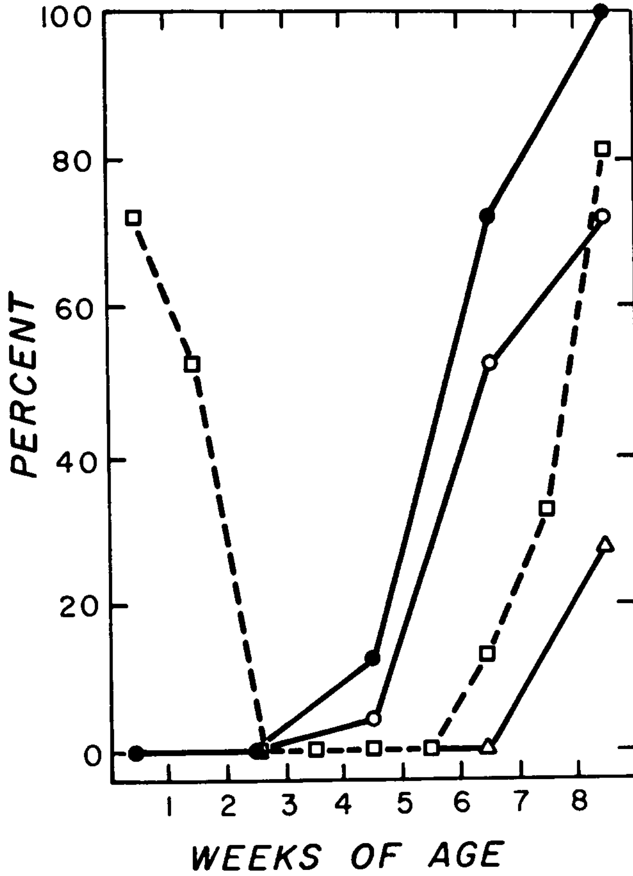

FIGURE 1. Response of a commercial broiler flock to infection with Marek's disease virus. □------------□ , precipitating antibody; ●——● , virus isolation; ○——○ , microscopic lesions; △——△ , gross lesions. (From Witter (54) by kind permission.)

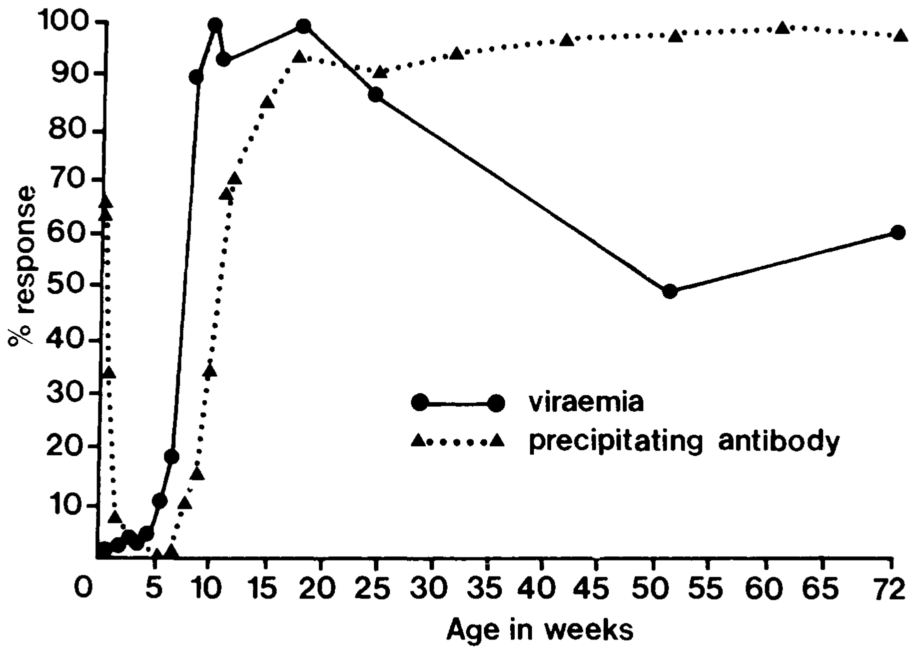

FIGURE 2. Incidence of viraemia and precipitating antibody in chickens exposed to Marek's disease virus. (From Jackson et al. (8) by kind permission.)

is a direct correlation between the presence of antibody in the hen, in her eggs and hatched chicks (37). The incidence of this antibody declines after hatching and has disappeared in most if not all chickens of a flock by 3 weeks of age (6-8). In progeny of vaccinated flocks titres of neutralizing antibody to MDV can be high with a geometric mean of up to 3.6 $\log_{10}$ and varying between individuals by as much as 200-fold (38). On exposure to MDV infection spreads rapidly and the incidence of infection rises steeply with an interval of between 4 and 5 weeks between the first isolation of MDV and the infection of all the chickens in the flock (6-8). The incidence of antibody rises at a similar rate but about 2 to 3 weeks later (6,7,8). Examples of a broiler flock are presented in Fig. 1 and a laying flock in Fig. 2. A high incidence of both viraemia and antibody can be present throughout the life of a laying flock (8, Fig. 2). The factors that affect the rate of spread of infection in a flock have not been determined but it is likely that the level of initial exposure is important. The larger number of chickens infected at initial exposure the higher the concentration of MDV shed and available to infect those which escaped infection initially. Also the higher the concentration of birds in a house or on a farm the more rapid spread of infection is likely to be. Mortality from MD can begin within 4 or 5 weeks of isolation of MDV from a sample of chickens in a flock.

4. FACTORS AFFECTING THE INCIDENCE OF MAREK'S DISEASE

Most, if not all, flocks become infected with MDV but the incidence of MD varies greatly (6). The factors involved can be considered as those concerned with the environment, with the causative virus and with the host.

4.1. The environment.

As already mentioned the concentration of virus at exposure will affect the rate of spread of infection in a flock. Since dose and age at infection influence the outcome of infection (39,40 and see Chapter 3) the rate of spread of infection in a flock will influence the resulting incidence of MD. There are factors which anecdotally have been associated with increasing the incidence of MD in an infected flock or initiating clinical disease. This is not an unreasonable concept since herpesvirus infections are commonly latent or persistent and the

exacerbation of infections by physiological and environmental factors are well documented for some herpesviruses e.g. herpes simplex, the cause of cold sores in man. Those that have been associated with exacerbating the effects of infection with MDV include stressing factors such as handling, re-housing, vaccination, debeaking and outbreaks of coccidiosis. The effects of stress have been investigated. The model used was social stress created by moving individuals in groups of chickens necessitating the establishment of new social hierarchies or "pecking orders". Chickens exposed to a high degree of social stress or selected on the basis of a high concentration of plasma corticosterone had an increased incidence of MD whereas chickens exposed to a low degree of social stress or selected on the basis of a low concentration of plasma corticosterone had a lower incidence of MD (41). The observations that coccidiosis was frequently associated with MD were correct but the suggestion that coccidiosis predisposed affected flocks to MD was not supported by experimentation. These studies provided no evidence for the transmission of MDV by coccidial oocysts or for coccidiosis increasing the incidence of MD in infected chickens (35,36,42). However, there is experimental evidence for infection with MDV resulting in increased incidence of coccidiosis (43) probably as a result of the immunosuppressive effect of infections with MDV.

4.2. Host

The genetic constitution of a flock of chickens will influence the incidence of MD after infection. The effects of genetic constitution on the susceptibility to the development of MD and the mechanisms involved are discussed in Chapter 11. Apart from recognised specific genetic factors controlling susceptibility to the development of MD, selection for rapid growth rate has been reported to be associated with susceptibility to MD (44).

Females are usually more susceptible to the development of MD than males and although there is no effect of age on infection the incidence of MD in a flock generally declines with increasing age at infection (40,45-47). The latter is associated with the development of immune competence and is related to genetically controlled resistance. These factors are discussed in Chapters 3 and 11.

Although passively acquired antibody does not prevent infection it

reduces the levels of infection, increases the latent period between infection and disease and reduces the incidence of MD (48,49). Actively acquired immunity in response to experimental infection with attenuated MDV and apathogenic viruses such as HVT and type 2 MDV does not prevent infection with pathogenic field virus but it does reduce the incidence of MD in flocks challenged with field pathogenic viruses (18,50-52).

4.3. Viruses.

The pathogenicity of the MDV to which a flock is exposed will affect the incidence of MD. Within type 1 MDV pathogenicity varies. Some virus strains are capable of producing high incidences of MD whereas others may only have the capability of producing a low incidence in a similar flock of birds. Virus isolates vary in their pathogenicity on a sliding scale between these extremes (see Chapter 4). However the incidence of MD in groups of chickens cannot be explained in such simple terms. The unpredictable nature of the incidence of MD has been one of its main features. The incidence of MD can vary greatly not only between sites but between houses on a site and between pens within a single house (6). The incidence of MD in chickens of the same genetic stock can vary between pens within a single house by as much as 13-fold (53). An investigation of this phenomenon (6,8,53) suggested that it was likely that immunization with viruses of little or no pathogenicity could occur naturally and that an interplay of viruses of varying pathogenicity could be responsible for the unexpected variation in incidence of MD. It was found that most if not all flocks were infected with strains of MDV of varying pathogenicity and that the incidence of MD was influenced by events at the time of infection. Events and environment subsequent to this period had little if any effect on the incidence of MD. It was found that the incidence of MD was associated with the predominant type of virus present in a group of chickens at the time of primary infection. Both pathogenic type 1 viruses and apathogenic type 2 viruses were present in each group of chickens but those groups where the pathogenic viruses predominated early after infection had a higher incidence of MD than those in which apathogenic viruses were predominant. It was concluded that it is the sequence of infection or dominance of isolates of a particular pathogenicity in the environment that influences the incidence of MD. Because individual chickens

can be infected by more than one MDV strain (18,19) it was postulated that where infection with an apathogenic strain preceded that by a pathogenic strain, natural vaccination occurred reducing the incidence of MD (6,8,53).

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13. CONTROL OF MAREK'S DISEASE BY THE POULTRY INDUSTRY : PRACTICAL CONSIDERATIONS

M. PATTISON

1. INTRODUCTION

Before the development of Marek's disease (MD) vaccines it was common to see 20% to 30% mortality in flocks during the growing period. This was particularly so during the late 1960's with the increased intensification of the poultry industry, and the disease was the major cause of mortality and financial loss. During this period, the application of sound principles of good hygiene was the only means of control of MD at the disposal of the industry.

Since the introduction of vaccination, which brought such remarkable control to a desperate situation, the basic principles of good hygiene have often been forgotten or ignored. This is regrettable as it will never be possible to achieve total control with vaccination. Marek's disease virus (MDV) is shed into the environment of the poultry house through the desquamation of feather follicle epithelial cells and survives in the litter and dust in the poultry house. The dust settles on the feeders, drinkers, beams, posts, pipes and ventilation ducts, which may contain fans, and can become wind-borne and spread infection from house to house especially at clean-out time or through the movement of birds, equipment or personnel.

2. CONTROL BY HYGIENE

The principles of hygiene which apply to the control of all infectious diseases of poultry are particularly appropriate to the control of MD. They can be summarized as follows: Cleaning routines for poultry houses should be the same and as thorough for those containing broilers as for those for housing replacement breeders or layers. In practice, they rarely are, because economic necessity dictates that broiler houses

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are refilled as quickly as possible. Cleaning methods vary but emphasis should be placed on efficient mechanical cleaning prior to disinfection. After the birds have been removed from the house, all items of movable equipment should be removed for cleaning outside. Provision of concrete aprons around houses makes this task more efficient. Litter is removed using a tractor and fore-loader and should be deposited at a site well away from other poultry houses. The inside of the building is washed down using a detergent solution delivered by a high pressure hose. It is essential to remove all deposits of dust and organic matter. This is particularly difficult in inlets or outlets containing fans. Disinfectant is then applied to clean surfaces by means of suitable spraying equipment. It is wise to apply an insecticide as it is known that insects such as the Alphitobius beetle can carry disease from one crop of birds to another. Finally the building should be fogged with formaldehyde vapour generated by heating paraformaldehyde, or by the addition of formalin to potassium permanganate crystals, or by spraying liquid formalin as a fine mist spray. Hložánek et al. (1) showed that cell-free MDV persisted in poultry house dust for over a year and the most successful disinfecting agent was a combination of formaldehyde vapour and a preparation based on iodine bound to organic carriers. The poultry house should be left empty for as long as possible before moving in the clean equipment and fresh wood shavings for deep litter houses. The longer the period between flocks, the better for removal of virus. This may be about 4 weeks for breeder flocks but may only be a few days for broiler flocks. In some countries, broiler houses are often stocked by successive crops for periods up to 2 years without clean out, often because of lack of a supply of wood shavings for litter. In such circumstances the principles of good hygiene are not being applied and vaccination becomes the only means of control.

In houses where litter is reused, it has been suggested that paper placed over the litter in the brooding area will cut down exposure to virus in the early life of the chicks (2).

MD control is much easier on single age sites which are completely depopulated prior to clean out. However, often for commercial reasons, and more so in the case of layers than of broiler breeders, several different ages may exist on one site. Then, young birds in the rearing

stage should be kept as far away as possible from the adults and looked after by separate attendants. If attendants look after more than one site they should be provided with separate overalls for each site.

It is important to avoid early exposure of young chicks to large amounts of MDV, as this can overwhelm the developing immunity from the vaccine given to the day-olds in the hatchery. Some multiage rearing/laying units have been forced to become single age units, because the incidence of MD made it the only economically sensible practice. For this reason also, hatcheries should be sited at least 2km from farms to avoid infection of day-old chicks. Basarab and Hall (3) showed the benefit of holding chicks in the clean environment of the hatchery for 24 hours after vaccination. However, in commerce it is difficult to put this principle into practice.

The conditions under which chicks are transported and the length of their journey from hatchery to farm are also important factors in determining whether they will succumb to MDV infection. Air transportation can result in excessive delays and holding of chicks in adverse temperatures. This causes stress and increases susceptibility.

Early brooding conditions are also important and stress at this stage can predispose chicks to infection. Good stockmanship is of vital importance. Temperature and ventilation control, clean water and fresh food within brooder surrounds are as important to MD control as to any other disease.

3. BREEDING FOR RESISTANCE

For many years it has been observed that certain breeds and strains survive better than others in situations of high challenge by MDV (see Chapter 11). Some breeding companies select for greater natural resistance to MD. This can be done by simply leaving primary breeding stock unvaccinated and breeding from survivors - a somewhat cavalier approach - or by having a test farm heavily seeded with MDV, where selection for the most resistant crosses can be carried out. Alternatively, test chicks may be placed in clean conditions and challenged with a controlled MDV infection by injection. A more scientific approach can be used, where genes for resistance that are well defined can be selected for. Selection is based on blood typing which can

detect those individuals homozygous for the particular allele conferring resistance, e.g. B^{21} in White leghorns. There will always be a commercial conflict of interest in any such selection programmes, as the breeder must select for other traits and resistance genes may show negative correlation with important commercial characteristics such as hatchability or growth rate.

4. CONTROL BY VACCINATION

The use of MD vaccine is virtually universal throughout the world. It is generally accepted that control of MD is not possible without it; however, control of the disease in vaccinated stock is often not complete without the good management practices already described.

As described in Chapter 8, vaccine is available in three forms: 1) Cell-associated MDV, 2) Cell-associated herpesvirus of turkeys (HVT), 3) Freeze-dried HVT.

4.1. Use of vaccine in commercial practice.

In general, MD vaccine is given as a single injection to day-old breeding stock and commercial layers before they leave the hatchery. In some situations, vaccine is also given to commercial broiler stock, for example, in the USA where the level of field challenge is likely to be very high due to continuous stocking. It may also be used when the broilers are likely to be taken to heavier roaster weights and therefore kept longer than the usual 6 to 7 weeks life span. Despite the extra care with storage and reconstitution of cell-associated vaccine, it is generally favoured in the UK for vaccination of breeding stock. This may be due to the apparently quicker onset of viraemia in the face of maternal antibody for cell-associated vaccine (3 to 4 days) than for freeze-dried vaccine (5 to 7 days).

4.2. Revaccination

Following the epidemic of MD in the UK in 1975/76, it became accepted practice to revaccinate broiler breeders at 2 to 3 weeks of age with freeze-dried vaccine. This is now an established routine for many companies and is successful, as judged by the lack of mortality from MD. The experimental data on revaccination does not explain why double vaccination should apparently be so successful in the field. Choi (4) showed that revaccination did not reduce significantly the development

of MD or increase vaccine viraemia and antibody response. There was, however, a slight trend toward reduction in MD mortality after challenge at different ages. Riddell _et al_. (5) showed that revaccination at 21 days of age did not produce a measurable beneficial effect on viraemia, antibody response or MD mortality when chickens were challenged at 6 weeks of age by contact with birds inoculated with MDV. Similarly Ball and Lyman (6) found that vaccination at 1 and 21 days did not reduce MD mortality over a single vaccination up to 378 days of age, when chicks were exposed to MDV from day-old. Cho _et al_. (7) also had similar findings when revaccination was delayed to 6 weeks of age. In practice, however, once a routine such as double vaccination becomes established and appears to work, it takes courage to change it! Some companies have used different vaccines for separate generations. Thus attenuated MDV vaccine may be used at the level of grandparent stock and HVT can be used for parent stock. In theory, there should be less interference with HVT vaccination in the parents because they will have only MDV maternal antibodies.

4.3. _Vaccine administration_.

Despite the availability and widespread use of semi-automatic machines, vaccine administration is still often carried out with a hand-operated automatic syringe with glass barrel (e.g. Avijector) (Fig. 1). One complete depression of the plunger delivers one dose of vaccine (0.1 to 0.2 ml) which is held in a 250 ml reservoir, connected to the syringe by a flexible tube. Vaccine may be given by intramuscular or subcutaneous routes and a skilled operator will achieve a rate of approximately 700 chicks/hour. Needles should be changed every 500 chicks or earlier if contamination occurs. Syringes should be calibrated at the start of each day's work by squirting 20 "doses" of distilled water from the reservoir into a measuring cylinder. The syringe is adjusted until the correct volume is being delivered. A regular check of vaccine usage should be made each day, recording the number of doses and the vaccine batch numbers.

4.3.1. _Freeze-dried vaccine (HVT)_. The vaccine is normally stored in a domestic refrigerator at 4°C. The diluent should also be kept in a cool place, at less than 18°C. The vaccine is reconstituted by injectting 1 ml of diluent with a sterile needle and syringe into the bottle

containing the pellet. The bottle should be rotated gently until the pellet dissolves. The reconstituted vaccine is then injected gently into the diluent bottle.

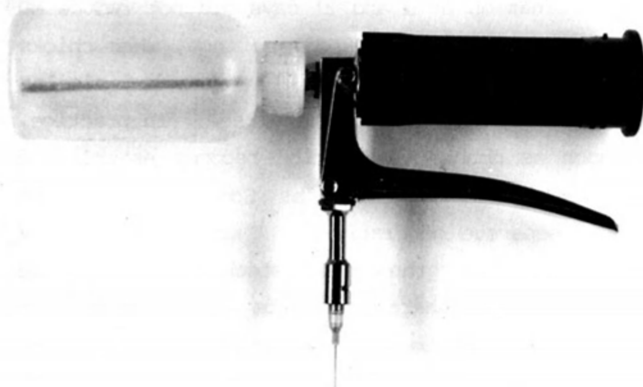

FIGURE 1. Hand-operated vaccinating gun.

4.3.2. Cell-associated vaccine (MDV or HVT). The vaccine is stored in liquid nitrogen and the level in the container should be checked daily to ensure that evaporation or leakage has not occurred. The diluent is stored in a refrigerator at 4°C. The vaccine is reconstituted by rapid thawing of the ampoule in water at 37°C. (The operator should wear eye protection during this procedure.) The vaccine is transferred to the diluent bottle as before. Reconstituted vaccine should be used within 1 hour and it is a good practice to keep it in an insulated box containing ice until use. Bottles containing cell-associated vaccine should be agitated during vaccination to keep the cells in suspension.

4.4. Mixing with other products.

Sometimes extra diluent is used in the hope of giving the chick extra fluid if it is to embark on a long journey. Various antibiotics have been included with the MD vaccine by some companies but this is not a generally recommended practice as it is usually an attempt to cover up some basic management problem, e.g. yolk sac infection. Eidson (8),

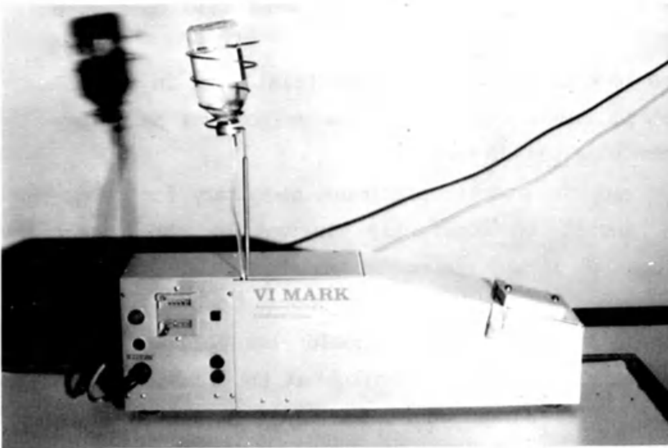

FIGURE 2. Semi-automatic vaccinating machine.

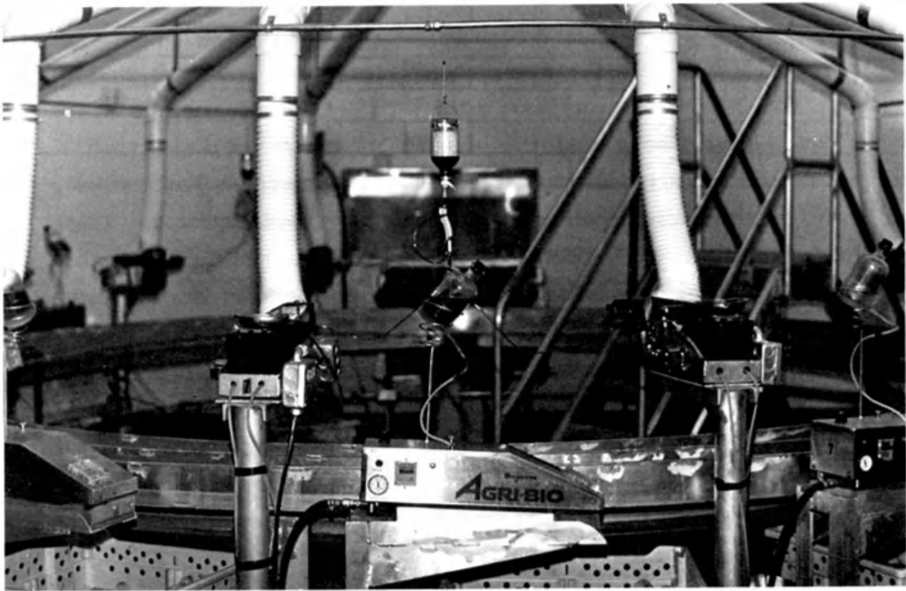

FIGURE 3. Carousel arrangement for several vaccinating machines operating together.

however, did list the antibiotics he felt were or were not compatible. Compatible antibiotics included dihydrostreptomycin sulphate, neomycin sulphate, potassium penicillin G, spectinomycin and tylosin tartrate. Chlortetracycline, oxytetracycline and erythromycin were said to have a deleterious effect.

Spray vaccination has been tried but extensive trial work in the USA in the mid-1970's failed to demonstrate that this method was as effective as injection, and the idea was dropped.

Vaccination technique and the aseptic procedure necessary for reconstitution of a vaccine should be constantly checked by the person responsible for quality control in any commercial hatchery.

4.5. Handling of vaccine

Vaccine manufacturers' recommendations should be followed precisely. Halvorson and Mitchell (9) have highlighted the possible loss of cell-associated vaccine titre that can occur through bad handling practices in the hatchery. For example, if ampoules of vaccine were removed from liquid nitrogen and held at room temperature for as little as 2½ minutes and then replaced in the liquid nitrogen, they lost 75% of their titre. If vaccine vials were left for 15 minutes in the thawing water, they could lose 50% of titre, and if the water used for thawing vaccine was too hot (40°C) or too cold (17°C), 30% of vaccine titre could be lost. They also showed the benefits of using vaccine within 30 minutes of reconstitution (as often happens in commercial hatcheries), rather than the maximum of 2 hours allowed by manufacturers. These observations emphasize the importance of continuous monitoring within hatcheries to ensure there is no departure from the correct handling, thawing and reconstitution of vaccine.

Sometimes if very large numbers of chicks are to be injected, as in broiler hatcheries, automated equipment must be used (Fig. 2). Several machines may be employed and situated with their operators in a carousel arrangement, which enables other operations such as debeaking and spray vaccinating for Newcastle disease and infectious bronchitis to be carried out at the same time (Fig. 3).

It cannot be emphazised too strongly that the efficiency of the vaccination procedure depends as much on constant alertness to operator efficiency as it does on consistent high quality of vaccine.

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